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The synergistic activation of Raf-1 kinase by phorbol myristate acetate and hydrogen peroxide in NIH3T3 cells

Michael Lee,[a,*] Gyorgy Petrovics,[b] and Wayne B. Anderson[c]

[a] *Korea Institute of Toxicology, Korea Research Institute of Chemical Technology, P.O. Box 123, Yusong, Daejeon 305-600, Republic of Korea*

[b] *Center for Prostate Disease Research (CPDR), Department of Surgery, Uniformed Services University of the Health Sciences, Rockville, MD 20852, USA*

[c] *Laboratory of Cellular Oncology, National Cancer Institute, NIH, Bethesda, MD 20892, USA*

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Abstract

We have previously demonstrated that a 33 kDa C-terminal fragment of c-Raf-1 underwent a mobility shift in response to hydrogen peroxide (H_2O_2) and phorbol myristate acetate (PMA), respectively. In this study, we have demonstrated that H_2O_2 induced the activation of N-terminal deletion mutant as well as full length Raf-1 kinase. The pharmacological PKC activator PMA also induced a weak increase in Raf-1 kinase activity through PKC-ε activation as determined by the transient expression of dominant negative mutants of PKC-ε-K436R. Interestingly, H_2O_2 produced synergistic increase of PMA-stimulated Raf-1 kinase activation after simultaneous treatment of PMA and H_2O_2. This synergistic activation of Raf-1 kinase was further enhanced by cypermethrin (an inhibitor of protein phosphatase 2B) and dephostatin (tyrosine kinase inhibitor) implying an inhibitory role for these phosphatases in the Raf-1 signaling pathway. Taken together, our data suggest that the synergistic activation of Raf-1 kinase in response to PMA and H_2O_2 occurs via mechanisms that involve an interaction of Raf-1 kinase and PKC-ε, along with a transient phosphorylation of both Raf-1 kinase and PKC.

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Raf-1, a ubiquitously expressed cytoplasmic serine/threonine protein kinase, plays an important role in the transmission of signals initiated at the plasma membrane in response to various mitogenic signals [1]. Earlier studies showed that Raf-1 couples Ras to the MAP kinase cascade involving the phosphorylation and subsequent activation of additional proteins, including mitogen-activated protein (MAP) kinase kinase (MEK) and MAP kinase, which ultimately lead to transcriptional activation and mitogenesis [2].

The likely involvement of protein kinase C (PKC) in the regulation of Ras/MAP kinase pathway has been demonstrated in a variety of systems ranging from yeast to higher eukaryotes [3]. PKC comprises a family of related serine/threonine protein kinases implicated in the regulation of various cellular processes, including proliferation and differentiation [4]. The PKC family is composed of at least 11 members which are classified into three major groups—conventional PKC (cPKC), including PKC-α, -β_1, -β_2, and -γ; novel PKC (nPKC), including PKC-δ, -ε, -ι, -θ, and -μ; and atypical PKC (aPKC), including PKC-ζ, and -λ [5]. It is believed that cPKC class is important in 'short-term,' receptor-mediated cellular responses to transient changes in cytosolic calcium, e.g., hormone secretion, neuromediator release while nPKC class is likely to be involved in 'long-term' cellular regulation, e.g., cell growth, differentiation, and tumor promotion, possibly relying for activation and DAGs generated by sustained phosphatidylcholine turnover [6]. Especially, cPKC-α and nPKC-ε have been shown to activate Raf-1 in vitro [7]. Evidence for an implication of the aPKC-λ and aPKC-ζ in the regulation of the MAP kinase pathway has also been presented [8,9].

Apart from the direct toxic action, reactive oxygen species exert multiple regulatory effects on cell behavior

* Corresponding author. Fax: +82-42-610-8172.
E-mail address: mikelee@kitox.re.kr (M. Lee).

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and function as a second messenger [10]. Furthermore, many cell types produce hydrogen peroxide (H_2O_2) in response to a variety of growth factors such as platelet-derived growth factor (PDGF) [11] and epidermal growth factor (EGF) [12]. Especially, it has been demonstrated that exogenously added H_2O_2 or other oxidant stresses can induce tyrosine phosphorylation in several cell types [13,14] and stimulate MAP kinase phosphorylation over a discrete range [15,16]. In addition, it has been recently reported that direct activation of $p21^{ras}$ may be a central mechanism by which reactive free radicals transmit their signal to the nucleus [17].

