Usage of Tautomycetin, a Novel Inhibitor of Protein Phosphatase 1 (PP1), Reveals That PP1 Is a Positive Regulator of Raf-1 in Vivo*

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Protein phosphatase type 1 (PP1), together with protein phosphatase 2A (PP2A), is a major eukaryotic serine/threonine protein phosphatase involved in regulation of numerous cell functions. Although the roles of PP2A have been studied extensively using okadaic acid, a well-known inhibitor of PP2A, biological analysis of PP1 has remained restricted because of lack of a specific inhibitor. Recently we reported that tautomycetin (TC) is a highly specific inhibitor of PP1. To elucidate the biological effects of TC, we demonstrated in preliminary experiments that treatment of COS-7 cells with 5 μM TC for 5 h inhibits endogenous PP1 by more than 90% without affecting PP2A activity. Therefore, using TC as a specific PP1 inhibitor, the biological effect of PP1 on MAPK signaling was examined. First, we found that inhibition of PP1 in COS-7 cells by TC specifically suppresses activation of ERK, among three MAPK kinases (ERK, JNK, and p38). TC-mediated inhibition of PP1 also suppressed activation of Raf-1, resulting in the inactivation of the MEK-ERK pathway. To examine the role of PP1 in regulation of Raf-1, we overexpressed the PP1 catalytic subunit (PP1C) in COS-7 cells and found that PP1C enhanced activation of Raf-1 activity, whereas phosphatase-dead PP1C blocked Raf-1 activation. Furthermore, a physical interaction between PP1C and Raf-1 was also observed. These data strongly suggest that PP1 positively regulates Raf-1 in vivo.

Protein phosphatases regulate numerous cellular functions and signal transduction pathways in cooperation with protein kinases (1, 2). Protein phosphatase types 1 and 2A, known as PP1 and PP2A, are two of four major protein serine/threonine phosphatases (PPs) that regulate diverse cellular events such as cell division, transcription, translation, muscle contraction, glycogen synthesis, and neuronal signaling (3–5). Okadaic acid (OA), a polyether fatty acid from the marine black sponge Halichondria okada, was first identified as a small molecular weight inhibitor of PP and has been studied extensively (6). More than 40 compounds that inhibit PP1 as well as PP2A have been identified. Using these natural compounds, numerous experiments have been performed to analyze the roles of PPs in various cellular events (6, 7). The IC50 values of such phosphatase inhibitors are almost identical for PP1 and PP2A, with the exception of compounds such as OA, TF-23A, and fosfotin (8–10). PP2A is selectively inhibited by OA, TF-23A, and fosfotin, and this selectivity has made it possible to analyze PP2A function in living cells. However, no known inhibitor inhibits PP1 specifically.

Okawa et al. (11) reported the total chemical synthesis of tautomycin (TM), a small molecular weight PP inhibitor originally isolated from Streptomyces spiroverticillatus. Using the synthesized TM and related compounds, we previously examined the structure-function relationship of TM and found that the left- and right-hand moieties of TM are required for inhibition of PP and induction of apoptosis, respectively (12). We also reported that the spiroketal structure in the right-hand moiety of tautomycin has nothing to do with phosphatase inhibition but rather induces apoptosis (13). These results strongly suggest that tautomycetin (TC), an antifungal antibiotic originally isolated from Streptomyces griseochromogenes (14), could be a potent PP1-specific inhibitor that would likely exhibit few nonspecific effects because it is structurally similar to TM but lacks a spiroketal structure (15). Recently, we demonstrated that TC is a specific PP1 inhibitor in vitro and proposed that TC may be used as a novel powerful probe to elucidate the physiological roles of PP1 in various biological events (16).

PP1 is composed of the catalytic subunit (PP1C) and a wide variety of targeting/regulatory subunits (4, 5). Thus far four serine/threonine protein phosphatase type 2B; PP, serine/threonine protein phosphatase; OA, okadaic acid; TC, tautomycetin; MAPK, mitogen-activated protein kinase; PP1C, PP1 catalytic subunit; TM, tautomycin; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; JNK, c-Jun NH2 kinase; p38, homologue of the budding yeast Hog1 protein; Me2SO, dimethyl sulfoxide; EGF, epidermal growth factor; GST, glutathione S-transferase; I-2, protein phosphatase inhibitor-2; TPA, 12-O-tetradecanoyl-13-phorbol acetate; HA, hemagglutinin.

