Src Tyrosine Kinase Inhibitor PP2 Markedly Enhances Ras-independent Activation of Raf-1 Protein Kinase by Phorbol Myristate Acetate and H₂O₂

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Recently we reported that simultaneous treatment of NIH 3T3 cells with the combination of phorbol myristate acetate (PMA) and hydrogen peroxide (H₂O₂) resulted in synergistic activation of Raf-1 kinase (Lee, M., Petrovics, G., and Anderson, W. B. (2003) Biochem. Biophys. Res. Commun. 311, 1026–1033). In this study we have demonstrated that PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butylypyrazolo[3,4-d]pyrimidine), a potent and selective inhibitor of the Src-family tyrosine kinase, greatly potentiated the ability of PMA and/or H₂O₂ to activate Raf-1 kinase, whereas it blocked the tyrosine phosphorylation of Raf-1. Unlike PMA/H₂O₂ treatment, which showed transient activation, PP2-mediated Raf-1 activation was sustained and continued to increase through 4 h of treatment. Transient transfection studies with a dominant-negative mutant of Ras (N19Ras) indicated that this PP2-induced activation of Raf-1 was Ras-independent. Moreover, PP2 showed no effect on platelet-derived growth factor-induced Raf-1 activation. Interestingly, mutation of the reported Raf-1 Src family tyrosine kinase phosphorylation site by conversion of tyrosines 340 and 341 to phenylalanine (YY340/341FF Raf) had limited effect on the ability of PP2 to induce significant stimulation of Raf-1 kinase activity. Taken together, our results suggest that a tyrosine phosphorylation event is involved in the negative feedback regulation of Raf-1. Inhibition of a Src family tyrosine kinase by PP2 appears to alleviate this tyrosine kinase-mediated inhibition of Raf-1 and allow activating modification(s) of Raf-1 to proceed. This PP2 effect resulted in significant and sustained Ras-independent activation of Raf-1 by PMA and H₂O₂.

Raf-1 is a cytoplasmic serine/threonine protein kinase that plays an important role in the transmission of signals initiated at the plasma membrane to modulate transcriptional activation and mitogenesis (1, 2). Raf-1 activation is mediated by an interaction with Ras-GTP, which recruits Raf-1 to the plasma membrane and induces a conformation change that relieves an inhibition imposed by the N terminus on the catalytic domain (3). This is, however, insufficient to activate Raf-1, and additional phosphorylations on tyrosine and serine/threonine residues are required for activation (4–7). In addition, dimerization and interaction with other proteins also play important roles in the regulation of Raf-1 activity (8, 9).

Protein kinase C (PKC) involvement in the regulation of the Ras/Raf/MAP kinase pathway has been demonstrated in a variety of systems ranging from yeast to higher eukaryotes (10–12). The PKC family is composed of at least 11 closely related isozymes that are classified into three major groups. The conventional PKCs (PKCζ, -β₁, -β₂, and -γ) are Ca²⁺-dependent and are regulated by diacylglycerol and phorbol 12-myristate 13-acetate (PMA). The novel PKCs (PKCδ, -ε, -ι, and -θ) are Ca²⁺-independent but exhibit similar response to diacylglycerol and PMA. In contrast, the atypical aPKCs (PKCζ and -λ) are insensitive to Ca²⁺, diacylglycerol, and PMA (13). Both conventional PKCa and novel PKCe have been shown to activate Raf-1 in vitro (4). Other studies also have reported that PKCe either directly or indirectly is involved in the activation of Raf-1 (14–16). In addition, evidence has been presented that implicates both aPKCa and aPKCi in the regulation of Ras/Raf/MAP kinase pathway (10, 17, 18).

In recent years it has become increasingly evident that the cellular generation of reactive oxygen species, including H₂O₂, plays an important role in cellular signal transduction and modulation of transmembrane signaling pathways (19–22). The oxygen radicals generated appear to act as second messengers to regulate transmembrane signaling and to modulate cellular functions such as cell proliferation, differentiation, and apoptosis. A number of cell types produce H₂O₂ in response to a variety of growth factors, including platelet-derived growth factor (23) and epidermal growth factor (24). Furthermore, the PDGF-induced activation of the ERK/MAP kinase mitogenic cascade was found to require the generation of H₂O₂ (25). Exposure of cells to H₂O₂ has been shown to increase the level of cellular tyrosine phosphorylation (26–28) and to stimulate the phosphorylation of MAP kinase (29–31). Moreover, we found that a 33-kDa C-terminal, kinase-inactive fragment of Raf-1 underwent a mobility shift in response to stimulation of NIH 3T3 cells with H₂O₂ (32), whereas Abe et al. (29) reported that treatment of bovine tracheal myocytes with H₂O₂ led to the activation of Raf-1 kinase. In related studies, treatment of cells with ionizing radiation was found to increase the level of...
membrane-bound, tyrosine-phosphorylated Raf-1 and stimulate Raf-1 kinase activity (29, 33).

Maximal activation of Raf-1 has been shown to require phosphorylation events (4–7). In particular, phosphorylation of serine 338 and tyrosine 341 are required for Ras-dependent activation of Raf-1 (34–36). Phosphorylation of tyrosine 341 has been reported to be carried out by Src family tyrosine kinases (35, 37, 38). The Src family of non-receptor tyrosine kinases plays an important role in the regulation of cell adhesion, growth, and differentiation through the activation of multiple intracellular signaling pathways (39, 40). Src kinase normally is maintained in an inactive state but can be transiently activated during cellular events such as mitosis or constitutively activated by abnormal events such as mutation (39). The activation of Src occurs as a result of disruption of negative regulatory processes that normally suppress Src tyrosine kinase activity (40).

