Tissue Inhibitor of Metalloproteinases-2 (TIMP-2) Suppresses TKR-Growth Factor Signaling Independent of Metalloproteinase Inhibition*

The tissue inhibitors of metalloproteinases (TIMPs) block matrix metalloproteinase (MMP)-mediated increases in cell proliferation, migration, and invasion that are associated with extracellular matrix (ECM) turnover. Here we demonstrate a direct role for TIMP-2 in regulating tyrosine kinase-type growth factor receptor activation. We show that TIMP-2 suppresses the mitogenic response to tyrosine kinase-type receptor growth factors in a fashion that is independent of MMP inhibition. The TIMP-2 suppression of mitogenesis is reversed by the adenylate cyclase inhibitor SQ22536, and implicates cAMP as the second messenger in these effects. TIMP-2 neither altered the release of transforming growth factor α from the cell surface, nor epidermal growth factor (EGF) binding to the cognate receptor, EGFR. TIMP-2 binds to the surface of A549 cells in a specific and saturable fashion (Kd = 147 pm), that is not competed by the synthetic MMP inhibitor BB-94 and is independent of MT-1-MMP. TIMP-2 induces a decrease in phosphorylation of EGFR and a concomitant reduction in Grb-2 association. TIMP-2 prevents SH2-protein-tyrosine phosphatase-1 (SHP-1) dissociation from immunoprecipitable EGFR complex and a selective increase in total SHP-1 activity. These studies represent a new functional paradigm for TIMP-2 in which TIMP suppresses EGF-mediated mitogenic signaling by short-circuiting EGFR activation.

In mature normal tissues, the structure and composition of the extracellular matrix (ECM)1 functions to maintain homeostasis and cellular quiescence. These anti-proliferative and differentiation promoting effects of the ECM are attributable both to its composition and three-dimensional spatial organization, as well as the presence of soluble growth inhibitors, such as TGF-β (1–4). Compelling evidence for these effects also comes from transgenic animal studies in which altered ECM expression or organization, disruption of ECM attachments, or proteolytic modification of ECM integrity results in altered developmental and disease-related phenotypes (5–7). The matrix metalloproteinases (MMPs) are a major determinant of ECM turnover in tissue morphogenesis. Altered expression of MMP activity is associated with a variety of pathologic conditions, including tumor progression and cancer invasion (5–8).

In addition to disrupting the structural organization of the ECM, MMP proteolysis of ECM can result in release and/or activation of sequestered growth factors (1, 3). In addition, MMP activity may expose cryptic sites in the ECM or directly modify cell surface receptors or ligands involved in both cell-matrix, as well as cell-cell adhesion (1, 3, 9). The endogenous metalloproteinase inhibitors, tissue inhibitors of MMPs (TIMPs), negatively regulate the proteolytic activity of MMPs during ECM turnover. Reduction or ablation of TIMP gene expression results in enhanced ECM proteolysis concomitant with up-regulation of cell invasive activity of nontransformed differentiated cells (10, 11). In comparison, TIMP overexpression results in decreased invasion of endothelial and tumor cells both in vitro and in vivo (12, 13). Recent transgenic animal studies have demonstrated that alteration of the MMP/TIMP balance in vivo in favor of TIMP-1 activity can block neoplastic proliferation in the SV40 T antigen-induced model of murine hepatocellular carcinoma (14). The mechanism of this TIMP-1 effect was mediated by direct inhibition of MMP processing of insulin-like growth factor-binding protein-3 (IGFBP-3), thereby preventing the release of insulin-like growth factor II and thus suppressing mitogenic activity. These and other studies demonstrate that, through inhibition of MMP activity and prevention of ECM turnover, TIMPs can suppress cell proliferation, invasion and reduce metastasis formation, i.e. TIMPs act as tumor suppressors. As a result of such studies, targeting MMP activity with synthetic MMP inhibitors has become an attractive strategy for therapeutic intervention in cancer progression (15). However, recent studies suggest that TIMPs may also directly modulate cell growth in an MMP-independent fashion, although many of these studies lack detailed mechanistic insight (16–20). Thus, in addition to their action as inhibitors of metalloproteinases, it is important to investigate whether TIMPs function to directly modulate cell growth and the potential mechanisms for these effects.

Epidermal growth factor receptor (EGFR) is highly expressed in human cancers and is detectable at low levels in many normal tissues (21). Overexpression of EGFR has been observed in a variety of human tumors, and EGFR-related growth factors play a role in human cancer growth through

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1 The abbreviations used are: ECM, extracellular matrix; TIMP, tissue inhibitor of matrix metalloproteinases; MMP, matrix metalloproteinase; Ala1-TIMP-2, amino-terminal alanine appended TIMP-2; TKR, tyrosine kinase-type receptor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGF-α, transforming growth factor α; PDGF, platelet-derived growth factor; ERK, extracellular regulated kinase; PTP, protein-tyrosine phosphatase; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline solution; MT-1-MMP, membrane-type matrix metalloproteinase-1; MOPS, 4-morpholinepropanesulfonic acid.
autocrine and paracrine mechanisms (22). Overexpression of EGFR (23) or structural alterations in the receptor protein, such as truncation of the cytoplasmic domain, may elicit ligand-independent signaling and autonomous cell growth (24). Ligand binding to EGFR initiates receptor dimerization, auto-phosphorylation of tyrosyl residues on the cytoplasmic domain of EGFR, and subsequently Src-mediated activation of the extracellular signal-regulated kinase mitogen-activated protein (MAP) kinase pathway (25). Mitogenic signaling of the EGFR seems to critically depend on activation of the extracellular signal-regulated kinase/mitogen-activated kinase cascade.

