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The C-Terminal Domain of Tissue Inhibitor of Metalloproteinases-2 Is Required for Cell Binding but Not for Antimetalloproteinase Activity

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We have generated a C-terminally-truncated form of recombinant tissue inhibitor of metalloproteinases-2 (designated rTIMP-2 delta) in which the region of the inhibitor extending from residue 128 to 194 and including 3 of the 6 disulfide bonds is deleted. rTIMP-2 and rTIMP-2 delta had similar inhibitory activities toward interstitial collagenase and inhibited the activation of the precursor form of matrix metalloproteinase-2 (proMMP-2). rTIMP-2 also bound with high affinity (Kd 0.99 nM) to HT1080 human fibrosarcoma cells treated with 12-O-tetradecanoyl-phorbol-13-acetate. However deletion of the C-terminal domain of TIMP-2 significantly lowered the cell surface binding affinity, with competition experiments indicating a 2 order of magnitude difference between rTIMP-2 and rTIMP-2 delta in the concentrations needed to displace ^{125}I-labeled rTIMP-2 binding. These data indicate that the C-terminal domain of TIMP-2 is not required for the antimetalloproteinase activity but plays a major role in the high affinity cell surface binding of the inhibitor.

Tissue inhibitors of metalloproteinases (TIMPs) are a family of natural inhibitors of the matrix metalloproteinases (MMPs), a class of proteases that degrade a broad spectrum of extracellular matrix components [1-4]. MMPs are produced in the extracellular space in an inactive proform and activation into an active form is achieved by elimination of the N-terminal prodomain which contains a Cys residue protecting the active Zn^{++} binding pocket [cysteine switch [5]]. This is a multistep process that can be initiated *in vitro* by destabilizing agents such as p-aminophenylmercuric acetate (APMA), and that involves in most cases an autoproteolytic cleavage [6,7]. *In vivo,* the mechanisms that activate proMMPs are more complex. In the particular case of progelatinase-A (proMMP-2), activation occurs at the cell surface and is mediated by MT1-MMP [8,9]. TIMPs exert a two-step control on the proteolytic activity of MMPs in the extracellular milieu, first by forming 1:1 stoichiometric irreversible complexes with the active enzyme [10], and secondly by inhibiting the autocatalytic activation of the proenzymes [11,12].

The amino acid sequence of TIMPs is characterized by the presence of 12 Cys residues placed in preserved positions among the 3 TIMPs and all involved in the formation of disulfide bonds [13]. The location of these disulfide bonds is such that the first 3 of the 6 disulfide bonds (1-72, 3-101 and 13-126) overlap in the N-terminal domain and the other 3 bonds overlap in the C-terminal domain [14]. In the case of TIMP-2, the N-terminal domain extends from Cys^{1} to Cys^{126} and is linked to the C-terminal domain (Cys^{128} to Pro^{194}) by Glu^{127}. Recently, a homonuclear two-dimensional ^{1}H nuclear magnetic resonance spectroscopy derived structure of the N-terminal domain of TIMP-2 has been reported [15]. The data have indicated that this domain contains a five-stranded antiparallel β-sheet that is rolled over on itself to form a closed β-barrel and 2 short helices that pack close to one another on the same barrel face. This structure is homologous to that observed in members of the oligosaccharide/oligonucleotide binding fold family. Mutagenesis studies performed on TIMP-1 and TIMP-2 have shown that the

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Abbreviations: TIMP, tissue inhibitor of metalloproteinases; rTIMP, recombinant TIMP; MMP, matrix metalloproteinase; APMA, p-aminophenylmercuric acetate; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; CHO, Chinese hamster ovary.

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N-terminal domain of TIMPs behaves as a fully active metalloproteinase inhibitor, whereas the C-terminal domain is involved in the binding of TIMPs to their respective proMMPs [16]. We have previously demonstrated that a 13.5 kDa proteolytic fragment of TIMP-2 generated by trypsin digestion and consisting of an uncleaved N-terminal region extending from residue 1 to residue 132 inhibits the catalytic activity of interstitial collagenase (MMP-1) and its autoproteolytic activation [17].

