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Cell surface binding of TIMP-2 and pro-MMP-2/TIMP-2 complex

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Abstract Tissue inhibitor of metalloproteinases (TIMP-2) is a low molecular weight proteinase inhibitor capable of inhibiting activated matrix metalloproteinases (MMPs). TIMP-2 is found both free and in a 1:1 stoichiometric complex with the pro-enzyme form of MMP-2 (pro-MMP-2/TIMP-2 complex). We have measured the binding of recombinant TIMP-2 to intact HT-1080 and MCF-7 cells. HT-1080 cells in suspension bound ^{125}I-labeled rTIMP-2 with a K_d of 2.5 nM and 30,000 sites/cell. Monolayers of MCF-7 cells were similarly found to bind [^{125}I]rTIMP-2 with a K_d of 1.6 nM and 25,000 sites/cell. Specific binding of MMP-2 alone to HT-1080 cells was not observed; however, pro-MMP-2/TIMP-2 complex was capable of binding to the surface of HT-1080 cells in a TIMP-2-dependent manner. Binding of rTIMP-2 was not competed by the presence of TIMP-1. These results suggest that rTIMP-2 alone binds directly to the cell surface of HT-1080 and MCF-7 cell lines, and TIMP-2 is capable of localizing MMP-2 to the surface of HT-1080 cells via interaction with a specific binding site.

Key words: Metalloproteinase; Tumor cell; Invasion; Activation; Tissue Inhibitor; TIMP-2

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes involved in extracellular matrix (ECM) turnover [1]. Members of the metalloproteinase family mediate ECM degradation during physiologic tissue remodeling, and have been implicated in a number of pathologic conditions including tumor invasion and osteoarthritis [2]. The regulation of MMPs is complex and occurs at many levels including gene expression, activation of secreted pro-enzyme forms, and inhibition of active enzyme forms [3]. MMP-2 (progelatinase A; 72 kDa type IV collagenase) is secreted from many cell lines in culture as a 72 kDa pro-enzyme which must be processed extracellularly to a 62 kDa active enzyme [2]. The active enzyme is capable of degrading several extracellular matrix components including type IV collagen and fibronectin [1].

Activity of MMPs can be modulated by interaction with specific proteinase inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). Three different TIMPs have been identified: TIMP-1, TIMP-2 and TIMP-3 [4–8]. TIMP-2 is a non-glycosylated, 192 amino acid protein of 21 kDa [4,5] which preferentially complexes with the pro-enzyme form of MMP-2, suggesting that it may selectively modulate activity of the enzyme [9]. A cell surface MMP-2 activating species has been described in HT-1080 fibrosarcoma cells which is capable of processing pro-MMP-2 to active MMP-2 [10]. The cell surface activating species has been proposed to be MT-MMP, a recently discovered member of the matrix metalloprotinease family[11]. The role of TIMP-2 in modulating pro-MMP-2 activation by these cells is not known.

Previous reports have shown specific TIMP-1 binding to cell lines in culture [12,13]. A recent report shows that TIMP-2 is also capable of direct cell surface binding [14]. In this study we examined the cell surface interaction of TIMP-2 and MMP-2/TIMP-2 complex with MCF-7 breast adenocarcinoma cells and HT-1080 fibrosarcoma cells. Our results show that both cell lines exhibit specific rTIMP-2 binding, and suggest that TIMP-2 binding may be important in localizing MMP-2/TIMP-2 complex to the cell surface.

2. Materials and methods

2.1. Proteins

Recombinant TIMP-2 (rTIMP-2) and C-terminally truncated TIMP-2 (Δ126–194) were prepared in a vaccinia virus mammalian cell expression system, as previously described [15]. Purified rTIMP-2 was generously provided by B. Bird, Oncologix Inc., Gaithersburg, MD. TIMP-1 expressing CHO cells were obtained from Genetics Institute (Cambridge, MA) and TIMP-1 purified from conditioned media by affinity chromatography. pro-MMP-2/TIMP-2 complex was purified from A2058 conditioned media as previously described [5]. The TIMP-2 C-terminal peptide, AWYRGAAPPKQEFLDIEDP, consisting of residues 176–194 of the mature TIMP-2 protein, was synthesized on a Biosearch 9600 peptide synthesizer and purified by reverse phase HPLC.