Here we study the role of TIMP-2 in the regulation of cell growth in response to tyrosine kinase-type receptor (TKR) growth factor stimulation. In addition to soluble ligand binding, membrane-anchored ligands can also stimulate TKR-mediated mitogenic responses at high cell densities or following proteolytic processing from the cell surface. Examples are the membrane-anchored EGFR ligands, which include heparin-bound epidermal growth factor, amphiregulin, transforming growth factor-α (TGF-α), and betacellulin, which are shed from the plasma membrane by proteolytic cleavage resulting in autocrine activation of the receptor (26, 27). Synthetic metalloproteinase inhibitors, such as BB-94 (Batimatstat), reduce cell proliferation in the human mammary epithelial cell line 184A1 by blocking TGF-α release (26). BB94 also inhibits EGFR trans-activation by G-protein-coupled receptors that occurs via a metalloproteinase directed cleavage of pro-heparin-bound EGF (27). These findings suggest that the metalloproteinase inhibitors prevent the release of membrane-anchored EGFR ligands (e.g. TGF-α, pro-heparin-bound EGF), thereby inhibiting autocrine activation of the receptor protein (26). However, if soluble ligands that do not require metalloproteinase processing (e.g. EGF) are present, BB-94 did not inhibit the mitogenic response in these experiments (26). Thus we have focused our experiments on TIMP inhibition of cellular responses to soluble mitogenic factors, in particular EGF.

We have examined the direct modulation of TKR growth factor-stimulated proliferation of human, A549 lung carcinoma, MCF7 mammary carcinoma, HT1080 fibrosarcoma, and Hs68 dermal fibroblast cells using both wild type TIMP-2 (wt-TIMP-2) and a null-inhibitor form of TIMP-2, Ala+TIMP-2. Both forms of TIMP-2 abrogate the TKR-mediated mitogenic responses in these cells. We also investigated the mechanism of the diminished mitogenic response following TIMP-2 pretreatment prior to growth factor stimulation. The results demonstrate that these suppressive effects are mediated by disruption of TKR activation proximal to the extracellular signal-regulated kinase pathway. To our knowledge this is the first demonstration that TIMP-2 can directly suppress activation of a mitogenic response through suppression of TKR activation in an MMP-independent fashion.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Recombinant human EGF, PDGF, and bFGF were obtained from R&D Systems, Minneapolis, MN. The following commercially available antibodies were obtained: human anti-EGFR, clone 52/8, mouse, monoclonal IgG<sub>2a</sub> (Santa Cruz Biotechnology, Santa Cruz, CA); human anti-phosphotyrosine, clone PY-20, mouse IgG<sub>1</sub>, monoclonal, (Transduction Labs, Lexington, KY); human anti-MT-1-MMP, clone 113-5B7 or 114-6G6 (catalytic), mouse, monoclonal (Chemicon International, Temecula, CA), human anti-Grb2, clone C-23, rabbit, polyclonal, (Santa Cruz, Santa Cruz, CA); human anti-SH-PTP1, clone C-19, rabbit, polyclonal (Santa Cruz Biotechnology); human anti-SH-PTP2, clone C-18, rabbit, polyclonal (Santa Cruz Biotechnology); mouse and rabbit IgG-horseradish peroxidase conjugate, (Santa Cruz); and goat anti-mouse IgG (H+L) (Kirkegaard & Perry, Gaithersburg, MD). MMP mouse IgG (H+L-horseradish peroxidase conjugate, (Santa Cruz); and goat anti-C-18, rabbit, polyclonal (Santa Cruz Biotechnology); human anti-SH-PTP2, clone C-19, rabbit, polyclonal (Santa Cruz Biotechnology); human anti-Grb2, clone C-23, rabbit, polyclonal, (Santa Cruz, Santa Cruz, CA); human anti-SH-PTP1, clone C-19, rabbit, polyclonal (Santa Cruz Biotechnology); human anti-SH-PTP2, clone C-18, rabbit, polyclonal (Santa Cruz Biotechnology); mouse and rabbit

**Cell Culture Conditions—**Human lung adenocarcinoma cells (A549; ATCC CCL 185), human fibrosarcoma cells (HT1080; ATCC CCL 121), human breast adenocarcinoma (MCF7; ATCC HTB 22), and human dermal fibroblasts (Hs68; ATCC CRL 1635) were obtained from American Tissue Culture Collection (Manassas, VA). Cells were grown to 80% confluence in Dulbecco’s modified Eagle’s medium (Life Technologies Inc.) containing 4500 mg/liter of α-glucose, γ-glutamine, sodium pyruvate, 100 units/ml penicillin-G, 100 μg/ml streptomycin sulfate, and 10% heat-inactivated fetal bovine serum, unless otherwise indicated. Cells were trypsinized using trypsin-EDTA (Life Technologies, Inc., Bethesda, MD).

**Cell Growth Assays—**A549, Hs68, HT1080, and MCF7 cells were plated at 5 × 10<sup>4</sup> cells/well on a 96-well Costar plate for 18 h in DMEM with 10% fetal bovine serum. The cells were then starved for 18 h in DMEM without serum to synchronize cells in G<sub>1</sub> (or G<sub>0</sub>) phase of the cell cycle. Fresh serum-free DMEM was added to the wells prior to treatment with TIMP-2. Cells were routinely incubated with TIMP-2 at the indicated concentrations for 30 min, followed by incubation in DMEM with or without EGF (100 ng/ml, R & D Systems), bFGF (50 ng/ml, R & D Systems), or PDGF (50 ng/ml, R & D Systems) and incubated for 24 h. TIMP-2 pretreatment could be reduced to 1 min prior to addition of growth factor without loss of an effect on growth factor stimulation. Following growth factor stimulation, the cells were incubated for 1 h with the CellTiter 96<sup>AQUAM</sup> One Solution reagent (Promega, Madison, WI) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, and phosphate-ethosulfate. The quantity of formazan product was determined by the 96 nm absorbance, and was directly proportional to the number of living cells in culture. The mean and S.D. for triplicate determinations were recorded for all incubation conditions.

Alternatively, the mitogenic response to growth factor stimulation with and without TIMP-2 pretreatment was quantitated by [<sup>3</sup>H]thymidine incorporation assays. Cells were synchronized in both serum-free and serum containing conditions and treated with TIMP-2 and growth factors as described above. Following growth factor treatment [<sup>3</sup>H]thymidine (0.1 μCi/ml; Amer sham Pharmacal Biotech) was added and incubated for 2 h at 37 °C. The percentage of thymidine incorporated in a 2-h pulse correlated in a linear fashion with the cell number. The culture medium was subsequently discarded, the wells were washed twice with phosphate-buff ered saline (PBS), and the cells were fixed in methanol/glacial acetic acid (3:1). The incorporated [<sup>3</sup>H]thymidine was extracted as described previously and quantitated by liquid scintillation counting (31). The mean and S.D. of triplicate assays were determined for all incubation conditions. SQ22536 or 9-tetrahydro-2-furylidenec (Calbiochem/Novabiochem) was solubilized in sterile deionized H<sub>2</sub>O and added to cells at a final concentration of 100 μM (32). H-89 was dissolved in sterile, deionized H<sub>2</sub>O and added to give a final concentration of 0.1 μM (33). The results of the growth assays are presented as the percentage of maximal growth factor response for the mitogen being tested after correcting for nonstimulated growth in basal medium. This allows comparison of the effects of TIMP-2 or Ala+TIMP-2 on the mitogenic response to various growth factors as well as growth factor stimulation.