Although TIMP-2 is primarily known as an inhibitor of MMPs, it also acts as a ligand and binds to the surface of a variety of cells including transformed fibroblasts [18], dermal fibroblasts [19], Raji cells [20], MCF7 and HT1080 cells [21]. At least 2 specific roles for this function have been proposed. First it may support the growth modulatory activity [20] and the erythroid potentiating activity [22] described for TIMP-2. Second, binding of TIMP-2 to the cell surface may be essential to mediate the activation of proMMP-2 by its cell surface associated activator MT1-MMP [23]. In an attempt to better understand the dual functions of TIMP-2 as an inhibitor of metalloproteinases and as a ligand, we have made a C-terminal truncated TIMP-2 mutant and examined the effect of this mutation on these two functions.

MATERIALS AND METHODS

Cloning and expression of rTIMP-2 delta cDNA. The strategy to generate a truncated form of recombinant TIMP-2 (rTIMP-2) in which the C-terminal region extending from Cys^{128} to Pro^{194} was deleted is described in Figure 1. This mutant was designated rTIMP-2 delta. A 270 bp cDNA fragment extending from a unique *Bam*HI site in the TIMP-2 cDNA sequence to codon 127 was generated by PCR using a 5′-CTATCAAGAGGATCCAGTATG-3′ sense primer (21 mer) and a 5′-AAGTCGCGGCCGCTTTATTGTGATCTTTCACTC-GCAGCC-3′ antisense primer (39 mer). This latter primer included the sequences for codon 125 to codon 127 followed by a stop codon (TGA), an AATAAA polyadenylation signal sequence and a *Not*I site (Figure 1, panel D). Using pCDNAHMI (HMI is identical to TIMP-2) as template [24], the PCR fragment was amplified and subcloned in orientation into pCDNAHMI digested with *Bam*HI and *Not*I to generate pCDNATIMP-2 delta. After verification for the mutation by sequencing, the truncated TIMP-2 delta cDNA was isolated by digestion of pCDNATIMP-2 delta with *Sac*I and *Xba*I and reinserted into pDSRα2 to generate pDSRα2TIMP-2 delta (Figure 1A). This plasmid was then transfected into dihydrofolate reductase deficient Chinese hamster ovary (CHO) cells which were selected for expression against increasing concentrations of methotrexate. Methotrexate resistant recombinant clones were established and examined for the secretion of a 14 kDa protein that cross-reacted with a polyclonal rabbit antiserum against human rTIMP-2. One positive clone (clone 4.2) that produced the largest amount of recombinant mutated protein was used to obtain purified rTIMP-2 delta from the cell culture supernatant.

Purification of rTIMP-2 delta. rTIMP-2 delta was purified essentially as described in a previously published procedure for rTIMP-2 [12]. The purity of the final preparation was verified by SDS-polyacrylamide gel electrophoresis and estimated to be greater than 95% by Coomassie Blue staining of the gel (Figure 1B). The final material contained a 14 kDa protein that was recognized by a polyclonal rabbit antiserum against *Escherichia coli* derived human rTIMP-2 in Western blot analysis (Figure 1C). The concentration of the protein was quantitated by absorbance at 280 nm using a calculated $\epsilon_{280}^{0.1\%}$ of 1.49 for rTIMP-2 and of 0.886 for rTIMP-2 delta. These values were verified by quantitative amino acid analysis.

Cell culture. CHO cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Gemini Bioproducts, Inc.), penicillin (50 units/ml) and streptomycin (50 μg/ml) (Irvine Scientific). HT1080 fibrosarcoma cells were cultured in Minimum Essential Medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine (Gibco BRL), penicillin (50 units/ml) and streptomycin (50 μg/ml).

