Characterization of the Role of the “MT-loop”

AN EIGHT-AMINO ACID INSERTION SPECIFIC TO PROGELATINASE A (MMP2) ACTIVATING MEMBRANE-TYPE MATRIX METALLOPROTEINASES*

William R. English‡§, Béatrice Holtz‡, Gavin Vogt‡, Vera Knäuper‡, and Gillian Murphy‡

From the §School of Biological Sciences, University of East Anglia, University Plain, Norwich, Norfolk NR4 7TJ, United Kingdom, ¶Commissariat à l’Energie Atomique, Département d’Ingenierie et d’Etudes des Protéines, 91191 Gif/Yvette Cedex, France, and the ¶Department of Biology, Bone and Joint Research, University of York, Healingston, York YO10 5YW, United Kingdom

Received for publication, August 14, 2001, and in revised form, September 10, 2001
Published, JBC Papers in Press, September 12, 2001, DOI 10.1074/jbc.M107783200

Progelatinase A (proGLA) activation is thought to be initiated almost exclusively by the type I transmembrane members of the membrane type matrix metalloproteinase family (MT-MMP): MT1, -2, -3, and -5-MMP (MMP14, -15, -16, and -24). One difference between these enzymes and the other MMP family members is the insertion of eight amino acids between strands βI and III in the catalytic domain. In MT1-MMP, the best characterized of these enzymes to date, these residues consist of KFAYFIREG. To investigate the role of this region of MT1-MMP on its catalytic activities, we have made a variety of mutations and deletions in both soluble and membrane-bound forms of the enzyme. Characterization of the activity of the soluble forms toward peptides and fibrinogen revealed that neither mutation nor deletion of residues 163–170 significantly impaired catalytic function, suggesting these residues have little influence on conformation of the active site cleft. Equally none of the mutants showed significant differences in K\textsuperscript{app} for the N-terminal inhibitory domain of TIMP2, again indicating that mutation or deletion of residues 163–170 has no major effect on the overall topology of the active site of MT1-MMP. However, characterization of the kinetics of activation of proGLA with and without its gelatin binding region by the mutants generated have shown that efficient activation of proGLA is, at least in part, through an interaction with residues 163–170 of MT1-MMP. The expression, localization, and processing from their ability to activate proGLA, MT1, -2, -3, and -5-MMP, which are all type I transmembrane proteins with a short cytoplasmic tail of ~20 amino acids, thought to be required for cellular localization (9, 10). These enzymes have all been shown to be efficient at activating proGLA either in vitro or in vivo (11–15). Detailed studies of the mechanism employed by MT1-MMP in a cell-based system have shown this to be potentiated by the binding of tissue inhibitor of metalloproteinases-2 (TIMP2) to both MT1-MMP and proGLA, suggesting that a ternary membrane-bound complex with an approximate stoichiometry 1:1:1 is formed. This is thought to cause a sufficiently high increase in the local concentration of proGLA to allow activation by TIMP2-free MT1-MMP and subsequent autocatalytic processing to the fully active form (14). Apart from their ability to activate proGLA, MT1, -2, -3, and -5-MMP also possess the ability to degrade a wide variety of extracellular matrix components in vitro (12, 13, 16). This has also been demonstrated for MT1-MMP in a variety of cell-based assays and in knockout mice, which suggests a major role for MT1-MMP in angiogenesis and bone remodeling mediated by its fibrinolytic and collagenolytic activities, respectively (16–18).

The second group of MT-MMPs, MT4-MMP and MT6-MMP, are not transmembrane proteins but are glycosylphosphatidylinositol-anchored (19, 20). These enzymes have been shown to...

Matrix metalloproteinases (MMPs) are primarily required...

GLA, gelatinase A; ΔGBR, deletion of residues Val191–Gln364 (gelatin binding region) of GLA; Mca, (7-methoxycoumarin-4-yl)acetyl; Dpa, N’-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Dns, dansyl-5-dimethylaminonapthalene-1-sulfonyldansyl; APMA, 4-aminophenylmercuric acetate; CHO, Chinese hamster ovary; WT, wild-type; DMEM, Dulbecco’s modified Eagle’s medium; NTA, nitroacetic acid; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; PCR, polymerase chain reaction; TPCK, t-1-tosylamido-2-phenyl ethyl chloromethyl ketone.

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
be more limited in their substrate range of extracellular matrix components and can be effectively inhibited by TIMPs 1–3 (23–24). Of particular interest is the finding that, despite existing as membrane-anchored MMPs and being able to bind TIMP2, these enzymes have been shown to be either unable to, or very inefficient at, activating proGLA in comparison with MT1-MMP (21–23). One structural feature in common within the group of transmembrane MT-MMPs, which is lacking from the glycosylphosphatidylinositol-anchored and soluble MMP members, is an insertion of eight amino acids in the catalytic domain. This forms a bulge in the structure distant from the active site between strands βII and βIII and has been termed the “MT-loop” by Fernandez-Catalan et al. (24). In MT1-MMP, this consists of residues 163PYAYIREG170. As these residues are unique to enzymes that efficiently activate progelatinase A, we have constructed deletion, point, and multiple mutants within this region of MT1-MMP to investigate the importance of the MT-loop in regards to proGLA activation, TIMP2 binding, and substrate cleavage.

