

# Pervanadate-triggered MAP kinase activation and cell proliferation are not sensitive to PD 98059

## Evidence for stimulus-dependent differential PD 98059 inhibition mechanism

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**Abstract** A tight and stable complex with corresponding protein kinases and phosphatases establishes coupling between activators and inactivators. One such example is emerging from the studies of the Ras-dependent MAP kinase cascade signaling pathway. Pervanadate, a potent inhibitor of protein tyrosine phosphatase, stimulates MAP kinase and elicits cell proliferation in cultured mouse fibroblasts which is insensitive to PD 98059, the major inhibitor of upstream MEK, whereas serum- or TPA-triggered proliferation is sensitive to PD 98059. It is suggested that imbalanced coordination between protein kinase and protein phosphatase determines the cellular responses such as cell proliferation. The PD 98059-insensitive cell proliferation upon protein tyrosine phosphatase inhibition is attributed to a MEK bypass pathway.

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**Key words:** Mitogen-activated protein kinase; Cell proliferation; PD 98059; Tyrosine phosphatase; Pervanadate

### 1. Introduction

Mitogen-activated protein (MAP) kinases, also known as extracellular signal-regulated kinases (ERKs), act at the end of a kinase cascade that is activated in response to extracellular signals via receptor tyrosine kinases [1], including the tumor promoter [2] 12-*O*-tetradecanoyl phorbol 13-acetate (TPA). The MAPK family members are key components of intracellular signaling cascades, and consist of at least 10 members [3–5]. MAPKK (MEK) is a dual specificity kinase that phosphorylates ERK on a threonine and a tyrosine residue in the catalytic domain. Upon activation, ERK/MAP translocates into the nucleus where it phosphorylates transcription factors and thereby alters gene transcription patterns [6,7]. Overactivation of protein kinases can lead to uncontrolled cell growth and tumorigenesis. There are a wide variety of protein phosphatases that keep the kinases in check by performing the opposite operation, dephosphorylation of the substrate. Thus, a coordinated action between protein kinases and protein phosphatases determines cellular responses [8]. For the activation of MAP kinase, phosphorylation on threonine as well as tyrosine residues is a prerequisite, but dephosphorylation of either one can render the enzyme inactive. Pervanadate (a potent inhibitor of tyrosine phosphatases) is sufficient to trigger the activation of Raf, MEK and MAP kinase [9]. Pervanadate treatment of C3H 10T1/2 mouse fibro-

blasts led to proliferation [2]. Pervanadate has also been reported to down-regulate gap junctional communication and to increase phosphorylation in connexin-43 in studies with hamster lung fibroblasts [10]. Some of the signals that activate MAP kinase are prevented by PD 98059, a compound that specifically inhibits the activation of MEK [11], the major upstream activator of MAPK. However, PD 98059-insensitive phosphorylation of connexin-43 has been reported when pervanadate stimulated MAP kinase. In the studies reported here, we directly examined the effect of PD 98059 on MAP kinase activation/translocation to the nucleus and the cell proliferation induced by stimulating protein kinase pathway (treating cells with serum or TPA) or by inhibiting protein tyrosine phosphatase (treating cells with pervanadate). The signals activating MAP kinase by inhibiting protein tyrosine phosphatase were found to be insensitive to PD 98059 which indicates that an additional mechanism of MAP kinase activation operates when tyrosine phosphatase is inhibited. These findings are relevant in view of the physical union of protein kinases and phosphatases that is considered crucial to guarantee fidelity of signal transduction pathways [8].

### 2. Materials and methods

#### 2.1. Materials

C3H 10T1/2 embryonic mouse fibroblasts were obtained from Dr. C.E. Wenner (Buffalo, NY) [ $\gamma$ -<sup>32</sup>P]ATP and 5-[<sup>125</sup>I]iodo-2'-deoxy uridine were purchased from Amersham, Anti-ACTIVE MAPK antibody was from Promega, anti-ERK-2 antibody was from Santa Cruz Biotechnology, PD 98059 was from Calbiochem, and all other reagents of highest purity grade were from Sigma, France. Pervanadate (phosphotyrosine phosphatase inhibitor) was prepared as described [2].