Recently we determined that exposure of NIH 3T3 cells to H2O2 also resulted in stimulation of Raf-1 kinase activity (41). Interestingly, treatment of cells with H2O2 in combination with PMA to stimulate PKCe resulted in synergistic activation of Raf-1 (41). Here we have employed the tyrosine kinase inhibitor PP2 (4-amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) to determine the possible involvement of tyrosine phosphorylation in the modulation of H2O2/PMA stimulation of Raf-1 kinase activity. PP2 is a potent inhibitor of Src family tyrosine kinases but only weakly inhibits ZAP-70 and JAK2 (42). Interestingly, treatment of cells with PP2 to inhibit Src family kinase was found to enhance PMA/H2O2-mediated activation of Raf-1. These results indicate that the generation of H2O2 along with the activation of PKCe and inhibition of Src family tyrosine kinase all play an important role in the Ras-independent, sustained activation of Raf-1 kinase. Furthermore, the results presented here suggest that tyrosine phosphorylation events may be involved in both the stimulation and down-regulation of Raf-1 kinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit polyclonal anti-Raf (C-12) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), whereas monoclonal mouse anti-Raf and anti-phosphotyrosine RC20H were from BD Biosciences Pharmingen. Protein A-agarose was from Roche Applied Science. Dulbecco’s modified Eagle’s medium, fetal calf serum, and penicillin-streptomycin were purchased from Invitrogen. Reagents for SDS-polyacrylamide gel electrophoresis were from Bio-Rad. (~)32P-ATP (3000 Ci/mmol) was purchased from PerkinElmer Life Sciences. PP2, GF 109203X, Go 6976, and wortmannin were purchased from Calbiochem. Monoclonal anti-FLAG M2, PMA, and H2O2 were obtained from Sigma. PM2 and PP2 were dissolved in Me2SO and freshly diluted for each experiment.

**Plasmid DNA**—The pcDNA vector encoding a dominant-negative mutant of Ras (N17Ras) was kindly provided by Dr. Toren Finkel (NIH, Bethesda, MD) with Dr. Silvio Gutkind’s (NIH, Bethesda, MD) permission. NIH 3T3/BBX Raf cells overexpressing N-terminally truncated and constitutively activated Raf-1 were produced by cloning into the pMTH vector (43). The pcDNA-FLAG-Raf construct, obtained from Dr. Jeffrey E. DeClue (NIH, Bethesda, MD) with Dr. Richard Kolesnick’s (Memorial Sloan-Kettering Cancer Center, New York, New York) permission (44), was used as the template to generate FLAG-tagged Raf-1 point mutants using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The presence of mutation points was verified by DNA sequence analysis.

**Mammalian Cell Culture and Transient Transfection**—Parental and v-Ha-ras-transformed NIH 3T3 cells were maintained at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin-streptomycin, and glutamine. Where indicated the cells were transiently transfected with the pcDNA vector encoding the indicated FLAG-tagged Raf-1 point mutants or expressing dominant-negative Ras (N17Ras) by the electroporation method as specified by the manufacturer (BTX). After 48 h the transfected cells were serum-deprived overnight before treatment with PMA, H2O2, and PP2 as indicated.

**Preparation of Cell Lysates**—The indicated treatments of cells were carried out at 37 °C in serum-free medium as described in the figure legends. After treatment whole cell lysates were prepared as follows. Cells were washed twice with ice-cold phosphate-buffered saline and harvested by scraping the cells into lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 20 mM β-glycerophosphate, and 2 mM sodium fluoride). Cell lysates were clarified by centrifugation at 15,000 × g for 10 min at 4 °C, and lystate protein concentrations were determined with a BCA protein assay reagent kit as described by the manufacturer (Pierce).

**Immunoprecipitation and Immunoblot Analysis**—Immunoprecipitation was performed on the whole cell lysates using either polyclonal anti-Raf or monoclonal anti-FLAG M2 and protein A-agarose beads. After incubation for 2 h at 4 °C, immunoprecipitates were washed twice with ice-cold lysis buffer. For immunoblotting, immunoprecipitates were denatured in Laemmli sample buffer and resolved by either 7.5% or 12% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose, and immunoblot analysis was performed using the antibody described in the figure legends. Endogenous Raf-1 kinase and the oncogenically active BXB Raf fragment (the C-terminal fragment starting with the amino acid 302 of c-Raf-1) were detected using antibodies described in the figure legends. Endogenous Raf-1 kinase and the oncogenically active BXB Raf fragment (the C-terminal fragment starting with the amino acid 302 of c-Raf-1) were detected using antibodies described in the figure legends.

**In Vitro Raf-1 Kinase Activity Assay**—Raf-1 proteins were specifically immunoprecipitated from lysates of NIH 3T3 cells, washed three times with wash buffer and once with kinase buffer (20 mM Tris, pH 7.4, 20 mM NaCl, 1 mM dithiothreitol, 10 mM MgCl2). Raf-1 kinase activity was measured by phosphorylation of recombinant MEK (Santa Cruz Biotechnology) as previously described (32). The washed immunoprecipitates were incubated in 40 μl of kinase buffer containing 10 mM ATP, 1 μg of the recombinant MEK, and 5 μCi of [γ-32P]ATP at 30 °C for 30 min in the presence of MEK inhibitor, PD 98059, which was used to inhibit the mitogen-activated protein kinase activity and diminished by the addition of gel-loading buffer. The samples were resolved by 7.5% SDS-PAGE, and phosphorylated MEK protein bands were visualized by autoradiography.