Collagenase inhibition assay. Collagenase inhibition assays were performed in 96 well plates coated with [^{14}C]-labeled rat skin type I collagen using the methods of Johnson and Wint [25]. Each well contained 50 μg of type I collagen (specific activity 194 cpm/μg) and was incubated for 5 hours at 37°C in the presence of APMA activated crude collagenase derived from the culture medium of TPA treated rabbit synovial cells [26]. TIMP-2 was added at indicated concentrations in a final volume of 200 μl of 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$ buffer and the collagenolytic activity was measured by counting the radioactivity released into the supernatant.

SDS-PAGE. SDS-PAGE was performed according to the method of Laemmli [27]. Zymography was performed on SDS-polyacrylamide gels in which gelatin (0.75 mg/ml) was added to the polymerization mixture. After electrophoresis, these gels were incubated for 30 minutes in 2.5% (v/v) Triton X-100 followed by overnight incubation at 37°C in substrate buffer (50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$). The gels were then stained in Coomassie Brilliant Blue and destained in acetic acid:methanol:water (50:40:10). In these gels, the presence of clear bands corresponding to digested gelatin indicated proteases with gelatinolytic activity. Immunoblotting was performed according to the method of Burnette [28] using a rabbit polyclonal antiserum raised against *Escherichia coli* derived human rTIMP-2 [12]. Immunocomplexes were visualized with the use of horseradish peroxidase linked anti-rabbit IgG antibody from donkey using luminol chemiluminescence (ECL, Amersham).

Iodination of rTIMP-2. Iodination was performed with ^{125}I according to the method of Hunter and Greenwood [29]. rTIMP-2 or rTIMP-2 delta protein (15 μg) was mixed with 0.06 M Sodium Phosphate, pH 7.4, 1 mCi of ^{125}I (Dupont, New England Nuclear, specific activity 17.4 Ci/mg), and 0.1 mg of chloramine T (Sigma) in a final volume of 50 μL. After 5 minutes of incubation on ice 20 μl of sodium metabisulfite (10 mg/ml) was added to stop the reaction. Samples were diluted with PBS and loaded onto a Sephadex G-25 desalting column. Protein containing fractions that eluted from the column were pooled and the amount of protein-bound ^{125}I was determined after precipitation in 10% (v/v) trichloroacetic acid.

Cell binding and competition assays. HT1080 cells were plated in 12-well culture plates (2.6×10^5 cells/cm^2). After 24 hours the cells were treated with 100 ng/ml of TPA in serum-free medium. After 16 hours the medium was replaced with medium containing 1 mg/ml of bovine serum albumin and 25 mM Hepes, pH 7.3, in the presence or absence of 100-fold excess of unlabeled rTIMP-2 or rTIMP-2 delta. ^{125}I-labeled rTIMP-2 or rTIMP-2 delta was then added to the medium at concentrations ranging from 0.5 to 400 nM and the plates were left on ice for 1 hour. The cells were then washed 6 times with ice cold PBS containing 1 mg/ml of bovine serum albumin and lysed in 200 μl of 0.4 M NaOH, 0.3% (v/v) SDS. The radioactivity in the cell lysate was determined by counting in a gamma counter (efficiency of 70%). Competition assays were performed by the same method. AccuFit™ binding software (Beckman, Fullerton, CA) was used to obtain the Scatchard plot and to derive Kd and binding capacity values.

