Interaction between tissue inhibitor of metalloproteinases-2 and progelatinase A: immunoreactivity analyses

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By immunoreactivity analysis using monoclonal antibodies, we showed that the C-terminal domain [R_{415–631}; R is residue] of progelatinase A [pro-matrix metalloproteinase-2 (proMMP-2); EC 3.4.24.24] affected the immunoreactivity of a one-step sandwich enzyme immunoassay (sandwich EIA) for tissue inhibitor of metalloproteinases-2 (TIMP-2) in exactly the same way as does proMMP-2 [Fujimoto, Zhang, Iwata, Shinya, Okada and Hayakawa (1993) Clin. Chim. Acta **220**, 31–45], confirming that the C-terminal domain ('tail' portion) of TIMP-2 participates in the binding with the C-terminal domain of proMMP-2. We also demonstrated that not only the C-terminal domain but also the N-terminal domain (R_{1–417}) of proMMP-2 bound to TIMP-2 in a 1:1 molar ratio. The binding of each individual domain to TIMP-2, however, was weak enough that either domain could be fully replaced by proMMP-2 itself, suggesting that either terminal domain binds to TIMP-2 through the same binding sites as does proMMP-2, and also that the high-order structure of proMMP-2 allows a more stable binding to TIMP-2. We further confirmed

that TIMP-2 complexed with the N-terminal domain of proMMP-2 had fully inhibitory activity against the collagenolytic activity of MMP-1. We also demonstrated that either the interstitial collagenase–TIMP-2 complex or the gelatinase B (MMP-9)–TIMP-2 complex was able to form a ternary complex with proMMP-2 in a 1:1 molar ratio, clearly indicating that there are two distinct binding sites, one specific for proMMP-2 and the other for active MMPs, on the TIMP-2 molecule. The C-terminal domain was able to bind to the MMP-9–TIMP-2 complex, but the binding seemed to be less stable than the binding with TIMP-2 alone. Even in the presence of a 10-fold molar excess of the N-terminal domain, ternary complex formation was not observed between the N-terminal domain and the MMP-9–TIMP-2 complex. These clear differences might be ascribed to some significant conformational change(s) evoked in the TIMP-2 molecule, or hindrance of a part of the N-terminal domain binding site of TIMP-2 by complex formation with MMP-9.

INTRODUCTION

Members of the matrix metalloproteinase (MMP) family, which consists of several zinc-dependent proteinases, have been recognized to participate in various important biological phenomena, such as ovulation, morphogenesis, angiogenesis, tissue remodelling, involution and wound healing as physiological events, and inflammation, tumour invasion and metastasis as pathological events [1].

This MMP activity is controlled *in vivo* by a family of inhibitors, tissue inhibitors of metalloproteinases, or TIMPs. So far, three distinct tissue inhibitors of metalloproteinases, TIMP-1, TIMP-2 and TIMP-3, have been isolated and well characterized [2–10]. These TIMPs interact with active forms of MMPs, such as interstitial collagenase (MMP-1; EC 3.4.24.7), gelatinase A (MMP-2; EC 3.4.24.24) and gelatinase B (MMP-9; EC 3.4.24.35) and stromelysin 1 (EC 3.4.24.17), with a 1:1 stoichiometry. K_d values of around 10^{-10} M [11] and their ability to inhibit MMPs suggest that they may play an important role in the regulation of MMP activity, effectively controlling the amount of connective-tissue breakdown. TIMP-1 and TIMP-2 specifically form complexes with proMMP-9 and proMMP-2 respectively, with a 1:1 stoichiometry and in a non-covalent fashion. It has been reported that the proMMP-2–TIMP-2 complex can autoactivate in the presence of organomercurials in a fashion similar to the free enzyme, although the specific activity is only 5–10% of that of uninhibited MMP-2, and that this activated enzyme can be

inhibited by the further addition of free TIMP-2, indicating the presence of two distinct TIMP-2 binding sites on MMP-2. The proMMP-2–TIMP-2 complex has also been shown to retain inhibitory activity against active MMP-1, neutrophil collagenase (MMP-8; EC 3.4.24.34) and MMP-9, which has led to the conclusion that there are two distinct MMP binding sites on TIMP-2 [12,13].

