Biochem. J. (1993) 289, 65–69 (Printed in Great Britain)

Characterization of the functional domain of tissue inhibitor of metalloproteinases-2 (TIMP-2)

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Analysis of the functional domain of tissue inhibitor of metalloproteinases-2 (TIMP-2) was performed using limited proteolytic degradation with trypsin. This treatment generated a 13.5 kDa fragment which was purified and shown to consist of an uncleaved N-terminal region extending from residue 1 to residue 132. The fragment retains the ability to inhibit activated interstitial collagenase and to block the autocatalytic activation of procollagenase.

INTRODUCTION

The matrix metalloproteinases are a family of Zn^{2+}-dependent endopeptidases which are implicated in connective tissue degradation and turnover, both normal and pathological [1–5]. They have several characteristic and highly conserved domains, and have been classified into three groups [1,2] which differ somewhat in their specificities toward extracellular matrix components such as collagens, fibronectin, laminin and proteoglycans. Interstitial collagenase is in group 1, the 72 kDa and 92 kDa gelatinases are in group 2, and the stromelysins are in group 3.

Regulation of the matrix metalloproteinases can occur post-translationally, since they are secreted in zymogen forms whose activation requires autoproteolytic removal of an 80-amino-acid N-terminal fragment [6]. Activaton can be initiated by organomercurials *in vitro*, and may involve the plasminogen activator/plasmin system *in vivo* [7,8]. Activation and activity can also be modulated by stoichiometric interaction with tissue inhibitor of metalloproteinases (TIMP or TIMP-1), a 184-amino-acid glycoprotein of about 28.5 kDa [9–13], and with the more recently described metalloproteinase inhibitor (MI) or TIMP-2, an unglycosylated 194-amino-acid protein [14–19].

TIMP-1 and TIMP-2 share a broad spectrum of inhibitory activities towards members of the matrix metalloproteinase family [14–16]. The inhibitors themselves are 39% identical [12,13,17,18], with the presence of 12 cysteine residues in conserved positions, all of which are involved in disulphide linkage [19,20]. The assignment of these six disulphide bonds has been recently described for TIMP-1 [20] and is likely to be the same for TIMP-2 (see Figure 1). The positions of these bonds separate the inhibitor into two regions, the first one extending from residue 1 to residue 126 and including three overlapping disulphide bonds, and the second one extending from residue 129 to the C-terminus and including the three remaining disulphide bonds. This unusually high number of disulphide bonds probably accounts for the marked stability of the inhibitors [9,11,14,21]. Functional distinctions between TIMP-1 and TIMP-2 include preferences for interaction with 92 kDa progelatinase and 72 kDa progelatinase respectively [15,16,22]. In addition, TIMP-2 can block the autoproteolytic activation of the proenzyme [19,23]. Recently, Murphy et al. [24] have shown that a truncated version of TIMP-1 consisting of amino acids 1–126, and therefore including the first three disulphide bonds, retains anti-metalloproteinase activity, suggesting that this domain of the molecule is involved in formation of the complex with the active enzyme. In the present paper we demonstrate that a similar domain exists in TIMP-2 and that this domain also prevents the autocatalytic activation of interstitial procollagenase.

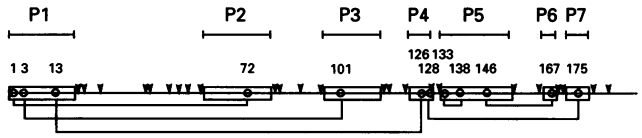

Figure 1 Linear map of TIMP-2

Positions of the Cys (C) residues are indicate across the top. Assignment of the disulphide bonds is based on the published data for TIMP-1 [20]. Potential cleavage sites for trypsin (Arg and Lys residues) are indicated by arrowheads. Tryptic peptides that contain Cys residues and could remain linked by disulphide bonds (analogous to the case with TIMP-1 [20]) are indicated by open boxes, and are designated P1–P7 (at the top).