EXPERIMENTAL PROCEDURES

Construction, Expression, and Refolding of Wild-type and Mutant Soluble Forms of MT1-MMP—Soluble MT1-MMP (WTαTM, see Fig. 1) was cloned from the human cDNA by PCR into pRSETb with BglII sites incorporated into the primers. Orientation was selected by digesting with XhoI and EcoRI, and positive clones were sequenced to detect PCR errors. This generated proMT1-MMP starting from Arg-Ser21-Ala-Leu-Ala-Ser-Leu . . . and ending . . . Cys-Pro-Ser-Gly22–Arg-Gly24, where amino acids in italics are those substituted by the addition of the BglII sites. The pRSETB vector also made the addition of an N-terminal sequence including a His6 affinity tag, the EXPRESS epitope, and an enterokinase cleavage site. Subsequent mutants were constructed using the overlap extension method. Overlapping primers for deletion of residues 163–170 (ΔL1ΔTM) and 171–173 (ΔHEKΔTM) and to make mutants ML1ΔTM, ML2ΔTM, ML3ΔTM, Y164SΔTM, and Y166S/167AΔTM (Fig. 1) were used in conjunction with primers annealing outside the multiple cloning site of vector pRSETB. The final PCR products were ligated into pRSETB after digestion with BglII and EcoRI before sequencing. All enzymes were then expressed in 18 h and assessing changes in activity by following hydrolysis of Mca-PLGL-Dpa-AR-NH2 and Mca-PLA-Nva-Dpa-AR-NH2, where concentrations of [S] < 1.5 μM, where [S] was estimated to be less than 3 μM and v was found to follow an apparent linear relationship with respect to [S]. Where kcat and Km were to be calculated, kinetics studies were performed as described previously (28). Briefly, degradation was performed in assay buffer A, for substrate concentrations ranging from 3.5 to 24.5 μM. The slopes of fluorescence increase under steady-state conditions were measured and corrected for inner filter effect. Kinetic parameters were then assessed according to the direct linear plot method (29, 30). ΔL1ΔTM showed effects of substrate inhibition with substrate Dns-PLA-Cys(OMeBn)-WAR-NH2, where v was given an initial increase followed by a decrease with increasing [S]. Estimates of kcat and Km were calculated using Equation 1.

\[
v = \frac{[E][S]k_{\text{cat}}}{K_m + [S] + [E][S]/K_m}
\]  

(Eq. 1)

v is the observed rate of hydrolysis at substrate concentration [S], [E], is the total enzyme concentration, and kcat is the first order rate constant describing the product formation after productive binding at the active site with the equilibrium constant Ka. Km is the equilibrium constant for the second nonproductive binding site, which prevents hydrolysis at the active site. Competitive product inhibition was eliminated by purification of the products on reverse phase-high performance liquid chromatography as described in Ref. 27 and following hydrolysis of 1 μM Mca-PLGL-Dpa-AR-NH2 by 200 nM ΔL1ΔTM in the presence of products at concentrations of up to 3 μM, where no inhibition was seen. Characterization of the Hydrolysis of Fibrinogen by Soluble Wild-type and Mutant ΔTM-MT1-MMP—Human plasma fibrinogen (Calbiochem) in assay buffer B (50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl2, 0.05% Brij v/v, pH 7.5) at a concentration of 0.25 mg ml−1 was incubated with 5, 10, and 20 ng of enzyme or assay buffer alone at 30 °C for 18 h. Reactions were stopped by boiling in reducing SDS-PAGE buffer. Hydrolysis was analyzed by reducing SDS-PAGE on 8% acrylamide gels and Coomassie Blue staining. The relative ability of the various soluble forms of MT1-MMP to degrade the Aα chain of fibrinogen was assessed by scanning the gels and performing densitometric analysis.

Characterization of the Interaction of Wild-type and Mutant ΔTM-MT1-MMP with TIMP2—The affinity for TIMP2 was calculated by incubating either 50 or 100 nM of all forms of MT1-MMP with TIMP2 at concentrations ranging from 0 to 20 nM in a volume of 2.5 ml of assay buffer B for ~18 h at 30 °C as described previously (31). Steady state rates of hydrolysis of 1 μM Mca-PLGL-Dpa-AR-NH2 were then plotted against inhibitor concentration, and KI was calculated using Equation 2 derived by Morrison and Walsh (32).

\[
v = (v_0/2E_0) [(K_{I}^p + I - E_0/4K_{I}^pE_0)^{1/2} - (K_{I}^p + I - E_0)]
\]  

(Eq. 2)

v0 is the rate of hydrolysis in the absence of inhibitor, E0 is the total enzyme concentration, and I is the total inhibitor concentration. The second order rate constant, describing the onset of inhibition in the presence of substrate assuming a simple bimolecular collision, was calculated for all soluble (ΔTM) forms of MT1-MMP at 30 °C in assay buffer B in the presence of 1 μM Mca-PLGL-Dpa-AR-NH2 as follows. Enzyme at 50–100 μM was rapidly mixed with TIMP2 at concentrations ranging from 0.32 to 1.9 μM and followed until enzyme activity reached a steady state. The progress curves were analyzed using Equations 3 and 4, which describe inhibition of enzyme under he conditions where \( I = E_0 \).

\[
P = v + (v_0 - v)/((1 - γ)\alpha\ln(1 - e^{-v/v_0}))
\]  

(Eq. 3)

\[
γ = (v_0 - v)/v_0E_0
\]  

(Eq. 4)

P is the product concentration at time t, v is the steady state rate and v0 is the rate in the absence of any inhibitor. E0 and I are the total enzyme and inhibitor concentrations. The factor γ accounts for depletion of free inhibitor due to binding. The parameter λ is the apparent pseudo-first-order rate constant for the association at each [I] and is...
MT-loop Required for Efficient Progelatinase A Activation

| Enzyme Name | 'MT-Loop' Sequence | Variant |
|-------------|--------------------|---------|
| WT | 163-IFYIREHEK-173 | ΔTM/TM |
| ΔL | 163-IFYIREKG-173 | ΔTM/TM |
| ΔHEK | 163-IFYIREKG-173 | ΔTM |
| ML1 | 163-GAGAAGHEK-173 | ΔTM/TM |
| ML2 | 163-GAGAAGREK-173 | ΔTM/TM |
| ML3 | 163-GAGAAGHEK-173 | ΔTM |
| Y164S | 163-PAYIREHEK-173 | ΔTM |
| Y166S/1167A | 163-PAYAIREHEK-173 | ΔTM |

Fig. 1. Diagram showing constructs used in this study. Wild-type (WT), deletion of the MT-loop (ΔL), and HEK are shown by underscore in the sequences. Mutations in the MT-loop (ML) are shown in bold. The soluble forms of the enzyme are the ΔTM constructs, which lack residues from 512 onward deleted and include a poly-His tag at the N terminus to aid purification. For mammalian cell expression, constructs with the transmembrane (TM) and cytoplasmic domains were used. The wild-type signal sequence was replaced by that of tissue plasminogen activator.

obtained from Equations 3 and 4. The apparent second-order rate constant for the onset of inhibition, $k_{on}$, is the obtained using Equation 5.

$$
\lambda = k_{on}[(E_i + E_f)^2 - 4E_iE_f]^{1/2} \quad \text{(Eq. 5)}
$$

Equation 5 is a slightly simplified version of that described by Williams and co-workers (33).