Recently we prepared monoclonal antibodies that react with TIMP-2 and both precursor and active forms of MMP-1, MMP-2 and MMP-9, as well as ones that discriminate between the N- and C-terminal domains of proMMP-2. Selecting appropriate combinations of these antibodies, we could set up one-step sandwich enzyme immunoassays (sandwich EIAs) for each of them, and also for TIMP-2 complexed with these MMPs [14–17] and the N- or C-terminal domain of proMMP-2. Taking advantage of these mild immunoreactions, we investigated the interaction between TIMP-2 and proMMP-2, looking especially for a possible weak one that might not be detected by other methods.

MATERIALS AND METHODS

Materials

BSA (fraction V), *o*-phenylenediamine and gelatin–agarose were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Abbreviations used: TIMP, tissue inhibitor of metalloproteinases; MMP, matrix metalloproteinase; HRP, horseradish peroxidase; R, residue; EIA, enzyme immunoassay; APMA, *p*-aminophenylmercuric acetate.

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Horseradish peroxidase (HRP; grade I) and pepsin from porcine gastric mucosa were from Boehringer Mannheim GmbH (Mannheim, Germany) and recombinant human interleukin-1 α and tumour necrosis factor α from Genzyme (Cambridge, MA, U.S.A.). Other materials and their sources were as follows: anti-human fibronectin antibody and HRP-labelled goat anti-(mouse immunoglobulin) antibody from Cappel Organon Teknika Co. (West Chester, PA, U.S.A.); 1 $\times$ 8-wells-Micro Well Module in frame from Nunc (Rockilde, Denmark and Naperville, IL, U.S.A.); *p*-aminophenylmercuric acetate (APMA) from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); Affigel Protein A-agarose from Bio-Rad (Richmond, CA, U.S.A.); Ultrogel AcA 54 and 44 from LKB (Villeneuve-la-Garenne, France); Sephadex G-25 and gelatin-Sepharose from Pharmacia Fine Chemicals AB (Uppsala, Sweden); *N*-(ϵ -maleimidocaproyloxy)succinimide, *S*-acetylmercaptosuccinic anhydride and other chemicals from Wako Pure Chemicals (Osaka, Japan).

Normal human skin fibroblasts CCD-41SK (ATCC No. 1505), human gingival fibroblasts Gin-1 (ATCC No. CRL-1292) and human fibrosarcoma cells HT1080 (ATCC No. CCL 121) were purchased from the American Type Culture Collection, Rockville, MD, U.S.A.

Purification of proMMPs and TIMPs

ProMMP-1 and proMMP-2 were purified from the conditioned medium of CCD-41SK cells treated with interleukin-1 α [14,17], and TIMP-1 and TIMP-2 were purified from human placenta as previously described [15,18]. ProMMP-9 was purified from the conditioned medium of HT1080 cells stimulated with tumour necrosis factor α [19]. Calculated M_r values of 70982 and 21600 were used for proMMP-2 and TIMP-2 respectively. Active MMP-1 was prepared by treating the purified proMMP-1 with 1 mM APMA for 3 h at 37 °C [17], and active MMP-9 was likewise prepared by incubation of the purified proMMP-9 with 1 mM APMA for 24 h at 37 °C [19]. M_r s of 46000 [17] and 67000 [19] were used for active forms of MMP-1 and MMP-9 respectively.

To examine the inhibitory activity of TIMP-2 and TIMP-2 complexed with the N-terminal domain of proMMP-2, a fixed amount (26 nmol) of TIMP-2 was mixed with the N-terminal domain at different molar ratios (0.5, 1.0 and 2.0) and pre-incubated for 3 h at room temperature. The mixture was added to the solution containing active MMP-1 (26 nmol) and then collagenolytic activity was determined by the method of Terato et al. [20] using [14 C]glycine-labelled collagen.

Purification of recombinant N-terminal domain of proMMP-2

Human recombinant N-terminal domain (R_{1-417} ; R is residue) of proMMP-2 was expressed in NSO mouse myeloma cells transfected with the vector PEE12 delta 418-631 proMMP-2, and was purified as reported previously [21]. The calculated M_r of 46809 was used for the N-terminal domain.

Purification of natural C-terminal domain of proMMP-2

The C-terminal domain ($R_{415-631}$) of proMMP-2 purified from medium conditioned by human gingival fibroblasts was prepared by the activation of proMMP-2 with 2 mM APMA for 16 h at 35 °C and was purified by gelatin-Sepharose and Ultrogel AcA 54 gel-filtration column chromatography as described by Ward et al. [22]. The calculated M_r of 24507 was used for the C-terminal domain.