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PP1C isoforms, α, γ1, γ2 and δ, have been identified that are widely expressed in mammalian tissues (17–21). Biochemical analysis using bacterially expressed PP1C isoforms of all four types has shown that they have similar properties (22). PP1C may be regulated by its interaction with a variety of subunits that appear to target PP1C to specific subcellular locations and define substrate specificity.

For three decades, we have extensively investigated neoplastic alterations in hepatomas of enzymes involved in glycogen metabolism and containing protein phosphatases. We first found that PP1 activity was markedly elevated in rat ascites hepatomas (23, 24). Then we observed that levels of both PP1α mRNA and PP1α protein were irreversibly increased in hepatomas and that PP1α protein accumulated in the non-nuclear membrane fraction and the nuclei (19, 25–27). The increase in PP1α mRNA expression seen in rat ascites hepatoma cells was due to the enhanced promoter activity of the PP1α gene (28, 29). In contrast to increases in PP1 expression seen in rat ascites hepatoma cells, PP2A and PP2C expression was not increased but rather was down-regulated (26). These results strongly suggested a positive involvement of PP1 in regulating cell growth of tumor cells. However, the mechanism underlying this role of PP1 remained unknown.

Activation of mitogen-activated protein kinase (MAPK) cascades plays a key role in transducing various extracellular signals to the nucleus (30–32). Three distinct MAPK cascades have been described: extracellular signal-regulated kinases (ERK), c-Jun NH2 terminal kinases (JNK), and homologues of the budding yeast HO1 protein (p38). Activation of MAPKs requires phosphorylation of conserved threonine and tyrosine residues by dual-specificity MAPK kinases, which in turn are activated by the phosphorylation of two serine residues by upstream MAPK kinases. The ERK pathway (Raf-MEK-1,2-ERK-1,2) is activated by mitogen via Ras and by phorbol esters via protein kinase C. The stress-activated MAPK pathways JNK (MEK kinase 1,3-SEK1,2-JNK1,2,3) and p38 (ASK1, TAK1-MKK3,6-p38α,β,γ,δ) are activated by cellular stress, e.g. UV light, osmotic and oxidative stress, and inflammatory cytokines (30–32). Phosphorylation of MAPKs results in their translocation to the nucleus, where they activate transcription factors by phosphorylation. Activities of MAPKs, MAPK kinases, and MAPK kinase kinases are also regulated by dephosphorylation at serine, threonine, and tyrosine residues by serine/threonine, tyrosine, and dual-specificity phosphatases, respectively (32–37). Numerous observations suggest that PP2A plays a major role in the down-regulation of JNK, MEK, and ERK activities. Therefore, the inhibition of intracellular PP2A by OA leads to the activation of these enzymes (6, 7, 35).

However, the involvement of PP1 in MAPK pathways has not been studied because of the lack of a PP1-specific inhibitor.

In the present study, we found that treatment of COS-7 cells with 5 μM TC selectively inhibited PP1 activity by more than 90% without affecting PP2A activity, demonstrating that TC is a useful tool for analysis of the biological function of PP1. Using TC, we then examined the involvement of PP1 in regulating MAPKs. These results are summarized as follows: (i) TC specifically inhibits activation of ERK among three MAPKs (ERK, JNK, and p38) upon treatment of cells with TPA and EGF; (ii) TC treatment suppresses activation of Raf-1 activity, which results in inactivation of the MEK-ERK pathway; (iii) overexpression of phosphatase-dead mutants of PP1C on the Raf-1-MEK pathway results in effects similar to those found in TC treatment of cells; (iv) PP1C physically interacts with Raf-1.

These results are the first demonstration that PP1 is a positive regulator of the Raf-MEK-ERK pathway.

EXPERIMENTAL PROCEDURES

Reagents—TC was prepared from S. griseochromogenes as described previously (14, 15). OA and TC were dissolved in dimethyl sulfoxide (DMSO) and stored at −80 °C. Rabbit skeletal muscle glycogen phosphorylase and epidermal growth factor (EGF) were purchased from Sigma. Purified protein phosphatase type 2B (PP2B)/calcineurin from bovine brain was purchased from Upstate Biotechnology. Phosphorylase kinase, recombinant GST-T-2 (a specific inhibitor of PP1), GST-PP5 (protein phosphatase type 5), GST-MEK-Hax6, GST-KNEK, and GST-PP1C were expressed in E. coli (low molecular mass double-specific phosphatase (pp1-C)) were prepared as described previously (22, 38, 39). An anti-MEK-1 antibody, H-8, was purchased from Santa Cruz Biotechnology. Other reagents were purchased from Wako (Osaka, Japan).