Activation of ProGLA and ProΔGBR by Soluble Wild-type and Mutant Forms of MT1-MMP in Vitro—ProGLA and proΔGBR were expressed from a mouse myeloma system and purified as described previously (26). The concentration of Gla and ΔGBR was calculated after activation with 1 mM APMA and active site titration with TIMP2 (26). ProGLA or proΔGBR were incubated in assay buffer B at a final concentration of 0.9 μM in the presence or absence of wild-type or mutant MT1-MMP at a molar ratio of 10:1 at 37°C. Samples were taken at intervals and diluted in assay buffer B before enzymatic activity was followed using the quenched fluorescent substrate Mca-PLGIL-Dpa-AR-NH₂, 37°C, with the final total concentration of GLA at 140 ps. After dilution, the activity of MT1-MMP was found to be negligible.

Construction of Wild-type and Mutant MT1-MMP for Mammalian Cell Expression and Immunolocalization in Transiently Transfected Cells—MT1-MMP lacking its signal peptide was subcloned using standard PCR methods from the cDNA. This was ligated into pRSETB using BglII and EcoRI introduced by PCR. Mutant soluble forms in pRSETB were digested with BglII and BamI, and this fragment was used to replace the corresponding fragment in the wild-type DNA in pRSETB. The resulting cDNAs were excised using BglII and EcoRI and ligated into pEE14 (CelltechChiroscience), which resulted in the addition of the tissue plasminogen activator signal peptide (Fig. 1). CHO L761H (CelltechChiroscience) were seeded onto 10-mm coverslips in 12-well dishes at 5 × 10⁴ cells/well overnight in DMEM, 10% FCS before transfection in the presence of serum with 2 μg of pEE14, WTTM, ΔLTM, ML1TM, or ML2TM DNA with Fugene 6 as recommended by the manufacturer. After 12 h the cells were washed three times with serum-free medium before the addition of serum-free medium containing 3 μg ml⁻¹ proGLA. Conditioned medium and cell lysates were taken after 0, 24, 48, and 72 h after the addition of proGLA. Cell lysates were probed for the presence of MT1-MMP using the sheep anti-human MT1-MMP polyclonal N175/6 by Western blot after 24 h. Activation of proGLA was analyzed in both the conditioned medium and cell lysates by gelatin zymography (34). Gelatin zymograms were scanned, and the relative abundance of the various forms at each time point was estimated using densitometric analysis. Only data obtained for the intermediate and active forms of GLA were used, as intensities of the pro-form were found to be outside the linear range of densitometric analysis.

RESULTS

Expression in E. coli and Refolding of Wild-type and Mutant Soluble MT1-MMP from Insoluble Inclusions—All soluble forms of MT1-MMP (ΔTM-MT1) lacking residues from 512 onward were expressed at high levels as insoluble inclusions in E. coli BL21 (DE3) pLyS3 upon induction with isopropyl-1-thio-β-D-galactopyranoside. All could then be purified under denaturing conditions first on DEAE-Sepharose, followed by Ni²⁺-NTA-agarose using the His₆ tag preceding the propeptide, to yield a single major band as determined by SDS-PAGE analysis (Fig. 2A). The wild-type enzyme (WTΔTM) and the mutants Y164SΔTM and Y166S/167ΔTM were refolded using a stepwise dilution in urea adopted from a method devised for the

Fig. 2. A, reducing Coomassie Blue-stained SDS-PAGE of the pure pro-enzymes after DEAE-Sepharose and Ni²⁺-NTA-agarose purification under denaturing conditions prior to refolding. B, an example of trypsin activation of refolded MT1-MMPs renatured with (WT) and without (ΔL) an intermediate folding step at 2.3 M urea. Samples were incubated with (+) and without (−) TPCK-treated trypsin as described under “Experimental Procedures” before analysis of the cleavage products on 12% reducing SDS-PAGE and Coomassie Blue staining. Pex, hemopexin domain; Cat, catalytic domain.
MT-loop Required for Efficient Progelatinase A Activation

 renaturation of MMP13. Refolding first into 2.3 M urea before dialysis to 0.1 M urea allows both the hemopexin and catalytic domains to fold independently (35). WT∆TM, Y164S∆TM, and Y166S/I167∆ATM all refolded to yield latent proenzymes, which could be activated by TPCK-treated trypsin or recombinant furin as determined by activity toward Mca-PLGL-Dpa-AR-NH2 (Fig. 2B and data not shown). Active site titration with TIMP2 indicated that ~50% of material refolded was catalytically active. However, mutants ∆L∆TM, ML1∆TM, ML2∆TM, ML3∆TM, and ∆HEK∆TM, all of which had either residues deleted or contained substantial mutations, did not refold using the stepwise renaturation in urea and <0.1% of material refolded was found to be catalytically active. This was remedied by refolding in a single step to 0.5 M urea, which activated latent enzymes, after which activation by TPCK-treated trypsin and TIMP2 titration allowed an estimated recovery of 5–10% folded protein. All soluble forms were then concentrated by dialysis versus PEG6000 and further dialed against the assay buffer to be used. Concentration of the proenzymes using PEG6000 was found to cause precipitation of a large proportion of the misfolded protein without loss of active material. Subsequent comparison of the digestion of WT and ∆L∆TM by TPCK-treated trypsin on reducing SDS-PAGE showed that refolding without the intermediate step in 2.3 M urea resulted in misfolding of the hemopexin domain of MT1-MMP. We have shown previously that trypsin digestion of ∆TM-MT1-MMP results in removal of the propeptide, generating the active catalytic domain of MT1-MMP, and also in processing within the hinge region linking the catalytic and hemopexin domains (36). This led to generation of 32- and 25-kDa forms consisting of the hemopexin and catalytic domains, respectively, when visualized on reducing SDS-PAGE (Fig. 2B, WT∆TM). After trypsin activation of ∆L∆TM, only the catalytic domain remains, indicating the hemopexin domain was trypsin sensitive and hence not folded (Fig. 2B). As the MT1-MMP hemopexin domain has not been shown to have a significant interaction with TIMP2 or proGLA, its presence or absence was not expected to have any influence on subsequent assays (37).