Table 1 Monoclonal antibodies against TIMP-2 and MMPs used

	Clone no.	Antibody-recognizing domains
TIMP-2	68-6H4	N-Terminal domain
	67-4H11	C-Terminal domain ('tail' portion)
MMP-2	43-3F9	Propeptide
	75-7F7	C-Terminal domain
MMP-1	78-12G8	Domain except N-terminus
MMP-9	73-18B3	Central domain

Preparation of mouse monoclonal antibodies against TIMP-2 and MMPs

Monoclonal antibodies specific for TIMP-2, clones 68-6H4 and 67-4H11, were prepared against oligopeptides R_{30-44} and $R_{178-193}$ respectively [15], and those for proMMP-2, clones 75-7F7 and 43-3F9, were prepared against the purified proMMP-2 and a synthetic oligopeptide consisting of 19 amino acids (R_{17-35}), representing a part of the propeptide sequence of MMP-2, respectively [14]. Monoclonal antibodies for MMP-1 (clone 78-12G8) [17] and MMP-9 (clone 73-18B3) [16], prepared with purified proMMP-1 and proMMP-9 respectively, were used as immunogens and recognized precursor and active forms of MMP-1 and a central domain of MMP-9 respectively. Each monoclonal antibody was further purified by ammonium sulphate fractionation, followed by Affigel Protein A-agarose column chromatography. The monoclonal antibodies used in this study are summarized in Table 1.

Preparation of Fab' from IgG

F(ab') $_2$ was prepared from purified monoclonal antibody (clone 67-4H11) by digestion with pepsin. The obtained F(ab') $_2$ was then reduced with 2-aminoethanethiol and applied to a Sephadex G-25 column [23]. The fractions containing Fab' were collected.

Preparation of HRP-labelled Fab' or IgG

Purified Fab' or IgG was conjugated with HRP by use of *N*-(ϵ -maleimidocaproyloxy)succinimide or *S*-acetylmercaptosuccinic anhydride by the method of Ishikawa et al. [23]. After the conjugation, the reaction mixture was applied to an Ultrogel AcA 44 column (1.5 $\times$ 90 cm) and fractions containing HRP-labelled Fab' or IgG were collected.

Sandwich EIAs for TIMP-2 and for MMP-TIMP-2 and MMP-TIMP-2-proMMP-2 complexes

Sandwich EIAs were carried out by previously established methods [14,15]. The combinations of monoclonal antibodies used were as follows: clones 68-6H4 and 67-4H11 for TIMP-2, clones 68-6H4 and 43-3F9 for proMMP-2-TIMP-2 and N-terminal domain-TIMP-2 complexes, clones 68-6H4 and 75-7F7 for proMMP-2-TIMP-2 and C-terminal domain-TIMP-2 complexes, clones 78-12G8 and 75-7F7 for MMP-1-TIMP-2-proMMP-2 complexes, clones 73-18B3 and 43-3F9 for MMP-9-TIMP-2-N-terminal domain complexes and clones 73-18B3 and 75-7F7 for MMP-9-TIMP-2-proMMP-2 and MMP-9-TIMP-2-C-terminal domain complexes.

Interaction between TIMP-2 and the N- or C-terminal domain of proMMP-2

The N- or C-terminal domain of proMMP-2 at different concentrations was incubated for 2 h at room temperature with 100 ng of TIMP-2, which had previously been coated to each well in 100 μ l of 50 mM Tris/HCl buffer (pH 7.5) containing 0.1 M NaCl and 10 mM CaCl_2 (buffer A), for 16 h at 4 °C; residual unoccupied well surface was blocked with buffer A containing 1 % BSA (buffer B). After the wells had been washed with saline, 1 μ g of HRP-labelled anti-MMP-2 IgG of clone 75-7F7 or Fab' of clone 43-3F9 in buffer B was reacted for 1 h at room temperature and the wells were again washed with saline. Colour development was carried out with 100 μ l of 2 mg/ml *o*-phenylenediamine and 0.02 % (v/v) hydrogen peroxide in 0.1 M sodium citrate buffer (pH 4.9) for 15 min at room temperature; 100 μ l of 2 M sulphuric acid was subsequently added to stop the reaction. The A_{492} was measured by a Micro Plate Reader (Tosoh, Japan).