#### 2.2. Cell cultures

C3H 10T1/2 embryonic mouse fibroblasts [12] were used at passage 15–22 at the post-confluence quiescent state. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% inactivated fetal calf serum in a 5% CO<sub>2</sub> atmosphere at 37°C. The culture medium was renewed 5 days after plating on Petri dishes and cells attained confluence at 9–10 days. Confluent cells were serum starved for 48 h and subsequently treated for 20 min with either 10% serum, TPA (160 nM) or pervanadate (12.5  $\mu$ M) to activate MAP kinase.

#### 2.3. Immunohistochemistry

For immunohistochemistry, cultures were treated with stimuli (pervanadate, TPA or fetal calf serum) or with the inhibitor (PD 98059). The primary antibody used in these experiments was the polyclonal Anti-ACTIVE MAPK antibody raised against the active form of p42/44 MAP kinase.

After a brief rinse with phosphate-buffered saline (PBS), cells were fixed in paraformaldehyde (4% in PBS) for 30 min followed by wash-

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ing three times with PBS. To permeabilize cell membranes, cultures were immersed 30 min in a 0.2% Triton X-100-supplemented PBS solution. After three additional rinses with PBS, the cells were incubated for 20 min in 10% normal goat serum in PBS to block non-specific binding. The cultures were then incubated overnight with the primary antibody (1/200 dilution) at 4°C. After three washes in the 5% goat serum-PBS solution, the cells were incubated for 1 h in a goat anti-rabbit secondary antibody (Jackson) fluorescein isothiocyanate-conjugated (1/200 in 5% goat serum-PBS). Cells were washed three times in PBS and mounted in Vectashield mounting medium (Vector) to reduce photobleaching. Proper controls were carried out while omitting the primary antibody in the incubation bath.

#### 2.4. Preparation of nuclear fraction

Post-confluent cells were deprived of serum for 48 h before stimulation with TPA or pervanadate or serum. Cells were pretreated with PD 98059 for 90 min as indicated. Following treatment, cells were rinsed gently three times with a medium containing 20 mM Tris-HCl, pH 7.5, 1 mM  $MgCl_2$  and 1 mM dithiothreitol (medium A). Petri dishes with attached cells were frozen at  $-80^{\circ}C$  for 2 h. After thawing on ice, cells were harvested in medium A, centrifuged at  $600\times g$  for 10 min at 4°C and the resulting pellet was homogenized in 2 volumes of medium A supplemented with 1.3 M sucrose. Adequate amounts of medium A supplemented with 2.4 M sucrose were added to this suspension so as to give a final concentration of 2.2 M sucrose in the