2.2. Binding experiments

rTIMP-2 and pro-MMP-2/TIMP-2 were iodinated using the lactoperoxidase method with Enzymobead radio-iodination reagent (Bio-Rad), following the manufacturer's procedure to a specific activity of $4–5 \times 10^6$ cpm/μg.

Binding assays of ^{125}I-labeled rTIMP-2 to HT-1080 cells (ATCC) in suspension were performed in triplicate as follows. Cells were grown to 80–90% confluency in DMEM supplemented with 10% FBS (Gibco BRL). Cells were then washed 3 times with cold PBS with 0.1% BSA in the absence of calcium, and incubated for 1 h in the same buffer. Cells were gently detached from the flask with a cell scraper, washed 3 times in calcium-free PBS with 0.1% BSA, and resuspended in calcium containing PBS with 0.1% BSA. 10 nM ^{125}I-labeled rTIMP-2 was added to the cells for 3 h at 4°C in the presence or absence of 1 μM unlabeled rTIMP-2. In some experiments, 1 μM unlabeled TIMP-1, 1 μM pro-MMP-2/TIMP-2 complex, or 10 μM of the TIMP-2 C-terminus peptide was added to the cells for 15 min prior to the addition of labeled rTIMP-2. Cells were washed 3 times with PBS with 0.1% BSA with calcium and counted in a gamma-counter. Specific binding was calcu-

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Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; rTIMP-2, recombinant TIMP-2; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; SDS, sodium dodecyl sulfate; DMEM, Dulbecc'os modified eagles medium; FBS, fetal bovine serum; dpm, disintegrations per minute.

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lated as the difference in bound ^{125}I-labeled rTIMP-2 in the presence or absence of unlabeled rTIMP-2, TIMP-1, pro-MMP-2/TIMP-2 complex, or C-terminus TIMP-2 peptide. Similar experiments with ^{125}I-labeled rMMP-2 and pro-MMP-2/TIMP-2 complex were also performed.

Binding assays were performed in triplicate with MCF-7 cells (ATCC) grown as a monolayer. Cells were first plated in a 24-well plate in DMEM supplemented with 10% FBS. After 1 day of culture, the cells were washed 3 times with DMEM containing 20 mM HEPES, 0.1% BSA. Saturation experiments were performed for 3 h at 22°C with various concentrations of [^{125}I]TIMP-2 (0.2–20 nM) in the presence or absence of 2 μM unlabeled rTIMP-2. Cell monolayers were then washed 3 times with PBS with 0.1% BSA, lysed with 0.1% SDS in 0.5 M NaOH, and the bound radioactivity was measured by gamma-counting. For the analysis of binding data, best fit-curves were determined using the MLAB program [16,17] with equations as previously described [18].

2.3. Electrophoresis

^{125}I-Labeled rTIMP-2 was incubated with HT-1080 cells in the presence or absence of 100-fold excess unlabeled rTIMP-2. Cells were washed 3 times with calcium containing PBS, and solubilized in calcium containing PBS with 1% Triton X-100 at 4°C in the presence of proteinase inhibitors. A portion of the initial supernatant of the sample with excess unlabeled TIMP-2 was added to the same cell preparation after solubilization such that the two samples (i.e. labeled rTIMP-2 ± excess unlabeled rTIMP-2) contained equivalent amounts of labeled rTIMP-2. Solubilized cells were centrifuged in a Beckman centrifuge at 100,000 × g for 1 h at 4°C. Supernatants were analyzed by electrophoresis and autoradiography using a 4–20% gradient polyacrylamide gel under non-denaturing, non-reducing conditions.

3. Results

Specific binding of ^{125}I-labeled rTIMP-2 to HT-1080 and MCF-7 cells was observed. The time course for the binding of TIMP-2 to an MCF-7 cell monolayer shows that equilibrium is reached at 3 h at 22°C (Fig. 1). The concentration dependence of TIMP-2 binding to the MCF-7 cells demonstrated specific and saturable binding characteristics (Fig. 2A). Scatchard analysis of binding data showed the existence of a single class of high-affinity binding sites with a K_d of 1.6 nM, and 25,000 sites per cell (Fig. 2B, Table 1). Similar results were obtained with isolated membrane preparations indicating the binding site localizes to the plasma membrane (results not shown).

