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Tissue Inhibitor of Metalloproteinase-2 Stimulates Fibroblast Proliferation via a cAMP-dependent Mechanism*

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In addition to inhibiting the proteolytic activity of the matrix metalloproteinases, tissue inhibitors of metalloproteinases (TIMPs) promote the growth of cells in the absence of other exogenous growth factors. TIMP-2 stimulates the proliferation of fibrosarcoma (HT-1080) cells and normal dermal fibroblasts (Hs68) in a dose-dependent manner. This response is evident as early as 2 h and persists up to 48 h after treatment with recombinant TIMP-2 (rTIMP-2). The specificity of this response is demonstrated by the ability of affinity-purified polyclonal anti-TIMP-2 antibodies to ablate TIMP-2 mitogenesis and by the lack of response to TIMP-1. This response is also blocked by the presence of an adenylate cyclase inhibitor, 9-(tetrahydro-2-furyl)adenine (SQ22536). Although SQ22536 did not affect untreated fibroblasts or fibrosarcoma cells, this inhibitor completely abrogates the proliferative response induced by rTIMP-2. Treatment of these cells with rTIMP-2 also stimulates the production of cAMP in a time-dependent manner that differs for the two cell lines. Moreover, treatment of purified cell membranes with rTIMP-2 suppresses cholera toxin-mediated ADP-ribosylation of the GTP-binding protein, Gsα subunit. These results indicate that the αβγ heterotrimer is dissociated by treatment with rTIMP-2, which may facilitate the Gsα-mediated activation of adenylate cyclase and subsequent production of cAMP. Since cAMP binds to the regulatory subunit of cAMP-dependent protein kinase and activates kinase activity, we evaluated how treatment with rTIMP-2 affected both these parameters. We demonstrate in this report that the cAMP produced in response to treatment with rTIMP-2 binds to the type I regulatory subunit of cAMP-dependent protein kinase and stimulates kinase activity. These results are the first demonstration that TIMP-2 directly activates adenylate cyclase to produce cAMP, which increases cAMP-dependent protein kinase activity, resulting in stimulation of fibroblast mitogenesis.

The matrix metalloproteinases are a family of zinc-dependent enzymes responsible for degrading the connective tissue matrix at physiological pH (1). Members of this protease family possess several conserved features including the consensus sequence for binding zinc at the active site and an activation locus responsible for the latency of the zymogen. The matrix metalloproteinase family is also defined by the ability of tissue inhibitor of metalloproteinases (TIMPs)[1] to inhibit proteolysis. Currently, there are three well defined members of the TIMP family that share 40% amino acid homology. These are TIMP-1 (2), TIMP-2 (3, 4), and TIMP-3 (5, 6). The TIMPs bind with high affinity in a 1:1 molar ratio to active matrix metalloproteinases, resulting in loss of proteolytic activity.

In addition to their function as matrix metalloproteinase inhibitors, a growing body of experimental evidence suggests that TIMPs behave as cytokines and stimulate cellular proliferation in the absence of other growth factors. In addition to their erythroid-potentiating activity (EPA) (7, 8), TIMP-1, and TIMP-2 are mitogens for a wide variety of cell types cultured in the absence of serum or exogenous growth factors (9–11). Recent studies have shown that SV40-transformed fibroblasts secrete TIMP-2 which is believed to mediate the survival of nontransformed fibroblasts in the absence of serum (12). TIMP-2 has also been shown to significantly increase the survival of pituitary folliculo-stellate cells cultured in the absence of serum (13).

These growth-promoting effects are presumably mediated by specific TIMP binding sites on the cell membrane, *i.e.* putative TIMP receptors. Recent studies have demonstrated selective cell surface binding of TIMP-2 to HT-1080 cells that is not competed by TIMP-1 (11, 14). However, neither the putative TIMP-1 or TIMP-2 receptor has been isolated or characterized. Furthermore, the signal transduction mechanisms involved in promoting growth by TIMPs have not been elucidated. Due to a growing body of evidence for the growth-promoting activities of the TIMPs, we have investigated the signal transduction mechanisms involved in TIMP-2-mediated growth stimulation of normal dermal fibroblasts and fibrosarcoma cells. We demonstrate that, in the absence of serum or exogenous growth factors, rTIMP-2 mediates a mitogenic response in normal dermal fibroblasts and fibrosarcoma cells by stimulating adenylate cyclase to produce cAMP which, in turn, activates cAMP-dependent protein kinase (PKA). This is the first report demonstrating that TIMP-2 stimulates growth by activation of PKA.

EXPERIMENTAL PROCEDURES

Culture Conditions—Fibrosarcoma cells (HT-1080; ATCC CLL 121) and normal human newborn foreskin fibroblasts (Hs68; ATCC CRL 1635) were obtained from American Tissue Culture Collection (Rockville, MD) at passage 19 and 14, respectively. The HT-1080 cells obtained from ATCC are derived from a fibrosarcoma and have an epi-

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[1] The abbreviations used are: TIMP, tissue inhibitor of metalloproteinase; rTIMP, recombinant TIMP; PKA, cyclic AMP-dependent protein kinase; PKI, inhibitor of PKA; CREB, cAMP responsive element binding protein; CRE, cAMP responsive element; G-protein, guanine nucleotide-binding proteins; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; EPA, erythroid-potentiating activity; CT, cholera toxin; PT, pertussis toxin; HPLC, high performance liquid chromatography; SQ22536, 9-(tetrahydro-2-furyl)adenine.

thelial-like morphology with a karyotype of $2n = 46$. We used the HT-1080 cells between passages 19 and 30 and Hs68 cells between passage 14 and 20. Cells were grown to 80% confluence in Dulbecco's modified Eagle's media (DMEM; Life Technologies, Inc.) containing 4500 mg/liter D-glucose, D-glutamine, sodium pyruvate, 100 units/ml penicillin-G, 100 μg/ml streptomycin sulfate, and 10% heat inactivated fetal bovine serum (FBS), unless otherwise indicated.