**RESULTS**

The Src-family Tyrosine Kinase Inhibitor PP2 Markedly Enhanced PMA/H2O2-mediated Activation of Raf-1 Kinase—Our previous studies showed that simultaneous treatment of cells with H2O2 and PMA resulted in synergistic activation of Raf-1 kinase (41). Since tyrosine phosphorylation has been shown to be an important event in Raf-1 activation (6, 7), and because one mechanism by which reactive oxygen species has been reported to modulate signal transduction pathway is via alteration in protein tyrosine phosphorylation (26, 45), several tyrosine kinase inhibitors were tested to determine their relative effects on Raf-1 kinase activation by the combination of PMA and H2O2. In these studies v-Ha-ras-transformed cells were used because these cells were found to be more susceptible to PMA and H2O2 activation than parental NIH 3T3 cells. In addition, NIH 3T3 cells expressing truncated BXB Raf also were used in these experiments. No apparent inhibition of PMA/H2O2-induced Raf-1 activation was observed with the tyrosine kinase inhibitors tested with the exception of AG490, an inhibitor of epidermal growth factor receptor tyrosine (Fig. 1A). Unexpectedly, PP2, an inhibitor of Src-family tyrosine kinases (42), significantly increased PMA/H2O2-mediated activation of Raf-1 kinase (Fig. 1A). Importantly, PP2 showed an even greater stimulatory effect of PMA and H2O2 activation of constitutively active BXB Raf (the N-terminal deletion mutant of Raf-1) (Fig. 1B). The kinetics of Raf-1 activation after exposure of v-Ha-ras-transformed cells to PP2 are shown in Fig. 1C. It was found that promotion of PMA/H2O2-induced activation of Raf-1 was optimal within 30 min of PP2 treatment, and this level of activation was sustained through at least 4 h of exposure to PP2. The ability of PP2 to enhance PMA/H2O2 activation...
tion of Raf-1 decreased after 12 h of treatment and approached control levels of Raf-1 kinase activity by 24 h of exposure to PP2 (inset, Fig. 1C).

**PP2 Blocked PMA and H₂O₂-induced Tyrosine Phosphorylation of Raf-1**—Next, studies were carried out to determine whether PP2 treatment inhibited the tyrosine phosphorylation of Raf-1 noted with exposure of v-Ha-ras-transformed NIH 3T3 cells to PMA and H₂O₂ (Fig. 2A). Subconfluent mock v-Ha-ras-transformed NIH 3T3 cells and BXB Raf-overexpressing NIH 3T3 cells were serum-deprived for 24 h along with exposure to the indicated tyrosine kinase inhibitors for the following time periods. Serum-deprived cells were treated with 60 μM genistein (tyrosine kinase inhibitor) for the final 60 min with 10 μM PP2 (Src kinase inhibitor) for the final 30 min with 50 μM AG490 (epidermal growth factor receptor kinase inhibitor) for the final 18 h and with 25 μM AG1296 (PDGF kinase inhibitor) for the final 4 h of serum deprivation. The cells were then exposed to 100 nM PMA and/or 1 mM H₂O₂ for 5 min. CTL, control. Un, untreated Panel B, PP2 treatment induced pronounced activation of constitutively active BXB Raf. Subconfluent membranes were serum-deprived for 24 h. The cells were then treated with 10 μM PP2 for the final 30 min of serum deprivation before treatment with 100 nM PMA and/or 1 mM H₂O₂ for 5 min with v-Ha-ras-transformed cells and for 30 s with BXB Raf-expressing cells. Panel C, time course of PP2-induced potentiation of Raf-1 kinase activation by PMA/H₂O₂. Serum-deprived v-Ha-ras-transformed NIH 3T3 cells were pretreated with 10 μM PP2 for the times indicated and then stimulated with 100 nM PMA, 1 mM H₂O₂ for 5 min. The lower inset extends the period of PP2 treatment through 12 and 24 h. In panels A–C in vitro Raf-1 kinase assays were performed on the immunoprecipitated Raf-1 and BXB Raf proteins using recombinant MEK-1 as substrate. The results presented are representative of at least three independent experiments carried out under the conditions described. Quantitation of MEK phosphorylation was carried out by densitometric scanning as described under “Experimental Procedures.” Numerical values given below each gel lane represent relative units of phosphorylation, with phosphorylation noted in unstimulated cells defined as 1.0.

