Tyrosine phosphorylation is crucial for growth signaling by tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2)

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Abstract **[^{3}H]Thymidine (TdR) incorporation by human osteosarcoma cell line MG-63 was significantly stimulated at as early as 3 h after the addition of either TIMP-1 or TIMP-2 alone. Maximum stimulation was attained at a concentration of either 20 ng/ml (0.71 nM) TIMP-1 or 1.0 ng/ml (46 pM) TIMP-2. Tyrosine kinase inhibitors such as genistein, erbstatin, and herbimycin A almost completely inhibited the [^{3}H]TdR incorporation stimulated by either of the TIMPs. However, essentially no effect was observed with H-89, H-7, bisindolylmaleimide and K-252a. These inhibition studies suggest a crucial role for tyrosine kinase in the signal transduction of TIMPs. Phosphotyrosine-containing proteins were significantly elevated by the treatment with both TIMPs. We also found that either TIMP stimulated an increase in mitogen-activated protein (MAP) kinase activity, suggesting that MAP kinase plays a role in TIMP-dependent growth signaling.**

Key words: Tissue inhibitor of metalloproteinase; Cell growth factor; Tyrosine kinase; Mitogen-activated protein (MAP) kinase; MG-63 cell

1. Introduction

Tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2, and TIMP-3) are now recognized as a family of intrinsic common inhibitors of matrix metalloproteinases (MMPs) represented by interstitial collagenase, and both TIMP-1 and TIMP-2 also inhibit the autoactivation step of progelatinase activation by forming complexes with progelatinases in a 1:1 stoichiometry [1,2]. It therefore seems that TIMPs play an important role in the regulation of MMP activity under both physiological and pathological conditions.

In addition to their functions as MMP inhibitors, both TIMP-1 and TIMP-2 are reported to have potent growth-promoting activity for a wide range of cells, and some findings support the proposition that both TIMPs are previously unrecognized cell-growth factors in serum [3,4]. The cell growth-promoting activity of both TIMPs appears to be a direct cellular effect mediated by cell-surface receptors [4–6], and is independent of their functions as MMP inhibitors. While growth signaling by a wide range of growth factors depends on tyrosine phosphorylation of cellular proteins and some of these factors activate the mitogen-activated protein (MAP) kinases [7,8], recent papers suggest that members of the Janus kinase (JAK) family activate signal transducers and activators of transcription (STATs) that are directly involved in the regulation of gene transcription [9,10]. Thus, it has yet to be elucidated whether tyrosine kinase and its downstream-signaling factors are involved in growth stimulation by TIMPs.

In this study, we report that the stimulation of DNA synthesis by either TIMP-1 or TIMP-2 is mediated through tyrosine phosphorylation following the activation of MAP kinase.

2. Materials and methods

2.1. Materials

The materials used and their sources were as follows: [*methyl*-^{3}H]thymidine ([^{3}H]TdR) from Amersham International plc (Bucks, UK); [γ-^{32}P]ATP (370 MBq/ml) from Dupont NEN (DE, USA); myelin basic protein (MBP) of bovine brain from Sigma Chemical Co. (MO, USA); genistein from Extrasynthese S.A. (Genay, France); erbstatin from Research Biochemicals International (MA, USA); herbimycin A from Kyowa Medex Co. (Tokyo, Japan); H-89, H-7 and K-252a from Biomol Research Laboratories, Inc. (PA, USA); Dulbecco's modified Eagle's minimal essential medium (D-MEM) and fetal calf serum (FCS) from Gibco Laboratories (Grand Island, NY); anti-extracellular-signal-regulated protein kinase 2 (ERK2) antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and human osteosarcoma cells (MG-63, CRL 1427) from the American Type Culture Collection (Rockville, MD). TIMP-1- and TIMP-2-free FCS was prepared as previously described [4]. Anti-phosphotyrosine (PTYR) polyclonal antibody was purchased from ICN Biomedicals, Inc. (CA, USA).