TIMP Proteins—The rTIMP-2 protein was expressed using a vaccinia virus expression system and purified as described previously (15). Affinity-purified rTIMP-2 was kindly provided by R. Bird (Oncologix, Inc., Gaithersburg, MD). Native TIMP-2 was purified from the human A2058 conditioned medium as described previously (3). rTIMP-1 was isolated from the conditioned medium of EPA-transfected Chinese hamster ovary cells (8/8 2G EPA2) (Genetics Institute, Cambridge, MA) as described previously (16) and then purified by HPLC gel permeation chromatography using 50 mM Tris-HCl, 150 mM NaCl, pH 7.5.

Thymidine Incorporation—HT-1080 and Hs68 cells were plated at 5×10^3 cells/well of a 96-well Costar plate for 18 h in DMEM with 10% FBS. The cells were then starved 18 h in DMEM without serum. Fresh serum-free DMEM was added to the wells prior to treatment with TIMP-2. After the indicated period of incubation, [^{3}H]thymidine (0.1 μCi/ml; Amersham Corp.) was added and incubated for 2 h at 37 °C. The percent of thymidine incorporated in a 2-h pulse correlated in a linear fashion with the number of cells. The medium was subsequently discarded, the wells were washed twice with phosphate-buffered saline (PBS), and the cells were fixed in methanol:glacial acetic acid (3:1). The incorporated [^{3}H]thymidine was extracted as described previously and quantitated by liquid scintillation counting (17). The mean and standard deviation of triplicate determinations were converted to percent control for graphic representation. SQ22536 or 9-(tetrahydro-2-furyl)-adenine (Calbiochem/Novabiochem) was solubilized in sterile deionized water and added to cells at a final concentration of 0.1 μM (18). Affinity-purified polyclonal anti-TIMP-2 antibodies (5 μg/ml) were incubated with 24 nM rTIMP-2 for 1 h at 4 °C and 1 h with anti-rabbit affinity gel (Oraganon Teknika, West Chester, PA). The supernatant from the immunoprecipitate was added to the cells followed by thymidine.

cAMP Determination—Hs68 and HT-1080 cells were plated at 1×10^5 cells/well of a 25-cm^2 Costar plate in 10% FBS for 18 h followed by an 18-h incubation in serum-free DMEM. After the indicated period of incubation at 37 °C, the medium was discarded and the cells were washed once with PBS. The cAMP was extracted by adding to the cells 200 μl of Amersham's cAMP assay buffer containing 7% perchloric acid and 1 mM 3-isobutyl-1-methylxanthine (Sigma). After a 10-min incubation at 4 °C, the cells were scraped, transferred to siliconized Eppendorf tubes (PGC Scientific, Gaithersburg, MD) and the precipitated protein pellet collected by a 10-min centrifugation in a refrigerated bench-top microcentrifuge. The supernatants were neutralized and the cAMP determined by ^{125}I-cAMP radioimmunoassay (Amersham) according to the manufacturer's instructions. The protein in the pellet was solubilized in 0.1 N sodium hydroxide, and the relative levels of protein were determined by the Bradford method (19). The cAMP levels were normalized to the protein concentration and expressed as fmol of cAMP per μg of protein.

Cholera Toxin ADP-ribosylation—HT-1080 cells were plated at 1×10^7 cells per 175-cm^2 Nunc tissue culture flask and cultured in DMEM containing 10% FBS for 18 h followed by 18 h in serum-free DMEM. The cells were washed in PBS, scraped, and homogenized in 250 mM sucrose buffer containing protease inhibitors (10 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). The homogenate was centrifuged for 5 min at 3000 rpm in a bench-top centrifuge, and the pellet was discarded. The supernatant was subjected to a 25-min high speed centrifugation (50,000 rpm) at 4 °C (TL100 Beckman ultracentrifuge). The pellet was resuspended in 100 μl of ribosylation buffer containing 100 mM Tris (pH 7.5), 1 mM $MgCl_2$, 100 μM GTP, 1 mM ATP, 10 mM thymidine, and protease inhibitors (same concentrations as above). rTIMP-2 (1 μg) was added to the membranes as indicated in the figure legends and incubated at 30 °C for 30 s prior to the addition of 1 μg of activated cholera toxin (CT; List Biological Laboratories, CA) and 20 μCi of [^{32}P]NAD (Dupont NEN; 2 mCi/ml). The reaction was then allowed to incubate for 20 min at 30 °C. The radioactive membrane proteins were subsequently pelleted in a bench-top centrifuge, washed twice in PBS, and resuspended in SDS-Laemmli sample buffer containing 1% β-mercaptoethanol. The proteins were heated in a boiling water bath for 2 min prior to loading and electrophoresis on a 10% Tris-glycine PAGE gel (Novex, San Diego, CA). Following electrophoresis, the proteins were transferred onto a 0.2-μm polyvinylidene difluoride (PVDF) membrane using 1× transfer buffer (Novex) containing 0.001% SDS. If CT was omitted from the reaction, the G-proteins were not labeled. The PVDF membrane was also assayed for Gsα protein immunoreactivity by Western blot. Nonspecific sites on the PVDF membrane were blocked using blocking buffer containing 0.9 M NaCl, 90 mM sodium citrate (pH 7.5), 1% non-fat dry milk, and 0.5% casein. The PVDF membrane was then incubated with anti-Gsα antibody (Upstate Biotechnology,Inc., Lake Placid, NY) followed by donkey anti-rabbit horse radish peroxidase antibody (Pierce), and the immunoreactive proteins were visualized by chemiluminescence (Amersham) according to the manufacturer's instructions. The extent of [^{32}P]ADP-ribosylation was quantitated by autoradiography of the Western blot using an Arcus flatbed scanner (Agfa-Gevaert AG, Germany) and Image 1.49 program (NIH, Bethesda, MD).