Our previous reports demonstrated that a 33 kDa C-terminal, kinase-inactive fragment of c-Raf-1 underwent a mobility shift in response to stimulation of NIH 3T3 cells with hydrogen peroxide (H_2O_2) and PMA, respectively [18]. Here we demonstrated that hydrogen peroxide and PMA individually activate Raf-1 kinase. Furthermore, they acted synergistically to stimulate Raf-1 kinase through a temporary alteration in the kinase–phosphatase balance after simultaneous treatment.

Materials and methods

Materials. A rabbit polyclonal anti-Raf (C-12) and anti-PKC-ε antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphos-ERK (Thr202/Tyr204) antibody was obtained from Cell Signaling Technology (Beverly, MA). Protein A–agarose was from Roche Diagnostics. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and penicillin–streptomycin were purchased from Gibco–Invitrogen (Carlsbad, CA). Reagents for SDS–polyacrylamide gel electrophoresis were from Bio-Rad (Hercules, CA). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Hydrogen peroxide, *N*-acetyl-L-cysteine, GF 109203X, and Gö 6976 were purchased from Calbiochem (San Diego, CA). Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma (St. Louis, MO) and PDGF was from R&D Systems (Minneapolis, MN). PMA was dissolved in DMSO and freshly diluted for each experiment. The kinase-inactive mutant of PKCε was constructed by converting lysine-437 within the catalytic domain to an arginine and then cloned into the pMTH vector, as described previously. The pcDNA vector encoding a dominant-negative mutant of Ras (N17Ras) was kindly provided by Dr. Torin Finkel (NIH, Bethesda, MD) with Dr. Silvio Gutkind's (NIH, Bethesda, MD) permission. NIH 3T3/BXB Raf cells overexpressing the N-terminally truncated, activated Raf-1 were produced by cloning into the pMTH vector [18].

Mammalian cell culture and transient transfection. Parental and its H-Ras transformed NIH 3T3 fibroblasts were maintained at 37 °C in DMEM supplemented with 10% fetal bovine serum, penicillin–streptomycin, and glutamine. For experimental purposes, cells were cultured in 150-mm tissue culture dishes until they reached ~80% confluence. The cells then were transiently transfected with pMTH vector encoding dominant negative mutants of PKC-ε-K436R or pcDNA vector encoding dominant negative vector (RasN17) by electroporation method as specified by the manufacturer (BTX). After 24 h, the transfected cells were serum-deprived overnight before stimulation.

Preparation of cell lysates. Chemical treatments were carried out at 37 °C in serum-free medium as described in the figure legends. The whole cell lysates were prepared as follows. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested by resuspension of the cell pellet in lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, containing 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 their protein concentrations were determined with the aid of a BCA protein assay reagent kit (Pierce; Rockford, IL).

Immunoprecipitation and immunoblot analysis. Immunoprecipitation was performed on the whole cell lysates using anti-Raf antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with 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 SDS–polyacrylamide gel. The proteins were transferred to nitrocellulose and immunoblot analysis was performed using antibody described in figure legends. The endogenous c-Raf-1 kinase and BxB fragment (oncogenically active form; the C-terminal fragment starting with the 302nd amino acids of Raf-1) were detected using a 1:1000 dilution of anti-Raf antibody (BD Biosciences Pharmingen, San Diego, CA). Immune complexes on nitrocellulose were detected by enzyme-linked chemiluminescence (Amersham Biosciences, Piscataway, NJ).

In vitro c-Raf-1 kinase assay. Raf-1 proteins were specifically immunoprecipitated from lysates of NIH3T3 cells and washed three times with lysis buffer, once with kinase buffer (20 mM Tris, pH 7.4, 20 mM NaCl, 1 mM dithiothreitol, and 10 mM $MgCl_2$). Raf kinase activity was measured by phosphorylation of the recombinant MEK (Santa Cruz Biotechnology) [19]. The washed immunoprecipitates were incubated in 40 μl kinase buffer containing 10 μM ATP, 1 μg of the recombinant MEK, and 5 μCi [γ-^{32}P]ATP at 30 °C for 30 min in the presence of MEK inhibitor, PD 98059, which was used to inhibit the autokinase activity of MEK1. Assays were terminated by the addition of gel-loading buffer. The samples were resolved by SDS–PAGE, phosphorylated Raf-1 bands were excised from the gels, and the radioactivity incorporated into the ^{32}P-labeled MEK proteins was determined by scintillation counting.

