

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

A KINETIC STUDY*

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We have used C-terminal domain mutants to further define the role of interactions of progelatinase A and membrane type 1 matrix 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 MMPs¹ 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 organomercurials initiates autolytic cleavages, leading to activation, as does proteolysis by either matrilysin 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

¹ The abbreviations used are: MMP, matrix metalloproteinase; MT1 MMP, membrane type 1 matrix metalloproteinase; MT1 MMP_{cat}, catalytic domain of MT1 MMP (Δ269–559); 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-GL.C-SL, gelatinase A (residues 1–417) fused to stromelysin-1 (residues 248–460); N-G.C-SGG, gelatinase A (residues 1–417) fused to stromelysin-1 (residues 248–305) then gelatinase A (residues 475–631); N-G.C-SGS, gelatinase A (residues 1–417) fused to stromelysin-1 (residues 248–305) followed by gelatinase A (residues 475–567) and stromelysin-1 (residues 400–460); E375A-gelatinase A, inactive mutant with active site Glu mutated to Ala; (Δ128–194)TIMP2, N-terminal three loops of TIMP2; (Δ186–194)TIMP2, TIMP2 lacking the C-terminal charged tail QEFLDIEDP; APMA, 4-aminophenylmercuric acetate; PAGE, polyacrylamide gel electrophoresis; HBSS, Hanks’ balanced saline solution; BSA, bovine serum albumin; CHO, Chinese hamster ovary.

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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-Leu³⁸, 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-Tyr⁸¹. Soluble forms of MT1 MMP have been prepared, and their ability to initiate this two step activation of progelatinase A has been confirmed (20, 21). In solution, this process is apparently not dependent upon TIMP2, which merely functions as an inhibitor if it is introduced into the system, nor does it require the presence of the C-terminal domain of progelatinase A. Since most cell-based studies of activation, namely propeptide processing, have been carried out using zymography as an assay, it is not clear to what extent functionally active gelatinase A is being generated. To resolve the differences in the data derived from the study of progelatinase A activation by membrane-associated versus soluble MT1 MMP, we have addressed the questions of the precise kinetics and concentration dependence of these events. We have also assessed the role of the C-terminal domain of progelatinase A in both the activation process and TIMP2 binding to determine whether they are related.

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-(S)-carbamoyl-2,2-dimethylpropyl)-N4-hydroxy-2-(R)-[3-(4-chlorophenyl)propyl]succinamide) was kindly donated by Dr. A. Docherty (Celltech Ltd., Slough, United Kingdom (UK)). The quenched fluorescent peptide (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH₂ and standard (7-methoxycoumarin-4-yl)acetyl-NH₂ were made by Dr. G. Knight (Strangeways Research Laboratory, UK).

Production of Enzymes and Inhibitors—The catalytic domain of MT1 MMP (Δ269–559; MT1 MMP_{cat}) was prepared as described previously (20). A version of MT1 MMP with the transmembrane and cytoplasmic domains deleted (Δ501–559; ΔTM MT1 MMP) was produced.² Progelatinase A, the N-terminal domain of progelatinase A (Δ418–631; N-GLA) and N-GL-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 475–567) and stromelysin-1 (residues 400–460), termed proN-G.C-SGS was constructed using polymerase chain reaction and subcloning techniques. Initially, DNA encoding Met⁴⁷⁵ to Lys⁵⁶⁷ of gelatinase A was amplified from pSP65-gelatinase A by polymerase chain reaction using oligonucleotides AAAAGCTTATGGGGCCCCCTGCTGGTGGCC and GGGGATCCATGGACTTCTTCACCTCATTGTATC. This created a HindIII site at the 5' end and a NcoI site at the 3' end of the fragment. The 290-nucleotide

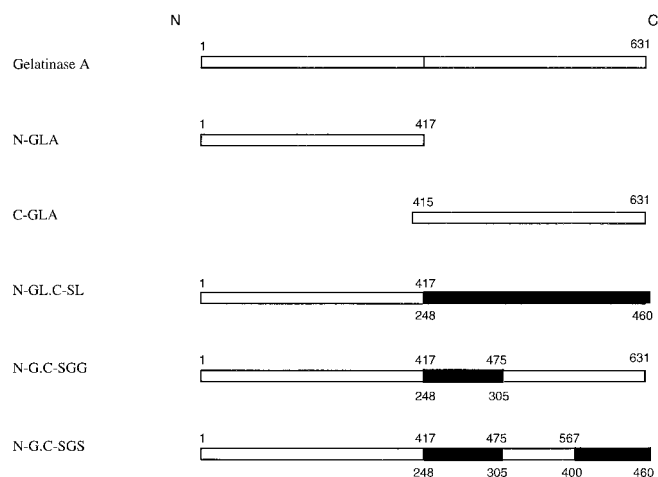


FIG. 1. Schematic diagram of gelatinase A protein constructs. Gelatinase A residues are shown above and stromelysin-1 residues are shown below each construct. Stromelysin-1 residues are shaded. Details of mutagenesis and expression are given under "Experimental Procedures."