2.2. Preparation of human TIMPs and their assays

Human recombinant TIMP-1 and TIMP-2 were prepared as reported previously [4]. TIMP-1 and TIMP-2 concentrations were determined by one-step sandwich enzyme immunoassays for TIMP-1 [11] and TIMP-2 [12].

2.3. [^{3}H]TdR incorporation assay

Cells were plated into 24-well plastic plates at 1×10^5 cells/well and then allowed to grow to confluence in 1 ml of D-MEM containing 10% FCS. The confluent cultures were then shifted to serum-free D-MEM for 48 h before the assay. The cells were incubated in D-MEM containing TIMP-1 or TIMP-2 for the indicated times, and incubation was extended for a further 5 h after the addition of 0.8 μCi/ml [^{3}H]TdR solution. The cultures were then washed three times with 1 ml of ice-cold phosphate-buffered saline. After having been left in ice-cold 5% trichloroacetic acid for 20 min, the precipitates were washed twice with 1 ml of the same trichloroacetic acid solution. The precipitates were subsequently solubilized with 0.5 M NaOH and [^{3}H]TdR incorporated was determined by use of a liquid scintillation spectrometer with 40% counting efficiency.

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Abbreviations: TIMP, tissue inhibitor of metalloproteinases; ^{3}H]TdR, [^{3}H]thymidine; PTYR, phosphotyrosine; MAP kinase, mitogen-activated protein kinase; D-MEM, Dulbecco's modified Eagle's minimal essential medium; FCS, fetal calf serum; ERK, extracellular-signal-regulated protein kinase

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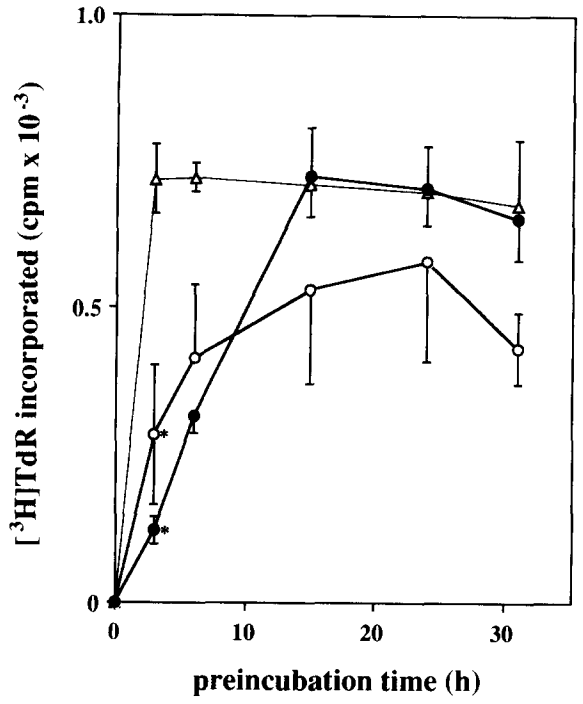

Fig. 1. Effects of preincubation time on TIMP-dependent [^{3}H]TdR incorporation by human osteosarcoma cell line MG-63. MG-63 cells grown to confluence were incubated in D-MEM containing either 20 ng/ml TIMP-1 or 1.0 ng/ml TIMP-2 for the indicated times. The incubation was extended for a further 5 h after the addition of 0.8 μCi/ml [^{3}H]TdR. [^{3}H]TdR incorporated into the cells was determined as described in Section 2. All the values with TIMP-1 (○) and TIMP-2 (●) are shown as net values after subtracting the corresponding control (D-MEM alone) value (△). *$p < 0.05$ from time 0. ←