Results

The effect of H_2O_2 on Raf-1 kinase activity in NIH 3T3 cells

Despite all available data on downstream signaling mediated by H_2O_2, and cellular function of Raf-1 kinase as H_2O_2 target, our knowledge of how H_2O_2 activates Raf-1 kinase is far from conclusive. Thus, experiments were carried out to determine the role of H_2O_2 in the Raf-1 kinase activation. First, to evaluate whether H_2O_2 stimulates Raf-1 kinase, serum-deprived NIH 3T3 cells were exposed to H_2O_2. As shown in Fig. 1A, H_2O_2 was found to activate Raf-1 kinase with induction first apparent at 5 min in parental NIH3T3 cells. Raf-1 kinase activity induced by H_2O_2 was maximal at 20–30 min and gradually decreased thereafter. Especially, an increase in Raf-1 kinase activity in response to H_2O_2 was more evident in H-Ras transformed NIH3T3 cells. We also investigated whether H_2O_2 further activates pre-activated N-terminal deletion mutant, BxB fragment (the C-terminal fragment starting with the 302nd amino acids of Raf-1). The kinase activity of BxB was increased approximately 2-fold or more by treatment of H_2O_2 (Fig. 1B). Interestingly, BxB kinase activity reached the peak as early as 30 s and then rapidly declined to normal

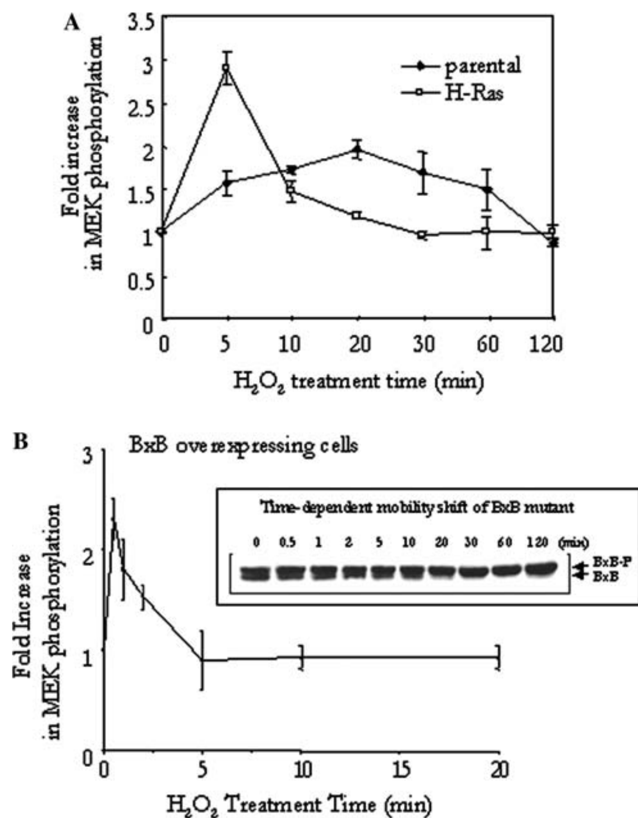

Fig. 1. The activation of Raf-1 protein kinase by hydrogen peroxide. (A) Time-dependent activation of Raf-1 protein kinase by H_2O_2. Subconfluent parental and H-Ras transformed NIH 3T3 cells. In vitro Raf-1 kinase assays were performed on the immunoprecipitated Raf-1 proteins using recombinant MEK as substrate. (B) Time-dependent activation of BXB (N-terminally truncated, oncogenically active Raf-1) by H_2O_2. BXB overexpressing cells were serum-deprived overnight and then exposed to 1 mM H_2O_2 for the indicated times. In vitro Raf-1 kinase assays were performed on the immunoprecipitated Raf-1 proteins using recombinant MEK as substrate. In the inset, BxB fragments were separated by electrophoresis on SDS–polyacrylamide gels (10%). The electrophoretic mobility of BxB was examined by immunoblot analysis. In (A) and (B), the in vitro ^{32}P-labeled MEK-1 protein was separated by 7.5% SDS–polyacrylamide gel electrophoresis and phosphorylation was determined by autoradiography. Activity is expressed as fold increase over unstimulated Raf-1 kinase activity. The values are the mean of three separate experiments with error bar representing the standard deviations.

level in 5 min. However, contrary to the previous reports, in which the mobility shift of Raf-1 kinase was an adequate monitor for the activation state of Raf-1, BxB proteins were completely up-shifted 5 min after returning of kinase activity to control level (Fig. 1B, inset), implying that hyperphosphorylated form is inactive form.