Activity of Wild-type and Mutant Forms of ∆TM-MT1 towards Peptide Substrates and Fibrinogen—To examine the effects of mutations within the MT-loop on catalytic activity, hydrolysis of a number of peptide substrates was examined. These experiments were conducted at 25 °C, pH 6.8 to allow comparison with previously obtained data. Initially kcat and Km were calculated for WT∆TM and ∆L∆TM, the most drastic mutation, for peptide substrates Dns-PLA-LWAR-NH2, Dna-PLA-Cys(OMeBn)-WAR-NH2, and Mca-PLGL-Dpa-AR-NH2 as described previously (27). Values obtained for peptides containing short side chains at the P1′ position, which fills the “specificity pocket” of MMPs, show that deletion of the MT-loop has very little effect on Ks (Table I). This would suggest that, overall, the affinity of the active site is not significantly altered. Differences observed in kcat/Km were found to largely result from changes in kcat (Table I). However, using the substrate Dns-PLA-Cys(OMeBn)-WAR-NH2, which has a long artificial P1′ residue thought to be able to completely occupy the S1′ subsite or specificity pocket, deletion of the MT-loop did show that some conformational changes had occurred. This substrate is efficiently hydrolyzed by WT∆TM, but not by ∆L∆TM, which was found to deviate significantly from Michaelis-Menten behavior, where plots of vs versus [S] showed characteristics of substrate inhibition. Furthermore, the purified products of Dns-PLA-Cys(OMeBn)-WAR-NH2 were found not to competitively inhibit hydrolysis of Mca-PLGL-Dpa-AR-NH2, even at high micromolar concentrations, confirming substrate inhibition. Although we were unable to demonstrate which mode of binding of Dns-PLA-Cys(OMeBn)-WAR-NH2 to the enzyme was unproductive, calculation of Ks and Ks′ using the simplified equation described under “Experimental Procedures” showed that Ks′, the affinity of the substrate for the enzyme in its unproductive binding mode, was significantly lower than the calculated Ks, the affinity of the substrate at the active site (Ks ≥ 9 × 10−5 M, Ks′ ≤ 1 × 10−9 M). Together, these results show that, with short naturally occurring side chains, the MT-loop does not effect catalysis at the active site, although deletion of the MT-loop does appear to alter the properties of the active site with respect to long P1′ side chains, perhaps indicating changes at the bottom of the deep MT-MMP S1′ subsite or overall conformational flexibility of the catalytic domain. Subsequently, kcat/Km for a variety of quenched fluorescent substrates was calculated for all soluble forms of MT1-MMP to see if any of the mutations or deletions of residues 171–173 significantly effected function of the active site. As expected from the more detailed investigation of ∆L∆TM hydrolysis of peptides, none of the remaining mutants showed any significant differences in kcat/Km for any of the substrates subsequently examined, all of which contain short side chains at the P1′ position (Table I). These results indicate that the MT-loop, which is an exo-site distinct from the active site, has little influence on hydrolysis of substrates that bind primarily to the active site cleft.

As the peptide substrates examined demonstrate only limited changes in interaction in the active site cleft, a comparison was made of the relative abilities of WT∆TM and the deleted and mutant forms to cleave a macromolecular substrate, fibrinogen. Cleavage of fibrinogen and fibrin by MT1-MMP has been proposed to be an integral part of angiogenesis (16–18). Hydrolysis was assessed by incubating increasing concentrations of ∆TM-MT1 with fibrinogen for 18 h at 30 °C (Fig. 3). As only the Aα chain showed significant hydrolysis (Fig. 3), densitometric analysis of this chain of fibrinogen was used to compare the extent of hydrolysis of WT and mutant ∆TM-MT1-MMPs with respect to the control incubation. Using the samples incubated with 5 ng of ∆TM-MT1-MMP for comparison, we found that WT∆TM, ML1∆TM, and ML2∆TM had all cleaved ~60–70% of the Aα chain after 18 h. ∆L∆TM, ∆HEK∆TM, ML3∆TM, Y164S∆TM, and Y166S/I167∆ATM had cleaved between 20 and 40% in comparison. The lowest rate of hydrolysis was found with Y166S/I167∆ATM, 20% over 18 h, or 3.5-fold slower than WT∆TM, is not a significant difference. As observed with the hydrolysis of quenched fluorescent peptide substrates, these results lend further weight to the argument that the MT-loop does not have a significant influence on the conformation of the active site cleft.

### Table I

| Substrate                  | kcat/Km calculated for Mca/Dpa substrates at 25 °C, pH 6.8 for wild-type and mutant soluble forms of MT1-MMP |
|----------------------------|-------------------------------------------------------------------------------------------------------------|
|                            | WT∆TM | ∆L∆TM | ∆HEK∆TM | ML1∆TM | ML2∆TM | ML3∆TM | Y164S∆TM | Y166S/I167∆ATM |
| Mca-PLGL-Dpa-AR-NH2         | 2.1   | 1.5   | 1.3     | 1.5    | 2.1    | 2.7    | 2.8      |               |
| Mca-PLA-Nva-Dpa-AR-NH2      | 0.86  | 1.0   | 0.42    | 0.4    | 0.96   | 1.03   | 0.88     |               |
| Mca-P-Cha-G-Nva-HA-Dpa-NH2  | 0.14  | 0.56  | 0.17    | 0.17   | 0.19   | 0.44   | 0.42     | 0.52          |
## Inhibition of Wild-type and Mutant Forms of ΔTM-MT1 by ΔTIMP2