2.4. Immunoblotting

Confluent cells were starved for 24 h in D-MEM, subsequently treated with acidic medium for 3 min [13], and then incubated for another 10 min in D-MEM. Finally, the cells were stimulated with 20 ng/ml TIMP-1 or 1.0 ng/ml TIMP-2 for the indicated times. Then the cells were washed with ice-cold phosphate-buffered saline containing 1.0 mM sodium orthovanadate, and quickly solubilized with buffer for gel electrophoresis containing 2% sodium dodecyl sulfate and 5% mercaptoethanol. The protein concentration was determined with a protein assay kit (Bio-Rad Laboratories), and 100 μg of proteins was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Millipore), probed with anti-PTYR antibody or anti-ERK2 antibody, and subsequently incubated with peroxidase-conjugated protein A (Amersham). Tyrosine-phosphorylated proteins or ERK2 was detected by an ECL system (Amersham). After transfer of the proteins from gels to membranes, the gels were stained with Coomassie blue to confirm the application of the proper amounts of proteins to the gels.

2.5. Assay for MAP kinase activity

This assay was performed using polyacrylamide gels containing MBP after SDS-PAGE according to a modified method [14] of Kameshita and Fujisawa [15]. Essentially the same procedure as men-

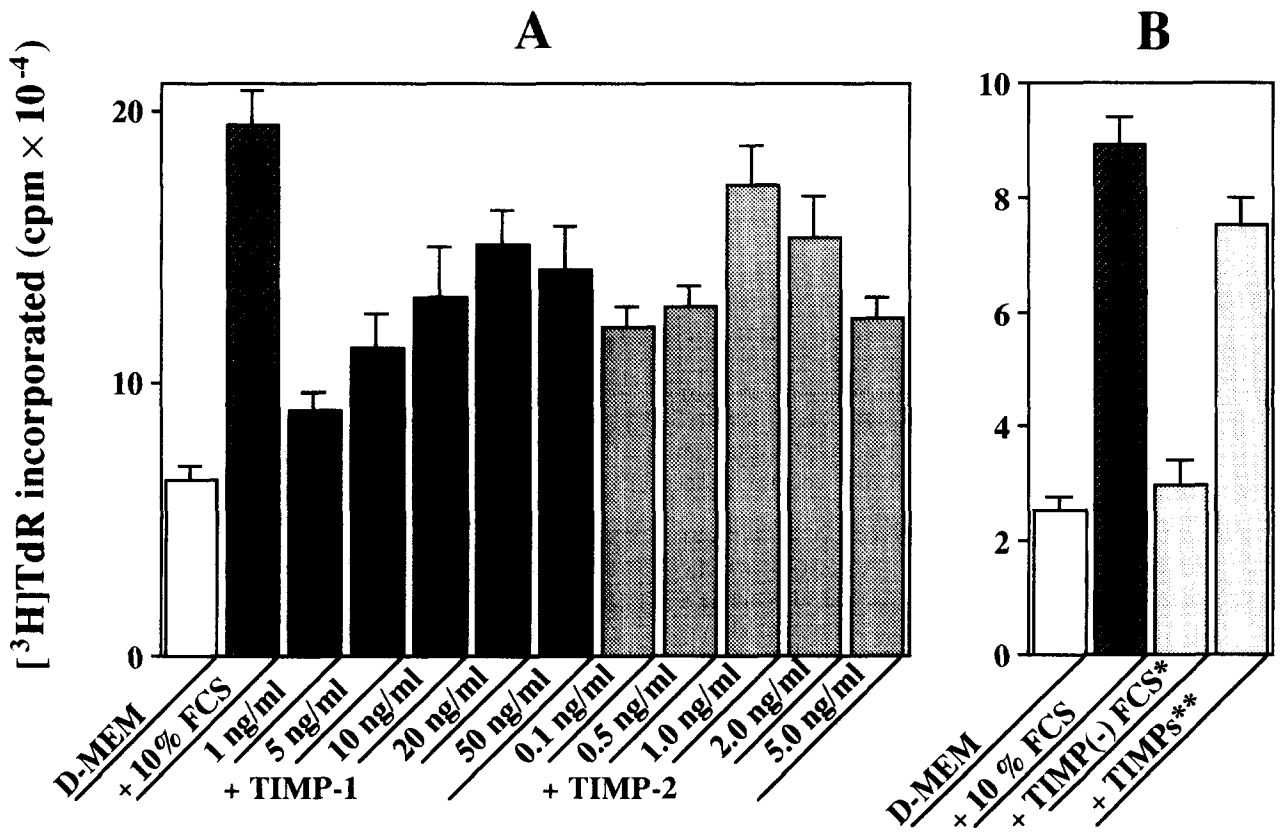

Fig. 2. Effects of addition of different concentrations of TIMP-1 and TIMP-2 (A) and depletion of both TIMPs from 10% FCS (B) on [^{3}H]TdR incorporation by MG-63 cells. MG-63 cells grown to confluence were incubated for 20 h in D-MEM containing the indicated amounts of either TIMP. The incubation was extended for a further 5 h after the addition of 0.8 μCi/ml [^{3}H]TdR. [^{3}H]TdR incorporated into the cells was determined as described in Section 2. *TIMP (−) FCS denotes 10% FCS freed of both TIMP-1 and TIMP-2. **The amounts of rTIMP-1 and -2 added were 38 and 32 ng/ml, respectively, which were the same as those originally contained in 10% FCS.