**Immunoprecipitation and Western Blotting—**HT1080, Hs68, A549, or MCF7 cells were grown in a 6-well Costar plate, pretreated with TIMP-2 or Ala+TIMP, followed by growth factors (described above). Following incubation with growth factor for 5 min, 37 °C, cells were washed with PBS and treated for 10 min at 4 °C with RIPA lysis buffer containing freshly added protease inhibitors (10 μg/ml aprotinin, 30 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, and 100 μM sodium Jolla, CA). The recombinant MT-1-MMP catalytic domain (150 units/mg) was purchased from Chemicon International and metalloproteinase activity was determined by the thioproteolysis assay as described previously (28). RIPA buffer consists of 50 mM Tris- HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS. The TIMP-2 protein was expressed using vaccinia virus expression system and purified as described (29). TIMP-2 was also expressed in Esherichia coli with the authentic sequence (wt) or with an alanine residue appended to the amino-terminal cysteine (Ala+TIMP-2) as described previously (28). TIMP-2 and Ala+TIMP-2 were purified by gel filtration in 4 x guanidine HCl, folded, and oxidized, and then purified by high performance liquid chromatography gel permeation chromatography using 50 mM Tris- HCl, 150 mM NaCl, pH 7.5. All TIMP preparations were endotoxin tested using the Limulus amoeobe cytosis assay and found to contain less than 2 EU/mg of protein.
TIMP-2 Suppresses EGFR Activation

**RESULTS**

**TIMP-2 Suppresses Tyrosine Kinase Growth Factor-stimulated Cell Proliferation**—Previous reports have shown that TIMP-2 and Ala+TIMP-2 can stimulate the growth of quiescent (serum starved) cells in culture (28, 34). However, this system does not represent a physiologic setting in which multiple stimuli, both positive and negative, are integrated to determine the cellular response. The effects of TIMP-2 on the mitogenic response to TKR growth factors in several cell lines, including Hs68, HT1080, A549, and MCF7 cells were examined in *vitro*. Treatment of these quiescent cells with a variety of TKR growth factors, including EGF, bFGF, and PDGF, results in a 2-fold stimulation of cell growth. A representative example of this growth stimulation is presented in the inset in Fig. 1A. Preincubation of quiescent A549, HT1080, Hs68, or MCF7 cells with increasing TIMP-2 concentrations followed by addition of a TKR growth factor, such as EGF, results in dose-dependent inhibition of the mitogenic response (Fig. 1A). Preincubation of cells with TIMP-2, prior to addition of growth factor, was routinely performed for 30 min at 37°C, but identical effects were observed with preincubation periods as short as 1 min, as previously reported (28). This suppressive effect on growth factor stimulation was not observed if TIMP-2 was added concurrent with EGF stimulation or after treatment of the cells with EGF (data not shown).

The observed effects of TIMP-2 on mitogenic response are not specific for A549 cells. The effects of TIMP-2 on the EGF-stimulated responses of HT1080 human fibrosarcoma and MCF7 human mammary carcinoma cells are essentially identical to those observed with the A549 cells (Fig. 1A). TIMP-2 maximally inhibited (student *p* < 0.01) the mitogenic response in all cell lines tested to 50–60% of the levels observed following EGF stimulation alone (Fig. 1A). Similarly, TIMP-2 reduced the mitogenic response to bFGF and PDGF in these cell lines (data not shown). The effects of TIMP-2 on the mitogenic responses were observed at low nanomolar concentrations (<10 nM) with the maximal suppression of growth factor-mediated proliferation obtained at 20–50 nM TIMP-2.
TIMP-2 Effect on Mitogenic Response Is Not Dependent on MMP Inhibition—To determine whether the effect of TIMP-2 on growth factor mitogenic response in these cells was dependent on inhibition of MMP activity, we examined the effect of other MMP inhibitors on the mitogenic response. We utilized the endogenous MMP-inhibitor, TIMP-1, a synthetic MMP inhibitor (BB-94, Batimastat), and Ala+TIMP-2, a form of TIMP-2 that lacks MMP inhibitor activity. Neither TIMP-1 nor the synthetic hydroxamate inhibitor, BB-94, demonstrated any modulating effects on mitogenic stimulation in any of the cell lines tested (data not shown, see below). However, Ala+TIMP-2 was effective at inhibiting the proliferative response stimulated by EGF, bFGF, and PDGF treatment of A549 and Hs68 cells (Fig. 1). Pretreatment with 50 nM Ala+TIMP-2 prior to exposure to bFGF or PDGF suppressed the growth factor-mediated mitogenic response of Hs68 and A549 cells (Fig. 1B). Ala+TIMP-2 suppressed cell growth to the levels observed with TIMP-2 stimulation alone, without addition of growth factors (Fig. 1B). The suppressive effect of Ala+TIMP-2 also demonstrated a dose dependence (Fig. 1C), however, Ala+TIMP-2 suppressed the mitogenic response to lower levels than those achieved with TIMP-2.