Inspection of the crystal structure of the MT1-MMP:TIMP2 catalytic domain complex reveals that the MT-loop generates a pocket in the MMP catalytic domain fold with which the AB-loop of TIMP2 interacts, although there is no direct interaction between the AB-loop and TIMP2 (24). Recent mutational studies of residues in the AB-loop of TIMP2 have shown that they are critical in modulating TIMP2:MT1-MMP affinity at equilibrium and during association (31). As changes in the MT-loop may affect the binding of TIMP2 to MT1-MMP, and hence both inhibition and progelatinase A activation. The equilibrium constant \(K_{pp}^{Δ TIMP2}\), and a second order association rate constant \(k_{on}\), describing the onset of inhibition of substrate hydrolysis, were calculated for wild-type and mutant forms of MT1-MMP. As the affinity of the complete TIMP2 molecule was found to be less than the minimum enzyme concentration at which activity could be detected \(K_{pp}^{Δ TIMP2} < 20 \text{ pm}\) for all forms of ΔTM-MT1), affinity measurements were made for the N-terminal inhibitory domain of TIMP2 because \(K_{pp}^{Δ TIMP2}\) could be accurately calculated. \(K_{pp}^{Δ TIMP2}\) was not found to change significantly for any of the mutants over wild-type MT1-MMP, indicating that the net effect on affinity of the TIMP2:MT1-MMP complex is not influenced by the MT-loop (Table III). \(k_{on}\) was also calculated for ΔTIMP2 rather than TIMP2, as it has been shown to be independent of the C-terminal TIMP2 interactions with MT1-MMP (11). Calculation of \(k_{on}\) for WTΔTM and mutants in and round the MT-loop showed that mutations in the region do have some effect on association (Table III). Mutations within the MT-loop, in particular those including mutations at the N-terminal portion (ML1–ML3 and Y164S) appear to decrease the association rate ~5–10-fold. In contrast, the deletion mutants ΔΔTM and ΔHEKΔTM and the double mutant Y166S/167AΔTM showed less significant effects on association when compared with WTΔTM (Table III).

### Activation of ProGLA and Pro3GBR by Wild-type and Mutant Forms of ΔTM-MT1 in Vitro—Activation of proGLA by WTΔTM and mutant ΔTM forms was studied to examine role of the MT-loop in this process. This was achieved by pre-incubation of proGLA in a 10-fold excess over MT1-MMP at micromolar concentrations and monitoring hydrolysis of Mca-PLGL-Dpa-AR-NH₂ after dilution of the reaction. These experiments were conducted at 37 °C, as the activation rate of proGLA was found to be substantially decreased at 30 °C and none of the mutants of MT1-MMP showed a significant decrease in activity over the time course examined. In the absence of MT1-MMP, no proGLA activation was seen (Fig. 4). Activation of proGLA by WTΔTM followed by this method showed the characteristic lag phase before an increase in activity is seen, corresponding to the production of the intermediate form of GLA and the subsequent autocatalytic step producing the fully active form, respectively. The lag phases for WTΔTM and ΔHEKΔTM were both similar, lasting ~15 min, with activation complete by 120 min (Fig. 4A). Using identical conditions, ΔΔΔTM showed an extended lag-phase of ~90 min and by 270 min only 50% of proGLA activation was achieved. A similar influence on proGLA activation was observed where the MT-loop was either completely replaced (ML1ΔΔTM and ML3ΔΔTM, Fig. 4B) or only the more conserved N-terminal residues were replaced (ML2ΔΔTM, Fig. 4B) where the most significant decrease in

### Table II

| Enzyme | \(K_M\) | \(k_{cat}\) | \(k_{cat}/K_M\) |
|--------|--------|---------|-----------|
| Mca-PLGL-Dpa-AR-NH₂ | 10⁻⁶ M | 10⁻³ s⁻¹ | 10³ M⁻¹ s⁻¹ |
| Dns-PLALWAR-NH₂ | 10⁻³ M | 10⁻⁹ s⁻¹ | 10⁹ M⁻¹ s⁻¹ |
| Dns-PLACys(OMeBn)-WAR-NH₂ | 10⁻⁶ M | 10⁻³ s⁻¹ | 10³ M⁻¹ s⁻¹ |

\(\text{NC}\) denotes not calculated.

### Table III

| Enzyme | \(k_{on}\) | \(K_{pp}\) |
|--------|---------|---------|
| WTΔTM | \(3.2 ± 0.1 \times 10^6\) s⁻¹ M⁻¹ | \(1.2 ± 0.1 \times 10^{-9}\) M |
| ΔΔTM | \(3.8 ± 0.5 \times 10^6\) s⁻¹ M⁻¹ | \(1.5 ± 0.1 \times 10^{-9}\) M |
| ΔHEKΔTM | \(0.95 ± 0.4 \times 10^6\) s⁻¹ M⁻¹ | \(1.0 ± 0.05 \times 10^{-9}\) M |
| ML1ΔΔTM | \(0.75 ± 0.2 \times 10^6\) s⁻¹ M⁻¹ | \(0.72 ± 0.03 \times 10^{-9}\) M |
| ML2ΔΔTM | \(0.19 ± 0.1 \times 10^6\) s⁻¹ M⁻¹ | \(0.95 ± 0.06 \times 10^{-9}\) M |
| ML3ΔΔTM | \(0.55 ± 0.2 \times 10^6\) s⁻¹ M⁻¹ | \(1.5 ± 0.2 \times 10^{-9}\) M |
| Y164SΔΔTM | \(0.57 ± 0.1 \times 10^6\) s⁻¹ M⁻¹ | \(1.3 ± 0.15 \times 10^{-9}\) M |
| Y166S/167AΔΔTM | \(1.6 ± 0.6 \times 10^6\) s⁻¹ M⁻¹ | \(1.5 ± 0.17 \times 10^{-9}\) M |

Errors are the experimental error derived from three separate experiments.