MATERIALS AND METHODS

Materials

Bovine pancreatic trypsin was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and kept in stock solution at a concentration of 1 mg/ml in sterile 20 mM Tris/HCl, pH 7.5, at −80 °C. Soybean trypsin inhibitor was purchased from Sigma (St. Louis, MO, U.S.A.) and kept in stock solution at 5 mg/ml in sterile 20 mM Tris/HCl, pH 7.5, at −80 °C. Interstitial procollagenase (specific activity 186 units/mg) was purified from the conditioned medium of phorbol 12-myristate 13-acetate-treated rabbit fibroblasts as previously described [19]. Recombinant TIMP-2 (rTIMP-2) was purified from the conditioned medium of Chinese hamster ovary cells transfected with vector pDSRα2 containing human TIMP-2 cDNA, as previously described [19]. rTIMP-2 in stock solutions was quantified by absorbance at 280 nm (using an $\epsilon_{280}^{0.1\%}$ of 1.82, which was arrived at by correlation of absorbance and quantitative amino acid analysis [19]). For rTIMP-2, the assay of Bradford [25] using BSA as standard leads to concentration values about the same

Abbreviations used: pAPMA, *p*-aminophenylmercuric acetate; TIMP, tissue inhibitor of metalloproteinases; RP-h.p.l.c., reverse-phase h.p.l.c.; rTIMP-2, recombinant TIMP-2.

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(greater by a factor of 1.03) as those determined by absorbance. The purified 13.5 kDa fragment of TIMP-2 was quantified in stock solutions by the Bradford assay with BSA as standard.

Collagenase inhibition assays

Assays were performed as previously described [26] using the ^{14}C-acetylated type I rat skin collagen film assay [27]. Rabbit interstitial procollagenase was activated by treatment with *p*-aminophenylmercuric acetate (pAPMA) (final concentration 5 mM) at 37 °C for 30 min, followed by incubation with inhibitor samples for 15 min at room temperature prior to testing for activity.

Polyacrylamide-gel electrophoresis

SDS/PAGE was performed according to the method of Laemmli [28]. Where indicated, sample preparation included reduction in the presence of dithiothreitol (final concentration 50 mM) and heating at 68 °C for 10 min. After electrophoresis, gels were stained with Coomassie Blue, or immunoblotted according to the method of Burnette [29] using a rabbit serum raised against human rTIMP-2 (dilution 1:200). Immunocomplexes were identified with a goat anti-(rabbit IgG) serum conjugated to horseradish peroxidase (Bio-Rad). Reverse zymography was performed as previously described [26]. Briefly, after electrophoresis in 12.5% (w/v) polyacrylamide gels containing gelatin (final concentration 0.1%, w/v) and SDS, the gels were incubated for 3 h with crude pAPMA-activated gelatinase obtained from rabbit fibroblasts and were stained with Coomassie Blue. In these gels, dark zones, corresponding to undigested gelatin, indicate the location of material with anti-gelatinase activity.

Reverse-phase (RP)-h.p.l.c.

RP-h.p.l.c. was performed using a Beckman Model 114M system with a C_4 column (Vydac; 214 Tp5415) which was equilibrated with buffer A [0.1% (v/v) trifluoroacetic acid/5% (v/v) acetonitrile]. Elution was at 0.8 ml/min for 10 min at 5% buffer B [0.1% (v/v) trifluoroacetic acid/100% (v/v) acetonitrile], followed by a linear gradient to 40% buffer B over 15 min and then to 100% buffer B over 90 min. Fractions of interest were collected manually with monitoring at 225 nm using a Kratos Model 757 variable-wavelength u.v. detector and concentrated by evaporation in a Speed Vac (Savant Instruments).

Amino acid sequencing

N-Terminal amino acid sequencing was performed by automated Edman degradation using an Applied Biosystem Model 477 pulsed liquid microsequencer. For C-terminal amino acid analysis, 5 μl of carboxypeptidase P (0.4 mg/ml; Boehringer Mannheim) was added to 50 μg (in 50 μl) of purified 13.5 kDa fragment in 40 mM sodium citrate/0.05% (w/v) Brij 35, pH 4. The reaction was carried out at room temperature, and aliquots (10 μl) were taken at 0, 5, 20 and 60 min and combined with 10 μl of 25% trifluoroacetic acid to stop the reaction. The last aliquot of the reaction mixture was then incubated at 37 °C for an additional 30 min and combined with 10 μl of 25% trifluoroacetic acid. The samples were dried, dissolved in 20 μl of 0.025% (w/v) K^+-EDTA, and analysed for free amino acids using an Applied Biosystems Model 420A derivatizer with an on-line narrow bore RP-h.p.l.c. The C-terminal anlaysis was performed on two separate preparations of purified 13.5 kDa fragment.