TIMP-2 Inhibition of Growth Factor Response Requires Adenylate Cyclase Activity—We previously reported that the mitogenic effects of TIMP-2 on quiescent Hs68 or HT1080 cell proliferation were dependent on activation of a heterotrimeric adenylate cyclase.
TIMP-2 Suppresses EGFR Activation

G protein, and a subsequent increase in cytosolic cAMP (34). In the present study, we examined the effects of an adenylate cyclase inhibitor (SQ22536) on TIMP-2 suppression of EGF-stimulated cell proliferation. Pretreatment of A549 cells with SQ22536 (100 μM), followed by TIMP-2 (50 nM) and then EGF, ablated the suppressive effect of TIMP-2, and restored the EGF-stimulated mitogenesis to levels observed in the absence of TIMP-2 (Fig. 2). In addition, the suppressive effects of TIMP-2 on mitogenesis were mimicked in these cells by use of nonhydrolyzable cAMP analogues, such as dibutryl-cAMP or S-p-cAMP (100 μM). Treatment of cells with cAMP analogues prior to stimulation with EGF suppressed the proliferative response in these cells to similar levels as observed with TIMP-2 or Ala1TIMP-2 (data not shown). These results are identical to our previous study (28) on the effect of TIMP-2 on the growth of serum-starved quiescent fibroblasts and suggest that G protein activation and stimulation of adenylate cyclase are common mechanisms for both effects.

**TIMP-2 Does Not Compete for EGF Binding but Binds to the Cell Membrane Independent of MT-1-MMP—**The mechanisms of the TIMP-2 mediated effects on stimulated mitogenesis were examined using A549 cells. Among possible mechanisms for the observed effects of TIMP-2 on cell growth are the inhibition of protease-mediated release of cell surface-bound EGF ligands or competition and displacement of exogenous EGF from its cognate receptor. An alternative possibility is the direct binding of TIMP-2 to the cell surface and activation of adenylate cyclase activity required for inhibition of growth factor stimulation.

The effect of TIMP-2 on shedding of TGF-α from the surface of A549 cells was examined by enzyme-linked immunosorbent assay measurement of TGF-α released from A549 cells. Incubation of A549 cells with 10–100 nM TIMP-2 resulted in no detectable decrease in soluble TGF-α concentration (<2.5 pg/ml). Whereas addition of 1–10 nM active gelatinase-A (MMP-2) resulted in an increase in soluble TGF-α (>8 pg/ml) released from A549 cells. Thus, TIMP-2 did not mediate growth suppressive effects by interfering with MMP-dependent proteolytic cleavage of membrane-anchored EGF ligands.

Next, we examined the effects of TIMP-2 on cell surface binding of EGF. Experiments with 125I-EGF showed that TIMP-2 does not directly compete with EGF binding to the EGFR (Fig. 3). Incubation of A549 cells with TIMP-2 or Ala1TIMP-2 (100 nM), followed by addition of 125I-EGF (0.2 nM), did not interfere with the binding of 125I-EGF to the EGFR protein. 25I-EGF binding was competed by addition of unlabeled EGF, as expected (Student’s t test, p < 0.01). Addition of TIMP-2 or Ala1TIMP-2 did not alter the competition of EGF for 125I-EGF bound to EGFR (Fig. 3). These results definitively demonstrate that TIMP-2 or Ala1TIMP-2 do not alter the mitogenic response in the cells tested by interfering with the binding of EGF to its cognate receptor.

The direct binding of TIMP-2 to the surface of A549 and MCF7 cells was quantified by use of a direct, fluorescent binding assay. Previous reports from several laboratories have shown that TIMP-2 can bind to the membrane-type matrix metalloproteinase-1 (MT-1-MMP) (8, 35–38). This interaction is mediated predominantly through interaction of the NH2-terminal inhibitory domain of TIMP-2 with the catalytic active site of MT-1-MMP. In our study of TIMP-2 binding to the surface of A549 cells we have utilized both TIMP-2 and the null inhibitor form Ala1TIMP-2. Data shown in Fig. 4A demonstrate that Ala1TIMP-2 does not inhibit the ability of MT-1-MMP to degrade synthetic peptide substrate, compared with the potent inhibitory activity of TIMP-2. These results are similar to our previous report that TIMP-2 inhibits MMP-2 activity, while Ala1TIMP-2 does not (28).

In the binding experiments, TIMP-2-BODIPY was added to a monolayer of cells, grown to 80–90% confluence, and the amount of bound (B) versus free (F) fluorescent TIMP-2-BODIPY was determined by quantitation of fluorescence (Fig. 4B). The concentration dependence of BODIPY-TIMP-2 binding was determined at each concentration in six replicate measurements in the presence (nonspecific binding) and absence (total binding) of unlabeled TIMP-2. The data were plotted as the amount of bound TIMP-2-BODIPY versus bound/free for Scatchard analysis (Fig. 4B). TIMP-2 bound to A549 cells in a specific and saturable fashion with a subnanomolar dissociation constant, Kd = 147 pm, and 115,00 receptors per cell. For comparative purposes the binding parameters for BODIPY-TIMP-2 interaction with MCF7 cells was also determined. For these cells the dissociation constant was low nanomolar Kd = 1.90 nM with 38,000 sites per cell. The data for TIMP-2 binding to MCF7 cells was in excellent agreement with data previously published by others (36, 38, 39), as well as our own laboratory (35), using 125I-TIMP-2 for determination of cell binding parameters. These findings suggest that our fluorescent-based method for determination of TIMP-2 binding was comparable.
in sensitivity and specificity to methods described previously.

The addition of a 10-fold excess of unlabeled Ala+TIMP-2 to A549 cells treated with TIMP-2-BODIPY leads to a statistically significant (Student’s t test, p < 0.001) reduction in binding of TIMP-2 to the cell surface. Ala+TIMP-2 competition results in a 65% decrease in BODIPY-TIMP-2 binding to A549 cells suggesting that it competes for binding to most, but not all, TIMP-2-binding sites (Fig. 4B). In contrast, addition of a broad spectrum, hydroxamate MMP inhibitor, BB-94 (0.5 μM), did not significantly compete for binding of BODIPY-TIMP-2 to the cell surface (Fig. 4B). This failure of BB-94 to compete with TIMP-2 cell surface binding was in contrast to the effects of synthetic hydroxamate MMP inhibitors which have been shown to inhibit the binding of TIMP-2 to MT-1-MMP (36, 38). Western blot analysis of A549 cell membranes demonstrated that these cells have low but detectable levels of MT-1-MMP compared with well characterized cell lines such as HT1080 (data not shown). However, addition of an MT1-MMP antibody, specific for the catalytic domain, maximally reduced the binding of TIMP-2 to cells by 35% (Fig. 4B). Together these data suggest that TIMP-2 binds to the cell surface and that this interaction may consist of at least two binding sites, an interpretation consistent with the Scatchard analysis shown in Fig. 4A, as well as previous reports (36).