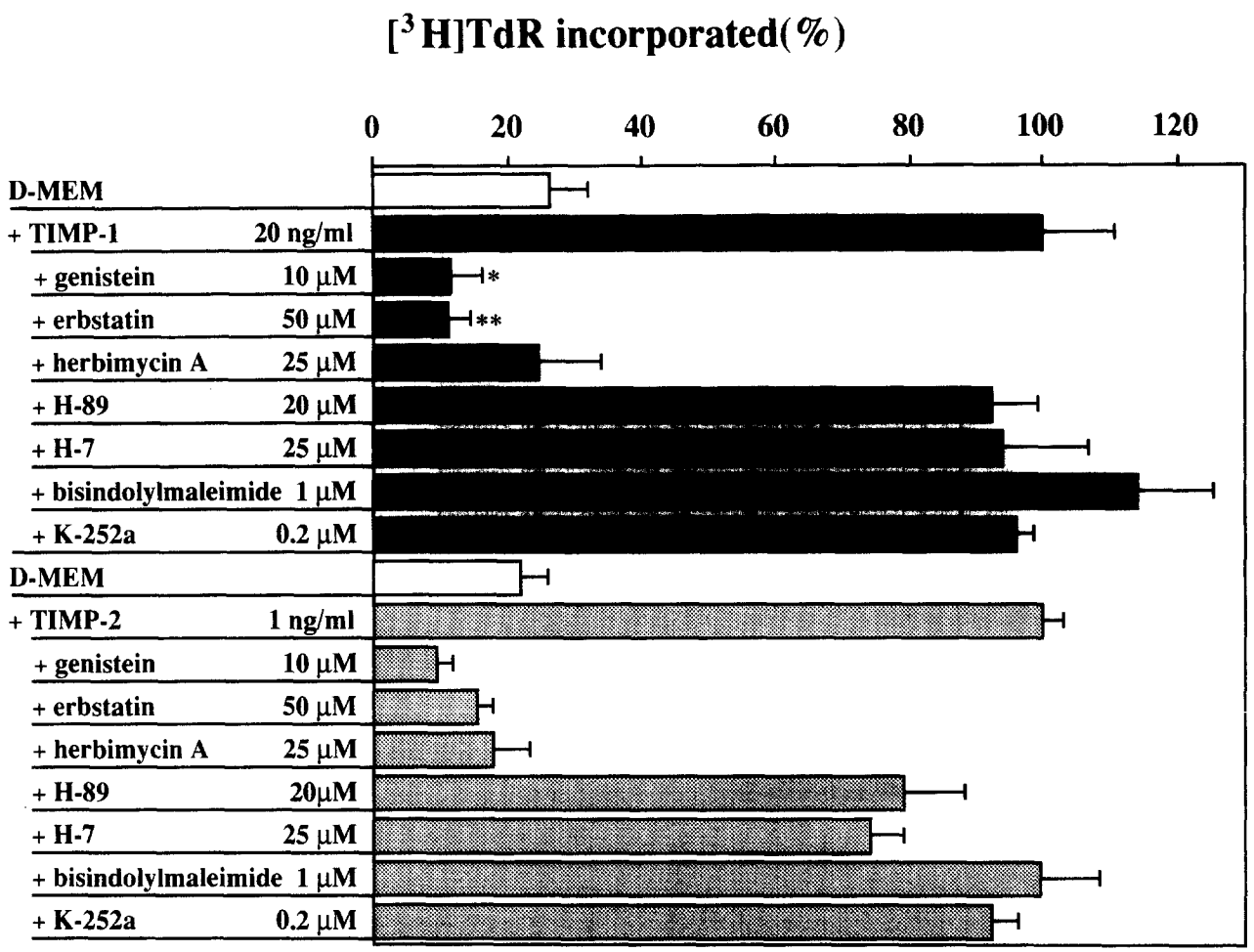

Fig. 3. Effects of protein kinase inhibitors on [^{3}H]TdR incorporation into MG-63 cells stimulated by both TIMPs. MG-63 cells having reached confluence were incubated for 16 h in D-MEM containing either of the TIMPs without or with inhibitors of the indicated concentrations. The incubation was extended for 5 h after the addition of 0.8 μCi/ml [^{3}H]TdR. Incorporation of radiolabel into the cells was determined as described in Section 2. $^*p < 0.005$ and $^{**}p < 0.02$ from D-MEM alone.

tioned above was used for the preparation of cell lysates except that the cells were incubated for 24 h instead of 10 min in D-MEM after the acid treatment prior to TIMP stimulation.

3. Results

We first examined the time course of TIMP-dependent [^{3}H]TdR incorporation by MG-63 cells. [^{3}H]TdR incorporation was significantly stimulated at 3 h after the addition of either TIMP-1 or TIMP-2, as shown in Fig. 1, and the incorporation remained linear up to 15–20 h. The effects of TIMPs on [^{3}H]TdR incorporation were dose dependent, showing maximum stimulation at a concentration of either 20 ng/ml TIMP-1 or 1.0 ng/ml TIMP-2 (Fig. 2A). The extent of stimulation with either TIMP was comparable to that obtained with 10% FCS. We also demonstrated that [^{3}H]TdR incorporation into MG-63 cells by 10% FCS was almost fully dependent on TIMPs in FCS (Fig. 2B).

To gain a clue as to the nature of the intracellular signal transduction, we next examined the effects of some protein kinase inhibitors on [^{3}H]TdR incorporation into the cells. As shown in Fig. 3, genistein, erbstatin and herbimycin A almost completely inhibited the incorporation of [^{3}H]TdR stimulated by either TIMP. However, essentially no effect was observed with H-89, H-7, bisindolylmaleimide or K-252a. These inhibition studies thus suggest the possible participation of tyrosine kinase in the signal transduction of TIMPs. To confirm this possibility, we examined the tyrosine-specific phosphorylation stimulated by either TIMP. As shown in Fig. 4A, tyrosine-specific phosphorylation in MG-63 cells was suppressed by serum depletion and was significantly increased in response to the addition of either TIMP.

When we examined the possible involvement of MAP kinases in the downstream signal transduction pathway, we found that both TIMPs significantly stimulated the activation of one of the MAP kinases, ERK2 (Fig. 4B), and the activity of this kinase (Fig. 4C) in MG-63 cells.