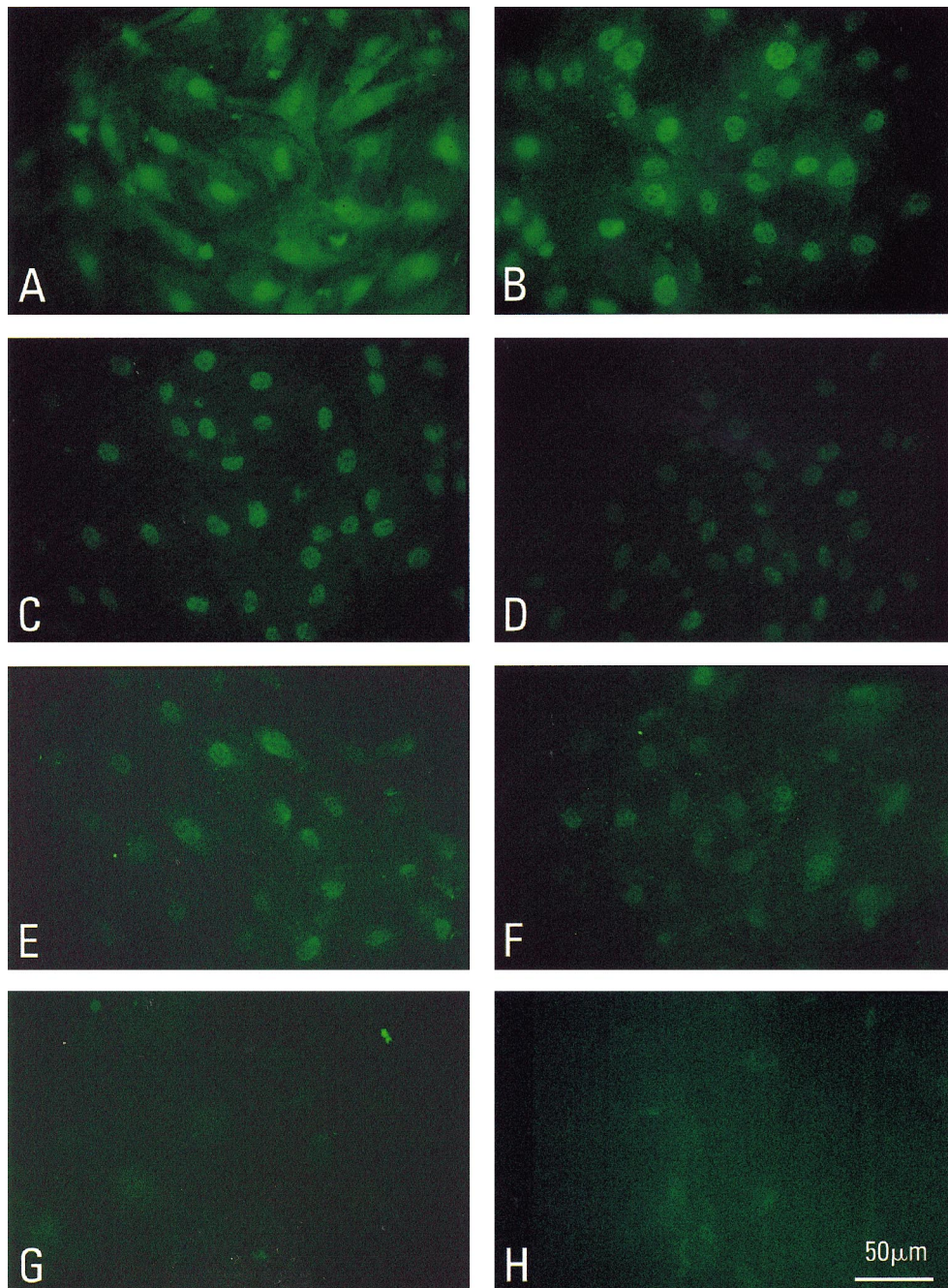


Fig. 1. Immunohistochemical detection of MAP kinase activation in C3H 10T1/2 fibroblasts. A direct visualization of active MAP kinase location was achieved using Anti-ACTIVE MAP kinase antibody. Cells were stimulated with 12.5  $\mu M$  pervanadate (A), 160 nM TPA (C), or 10% fetal calf serum (E) or were unstimulated, i.e. without any treatment (G). The inhibitory effect of 50  $\mu M$  PD 98059 was tested on cells stimulated with 12.5  $\mu M$  pervanadate (B), TPA (D) or serum (F). Control (H) was obtained by omitting the primary antibody from the immunohistochemical staining.

medium. This was homogenized with up and down strokes in a Dounce homogenizer and centrifuged at  $100\,000\times g$  for 20 min at  $4^{\circ}\text{C}$  in a Beckman TL 100 ultracentrifuge. The resulting pellet consisted of isolated nuclei which were devoid of any plasma membrane contaminants as attested by marker enzyme activity [13]. The isolated nuclei were resuspended in a medium containing 20 mM Tris-HCl, pH 7.5, 4 mM  $\text{MgCl}_2$ , 2 mM EDTA, 10% glycerol, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 2 mM  $\text{Na}_3\text{VO}_4$ , 20 mM NaF, 10  $\mu\text{g}/\text{ml}$  leupeptin and aprotinin, 25  $\mu\text{g}/\text{ml}$  trypsin inhibitor, sonicated three times (5 s each) with a 1 min interval between two sonications and centrifuged at  $100\,000\times g$  for 20 min. The supernatant constituted the nuclear fraction.