Confocal, laser fluorescent microscopy was utilized to examine the localization of TIMP-2 on the cell surface and colocalization with MT-1-MMP. Confocal fluorescent microscopy demonstrated fluorescent-labeled (BODIPY) TIMP-2 or Ala+TIMP-2 bound to the surface of A549 cells (Fig. 5A). Analysis of Nemarsky optic images of A549 cells with or without TIMP-2 or Ala+TIMP-2 treatment revealed no significant morphologic changes following short term (30 min) exposure to TIMP-2 (Fig. 5, A, top and middle left panels; B, top two panels). Fluorescence localization of BODIPY-TIMP-2 or Ala+TIMP-2 demonstrates a linear, punctate pattern consistent with cell surface localization (Fig. 5, A, lower panels, B, second panels from top). Xz-axis analysis of the confocal images (Fig. 5A, bottom panel) confirms that TIMP-2 binding occurs on the surface of A549 cells. By fluorescent antibody staining the cells with rhodamine anti-MT-1-MMP antibody complexes, the majority of TIMP-2 binds to the surface of A549 cells independent of MT1-MMP localization (Fig. 5B, bottom panel right, and arrowheads). A minor component of the TIMP-2 on the cell surface colocalized with MT-1-MMP (Fig. 5B, bottom panel, and arrows). The MT-1-MMP colocalization was markedly less apparent when the binding of Ala+TIMP-2 was examined (Fig. 5B, bottom panel, left). Ala+TIMP-2 colocalization with MT1-MMP (Fig. 5, bottom panel, left, and arrows) was reduced compared with that observed with wtTIMP-2 (Fig. 5, bottom panel, right, and arrows). These findings are consistent with our in vitro observations that the Ala+TIMP-2 mutant does not inhibit MT-1-MMP activity (Fig. 4A) (i.e., Ala+TIMP-2 does not bind to the MT-1-MMP active site).

TIMP-2 Disrupts EGFR Phosphorylation and Grb-2 Association

**Fig. 4.** TIMP-2 and Ala+TIMP-2 activity against MT-1-MMP catalytic domain and cell binding experiments. A, measurement of Ala+TIMP-2 suppressive activity against MT-1-MMP. TIMP-2 and Ala+TIMP-2 inhibition of MT-1-MMP activity were determined using the thiopeptolide assays as described previously (28). These assays were performed in 50 mM MOPS, 150 mM NaCl, 1 mM CuCl2, and 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) at pH 7.0. Concentrations of the catalytic domain and cell binding experiments.

A549 cells were seeded onto 96-well plates and treated with TIMP-2-BODIPY (0–100 nM) for 30 min, 37 °C. Supernatant was transferred to new wells and measured as the amount of unbound TIMP-2-BODIPY. Cells were washed with PBS and the amount of TIMP-2-BODIPY was measured as the amount of fluorescence remaining on the A549 cell surface. Concentration of bound TIMP-2-BODIPY was determined with a standard curve (TIMP-2-BODIPY concentration versus fluorescence units) and plotted against Bound/Free, to give the resulting Scatchard plot. Scatchard analysis was performed as described previously (35). Unlabeled TIMP-2 was added to cells to determine specific binding from total and nonspecific binding. C, a 10-fold excess of Ala+TIMP-2 (unlabeled) was added to cells, resulting in a reduction of bound fluorescence (Student’s t test, p < 0.01), whereas addition of BB94 (0.5 μM) did not result in any significant change. However, addition of 10 μg/ml anti-MT1-MMP (clone 114–6G6) resulted in a reduction in bound TIMP-2-BODIPY fluorescence (Student’s t test, p < 0.05).
From these studies so far, we have shown that TIMP-2 suppresses EGF mitogenesis without the requirement for MMP inhibition. TIMP-2 binds to the plasma membrane thereby activating an adenylate cyclase signaling pathway. This binding is independent of MT1-MMP, and does not compete for EGF ligand binding to the EGFR. These findings suggest that the effect of TIMP-2 on mitogenic stimulation should be rapid and proximal in the EGF signaling pathway. To further study the mechanism of TIMP-2 effects on the mitogenic response, we have examined the activation status of the EGFR receptor (phosphorylation status, Grb-2 association), as well as phosphatase activity that influences the state of EGFR activation.

Ligand binding induced activation of the EGFR initiates autophosphorylation of the receptor on the cytoplasmic, SH2 domain. The amount of phosphorylated EGFR was measured by Western blot analysis of EGFR immunoprecipitates prepared from equivalent numbers of A549 lung adenocarcinoma or HT1080 fibrosarcoma cells. The total quantity of immunoprecipitable EGFR did not change in response to treatment with TIMP-2 or Ala\(^{1}\)TIMP-2 and therefore served as a loading control for these experiments (Fig. 6A, lower gel panel). This finding also demonstrates that the effect of TIMP-2 or Ala\(^{1}\)TIMP-2 occurs in the absence and/or prior to any change in the level of EGFR on the cell surface (i.e. EGFR internalization and/or turnover). TIMP-2 pretreatment prior to EGF stimulation of HT1080 cells results in a dose-dependent decrease in EGFR-associated tyrosine phosphorylation (Fig. 6A). At the highest concentration of TIMP-2 tested (200 nM) in these experiments, the level of EGFR phosphorylation approached that of basal levels under serum-free conditions. It should be noted that under basal conditions the level of EGFR phosphorylation in these HT1080 cells was low but significantly increased (greater than 10-fold) following EGF stimulation of cell growth.
TIMP-2 and Ala + TIMP-2 also showed a dose-dependent inhibition of EGFR phosphorylation in MCF7 and A549 cells stimulated with EGF (data not shown). Inhibition of EGFR tyrosine phosphorylation was not observed following treatment with BB-94, or TIMP-1 prior to stimulation with EGF (Fig. 6B). This finding supports the conclusion that TIMP-2 exhibits an early and immediate effect on EGFR activation by a mechanism independent of MMP-inhibition.

