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Both tissue inhibitors of metalloproteinases-1 (TIMP-1) and TIMP-2 activate Ras but through different pathways

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Abstract

Tissue inhibitors of metalloproteinases-1 (TIMP-1) and TIMP-2 have growth-stimulating activity for a wide range of cell types. Ras, which comprises a family of three members, i.e, Ha-Ras, Ki-Ras, and H-Ras, is known to participate in growth control in all its facets, including cell proliferation, transformation, differentiation, and apoptosis. In this study, we tested the hypothesis that Ras might be involved in the cell growth-promoting activity of TIMPs. Using MG-63 human osteosarcoma cells, we demonstrated that both TIMP-1 and TIMP-2 caused an increase in the Ras–GTP level in a dose-dependent manner. Our previous results indicated that TIMP-1 activity is mediated through the tyrosine kinase (TYK)/mitogen-activated protein kinase (MAPK) pathway. Here, we demonstrated that Ras activation by TIMP-1 was inhibited by a specific TYK inhibitor, herbimycin A, suggesting that the TYK/MAPK signaling pathway was involved in Ras activation by TIMP-1. However, the activation of Ras by TIMP-2 was inhibited by an inhibitor specific for cyclic AMP-dependent protein kinase (PKA), H89, suggesting the involvement of the PKA-mediated pathway. Furthermore, TIMP-2 promoted the formation of a complex between Ras–GTP and phosphoinositide 3-kinase.

Keywords: TIMP-1; TIMP-2; Ras; Ras–GTP; Tyrosine kinase; Cyclic AMP; cAMP-dependent protein kinase; Phosphoinositide 3-kinase; Human osteosarcoma (MG-63) cells

Tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) [1–4] are now recognized as a family of intrinsic inhibitors of matrix metalloproteinases (MMPs). In addition to their functions as MMP inhibitors, TIMP-1 and TIMP-2 were reported to have potent growth-promoting activity for a wide range of cells [1,4], and some findings support the proposition that TIMP-1 and TIMP-2 are previously unrecognized cell-growth factors in serum [5,6]. The cell growth-promoting activity of both TIMP-1 and TIMP-2 appears to be a direct cellular effect mediated by cell-surface receptors [6–9] and is independent of their functions as MMP inhibitors [6,10]. Corcoran and Stetler-Stevenson [11] demonstrated that TIMP-2 mediated a mitogenic response in normal dermal fibroblasts and fibrosarcoma cells by stimulating adenylate cyclase to produce cAMP, which then activated PKA. However, Yamashita et al. [12] found that both TIMP-1 and TIMP-2 at low concentrations stimulated cell growth via the TYK/MAPK pathway.

A large variety of extracellular signals that activate receptors with TYK activity have been described to require Ras, which comprises a family of three members, i.e., Ha-Ras, Ki-Ras, and H-Ras, to exert their effects [13]. Moreover, receptors not directly associated with TYKs, such as T-cell receptors, heterotrimeric G protein-coupled receptors, and lysophosphatidic acid also activate Ras. Ras is a small GTP-binding protein having both GDP/GTP-binding and GTPase activities. Ras was well characterized to directly bind to and activate Raf, which then induces gene expression through the MAPK cascade in response to various extracellular signaling molecules. Ras regulates not only cell proliferation but also differentiation, morphology, and apoptosis. A variety of candidate Ras effectors have been reported in addition to Raf. These

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include RalGDS [14–16], RIN1 [17], and phosphoinositide 3-kinase (PI3K) [18]. Recently, TSH was shown to activate Ras through a cAMP-mediated pathway [19]; and PI3K, to be an important effector of Ras in cAMP-induced proliferation [20]. In this study, we present evidence that both TIMP-1 and TIMP-2 activate Ras. Furthermore, we found that TIMP-1 stimulated the activation of Ras, which is an upstream regulator of the TYK/MAPK cascade, whereas TIMP-2 activated Ras in a PKA-dependent manner and was shown to be directly involved in the formation of a Ras/PI3K complex.