8-Azido PKA Labeling and Kinase Assay—Hs68 and HT-1080 cells (1×10^7) were plated on 175-cm^2 flasks in DMEM containing 10% FBS for 18 h followed by incubation in serum-free DMEM for 18 h. Prior to rTIMP-2 treatment, the cells were washed in serum-free DMEM. Cells were then incubated at 37 °C with or without 48 nM rTIMP-2 (1 μg/ml) for 5 or 10 m. The cells were then washed in PBS, scraped, and homogenized in buffer containing 1% Triton X-100, 10 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM 3-isobutyl-1-methylxanthine, and proteinase inhibitors (described above). 8-Azido-cAMP binding experiments utilized 2.5 μg of Hs68 and 4.5 μg of HT-1080 cell lysates. The cell lysates and 1 μg of the positive controls were incubated with 20 μM azido-[^{32}P]cAMP (ICN, Costa Mesa, CA; 10 μCi/ml) for 30 min at 30 °C. The positive controls used were bovine heart (contains 54- and 56-kDa type II regulatory subunits of PKA), and rabbit skeletal muscle (contains the 49-kDa type I regulatory subunit of PKA) (Sigma). Following incubation, the azido group was activated by a 30-s irradiation at 254 nm UV light (20). If the UV irradiation step is omitted, proteins were not labeled. The reaction was stopped by adding SDS-Laemmli sample buffer containing 1% β-mercaptoethanol and heated in a boiling water bath for 2 min prior to loading onto 8–16% Tris-glycine PAGE gel (Novex). After electrophoresis, the proteins were Western blotted onto PVDF membrane using 1 × transfer buffer containing 0.001% SDS. The ^{32}P-labeled proteins were visualized by autoradiography of the PVDF membrane and quantitated by densitometric analysis as described above.

PKA activity was determined by using the PKA assay system from Life Technologies, Inc. with slight modification. Hs68 and HT-1080 cells (1×10^6) were plated on 75-cm^2 flasks in DMEM containing 10% FBS for 18 h followed by 18 h in serum-free DMEM. After treatment with 24 or 48 nM rTIMP-2 for either 5 or 10 min, the cells were washed once with PBS, scraped, and homogenized in 500 μl of extraction buffer (Life Technologies, Inc.) containing 1 mM 3-isobutyl-1-methylxanthine and protease inhibitors (described above). The cell lysates (20 μl) from the samples were aliquoted in quadruplicate on a 96-well microtiter plate (Nunc, Denmark) and incubated for 10 min with either PKA inhibitor (PKI) or PKI plus cAMP (Life Technologies, Inc.). Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide; Life Technologies, Inc.) and [γ-^{32}P]ATP (20 μCi/ml; Amersham) were added to the reaction and incubated for 10 min at 30 °C. Twenty μl from each reaction were spotted onto phosphocellulose disks and extracted with 1% phosphoric acid and water. The incorporated radioactivity was quantitated by liquid scintillation. The percent active PKA is derived from the picomoles of phosphate incorporated into Kemptide per min as follows: (picomoles of phosphate/min inhibited by PKI in the absence of cAMP) divided by (picomoles of phosphate/min inhibited by PKI in the presence of cAMP) multiplied by 100 as per the manufacturer's instructions.

RESULTS

Effect of TIMP-2 on Thymidine Incorporation—Since TIMPs stimulate the growth of a variety of cells, we determined whether TIMP-2 was mitogenic to normal human foreskin fibroblasts (Hs68) and fibrosarcoma cells (HT-1080). Both cells respond to rTIMP-2 exposure with a dose dependent increase in [^{3}H]thymidine incorporation. Two hours after exposure to rTIMP-2, the maximal stimulation in Hs68 cells is 2.5-fold over basal rate of thymidine incorporation and occurs at a narrow range of low nM concentration of TIMP-2 (24 nM or 500 ng/ml) (Fig. 1*A*, ■). TIMP-2 concentrations 2-fold greater than the maximally stimulating concentration also increases thymidine incorporation, but to a slightly lesser degree. Further increases in TIMP-2 concentrations above 48 nM markedly diminished the mitogenic response in Hs68 fibroblasts. Increasing concentrations of rTIMP-2 results in a dose dependent increase of

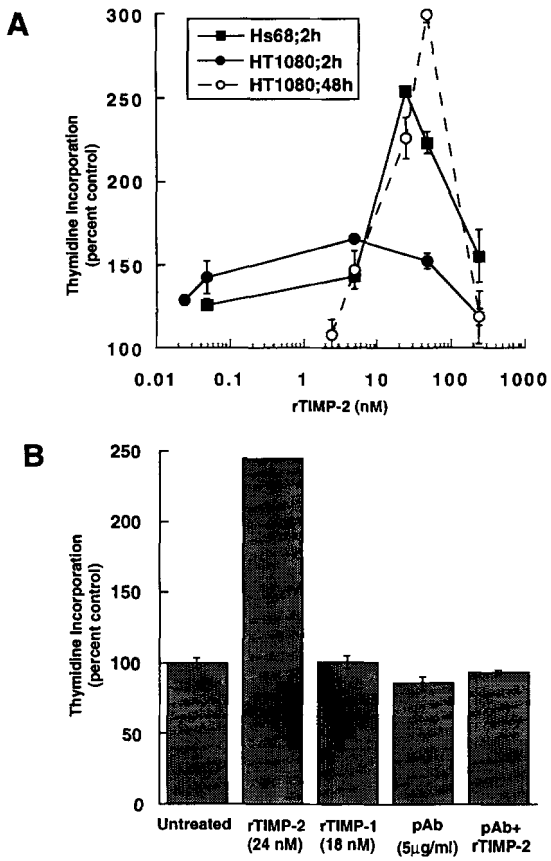

FIG. 1. **TIMP-2 mediated proliferation of normal dermal fibroblasts and fibrosarcoma cells.** Hs68 fibroblasts and HT-1080 fibrosarcoma cells were plated at 3×10^3/well of a 96-well Costar plate in DMEM with 10% FBS for 18 h and 18 h without serum prior to treatment with rTIMP-2. [^{3}H]Thymidine was added either 2 or 48 h after treatment, and the percent thymidine incorporation was measured as described under "Experimental Procedures." *Panel A* represents the dose response of Hs68 (■) or HT-1080 cells (●) after 2-h treatment with rTIMP-2 and HT-1080 cells (○) following 48-h incubation with rTIMP-2. *Panel B* depicts the ability of affinity-purified polyclonal anti-TIMP-2 antibodies (5 μg/ml) to block proliferation of HT-1080 cells following a 24-h treatment with 24 nM rTIMP-2.