DNA fragment was digested with HindIII and NcoI and ligated to a 515-nucleotide fragment (NcoI-BamHI) containing the 3' end of the stromelysin cDNA encoding Ser⁴⁰⁰ to Cys⁴⁶⁰ (22). The ligation mixture was digested with HindIII and BamHI, and the 805-nucleotide fragment was purified using low gelling temperature agarose. The fragment was cloned into pSP64 using the HindIII and BamHI sites and the nucleotide sequence of the amplified region was confirmed. The 805-nucleotide HindIII-BamHI fragment was subcloned into pEE12, and a HindIII fragment from the proN-GL-C-SL hybrid encoding the N-terminal portion of progelatinase A and residues Pro²⁴⁸ to Leu³⁰⁵ of stromelysin (22) was inserted at the HindIII site. Transformants containing the appropriately oriented fragment were identified by restriction mapping.

A second hybrid construction was made by replacement of the C-terminal-encoding region from the KpnI to EcoRI sites with the corresponding fragment from pEE12.GLA. This was done in the intermediate (HindIII-BamHI) subclone in pEE12 due to the presence of an additional KpnI site in the remaining stromelysin-encoding DNA. Once again, the N-terminal HindIII fragment was inserted and an appropriately oriented clone was identified. This plasmid was designated pEE12.N-G.C-SGG and encoded gelatinase A (residues 1–417), stromelysin (residues 248–305), and gelatinase A (residues 475–631).

The pEE12.N-G.C-SGS and pEE12.N-G.C-SGG were transfected into NS0 myeloma cells by electroporation (25), and colonies were screened for the production of metalloproteinase activities that degraded gelatin. The most productive cell lines were expanded and used to produce conditioned medium containing the hybrid proteins. Purification of the proteins was as described previously using gelatin-Sepharose (8, 22) and resulted in preparations of proenzyme which ran identically to progelatinase A on SDS-PAGE (M_r 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, pGWIHG (26), and overexpressing clones were selected with mycophenolic acid. The highest MT1 MMP-expressing clone was expanded and cultured in the presence of 1 μ M 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 proteinase inhibitors, phenylmethanesulfonyl fluoride (100 μ M), pepstatin (1 μ g/ml), and L-transexpoxysuccinic acid (1 μ g/ml).

NS0 mouse myeloma cells were stably co-transfected with MT1 MMP and TIMP2 using the method described previously for progelatinase A (25). Positive clones were identified by reverse gelatin zymography for the presence of TIMP2 and for their ability to activate exogenously added progelatinase A. Crude plasma membranes were prepared from the positive clones using the method described previously (10).

Binding of Proenzymes to TIMP2—Two μ g of each proenzyme was incubated for 1–2 h at room temperature with 1 μ g of TIMP2 in TCAB buffer (50 mM Tris-HCl, pH 7.5, 10 mM CaCl₂, 0.025% Brij 35, 0.02%

² d'Ortho, M.-P., Will, H., Atkinson, S., Butler, G., Messent, A., Gavrilović, J., Smith, B., Timpl, R., Zardi, L., and Murphy, G. (1997) *Eur. J. Biochem.*, in press.

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 fibril assay (27).

Activation of MT1 MMP—The catalytic domain of MT1 MMP (MT1 MMP_{cat}) was activated at 30 μg/ml with 5 μg/ml trypsin (20). ΔTM MT1 MMP was activated with 1:20 w/w active MT1 MMP_{cat} 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 spectrofluorimeter using 1 μM quenched fluorescent peptide substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH₂ (28). The machine was calibrated using a known concentration of the standard (7-methoxycoumarin-4-yl)acetyl-NH₂.

Estimation of Association Rate Constants—Association rate constants (k_{on}) 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).

Activation of Progelatinase A and C-terminal Domain Mutants in Solution—Proenzyme concentrations were estimated by activating each enzyme at 100 μg/ml for 4 h at 25 °C with 2 mM APMA, ensuring complete activation (confirmed by SDS-PAGE and silver staining). The rate of cleavage of 1 μM fluorescent peptide was measured, and the enzyme concentration was calculated using a k_{cat}/K_m value of $6.29 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (28) for each of the gelatinase constructs (since the catalytic domain is identical in each and the hemopexin domain is not involved in the hydrolysis of peptide substrates; Ref. 8). Equimolar amounts of each proenzyme were activated at 37 °C in FAB buffer, either with 2 mM APMA or with MT1 MMP at molar ratios detailed in the text. Heparin was included in some cases and is described under "Results." Aliquots of the activation mixture were removed at intervals and diluted into ice-cold FAB buffer. Activity was measured against the fluorescent peptide substrate at 37 °C, or aliquots were used for SDS-PAGE analysis.