4. Discussion

We reported earlier that human TIMP-1 and TIMP-2 have potent growth-promoting activity for a wide range of cells [3,4]. TIMP-3, produced by chicken embryo fibroblasts, was also shown to have a cell growth-promoting activity [16], leading us to conclude that such activity is a common feature of members of the TIMP family. The cell growth-promoting activity of both TIMP-1 and -2 is suggested to be a direct cellular effect mediated by cell surface receptors [4–6], rather

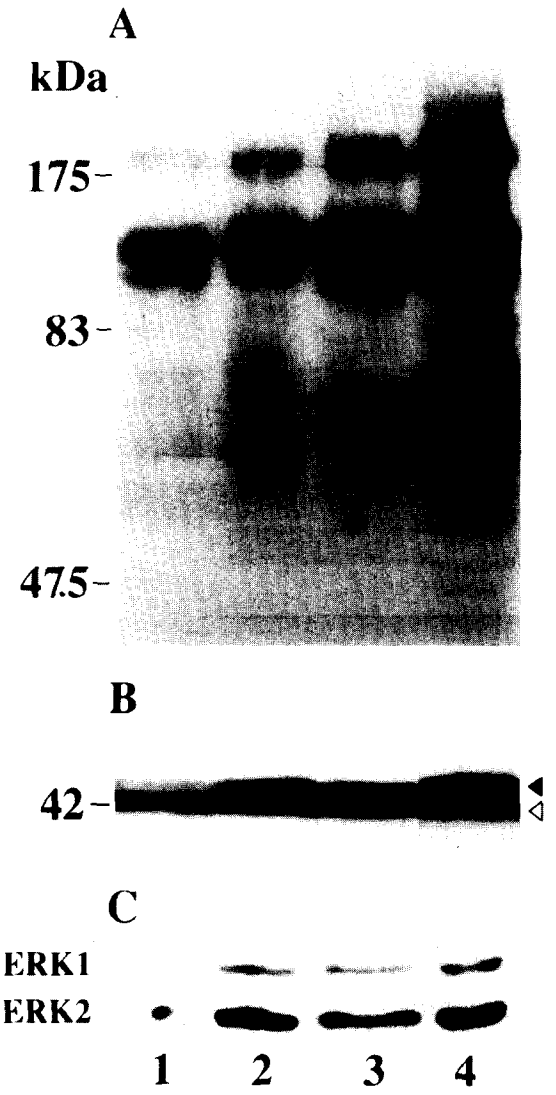

Fig. 4. Increase in amount of PTYR-containing proteins (A), activation of MAP kinase (B), and detection of the kinase activity (C) in MG-63 cells treated with either TIMP-1 or TIMP-2. Confluent cell cultures were starved in serum-depleted medium and subsequently stimulated with either TIMP for 10 min. Cell lysates from MG-63 cells were subjected to SDS/PAGE and examined by immunoblotting with anti-PTYR (A) or anti-ERK2 (B) antibody, as described in Section 2. The cell lysates were also electrophoresed in SDS-PAGE containing MBP. After renaturation of proteins in the gels, the kinase activity was determined (C), as described in Section 2. 1, D-MEM alone; 2, 1+20 ng/ml TIMP-1; 3, 1+1.0 ng/ml TIMP-2; 4, 1+10% FCS. Activity bands corresponding to ERK2, 1–4 in (C), were quantified by the use of an image analyzer (Fuji BAS 2000) to give relative values of 1.0, 3.8, 3.3, and 3.7, respectively. Open and solid arrowheads in (B) denote non-activated and activated, respectively.

than being due to the inhibition of MMPs. It has also been demonstrated that the binding of TIMP-2 to the cell surface is not competed by TIMP-1, suggesting that both TIMPs have their own specific receptors [4,17].