**Fig. 1. Treatment of cells with PP2 markedly potentiated PMA- and H₂O₂-mediated activation of Raf-1 kinase.** Panel A, modulation of PMA/H₂O₂ activation of Raf-1 kinase with different tyrosine kinase inhibitors. Subconfluent v-Ha-ras-transformed NIH 3T3 cells were serum-deprived for 24 h along with exposure to the indicated tyrosine kinase inhibitors for the following time periods. Serum-deprived cells were treated with 60 μM genistein (tyrosine kinase inhibitor) for the final 60 min with 10 μM PP2 (Src kinase inhibitor) for the final 30 min with 50 μM AG490 (epidermal growth factor receptor kinase inhibitor) for the final 18 h and with 25 μM AG1296 (PDGF kinase inhibitor) for the final 4 h of serum deprivation. The cells were then exposed to 100 nM PMA and/or 1 mM H₂O₂ for 5 min. CTL, control. Un, untreated Panel B, PP2 treatment induced pronounced activation of constitutively active BXB Raf. Subconfluent mock v-Ha-ras-transformed NIH 3T3 cells and BXB Raf-overexpressing NIH 3T3 cells were serum-deprived for 24 h. The cells were then treated with 10 μM PP2 for the final 30 min of serum deprivation before treatment with 100 nM PMA and/or 1 mM H₂O₂ for 5 min with v-Ha-ras-transformed cells and for 30 s with BXB Raf-expressing cells. Panel C, time course of PP2-induced potentiation of Raf-1 kinase activation by PMA/H₂O₂. Serum-deprived v-Ha-ras-transformed NIH 3T3 cells were pretreated with 10 μM PP2 for the times indicated and then stimulated with 100 nM PMA, 1 mM H₂O₂ for 5 min. The lower inset extends the period of PP2 treatment through 12 and 24 h. In panels A–C in vitro Raf-1 kinase assays were performed on the immunoprecipitated Raf-1 and BXB Raf proteins using recombinant MEK-1 as substrate. The results presented are representative of at least three independent experiments carried out under the conditions described. Quantitation of MEK phosphorylation was carried out by densitometric scanning as described under “Experimental Procedures.” Numerical values given below each gel lane represent relative units of phosphorylation, with phosphorylation noted in unstimulated cells defined as 1.0.

**Fig. 2. PP2 Blocked PMA and H₂O₂-induced Tyrosine Phosphorylation of Raf-1**—Next, studies were carried out to determine whether PP2 treatment inhibited the tyrosine phosphorylation of Raf-1 noted with exposure of v-Ha-ras-transformed (Fig. 2A) and BXB Raf-overexpressing (Fig. 2B) NIH 3T3 cells to PMA and H₂O₂. In this experiment both Raf-1 and the BXB Raf fragment were immunoprecipitated using anti-Raf antibody, and the presence of phosphorytrosine was detected by immunoblotting with anti-phosphotyrosine antibody. As shown, the presence of PP2 blocked the slight increase in Raf-1 (Fig. 2A) and BXB Raf (Fig. 2B) tyrosine phosphorylation...
BXB Raf-overexpressing NIH 3T3 cells serum-deprived for 24 h were treated with 1 mM H2O2 for 5 min. Raf-1 activation by OAG/H2O2 was markedly enhanced by pretreatment of cells with 100 nM PMA and 1 mM H2O2 for 5 min. In Panels A and B, BXB Raf-overexpressing NIH 3T3 cells serum-deprived for 24 h were pretreated with and without 10 μM PP2 for 30 min before stimulation with the combination of 100 nM PMA and 1 mM H2O2 for 5 min. In Panels A and B, Raf-1 and BXB Raf proteins were immunoprecipitated from cell lysates with anti-Raf antibody, and the immunoprecipitates were resolved by 7.5% SDS-PAGE. The absence of phosphotyrosine was detected by immunoblotting with anti-phosphotyrosine antibody RC20H. The same blots were stripped and then reprobed with anti-Raf antibody to determine the amount of Raf-1 and BXB Raf protein present in each lane. The results are representative of three independent experiments.

induced by PMA and H2O2. There was no change in the levels of either Raf-1 or BXB Raf protein under these conditions, as determined by reprobing the stripped immunoblots with anti-Raf antibody.

The Stimulatory Effect of PP2 Is Ras-independent—The phorbol ester tumor promoter PMA serves as a potent, non-physiological activator of PKC by acting as a mimic of diacylglycerol, an endogenous activator of PKC. Thus, studies were carried out with the diacylglycerol analogue, 1-oleoyl-2-acyl-sn-glycerol (OAG) to determine whether OAG can replace PMA to induce synergistic activation of Raf-1 in combination with H2O2. Although not as effective as PMA, simultaneous treatment of cells with OAG along with H2O2 did result in transient activation of Raf-1 (Fig. 3A). Raf-1 activation by OAG/H2O2 reached its peak within 5–10 min after treatment and was found to be markedly enhanced by pretreatment of cells with PP2. Conversely, PP2 showed no effect on PDGF-induced Raf-1 kinase activation, which is Ras-dependent (Fig. 3B). Thus, experiments were carried out to determine whether Ras is required for PP2 stimulation of Raf-1 activation induced by PMA/H2O2. Inhibition of Ras function was achieved by transient transfection with a vector encoding dominant-negative Ras (RasN17). As shown in Fig. 3C, expression of dominant-negative RasN17 to block Ras function did not inhibit the PMA/H2O2-induced activation of Raf-1 noted in the presence of PP2. However, in experiments carried out to establish that expression of RasN17 in these cells effectively blocked Ras function, as determined by inhibition of PDGF-induced ERK phosphorylation, it was found that the elevated levels of ERK phosphorylation noted with v-Ha-ras-transformed cells made it difficult to detect consistent, significant stimulation of ERK phosphorylation induced by PDGF alone. Thus, parental NIH 3T3 cells were used to establish that expression of RasN17 did block Ras function, as determined by inhibition of PDGF-induced stimulation of ERK phosphorylation, but had a much less pronounced effect on PMA/H2O2-induced ERK activation (Fig. 3C, inset). Importantly, transient transfection of RasN17 into NIH 3T3 cells did not significantly block PMA/H2O2/PP2-induced activation of Raf-1. These results established that PMA/H2O2-induced activation of Raf-1 was Ras-independent. Of added interest was the finding that the presence of PP2 did not enhance PMA/H2O2-induced ERK activation with v-Ha-ras-transformed cells, as determined by increased ERK phosphorylation, despite the noted hyperactivation of Raf-1 under these conditions (Fig. 3D). These results suggested that PMA/H2O2/PP2-mediated activation of Raf-1 did not appear to potentiate signaling through the Ras/MEK/ERK pathway.