Materials and methods

Human recombinant TIMP-1 and TIMP-2 were prepared as reported previously [6]. TIMP-1 and TIMP-2 concentrations were determined by one-step sandwich enzyme immunoassays for TIMP-1 [21] and TIMP-2 [22]. MG-63 cells (CRL 1427), a human osteosarcoma cell line, were grown to confluence in Dulbecco's modified Eagle's essential medium (DMEM; Sigma) supplemented with 10% fetal calf serum (Sigma). The cells were then starved for 18 h in DMEM without serum and subsequently stimulated by either TIMP-1 or TIMP-2 for the desired times. Herbimycin A (HA; Funakoshi, Tokyo, Japan) and H89 (Sigma) were added 18 and 1 h, respectively, prior to the stimulation by TIMPs.

Immunoprecipitation assays were performed after stimulation with reagents. Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% deoxycholic acid, 1% NP-40, 0.1% SDS, 25 mM NaF, 2 mM Na_3VO_4, and a protease inhibitor cocktail. Lysates were clarified by centrifugation. To detect PI3K/Ras or Ras/Raf-1 complexes, we incubated cell lysates with 5 μg anti-p21Ras or anti-Raf-1 antibody overnight at 4 °C. At the end of incubation, 40 μl of a 50% suspension of protein A–Sepharose was added and the incubation was continued for an additional 30 min. To isolate Ras–GTP, we incubated lysates overnight at 4 °C with 30 μg of GST-RalGDS RBD fusion protein precoupled to glutathione–Sepharose [23]. Proteins were electrophoresed and then transferred onto nitrocellulose filters. PI3K subunits, pRaf-1, and active Ras were detected by using their specific antibodies (Santa Cruz).

Western blot analysis for Ras and Raf-1 was performed by using whole cell protein extracts in lysis buffer. Protein samples were run on SDS–PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked at 4 °C overnight with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.05% Tween 20 and probed for 1 h with anti-Pan-Ras (Santa Cruz) or anti-Raf-1 (Santa Cruz) antibody in TBS containing 0.1% BSA and 0.05% Tween 20. After the membranes had been washed three times with TBS containing 0.05% Tween 20, they were incubated with an HRP-coupled secondary antibody (Santa Cruz) for 45 min. The blots were then washed three times with the same buffer mentioned above and developed by using ECL (Amersham) according to manufacturer's instructions.

Intracellular cAMP measurements were performed by incubation of MG-63 cells in 96-well plate for 18 h in DMEM without serum, after which the cells were stimulated with TIMP-1 or TIMP-2 for the desired period. The intracellular cAMP in the sample was determined by using an enzyme immunoassay (EIA) system (Amersham), based on the competition between unlabeled cAMP and a fixed quantity of peroxidase-labeled cAMP for the limited number of binding sites on a cAMP-specific antibody. The cAMP levels were expressed as fmol cAMP/10^4 cells.

Results and discussion

To determine whether Ras is a mediator in the signaling pathway involved in promoting cell growth by TIMPs, we first examined Ras activation in MG-63 cells stimulated with either TIMP-1 or TIMP-2. Either TIMP-1 or TIMP-2 stimulated the complex formation between Ras and GTP in a dose-dependent manner, giving maximum responses at 20 ng/ml TIMP-1 (Fig. 1A) and 100 ng/ml TIMP-2 (Fig. 1B). The important role of Ras has been suggested in signal transduction pathways for proliferation or differentiation through growth factor receptors with tyrosine kinase activity such as those for PDGF, EGF, insulin, and NGF [24–28]. Yamashita et al. [12] demonstrated that the cell growth-promoting activity of either TIMP-1 or TIMP-2 at low concentrations was mediated through the TYK/MAPK pathway. To determine whether tyrosine kinase (TYK) is involved in the activation of Ras, we treated MG-63 cells with herbimycin A (HA), prior to the stimulation with either TIMP. As shown in Fig. 2A, HA impaired TIMP-1-stimulated Ras activation, indicating that TIMP-1 activated Ras mostly through TYK; whereas HA had no effect at all on TIMP-2-stimulated Ras activation.