The effect of PMA on Raf-1 kinase activity in H-Ras transformed NIH 3T3 cells

Since PKC has been known to interact with Raf-1 kinase, we also investigated the effect of the pharmacological PKC activator PMA on Raf-1 kinase activation. Especially, a paper by Marais et al. [20] suggests that activation of PKC can cause both accumulation of Ras-GTP via a hitherto unidentified mechanism and subsequent phosphorylation-dependent activation of Raf-1. For this experiment, H-Ras transformed cells, in which Raf-1 is believed to be already associated with cell membrane by activated Ras proteins, were used because these cells were found to be more susceptible to PMA activation than parental NIH3T3 cells. At usual concentration (100 nM) for PKC activation, PMA induced a weak increase in Raf-1 kinase activity (Fig. 2A). A maximum stimulation of Raf-1 kinase was observed as early as 5 min.

To further characterize the PKC isotypes responsible for Raf-1 kinase regulation, two PKC inhibitors were utilized. In this study, pretreatment of cells for 2 h with Gö 6976 (conventional PKC inhibitor) did not interfere with activation of Raf-1 kinase by PMA. PMA-induced Raf-1 kinase activation was, however, effectively blocked by GF 109203X (novel and conventional PKC inhibitor), suggesting a critical role for novel PKCs. Among several novel PKC isotypes, we are especially interested in nPKC-ε, which is the only isoform that has oncogenic potential mediated through its interaction with Raf-1 kinase. Actually, PMA-induced activation of Raf-1 kinase was suppressed in cells expressing kinase-deficient, dominant negative (DN) mutants of PKC-ε-K436R (Fig. 2B). Replacement of the critical lysine at the ATP-binding site by an arginine has been shown to result in kinase-defective PKC mutant that competes with the endogenous wild-type enzymes and acts as an isoform selective dominant negative inhibitor [21]. We confirmed that DN mutant of PKC-ε was not phosphorylated by PMA treatment (Fig. 2B, upper inset). In accordance with the data obtained with the DN mutant of PKC-ε, the transient transfection of the antisense oligonucleotides specific for PKC-ε showed an inhibitory effect on the activation of Raf-1 kinase by PMA (data not shown).

The role of Ras in H_2O_2- and PMA-induced Raf-1 kinase activation

Next, we investigated if Ras, which is an upstream activator of Raf-1, is involved in synergistic activation. Since MEK1/ERK pathway is dependent on Raf-1 kinase activation, we determined ERK phosphorylation using a phospho-specific anti-ERK antibody that detects phosphorylated threonine 202 and tyrosine 204 of ERK1 and ERK2. The inhibition of Ras function was achieved by either transient transfection with vectors encoding dominant negative Ras (RasN17), which blocks endogenous Ras function interfering with upstream activation of Ras proteins [22]. As previously reported [23,24], the expression of a dominant negative mutant of Ras demonstrated inhibitory effects on ERK