The Stimulatory Effect of PP2 on the Synergistic Activation of Raf-1 by H2O2/PMA Was More Pronounced in Src-transformed Cells—Because PP2 is an Src-family tyrosine kinase inhibitor, studies were carried out to determine whether PP2 treatment of v-src-transformed NIH3T3 cells would result in even more pronounced activation of Raf-1 kinase. The kinetics of Raf-1 activation after exposure of v-src-transformed cells to PP2 is presented in Fig. 4. Compared with the results obtained with v-Ha-ras-transformed cells (see Fig. 1C), PP2 treatment of v-src-transformed cells resulted in delayed, but more dramatic, stimulation of PMA/H2O2-induced activation of Raf-1 (Fig. 4). Exposure of v-src-transformed cells to PP2 alone for 5 and 10 min resulted in a slight activation of Raf-1 kinase activity, but the presence of PMA/H2O2 for the final 5 min of PP2 treatment did not result in further activation of Raf-1. However, exposure of v-src-transformed cells to PP2 for 20 and 30 min dramatically enhanced PMA/H2O2 activation of Raf-1. This delay in the ability of PP2 to potentiate PMA/H2O2-induced activation of Raf-1 in v-src-transformed cells likely is due to the elevated level of Src-mediated tyrosine phosphorylation present in these cells.

Point Mutation of Raf-1 Tyrosines 340/341 Did Not Block PP2-mediated Activation of Raf-1—Src-family tyrosine kinases previously have been slow to phosphorylate Raf-1 on tyrosine 341 (35, 38). Thus, mutational analysis was performed to determine whether phosphorylation/dephosphorylation of tyrosines 340/341 was involved in PP2-mediated Raf-1 activation. For this experiment, v-Ha-ras-transformed cells were transiently transfected with either FLAG-tagged wt-Raf-1 or FLAG-tagged Raf mutant (YY340/341FF) in which tyrosines 340 and 341 were changed to phenylalanines. After the treatments indicated with PMA and H2O2 in the presence and absence of PP2, the FLAG-tagged Raf proteins were immunoprecipitated using anti-FLAG antibody to separate epitope-tagged proteins from endogenous wild-type Raf-1 proteins. The data presented in Fig. 5A revealed that loss of the Src phosphorylation site in the YY340/341FF mutant rendered Raf-1 essentially unresponsive to stimulation by PMA alone or by the combination of PMA/H2O2, although a low level of intrinsic activity was detectable. Importantly, mutation of the YY340/341FF tyrosine phosphorylation site did not significantly alter...
FIG. 3. 

Panel A, PP2 also enhanced Raf-1 kinase activation by the combination of the diacylglycerol analogue OAG and H2O2. Subconfluent v-Ha-ras-transformed cells were serum-deprived for 24 h and then exposed to 10 μM PP2 where indicated for 30 min before treatment with the combination of 50 μM OAG and 1 mM H2O2 for the times indicated. Panel B, PP2 does not enhance the ability of PDGF to stimulate Raf-1 kinase. Subconfluent NIH 3T3 cells were serum-deprived for 24 h along with exposure to the indicated tyrosine kinase inhibitors for the following time periods. The serum-deprived cells were treated with 60 μM genistein for the final 60 min, with 10 μM PP2 for the final 30 min, with 50 μM AG1296 (PDGFR kinase inhibitor) for the final 4 h, and with 50 μM AG1478 (epidermal growth factor receptor kinase inhibitor) for the final 30 min before exposure to 20 ng/ml PDGF for 2 min. 

Panel C, PP2 stimulation of Raf-1 kinase by PMA and H2O2 is Ras-independent. v-Ha-ras-transformed cells were transfected with mock control pcDNA vector or with the pcDNA vector encoding dominant-negative Ras (RasN17). After 24 h of transfection, the cells were washed twice with phosphate-buffered saline and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum for an additional 24 h. Cells then were washed once with phosphate-buffered saline and incubated with serum-free Dulbecco's modified Eagle's medium for 24 h. PP2 (10 μM) was added for the final 30 min of incubation before exposure to the combination of 100 nM PMA and 1 mM H2O2 for 5 min. Inset, parental NIH 3T3 cells were first transfected for 24 h with control vector or dominant-negative RasN17 and then incubated with serum-free medium for 24 h as described above before treatment. Inset, left panel, PP2 (10 μM) was added for the final 30 min of incubation before exposure to the combination of 100 nM PMA and 1 mM H2O2 for 20 min. Inset, right panel, either PDGF (20 ng/ml) or the combination of 100 nM PMA and 1 mM H2O2 was added for 20 min as indicated. Panel D, PP2 treatment did not potentiate the PMA- and H2O2-induced activation of endogenous ERK. Subconfluent v-Ha-ras-transformed NIH 3T3 cells were serum-deprived for 24 h and then treated
the ability of PP2 to potentiate mutant YYFF Raf activation by PMA and H$_2$O$_2$. Western blot analysis of the FLAG-tagged immunoprecipitates with anti-Raf-1 antibody showed that treatment of these cells with PMA, H$_2$O$_2$, and PP2 had no effect on the cellular levels of either of these expressed FLAG-tagged proteins.