Competition between the N- and C-terminal domains of proMMP-2 for binding to TIMP-2

First, the N-terminal domain (1 μ g) of proMMP-2 was transferred to each well previously coated with 50 ng of TIMP-2, and the plate was incubated for 2 h at room temperature as described above. After the wells had been washed with saline, different amounts of the C-terminal domain were reacted with the N-terminal domain-TIMP-2 complex in the wells for 2 h at room temperature. The N- or C-terminal domain bound to TIMP-2 was then detected by use of a monoclonal antibody, clone 43-3F9 or 75-7F7 respectively, as described earlier.

Comparison of the immunoreactivity between the proMMP-2-TIMP-2 complex and the N- or C-terminal domain-TIMP-2 complex

A fixed amount (500 ng) of TIMP-2 was mixed with either proMMP-2 or the N-terminal domain in buffer B to give the indicated molar ratio and incubated for 2 h at room temperature. The reactant was measured by a sandwich EIA using anti-TIMP-2 antibody (clone 68-6H4) as the solid phase and anti-proMMP-2 antibody (clone 43-3F9) as the HRP-labelled phase.

In a similar type of experiment, 250 ng of TIMP-2 was mixed with either proMMP-2 or the C-terminal domain in buffer B to give the indicated molar ratio and incubated for 2 h at room temperature. The reactant was measured by a sandwich EIA using anti-TIMP-2 antibody (clone 68-6H4) as the solid phase and anti-proMMP-2 antibody (clone 75-7F7) as the HRP-labelled phase.

Competition between proMMP-2 and its N- or C-terminal domain for binding to TIMP-2

First, 1 μ g of the N-terminal domain or 2 μ g of proMMP-2 in buffer B was transferred to individual wells previously coated with 50 ng of TIMP-2, and then the plate was incubated for 2 h at room temperature as described previously. After the wells had been washed with saline, the N-terminal domain or proMMP-2 bound to TIMP-2 in each individual well was reacted with various amounts of proMMP-2 or the N-terminal domain respectively, for 2 h at room temperature. After the wells had been washed again with saline, both proMMP-2 and the N-terminal domain bound to TIMP-2 were measured by use of HRP-labelled anti-MMP-2 antibody (clone 43-3 F9 or 75-7F7) and the colour was developed as described earlier. In a similar

type of experiment, 500 ng of the C-terminal domain was used instead of the N-terminal domain. In this case, both proMMP-2 and the C-terminal domain bound to TIMP-2 were measured by use of HRP-labelled anti-MMP-2 antibody (clone 75-7F7 or 43-3F9) followed by colour development.

Binding of proMMP-2 to the MMP-1-TIMP-2 or MMP-9-TIMP-2 complex

Active MMP-1 (8.3 pmol) or active MMP-9 (4 pmol) was incubated with equimolar TIMP-2 in buffer B for 3 h at room temperature. ProMMP-2 at different molar ratios was then added to the reaction mixture and the plate was incubated for 2 h at room temperature. A ternary complex, either MMP-1-TIMP-2-proMMP-2 or MMP-9-TIMP-2-proMMP-2, formed in the reaction mixture was measured by the respective sandwich EIA.

Binding of the N- or C-terminal domain of proMMP-2 to the MMP-9-TIMP-2 complex

MMP-9-TIMP-2 complex (24 pmol) was incubated with either the N- or C-terminal domain of proMMP-2 at different molar ratios in buffer B overnight at 4 °C. Then the ternary complex, either MMP-9-TIMP-2-N-terminal domain or MMP-9-TIMP-2-C-terminal domain, formed in the reaction mixture was measured by a combination of HRP-labelled anti-MMP-2 antibody (clone 43-3F9) or HRP-labelled anti-MMP-2 antibody (clone 75-7F7) respectively, and anti-MMP-9 antibody (clone 73-18B3).

Determination of protein concentrations

The protein concentrations of the purified monoclonal antibodies, MMPs, TIMPs and the N- and C-terminal domains of proMMP-2 were determined by a Micro BCA Protein Assay Reagent (BCA is bicinchoninic acid) with BSA used as the standard protein, according to the instructions of Pierce (Rockford, IL, U.S.A.).