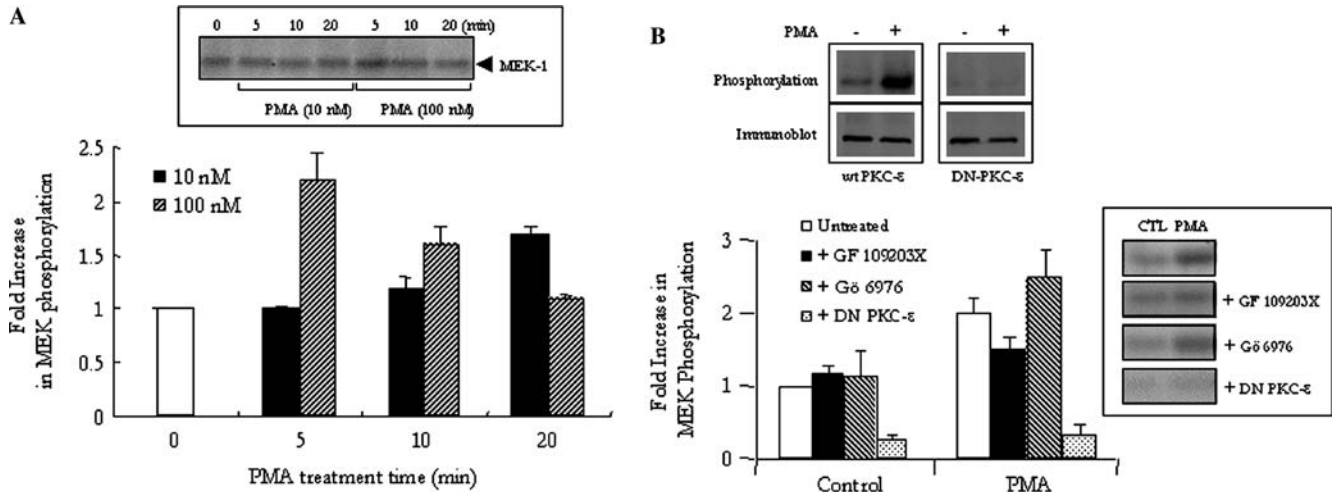

Fig. 2. The activation of Raf-1 protein kinase by PMA. (A) Time- and dose-dependent activation of Raf-1 protein kinase by PMA. Subconfluent H-Ras transformed NIH 3T3 cells were serum-deprived for 24 h and then exposed to PMA for the indicated times. In vitro Raf kinase assays were performed on immunoprecipitated Raf-1 proteins using recombinant MEK as substrate. Activity is expressed as fold increase over unstimulated Raf-1 kinase activity. The values are the mean of three separate experiments with error bar representing the standard deviations. The inset shows a representative autoradiograph of ^{32}P-labeled MEK separated by SDS–PAGE. (B) Inhibition of PMA-induced Raf-1 kinase activation by overexpression of dominant negative PKC-ε (DN-PKC-ε). H-Ras transformed NIH3T3 cells were pretreated (+) or not (−) with either GF 109203X (1 μM), or G 6976 (1 μM) for 2 h before stimulation with 100 nM PMA for 5 min, after which cells were harvested. Also, for identification of the effect of DN-PKC-ε, H-Ras transformed NIH3T3 cells were transiently transfected with pMTH-DN-PKC encoding dominant negative PKC-ε by electroporation method as specified by the manufacturer (BTX). After 48 h, the transfected cells were serum-deprived overnight before stimulation with 100 nM PMA. The in vitro ^{32}P-labeled MEK-1 protein was separated by 7.5% SDS–polyacrylamide gel electrophoresis and phosphorylation was determined by autoradiography. Activity is expressed as fold increase over unstimulated Raf-1 kinase activity. The values are the mean of three separate experiments with error bar representing the standard deviations. The right inset shows a representative autoradiograph of ^{32}P-labeled MEK separated by SDS–PAGE. The upper inset shows that DN-PKC-ε was not phosphorylated by PMA treatment.

activation induced by PDGF used as a positive control, indicating that the transfected cells expressed RasN17 proteins sufficient for inhibiting Ras function. However, transient transfection of RasN17 had little inhibitory effect on H_2O_2- and PMA-induced ERK activation (Fig. 3), suggesting Ras-independent Raf-1 activation.

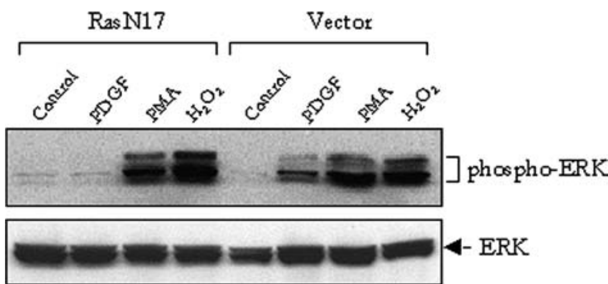

Fig. 3. Ras is not involved in H_2O_2- and PMA-induced ERK activation. Parental NIH3T3 cells were transfected with control vector or dominant negative Ras (RasN17). After 24 h of transfection, the cells were washed twice with PBS and maintained in DMEM containing 10% FBS for 48 h. Cells were then washed once with PBS and incubated with serum-free DMEM for 24 h before treatment with either PDGF (20 ng/ml), H_2O_2 (1 mM) or PMA (100 nM) for 20 min. The whole cell lysates were analyzed by immunoblot. The phosphorylated forms of ERK were detected with immunoblotting using anti-phospho-ERK (Thr202/Tyr204) antibody. The additional blot was probed with anti-ERK antibody to show similar expression level of ERK protein in all lanes. Results of one representative experiment of three are shown.

The synergistic activation of Raf-1 kinase by H_2O_2 and PMA

Since H_2O_2 has been reported to induce prolonged activation of PKC [25], we next determined the possible interaction between Raf-1 kinase activation of H_2O_2 and PMA. As shown in Fig. 4, after simultaneous treatment of PMA and H_2O_2, Raf-1 kinase activity was greatly enhanced. The synergistic activation of Raf-1 kinase reached maximum level at 20 min in control cells. Especially, in H-Ras transformed cells, this synergistic activation of Raf-1 kinase was more evident, reaching the peak as early as 5 min.