We also examined the effects of an adenylyl cyclase inhibitor, SQ22536, on phosphorylation of EGFR tyrosyl residues. Preincubation of A549 cells with SQ22536 (100 μM), followed by TIMP-2 (100 nM), and then stimulation with EGF, blocks the TIMP-2 suppressive effects on EGFR phosphorylation (data not shown). When cells were preincubated with SQ22536, the amount of phosphorylated EGFR remains similar to levels obtained with EGF stimulation alone. Thus, the effect of TIMP-2 on EGFR phosphorylation, like the growth suppressive effect reported in Fig. 2 depends on activation of adenylyl cyclase.

Following ligand-induced dimerization and autophosphorylation of EGFR, there is specific recruitment of Grb-2 to phosphorylated EGFR (40). Therefore, we determined the level of Grb-2 associated with EGFR to confirm the effects of TIMP-2 on EGFR phosphorylation. Cells were pretreated with TIMP-2 or Ala + TIMP-2 for 30 min, followed by incubation with EGF for 5 min at 37 °C. Cells were lysed using RIPA buffer and EGFR immunoprecipitates were prepared as before. The amount of 25-kDa Grb-2 bound to EGFR was determined by Western blot. The effects of TIMP-2 on PTP association with EGFR, as detected by anti-Grb-2, of EGFR immunoprecipitates. Ad-}

\[ \text{Hours} \]

\[ \text{EM} \]

\[ \text{H89} \]

\[ \text{TIMP-2} \]

\[ \text{EGF} \]

\[ \text{Phosphorylated EGFR-P} \]

\[ \text{TIMP-2} \]

\[ \text{Ala + TIMP-2} \]

\[ \text{EGF} \]

\[ \text{Grb-2} \]

\[ \text{associated with EGFR} \]

\[ \text{observed following TIMP-2 pretreatment. The results demonstrate that TIMP-2 and Ala + TIMP-2 pretreatment results in a rapid (within 5 min of EGF treatment) reduction in EGFR phosphorylation, that in turn results in decreased Grb-2 association with EGFR.} \]

**Role of PKA in TIMP-2 Effects on TKR-stimulated Proliferation and EGFR Phosphorylation**—Pretreatment of A549 cells with the PKA inhibitor, H89 (0.1 μM), prior to EGF stimulation results in a 60% reduction of the mitogenic response that is unchanged by the addition of TIMP-2 prior to EGF stimulation (Fig. 7A). Although H89 did not appear to reverse the suppressive effect of TIMP-2 on EGF-stimulated growth, the reduction in the EGF-induced mitogenic response by H89 alone prevents exclusion of a role for PKA in mediating the TIMP-2 suppression of EGF-stimulated growth.

H89 was also used to investigate the possible role of PKA in the TIMP-2 reduction in EGFR phosphorylation observed following EGF stimulation. The direct serine phosphorylation of the EGFR receptor by PKA catalytic subunit reportedly results in down-regulation of EGFR mitogenic signaling (41). We speculated that if PKA is required for the TIMP-2 down-regulation of EGFR tyrosine phosphorylation, that H89 would reverse the effect of TIMP-2 in reducing EGFR phosphorylation. Fig. 7B presents the results of these experiments. The data showed that the effects of H89 on reversal of TIMP-2 reduction in EGFR phosphorylation are at best only partial. The data from the two experiments on cell proliferation and EGFR phosphorylation using the PKA inhibitor H89 do not definitively demonstrate a clear-cut requirement for PKA activity in the growth suppressive effects of TIMP-2. However, the data suggest that an alternative pathway that is not dependent on PKA activation may also function to mediate the effects of TIMP-2 on cell growth and EGFR phosphorylation.

**TIMP-2 Induces SH2 Protein Phosphatase-1 (SH-PTP1) Activity and Association with EGFR**—The level of phosphorylation of activated growth factor receptors with endogenous tyrosine kinase activity depends upon the net result of both tyrosine specific autophosphorylation and rapid dephosphorylation by phosphotyrosine phosphatases (PTPs). Receptor dephosphorylation attenuates signaling downstream from the activated receptor. The SH2-domain containing PTPs (SH2-PTPs) have been shown to interact with multiple growth factor receptors, including EGFR (42–44). Generally, SHP-1 (also known as SH2-P2-1 and PTP-1C) negatively regulates receptor signaling, while SHP-2 (also known as SH2-P2-1 and PTP-1D) reportedly enhances positive signaling, although negative modulation of receptor signaling by SHP-2 has also been reported (42–44).

In this experiment, we determined if SHP-1 or SHP-2 was bound to EGFR by immunoprecipitation of the EGFR complex by Western blot. The effects of TIMP-2 on PTP association with this receptor complex were examined. Cells (A549 or HT1080) were preincubated with and without TIMP-2 (100 nM), and

**Fig. 7. Inhibition of PKA activity reduces EGF-stimulated proliferation of A549 cells and only partially reverses TIMP-2 reduction of EGFR phosphorylation.** A, cells were pretreated with H-89 (0.1 μM) for 30 min, prior to treatment with TIMP-2 (100 nM) for 30 min, and stimulation with EGF (200 ng/ml), for 24 h, 37 °C. Proliferation was measured as described under “Experimental Procedures.” B, serum-starved A549 cells were treated with H89 (0.1 μM) for 30 min, followed by TIMP-2 (50 nM), 30 min, and then EGF (200 ng/ml), 5 min. EGFR immunoprecipitates were prepared as described in the legend to Fig. 6. Each data point represents the average of three replicate measurements ± S.D.
stimulated with EGF as above. Analysis of SH2-PTPs associated with EGFR immunoprecipitates demonstrates that, compared with EGF treatment alone, TIMP-2 pretreatment preserves the association of SHP-1 with EGFR immunoprecipitates to levels that are essentially identical with those observed in the basal state. The levels of SHP-1 associated with EGFR complexes were inversely correlated with the level of EGFR phosphorylation previously observed (Figs. 6A and 8A and B). In contrast, the association of SHP-2 with EGFR complex remains unchanged following either TIMP-2 pretreatment or EGF stimulation of cells (Fig. 8A and B). We also assayed total cytoplasmic SHP-1 and SHP-2 activity utilizing an in vitro tyrosine phosphatase assay following selective immunoprecipitation of total SHP-1 and SHP-2. The results of these experiments demonstrate a significant (Student’s t test, p < 0.01) increase in total SHP-1 activity in cells pretreated with TIMP-2 prior to EGF stimulation (Fig. 8C, decrease in optical density for phosphotyrosine staining reflects enhanced phosphotyrosine activity). No increase in SHP-2 activity was observed following TIMP-2 preincubation. In fact, EGF stimulation alone results in a significant induction of SHP-2 activity and a slight inhibition of SHP-2 activity was noted following TIMP-2 pretreatment (Fig. 8C). These results demonstrate that TIMP-2 suppression of EGFR activation was mediated, at least in part, through persistent association of SHP-1 with EGFR and a selective increase total SHP-1 activity.