Binding of Enzymes to Heparin-Agarose—One to 2 μg of each proenzyme was loaded onto heparin-agarose in TCAB, and columns were washed extensively using the same buffer. Bound material was eluted using a higher salt buffer and analyzed by SDS-PAGE (gelatin zymography or silver stain). Binding of both pro and active forms of ΔTM MT1 MMP or MT1 MMP_{cat} was tested in TCAB. Protein was detected by Western blotting with a sheep polyclonal antibody to MT1 MMP.³

Activation of Progelatinase A and C-terminal Domain Mutants by Membrane-bound MT1 MMP—The amount of active MT1 MMP present on the membranes was assessed using the quenched fluorescent peptide assay and the concentration was calculated using a k_{cat}/K_m value of $1.9 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ obtained for soluble ΔTM MT1 MMP (this study). The activity could be inhibited by TIMP2, but not by TIMP1, and was attributed to MT1 MMP alone. NSO cells do not produce detectable levels of endogenous TIMP2. Endogenous levels of MT1 MMP are also negligible (0.03 pmol/mg of membrane protein). Cotransfection with MT1 MMP and TIMP2 resulted in approximately 0.10 pmol of active MT1 MMP/mg of membrane protein. Membranes from HT1080 cells overexpressing MT1 MMP and depleted of TIMP2 were found to contain no bound gelatinase A by zymographic analysis, and TIMP2 was below the level of detection of Western blotting (data not shown). Membrane preparations were incubated with either progelatinase A or the various mutants at 37 °C, and aliquots of the incubation were removed at intervals for analysis by gelatin zymography or in fluorescent peptide assays.

Potential of Progelatinase A Activation by TIMP2—Various molar ratios of TIMP2 were incubated for approximately 1 h at room temperature with TIMP2-depleted HT1080 membranes, containing known amounts of MT1 MMP, in TCAB buffer. Progelatinase A was added, and the reaction was incubated at 37 °C. Aliquots of the reaction were removed at intervals and diluted into ice-cold FAB buffer. Samples were assayed at 37 °C in duplicate for activity against the fluorescent peptide substrate. For zymographic analysis, aliquots of progelatinase A were incubated with HT1080 cell membranes that had been preincubated with TIMP2 as before. After 18 h at 37 °C, the reactions were termi-

TABLE I

Binding of proenzymes to TIMP2

Two μg of each proenzyme was incubated for 1–2 h at room temperature with 1 μg of TIMP2. Complexes of TIMP2 bound to proenzyme were isolated on gelatin-Sepharose. TIMP2 activity in the bound and unbound fractions was measured in a rabbit collagenase diffuse fibril assay (27).

Incubation	Unbound	Bound
	%	%
TIMP2	99.9	0.1
Progelatinase A + TIMP2	0.1	99.9
ProN-GLA + TIMP2	99.8	0.2
C-GLA + TIMP2	99.9	0.1
ProN-GL.C-SL + TIMP2	99.8	0.2
ProN-G.C-SGS + TIMP2	99.9	0.1
ProN-G.C-SGG + TIMP2	0.0	100.0
Progelatinase A + (Δ128–194)TIMP2	99.8	0.2

TABLE II

Association constants for TIMP2 and gelatinase A or C-terminal domain mutants

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; *, data taken from 23).

	k_{on}	
	TIMP2	(Δ186–194)TIMP2
	$\text{M}^{-1} \cdot \text{s}^{-1} (\times 10^{-6})$	
Gelatinase A	38.0 ($n > 4$)*	7.18 ± 0.43 ($n = 2$)
N-GLA	0.30 ($n > 4$)*	ND
N-GL.C-SL	0.83 ($n > 4$)*	ND
N-G.C-SGS	0.17 ± 0.01 ($n = 4$)	0.18 ± 0.003 ($n = 2$)
N-G.C-SGG	33.17 ± 0.33 ($n = 4$)	4.52 ± 0.04 ($n = 2$)

nated with non-reducing sample buffer and the samples were analyzed by gelatin zymography.

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 Dulbecco's modified Eagle's medium and then incubated on ice for 30 min in Hanks' balanced salt solution (HBSS) containing 0.5% BSA. After three washes with HBSS/0.5% BSA, the cells were incubated with 5 μg/ml 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 previously been 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, QEFLDIEDP (kindly donated by Dr. Kazushi 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 preincubated 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 ($k_{on} 38.0 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$; Table II). The mutant N-G.C-SGG was the only

³ M.-P. d'Ortho and R. M. Hembray, manuscript in preparation.