Analysis of ^{125}I-labeled rTIMP-2 binding to HT-1080 cells in suspension was performed at 22°C for 3 h based on the TIMP-2 binding experiments performed with MCF-7 cells. Again, the concentration dependence of this binding showed specific and saturable binding of TIMP-2 to the HT-1080 cells (data not shown). Scatchard analysis of the TIMP-2 binding to HT-1080 cells in suspension gave results (Table 1) that are in excellent agreement with those obtained for MCF-7 cell monolayers. HT-1080 cells had a dissociation constant for TIMP-2 binding K_d = 2.5 nM, with approximately 30,000 sites per cell.

Based on the Scatchard analysis of binding, further exploration of the nature of the TIMP-2 binding to HT-1080 cells was performed using 10 nM concentration of ^{125}I-labeled rTIMP-2. Addition of 100-fold molar excess of pro-MMP-2/TIMP-2 complex, or C-terminally truncated TIMP-2 completely abolished binding of rTIMP-2 to the cells (Fig. 3). Under these conditions non-specific binding of TIMP-2 was minimized to less than 15% of total binding. The specific binding of ^{125}I-labeled rTIMP shown in Fig. 3 represents the difference between total ^{125}I-labeled rTIMP bound (3823 ± 35 dpm) and ^{125}I-labeled rTIMP bound in the presence of a 100-fold molar excess of unlabeled rTIMP-2 (615 ± 14 dpm). However, competition with 100-fold molar excess of TIMP-1 did not affect rTIMP-2 binding (Fig. 3) nor did the addition of 1000-fold molar excess of the linear peptide, AWYRGAAPPK-QEFLDIEDP, representing the C-terminus of TIMP-2 (Fig. 3). These findings are consistent with the known interactions of pro-MMP-2 and TIMP-2 which are mediated by direct C-terminal interactions, and suggest that the cell-surface binding domain of TIMP-2 is located elsewhere within the TIMP-2 molecule.

Table 1
MCF-7 and HT-1080 cell binding data

Cell line	TIMP-2 binding		MMP-2 binding
	K_d	Sites/cell	
HT-1080	2.5 nM	30,000	Mediated by TIMP-2
MCF-7	1.6 nM	25,000	Direct?

The two cell lines show similar TIMP-2 binding characteristics, but differ in the binding and activation of MMP-2.

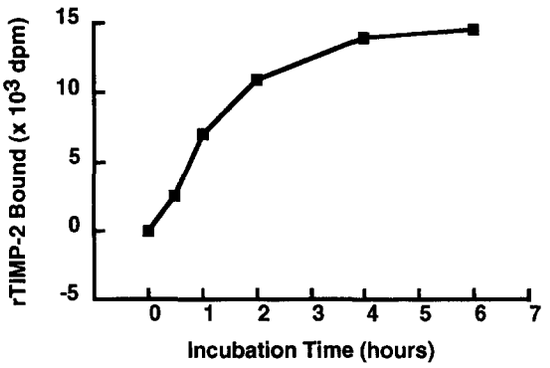

Fig. 1. Time course of binding of rTIMP-2 to MCF-7 cells. Steady-state binding was achieved after 3 h of incubation as described in section 2.

^{125}I-Labeled pro-MMP-2/TIMP-2 complex exhibited specific binding to HT-1080 cells in suspension, but ^{125}I-labeled pro-rMMP-2 alone did not (data not shown). The binding of ^{125}I-labeled pro-MMP-2/TIMP-2 complex was not affected by the presence of 100-fold molar excess of TIMP-1, but was completely abolished in the presence of excess rTIMP-2, or C-terminally truncated TIMP-2 (Fig. 4). The binding of both components of the ^{125}I-labeled pro-MMP-2/TIMP-2 complex to the cells was confirmed by SDS-PAGE and autoradiography (results not shown). These results are again consistent with the cell-surface binding of TIMP-2 being mediated by peptide sequence that is not located in the final C-terminal peptide loop or tail of the TIMP-2 peptide.

Native gel electrophoresis performed on solubilized HT-1080 cells after binding with labeled rTIMP-2 showed a specific shift in mobility of the rTIMP-2 (Fig. 5). Under non-denaturing, non-reducing conditions, rTIMP-2 alone migrates as a diffuse band (Fig. 5; lane 1). Electrophoresis performed after HT-1080 binding and solubilization shows the labeled rTIMP-2 shifted to a compact band which migrated less distance in the gel, due to association with the HT-1080 binding moiety (Fig. 5; lane 2). Competition with excess unlabeled rTIMP-2 dissociates the ^{125}I-labeled binding complex and the ^{125}I-labeled rTIMP-2 migrates with the free rTIMP-2 (Fig. 5; lane 3).