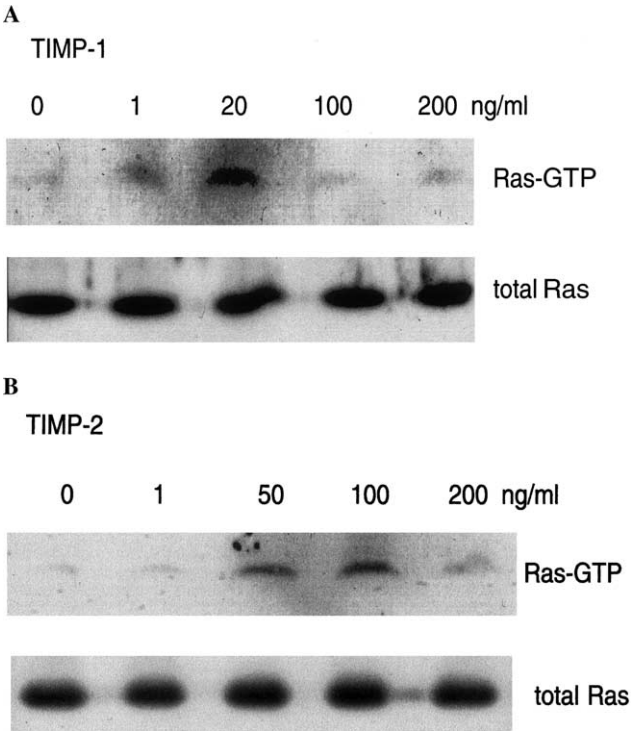

Fig. 1. TIMP-1 and TIMP-2 activate Ras. MG-63 cells grown to confluence were incubated for 18 h in DMEM without FCS. The cells were then stimulated with the indicated concentrations of TIMP-1 (A) or TIMP-2 (B) for 10 min. Cell lysates were incubated with glutathione–Sepharose-coupled peptides corresponding to RalGDS RBD. Bound GTP–Ras was eluted from the beads and analyzed by immunoblotting with anti-Pan-Ras antibody (Santa Cruz). Whole cell extracts were analyzed in parallel with anti-Pan-Ras antibody.

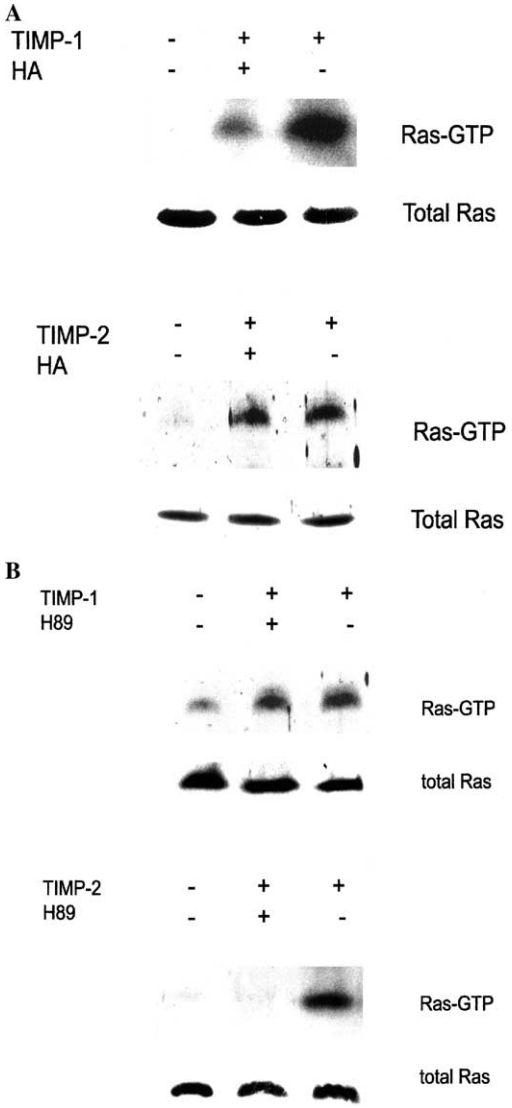

Fig. 2. Effects of inhibitors on Ras activation stimulated by TIMPs. MG-63 cells were pretreated with 1 μM herbimycin A (HA) for 18 h and then stimulated with either 20 ng/ml TIMP-1 or 100 ng/ml TIMP-2 for 10 min (A), or pretreated with 25 μM H89 (Sigma) for 1 h, prior to stimulation with either TIMP (B). Ras activation was monitored by using RalGDS RBD beads and detected with anti-Pan-Ras antibody (Santa Cruz). Whole cell extracts were analyzed in parallel with anti-Pan-Ras antibody.