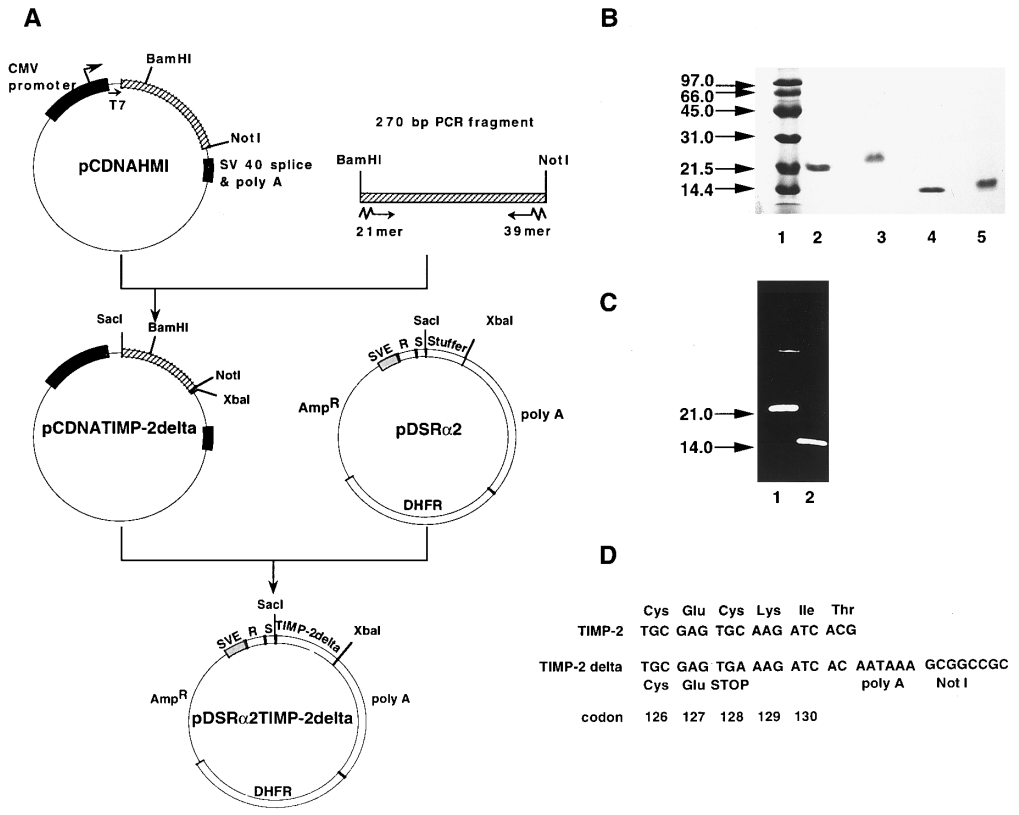

FIG. 1. Cloning and expression of rTIMP-2 delta. (A) A 270 bp PCR fragment in which codon Cys^{128} was replaced by a stop codon was subcloned into pCDNAHMI digested with *Bam*HI and *Not*I as indicated in Materials and Methods. The truncated cDNA was further subcloned into the mammalian expression vector pDSRα2 to generate pDSRα2TIMP-2 delta. (B) SDS-polyacrylamide gel showing the purified rTIMP-2 and rTIMP-2 delta. rTIMP-2 (2 μg, lanes 2 and 3) and rTIMP-2 delta (2 μg, lanes 4 and 5) were electrophoresed on a 0.1% (v/v) SDS, 12.5% (w/v) polyacrylamide gel in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of 10 mM dithiothreitol. The gel was stained with Coomassie Brilliant Blue. Reduced molecular weight standards are shown in lane 1, and molecular mass (in kDa) is indicated by arrows on the left. (C) Western blot analysis of rTIMP-2 and rTIMP-2 delta. rTIMP-2 wt (100 ng) (lane 1) and rTIMP-2 delta (100 ng) (lane 2) were electrophoresed as described in (B). The gel was then immunoblotted with a polyclonal rabbit antiserum against rTIMP-2 as described in Materials and Methods. The figure is a negative of the chemoluminescence reaction generated in the presence of Luminol. (D) Sequence of the mutation introduced to generate a 270 bp PCR fragment containing rTIMP-2 delta.