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 Graftit (Erithacus Software) (ND, not determined; n , number of assays).

	k_{on}		
	TIMP2	($\Delta 128-194$)/TIMP2	($\Delta 186-194$)/TIMP2
		$M^{-1} \cdot s^{-1} (\times 10^{-6})$	
MT1 MMP _{cat}	4.18 ± 0.20 ($n = 6$)	2.80 ± 0.45 ($n = 6$)	ND
Δ TM 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 ($33.2 \times 10^6 M^{-1} \cdot s^{-1}$). Mutants N-G.C-SGS, N-GLA, or N-GL.C-SL bound to TIMP2 between 45 and 200 times more slowly than wild-type gelatinase A. ($\Delta 186-194$)/TIMP2, 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 Δ TM MT1 MMP cleavage of the fluorescent peptide at 37 °C and pH 7.5 was $1.92 \times 10^5 M^{-1} \cdot s^{-1}$, which is comparable with the k_{cat}/K_m for MT1 MMP_{cat} (20). Estimates of the association constant for wild type TIMP2, or mutant forms lacking the charged tail ($\Delta 186-194$) or the C-terminal domain three loops ($\Delta 128-194$) with soluble MT1 MMP (Δ TM or catalytic domain) were similar, ranging from 2.8 to $4.2 \times 10^6 M^{-1} \cdot s^{-1}$ (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-GL.C-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 progelatinase A or the domain mutants were activated under the same conditions with a 10:1 molar ratio of proenzyme: Δ TM 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 MMP_{cat} (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 MT1 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: Δ TM MT1 MMP, a characteristic sigmoidal activation curve resulted, where a 30–60-min lag was followed by a phase of rapid activation and then a plateau. As the molar ratio of progelatinase A: Δ TM 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%, 24% of that cleaved by gelatinase A incubated in a 6:1 molar ratio with Δ TM MT1 MMP. The rate of activation of progelatinase A was similarly dependent upon the relative concentration of MT1 MMP_{cat} (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

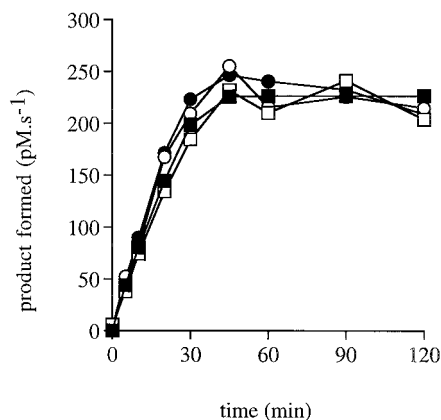


FIG. 2. Wild-type progelatinase A and C-terminal domain mutants are activated with comparable kinetics in the presence of APMA. A total of 320 nM progelatinase A (●), proN-GLA (○), proN-G.C-SGG (□), or proN-G.C-SGS (■) were activated with 2 mM APMA at 37 °C in FAB buffer. At intervals, activity was measured against the fluorescent peptide substrate.

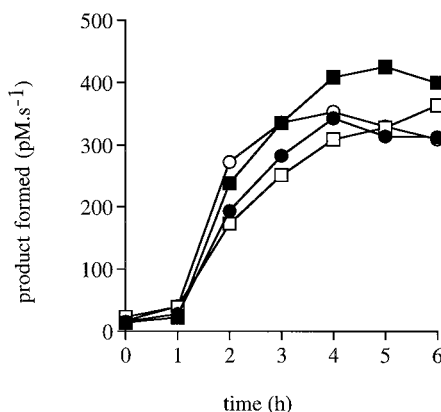


FIG. 3. Wild-type progelatinase A and C-terminal domain mutants are activated with comparable kinetics by soluble MT1 MMP. A total of 320 nM progelatinase A (●), proN-GLA (○), proN-G.C-SGG (□), or proN-G.C-SGS (■) were activated with 10:1 molar ratio Δ TM MT1 MMP at 37 °C in FAB buffer. Aliquots were removed at intervals, and activity was measured using the fluorescent peptide substrate.

reactants at the cell surface. The binding of pro and active forms of MT1 MMP_{cat} and Δ TM MT1 MMP to heparin-agarose in TCAB was tested (Fig. 5). The recombinant Δ TM 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 Δ TM MT1 MMP still bound to heparin, as did the lower molecular weight doublet. A smaller doublet (M_r 28,000–30,000) did not bind. The proform of MT1 MMP_{cat}, 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.

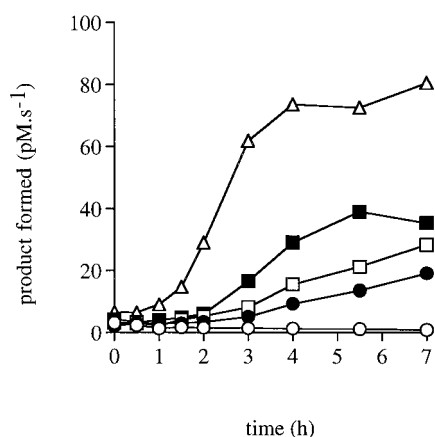


FIG. 4. The rate of activation of progelatinase A by soluble MT1 MMP is dependent upon the concentration of MT1 MMP. A total of 123 nM progelatinase A was activated with various ratios of Δ TM MT1 MMP at 37 °C in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM CaCl_2 , 0.025% Brij 35. Aliquots were removed at intervals and diluted into ice-cold FAB buffer. Activity against the fluorescent peptide substrate was measured. Ratios of Δ TM MT1 MMP:progelatinase A were as follows: \circ , 0:1; \bullet , 1:125; \square , 1:60; \blacksquare , 1:30; \triangle , 1:6.