Mammalian Expression Vectors—pSRa-HE-ERK2, pSRa-HA-JNK1, pMT3-HA-p38a, pBEG-MEK1 (GST-MEK1), pBEG-SEK1 (GST-SEK1), pBEG-MKK6 (GST-MKK6), pHS-Flag-Raf-1, pcDNA3-Myc-PP1α, and pcDNA3-Myc-PP1γ were described previously (36–38, 40).

Cell Culture—COS-7 and 293-T cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) or RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum, 1.9 g/liter sodium bicarbonate, 100 μg/ml streptomycin, and 20 units/ml penicillin G (termed “complete medium”) at 37 °C under 5% CO2.

Cell Treatment and Phosphatase Assays—8–9 × 104 cells were cultured in 1 ml of complete medium in 35-mm dishes. Five μl of MeSO or MeSO plus OA or TC was added, and the cells were incubated at 37 °C for 5.5 h. Adherent cells were washed with 1 ml of phosphate-buffered saline (divalent, cation-free) on ice and scraped in 100 μl of hypotonic buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% β-mercaptoethanol, 10 μg/ml benzamidine, 10 μg/ml trypsin inhibitor, and 10 μg/ml aprotonin) at 4 °C. Suspensions were subjected to three cycles of freezing in liquid nitrogen followed by thawing at 30 °C and were then centrifuged at 4 °C for 10 min at 15,000 rpm. The resulting supernatants were used as enzymes.

Phosphorylase was 32P-labeled by phosphorylase kinase to 1 mol of phosphate/mol of phosphorylase and used at 5 μM in the assay (16, 41). PP1 and PP2A activities were measured as described previously (16, 41) with a slight modification. Briefly, 10 μl of cell-free extracts were diluted with solution A (50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 0.1% (v/v) β-mercaptoethanol) containing 1 mg/ml bovine serum albumin. The diluted enzymes were then preincubated with 30 μl of solution A containing 0.33 mg/ml bovine serum albumin and 0.02% (w/v) Brij-35 with or without the inhibitor for 15 min at 30 °C. The reaction was initiated with 32P-labeled substrate in 20 μl of solution A containing 15 mM caffeine. After 10 min at 30 °C, the reaction was stopped by adding 50 μl of 10 mM H2SO4 acid solution containing 20 mM silicotungstic acid, and the solution was centrifuged. Subsequent procedures were essentially the same as those described previously (16, 41). One unit of the enzyme was defined as the amount of enzyme required to catalyze the release of 1 μmol of phosphate/min. The activity of calcineurin was measured as described previously (42).

Transient Transfection and Stimuli—COS-7 and 293-T cells in 35-mm dishes were co-transfected with 1 μg of pEG-MEK1, pBEG-SEK1, or pBEG-MKK6 together with 1 μg of pSRa-HE-ERK2, pSRa-HA-JNK1, or pMT3-HA-p38a, respectively. For transient assays, cells were transfected using Fugene-6 (Roche Diagnostics Inc., Mannheim, Germany) according to the manufacturer’s recommendation. Forty hours after transfection, cells were maintained with or without phosphatase inhibitors for 4.5–5.5 h and then stimulated with either 12-O-tetradecanoyl-13-phorbol acetate (TPA) or EGF for ERK2 activation. For JNK1 and p38a activation, cells were stimulated with 0.4 mM sorbitol for 30 min.