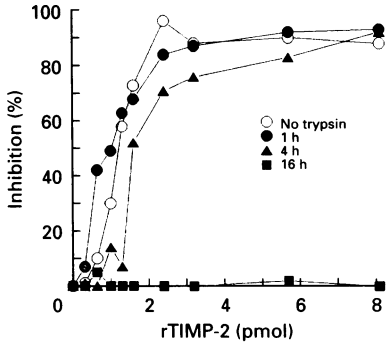

Figure 2 Effect of trypsin on anti-collagenase activity of rTIMP-2

rTIMP-2 (1.2 μg; 56 pmol) was incubated with trypsin (0.12 μg; 5.7 pmol) at 37 °C in 200 μl of 50 mM Tris/HCl, 200 mM NaCl and 10 mM $CaCl_2$, pH 7.5. At the indicated times, portions of the reaction mixture were taken and a 5-fold excess (0.6 μg) of soybean trypsin inhibitor was added to stop the reaction. Increasing amounts of the incubated mixtures were tested for collagenase-inhibiting activity.

RESULTS

Sensitivity of rTIMP-2 to trypsin degradation

The effect of trypsin on the inhibitory activity of rTIMP-2 was first examined. At an rTIMP-2/trypsin molar ratio of 10:1, a progressive loss of anti-collagenase activity was observed (Figure 2). No loss of activity was detected after 1 h of incubation, a 45% loss was seen after 4 h (the amount of inhibitor that caused 50% inhibition increased from 1 pmol to 1.6 pmol) and 100% loss was seen after 16 h. Analysis of the reaction products at these times was performed by SDS/PAGE under non-reducing conditions. The 21.5 kDa band representing intact inhibitor was lost after 1 h, and the presence of new bands with apparent molecular masses of 19 kDa and 13.5 kDa (unreduced) was noted (Figure 3a). After 4 h the 19 kDa band was not detected but the 13.5 kDa band was still present, although with lower intensity. After 16 h, no proteolytic fragments could be detected. Reverse zymogram analysis of the same reaction samples revealed a zone of undigested gelatin at the position of the 13.5 kDa band (when present, i.e. at the 1 h and 4 h points), indicating that this proteolytic fragment retained inhibitory activity (Figure 3b). This observation is consistent with the detection of inhibitory activity after incubation for 1 h and 4 h with trypsin. The 13.5 kDa proteolytic fragment was also detectable by immunoblot analysis (Figure 3c) and therefore has one or more antigenic site(s). These results suggested that there is a fragment of the TIMP-2 molecule, generated during trypsin digestion, which retains anti-collagenase activity.

Purification and sequencing of the 13.5 kDa proteolytic fragment of TIMP-2

We selected a 2 h incubation time to generate 13.5 kDa TIMP-2 fragment for further characterization. Purification of the 13.5 kDa fragment was achieved by RP-h.p.l.c. using a gradient of acetonitrile. The fragment eluted at an acetonitrile concentration of 48% and was well separated from other smaller proteolytic fragments and from trypsin (Figure 4a). The apparent molecular mass of 13.5 kDa was obtained on SDS/PAGE with the fragment unreduced. After reduction it migrated with a