Effects of protein phosphatase inhibitors on the synergistic activation of Raf-1 kinase by PMA/H_2O_2

H_2O_2 may serve to transiently allow for a temporary alteration in the kinase–phosphatase balance [26]. Thus, several inhibitors of protein phosphatase were tested to determine the relative effects on Raf-1 kinase activation by PMA/H_2O_2. As shown in Fig. 5, PMA/H_2O_2-induced

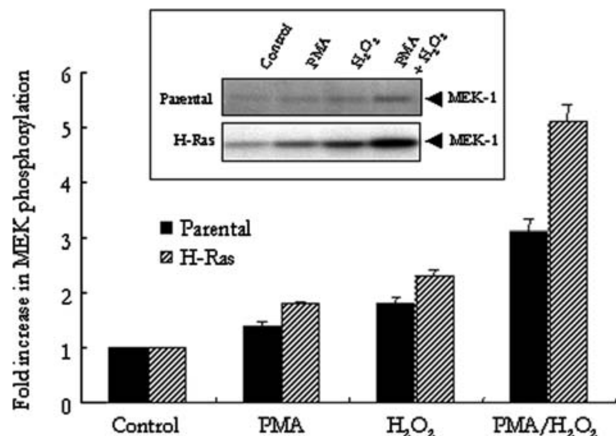

Fig. 4. The synergistic effect of H_2O_2 and PMA on Raf-1 kinase activity. Subconfluent NIH3T3 cells were serum-deprived for 24 h and then exposed to 100 nM PMA and/or 1 mM H_2O_2 for 20 min in control cells and for 5 min in H-Ras transformed cells. In vitro Raf kinase assays were performed on the immunoprecipitated Raf-1 proteins using recombinant MEK as substrate. Activity is expressed as fold increase over unstimulated Raf-1 kinase activity. The values are the mean of three separate experiments with error bar representing the standard deviations. The inset shows a representative autoradiograph of ^{32}P-labeled MEK separated by SDS–PAGE.

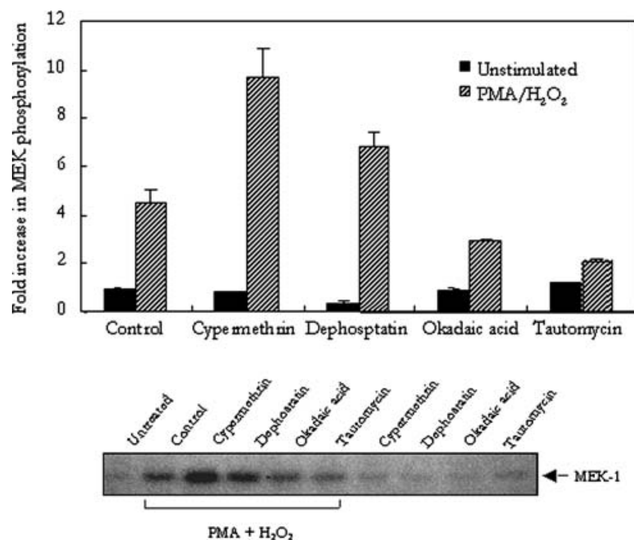

Fig. 5. The effect of protein phosphatase inhibitors on synergistic activation of Raf-1 protein kinase by H_2O_2 and PMA in H-Ras-transformed NIH3T3 cells. Subconfluent H-Ras transformed NIH 3T3 cells were serum-deprived for 24 h and exposed to 1 μM cypermethrin (PP2B inhibitor), 20 μM dephostatin (tyrosine phosphatase inhibitor), 1 μM okadaic acid (PP2A inhibitor), or 1 μM tautomycin (serine/threonine phosphatase 1 inhibitor) for the final 30 min prior to exposure to 100 nM PMA and/or 1 mM H_2O_2 for 5 min. In vitro Raf kinase assays were performed on the immunoprecipitated Raf-1 proteins using recombinant MEK as substrate. Activity is expressed as fold increase over unstimulated Raf-1 kinase activity. The values are the mean of three separate experiments with error bar representing the standard deviations. The lower panel presented is representative of three independent experiments.

activation of Raf-1 kinase was effectively inhibited by okadaic acid and tautomycin at concentrations that affect protein phosphatase 2 A (PP2A) and protein phosphatase 1 (PP1) activities, respectively. Conversely, this synergistic activation of Raf-1 kinase was further enhanced by cypermethrin, an inhibitor of protein phosphatase 2B (PP2B), implying an inhibitory role for this phosphatase in the Raf-1 signaling pathway. Dephostatin, tyrosine phosphatase inhibitor, also had an enhancing effect on the synergistic activation of Raf-1 kinase by PMA/H_2O_2.