cAMP has been reported to activate Ras through PKA-independent signaling pathways [19,29], but Ambrosini et al. [30] reported that Ras was activated at least in part by PKA. Corcoran and Stetler-Stevenson [11] reported that TIMP-2 at high concentrations (0.5–1 μg/ml) stimulated the proliferation of fibrosarcoma cells and normal dermal fibroblasts via a cAMP/PKA pathway. So, first we confirmed that TIMP-2 at 100 ng/ml stimulated the production of intracellular cAMP in a time-dependent manner (Fig. 3). Then, we tested whether H89, a typical PKA inhibitor, would affect Ras activation. H89 potently inhibited TIMP-2-

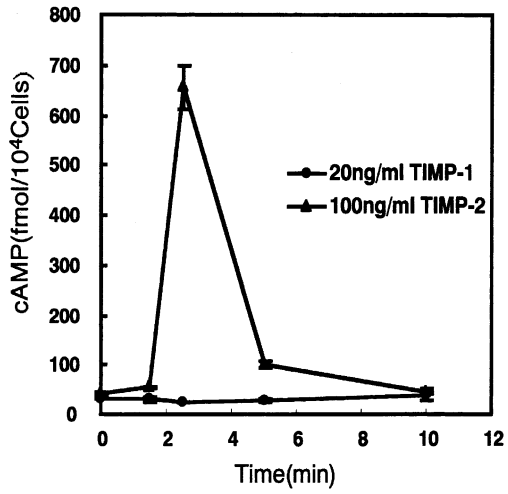

Fig. 3. TIMP-2 stimulates the production of intracellular cAMP. MG-63 cells were incubated in a 96-well plate for 18 h in DMEM without serum and subsequently stimulated by either 20 ng/ml TIMP-1 or 100 ng/ml TIMP-2 for the indicated periods. The intracellular cAMP in each sample was determined by use of the EIA system (Amersham). Results are the means of three experiments, along with SD.

mediated Ras activation, suggesting the involvement of PKA in Ras activation in MG-63 cells (Fig. 2b). However, H89 had essentially no effect on TIMP-1-mediated Ras activation.

It has been well documented that Raf-1 functions downstream of Ras and is at the heart of signaling networks that control proliferation, differentiation, and cell survival [31]. So we next examined whether TIMP-1 or TIMP-2 would activate Raf-1. Fig. 4 shows that TIMP-1 stimulated the phosphorylation of Raf-1, and also increased the amount of Ras–GTP complex immunoprecipitated together with Raf-1, suggesting that TIMP-1 enhanced the binding between Ras–GTP and Raf-1. However, pRaf-1 was not detected in TIMP-2-stimulated cell lysates (data not shown). As another downstream effector of Ras, PI3K has been implicated in the regulation of various cellular activities, including proliferation, differentiation, membrane ruffling, prevention of apoptosis, and insulin-stimulated glucose transport [32–34]. It is well known that PI3K is composed of an 85-kDa regulatory subunit (p85α, β) and 110-kDa catalytic subunit (p110α, β, γ). Recently, several truncated isoforms of the PI3K adapter subunit have been identified including a 55-kDa variant of p85 (p55γ or p55PIK) and two splice variants of the p85α gene, i.e., a 53–55-kDa form (AS53 or p55α) and a 50-kDa form (p50α) [32]. In this study, TIMP-2 increased the amount of proteins immunoprecipitated together with Ras showing immunoreactive bands at 55, 53, and 50 kDa (Fig. 5), which correspond closely to the reported multiple protein products of p55PIK [35], indicating that TIMP-2 enhanced the complex formation between Ras and p55PIK. The in vivo products of