* Data published (29).
MT-loop Required for Efficient Progelatinase A Activation

The catalytic domain of proGLA differs from most MMP catalytic domains in several key ways. First, the propeptide contains a disulfide bond constraining the region hydrolyzed by MT1-MMP; second, it contains three type II fibronectin repeats, or gelatin binding region, inserted between residues Val191 and Gln364. This additional domain in the MMP catalytic fold also interacts with the extended N terminus of the propeptide, possibly causing further stabilization (38). To examine the role of the gelatin binding region in the activation of proGLA by MT1-MMP, the activation of proGLA by the key mutant forms of soluble MT1-MMP was examined in comparison with WTΔTM under identical conditions to proGLA. Activation of proΔGBR by WTΔTM was found to be approximately 2-fold slower than the rate at which proGLA was activated (Fig. 5). ΔLΔTM was found to activate proΔGBR with virtually identical kinetics to WTΔTM (Fig. 5), suggesting that residues 163–170 WTΔTM interact with the gelatin binding type II fibronectin repeats of proGLA and maybe responsible for the faster rate of activation. However, replacement of the residues 163–166 (ML2ΔTM) or 163–169 (ML3ΔTM and ML4ΔTM) resulted in impaired ability to activate proΔGBR. That these replacement mutants are also impaired in TIMP2 association is of interest. As neither affinity for TIMP2 nor activity of the active site is impaired, one hypothesis to explain these observations is that (a) significant mutations of the MT-loop sequence are generating a loop with increased flexibility, which is sterically hindering TIMP2 association and proGLA interaction, or (b) these mutations cause structural alterations in the TIMP2 AB-loop binding pocket of MT1-MMP, and that the correct conformation of the TIMP2 AB-loop binding pocket of MT1-MMP is also required for proGLA activation.

Fig. 4. Activation of 0.9 μM proGLA by 0.09 μM ΔTM-MT1-MMP at 37 °C in vitro. Samples were taken at the time points indicated and diluted before activation was monitored by changes in fluorescence on hydrolysis of Mca-PLGL-Dpa-AR-NH₂. Rate of hydrolysis was then converted to percentage of activation of proGLA using APMA-activated GLA to determine 100% activation. All time points are the average of three independent experiments. A, WTΔTM (■), ΔHEK△TM (○), ΔLΔTM (▲), and proGLA in the absence of ΔTM-MT1 (△). B, WTΔTM (■), ML1△TM (○), ML2△TM (▲), ML3△TM (□), and proGLA in the absence of ΔTM-MT1 (△). C, WTΔTM (■), Y164S/166I△TM (▲), Y169S/167A△TM (□), and proGLA in the absence of ΔTM-MT1 (△). Lines through the data are a spline fit.

Fig. 5. Activation of 0.9 μM proΔGBR by 0.09 μM ΔTM-MT1 at 37 °C in vitro as described in Fig. 4. Activation was by WTΔTM (■), ΔLΔTM (▲), ML1△TM (○), ML2△TM (▲), ML3△TM (□), and proΔGBR alone (△). Lines through the data are a spline fit.

Activation was seen. These results show that the MT-loop is required for efficient activation of proGLA, and that deletion of residues after the MT-loop has no influence on activation. Equally, replacement of the more conserved residues in the MT-loop suggests that these are the more important in proGLA activation. This is also born out when Tyr164 is mutated to Ser, which changes the size of the residue but preserves its polar nature, as this single mutation has a lesser, but noticeable effect on activity (Fig. 4C). In contrast the double mutant Y166S/167A△TM shows very little decrease in ability to activate proGLA as compared with WTΔTM (Fig. 4C), again indicating it is the structure of the more conserved N-terminal end of the MT-loop that is required for efficient proGLA activation.
Expression of Wild-type and Mutant Forms of MT1-MMP in CHO Cells: Comparison of Expression, Processing, Localization, and Activation of ProGLA—By examining proGLA activation of the complete membrane-bound forms of the MT1-MMP mutants in transfected cells, the role of the MT-loop in proGLA activation can be examined in the TIMP2-dependent “receptor” model of activation. ML3TM was not investigated, as it was not expected to give a different result from ML1TM. Similarly, mutants Y164S and Y166S/I167A were not transfected as their deficiencies in proGLA activation in vitro were thought to be too small to be detectable in this system. WTTM, ΔLTM, ML1TM, and ML2TM were transfected into CHO L761H cells in the presence of serum overnight. The serum-containing medium was removed and substituted with serum-free medium supplemented with proGLA. Cell lysates and samples of the conditioned medium were then harvested at 0, 24, 48, and 72 h after addition of proGLA. All enzymes were found to be expressed at similar levels and showed virtually identical levels of processing when cell lysates were examined by Western blot using a sheep anti-human MT1-MMP polyclonal antibody (Fig. 6). That WTTM, ΔLTM, ML1TM, and ML2TM are all processed equally to the 45-kDa form indicates that this processing is not influenced by the MT-loop. Immunolocalization was performed in transiently transfected CHO L761H cells, and all forms of MT1-MMP were found to show similar patterns of staining characteristic of plasma membrane localization (Fig. 7).

When examining proGLA activation by WT and mutant forms of MT1-MMP by gelatin zymography, it was found that all three mutants were deficient in proGLA activation when compared with WTTM MT1-MMP. ProGLA activation was monitored by running cell lysates and conditioned medium on zymograms, followed by densitometric analysis to allow comparisons of the rates of GLA activation. Only the intermediate and active forms of GLA could be analyzed, as the levels of proGLA were outside the linear range of the assay. In the cell lysate, significant levels of intermediate and active forms of GLA could be detected in cells transfected with WTTM at 24 h after proGLA addition over vector control-transfected cells (Fig. 8). The mutant forms of MT1-MMP were found to be kinetically impaired in their generation of the intermediate and active forms of GLA when cell lysates were examined (Fig. 8). In comparison, the accumulation of intermediate and active forms of GLA in the conditioned medium of WTTM transfected cells appeared to occur later than in the cell lysates. Detectable levels of the intermediate form of GLA were observed at 48 h for WTTM, and the mutants at 72 h, an apparent delay of ~24 h in each case. In the conditioned medium, the levels of active GLA produced were indistinguishable between the mutant and control transfected cells (Fig. 8). These results show that activation at the cell surface precedes the accumulation of active GLA in the conditioned medium and differences in activity caused by the mutations in the MT-loop seen in the TIMP2 cell-free system are also observed in a TIMP2-dependent cellular activation model.