Detection of Activated MAPKs and MEK by Immunoblot—Transfected cells were washed with 1 ml of phosphate-buffered saline on ice and lysed by sonication in MAPK lysis buffer (20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.5% deoxycholate, 10% glycerol, 157 mM NaCl, 5 mM EDTA, 50 mM β-glycerophosphate, 2 mM orthovanadate, 20 mM NaF, 1 mM dithiothreitol, 0.5 mM benzamide, 10 μg/ml leupeptin, and 10 μg/ml aprotonin). Extracts were prepared by centrifugation at 20,000 × g for 10 min. Each sample was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Amer sham Biosciences). The phosphorylation status of activated MAPKs, MEK1, and SEK1 was monitored by anti-phospho-ERK antibodies (New England Biolabs (NEB)), anti-ACTIVE JNK antibody (Promega), an anti-phospho p38 antibody (NEB), an anti-phospho MEK1/2 antibody (NEB), or an anti-phospho SEK1/MKK4 antibody (NEB) followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Chemicon International, Temecula, CA).
The expression levels of HA-tagged MAPKs and GST-tagged MAPK kinases were monitored by anti-HA (12CA5) monoclonal (Roche Diagnostics) anti-GST monoclonal antibodies (CG1B), respectively, followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Signals were detected using the enhanced chemiluminescence reagent (ECL, Amersham Biosciences).

**Assay for Raf Kinase Activity**—Transfected or treated cells on 35-mm dishes were lysed by sonication in MPAK lysis buffer. After centrifugation of cell lysates at 20,000 × g for 10 min, the supernatant was used as a cellular extract. FLAG epitope-tagged Raf-1 in extracts was immunoprecipitated with an anti-FLAG M2 monoclonal antibody (Sigma). Raf kinase activity was determined by incubating immunoprecipitates in the presence of GST-MEK (0.1 μg) and GST-KNERRK (1.5 μg) in 40 μl of kinase reaction mixture (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 20 mM β-glycerophosphate, 0.4 mM benzamidine, 5% glycerol, and 100 μM ATP) for 8–25 min at 30–35 °C. After incubation, proteins in the reaction mixture were separated by SDS-PAGE. Phospho-ERK and Raf-1 were detected by immunoblotting with anti-phospho-ERK antibody, anti-Raf-1 antibody C12, or anti-Raf-1 antibody E10 (Santa Cruz Biotechnology). The amounts of phospho-ERK and Raf-1 were quantified using a luminescent image analyzer, LAS-1000 Plus (Fujifilm, Tokyo, Japan).

**Co-immunoprecipitation**—Transfected cells were lysed in 300 μl/100-mm plate co-immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 30 °C, 4 mM EDTA, 5% glycerol, 0.1% Triton X-100, 1 mM benzamidine, 50 mM β-glycerophosphate, 2 mM orthovanadate, 0.1% β-mercaptoethanol) containing 10 μg/ml leupeptin and 10 μg/ml aprotinin. Cell lysates were centrifuged at 20,000 × g for 10 min. 150 μl of supernatant was incubated with anti-Raf-1 antibody (1.6 μg) or anti-Myc antibody (14 μg). After rotation for 30 min at 4 °C, 5 µl of protein G-Sepharose 4 fast flow (Amersham Biosciences) was added to the mixture. After rotation for 1.5 h at 4 °C, the beads were washed with 1 ml of co-immunoprecipitation buffer. The immunoprecipitates were resuspended in 25 μl of 1.25% Laemmli's SDS sample buffer, boiled for 5 min, separated by SDS-PAGE on 9% gels, and transferred to a nitrocellulose membrane (Amersham Biosciences). Raf-1 or Myc-tagged proteins were detected by immunoblotting with the respective antibodies.

**Protein Measurement**—The protein concentration was measured by a modification of the method of Bradford using bovine serum albumin as a standard (43). Briefly, cell lysates were diluted with 0.01% Triton X-100. 50 μl of diluted lysate was added to 0.95 ml of a mixture of 0.25 M Tris-HCl, pH 6.8, 6.25 M glycerophosphate, 2 mM orthovanadate, 0.1% β-mercaptoethanol, and 0.1% SDS sample buffer, boiled for 5 min, separated by SDS-PAGE on 9% gels, and transferred to a nitrocellulose membrane. The immunoblots were incubated with the respective antibodies.