Discussion

The generation of H_2O_2 has been known to be required for a variety of growth factor signal transduction [11,27,28]. In this report, we found that exposure of NIH3T3 cells to H_2O_2 resulted in stimulation of Raf-1 kinase (Fig. 1). However, the molecular mechanism by which H_2O_2 transmits the signal remains unclear. A direct treatment of H_2O_2 to Raf-1 immunoprecipitates did not activate Raf-1 kinase (data not shown). One suggested mechanism is that oxygen free radicals directly activate critical signaling molecules such as Ras and thus trigger downstream events [15,17,29]. However, Ras binding is highly unlikely to be involved in H_2O_2-induced Raf-1 activation because an activated Raf-1 mutant (BxB) lacking Ras binding site was further activated by treatment of H_2O_2. Moreover, H_2O_2-induced Raf-1 kinase activation was found to be Ras-independent as determined by transient transfection with dominant negative Ras. Instead, H_2O_2 was shown to serve to transiently inactivate intracellular protein phosphatase, allowing for a temporary alteration in the kinase–phosphatase balance [26]. Therefore, activation of deletion mutant BxB by H_2O_2 may be due to phosphorylation of C-terminal region besides release from negative regulation by N-terminal region.

Consistent with the previous report [30], we also found that the pharmacological PKC activator PMA had a detectable stimulatory effect on Raf-1 kinase. Although an unequivocal role for distinct PKC isotypes in Raf-1 regulation was not clearly defined, PKC-α was shown to be able to activate the MAP kinase pathway by phosphorylating and thus activating Raf [31]. Sö zeri et al. [32] also demonstrated that PKC-β and -γ were able to phosphorylate Raf-1 [32], whereas others found Raf-1 phosphorylation by PKC-ζ [33]. Our PKC inhibitor studies clearly show that the activation of Raf-1 kinase is dependent upon novel PKC isotypes in NIH3T3 cells, which express the phorbol ester-sensitive isotypes PKC-α, PKC-ε, and PKC-δ [34,35]. Especially, a transient expression of a dominant negative PKC-ε-K436R inhibited the activation of Raf-1 (Fig. 2B). These findings are in line with the work of Perletti et al. [36],

who found a marked increase of Raf-1 phosphorylation in PKC-ε-transformed colon epithelial cells. Furthermore, PKC-ε expressed in NIH3T3 cells could phosphorylate and stimulate Raf-1 kinase through a direct interaction of Raf and PKC-ε [7,37], although other data suggest that the effect is indirect, via the secretion of autocrine growth factors [38].

On the other hand, PMA alone, even when added at a maximally effective concentration (100 nM), had only very small effects on Raf-1 kinase activation. However, H_2O_2 produced synergistic increase of PMA-stimulated Raf-1 kinase activation. Notably, H-Ras transformed cells, which produce high levels of reactive oxygen species (ROS) by inducing the NADH-oxidase system [39,40], had a higher basal activity of Raf-1 kinase compared to parental cells and showed a dramatic increase of Raf-1 kinase activity in response to PMA. This synergistic activation of Raf-1 kinase was further enhanced by cypermethrin, an inhibitor of protein phosphatase 2B (PP2B), implying a role for this phosphatase in the inhibition of Raf-1 signaling pathway. Among three highly homologous serine/threonine phosphatases, PP1, PP2A, and PP2B used in this study, PP2B is the only major serine/threonine phosphatase that is a sensitive target for inhibition by reactive oxygen species [41]. Thus, H_2O_2-mediated inhibition of PP2B is likely to enhance Raf-1 kinase activity. In addition, dephostatin, tyrosine phosphatase inhibitor, also had an increasing effect on the synergistic activation of Raf-1 kinase by PMA/H_2O_2. It is believed that tyrosine phosphorylation is required for the activation of Raf-1 kinase as well as PKC although, in the case of PKC, there is a report showing that isozymes other than PKC-δ showed little tendency to become phosphorylated on tyrosine [42].