RESULTS AND DISCUSSION

Effect of proMMP-2 and its C-terminal domain on the one-step sandwich EIA for TIMP-2

It has been well recognized that TIMP-2 binds to the C-terminal domain of MMP-2 [24,25]. Recently, Willenbrock et al. [26] suggested that there are ionic interactions between the C-terminal peptide of TIMP-2, but not of TIMP-1, and proMMP-2.

Earlier, we obtained two monoclonal antibodies, clone 68-6H4, which recognizes a part (R_{30-44}) of the N-terminal domain of TIMP-2, and clone 67-4H11, which recognizes the C-terminal domain ($R_{178-193}$). Using these two antibodies, we set up a sandwich EIA for TIMP-2 and demonstrated that the immunoreactivity of TIMP-2 with clone 67-4H11 is abolished in the presence of proMMP-2, suggesting that the C-terminal domain of TIMP-2 interacts with the proMMP-2 molecule [15].

In the present study, we examined whether the C-terminal domain of proMMP-2 affects the immunoreactivity of the sandwich EIA for TIMP-2 and compared the results with the effect of proMMP-2 itself. As shown in Figure 1, the C-terminal domain affected the immunoreactivity in exactly the same fashion as proMMP-2 did, suggesting that the C-terminal domain ('tail' portion) of TIMP-2 participates in the interaction with the C-terminal domain of proMMP-2 [24-26].

Figure 1 Effect of proMMP-2 and its C-terminal domain on the sandwich EIA for TIMP-2

A fixed amount (30 ng) of TIMP-2 was mixed with either proMMP-2 (○) or its C-terminal domain (●) to give the indicated molar ratios, and was then subjected to sandwich EIA for TIMP-2 utilizing HRP-labelled clone 67-4H11 monoclonal antibody.

Figure 2 Binding of the N- and C-terminal domains of proMMP-2 to TIMPs

The N- (○) or C-terminal domain (●) at the indicated concentrations was transferred to each micro-well previously coated with TIMP-2 (4.6 pmol/well). Then HRP-labelled antibody, clone 43-3F9 or 75-7F7, was used to detect the N- or C-terminal domain, respectively, bound to TIMP-2. The same experiment with the N-terminal domain (△) was done with micro-wells coated with TIMP-1 (4.6 pmol/well) instead of TIMP-2.

Interaction between TIMP-2 and the N- or C-terminal domain of proMMP-2

We investigated the interaction between TIMP-2 and the N- or C-terminal domain of proMMP-2 by incubating TIMP-2 coated onto microwells with the N- or C-terminal domain of proMMP-2 and then detecting the N- or C-terminal domain bound to TIMP-2 using HRP-labelled anti-MMP-2 antibody of clone 43-3F9 or 75-7F7 respectively. Not only the C- but also the N-terminal domain of proMMP-2 was found to bind dose-dependently to TIMP-2 (Figure 2). Next we investigated the interaction between TIMP-1 and the N-terminal domain of proMMP-2. As shown in Figure 2, only less than 10% of TIMP-1 formed a complex with the N-terminal domain, strongly suggesting that the N-terminal domain binds exclusively to TIMP-2. We further tried to confirm that the complex had inhibitory activity toward active MMP-1. All these mixtures retained the same inhibitory activity as free TIMP-2 (Table 2). By this experiment we confirmed that the N-terminal domain binds TIMP-2 at site(s) different from the inhibitory site.

Table 2 Effect of TIMP-2 and its complex with the N-terminal domain of proMMP-2 on the collagenolytic activity of MMP-1

MMP-1 activity was determined as described in the Materials and methods section. Molar ratio = N-terminal domain:TIMP-2 (26 nmol).

	Molar ratio	Activity (m-units)	Activity (%)
Active MMP-1 (26 nmol)	—	44.6	100
+ TIMP-2 (26 nmol)	—	9.8	22
+ N-Terminal domain–TIMP-2	0.5:1	10.0	22
+ N-Terminal domain–TIMP-2	1:1	6.9	15
+ N-Terminal domain–TIMP-2	2:11	8.0	18

Figure 3 Competition between the N- and C-terminal domains of proMMP-2 for binding to TIMP-2

First, the N-terminal domain (1 µg) was transferred to each well previously coated with TIMP-2 (50 ng). After the wells had been washed with saline, the C-terminal domain at the indicated amounts was reacted with the complex. An antibody, clone 43-3F9 (○) or 75-7F7 (●), was used to detect specifically the N- or C-terminal domain, respectively.