RESULTS

Inhibition of activated MMP-1 by rTIMP-2 and rTIMP-2 delta. We first examined the effect of the C-terminal truncation of TIMP-2 on its anticollagenase activity by comparing the ability of rTIMP-2 and rTIMP-2 delta to inhibit the degradation of [^{14}C]-labeled type I collagen by APMA-activated rabbit fibroblast derived interstitial collagenase (MMP-1). The data (Figure 2) show similar inhibitory activities for both proteins with an IC_{50} of 2.5 nM for rTIMP-2 delta and 3.0 nM for rTIMP-2. Thus, the N-terminal domain of TIMP-2 has full inhibitory activity for interstitial collagenase.

Inhibition of proMMP-2 activation. To compare the ability of these inhibitors to inhibit the activation of proMMP-2, we performed experiments in which HT1080 cells were treated with TPA in the presence of rTIMP-2 or rTIMP-2 delta. It has been previously demonstrated that TPA induces proMMP-2 activation at the cell surface [30] and increases MT1-MMP mRNA expression [31]. These experiments (Figure 3) demonstrated a similar inhibitory activity for rTIMP-2 and rTIMP-2 delta, which both prevented the conversion of the 68 kDa proMMP-2 to the 64 kDa intermediate and the fully activated 62 kDa MMP-2 when added to the culture medium at concentrations ranging from 50 to 500 nM. These observations are consistent with the assignment of the antimetalloproteinase activity of TIMP-2 to its N-terminal domain, and with the concept that the control that TIMP-2 exerts on the activation of proMMPs is related to its antimetalloproteinase activity.

Binding studies. The binding of ^{125}I-labeled rTIMP-2 to the surface of human HT-1080 cells was examined. We observed little specific cell surface binding of rTIMP-2 unless these cells were pretreated with TPA which led to a saturable binding when a concentration

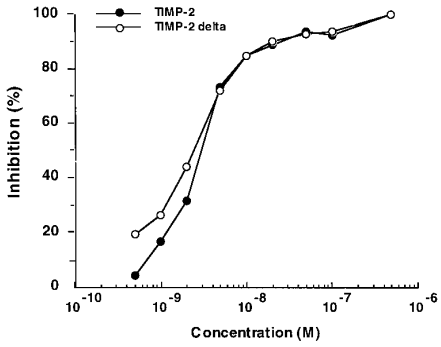

FIG. 2. Inhibition of interstitial collagenase (MMP-1) by rTIMP-2 and rTIMP-2 delta. Data represent the percentage inhibition of [^{14}C]-labeled-type I collagen degradation by APMA activated crude interstitial collagenase incubated in the presence of increasing amounts of rTIMP-2 (closed circles) and rTIMP-2 delta (open circles). The data represent the mean of triplicate samples for each rTIMP-2 concentration.

of ^{125}I-labeled TIMP-2 of 50 nM was approached (Figure 4A). The specificity of the cell binding was confirmed by the addition of 100 fold excess of nonradiolabeled rTIMP-2. Scatchard plot of the data indicated a Kd of 0.99 nM and a capacity of 30,000 sites per cell. Similar experiments performed with ^{125}I-labeled rTIMP-2 delta indicated an absence of saturable specific binding even at concentrations of rTIMP-2 delta as high as 400 nM and a large degree of non-specific binding such that the data were difficult to interpret (Figure 4B). Therefore we compared the ability of unlabeled rTIMP-2 delta