DISCUSSION

Efficient activation of proGLA is limited to a small subset of the MMP family, the type I transmembrane-anchored MMPs, MT1, -2, -3, and -5. As the isolated catalytic domain of MT1-MMP is sufficient itself to activate proGLA (39) and the hemopexin domain is not thought to participate in activation (37), we hypothesized that there is a specific adaptation within this domain that allows this to occur. The most obvious of motif is the insertion of eight amino acids between strands βII and βIII in MT1, -2, -3, and -5-MMP, absent from the other MMPs (24). Defects in human MT2-MMP activation of proGLA when transfected in COS-1 cells, not seen with mouse MT2-MMP, were attributed to differences in the sequence of this region by a report of Miyamori et al. (39). However, the investigators did not establish whether hMT2-MMP in these cells is correctly processed or localized at the plasma membrane, as the isolated catalytic domain of hMT2-MMP has been shown to activate proGLA (11). To further investigate the role of this loop, we have either deleted or mutated residues 163–171, or “MT-loop” in MT1-MMP, the most thoroughly characterized of the MT-MMP family to date. As expected for an exo-site, these mutations were found to have little effect on hydrolysis of peptide substrates or on hydrolysis of fibrinogen, a macromolecular...
substrate of MT1-MMP, the cleavage of which is thought to play a role in angiogenesis (16–18). This is in agreement with a report made by Terp et al. (40), which used homology modeling of the MMP catalytic domains to investigate structure-function relationships, predicts that the MT-loop would not be expected to significantly influence the activity of the catalytic site of MT1-MMP.

The current cellular model of proGLA activation by MT1-MMP takes into account the need for proGLA concentrations to be elevated at the cell surface to allow efficient activation. This process requires TIMP2, which is hypothesized to form a ternary complex with MT1-MMP and proGLA through N- to N-terminal and C- to C-terminal interactions, respectively. MT1-MMP not inhibited by TIMP2 would then be expected initiate activation. However, Miyamori et al. (41) have shown recently that, in cells where TIMP2 levels are not detectable, MT1-MMP can efficiently initiate activation of proGLA to its intermediate form but further processing to active GLA does not occur without addition of TIMP2. We have also observed similar effects on the activation of proGLA with isolated membranes depleted of TIMP2 and highly enriched in MT1-MMP. These more recent findings would indicate that it is the final autocatalytic activation step that has a greater requirement for a MT1-MMP:TIMP2:proGLA receptor rather than the initial attack by MT1-MMP.

MT1-MMP also undergoes processing to a truncated form consisting predominantly of the hemopexin-transmembrane-cytotail (42, 43). Production of this 45-kDa processed form is thought to be concomitant with proGLA activation and is produced thorough autocatalytic cleavage as a means to down-regulate MT1-MMP activity. Although the N terminus of a corresponding processed soluble form has been identified, this is not at a site in the catalytic domain that is particularly solvent-accessible to proteolytic cleavage (42). This raises the possibility of an initial cleavage event further toward the N terminus, with the MT-loop being an ideal candidate site. Interestingly, Imper and Van Wart (44) have described a peptide substrate for MT1-MMP that is similar in sequence to residues 163–170 of MT1-MMP. That mutation of the MT-loop does not appear to effect the processing to the 45-kDa form indicates that this region is unlikely to be an initial site of cleavage.

All MT1-MMP mutants in residues 163–170 investigated in this study are processed equally, regardless of the level of activation of proGLA achieved. In contrast, we have observed that the proportion of 45-kDa form to the other forms of MT1-MMP is dependent on the level of transfection of MT1-MMP, i.e. the greater the transfection level, the more abundant the 45-kDa form is. This lends further weight to the argument that generation of the 45-kDa form is an autocatalytic event, but it may not be directly linked to proGLA activation per se.

2 W. R. English and G. Murphy, unpublished observations.
Acknowledgments—We thank Daniel Leongamornlert, Peter Greaney, and Mike Hutton for technical assistance and Vincent Dive for helpful discussion.