To examine the possible involvement of other reported Raf-1 phosphorylation sites in PP2 modulation of Raf-1 kinase, we tested the ability of PP2 to enhance PMA/H$_2$O$_2$-induced Raf-1 activation in cells expressing FLAG-tagged Raf-1 protein with point mutations at these known sites of phosphorylation (Fig. 5B). Phosphorylation of Ser-621 (46) and also of Ser-624 (32) appear to be essential for Raf-1 activation. As presented in Fig. 5B, it was found that mutation of Ser-621 (S621A) and of Ser-624 (S624A) to alanine as well as the tandem mutation of the serines at positions 621 and 624 (Raf-1 S621A/S624A) resulted in loss of Raf-1 kinase activity. These inactive mutants did not respond to treatment with PP2. Phosphorylation of Ser-499 previously has been implicated in the PKC-mediated activation of Raf-1 (47). Mutation of Ser-499 to alanine (Raf-1 Ser-499 previously has been implicated in the PKC-mediated did not respond to treatment with PP2. Phosphorylation of Ser-499 has been implicated in the PKC-mediated activation of Raf-1 (47). Mutation of Ser-499 to alanine (Raf-1 S499A) did not alter the ability of PMA/H$_2$O$_2$ and PP2 to stimulate Raf-1 kinase activity. Phosphorylation of Ser-338 is an essential regulatory event for Raf-1 activation, and mutation of Ser-338 (Raf-1 S338A) has been shown to markedly impair both the basal and induced activities of Raf-1 (34, 48). In agreement with these reports, the results presented in Fig. 5B also show that mutant Raf S338A exhibits no detectable kinase activity when cells are treated with PMA/H$_2$O$_2$. However, treatment with PP2 along with the combination of PMA/H$_2$O$_2$ did result in slight activation of Raf S338A. Finally, Ser-259 has been reported to be a site of inhibitory phosphorylation of Raf-1 by PKA (49) and Akt/PKB (50). Mutation of Ser-259 (Raf-1 S259A) did enhance the ability of Raf-1 S259A to phosphorylate MEK-1 and also potentiated PMA/H$_2$O$_2$ and PP2 stimulation of MEK-1 phosphorylation above that noted with wt-Raf (Fig. 5B).

DISCUSSION

Raf-1 protein kinase has been reported to play a critical role in the Ras/MAP kinase signaling pathway (1, 2, 51). An important mechanism involved in regulating cellular signaling cascades is the reversible phosphorylation of proteins by protein kinases and phosphoprotein phosphatases to mediate protein-protein interactions and protein activities. Although a number of factors have been reported to mediate activation of Raf-1 kinase, including Ras, Src-like tyrosine kinases, various PKC isoforms, 14-3-3 protein, phosphoprotein phosphatases, and phospholipids (1, 2, 9, 51), the molecular mechanism(s) involved in the cellular regulation of Raf-1 remain to be fully elucidated. In a recent report we showed that exposure of cells to H$_2$O$_2$ resulted in the activation of Raf-1 kinase (41). The PKC activator PMA also induced a weak increase in Raf-1 kinase activity. Importantly, simultaneous treatment of cells with the combination of PMA and H$_2$O$_2$ resulted in synergistic activation of Raf-1. Studies with selective PKC isotype inhibitors and with the expression of a kinase-deficient, dominant-negative mutant of PKCc (K436R), established that the PKC isozyme was involved in mediating this PMA-induced activation of Raf-1 (41). Furthermore, transient transfection studies with a dominant-negative mutant of Ras (N19Ras) demonstrated that both H$_2$O$_2$ and PKC-mediated activation of Raf-1 kinase were Ras-independent.

Since tyrosine phosphorylation has been reported to be an important regulatory parameter of Raf-1 kinase (6, 7), studies were carried out here to determine the effect of tyrosine kinase inhibitors on the ability of PMA and H$_2$O$_2$ to stimulate Raf-1. It was found that treatment of cells with PP2, a selective inhibitor of Src-like tyrosine kinases, caused a marked and sustained increase in Raf-1 susceptibility to PMA/H$_2$O$_2$-induced activation (Fig. 1). The moderate increase in Raf-1 kinase activity observed in response to treatment of v-Ha-ras-transformed cells with PP2 alone is likely due to the presence of elevated levels of activated PKC (52, 53) and reactive oxygen species (54) found in these cells. The ability of PP2 to potentiate PMA/H$_2$O$_2$-induced activation of Raf-1 was optimal within 30 min of treatment with PP2 and was sustained through at least 4 h of exposure to PP2. Treatment of NIH 3T3 cells expressing truncated, constitutively active BBX Raf kinase with PP2 also stimulated BBX Raf activity and enhanced PMA and H$_2$O$_2$ activation of BBX Raf. Since BBX Raf is constitutively active due to deletion of the N-terminal region responsible for Ras binding and negative regulation of Raf-1, these results indicated that PP2/PMA/H$_2$O$_2$ activation of Raf-1 kinase was Ras-independent and involved regulatory events within the C-terminal catalytic region.