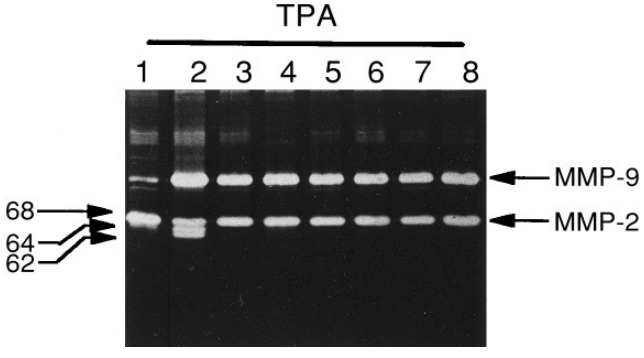

FIG. 3. Inhibition of MT1-MMP mediated proMMP-2 activation by rTIMP-2 and rTIMP-2 delta. Serum free conditioned media from HT1080 cells treated with TPA (100 ng/ml) in the presence or absence of rTIMP-2 or rTIMP-2 delta for 16 hours were collected, concentrated (20×), and loaded on a gelatin-containing SDS-polyacrylamide gel as indicated in Materials and Methods. The volume loaded in each lane was adjusted to represent 5 μg of proteins. Lane 1, conditioned medium from untreated HT1080 cells; lane 2, conditioned medium from cells treated with TPA; lanes 3 and 4, conditioned media from TPA-treated HT1080 cells after addition of rTIMP-2 (lane 3) and rTIMP-2 delta (lane 4) at concentrations of 50 nM; lanes 5 and 6, same as lanes 3 and 4 in the presence of 250 nM TIMP-2 (lane 5) and TIMP-2 delta (lane 6); lanes 7 and 8, same as lanes 3 and 4, in the presence of 500 nM TIMP-2 (lane 7) and TIMP-2 delta (lane 8).