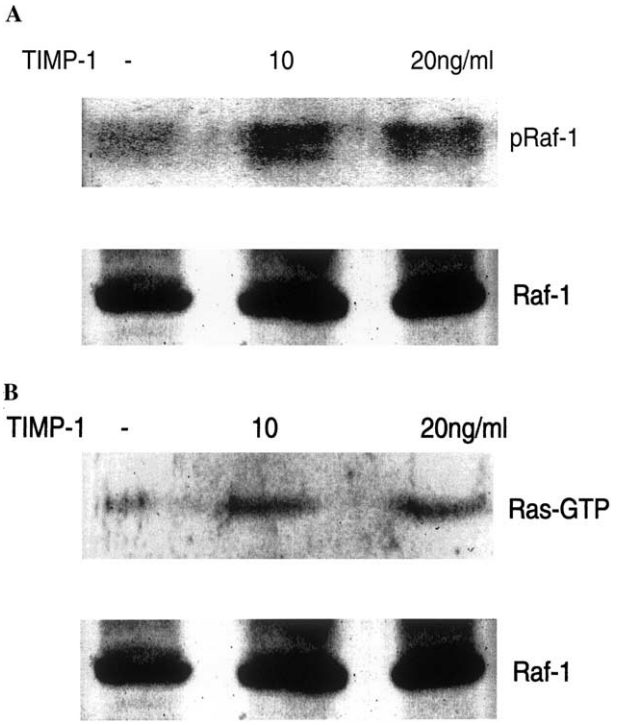

Fig. 4. TIMP-1 stimulates the formation of the Ras/Raf-1 complex. MG-63 cells were stimulated with TIMP-1 for 10 min. Cell lysates were immunoprecipitated with an antibody against Raf-1 (Santa Cruz), resolved on an SDS gel, transferred to nitrocellulose membranes, and blotted with (A) an antibody against pRaf-1 (Santa Cruz) or (B) Pan-Ras (Santa Cruz). The bottom panel in "B" shows a Western blot with the anti-Raf-1 antibody (Santa Cruz).

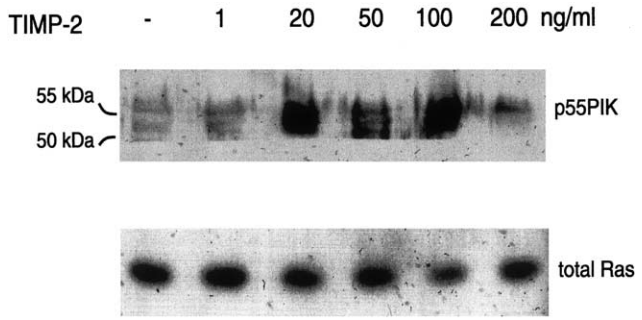

Fig. 5. TIMP-2 stimulates the formation of Ras/PI3K complex. MG-63 cells were stimulated with the indicated concentrations of TIMP-2 for 10 min. Cell lysates were immunoprecipitated with an antibody against Ras (Santa Cruz), resolved on a SDS gel, transferred to nitrocellulose membranes, and blotted with antibodies reactive with p85α, p85β, and p55PIK subunits of PI3K (Santa Cruz). The bottom panel is a Western blot with an anti-Ras antibody.

PI3K activity, phosphatidylinositol 3, 4, 5-triphosphate [PtdIns$(3,4,5)P_3$], targets p70 ribosomal S6 kinase (p70S6K), Akt/protein kinase B (PKB), Burton's tyrosine kinase (Btk), phosphoinositide-dependent kinase (PDK), integrin-linked kinase (ILK), atypical protein kinase C/PKC-related kinase (PRK), phopholipase Cγ, and more [32,36]. An investigation is underway in our laboratory to elucidate what pathway located downstream of PI3K is activated by TIMP-2.

In summary, TIMP-1 activated Ras through a TYK-mediated pathway in MG-63 cells and the activated Ras, Ras–GTP, formed a complex with Raf-1 as an effector for Raf-1 activation. TIMP-2 also activated Ras, but through a PKA-mediated pathway; and this activated Ras formed a complex with PI3K.

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