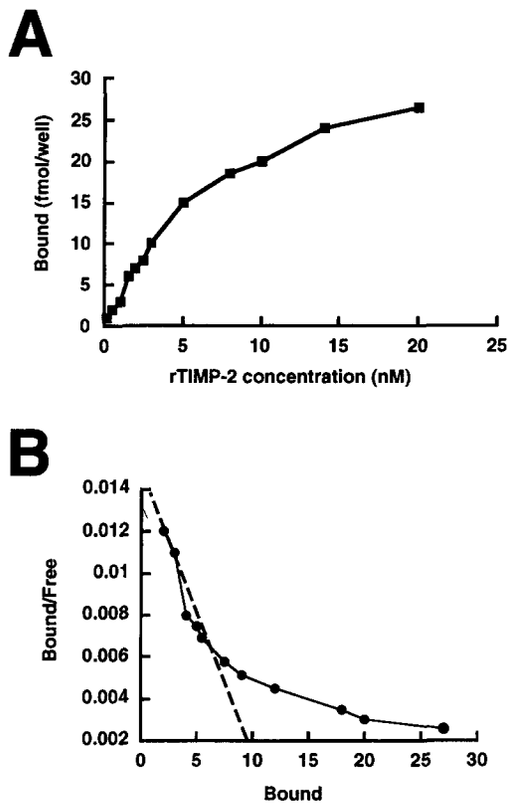

Fig. 2. Steady-state binding of ^{125}I-labeled rTIMP-2 to monolayers of MCF-7 cells. Binding was carried out in triplicate using various concentrations of ^{125}I-labeled rTIMP-2 for 3 h at 22°C. Specific binding was calculated as the difference in bound radioactivity in the presence or absence of excess unlabeled rTIMP-2. Fig. 2. (A) Binding curve showing saturation of binding in MCF7 monolayers (specific activity of ^{125}I-labeled rTIMP-2 = 4,800 cpm/ng; each well contained 2.1×10^5 cells per well). B: Scatchard plot of the binding data. Analysis of the data indicated MCF-7 cells contain 25,000 sites/cell and bind to TIMP-2 with a K_d of 1.6 nm.

4. Discussion

In the present study we describe the identification of a binding site for rTIMP-2 on HT-1080 fibrosarcoma and MCF-7 breast adenocarcinoma cells. Similar binding characteristics were observed with both cell types. TIMP-2 dependent binding of pro-MMP-2/TIMP-2 complex to HT-1080 cells was also observed, representing a mechanism for localizing MMP-2 to the cell surface. The TIMP-2-mediated binding of MMP-2 to HT-1080 cells and apparent independent binding of TIMP-2 and MMP-2 [18] directly to MCF-7 cells may represent two models of metalloproteinase cell surface interaction (Table 1).

HT-1080 cells produce endogenous MMP-2 and TIMP-2, while MCF-7 cells produce only low levels of endogenous TIMP-2. Both MCF7 and HT1080 cells are capable of directly binding TIMP-2 and pro-MMP-2/TIMP-2 complex, and we have shown previously that MCF-7 cells can possibly bind MMP-2 directly [19]. However, in these earlier experiments we did not directly asses MCF-7 production of endogenous TIMP-2 which could mediate the binding of exogenous MMP-2. Furthermore, the exogenous MMP-2 preparation used in these studies was purified from the conditioned media of BeWo cells and contains pro-MMP-2/TIMP-2 complex. The similarity of the dissociation constants and number of binding sites reported previously for MMP-2 on MCF-7 cells and in the present study for TIMP-2 suggests that TIMP-2 mediated binding of MMP-2 in MCF-7 cells is a distinct possibility. Although we cannot presently rule out that MMP-2 may bind to some cells independently of TIMP-2. HT-1080 cells cannot bind MMP-2 alone and compared to MCF-7 cells they are unique in their ability to synthesize, secrete and activate the enzyme.