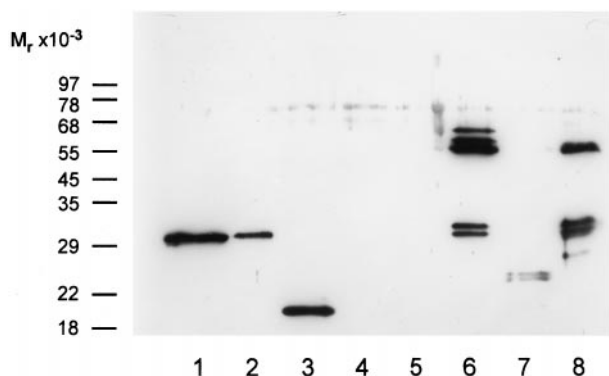


FIG. 5. Δ TM MT1 MMP binds to heparin-agarose. A total of 1–2 μg of pro or active Δ TM MT1 MMP or MT1 MMP_{cat} were applied to heparin-agarose in TCAB buffer. Columns were washed extensively, and bound material was eluted in a high salt buffer. Fractions were run on SDS-PAGE and Western-blotted. Protein was detected using a sheep polyclonal antibody raised against MT1 MMP. Lanes contained (unbound and bound fractions, respectively) the following: lanes 1 and 2, proMT1 MMP_{cat}; lanes 3 and 4, active MT1 MMP_{cat}; lanes 5 and 6, pro Δ TM MT1 MMP; lanes 7 and 8, active Δ TM MT1 MMP.

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-GL.C-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-GL.C-SL or proN-G.C-SGS was not affected by the presence of heparin.

Since both progelatinase A and Δ TM 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: Δ TM MT1 MMP reduced the length of the lag phase (Fig. 6A). The rate of gelatinase A activation was increased by the addition of 10 $\mu\text{g}/\text{ml}$ heparin and was maximal at 50 $\mu\text{g}/\text{ml}$. Addition of

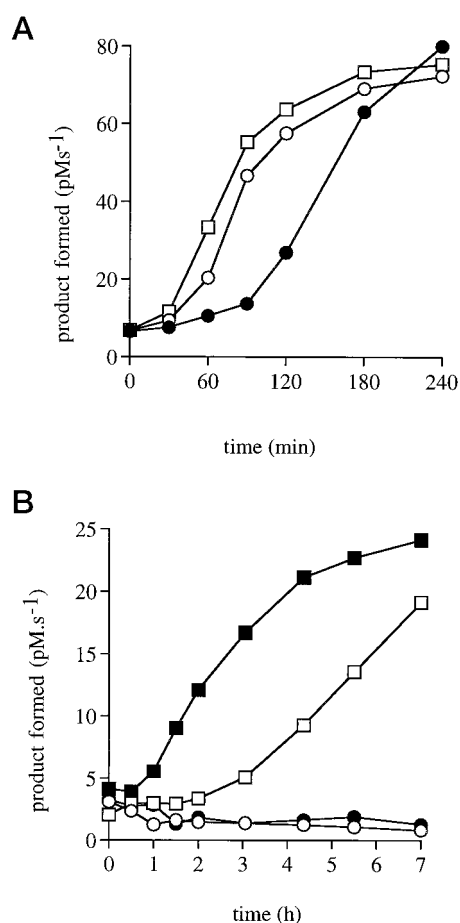


FIG. 6. Heparin potentiates the rate of progelatinase A activation by soluble MT1 MMP. A, 123 nM progelatinase A was activated with Δ TM MT1 MMP (6:1 molar ratio) at 37 °C in 50 mM Tris-HCl, 50 mM NaCl, 5 mM CaCl_2 , 0.025% Brij 35. Various concentrations of heparin were included in the activation: no heparin, \bullet ; 10 $\mu\text{g}/\text{ml}$, \circ ; 50 $\mu\text{g}/\text{ml}$, \square . One hundred, 200, and 500 $\mu\text{g}/\text{ml}$ heparin gave the same curve as 50 $\mu\text{g}/\text{ml}$ and are not represented here. B, 123 nM progelatinase A was incubated alone (\circ) or with 125:1 molar ratio Δ TM MT1 MMP (\square) at 37 °C in 50 mM Tris-HCl, 50 mM NaCl, 5 mM CaCl_2 , 0.025% Brij 35 with (shaded) or without (unshaded) 50 $\mu\text{g}/\text{ml}$ heparin. Aliquots were removed at intervals and diluted into ice-cold FAB buffer. Activity against the fluorescent peptide substrate was measured.

higher concentrations of heparin (up to 500 $\mu\text{g}/\text{ml}$, 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) was increased by heparin (Fig. 6B). An enhanced rate in the presence of heparin was also observed when MT1 MMP_{cat} was used (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 Δ TM 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 Δ TM MT1 MMP was not affected by heparin (data not shown). We conclude that heparin accelerates the second, autolytic, cleavage step in the activation of progelatinase A by concentration of the proenzyme, even when the relative concentration of MT1 MMP is low.