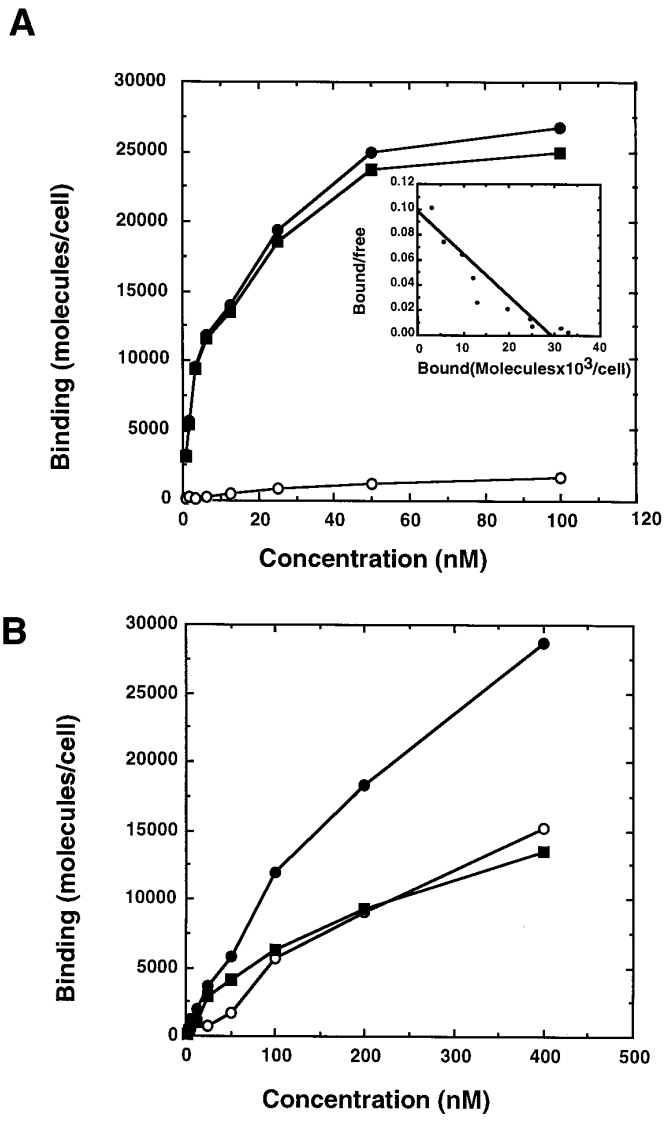

FIG. 4. Binding of ^{125}I-labeled rTIMP-2 and rTIMP-2 delta to the surface of TPA treated HT1080 cells. (A) The data represent specific binding (in molecules/cell) of ^{125}I-labeled rTIMP-2 to TPA treated HT1080 cells in the presence of increased concentrations of inhibitor. The specific binding (closed squares) was calculated by subtracting binding in the presence of 100 fold excess of nonradiolabeled TIMP-2 (open circles) from binding in the absence of unlabeled competitor (closed circles). Data represent the mean of 2 measurements. (Inset) Scatchard plot of bound/free over bound (in molecules per cell) from binding data for TIMP-2 shown in (A). (B) Binding of radiolabeled TIMP-2 delta to TPA treated HT1080 cells in the presence of increased concentrations of inhibitor. As in (A), closed squares represent specific binding values after subtraction of non-specific binding values (open circles).

and rTIMP-2 to compete with the binding of ^{125}I-labeled rTIMP-2 to TPA treated HT1080 cells. These competition experiments indicated that unlabeled rTIMP-2 began to compete with the binding of radiolabeled rTIMP-2 at equimolar concentration (50 nM), whereas even at 100 fold molar excess concentration, unlabeled rTIMP-

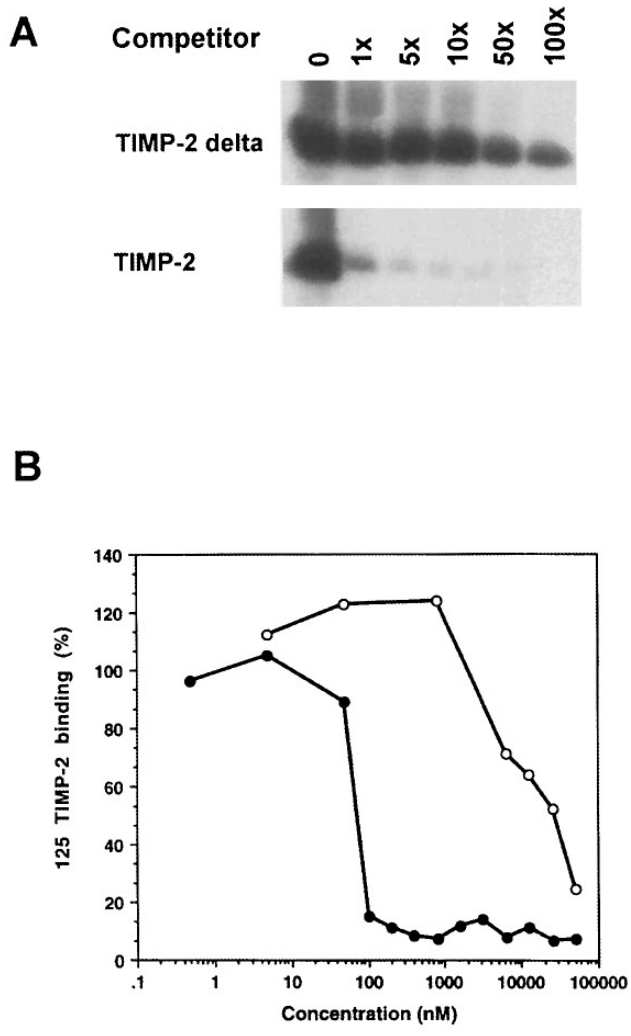

FIG. 5. Competition of ^{125}I-labeled rTIMP-2 binding to TPA-treated HT1080 cells by rTIMP-2 and rTIMP-2 delta. Radiolabeled TIMP-2 (50 nM) was incubated with TPA-treated HT1080 cells in the presence of indicated concentrations of non-radiolabeled rTIMP-2 or rTIMP-2 delta, as described in Materials and Methods. (A) Autoradiography of aliquots of the cell extracts corresponding to 10^6 cells loaded on a 0.1% (v/v) SDS, 12.5% (w/v) polyacrylamide gel. Numbers on the top of (A) indicate the excess of non-radiolabeled rTIMP-2 or rTIMP-2 delta added to the incubation mixture. (B) Competition curve showing the percent of ^{125}I-labeled TIMP-2 bound to the cell in the presence of increased concentrations of non-radiolabeled rTIMP-2 (solid circles) or non-radiolabeled rTIMP-2 delta (open circles).