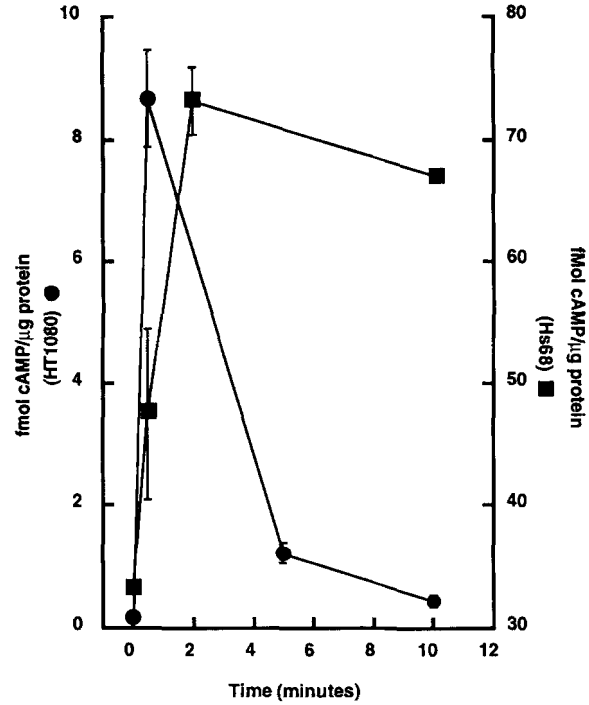

FIG. 2. **TIMP-2 increases cAMP.** HT-1080 (●) and Hs68 (■) cells were plated on Costar six-well plates (1×10^5/well) in DMEM with 10% FBS for 18 h and in serum-free DMEM for 18 h. At the indicated time points, 200 μl of 7% perchloric acid were added to the wells. The total cAMP in the sample was determined by radioimmunoassay and normalized to the protein concentration of the sample. The results are expressed as femtomoles of cAMP/μg of protein as described under "Experimental Procedures." The *left vertical axis* represents the response of HT-1080 fibrosarcoma cells, and the *right vertical axis* represents the response of Hs68 normal dermal fibroblasts.

thymidine incorporation in HT-1080 cells similar to Hs68 fibroblasts except that the maximal thymidine incorporation at 2 h was 1.5-fold over the basal level of incorporation (Fig. 1*A*, ●). Incubating HT-1080 cells with 24 nM rTIMP-2 and native TIMP-2 (data not shown) for 24 h also stimulates a 2.5-fold increase in mitogenesis (Fig. 1*B*). After a 48-h incubation with 48 nM rTIMP-2, a nearly 3-fold induction in thymidine incorporation is observed in these cells (Fig. 1*A*, ○). In Hs68 normal dermal fibroblasts, the response after a 2-h treatment with rTIMP-2 is much more dramatic compared to that of HT-1080 fibrosarcoma cells, but this difference is reduced by 24 h. These differences probably reflect the degree of synchronization induced by serum starvation, as well as inherent differences in the cell cycle times for these cell lines.

The specificity of TIMP-2 inducing this proliferative response in HT-1080 (Fig. 1*B*) and Hs68 cells (data not shown) is demonstrated by the ability of affinity purified anti-TIMP-2 antibodies to ablate this response and inability of TIMP-1 to stimulate proliferation (Fig. 1*B*). Thus, the increase in thymidine incorporation mediated by TIMP-2 is evident as soon as 2 h after treatment and persists up to 24–48 h in HT-1080 cells. These results demonstrate that TIMP-2 specifically mediates a proliferative response that is not due to a contaminant in the TIMP-2 preparations utilized for these experiments.

TIMP-2 Stimulates the Production of cAMP—Previously, mitogenic responses have been observed by treating fibroblasts with agents that either increase intracellular cAMP (21, 22) or suppress the cyclic nucleotide phosphodiesterase activity (21, 23). The mitogenic response observed by treating fibroblasts with agents such as platelet-derived growth factor (24), interleukin-10 (25), mastoparan (26), bombesin (27, 28), or vasoactive intestinal peptide (22) has been attributed to an increased production of cAMP. In addition, reports have documented that ATP binds to the A_2-adenosine receptor and stimulates proliferation in fibroblasts by increasing cAMP levels and PKA activity (29–31). The responses observed in both the HT-1080 and Hs-68 cell lines following TIMP-2 treatment are similar in magnitude to that observed if both cell lines are treated with agents that are known to increase or mimic endogenous cAMP, such as cholera toxin or low concentrations of dibutyryl cAMP. Hence, we evaluated whether exposure to rTIMP-2 may stimulate the production of cAMP.

rTIMP-2 stimulates the production of cAMP in HT-1080 and Hs68 cell lines in a time-dependent manner. Treatment of HT-1080 cells with 48 nM rTIMP-2 stimulated an approximately 50-fold increase in the steady state levels of cAMP as early as 30 s compared to untreated cells (Fig. 2, ●). This rapid increase in the steady state levels of cAMP quickly decreases to normal levels by 10 min. The kinetics of cAMP production by Hs68 normal dermal fibroblasts in response to rTIMP-2 treatment is different from that observed with HT-1080 fibrosarcoma cells. Addition of 48 nM rTIMP-2 stimulates the production of cAMP as early as 30 s after treatment (Fig. 2, ■). The levels of cAMP in Hs68 cells stimulated by a 2-min incubation with 48 nM rTIMP-2 are approximately 2.6-fold over basal cAMP levels. In these cells, the cAMP levels remain elevated