DISCUSSION

The tissue microenvironment is known to exert a profound influence on cell proliferation and differentiation (45–47). Cell
TIMP-2 Suppresses EGFR Activation

fate is the net result of cellular integration of multiple signals derived from soluble factors and adhesive interactions present in the microenvironment (48–50). Evidence for the influence of this integrative process on cell behavior is derived from studies using reconstituted ECM, as well as alteration in the expression of cell adhesion molecules, or the introduction of proteases to disrupt the structure and/or composition of the microenvironment (3, 6, 46, 49). ECM turnover is a critical event in development, morphogenesis, and tissue remodeling (3, 6, 46, 49). Enhanced MMP activity results in altered neonatal developmental and progression of pathologic conditions (6, 49). ECM turnover induces apoptosis (58, 59), an effect variably reproduced by synthetic MMP inhibitors and possibly related to stabilization of TNF-α receptors or inhibition of TNF-α converting enzyme (60).

In the present report we examine how TIMP-2 induction of intracellular signaling is integrated with TKR growth factor induction of mitogenic signals. TIMP-2 abrogates the mitogenic response of a variety of cell types to several different TKR-type growth factors. The TIMP-2 inhibition of growth factor-stimulated mitogenesis occurs in a concentration range identical to that observed for the maximal effect on stimulation of growth in quiescent, dermal fibroblasts (28, 34). These TIMP-2 concentrations are only slightly lower than reported for the growth suppressive effect of TIMP-2 on FGF-stimulated endothelial cells (31). Mitogenic stimulation of various cell lines at sub-nanomolar concentrations of TIMPs has also been reported (19, 53, 55). The differences in effective TIMP-2 concentrations between these reports are possibly due to variation in the innate sensitivity of the cell lines tested, or, alternatively, different TIMP preparations may have altered potency or contaminants (e.g. endotoxin). All of the TIMP-2 preparations in this study are known to be free of significant endotoxin contamination (see “Experimental Procedures”). It is also possible that differences in the levels of active MMPs produced by the cell lines may influence responsiveness to the growth modulating activity of TIMP-2. This is because high concentrations of available MMP active sites could sequester TIMP-2 and prevent it from binding to the cell surface.

The suppressive effect of TIMP-2 on growth factor-stimulated mitogenesis is specific and independent of MMP inhibition. No effects on stimulated cell growth are observed with TIMP-1 or BB-94. Ala+TIMP-2, lacking MMP inhibitor activity, remains active in abrogating the TKR-stimulated response. No evidence of cell death or apoptosis was observed in these experiments, and the cells remained viable following exposure to TIMP-2 or Ala+TIMP-2 alone. In fact, TIMP-2 or Ala+TIMP-2 treatment without growth factor stimulation reproduces the modest mitogenic stimulation of quiescent cells as previously reported (28, 34). Ala+TIMP-2 shows a somewhat more potent effect on inhibiting EGF-stimulated growth compared with the wild type protein. This is specific for EGF-stimulated growth and not observed with the other growth factors utilized in this study. Possible explanations for this observation include intrinsic differences in the growth factor-specific responses and/or greater availability of Ala+TIMP-2, which did not bind effectively to the MT-1-MMP active sites (see below).

TIMP-2 regulation of TKR-induced mitogenesis occurs immediately downstream of receptor-ligand interaction during receptor activation. Previous reports demonstrate that metalloproteinases contribute to shedding of EGFR ligands, such as TGF-α, as well as EGFR (61). TIMP-2 treatment did not alter EGFR levels, compete for EGFR binding to EGFR, or alter TGF-α release. These findings are again consistent with TIMP-2 growth modulation that is independent of inhibition of MMP activity required for either ligand or receptor processing.

TIMP-2 binds directly to the cell surface (19, 34, 36, 55). However, the identification of cell surface binding proteins for TIMP-2 is complicated by the presence of MT-MMPs. These integral membrane proteins contain a transmembrane domain and catalytic site that is oriented toward the ECM (8, 37). Binding of TIMP-2 to MT-1-MMP involves interaction of the NH2-terminal of TIMP-2 with the catalytic site of MT-1-MMP (38), and is reportedly sensitive to synthetic, hydroxamate MMP inhibitor (36). Our in vitro analysis confirms that Ala+TIMP-2 does not inhibit MT-1-MMP activity, as we have previously reported for MMP-2 (28). This finding suggests that Ala+TIMP-2 will not bind to the MT-1-MMP catalytic site on the cell surface.

We demonstrate specific and saturable binding of TIMP-2 and Ala+TIMP-2 to the surface of A549 and MCF7 cells. Cell surface binding of TIMP-2 to A549 cells was not competed by TIMP-1 or BB-94. Anti-MT-1-MMP catalytic domain antibodies reduce TIMP-2 binding by only 35%, compared with the 65% reduction following addition of excess unlabeled Ala+TIMP-2. In fluorescence confocal microscopy experiments, minor colocalization of TIMP-2 and MT-1-MMP in A549 cells is observed, but is essentially absent when Ala+TIMP-2 and MT-1-MMP are visualized. Collectively our studies on TIMP-2 binding and fluorescence co-localization confirm the presence of a high affinity, TIMP-2-binding site on A549 cells that is independent of MT-1-MMP. This is a principal binding site for TIMP-2 in A549 cells that is specifically competed by Ala+TIMP-2, but is not blocked by synthetic hydroxamate MMP inhibitors nor anti-MT-1-MMP antibody. The presence of such sites has been suggested in previous studies that demonstrated TIMP-2 binding in the presence of synthetic MMP inhibitor (36), but such sites have remained poorly characterized. From these studies we conclude that TIMP-2 binds to the cell surface and this interaction may consist of at least two binding sites, one MT-1-MMP independent, as well as a MT-1-MMP site. This interpretation is consistent with Scatchard analysis, as well as previous reports of multiple TIMP-2-binding sites (36). TIMP-2 binding to the MT-1-MMP-independent, high affinity site presumably results in activation of adenylate cyclase that is required for TIMP-2 inhibition of TKR-stimulated cell growth. TIMP-2 binding results in heterotrimeric G protein activation and an increase in cytosolic cAMP level (34).