In vivo, cells like HT-1080 would be capable of producing and activating pro-MMP-2 and/or pro-MMP-2/TIMP-2 complex independently. On the other hand, cells like MCF-7 would bind the active enzyme, or active enzyme complex to the cell surface and effect degradation of ECM molecules. This model is consistent with in vivo studies showing that stromal fibroblasts produce MMP-2 mRNA, but the protein product is localized to nearby epithelial cells [20–22]. The significance of producing active MMP-2 versus active MMP-2/TIMP-2 complex is unclear. Activated MMP-2 alone is more proteolytically active than MMP-2/TIMP-2 complex, but has a shorter half-life than enzyme complex due to autolysis [23]. The cells could potentially produce short bursts of high proteolytic activity by secreting and activating MMP-2 alone, as opposed to activating MMP-2/TIMP-2 complex and producing sustained but lower levels of proteolytic activity.

Binding of free TIMP-2 may also serve an important protective function for cells in the vicinity of ECM degradation. Placement of the inhibitor at the cell surface may allow stromal or epithelial cells to actively participate in tissue remodeling without extensive degradation of cell surface molecules. Normal wound healing, for example, consists of sequential steps of tissue resorption and regeneration in which stromal and epithelial cells are in close proximity to regions of ECM undergoing extensive proteolysis. Unrestricted proteolysis of cellular proteins would presumably be deleterious to cells mediating this process. Localization of free TIMP-2 to the membranes of these cells would be an effective inhibitor of cell surface proteolysis mediated by any members of the MMP class.

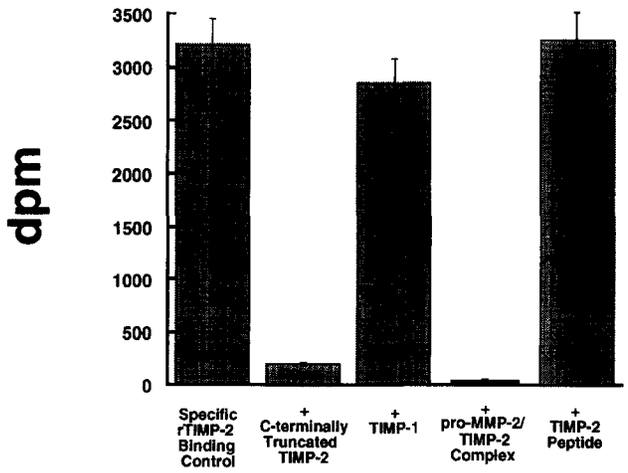

Fig. 3. Specific binding of rTIMP-2 to HT-1080 cells in suspension. Binding was inhibited by 100 fold molar excess of C-terminally truncated TIMP-2, or 100-fold molar excess of MMP-2/TIMP-2 complex. The C-terminally truncated TIMP-2 is missing the domain responsible for interaction with pro-MMP-2. 100-fold molar excess TIMP-1, and 1000-fold molar excess of TIMP-2 C-terminal peptide did not demonstrate significant competition for TIMP-2 binding.

TIMP-2 is a bifunctional protein with growth stimulatory as well as proteinase inhibitory activity. An additional function of TIMP-2 cell surface binding may be in mediating cell proliferation. Several previous reports have shown that both TIMP-1 and TIMP-2 can stimulate a proliferative response, and recent findings suggest that rTIMP-2 is growth stimulatory for HT-1080 cells (Corcoran, M.L. and Stetler-Stevenson, W.G., unpublished results). Cell surface binding sites for TIMP-1 and TIMP-2 have been reported, however, cell surface receptors for these molecules have not been isolated [12–14]. HT-1080 cells show increased cAMP levels when treated with rTIMP-2 suggesting that TIMP-2 modulation of cell growth is mediated via a receptor/second messenger system (Corcoran, M.L. and Stetler-Stevenson, W.G., unpublished results). The relationship between the binding moiety identified in the present study and the putative rTIMP-2 receptor responsible for HT-1080 cell proliferation is presently not known. Thus, TIMP-2 is capable of mediating several important physiologic processes involving cellular interactions with the ECM. In normal physiologic processes such as tissue remodeling and repair, TIMP-2 has the potential to modulate MMP-2 activity during ECM degradation, protect cell surface molecules from extensive degradation, inhibit all active MMP's after sufficient ECM degradation has occurred, and stimulate proliferation of fibroblasts during tissue reconstitution.