### 2.5. Cellular protein extraction

After treatment with different stimuli, cells were washed with PBS and subsequently lysed with an extraction buffer (20 mM  $\beta$ -glycerol-phosphate pH 7.5, 2 mM EDTA, 1 mM DTT, 2 mM  $\text{MgCl}_2$ , 10% glycerol, 0.1% Triton X-100, 20 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 2 mM  $\text{Na}_3\text{VO}_4$ , 10  $\mu\text{g}/\text{ml}$  leupeptin, sonicated four times (5 s each) on ice with a 1 min interval between sonications. The lysate was clarified by centrifugation at  $12\,000\times g$  for 10 min.

### 2.6. MAP kinase activity

The kinase assay medium contained: 20 mM HEPES, pH 7.5, 20 mM  $\beta$ -glycerophosphate, pH 7.5, 20 mM NaF, 2 mM EDTA, 2 mM  $\text{Na}_3\text{VO}_4$ , 10 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$  ATP, 0.28 mg/ml myelin basic protein (MBP) and  $3\text{--}5\times 10^5$  cpm  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . An appropriate amount of cellular fractions was assayed for MAP kinase activity. After incubation (15 min at  $30^{\circ}\text{C}$ ), labelled MBP was precipitated by TCA (20%) and the  $^{32}\text{P}$  incorporated into MBP was determined by Cerenkov spectrometry. The protein in each fraction was determined by the Bradford procedure [14] using the Bio-Rad protein assay dye reagent.

### 2.7. Western blotting

Nuclear fractions were mixed with electrophoresis sample buffer and boiled for 5 min at  $90^{\circ}\text{C}$ . Proteins were separated on SDS-PAGE (10% acrylamide). After electrophoresis, proteins were transferred to nitrocellulose membrane using a Bio-Rad semi-dry apparatus. The immunoblot was revealed using anti-ERK-2 antibody

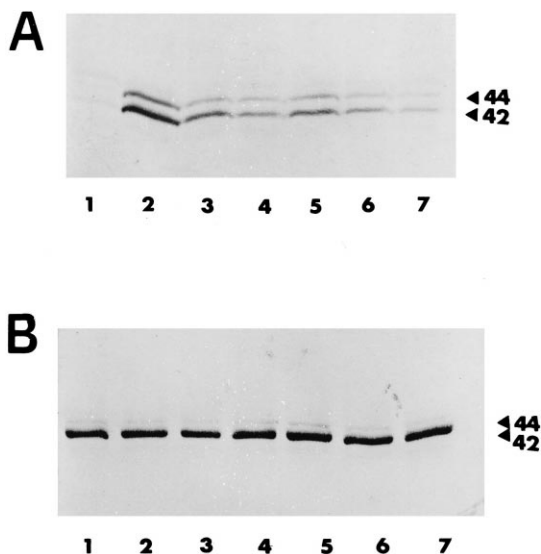


Fig. 2. Western blotting of nuclear fractions. Nuclear fractions (A, B) were separated on SDS-PAGE and transferred to nitrocellulose membrane. The nitrocellulose membranes were subjected to Western blotting using Anti-ACTIVE MAPK antibodies (A) or anti-Erk2 antibodies (B). Lane 1, untreated cell; lane 2, 12.5  $\mu\text{M}$  pervanadate (20 min); lane 3, 160 nM TPA (30 min); lane 4, 10% serum; lane 5, cells treated with 50  $\mu\text{M}$  PD 98059 and 12.5  $\mu\text{M}$  pervanadate; lane 6, cells treated with 50  $\mu\text{M}$  PD 98059 and 160 nM TPA; lane 7, cells treated with PD 98059 and 10% serum. 20  $\mu\text{g}$  protein was loaded on each lane in A and 30  $\mu\text{g}$  in B.