We have further confirmed here the direct action of both TIMPs on the cells as cell-growth factors by showing that [^{3}H]TdR incorporation was significantly stimulated at 3 h after the addition of either TIMP and that the incorporation was linear up to 15–20 h.

The dose-response curve for either TIMP-1 or TIMP-2 showed a bell-shape profile for [^{3}H]TdR incorporation, which is consistent with TIMP-dose curves for cell proliferation [4], with maximum incorporation at 20 ng/ml (0.71 nM) for TIMP-1 and 1.0 ng/ml (46 pM) for TIMP-2 (Fig. 2A). The maximum response to either TIMP-1 or TIMP-2 was comparable to that found with 10% FCS.

Tyrosine kinase inhibitors, such as genistein, erbstatin, and herbimycin A, effectively blocked the [^{3}H]TdR incorporation into MG-63 cells (Fig. 3), indicating that activation of tyrosine kinase is required for the stimulation of DNA synthesis mediated by either TIMP-1 or TIMP-2. Either genistein or erbstatin inhibited [^{3}H]TdR incorporation significantly to a level below that in D-MEM alone, indicating that they also inhibited the autocrine stimulation by TIMP-1 produced by the cells themselves during the 21 h incubation. Both TIMPs stimulated tyrosine-specific phosphorylation of some cellular proteins ($\sim$200 and $\sim$130 kDa, Fig. 4A). The effects of many growth factors, such as EGF, PDGF, insulin, and IGF-I, are known to be mediated by high-affinity receptor tyrosine kinases. Both TIMP-1 and TIMP-2 seem to be new ligands that may be categorized in this group, even though it is not yet clear what type of tyrosine kinases are involved in the signaling pathways induced by them [7,18].

MAP kinases have been characterized as ubiquitous components of signal transduction pathways from yeasts to vertebrates, and thus have been implicated in the control of a broad spectrum of cellular events, particularly in view of their roles in cell proliferation and/or differentiation [8,19]. Recently, activation of constitutive MAP kinase by MAP kinase kinase was shown to be necessary and sufficient for cell differentiation or proliferation depending on the cellular context [20]. At least four distinct MAP kinases have been purified and studied in depth [19,21]. In this study we demonstrated the activation of at least one of the group, ERK2 (Fig. 4B), and detected MAP kinase activity in lysates of TIMP-treated cells in SDS-PAGE containing MBP (Fig. 4C), suggesting that MAP kinase transduces TIMP-dependent growth signaling.

During the course of this work, Corcoran and Stetler-Stevenson [22] reported that TIMP-2 stimulated the proliferation of fibrosarcoma cells and normal dermal fibroblasts via a cAMP-dependent intracellular signal transduction pathway. In this study we showed that H-89, H-7, bisindolylmaleimide, and K-252a, known to be potent inhibitors of protein kinase A, C, and G, and myosin light chain kinase [23], had essentially no inhibitory activity on [^{3}H]TdR incorporation into MG-63 cells stimulated by either TIMP (Fig. 3). One obvious difference between our two studies is in the TIMP-2 concentration used; i.e. they used 0.5–1 μg/ml TIMP-2, which is 500–1000-times higher than the level we used. By using Raji cells, which express neither TIMP-1 nor TIMP-2, we demonstrated the presence of both high (K_d = 0.15 nM) – and low (35 nM) – affinity binding sites on the cell surface [4]. Taking into account these K_d values, we may speculate that we are looking at signal transduction evoked through TIMP-2 binding to high-affinity binding sites, whereas Corcoran and Stetler-Stevenson studied another pathway involving transduction through low-affinity binding sites. Experiments with this speculation in mind are now under way in our laboratory to reconcile these inconsistent findings.

In conclusion, we, for the first time, have demonstrated that either TIMP-1 or TIMP-2 at the concentration expressing growth factor activity induced tyrosine phosphorylation, acti-

vated MAP kinase, and stimulated DNA synthesis in the absence of other exogenous growth factors.

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