**Results**

**Tautomycetin Specifically Inhibits PP1 Activity in COS-7 Cells**—We recently demonstrated that tautomycetin is the most specific PP1 inhibitor among more than 40 phosphatase inhibitors assayed in vitro; however, its biological effect remained to be examined (16). To do so, we first measured the activity levels of PP1 and PP2A in extracts prepared from COS-7 cells pretreated with TC or okadaic acid, an inhibitor of PP2A (Fig. 1). From preliminary experiments of dose responsiveness, the concentrations required for complete inhibition of PP1 and PP2A in COS-7 cells were 5 μM TC and 100 nM OA, respectively (data not shown). To differentiate between residual phosphatase activity in cell extracts, phosphatase activity levels were measured using phosphorylase a as a substrate in the presence or absence of 1 nM OA to specifically inhibit PP2A or 167 nM I-2 to specifically inhibit PP1. Total phosphatase activities in extracts of OA-treated cells were resistant to 1 nM OA, and ~90% of the phosphatase activity was inhibited by 167 nM I-2, showing that OA treatment specifically inhibits intracellular PP2A. Residual phosphatase activity in extracts from TC-treated cells was resistant to 167 nM I-2 and completely inhibited by 1 nM OA. These results strongly suggest that TC penetrates cell membranes and binds to and specifically inhibits PP1, and the binding between TC and PP1C is tight enough to resist dissociation during preparation of cell extracts. We then examined the effects of TC on other phosphatases. TC had no effect on purified calcineurin and LDP-1 up to 1 μM. The IC₅₀ for PP5 is ~1 μM higher than for PP2A (data not shown). From these results, we concluded that TC could be used as a tool to investigate the biological function of PP1.

**Tautomycetin Inhibits Activation of ERK in Cells**—It is known that PP2A inhibits the activation of ERK and JNK, based on observations that treatment of cells with OA results in hyperphosphorylation/activation of ERK and JNK (6, 35). Because we were interested in the role of PP1 in the regulation of MAPK activation, we compared the effects of OA and TC on activation of HA-ERK2, HA-JNK1, or HA-p38α expressed in COS-7 and 293T cells (Fig. 2). As expected, OA at 100 nM increased the phosphorylation levels of HA-ERK2 in unstimulated COS-7 and 293T cells (Fig. 2A, lanes 3 and 9). However, in contrast, TC at 5 μM dramatically decreased the phosphorylation levels of HA-ERK2 activated by TPA in COS-7 and 293T cells (Fig. 2A, lanes 6 and 12). On the other hand, TC and OA showed similar positive effects on JNK activation (Fig. 2B), but neither TC nor OA showed any effect on p38 activation (Fig. 2C). These results suggest that inhi-
bition of PP1 by pretreatment of cells with TC specifically blocks ERK activation.

**Tautomycetin Inhibits Activation of MEK in Cells**—ERK is phosphorylated directly by MEK. To clarify whether inhibition of ERK activation is due to suppression of MEK activation, we compared the effect of TC on GST-MEK activation with that on HA-ERK upon stimulation at various concentrations of TPA in COS-7 cells. As shown in Fig. 3A, TC decreased phosphorylation/activation of GST-MEK substantially. The rates of decrease in phospho-GST-MEK by TC treatment were similar to those seen with phospho-GST-MEK (Fig. 3A, compare lanes 14 and 15 with lanes 4 and 5), suggesting that inhibition of ERK activation results from inhibition of MEK activation. Under these conditions, OA enhanced phosphorylation of MEK as well as ERK (6, 7, 32).

**Dose-dependent Inhibition of MEK by Tautomycetin**—Dose-dependent inhibition of EGF- and TPA-induced MEK activation by TC was analyzed. As shown in Fig. 4, TC decreased phosphorylation levels of MEK induced by EGF and TPA in a dose-dependent manner. It is noteworthy that 5 μM TC, a concentration sufficient for complete inhibition of intracellular PP1 (Fig. 4), is enough to reduce the phosphorylation level to

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**Fig. 3. Effects of OA and TC on activation of ERK and MEK in COS-7 cells.** COS-7 cells were transfected with pSRα-HA-ERK2 and pEBG-MEK1. The transfected cells were incubated for 5.5 h with vehicle (None: lanes 1–6), 100 nM OA (lanes 7–12), or 5 μM TC (lanes 13–18). Cells were then stimulated with either TPA for 10 min (A) or EGF for 4 min (B) at the indicated concentrations. Immunoblots were performed as described under “Experimental Procedures.” Similar results were obtained in four separate experiments.
background (compare lanes 17 and 26 to lane 1 in Fig. 2). Under these conditions, OA increased phosphorylation levels of MEK in a dose-dependent manner with a plateau at 50–100 nM, which is enough to inhibit PP2A in vivo, as shown in Fig. 1. These results suggest that inhibition of PP1 causes suppression of MEK activation.