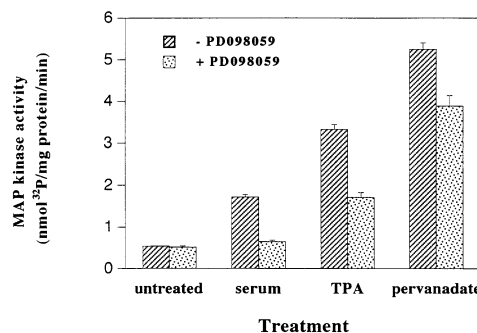


Fig. 3. Effect of PD 98059 on p42/44 MAP kinase activity. MAP kinase activity was determined in whole cell lysate after pretreatment with or without 50  $\mu\text{M}$  PD 98059 for 90 min followed by stimulation with 10% serum, 12.5  $\mu\text{M}$  pervanadate or 160 nM TPA for 20 min.

(1:1000 dilution), or Anti-ACTIVE MAPK antibody (1:20 000 dilution) as primary antibody, employing alkaline phosphatase-conjugated secondary antibody. The immunoblot was developed using NBT-BCIP reagent.

### 2.8. Cell proliferation

For the  $^{125}\text{I}$ -labeled uridine incorporation assay, cells were plated in 24-well plates (Falcon, Oxnard, CA) at a density of  $1.6\times 10^6$  cells per well. The DNA synthesis as well as the number of cells were counted employing trypan blue staining as described [2].

## 3. Results

Employing Anti-ACTIVE MAP kinase antibody revealed immunolabeling of nuclei upon treatment of cells in culture with pervanadate, serum or TPA (Fig. 1). The labeling was more intense when cells were treated with pervanadate (Fig. 1A) as compared with TPA (Fig. 1C) or serum (Fig. 1E) treatment. Preincubating cells with the medium supplemented with PD 98059 followed by pervanadate stimulation did not change the fluorescence (Fig. 1B), whereas weak fluorescence was seen with TPA (Fig. 1D) or serum (Fig. 1F) treatment. In unstimulated cells (Fig. 1G) no active MAP kinase nuclear labeling was seen. Immunocytochemical data were fully supported by Western blot analysis of the nuclear fractions obtained from cells variously stimulated. PD 98059 insensitivity was seen when signals activating MAP kinase were derived from pervanadate treatment (Fig. 2) while TPA- or serum-mediated MAP kinase activation was sensitive to PD 98059. It may be noted here, consistent with the current notion, that pervanadate was the most potent stimulus of MAP kinase activation (Fig. 3). The proliferation was studied by determining DNA synthesis (Fig. 4A) and cell counts (Fig. 4B). The degree of proliferation due to pervanadate treatment of cells ran parallel to MAP kinase activation. Pervanadate-mediated cell proliferation was not sensitive to PD 98059 (Fig. 4B).

## 4. Discussion

Cellular responses to external signals require coordinated control of protein kinases and protein phosphatases. Thus a picture is emerging where multiple complexes containing both kinases and phosphatases [15,16] are strong candidates for the regulation and specificity of signaling pathways. Any process that imbalances such a fine coordination results in an over-activated cellular response. In this scenario pervanadate, a