Competition between the N- and C-terminal domains of proMMP-2 for binding to TIMP-2

When the N-terminal domain was incubated with TIMP-2 in the first reaction and the C-terminal domain was then added at various concentrations, the observed signal of the N-terminal domain was not changed, but that of the C-terminal domain increased dose-dependently (Figure 3). This result indicates that both terminal domains independently interact with TIMP-2. Willenbrock et al. [26] proposed a working model of various points of interaction between TIMP-2 and MMP-2, that is: (1) interactions between the N-terminal domain of TIMP-2 and the active site of MMP-2; (2) ionic interactions between the N-terminal domain of TIMP-2 and MMP-2; (3) low-affinity interactions between the C-terminal domain of TIMP-2 and MMP-2; and (4) electrostatic interactions between the C-terminal domain ('tail' portion) of TIMP-2 and the C-terminal domain of MMP-2. We demonstrated that there are at least two distinct binding sites on the TIMP-2 molecule for proMMP-2, that is, for the N- and C-terminal domains of proMMP-2. These results are consistent with the working model mentioned above.

Comparison of the immunoreactivity between the proMMP-2–TIMP-2 complex and the N- or C-terminal domain–TIMP-2 complex

Both proMMP-2–TIMP-2 and N-terminal domain–TIMP-2 complexes were subjected to a sandwich EIA using anti-TIMP-2 antibody (clone 68-6H4) as the solid phase and anti-

Figure 4 Comparison of the immunoreactivity between the proMMP-2–TIMP-2 complex and the N- or C-terminal domain–TIMP-2 complex

Top: a fixed amount (500 ng) of TIMP-2 was mixed with either proMMP-2 (○) or the N-terminal domain (●) to give the indicated molar ratios, and the complexes were then subjected to sandwich EIA using anti-TIMP-2 antibody (clone 68-6H4) as the solid phase and anti-proMMP-2 antibody (clone 43-3F9) as the HRP-labelled liquid phase. Bottom: a fixed amount (250 ng) of TIMP-2 was mixed with either proMMP-2 (○) or the C-terminal domain (●) to give the indicated molar ratios, and the complexes were then subjected to sandwich EIA using anti-TIMP-2 antibody (clone 68-6H4) as the solid phase and HRP-labelled anti-proMMP-2 antibody (clone 75-7F7) as the conjugate.

proMMP-2 antibody (clone 43-3F9) as the HRP-labelled liquid phase. As shown in Figure 4 (top), the N-terminal domain–TIMP-2 complex showed higher colour development than the proMMP-2–TIMP-2 complex. As the antibody (clone 43-3F9) was prepared against an oligopeptide representing a part of the propeptide sequence of MMP-2, the difference of immunoreactivity is probably due to some difference in the conformation of the propeptide portion, which is not difficult to imagine, between proMMP-2 and its N-terminal domain. Through this experiment, we further confirmed that the N-terminal domain of proMMP-2 formed a complex with TIMP-2 in a 1:1 molar ratio (Figure 4, top). It has been reported, however, that the N-terminal domain of proMMP-2 could not bind TIMP-2 [21,25]. This inconsistent result can be explained as follows: the binding affinity between the N-terminal domain of proMMP-2 and TIMP-2 seems to be unable to withstand chromatography on gelatin–Sephacrose [21] or precipitation with Protein G coupled to Sepharose beads [25], and thus the complex breaks down. In a similar type of experiment, both proMMP-2–TIMP-2 and C-terminal domain–TIMP-2 complexes were subjected to a sandwich EIA using anti-TIMP-2 antibody (clone 68-6H4) as the solid phase and anti-proMMP-2 antibody (clone 75-7F7) as the HRP-labelled liquid phase. As shown in Figure 4 (bottom), the proMMP-2–TIMP-2 complex showed higher colour development than the C-terminal domain–TIMP-2 complex. As the antibody used for this experiment is the one (clone 75-7F7)

Figure 5 Competition between proMMP-2 and its N- or C-terminal domain for binding to TIMP-2, as assessed by displacement assay