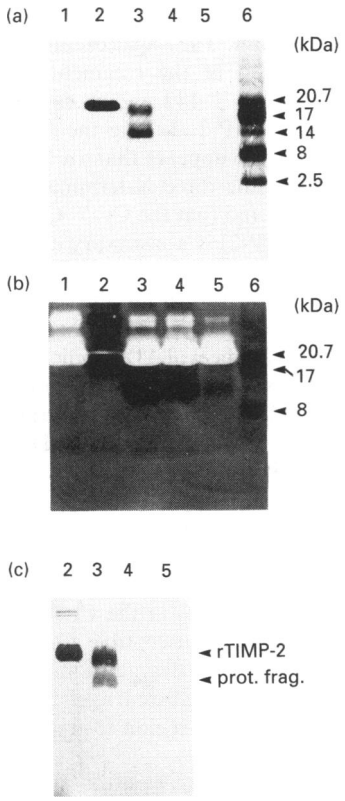

Figure 3 Analysis of the proteolytic products of rTIMP-2 by SDS/PAGE

rTIMP-2 (3 μg) was incubated in the presence of trypsin (0.3 μg) in 5 μl of 50 mM Tris/HCl, 200 mM NaCl and 10 mM $CaCl_2$, pH 7.5. At the indicated times, the reaction was terminated by addition of gel sample treatment buffer (lacking reducing agent) and freezing. Samples (obtained in triplicate) were electrophoresed on an SDS/polyacrylamide gel (20%, w/v) (gels **a** and **c**) or on a gelatin (0.1%, w/v)/SDS-containing polyacrylamide gel (zymogram; gel **b**). Gels (**a**) and (**b**) were stained with Coomassie Blue; gel (**c**) was immunoblotted using a rabbit anti-rTIMP-2 antiserum. Lanes 1, trypsin (0.3 μg); lanes 2, rTIMP-2 (3 μg); lanes 3, rTIMP-2 incubated with trypsin for 1 h; lanes 4, same as lane 3 but incubated for 4 h; lanes 5, same as lanes 3 and 4 but incubated for 16 h; lanes 6, reduced molecular mass markers, with molecular mass values indicated on the right in kDa. The positions of rTIMP-2 and the 13.5 kDa proteolytic fragment (prot. frag.) are indicated by arrowheads on the right of gel (**c**). The clear zone in lanes 1, 3, 4 and 5 of gel (**b**) are indicative of gelatin degradation by trypsin present in the gel samples for those lanes. High-molecular mass bands of inhibitory activity are apparent in lane 2 of gel (**b**). These reflect small amounts of rTIMP-2 which are aggregated (probably by interchain disulphide linkage; note that the gel samples are unreduced). These are apparent in lane 2 of gel (**c**) (immunoblot), and are visualized to a disproportionate degree in the gel (**b**) zymogram.

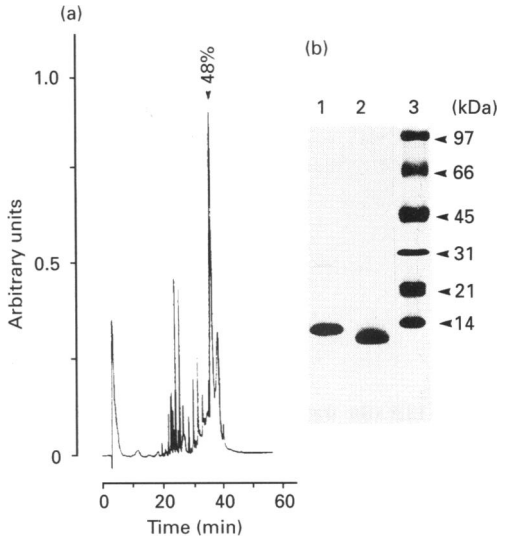

Figure 4 Purification of the 13.5 kDa proteolytic fragment by RP-h.p.l.c.

(**a**) Trypsin-treated rTIMP-2 (200 μg) was subjected to RP-h.p.l.c. (see the Materials and methods section). (**b**) An aliquot (1 μg) of the material eluting at 48% acetonitrile was electrophoresed on an SDS/polyacrylamide gradient gel (10–20%, w/v). Lane 1, sample unreduced; lane 2, sample reduced; lane 3, positions of reduced molecular mass markers (kDa).