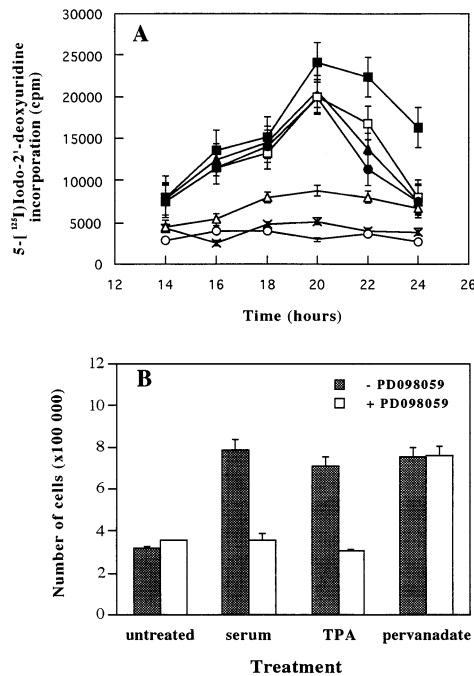


Fig. 4. Effect of PD 98059 on cell proliferation. A: DNA synthesis was determined by [ $^{125}$ I]deoxyuridine incorporation. PD 98059 (50  $\mu$ M) was added in the incubation medium of C3H 10T1/2 cells for 90 min before treatment with pervanadate, TPA or serum. ■, 12.5  $\mu$ M pervanadate; □, 50  $\mu$ M PD+12.5  $\mu$ M pervanadate; ▲, 160 nM TPA; △, 50  $\mu$ M PD+160 nM TPA; ●, 10% serum; ○, 50  $\mu$ M PD+10% serum; ×, untreated cells. B: The numbers of C3H 10T1/2 cells were counted after 40 h of incubation with or without PD 98059 (50  $\mu$ M) pretreatment followed by different effector treatments.

potent inhibitor of protein tyrosine phosphatase, has been recognized as a powerful activator of the Ras-Raf-MAP kinase cascade. Phosphorylation of Stat proteins in the nucleus [17], overphosphorylation of connexin-43 [18] and proliferation of C3H 10T1/2 mouse fibroblasts [2] are some of the examples of pervanadate-mediated preferred signal activation as contrasted to signals generated due to the classical protein kinase activation mechanism.

Against this background we investigated the role of PD 98059 in inactivating MAP kinase and cell proliferation triggered by pervanadate with due comparison to similar effects elicited by serum or TPA treatment of cultured mouse fibroblasts. PD 98059 inhibited MAP kinase activation/translocation to the nucleus and proliferation induced by serum or TPA. It may be recalled here that both serum and TPA act via the Ras-dependent protein kinase cascade and PD 98059 inhibits MEK, the upstream activator of MAP kinase. And therefore the action of PD 98059 in rendering MAP kinase inactive is expected. In addition we report here that PD 98059 treatment of cells in culture abrogates serum- or TPA-triggered proliferation. Thus a direct action of PD 98059 on reversal of TPA- or serum-mediated proliferation is observed. Our results are in complete agreement with the PD 98059-sensitive proliferation mediated by PDGF [19] or leptin [20].

Yet another important message that has emerged from these studies concerns PD 98059-insensitive MAP kinase activation and cell proliferation. Interestingly, pervanadate-trig-

gered MAP kinase action (proliferation as well) has not been found to be sensitive to PD 98059. This clearly demonstrates that the stable complex between MEK and its phosphatase is destabilized due to inhibition of tyrosine phosphatase and this imbalance causes overstimulation of downstream MAP kinase in a PD 98059-insensitive manner. Alternatively, inhibition of dual specificity MAP kinase phosphatase [21] targeting ERK by pervanadate keeps ERK (42/44 kinase as seen here) activated even when cells are inhibited by PD 98059. And therefore for cell proliferation, ERK activation is crucial and that is why PD 98059 is not able to reverse proliferation elicited by pervanadate. The selectivity of dual specificity phosphatase MKP-3 in tight binding to ERKs and not other MAP kinase family members such as JNK/SAPKs or p38 is well established [16].

In summary, these observations clearly demonstrate that the PD 98059-insensitive pathway operates in cellular responses such as cell proliferation (at least in mouse fibroblasts) and MAP kinase activation/translocation to the nucleus. In this context pervanadate has emerged as a major tool for understanding the tight association of protein kinases and protein phosphatases.

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