REFERENCES

1. Werb, Z. (1997) Cell 91, 439–442
2. Weevers, J. F., and Nagase, H. (2000) Matrix Metalloproteinases and TIMPs, Oxford University Press Inc, New York
3. Murphy, G., Knauper, V., Atkinson, S., Gavrilovic, J., and Edwards, D. (2000) Fibrinolysis Proteolysis 14, 165–174
4. Sang, Q. X. (1998) Cell Res. 8, 171–177
5. Kellerbroek, S. M., and Stack, M. S. (1999) Bioessays 21, 940–949
6. Nagase, H. (1998) Cell Res. 8, 179–186
7. Ha, H. Y., Moon, H. B., Nam, M. S., Lee, J. W., Ryu, Z. Y., Lee, T. H., Lee, K. K., So, B. J., Sato, H., Seiki, M., and Yu, D. Y. (2001) Cancer Res. 61, 984–990
8. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994) Nature 370, 61–65
9. Nakahara, H., Howard, L., Thompson, E. W., Sato, H., Seiki, M., Yeh, Y. Y., and Chen, W. T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7959–7964
10. Lehti, K., Valtanen, H., Wickstrom, S., Lehi, J., and Keski-Oja, J. (2000) J. Biol. Chem. 275, 15006–15013
11. Butler, G. S., Will, H. Atkinson, S. J., and Murphy, G. (1997) Eur. J. Biochem. 244, 653–657
12. Shofuda, K., Yasumitsu, H., Nishihashi, A., Miki, K., and Miyazaki, K. (1997) J. Biol. Chem. 272, 9479–9474
13. Llano, E., Penda, K., Caterina, J., Yamada, S., and Murphy, G. (1997) EMBO J. 17, 5288–5294
14. Butler, G. S., Butler, M. J., Atkinson, S. J., Will, H., Tamura, T., Van Westrum, S. S., Crabbe, T., Clements, J., D’Ortho, M. P., and Murphy, G. (1998) J. Biol. Chem. 273, 871–880
15. D’Ortho, M.-P., Will, H., Atkinson, S., Butler, G. S., Messent, A., Gavrilovic, J., Smith, B., Timpl, R., Zard, L., and Murphy, G. (1997) Eur. J. Biochem. 259, 751–757
16. Hirao, N., Allen, E., Apel, I. J., Gyetko, M. R., and Weiss, S. J. (1998) Cell 95, 365–377
17. Holmbeck, K., Bianco, P., Caterina, J., Yamada, S., Kromer, M., Kuznetsov, S. A., Mankani, M., Robey, P. G., Postle, A. H., Pidoux, I., Ward, J. M., and Birkedal-Hansen, H. (1999) Cell 99, 81–92
18. Zhou, Z., Apte, S. S., Soo, H., Cao, R., Kaldstrøm, G. Y., Rauzer, R. W., Wang, J., Cao, Y., and Tryggvason, K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4052–4057
19. Inoh, Y., Kaitsa, M., Kino, H., Mori, H., Okada, A., and Seiki, M. (1999) J. Biol. Chem. 274, 34260–34266
20. Kojima, S.-I., Yoshifumi, I., Matsumoto, S.-I., Mashulo, Y., and Seiki, M. (2000) FEBS Lett. 480, 142–146
21. English, W. R., Puente, X. S., Freije, J. M. P., Knauper, V., Amour, A., Merryweather, A., Lopez-Otin, C., and Murphy, G. (2000) J. Biol. Chem. 275, 14046–14055
22. English, W. R., Velasco, G., Straciek, J. O., Knauper, V., and Murphy, G. (2001) FEBS Lett. 491, 137–142
23. Velasco, G., Cal, S., Merlos-Suarez, A., Ferrando, A. A., Alvarez, S., Nakano, A., Arribas, J., and Lopez-Otin, C. (2000) Cancer Res. 60, 877–882
24. Fernandez-Catalan, C., Bode, W., Huber, R., Turk, D., Calvete, J. J., Lichte, A., Tschesse, H., and Maskos, K. (1998) EMBO J. 17, 5288–5294
25. Gill, S. C., and Von Hippy, P. H. (1989) Anal. Biochem. 182, 319–326
26. Murphy, G., and Willenbrock, F. (1996) Methods Enzymol. 246, 496–510
27. Mucka, A., Cuniasse, P., Kannan, R., Beau, F., Yiotakis, A., Basset, P., and Dive, V. (1998) J. Biol. Chem. 273, 2763–2768
28. Holtz, B., Cuniasse, P., Boulay, A., Kannan, R., Mucha, A., Beau, F., Basset, P., and Dive, V. (1999) Biochemistry 38, 12174–12179
29. Eisenhal, R., and Cornish-Bowden, A. (1994) Biochem. J. 290, 715–720
30. Cornish-Bowden, A., and Eisenhal, R. (1974) Biochem. J. 199, 248–246
31. Butler, G. S. H., Hutton, M., Wattam, B. A., Williamson, R. A., Knauper, V., Willenbrock, F., and Murphy, G. (1999) J. Biol. Chem. 274, 20391–20396
32. Morrison, J. F., and Walsh, F. S. (1998) Adv. Enzymol. Relat. Areas Mol. Biol. 61, 201–301
33. Williams, J. W., Morrison, J. F., and Duggleby, R. G. (1979) Biochemistry 18, 2567–2573
34. Ward, R. V., Atkinson, S. J., Slocombe, P. M., Docherty, A. P., Reynolds, J. J., and Murphy, G. (1991) Biochem. Biophys. Acta 1079, 242–246
35. Zhang, Y., and Gray, R. D. (1996) J. Biol. Chem. 271, 8015–8021
36. Will, H., Atkinson, S. J., Butler, G. S., Smith, B., and Murphy, G. (1996) J. Biol. Chem. 271, 17119–17123
37. Overall, C. M., Tam, E., McQuibban, A., Morrison, C., Wallon, U. M., Bigg, H. F., King, A. E., and Roberts, C. R. (2000) J. Biol. Chem. 275, 39497–39506
38. Margunova, E., Tuuttila, A., Bergmann, U., Isupov, M., Lindqvist, Y., Schneider, G., and Tryggvason, K. (1998) Science 284, 1667–1670
39. Miyamori, H., Takino, T., Seki, M., and Sato, H. (2000) Biochem. Biophys. Res. Commun. 267, 796–800
40. Tzobcheva, S. T., Tzotcheva, T. I., and Jorgensen, F. S. (2000) J. Biomol. Struct. Dyn. 17, 933–946
41. Miyamori, H., Takino, T., Kobayashi, Y., Tokai, H., Itoh, Y., Seki, M., and Sato, H. (2001) J. Biol. Chem. 276, 28204–28211
42. Lehti, K., Lehi, J., Valtanen, H., and Keski-Oja, J. (1998) Biochem. J. 334, 345–353
43. Stanton, H., Gavrilovic, J., Atkinson, S. J., d’Ortho, M. P., Yamada, K. M., Zard, L., and Murphy, G. (1998) J. Cell Sci. 111, 2789–2798
44. Imper, V., and Van Wart, H. E. (1998) in Matrix Metalloproteinases (Parks, W. C., and Meecham, R. P., eds) pp. 219–238, Academic Press, Orlando, FL
Characterization of the Role of the "MT-loop": AN EIGHT-AMINO ACID INSERTION SPECIFIC TO PROGELATINASE A (MMP2) ACTIVATING MEMBRANE-TYPE MATRIX METALLOPROTEINASES
William R. English, Béatrice Holtz, Gavin Vogt, Vera Knäuper and Gillian Murphy

J. Biol. Chem. 2001, 276:42018-42026.
doi: 10.1074/jbc.M107783200 originally published online September 12, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107783200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 22 of which can be accessed free at http://www.jbc.org/content/276/45/42018.full.html#ref-list-1