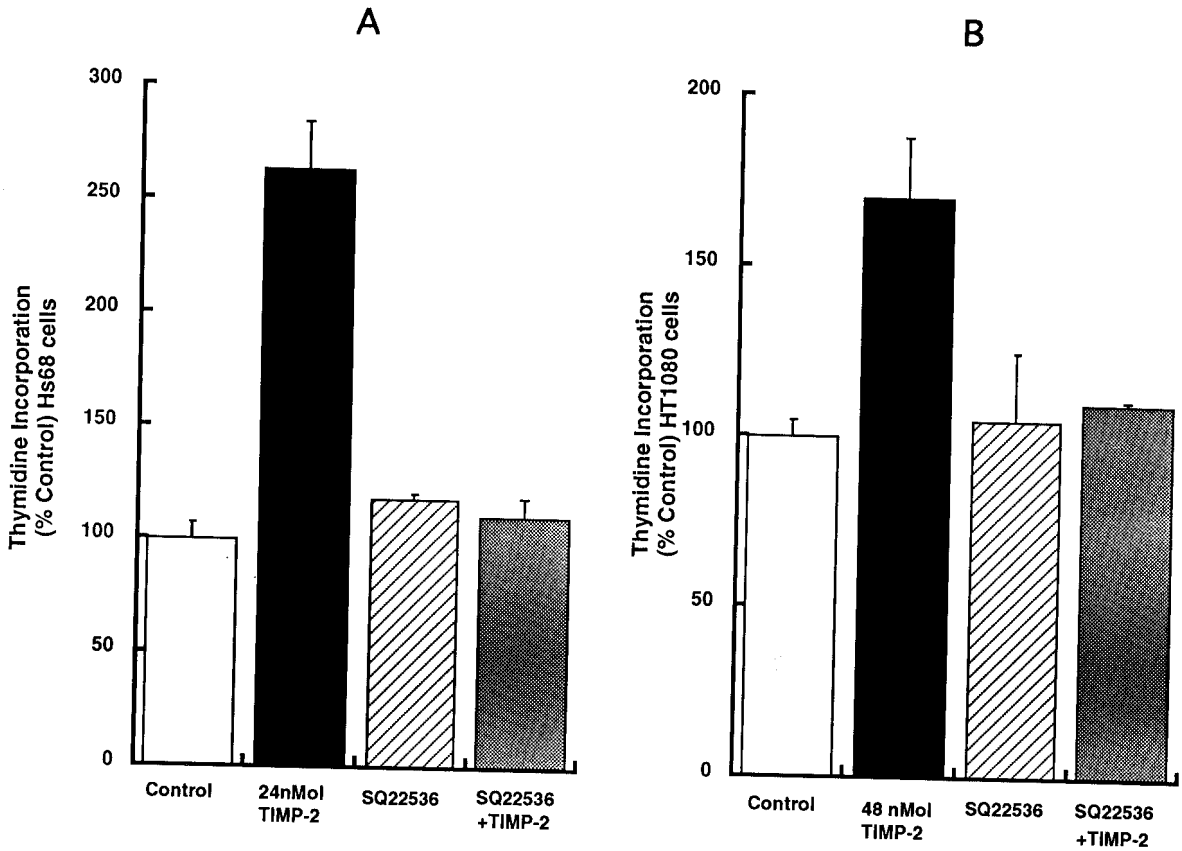

FIG. 3. **Effect of adenylate cyclase inhibitor on TIMP-2-mediated proliferation.** Hs68 (*Panel A*) and HT-1080 (*Panel B*) cells (3×10^3/well) were plated on 96-well plates in DMEM with 10% FBS for 18 h and in serum-free DMEM for 18 h. Prior to stimulation, the cells were washed with DMEM without FBS. 10^{-4} M SQ22536, rTIMP-2, and [^{3}H]thymidine were sequentially added to the cells and incubated at 37 °C for 2 h. The percent of thymidine incorporated was measured as described under "Experimental Procedures." *Panel A*, percent control of thymidine incorporation in Hs68. *Panel B*, percent control of thymidine incorporation in HT-1080.

up to 10 min after addition of rTIMP-2. Clearly, the production of cAMP in both cells is rapidly increased upon initial exposure to low nanomolar concentrations of rTIMP-2. However, the rate of cAMP breakdown following the initial stimulation differs dramatically between these cells, presumably due to differential regulation of phosphodiesterase activity. These kinetics may partially explain the differences in the time course of the proliferative responses of the two cell lines following rTIMP-2 treatment observed in our initial [^{3}H]thymidine incorporation experiments (Fig. 1*A*). Nevertheless, these results clearly demonstrate that TIMP-2 stimulates the production of cAMP which may contribute to the mitogenic response in these cells. HPLC purified rTIMP-1 did not stimulate cAMP production when measured in HT-1080 cells consistent with the lack of growth stimulatory response in both cell lines to this inhibitor (Fig. 1*B*).

Inhibition of Adenylate Cyclase Blocks TIMP-2-mediated Proliferation—To determine whether the increase in the steady state production of cAMP directly contributed to the TIMP-2 mitogenic responses, we evaluated whether blocking the production of cAMP with a specific adenylate cyclase inhibitor, SQ22536, affected the stimulation of proliferation by rTIMP-2. SQ22536 is a substituted adenine derivative that binds to the P-site of adenylate cyclase and suppresses prostaglandin-mediated proliferation. This analogue has been used in biological systems to demonstrate that prostaglandins and neurotransmitters (31, 32) stimulate adenylate cyclase activity. Treatment with SQ22536 (10^{-4} M) was not toxic to cells and did not affect basal levels of proliferation (Fig. 3). However, pretreatment of Hs68 (Fig. 3*A*) and HT-1080 (Fig. 3*B*) with SQ22536 prior to rTIMP-2 ablated the mitogenic response in both cell lines. These results demonstrate that stimulation of proliferation by TIMP-2 is directly dependent on activation of adenylate cyclase which increases the production of cAMP in these cells.