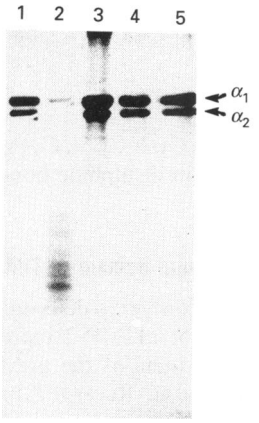

Figure 5 The 13.5 kDa fragment inhibits type I collagen degradation by interstitial collagenase

^{14}C-labelled type I rat skin collagen (10000 c.p.m.) was incubated in the presence of pAPMA-activated interstitial collagenase and inhibitor as indicated in 100 μl of 50 mM Tris/HCl, 200 mM NaCl, 10 mM $CaCl_2$ and 10 mM *N*-ethylmaleimide, pH 7.5, at 37 °C for 16 h. Samples containing reaction mixture were electrophoresed (unreduced) on an SDS/-polyacrylamide gradient gel (5–15%, w/v). After electrophoresis, the gel was soaked in Enhance (New England Nuclear), dried and subjected to autoradiography for 24 h at −80 °C. Lane 1, ^{14}C-labelled type I collagen; lane 2, as lane 1 plus pAPMA-activated collagenase (1.3 μg; 25 pmol); lane 3, same as lane 2 plus EDTA (final concentration 20 mM) added prior to incubation; lane 4, same as lane 2 plus purified 13.5 kDa fragment (1 μg; 74 pmol) added prior to incubation; lane 5, same as lane 2 plus rTIMP-2 (1 μg; 48 pmol) added prior to incubation. Positions of α_1 and α_2 collagen chains are indicated on the right. Type I collagen degradation products are seen in lane 2.

slightly lower apparent molecular mass of 12 kDa (Figure 4b). This observation suggests that the fragment is a continuous region of polypeptide, i.e. it contains intramolecular disulphide bonds but does not represent multiple peptides interlinked by disulphide bonds. N-Terminal sequencing revealed a single sequence matching that of intact TIMP-2: (C)-S-(C)-S-P-V-H-(P)-(Q)-Q-A-F-(C)-N-A-(D)-(I)-V-I-R... (residues in parentheses represent expected amino acids that were not actually assigned). The absence of multiple N-terminal sequences further indicates that the fragment consists of a large N-terminal region with uncleaved Lys and Arg bonds. To locate its C-terminus, the fragment was treated with carboxypeptidase P. The amino acids Arg, Thr, Ile, Lys, Cys and Glu were released; these would appear to correspond to the region from Glu^{127} to Arg^{132} (see Figure 1 and [17]). Thus the fragment includes a polypeptide extending from residue 1 to residue 132. It may be that the small peptide P7 is linked to polypeptide-(1–132) by way of disulphide bond Cys^{128}–Cys^{175} (see Figure 1). However, P7 was not detected by either the N-terminal or the C-terminal sequencing, and the increased mobility of the fragment on SDS/polyacrylamide gels upon reduction could be due to breakage of intrachain disulphide bonds within polypeptide-(1–132). The fragment probably con-

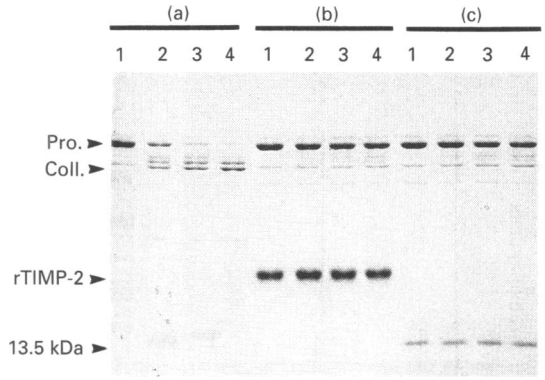

Figure 6 The 13.5 kDa fragment inhibits autoproteolytic activation of interstitial procollagenase

Interstitial procollagenase (0.84 μg; 16 pmol) in 5 μl of 50 mM Tris/HCl, 200 mM NaCl and 10 mM $CaCl_2$, pH 7.5, was incubated with or without inhibitor for 15 min at 22 °C prior to pAPMA addition (final concentration 5 mM) and incubation at 37 °C. At the indicated times, the reactions were blocked by the addition of EDTA to 20 mM. Samples were then heated at 68 °C for 10 min in the presence of gel sample buffer containing 50 mM dithiothreitol and electrophoresed on an SDS/polyacrylamide gel (12.5%, w/v). The gel was stained with Coomassie Blue. (**a**) Procollagenase activated with pAPMA in the absence of inhibitor, with the conversion of the procollagenase (52 kDa) to activated forms (44 kDa and 42 kDa) and a 27-kDa proteolytic fragment [32]. (**b**) As (**a**), except that rTIMP-2 (0.57 μg; 27 pmol) was added prior to activation with pAPMA. (**c**) Same as (**a**), except that purified 13.5 kDa fragment (0.28 μg; 20 pmol) was added prior to activation with pAPMA. Lanes 1, reactions blocked at 0 min; lanes 2, reactions blocked at 10 min; lanes 3, reactions blocked at 20 min; lanes 4, reactions blocked at 30 min. Arrowheads on the left indicate positions of procollagenase (52 kDa; Pro), activated collagenase (42 kDa; Coll), rTIMP-2 and 13.5 kDa fragment.