Activation of Progelatinase A and C-terminal Domain Mu-

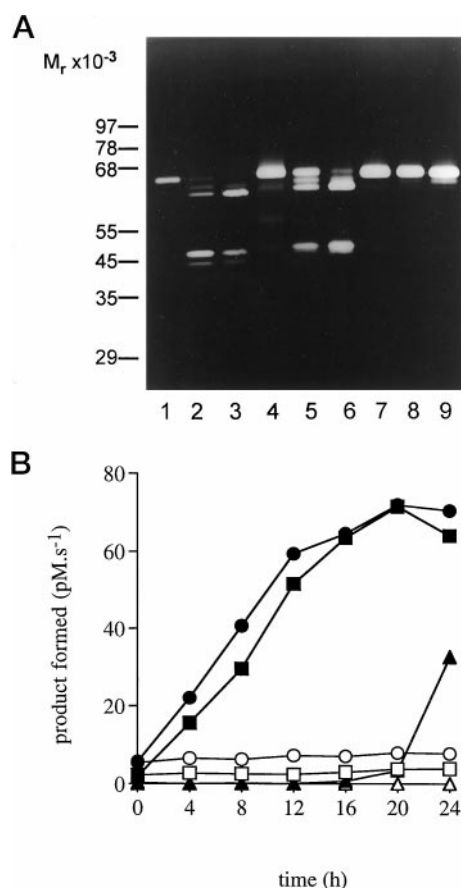


FIG. 7. Progelatinase A and C-terminal domain mutants are differentially activated by cell membrane-associated MT1 MMP. A, 7 nM proenzyme was incubated with 0, 0.5 mg/ml, or 4 mg/ml MT1 MMP-expressing NS0 membrane preparations for 18 h at 37 °C (shown in the figure from left to right). Products of the 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-SGS. 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-SGS (△) 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.

tants by Cell Membranes—Gelatin zymography of the reaction products from incubations of progelatinase A or the C-terminal domain mutants with isolated NS0 cell membranes containing known amounts of active MT1 MMP (see “Experimental Procedures”) revealed that only wild-type progelatinase A and mutant proN-G.C-SGG were activated in a dose-dependent manner by the membrane preparations. Mutant proN-G.C-SGS was hardly processed at all under these conditions (Fig. 7A). In fluorimetric assays, conducted using higher concentrations of progelatinase A, both progelatinase A and proN-G.C-SGG were activated rapidly by membranes derived from NS0 cells cotransfected with MT1 MMP and TIMP2, with maximal activity after 20 h (Fig. 7B). In contrast, after 20 h, proN-G.C-SGS was barely activated. A similar result was obtained using membranes from HT1080 cells transfected with MT1 MMP (data not shown). NS0 cell membranes from vector control transfections did not activate any of the proenzymes in the 24-h period studied.

Potential of Activation by TIMP2—Membrane preparations from HT1080 cells were prepared essentially free from endogenous TIMP2 as described under “Experimental Procedures.” Membranes from HT1080 cells transfected with vector

alone contained 0.13 pmol of active MT1 MMP/mg of membrane protein, (estimated as described under “Experimental Procedures”), whereas those transfected with MT1 MMP cDNA contained approximately 1.52 pmol/mg of membrane protein. Equal amounts of each membrane preparation were preincubated with 0, 1 nM, or 4 nM titrated TIMP2 (giving a molar ratio of 1:0, 2:1, and 1:2 for MT1 MMP:TIMP2 for the membranes derived from cells overexpressing MT1 MMP). Progelatinase A was added, and activation was monitored using the fluorescent peptide assay. After 6.25 h, in the absence of added TIMP2, the membranes derived from HT1080 cells transfected with MT1 MMP processed the progelatinase A to give an activity of 12.1 pM product·s⁻¹ (Fig. 8A). When TIMP2 was added in a 2:1 MT1 MMP:TIMP2 molar ratio, potentiation of gelatinase A activation was observed, resulting in an activity of 18.3 pM product·s⁻¹. Progelatinase A activation was inhibited when TIMP2 was added in a ratio of 1:2 MT1 MMP:TIMP2. Results from a similar experiment are also shown using zymography (Fig. 8B). The control membranes from HT1080 cells transfected with vector DNA activated the progelatinase A to give 5.2 pM product·s⁻¹, probably reflecting the endogenous MT1 MMP activity. However, when 1 nM or 4 nM TIMP2 was added, inhibition of activation was observed in both cases. Membrane preparations (data not shown) or progelatinase A incubated alone had no detectable activity against the fluorescent substrate. When (Δ128–194)TIMP2 was added to the membranes at the same molar ratios, progelatinase A activation was inhibited (data not shown); no potentiation of activation was seen at any concentration.