**Tautomycin Prevents Raf-1 Activation**—To clarify how activation of MEK is inhibited by TC, we analyzed the effect of TC on the activation of Raf-1, which is a major MEK kinase in vivo. As shown in Fig. 5A, FLAG-Raf-1 activity was increased with a peak at 5–10 min upon EGF treatment. Under these conditions, treatment of cells with 5 μM TC inhibited FLAG-Raf-1 activation by 71% at 5 min, whereas treatment with 100 nM OA inhibited FLAG-Raf-1 by 95% at 5 min. We then compared the time courses of Raf-1 activity (Fig. 5A) and MEK phosphorylation (Fig. 5B) with or without inhibitors upon EGF treatment. As shown in Fig. 5, TC inhibition of activation of Raf-1 and MEK was almost the same in terms of the time course and rate of inhibition, suggesting that inhibition of PP1 by TC negatively regulates Raf-1 activation. Inhibition of PP2A by OA was shown to affect Raf-1 negatively and MEK positively.

**The Catalytic Subunit of PP1 Inhibits Activation of MEK and Raf-1 in Vivo**—To elucidate a physiological role of PP1 in Raf-1 activation, wild type and a catalytically inactive form (H125A) of PP1C (Myc-tagged) were transiently expressed in COS-7 cells. Cell extracts were then prepared, and the phosphorylation level of GST-MEK upon EGF activation was examined by immunoblot (Fig. 6A). Compared with extracts from control cells transfected with the empty vector (mock), extracts from cells transfected with pcDNA3/Myc-PP1C exhibited an increase in the phosphorylation level of GST-MEK with or without EGF stimulation. By contrast, extracts from cells transfected with pcDNA3/Myc-PP1C(H125A) showed marked decreases in the phosphorylation level of GST-MEK. In order to examine the effect of PP1 on Raf-1 activation, FLAG-Raf-1 was co-expressed with Myc-PP1C or Myc-PP1C(H125A). Compared with extracts from control cells, extracts expressing Myc-PP1C exhibited marked increases in Raf-1 activity with or without EGF stimulation. By contrast, extracts from cells expressing Myc-PP1C(H125A) showed a marked decrease in Raf-1 activity, suggesting that Myc-PP1C(H125A) works as a dominant negative in Raf-1 activation. Activation rates of FLAG-Raf-1 and GST-MEK by Myc-PP1C were similar, and inhibition rates of FLAG-Raf-1 and GST-MEK by Myc-PP1C(H125A) were also similar, indicating that the target of PP1 is not MEK but Raf-1 or an effector upstream of Raf-1. Similar results were obtained using other isoforms of PP1C such as PP1α and PP1δ (data not shown).

**Interaction of PP1γ1 and Raf-1**—Because PP1 was shown to be a positive regulator of Raf-1 activity, we examined whether PP1 physically associates with Raf-1 (Fig. 7). FLAG-Raf-1 was detected in Myc-PP1C immunoprecipitates, and Myc-PP1C was detected in Flag-Raf-1 immunoprecipitates, demonstrating that PP1C interacts physically with Raf-1 in vivo.

**DISCUSSION**

The present study demonstrates that using TC and OA, the functions of PP1 and PP2A can be differentiated in vivo. We recently reported that TC has a 40-fold preference for purified PP1 compared with purified PP2A in vivo, suggesting that TC is a useful tool in elucidating the physiological function of PP1 in various biological events (16). Here we have demonstrated that treatment of cells with 5 μM TC causes a complete inhibition of PP1 activity without affecting PP2A activity. TM has been used to analyze PP1 in vivo because of its relatively high affinity for PP1 among PP inhibitors. However, 10 μM TM, a minimum concentration sufficient to induce complete inhibition of PP1, also induced inhibition of PP2A by 50% (44). Therefore, it is a great advantage for in vivo analysis of PP1 and PP2A that 5 μM TC and 100 nM OA can differentiate between the functions of PP1 and PP2A in cells, respectively. This method could be used widely to reveal the unknown functions of PP1 in vivo.