Top: 1 μ g of the N-terminal domain was transferred to individual wells previously coated with TIMP-2 (50 ng) and the plate was incubated for 2 h at room temperature. After a saline wash, the wells with N-terminal domain were reacted with the indicated amounts of the proMMP-2. Both the N-terminal domain and proMMP-2 bound to TIMP-2 were detected by use of HRP-labelled antibodies, clones 43-3F9 (○) specific to the propeptide and 75-7F7 (△) specific to the C-terminus. Similarly, 2 μ g of proMMP-2 was incubated with TIMP-2 previously coated to wells. After washing, the wells with proMMP-2 were then reacted with indicated amounts of the N-terminal domain. Both proMMP-2 and the N-terminal domain bound to TIMP-2 were detected by use of HRP-labelled anti-proMMP-2 antibodies, clones 43-3F9 (●) and 75-7F7 (▲). Bottom: 500 ng of the C-terminal domain was transferred to wells previously coated with TIMP-2 (50 ng) and the plate was incubated for 2 h at room temperature. The wells with bound C-terminal domain were reacted with the indicated amounts of the proMMP-2. Both proMMP-2 and the C-terminal domain bound to TIMP-2 were detected by use of HRP-labelled anti-proMMP-2 antibodies, clones 75-7F7 (△) and 43-3F9 (○). Similarly, 2 μ g of proMMP-2 was incubated with TIMP-2 (50 ng) previously coated on to wells. The wells were then reacted with the indicated amount of C-terminal domain. Both proMMP-2 and the C-terminal domain bound to TIMP-2 were detected by use of HRP-labelled anti-proMMP-2 antibodies, clones 75-7F7 (▲) and 43-3F9 (●).

prepared against intact proMMP-2, it is quite reasonable that native proMMP-2 would show more stable binding to the antibody than the C-terminal domain, the conformation change of which might be provoked by its cleavage from the N-terminal domain. This result confirmed again that the C-terminal domain bound to TIMP-2 in a 1:1 molar ratio.

Competition between proMMP-2 and its N- or C-terminal domain for binding to TIMP-2

Utilizing the difference in colour development of the sandwich EIA between the proMMP-2–TIMP-2 complex and either the N- or C-terminal domain–TIMP-2 complex (Figure 4), we carried out a competition study between proMMP-2 and the N- or C-terminal domain on binding to TIMP-2. As shown in Figure 5, we demonstrated that proMMP-2 fully replaced either the N- or C-terminal domain that had previously bound to TIMP-2 by

Figure 6 Binding of proMMP-2 to MMP-1-TIMP-2 or MMP-9-TIMP-2 complexes

Active MMP-1 (8.3 pmol) or active MMP-9 (4 pmol) was incubated with an equimolar amount of TIMP-2, and then proMMP-2 at different molar ratios was added to the reaction mixture. MMP-1-TIMP-2-proMMP-2 (○) and MMP-9-TIMP-2-proMMP-2 (●) complexes in the reaction mixture were measured by the respective EIAs.

detecting newly formed complexes with monoclonal antibodies (clones 43-3F9 and 75-7F7), but not vice versa. The quantity of proMMP-2 required to replace 50% of the N- or C-terminal domain bound to TIMP-2 was less than 25 ng/well (Figure 5, top) or approx. 100 ng/well (Figure 5, bottom) respectively, indicating that the relative contribution of the N-terminal domain to TIMP-2 binding is weaker than that of the C-terminal domain. These facts, together with the results shown in Figure 3, suggest that either the N- or C-terminal domain binds to TIMP-2 through the same binding sites as those of proMMP-2 and also that the binding affinity of TIMP-2 for proMMP-2 is much stronger than that for either the N- or C-terminal domain. This would indicate that the high-order structure of proMMP-2 might be involved in the stable binding to TIMP-2 [26,27].

Furthermore, the effect of the reductive alkylation of the C-terminal domain of proMMP-2 on the binding affinity for TIMP-2 was examined and essentially no effect was observed (results not shown), indicating that a disulphide bond in the C-terminal domain does not affect the binding affinity.