2 delta failed to entirely displace ^{125}I-labeled TIMP-2 (Figure 5A). This resulted in a 2 orders of magnitude difference in the competition curves (Figure 5B). The data indicate a loss of high affinity binding when the C-terminal domain of TIMP-2 is absent.

DISCUSSION

Recently it has become apparent that TIMP-2 is a dual function protein that not only controls the activity of MMPs but also acts as ligand at the cell surface. Whereas the function of TIMP-2 as inhibitor of MMPs has been extensively studied, its function as ligand is not as well understood. In this report we demonstrate that the N-terminal domain of TIMP-2 behaves as a fully active inhibitor of MMPs. This observation is consistent with our previous report indicating that a trypsin generated proteolytic fragment of TIMP-2 consisting of an uncleaved N-terminal region extending from residue 1 to residue 132 plus a small C-terminal fragment linked by the disulfide bond Cys^{128}-Cys^{175} inhibits activated MMP-1 [17]. It is also consistent with the work of others who have demonstrated that C-terminally-truncated TIMP-1 and TIMP-2 effectively inhibit matrilysin and the catalytic activity of stromelysin and gelatinase A [16]. We also demonstrate that the N-terminal domain of TIMP-2 inhibits the activation of proMMP-2 in HT1080 cells treated with TPA. ProMMP-2 activation occurs via a two-step process that involves an initial cleavage of the Asn^{37}-Lys^{38} by MT1-MMP that converts the 68 kDa proenzyme to a 64 kDa intermediate. This cleavage is followed by an autocatalytic conversion of the intermediate into a 62 kDa active enzyme [9]. Since in the presence of rTIMP-2 and rTIMP-2 delta both activation steps were inhibited, the data suggest that the N-terminal domain of TIMP-2 can inhibit the cleavage of the prodomain of MMP-2 by MT1-MMP. These observations are consistent with the concept that activation of these proMMPs involves metalloproteinase dependent steps which can be effectively blocked by the N-terminal domain of TIMP-2.

Comparison between the binding activities of rTIMP-2 and rTIMP-2 delta at the surface of HT1080 cells brings some novel insight into the binding function of TIMP-2. Whereas we report a Kd value of 0.9 nM for rTIMP-2 binding to HT1080 cells, Strongin *et al.* [23] and Emmert-Buck *et al.* [21] have reported Kd values of 2.5 and 2.54 nM, respectively, for the same cells and a Kd of 0.35 nM for Raji cells was reported by Hayakawa *et al.* [20]. Based on the observation that binding of rTIMP-2 could be displaced in the presence of 100 fold excess rTIMP-2 delta, Emmert-Buck *et al.* concluded that the N-terminal domain of TIMP-2 contains the full ligand domain [21]. However, we show that this is most likely not the case because our competition experiments covering a range of concentrations demonstrated a significant difference between the N-terminal domain and the full length protein. Thus our data suggest that the binding site is comprised of a region that includes both the C-terminal and the N-terminal domains. Therefore the C-terminal domain which is not required for the antimetalloproteinase function of TIMP-2, appears essential for the high affinity binding of the inhibitor to the cell surface.

The exact nature of the TIMP-2 receptor and its specific role remain to be further investigated. It has been proposed that MT1-MMP is a receptor for TIMP-2

based on the isolation of trimolecular complexes between TIMP-2, MT1-MMP and the C-terminal domain of proMMP-2 [23]. However the possibility of another TIMP-2 binding protein located at the cell surface cannot be eliminated. The availability of the mutant described in this study will help to clarify the actions of TIMP-2 at the cell surface.

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