Raf-1 activation induced by activated Src tyrosine kinase is accompanied by phosphorylation of Raf-1 tyrosine residues 340/341 (5, 35). However, in a number of cell systems Raf-1

|        | 5 min | 10 min | 20 min | 30 min |
|--------|-------|--------|--------|--------|
| Untrat | +     | +      | +      | +      |
|        | -     | +      | +      | +      |
| PMA    | +     | +      | -      | +      |
| H$_2$O$_2$/PMA | 1.0   | 0.9    | 2.8    | 1.6    |
| PP2    | 3.1   | 3.2    | 3.0    | 3.0    |
| MEK-1  | 2.9   | 2.9    | 12.4   | 4.0    |

FIG. 4. PP2 stimulation of PMA/H$_2$O$_2$-induced activation of Raf-1 kinase was delayed but more pronounced in v-src-transformed NIH 3T3 cells. Subconfluent v-src-transformed NIH 3T3 cells were serum-deprived for 24 h and then pretreated with 10 $\mu$m PP2 for the times indicated before stimulation with the combination of 100 nM PMA and 1 mM H$_2$O$_2$ for the final 5 min of exposure to PP2. In vitro Raf-1 kinase assays were performed on the immunoprecipitated Raf-1 proteins using recombinant MEK-1 as substrate. The results presented are representative of three independent experiments. Numerical values given below each gel lane represent relative units of MEK phosphorylation determined by densitometric scanning as described in the Legend to Fig. 1.

with and without 10 $\mu$m PP2 as indicated for the final 30 min before exposure to 100 nM PMA and/or 1 mM H$_2$O$_2$ as described under “Experimental Procedures.” Quantitation of MEK phosphorylation was carried out by densitometric scanning as described in the Legend to Fig. 1.
kinase activation appears to occur in the absence of tyrosine phosphorylation (55, 56). Here, we found little detectable phosphotyrosine present in Raf-1 immunoprecipitates from cells treated with PMA alone (Fig. 2). Conversely, low levels of Raf-1 tyrosine phosphorylation were detected in cells treated with H$_2$O$_2$ alone. This H$_2$O$_2$-induced increase in cellular tyrosine...
Stimulation of Raf-1 by Src Kinase Inhibitor PP2

**Figure 6.** Hypothetical model for the sustained activation of Raf-1 by the Src-family tyrosine kinase inhibitor PP2. Raf-1 kinase has three highly conserved regions termed CR1, CR2, and CR3. The CR1 region contains the binding site for activated Ras protein. The CR1 and CR2 regions act to negatively regulate the Raf-1 kinase catalytic domain (CR3), which is the C terminal domain of Raf-1. Src, Src-family tyrosine kinase; PP2, Src-family tyrosine kinase inhibitor; see “Discussion” for details. This is adapted from a model of Raf-1 activation proposed by Kolch (9).

Phosphorylation is likely due to inhibition of phosphotyrosine phosphatase(s) rather than to activation of tyrosine kinase(s) (17–19). Importantly, exposure of cells to PP2 completely blocked the tyrosine phosphorylation of Raf-1 and BXB Raf under conditions that enhance activation by PMA and H2O2. The PP2-induced modulation of Raf-1 was found to be specific to the Ras-independent stimulation of Raf-1 noted with either PMA or OAG in combination with H2O2 (Fig. 3). When cells were treated with PDGF to activate Raf-1, the presence of PP2 inhibited rather than enhanced PDGF-induced stimulation of Raf-1 kinase. In addition, expression of RasN17 to block Ras function did not decrease PMA/H2O2-induced activation of Raf-1 in either the presence or absence of PP2. Moreover, PP2 did not potentiate PMA/H2O2-induced activation of ERK (Fig. 3). Together, these results suggested that PP2-mediated activation of Raf-1 did not potentiate signaling through the Ras/MEK/ERK pathway. Rather, altered tyrosine phosphorylation within the Raf scaffolding complex in response to PP2 inhibition of a Src-like tyrosine kinase may serve as a switch between Ras-dependent and Ras-independent activation of Raf-1.

Mutational analysis carried out to determine whether reported Raf-1 phosphorylation sites might be involved in the PP2-mediated activation of Raf-1 showed that mutation of the Src tyrosine kinase phosphorylation site on tyrosine 341 (YY340/341FF) decreased but did not block the ability of PP2 to induce Raf-1 activation in the presence of PMA/H2O2 (Fig. 5). Phosphorylation of essential sites Ser-621 and Ser-624, however, was required as mutation of each of these sites to alanine resulted in an inactive form of Raf-1 that was not receptive to activation by PP2/PPMA/H2O2. Likewise, mutation of the activating phosphorylation site at Ser-388 to alanine resulted in an inactive form of Raf-1 that exhibited only slight activation in response to PP2/PPMA/H2O2 treatment. Conversely, mutation of the Raf-1 inhibitory phosphorylation site at Ser-259 resulted in only a slight (−60%) enhancement in the activation of Raf-1 S259A by PMA/H2O2 and an even lesser (−30%) effect on Raf-1 S259A stimulation by PP2/PPMA/H2O2. Replacement of a putative PKC phosphorylation site at Ser-499 with alanine had no effect on the ability of PP2 to stimulate MEK phosphorylation by Raf-1 S499A. These results indicated that phosphorylation of Raf-1 residues Ser-338, Ser-621, and Ser-624 was required for optimal Ras-independent activation of Raf-1 by the combination of the Src-like tyrosine kinase inhibitor PP2 and PMA/H2O2.