TIMP-2 Stimulates Dissociation of Heterotrimeric Gsα Protein—Adenylate cyclase activity is stimulated by a receptor-mediated activation of the GTP-binding protein, Gsα (33). ADP-ribosylating toxins such as pertussis toxin (PT) and CT have been useful tools in determining the activation state of G-proteins, because it has been previously demonstrated that ADP-ribosylation of Gsα protein occurs only in the presence of the βγ subunit (34, 35). Previously, suppression of PT ADP-ribosylation has been employed to characterize the receptor and second messenger systems (24) stimulated by thrombin (36, 37) and vasopressin. Because ADP-ribosylating toxins, such as CT and PT, more efficiently ADP-ribosylate the heterotrimeric αβγ complex, the inactivation state of the G-protein (38), we employed CT as a tool to evaluate whether treatment with rTIMP-2 affected the activation state of the Gsα protein. Membranes from HT-1080 cells were isolated and pretreated with rTIMP-2 prior to ADP-ribosylation with CT. The Gsα proteins in these cells were identified based on susceptibility to CT-mediated ADP-ribosylation, molecular weight, and immunoreactivity with specific Gsα antibodies. Although two molecular mass species (46- and 52-kDa) were identified by Gsα antibody staining (Fig. 4*B*), CT-mediated ADP-ribosylation favored the 46-kDa isoform (Fig. 4*A*). rTIMP-2 treatment (Fig. 4*A*, *lane 2*) resulted in an approximately 40% decrease in CT-mediated [^{32}P]ADP-ribosylation of both the 46- and 52-kDa Gsα proteins when compared to untreated cell membranes (Fig. 4*A*, *lane 1*). Western blot analysis of this PVDF membrane (Fig. 4*B*) not only shows that both Gsα proteins are expressed but also confirms that equal amounts of the protein were tested

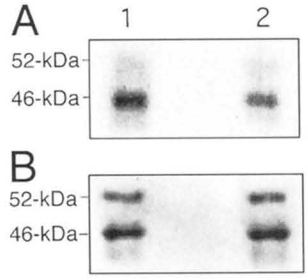

FIG. 4. **CT ADP-ribosylation is suppressed by TIMP-2.** HT-1080 cells (1×10^7) were plated on 175 cm^2 flasks with FBS for 18 h and DMEM without FBS for 18 h. The cells were washed in PBS, scraped in 250 mM sucrose buffer, and homogenized. Equal amounts of membranes were resuspended in 100 μl of ribosylation buffer and incubated with (*lane 2*) or without (*lane 1*) 1 μg of rTIMP-2 for 30 s prior to addition of [^{32}P]NAD and 1 μg of CT. The reaction was allowed to incubate for 20 m at 30 °C. The membrane proteins were subsequently washed in ribosylation buffer, resuspended in Laemmli SDS sample buffer containing β-mercaptoethanol, and boiled for 2 min. The proteins were separated on a 10% Tris-glycine PAGE gel and transferred onto a PVDF membrane as described under "Experimental Procedures." The relative quantity of the radioactivity incorporated into the proteins was visualized by autoradiography. Two proteins, 46 and 52 kDa, are ADP-ribosylated by CT (*Panel A*) and recognized by the Gsα-specific antibody (*Panel B*). The relative intensity of the ^{32}P-labeled proteins (*Panel A*) and immunoreactive bands (*Panel B*) was quantitated by scanning the images on an Arcus flatbed scanner, and the relative integrated intensity was quantitated as described under "Experimental Procedures."

in this assay. The decrease in the radioactive labeling of these proteins (Fig. 4*A*) suggests that treatment with rTIMP-2 (Fig. 4*A*, *lane 2*) results in the dissociation of the Gsα from the $\beta\gamma$ subunits, a critical step in the subsequent activation of adenylate cyclase.

TIMP-2 Treatment Results in cAMP Binding and Activation of PKA—We have demonstrated that stimulation of cAMP production by TIMP-2 is a critical step for the mitogenic stimulation in Hs68 and HT-1080 cells. This signal transduction cascade can result in the activation of the PKA (39). Hence, we evaluated if the cAMP, produced in response to TIMP-2, bound to the regulatory subunit of PKA. The photo-activable cAMP analog, 8-azido-[^{32}P]cAMP, was utilized for these studies not only to radioactively label the type of regulatory subunit present in these cells but also to test whether the endogenous cAMP produced by rTIMP-2 treatment could interfere with the binding of this analog to the sites on the regulatory subunit of PKA. Hs68 and HT-1080 cells were untreated or treated with rTIMP-2, and cell lysates were generated from these cells. Equal amounts of protein from these lysates were incubated with 8-azido-[^{32}P]cAMP and cross-linked to the regulatory subunit by UV irradiation. The proteins were separated by PAGE and transferred to PVDF as described under "Experimental Procedures." Autoradiography revealed that both Hs68 (data not shown) and HT-1080 (Fig. 5, *lane A*) cell lysates contained a single γ-azido-[^{32}P]cAMP-labeled protein that co-migrated with the 49-kDa rabbit skeletal (Fig. 5, *lane D*) type I regulatory subunit of PKA. Pretreatment of HT-1080 cells for 30 s (data not shown) or 2 min (Fig. 5, *lane B*) with 48 nM rTIMP-2 decreased the labeling of this protein. However, 10 min after treatment with rTIMP-2 (Fig. 5, *lane C*), the labeling of this protein was back to the levels observed without treatment (Fig. 5, *lane A*). Addition of dibutyryl-cAMP to these lysates blocks the ^{32}P-labeling of these proteins by 8-azido-cAMP (data not shown). These results show that the cAMP produced in response to treatment with TIMP-2 binds to the type I regulatory subunit of PKA in these cells. The kinetics of the cAMP response as determined by competition for 8-azido-cAMP binding to the regulatory subunit of PKA (Fig. 5) correlate with the results obtained by a direct measurement of cAMP following