tains disulphide bonds Cys^{1}–Cys^{72}, Cys^{3}–Cys^{101} and Cys^{13}–Cys^{126} (Figure 1) as intrachain disulphide bonds.

Function of the N-terminal domain of TIMP-2

Experiments were performed to determine whether the 13.5 kDa proteolytic fragment of rTIMP-2 representing the N-terminal domain had retained some of the previously described [14,19] functions of the inhibitor. Reverse zymogram analysis (Figure 3b) had shown that the fragment retained anti-gelatinase activity. We further demonstrated that the fragment inhibited the cleavage of type I collagen by interstitial collagenase (Figure 5), and also blocked the autoproteolytic activation of interstitial procollagenase initiated in the presence of pAPMA (Figure 6).

DISCUSSION

Until recently, structural studies of both TIMP-1 and TIMP-2 had suggested that most or all of the primary structure is required for inhibitory functions, and that the six disulphide bonds contribute to a stable compact tertiary structure. Reduction/alkylation destroys the activity of TIMP-1 [10,21], and that of TIMP-2 as well [14]. A truncated form of TIMP-1 consisting of amino acids 1–122 had been shown to be inactive [30]; this version lacked the Cys^{13}–Cys^{124} disulphide bond. However, recently Murphy et al. [24] have shown that a truncated TIMP-1 form containing amino acids 1–126 possesses inhibitory activity. In addition, the present data demonstrate that the 13.5 kDa fragment of TIMP-2, generated by limited trypsin treatment, also retains inhibitory activity. The finding that the fragment has inhibitory activity in reverse zymogram analysis initially indicated that the entire structure of TIMP-2 is not required for its function. The isolation and structural and functional characterization of the fragment have shown that it corresponds to residues 1–132 and is capable of the primary functions of intact TIMP-2. Taking the findings with TIMP-1 and TIMP-2 together, it appears that an N-terminal portion of the TIMPs including the three N-terminal disulphide bonds is sufficient for activity, and that the Cys^{13}–Cys^{124} disulphide bond (Cys^{13}–Cys^{126} in TIMP-2) is a necessary component.

Our observations provide some new insight into the degradation of TIMPs by trypsin. Both TIMP-1 [9,10] and TIMP-2 [14] can be inactivated by trypsin, but high ratios of trypsin to TIMP are required. Okada et al. [31] studied the effects of trypsin on TIMP-1 as a function of time; at points where inhibitory activity was largely lost, intermediate degradation products of 18 kDa and 14 kDa were apparent on SDS/PAGE, but only under reducing conditions. In the present study with TIMP-2, we show that intermediate degradation products including the 13.5 kDa fragment are apparent on SDS/PAGE under non-reducing conditions at a point in the time course of trypsin treatment when the inhibitory activity of the TIMP-2 preparation is not decreased at all. Bonds in the C-terminal portion of the TIMP-2 are initially more susceptible to trypsin. With longer exposure to trypsin, complete degradation of the TIMP-2 results. It may be that the C-terminal cleavages indirectly increase the sensitivity of the N-terminal region to trypsin.

This work was supported by Grant CA 42919 from the National Institutes of Health, Department of Health and Human Services (U.S.A.), and by Grant BE 84 from the American Cancer Society. We thank Solly Weiler for the C-terminal amino acid analysis, K. Cheng-Chen for the preparation of Figures, and Joan Bennett for typing the manuscript.

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Received 29 April 1992/25 June 1992; accepted 2 July 1992