The Ras-Raf-MEK-ERK cascade plays a central role in mediating various extracellular stimuli into the nucleus. The duration and magnitude of ERK activation are regulated at multiple points in the signaling cascade. The activity of ERK reflects a balance between the activities of upstream activating kinases and protein phosphatases. Previously, the involvement of PP1 in the ERK pathway had not been established, whereas investigations of the role of PP2A in the ERK pathway have produced contradictory results. In vitro, PP2A dephosphorylates and inactivates MEK and ERK, and both MEK and ERK are activated by OA treatment in vivo (6). Expression of SV40 small T antigen, which inhibits PP2A, results in activation of MEK and ERK (45). On the other hand, genetic analysis suggests a positive role of PP2A in the regulation of the ERK pathway during Drosophila photoreceptor development (46). To dissect PP1 and PP2A functions in the ERK pathway, we compared the PP1 and PP2A functions on Raf-1, MEK, and
ERK by differential usage of TC and OA. The present data suggest that PP1 is required for Raf-1 activation but is not involved in dephosphorylation of MEK or ERK in vivo. PP2A is required for Raf-1 activation but is involved in dephosphorylation of MEK and ERK. We conclude that PP1 is a positive regulator of Raf-1, whereas PP2A can function as a positive regulator of Raf-1 but a negative regulator of MEK and ERK. It is of note that activation of HA-ERK by OA is less than activation of GST-MEK (Fig. 3A). It is possible that ERK is dephosphorylated not only by PP2A but also by dual-specificity or tyrosine phosphatases activated by OA.

The present results obtained using TC, a novel PP1-specific inhibitor, indicate that PP1 is a positive regulator of cell growth. The up-regulation of Raf-MEK-ERK by PP1 was observed not only in COS-7 and 293T cells but also in HeLa and HepG2 cells (data not shown), suggesting a general role of PP1 as a positive regulator in cell growth regulation. Through systematic experiments of protein phosphatase activity, we concluded previously that PP1α in hepatoma cells is irreversibly
up-regulated at the transcriptional level (19, 25–29). PP1 in hepatoma cells accumulates both in the non-nuclear particulate fraction and in the nuclei. Our present results strongly suggest that increased PP1 in hepatomas plays a role in their rapid growth rate, whereas the significance of increased PP1 levels in the nucleus of hepatomas remains unclear. Increased nuclear PP1 is thought to inhibit rather than accelerate the cell cycle through dephosphorylation of Rb (47, 48). We also previously reported that NIPP-1, a potent nuclear inhibitor of PP1, is markedly increased in rapidly growing hepatomas, suggesting that the increased activity of the nuclear PP1 in hepatoma cells is suppressed (49).

Raf-1 is present in cells in a multiprotein complex containing 14-3-3, Ras, KSR (kinase suppressor of Ras), and Hsp90 (50, 51). Raf-1 contains several phosphorylation sites, and its activation state of multiple sites (52, 53). Residues such as Ser-259 phosphorylation appears to be a main target for the inhibitory phosphorylation of Raf-1 (52, 53). Here we have demonstrated that PP1 is targeted by PP1. It is interesting that myosin-binding subunit 7 (Myb) (52, 53). Thus is reported that Ser-259 phosphorylation appears to be a main target for the inhibitory phosphorylation of Raf-1 (52, 53). Recently, it is reported that Raf-1 is present in a multicomplex with Raf-1 and positively regulates activation (52, 53). Here we have demonstrated that PP1 is present in a multicomplex with Raf-1 and positively regulates Raf-1 activity. These data suggest that PP1 phosphorylates Raf-1 or associated proteins such as KSR and SOS (Son of Sevenless), which results in the activation of Raf-1. Recently, it is reported that Ser-259 phosphorylation appears to be a main target for the inhibitory phosphorylation of Raf-1 (52, 53). Thus it is possible that Ser-259 on Raf-1 is a phosphorylation site targeted by PP1. It is interesting that myosin-binding subunit (M110), a PP1 regulatory protein, was reported to interact with Raf-1 in vivo and to be phosphorylated by Raf-1 in vitro (54). Therefore, it is possible that PP1C associates with Raf-1 and that it regulates each other in vivo. Further in vivo analyses of the complex of PP1 and Raf-1 are necessary to clarify the precise mechanism by which PP1 is targeted to Raf-1 and regulates its activity.

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Usage of Tautomycetin, a Novel Inhibitor of Protein Phosphatase 1 (PP1), Reveals That PP1 Is a Positive Regulator of Raf-1 in Vivo
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