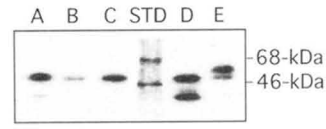

FIG. 5. **Binding of 8-azido-cAMP to type I regulatory subunit of PKA.** HT-1080 (1×10^7) cells were plated on 175-cm^2 Nunc flasks in DMEM with 10% FBS for 18 h and DMEM without FBS for 18 h. Prior to treatment with rTIMP-2, the cells were washed in DMEM without FBS. Cells were either untreated (*lane A*) or treated with 48 nM rTIMP-2 for 2 min (*lane B*) or 10 min (*lane C*). 4.5 μg of the HT-1080 cell lysates or 1 μg of positive controls were incubated with 20 μM 8-azido-[^{32}P]cAMP for 20 min at 30 °C. The samples were irradiated with 254 nm for 30 s, and the reaction was stopped by adding Laemmli-SDS sample buffer containing 1% β-mercaptoethanol and boiling. The proteins were separated on a 10% Tris-glycine PAGE gel and transferred onto a PVDF membrane, and the radioactive proteins were visualized by autoradiography as described under "Experimental Procedures." *Lane D* represents the labeling of 1 μg of rabbit skeletal protein which contains the 49-kDa type I regulatory subunit of PKA. *Lane E* represents 1 μg of bovine heart protein which predominately contains the 54-kDa type II regulatory subunit of PKA. The relative integrated density units of the bands are as follows: *lane A*, 0.7; *lane B*, 0.3; and *lane C*, 0.7.

rTIMP-2 treatment (Fig. 2).

Binding of cAMP to the regulatory subunit of PKA results in the release of the catalytic subunit and increased protein kinase activity (39). Therefore, we evaluated if rTIMP-2 increased the kinase activity of PKA. In order to specifically analyze for PKA activity, we utilized an assay system that measures the relative phosphorylation of Kemptide in the presence and absence of a specific PKA inhibitor, PKI. By using PKI in the presence and absence of cAMP, the contribution of other kinases that may phosphorylate kemptide was eliminated. Treatment of Hs68 normal dermal fibroblasts with 24 nM rTIMP-2 increased by 2- and 3-fold the kinase activity of PKA at 5 and 10 min, respectively (Table I). Stimulation of the HT-1080 cells with 48 nM rTIMP-2 for 5 min also increases the kinase activity 2-fold (Table I). However, 10 min after treatment with 48 nmol of rTIMP-2, the PKA activity in HT-1080 cells falls back to normal levels. These results are consistent with the cAMP determinations (Fig. 2) and the 8-azido-cAMP competition studies (Fig. 5).

DISCUSSION

Growth factors can stimulate cell proliferation through a variety of different mechanisms including activation of tyrosine or serine/threonine protein kinases (39). Although TIMP-2 has previously been shown to possess growth-promoting activity (8, 11–13), the mechanism by which TIMP-2 transduces these growth signals has not been previously elucidated. Identification of the signaling pathways utilized in growth promotion is helpful not only in understanding the nature of the growth factor response but also in characterization of the receptor. This report gives the first description of the signal transduction events that occur following treatment of normal dermal fibroblasts (Hs68) and fibrosarcoma cells (HT-1080) with TIMP-2. We demonstrate in this study that, in the absence of other exogenous growth factors, rTIMP-2 induces a proliferative response in normal dermal fibroblasts and HT-1080 fibrosarcoma cells. This response to TIMP-2 treatment has also been previously observed in Raji lymphoma cells, with the maximal effect in these cells observed at sub-nanomolar concentrations (11). These investigators also demonstrate that TIMP-2 can stimulate proliferation of human Burkitt lymphoma cells following exposure in culture for 3–7 days. Moreover, the ability of TIMP-2 to increase thymidine uptake in HSF4 fibroblasts has been shown to directly correlate with the percent of cells in the S phase as determined by flow cytometric DNA content analysis (12).

The differences in the maximal effective concentrations are

TABLE I
TIMP-2 increases PKA activity

Cells were treated with the maximally stimulating dose of TIMP-2 (48 nM for HT-1080 and 24 nM for Hs68). Cell lysates (20 μl) from each treatment were tested on a Nunc 96-well plate in quadruplicates and incubated at 30 °C for 10 min with either PKI or cAMP or both as described under "Experimental Procedures." Kemptide and γ-[^{32}P]ATP was subsequently added and incubated for 10 min at 30 °C. Aliquots (20 μl) from each reaction solution were spotted on phosphocellulose disks and were washed twice with 1% phosphoric acid and twice with water. The picomoles phosphate incorporated into Kemptide/min by PKI-sensitive kinase activity was determined by liquid scintillation. This assay demonstrated a 1–6% error in the quadruplicate determinations for phosphorylated Kemptide. The percent active PKA is the TIMP-2-activated kinase activity (picomoles phosphate incorporated/min) divided by the AMP-activated kinase activity (picomoles phosphate/min) multiplied by 100 as per the manufacturer's instructions. The relative PKA activity column represents the kinase activity as a percentage of the maximally stimulated activity obtained by adding cAMP to the cell lysates.

Cell	TIMP-2 treatment	Duration	Relative PKA activity
	nM	*min*	*% inducible by cAMP*
HT1080	0	Basal	4.3
	48	5	9.4
	48	10	3.2
Hs68	0	Basal	11.4
	24	5	23.4
	24	10	35.0

probably due to differences in cell line responsiveness and/or inherent differences in the receptor affinities. The observed stimulation of proliferation in Hs68 and HT-1080 fibrosarcoma cells is dependent on the concentration of rTIMP-2 and is selectively blocked by affinity purified polyclonal anti-TIMP-2 antibodies and pretreatment of the cells with a specific inhibitor of adenylate cyclase, SQ22536. HPLC purified TIMP-1 did not modulate cell growth, thymidine incorporation, or cAMP production in these cell lines. This selective growth modulating activity of TIMP-2 compared with TIMP-1 has been observed previously in our laboratory (17).