A further study was carried out in which the concentration of the putative TIMP2-MT1 MMP receptor was increased but the ratio of progelatinase A to active MT1 MMP was kept constant. Increasing amounts of TIMP2-depleted membranes from HT1080 cells transfected with MT1 MMP were preincubated with increasing amounts of TIMP2 such that the molar ratio of MT1 MMP:TIMP2 ranged from 1:0 to 1.5:0.5, 2:1, 3:2, and 7:6, but the concentration of free active MT1 MMP remained constant at 3.8 nM. The complexed membranes were incubated with progelatinase A at a ratio of progelatinase A:active MT1 MMP of approximately 4:1. Progelatinase A activation was monitored using the fluorescent substrate (Fig. 8C). After 20 h, the HT1080 membranes alone activated progelatinase A to give 32 pM product·s⁻¹. The addition of 0.5, 1, or 2 molar equivalents of MT1 MMP:TIMP2 resulted in an increase in gelatinase A activity to approximately 50 pM product·s⁻¹. There was no increase in gelatinase A activity when a 7:6 molar ratio of MT1 MMP:TIMP2 was used.

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-SGG and proN-G.C-SGS 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-

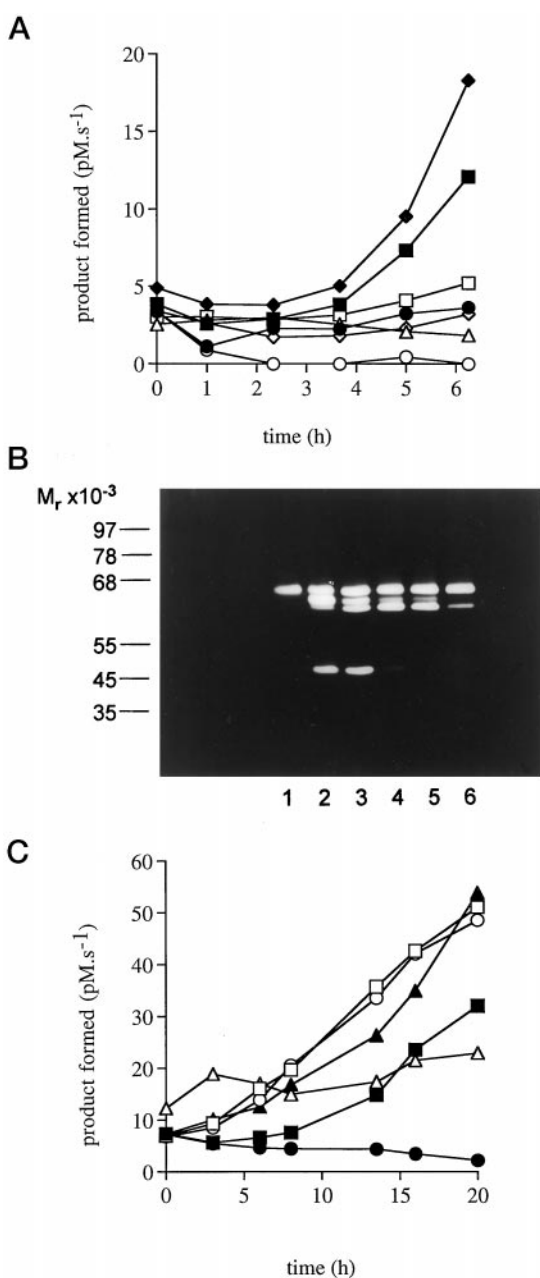


FIG. 8. TIMP2 potentiates the activation of progelatinase A by MT1 MMP on cell membranes. A, 1.25 mg/ml plasma membrane preparation derived from HT1080 cells transfected with MT1 MMP cDNA, depleted of TIMP2 as described under "Experimental Procedures" (closed shapes) or vector DNA (open shapes) were incubated for 1 h with titrated TIMP2: 0 nM (□), 1 nM (◇), or 4 nM (○) (equivalent to a molar ratio of 1:0, 2:1, and 1:2 MT1 MMP:TIMP2 for the membranes derived from cells overexpressing MT1 MMP). Then 200 nM progelatinase A was added or incubated alone (Δ), and the reaction was incubated at 37 °C. Aliquots were removed at intervals, and activity against the fluorescent substrate was assessed. B, 300 μg/ml TIMP2-depleted HT1080 membrane preparations (9.7 nM MT1 MMP) were incubated for 1 h at 25 °C with varying molar ratios of exogenous TIMP2. Proenzyme activation was assessed by addition of wild-type progelatinase A or the C-terminal domain mutants (7 nM) and incubation at 37 °C for 18 h. Products of the reaction were analyzed on 8% polyacrylamide/gelatin zymograms (0.5 mg/ml gelatin). The figure shows progelatinase A incubated alone (lane 1) or with membrane-bound MT1 MMP:TIMP2 in the following molar ratios: 1:0, lane 2; 4:1, lane 3; 2:1, lane 4; 1:1, lane 5; 1:2, lane 6. C, 2.5 mg/ml HT1080 membranes (3.8 nM MT1 MMP) derived from cells transfected with MT1 MMP cDNA and grown in the presence of the inhibitor CT1746 were washed thoroughly and incubated with 15 nM progelatinase A in TCAB at 37 °C with various amounts of 1:1 molar ratio TIMP2:MT1 MMP (membrane-bound). Aliquots were removed at intervals, and activity was tested in duplicate