Two binding sites on MMP-2 have been described for TIMP-2 [9,24,25]. The C-terminal region of TIMP-2 binds to the C-terminus of pro-MMP-2 to form the pro-MMP-2/TIMP-2 complex, and the N-terminal region of TIMP-2 can interact with the active site of the enzyme to inhibit proteolysis [9,23–26]. The results of the present study suggest that a third domain in rTIMP-2 mediates the binding to HT-1080 cells. C-Terminally truncated TIMP-2 inhibited binding of labeled rTIMP-2 to HT-1080 cells, and the TIMP-2 C-terminus peptide did not inhibit binding suggesting that TIMP-2 does not bind to HT-1080 cells via the C-terminal region. Since the HT-1080 binding site is capable of binding TIMP-2, but not TIMP-1, we can postulate that the binding moiety is not an active MMP which

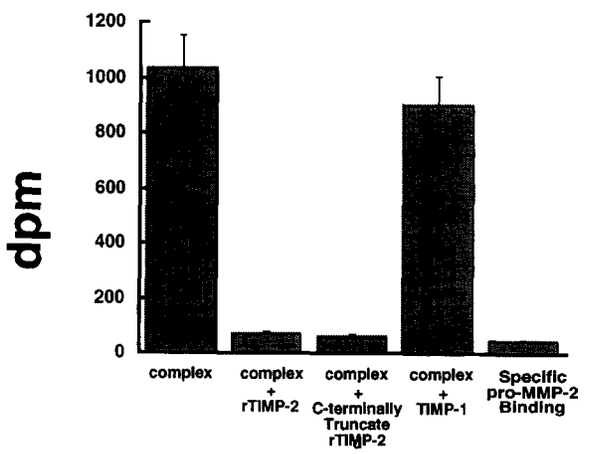

Fig. 4. Specific binding of pro-MMP-2 and pro-MMP/TIMP-2 complex to HT-1080 cells in suspension. Binding of th pro-MMP-2/TIMP-2 complex was competed with both 100-fold molar excess TIMP-2, as well as 100-fold molar excess of C-terminally truncated TIMP-2. Excess TIMP-1 did not inhibit binding of the pro-MMP-2/TIMP-2 complex. Binding of complex was not competed by the addition of cold pro-MMP-2 and direct binding of [^{125}I]pro-MMP-2 alone was not observed.

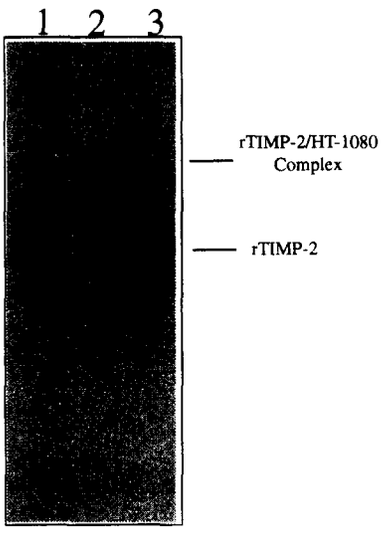

Fig. 5. Electrophoresis of TIMP-2-cell surface complex. Polyacrylamide gel electrophoresis analysis of TIMP-2 bound to HT-1080 cells followed by solubilization with 1% triton X-100. Lanes represent: (1) radiolabeled rTIMP-2 alone, (2) radiolabeled rTIMP-2 bound to solubilized HT-1080 cells, and (3) radiolabeled rTIMP-2 bound to solubilized HT-1080 cells in the presence of excess unlabeled rTIMP-2. Electrophoresis was run under non-denaturing, non-reducing conditions. The shift in electrophoretic mobility observed in lane 2 suggests the rTIMP-2 binds to a specific HT-1080 cell surface molecule.

is interacting with the N-terminal inhibitory domain of TIMP-2. All members of the MMP family to date are capable of being inhibited by both TIMP-1 and TIMP-2 [1,26]. Thus, interaction of TIMP-2 with the HT-1080 cell surface binding site is most likely mediated by a third functional domain in the inhibitor. The selective binding of TIMP-2 to HT-1080 cells also suggests that TIMP-2 and TIMP-1, which are closely related proteins, possess distinct cell-surface binding domains.

In summary, we describe a binding site for rTIMP-2 on the surface of two human cell lines. The binding site may be important in mediating several TIMP-2 dependent actions including cell proliferation, and cell surface modulation/localization of MMP-2.

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