Interaction between proMMP-2 and either the MMP-1-TIMP-2 or MMP-9-TIMP-2 complex

Signals of ternary complex formation increased dose-dependently, eventually reaching a plateau, on the addition of proMMP-2 to either the MMP-1-TIMP-2 or MMP-9-TIMP-2 complexes (Figure 6), suggesting that either the MMP-1-TIMP-2 or MMP-9-TIMP-2 complex was able to form a ternary complex with proMMP-2 in a 1:1 molar ratio and indicating that there are two distinct MMP binding sites, one specific for the active MMPs and the other for proMMP-2, on the TIMP-2 molecule. Our results are consistent with the results reported by Kolkenbrock et al. [13], who demonstrated that the proMMP-2-TIMP-2 complex has the ability to inhibit other MMPs, such as MMP-1, MMP-8 and MMP-9.

Interaction between the N- or C-terminal domain of proMMP-2 and the MMP-9-TIMP-2 complex

Finally, we examined whether the N- or C-terminal domain of proMMP-2 can bind to MMP-9-TIMP-2 complexes. As shown

Figure 7 Binding of the N- or C-terminal domain of proMMP-2 to the MMP-9-TIMP-2 complex

MMP-9-TIMP-2 complex (24 pmol) was incubated with either the N- or C-terminal domain at different molar ratios overnight at 4 °C. Then the MMP-9-TIMP-2-N-terminal domain (○) and MMP-9-TIMP-2-C-terminal domain (●) complexes in the reaction mixture were determined by the respective EIAs.

in Figure 7, even in the presence of a 10-fold molar excess of the N-terminal domain of proMMP-2, ternary complex formation was not observed between the N-terminal domain and the MMP-9-TIMP-2 complex, suggesting that the N-terminal domain was not able to bind to this complex. This result is in contrast with that obtained from the binding study between the N-terminal domain of proMMP-2 and free TIMP-2 (Figure 4, top). Though the C-terminal domain was able to bind to the complex, the binding seemed to be less stable than the binding with TIMP-2 alone. These significant differences might be due to a conformational change in the TIMP-2 molecule evoked by complex-formation with MMP-9. These results possibly indicate that the binding site(s) for both, especially the N-terminal domain of proMMP-2 on the TIMP-2 molecule, might be directly occupied by MMP-9 or sterically hindered indirectly by complex-formation with MMP-9. A similar observation has been reported about the nature of TIMP-1 binding sites on the MMP-2 molecule, that is, binding to one site may decrease the affinity of TIMP-2 binding to the second site [27].

Conclusions

Taking advantage of mild immunoreactions, we have initially demonstrated that not only the C- but also the N-terminal domain of proMMP-2 binds exclusively to TIMP-2 in a 1:1 molar ratio, but not to TIMP-1 (Figures 2 and 4). As the N-terminal domain complexed with TIMP-2 can be replaced easily with proMMP-2 (Figure 5), the binding affinity between the N-terminal domain and TIMP-2 seems to be unable to withstand chromatography on gelatin-Sepharose [21] or immunoprecipitation with a monoclonal antibody to TIMP-2 and Protein G complexed to Sepharose beads [25]; under these conditions complex-formation could not therefore be demonstrated. We have further confirmed that both N- and C-terminal domains of proMMP-2 interact independently with TIMP-2 (Figure 3), indicating the presence of at least two distinct binding sites on the TIMP-2 molecule for proMMP-2. These results are consistent with the working model proposed by Willenbrock et al. [26]. It is quite natural to imagine that protein-protein interaction, like that between proMMP-2 and TIMP-2, might occur between two faces, not points, covering a relatively large portion of each protein. So, even if the primary binding site is located on the C-terminal domain of proMMP-2, it is not surprising that the

N-terminal domain may also participate in the interaction with TIMP-2.

It is known that TIMP-2, when associated with proMMP-2, can decrease proteolytic activity and autodegradation *in vitro* [24,27,28], although the N-terminal conversion is still possible. Recently, Bergmann et al. [29] demonstrated that TIMP-2 protects the autocatalytic cleavage at Pro³⁹⁴–Ile³⁹⁵ and Glu⁴¹⁴–Leu⁴¹⁵ linkages of proMMP-2, which confirms that TIMP-2 has binding site(s) close to the catalytic domain. These results are consistent with our findings that suggest that TIMP-2 interacts with not only the C-terminal domain (R_{415–631}) of proMMP-2 but also the N-terminal domain (R_{1–417}), which contains both cleavage sites, Pro³⁹⁴–Ile³⁹⁵ and Glu⁴¹⁴–Leu⁴¹⁵.

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