Conversely, okadaic acid, PP2A inhibitor, and tautomycin, PP1 inhibitor effectively inhibited PMA/H_2O_2-induced activation of Raf-1 kinase. PP1 and PP2A are known to be positive regulators of Raf-1 kinase [43,44] but having inhibitory effect on PKC-α, -β, and -ε [45,46]. In general, hyperphosphorylation has been suggested to play a role in the shift in electrophoretic mobility of activated Raf-1 on SDS–polyacrylamide gels [47]. The recent reports suggested that the mobility shift may in fact reflect a feedback mechanism to turn off Raf-1, and the more highly modified (slower migrating) form of Raf-1 actually be inactive [48]. Also, our previous results have shown that the region of Raf-1 responsible for the gel mobility is localized to a catalytically inactive C-terminal fragment of Raf-1 [18], and the protein feedback loop may lead to modification of serine 624, resulting in a change in Raf-1 electrophoretic mobility [19]. Notably, our study showed that H_2O_2-induced BxB activation actually occurred prior to the mobility shift, and the decrease in BxB protein mobility remained apparent even after returning of kinase activity to the control level.

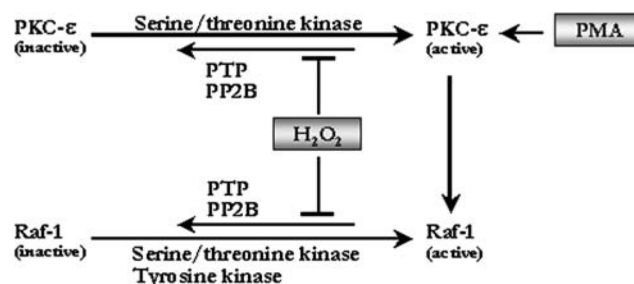

Fig. 6. Model of the synergistic activation of Raf-1 by PMA and H_2O_2. When growth factors activate the protein kinase, concurrent inhibition of protein phosphatase may be necessary in order to increase the steady-state level of protein phosphorylation. The reactive oxygen species such as H_2O_2 may serve to transiently inactivate intracellular protein phosphatase, allowing for a temporary alteration in the kinase-phosphatase balance. On the other hand, PMA treatment induces the phosphorylation of serine 499 within the activation loop of Raf-1 kinase, which has been proposed to be responsible for Raf-1 activation by PKC. Thus, increased phosphorylation of catalytic domain through the combined signals of coming from PMA and H_2O_2 may maintain the highly activated form of Raf-1 kinase. *Abbreviations:* PTP, protein tyrosine phosphatase; PP2B, protein phosphatase 2B.

Thus, these results suggest that PP2A and PP1 protein phosphatases induce the active form of Raf susceptible to activation by PMA and H_2O_2 by dephosphorylating the inhibitory phosphorylation, which may keep inactive state of Raf-1 kinase and be responsible for the electrophoretic mobility of Raf-1 kinase.

Our working model for the synergistic activation of Raf-1 kinase by PMA/H_2O_2 is summarized in Fig. 6. Our data suggest that at least two different mechanisms are involved in the regulation of Raf-1 kinase in response to PMA and H_2O_2: (1) multiple phosphorylations of the amino-terminal region of the catalytic domain through a temporary alteration in the kinase-phosphatase balance and (2) the direct interaction of Raf-1 and PKC-ε. The balance between phosphatases and kinases is important in control of Raf-1 and PKC. Especially in case of tyrosine phosphorylation, the specific activities of PTPases in vitro are 10–1000 times greater than those of protein tyrosine kinase in most cells [49]. Thus, when growth factors activate the protein kinase, concurrent inhibition of phosphatases may be necessary in order to increase the steady-state level of protein phosphorylation. The reactive oxygen species such as H_2O_2 may inactivate intracellular protein phosphatase, resulting in a dramatic shift to kinase in the kinase–phosphatase balance. On the other hand, the primary effect of PMA treatment is to phosphorylate serine 499 within the activation loop of Raf-1 kinase, which has been proposed to be responsible for Raf-1 activation by PKC [31]. Thus, the increased phosphorylation of catalytic domain of Raf-1 kinase by the combined signals of coming from PMA and H_2O_2 may maintain the highly activated form of Raf-1 kinase. Undoubtedly more work is necessary to determine the

precise mechanism by which Raf-1 enzymatic activity is affected by reactive oxygen species and PKC in physiological nature. We are currently examining the relationship between regulation and phosphorylation of Raf-1 kinase in response to PMA and H_2O_2 with respect to cell growth in NIH3T3 cells.

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