G.C-SGS (or proN-GLA and proN-GL.C-SL; data not shown) did not bind to MT1 MMP-transfected cells (panel C). The binding of progelatinase A or proN-G.C-SGG to the cells abolished the signal from the antibody directed against the C-terminal epitope of TIMP2 (panels G and H), but the binding of the polyclonal antibody to the whole TIMP2 molecule was unaffected (panels D, E, and F).

DISCUSSION

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.

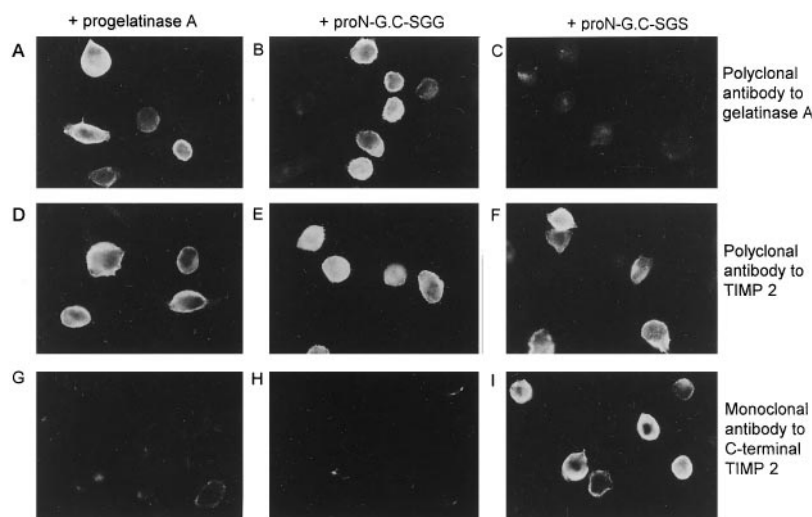
The binding of TIMP2 to progelatinase A required the C-terminal region of both the inhibitor and proenzyme as demonstrated previously (8) and substitution of the C-terminal domain of gelatinase A with that of stromelysin-1 abolished binding of proN-GL.C-SL to TIMP2. TIMP2 was able to bind to proN-G.C-SGG, 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.

against the quenched fluorescent substrate. Progelatinase A (●); MT1 MMP:TIMP2 molar ratio: 1:0 (■), 1.5:0.5 (□), 2:1 (○), 3:2 (▲), 7:6 (△).

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 progelatinase 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 Δ TM MT1 MMP and MT1 MMP_{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 progelatinase 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.² This domain appears to be responsible for the binding of active Δ TM 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 progelatinase 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 progelatinase 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_{cat} in a fluorimetric assay,⁵ 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 Δ TM MT1 MMP or MT1 MMP_{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. Δ TM MT1 MMP was previously purified on heparin-Sepharose (21); in this study, pro and active Δ TM MT1 MMP bound to heparin-agarose, whereas active MT1 MMP_{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 Δ TM MT1 MMP and progelatinase A bind to heparin, experiments with MT1 MMP_{cat} and proE375A-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-

⁵ G. Butler and G. Murphy, unpublished results.

count 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)⁶ or heparan sulfate proteoglycans which might localize progelatinase A at the cell surface. The integrin, $\alpha_v\beta_3$, 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 MT1 MMP concentration. ($\Delta 128-194$)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 MT1 MMP:TIMP2 to free 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 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

the C-terminal domain interaction with progelatinase A acts to colocalize and concentrate the progelatinase A in the vicinity of active MT1 MMP molecules. Until now, the role of TIMP2 in the activation of progelatinase A at the cell surface has been unclear, and the possibility that a multicomponent system was required in which MT1 MMP, TIMP2 and progelatinase A interact with other unknown membrane components had not been ruled out (42). We found no evidence for the involvement of membrane proteins other than MT1 MMP. Our studies suggest that the amount of TIMP2 present determines the balance between the level of activator (free MT1 MMP) and of receptor (MT1 MMP·TIMP2 complex), which regulates the degree of activation of progelatinase A. Hence, TIMP2 is novel in its dichotomous mode of action. On the one hand, TIMP2 acts as a specific inhibitor of metalloproteinases, while on the other, it is intricately involved in the mechanism of progelatinase A activation. It is anticipated that other members of the TIMP family such as TIMP3 and TIMP4 may also have as yet undiscovered functions.

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