That truncated BXB Raf also was stimulated by PP2 treatment indicated that inhibition of a Src-like tyrosine kinase resulted in the altered modification of, or in the release of a regulatory protein from the CR3 catalytic domain of Raf-1. This could result in the generation of the Ras-independent form of Raf-1 reported here that is susceptible to marked and sustained activation by PKCε and H2O2. This is similar to results reported by Zimmermann et al. (57) of Raf-1 activation by a positive feedback mechanism through MEK-1 that is independent of Ras, Src, and tyrosine phosphorylation of Raf-1. In turn, PP2-mediated modification of Raf-1 and its associated proteins may result in the targeting of these different Raf-1 complexes to specific subcellular sites and to distinct lipid raft membrane domains (58, 59). Localization at different sites within the cell likely would alter the regulatory parameters (i.e. PKCε and reactive oxygen species) required to generate and maintain an active Raf-1 complex. In addition, altered localization also would likely dictate which protein substrates are recognized and which signaling pathways are regulated by different forms of the Raf-1 complex. Several new targets have been described, including cell cycle proteins Rb, p53, and cdc25 (60). In fact, mitotic activation of Raf-1 does not lead to stimulation of ERK, suggesting that the mechanism(s) responsible for activating Raf-1 during mitosis and the subsequent downstream effects are distinct from those involved in growth factor-induced stimulation of Raf-1 and the modulation of ERK activation (61, 62). The Raf-1 knockout and knockin mice studies of Mikula (63) and Hüber (64) lend support to the suggestion that different Raf-1 signaling complexes may exist to target alternate signaling pathways. As outlined by Murakami and Morrison (65), the results of these Raf-1 knockout studies suggested that Raf-1 might be dispensable for MEK/MAP kinase signaling. Rather, the primary function of Raf-1 is to protect cells from apoptosis, and the MEK/MAP kinase cascade does not mediate this anti-...
apoptotic effect. Rather, these studies provide evidence for additional roles for Raf-1 and suggest that other relevant substrates of Raf-1 likely exist (65).

Studies have established that in most cases Raf-1 kinase activation is dependent on phosphorylation events and on the interaction between Raf and GTP-Ras to localize Raf-PK to the plasma membrane, where it then is converted to a catalytically active form (6, 9). This is followed by an as yet unidentified negative feedback mechanism, likely triggered by activated Raf-1, to release Raf-1 from Ras to terminate Raf-1 activation and return it to an inactive state (7, 9). As depicted in the model presented in Fig. 6, one possible mechanism for PP2-induced activation of Raf-1 is that it acts to prevent or reverse the generation of an inactive Raf-1 complex. A number of proteins that interact with and regulate Raf-1 have been identified, and the ability of these proteins to complex with Raf-1 often is influenced by phosphorylation events (7). For example, the binding of 14-3-3 protein to Raf-1 is modulated by the phosphorylation of Raf-1 residues Ser-259 and Ser-621 (9). Kinase suppressor of Ras (KSR) also has been identified as a scaffold phosphoprotein specific for the Raf/MEK/ERK pathway (66). Recently, it was shown that protein phosphatase 2A (PP2A) acts to dephosphorylate KSR and Raf-1 to coordinate the assembly of an active signaling complex at the membrane (67). Raf kinase inhibitor protein (RKIP) is another protein reported to interact with Raf-1 (7). Corbit et al. (68) recently reported the interesting finding that classical and atypical PKC isoforms, but not novel isoforms (i.e., PKCe), phosphorylate RKIP. This PKC-mediated phosphorylation of RKIP was shown to release RKIP from Raf-1 to relieve inhibition of the Ras/MAP kinase signaling cascade. Here, we consider the possibility that an as yet unidentified Raf-1-binding protein involved in the negative regulation of Raf-1 may be a potential target for tyrosine phosphorylation by an Src-like tyrosine kinase. The PP2 inhibitor could act by blocking tyrosine phosphorylation of this putative regulatory protein to either prevent or alleviate inhibition of Raf-1. The nature of this putative regulatory protein remains to be identified. Possible candidates may reside within the family of Sprouty (Spry 1–4) and Sprouty-related EVH1 domain (Sprd 1–3) proteins (69). Sprouts are a family of membrane-bound phosphoproteins that act as negative regulators of ERK activation (70, 71). Recent studies have established that phosphorylation of a tyrosine residue within the N terminus of Sprouts may be involved in mediating their binding to a number of signaling molecules to interfere with signal transduction pathways (72–74). Interestingly, Sprouty 4 recently was shown to inhibit Ras-independent, but not Ras-dependent activation of Raf-1 (75). This selectivity was a result of the direct binding of Sprouty 4 to Raf-1 through its C-terminal cysteine-rich domain. Since cysteine-rich regions are highly susceptible oxidative regulation (76), it is conceivable that Sproutys or similar regulatory proteins also may be susceptible to H2O2-induced modification.

In conclusion, the results presented here indicate that treatment of cells with PP2, a selective inhibitor of Src-like tyrosine kinases, altered the Raf-1 signaling complex to a form that exhibited a marked and sustained increase in susceptibility to stimulation by the combination of activated PKC and H2O2. This PP2-modified form of activated Raf-1 recognized MEK as a substrate in vitro but did not mediate activation (phosphorylation) of ERK in intact cells. Conceivably, modulation by PP2 may alter Raf-1 to a form that recognizes potential substrates other than or in addition to MEK.

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