A rapid increase in the production of cAMP is observed by treating both cell lines with rTIMP-2. Two lines of experimental evidence presented in this report suggest that TIMP-2 mediates production of cAMP by activation of adenylate cyclase instead of suppressing phosphodiesterase activity. These are the kinetics of cAMP production in HT-1080 cells following rTIMP-2 treatment and the suppression of mitogenesis by an adenylate cyclase inhibitor, SQ22536. Although both cell lines respond to rTIMP-2 by initially increasing the production of cAMP, the kinetics of the breakdown of cAMP differs dramatically between these cells. HT-1080 cells treated with 48 nM rTIMP-2 results in an immediate increase in the steady state levels of cAMP as soon as 30 s after treatment. The rapid decline of cAMP observed in HT-1080 cells probably reflects the immediate activation of a phosphodiesterase activity. The rapid spike in steady state levels of cAMP (Fig. 2), followed by a return to basal levels at 10 min in the HT-1080 cells, is consistent with the observations in the 8-azido-cAMP-labeling experiment (Fig. 4) and direct measurements of PKA activity (Table I) in this same cell line. Treatment of Hs68 cells with 48 nM rTIMP-2 also results in a 2.6-fold production of cAMP as early as 2 min after treatment. However, the production of cAMP in these cells remains elevated up to 10 min after treatment with rTIMP-2. The persistence of elevated cAMP steady state levels in these cells correlates with the 8-azido-cAMP labeling experiment (not shown) and the percent active PKA (Table I) observed at 10 min following rTIMP-2 treatment. Moreover, the results obtained from the 8-azido-cAMP labeling experiment demonstrate that only one type of regulatory subunit is labeled in these cell lysates and that this subunit binds to the cAMP produced in response to treatment with rTIMP-2. Since binding of cAMP to the regulatory subunit results in activation of PKA, we also demonstrate that the catalytic activity of PKA is stimulated by treatment with TIMP-2.

The differential responses of the Hs68 fibroblasts and HT-1080 fibrosarcoma cells to TIMP-2 observed in several assays may be due to a number of possibilities. These include differences in the cell cycle synchronization following serum starvation as well as differences in entry into S phase of the cell cycle. Alternative explanations for the persistent difference in TIMP-2 responses between these cell lines include the genetic backgrounds of these cells, differential expression of the receptor for TIMP-2, differential regulation of phosphodiesterase activity, and/or their inherent response to increases in PKA activity. Stimulation of HT-1080 and Hs68 cells with rTIMP-2 results in the production of cAMP and enhanced catalytic activity of PKA which may mediate a mitogenic response by phosphorylating cytoplasmic and nuclear proteins targets (40). Previous reports have shown that cAMP-mediated activation of PKA results in translocation of the catalytic subunit of the PKA into the nuclei of fibroblasts (41). The translocation of the PKA catalytic subunit in NIH 3T3 fibroblasts has been previously reported to increase the phosphorylation of transactivating proteins such as CREB, CREM, and ATF-1 which facilitates their binding to cAMP responsive elements (CREs: TGACGTCA) (42). Repressor molecules of the CRE such as CREM-α, -β, -γ, ICER, and CREB-2 are also activated and induced by cAMP and PKA. The activation of both activators and repressors functions to fine tune and coordinate cellular responses to cAMP production. Depending on the cell type and the concentration of cAMP, these events may result in differentiation or proliferation (39, 43).

Our findings demonstrate that TIMP-2 mediates proliferation by binding a putative G-protein coupled receptor which activates adenylate cyclase to produce cAMP and ultimately activates PKA (44). It is postulated that when a ligand binds a G-protein coupled receptor, GDP is exchanged for GTP on the Gα subunit which results in its dissociation from Gβγ. The free Gα subunit, in turn, activates effector functions such as the adenylate cyclase activity (33). Toxins have been useful in delineating the activation state of G-proteins because they selectively recognize the inactive or heterotrimeric G-protein and transfer an ADP-ribose moiety from NAD to the Gα subunit (34, 35). The ability of ligand treatment to suppress toxin ADP-ribosylation is believed to be due to dissociation of the heterotrimeric complex or activation of Gα subunit. Therefore, the decrease in CT-mediated ADP-ribosylation of the Gsα by treatment with rTIMP-2 suggests that a receptor-ligand interaction has induced the dissociation of Gsα from Gβγ. The dissociated Gsα may subsequently activate adenylate cyclase to produce cAMP. These results imply that the putative TIMP-2 receptor may be a G-protein-coupled receptor. Confirmation of this observation awaits isolation and characterization of these receptors.

This report is the first demonstration that rTIMP-2 induces proliferation in normal dermal foreskin fibroblasts and fibrosarcoma cells by increasing cAMP and the kinase activity of PKA. However, the overall significance of these findings are presently not understood. Since TIMP-2 initiates a cascade of events that result in the proliferation of neoplastic and normal fibroblasts in the absence of exogenous growth factors, this protein can be viewed as a growth factor for these cells. In the absence of other exogenous growth factors, TIMP-2 could support the survival and growth of normal fibroblasts, which may promote wound healing (45). The ability of TIMP-2 to stimulate

fibrosarcoma cell proliferation suggests that TIMP-2 could also contribute to the autocrine-stimulated neoplastic cell growth. However, recent studies have demonstrated that increasing cAMP in HT-1080 cells decreases the invasive potential of these cells when evaluated by *in vitro* invasion assays (46). Other studies have shown that TIMP-2 suppresses the *in vitro* invasive activity of HT-1080 cells presumably through direct inhibition of the required matrix metalloproteinase-2 activity (47). The present study suggests that TIMP-2 may suppress the invasive phenotype not only by directly inhibiting protease activity but also by stimulating the endogenous production of cAMP.

Neither tumor cells nor fibroblasts *in vivo* are stimulated by a single growth factor, such as TIMP-2. Growth of these cells is a result of the delicate integration of a number of second messenger systems, not only from growth factors and their receptors but also from extracellular matrix components and their receptors. Thus, the ability of a cell to grow in response to TIMP-2 may depend on its sensitivity to cAMP at the time of TIMP-2 stimulation. The effects of TIMP-2 on cell growth under these *in situ* conditions may be very different. In fact, we have recently demonstrated that TIMP-2 selectively blocks the mitogenic effects of basic fibroblast growth factor on human microvascular endothelial cells (17). Thus, TIMP-2 may have distinctive effects on cell growth depending on the specific cell type and presence of other growth factors. We are currently investigating the interaction of signaling pathways involved in the disparate effects of TIMP-2 on cell growth.

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