Activation of downstream signaling cascades by TKR(s) is dependent on the net level of receptor phosphorylation. Net phosphorylation is dependent on the level of autophosphorylation induced by ligand binding and the level of associated protein-tyrosine phosphatase activity (62, 63). Cells treated with TIMP-2 or Ala+TIMP-2, prior to EGF, down-regulate the level of EGFR autophosphorylation in a dose-dependent fashion. This effect is again specific for TIMP-2 and is not observed with synthetic, hydroxamate MMP inhibitor, BB94, or TIMP-1. Furthermore, the decrease in EGFR phosphorylation is confirmed by a concomitant decrease in Grb-2 association with EGFR. This effect of TIMP-2 on EGFR autophosphorylation is dependent upon adenylate cyclase activity, but is not com-
TIMP-2 Suppresses EGFR Activation

Activated, TKR(s) are rapidly dephosphorylated resulting in down-regulation of their signaling activity (62, 63). The SH2-PTPs are known to interact with multiple receptor systems, including the erythropoietin receptor and interleukin-3 receptor in hematopoietic cells, as well as the EGFR and vascular endothelial growth factor (VEGF) receptor (Flt, KDR) in epithelial and endothelial cells, respectively (42–44). The SH2-PTP, SHP-1, is involved in receptor dephosphorylation and negative regulation of receptor signaling. SHP-2 has little effect on receptor phosphorylation and positively mediates receptor signaling via mechanisms that are not well understood. The direct interaction of SHP-1 with the EGFR receptor has been demonstrated in vitro (42–44). The catalytic domain of SHP-1 is important for EGFR dephosphorylation and cannot be substituted by the catalytic domain in SHP-2 (43). It is not known if SHP-1 displays selectivity with respect to dephosphorylation of individual phosphotyrosine residues on EGFR (43). Also, EGFR dephosphorylation does not absolutely correlate with SHP-1 binding (43). This has been interpreted to suggest that the SHP-1 activity site may be sterically hindered with respect to some phosphotyrosine sites on EGFR, and/or not all SHP-1 activity is directly bound to EGFR in vivo. SHP-1 may also associate with the EGFR complex via an intermediary docking protein.

In our experiments TIMP-2 prevents dissociation and/or promotes association of SHP-1 with the EGFR complex in a fashion that correlates with the decrease in EGFR phosphorylation. These findings are confirmed by direct measurement of phosphatase activity in SHP-1 and SHP-2 immunoprecipitates prepared from whole cell lysates. Comparison of the results of SHP-1 levels associated with EGFR in the basal and TIMP-2-treated cells with the direct assay of SHP-1 activity is consistent with previous reports that suggest not all SHP-1 activity is bound to EGFR (43). Alternatively there may be a selective increase in the specific activity of the SHP-1 associated with EGFR following TIMP-2 treatment. The observed increase in SHP-2 activity with EGF treatment is consistent with the reported positive modulation of EGFR signaling reported for this PTP (44). Our findings are consistent with previous reports demonstrating that SHP-1 can negatively modulate EGFR activation and prevent downstream signal propagation from this receptor (43, 44). Also, TIMP-2 may induce a selective increase in SHP-1 activity that is not directly bound to EGFR, but may require some auxiliary docking protein that is as yet unidentified. Little is known about regulation of SHP-1 levels or activity, but one report does suggest that a cAMP-regulated pathway involving phosphorylation of SHP-1 may function to regulate this activity (64).

In summary, TIMP-2 suppresses the mitogenic response to TKR growth factor stimulation. TIMP-2 binds to the surface of A549 cells independently of MT-1-MMP. TIMP-2 binding to the cell surface results in activation of adenylate cyclase and increased cAMP levels in the cytosol, presumably secondary to receptor-mediated activation of the GTP-binding protein Gαs. TIMP-2 modulates the phosphorylation of the EGFR following growth factor stimulation and this effect is mediated by SHP-1. These effects of TIMP-2 on the mitogenic response are observed with several different growth factors (EGF, bFGF, and PDGF) and in multiple cell types (including neoplastic cells as well as dermal fibroblasts). Based on these findings, we propose TIMP-2 functions to suppress inappropriate growth factor stimulation of cells in G1/G0 phase of the cell cycle, as well as to inhibit MMP-mediated ECM turnover. Consistent with our observation that TIMP-2 needs to be available prior to growth factor stimulation, and the proximal inhibition or down-regulation of EGFR activation (phosphorylation), TIMP-2 must function during early G1 prior to entry in the restriction point late in G1. Both this G1 regulator-type and protease inhibitor functions of TIMP promote tissue homeostasis. The G1 regulator-type function may be a feedback mechanism that informs cells that MMP-mediated remodeling of the ECM is complete. In this proposal TIMP-2 would not function to suppress mitogenesis until ECM remodeling is shutdown by inhibition of MMP activity. Saturation of available MMP active sites in the remodeling matrix and/or on the cell surface would block ECM turnover, only then would excess free TIMP-2 begin to suppress cell responsiveness to residual growth factor stimulation. Our proposal is consistent with the recent finding that tipm-2-deficient mice display no abnormalities in fertility or development (65, 66). As a G1 cell cycle phase regulator, one would expect that TIMP-2 would be a nonessential gene and that elimination from the germ line would not necessarily result in disruption of fetal development (67). The results of the present study demonstrate that TIMP-2 is multifunctional in suppressing growth factor stimulation, modulating MT-1-MMP activation of pro-MMP-2, in addition to the well established role of MMP inhibition, which prevents ECM remodeling. The growth suppression and MMP inhibitor functions both act to preserve tissue homeostasis. The control and integration of these TIMP-2 functions is poorly understood and warrants further investigation.

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