

A synthetic inhibitor of the mitogen-activated protein kinase cascade

(protein phosphorylation/growth factor/cell proliferation)

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ABSTRACT Treatment of cells with a variety of growth factors triggers a phosphorylation cascade that leads to activation of mitogen-activated protein kinases (MAPKs, also called extracellular signal-regulated kinases, or ERKs). We have identified a synthetic inhibitor of the MAPK pathway. PD 098059 [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one] selectively inhibited the MAPK-activating enzyme, MAPK/ERK kinase (MEK), without significant inhibitory activity of MAPK itself. Inhibition of MEK by PD 098059 prevented activation of MAPK and subsequent phosphorylation of MAPK substrates both *in vitro* and in intact cells. Moreover, PD 098059 inhibited stimulation of cell growth and reversed the phenotype of *ras*-transformed BALB 3T3 mouse fibroblasts and rat kidney cells. These results indicate that the MAPK pathway is essential for growth and maintenance of the *ras*-transformed phenotype. Further, PD 098059 is an invaluable tool that will help elucidate the role of the MAPK cascade in a variety of biological settings.

Many diverse extracellular stimuli—including growth factors, hormones, osmolar shock, stress, and elevated temperature—result in activation of phosphorylation cascades utilizing mitogen-activated protein kinases (MAPKs) (1–8). MAPKs (sometimes called extracellular signal-regulated kinases, or ERKs) comprise a family of related protein kinases that are themselves activated by phosphorylation on threonine and tyrosine residues. The MAPK-activating enzymes (MAPK/ERK kinases, or MEKs) are unusual in their ability to catalyze phosphorylation on both threonine and tyrosine residues (9, 10). MEKs are in turn activated by phosphorylation on serine residues by upstream kinases. These MEK kinases, which appear to require activation by the *ras* protooncogene product (11, 12), include members of the Raf family (13–15), a mammalian homologue of the yeast *STE11* gene product (16), the *tpl12* protooncogene product (17), and a growth-factor sensitive enzyme derived from PC12 rat pheochromocytoma cells (18). However, the precise specificity of these kinases *in vivo* is unclear, since some of them may participate in cascades leading to activation of the related stress-activated protein kinases (19, 20).

While the MAPK pathway is activated under many circumstances in tissue culture cells, the exact role of this pathway *in vivo* remains undefined. Approaches using dominant negative interfering mutant constructs of MEK have indicated that this pathway is required for nerve growth factor-dependent differentiation of PC12 cells. Furthermore, expression of constitutively activated mutants has resulted in transformation (21, 22). We sought a more widely applicable method to determine the physiological role of this pathway by identifying selective inhibitors of specific components of the MAPK cascade.

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MATERIALS AND METHODS

***In Vitro* Kinase Assay.** Incorporation of ³²P into myelin basic protein (MBP) was assayed in the presence of glutathione S-transferase (GST) fusion proteins containing the 44-kDa MAPK (GST-MAPK) or the 45-kDa MEK (GST-MEK1). Assays were conducted in 50 μl of 50 mM Tris, pH 7.4/10 mM MgCl₂/2 mM EGTA/10 μM [γ-³²P]ATP containing 10 μg of GST-MEK1, 0.5 μg of GST-MAPK, and 40 μg of MBP. After incubation at 30°C for 15 min, reactions were stopped by addition of Laemmli SDS sample buffer. Phosphorylated MBP was resolved by SDS/10% PAGE.

For direct evaluation of MEK activity, 10 μg of GST-MEK1 was incubated with 5 μg of a GST fusion protein containing 44-kDa MAPK with a lysine-to-alanine mutation at position 71 (GST-MAPK-KA). This mutation eliminates kinase activity of MAPK, so that only kinase activity attributed to the added MEK remains. Similar incubations were performed with 5 μg of a fusion protein containing artificially partially activated MEK with serine-to-glutamate mutations at positions 218 and 222 (GST-MEK-2E). These assays utilized the same buffer and incubation conditions as described above. Phosphorylated MAPK-KA was resolved by SDS/10% PAGE and detected by autoradiography.

Immunoprecipitation and Immunoblot Analysis. Tyrosine-phosphorylated MAPK-KA was determined by using the same incubation protocol as for phosphorylation, but without radiolabeled ATP. After electrophoresis, proteins on the gel were transferred to a nitrocellulose membrane, and nonspecific binding sites on the membrane were blocked by incubation with 1% ovalbumin/1% bovine serum albumin/150 mM NaCl/10 mM Tris, pH 7.4/0.05% Tween 20. The membrane was then incubated with a commercially available antibody directed against phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY). Antibody bound on the membrane was detected by incubation with ¹²⁵I-labeled protein A, followed by autoradiography.

To determine the state of tyrosine phosphorylation of cellular MAPK, Swiss 3T3 cells were serum-deprived overnight and then treated with the indicated concentrations of PD 098059 for 30 min, followed by platelet-derived growth factor (PDGF, 100 ng/ml) for 5 min. Cells were harvested by scraping and pelleted at 13,000 × *g* for 2 min. The resulting cell pellet was suspended and dissolved in 100 μl of 1% SDS containing 1 mM Na₃VO₄. After denaturation and dilution in RIPA buffer [50 mM Tris, pH 7.4/150 mM NaCl/1% (vol/vol) Triton X-100/0.1% (wt/vol) sodium deoxycholate/10 mM EDTA], MAPK was immunoprecipitated from the lysate by use of a polyclonal antiserum raised against a fragment of MAPK (18). The immunoprecipitate was resolved in a 10%

Abbreviations: EGF, epidermal growth factor; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; MAP2, microtubule-associated protein 2; MBP, myelin basic protein; MEK, MAPK/extracellular signal-regulated kinase kinase; PDGF, platelet-derived growth factor.

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acrylamide gel. Proteins on the gel were transferred to a nitrocellulose membrane, and phosphotyrosine-containing bands were visualized as described above.

Phosphorylation Assay with Microtubule-Associated Protein 2 (MAP2). Confluent cells were serum-deprived overnight and treated with PD 098059 and growth factors as described above. Cells were washed with phosphate-buffered isotonic saline and lysed in 70 mM NaCl/10 mM Hepes, pH 7.4/50 mM glycerol phosphate/1% Triton X-100. Lysates were clarified by centrifugation at $13,000 \times g$ for 10 min. Samples (5 μ g) of the resulting supernatants were incubated with 10 μ g of MAP2 for 15 min at 30°C in 25 μ l of 50 mM Tris, pH 7.4/10 mM MgCl₂/2 mM EGTA/30 μ M [γ -³²P]ATP. Reactions were terminated by addition of Laemmli sample buffer. Phosphorylated MAP2 was resolved in a 7.5% acrylamide gel and incorporated radioactivity was determined by autoradiography and subsequent excision of the bands for scintillation counting.

Thymidine Incorporation. Cells plated in multiwell plates were serum-starved for 24 hr and then incubated with PDGF (10 ng/ml) and various concentrations of PD 098059. PDGF and inhibitor incubations continued for an additional 24 hr; during the final 2 hr, cultures were supplemented with 1 μ Ci (37 kBq) of [*methyl*-³H]thymidine. Cultures were washed with phosphate-buffered saline and then fixed with 5% trichloroacetic acid. Cultures were extensively washed with water, and insoluble material was solubilized with 2% SDS before quantitation of ³H by scintillation counting.

Growth Assays. For monolayer growth, cells were plated into multiwell plates at 10,000–20,000 cells per ml. Forty-eight hours later, various concentrations of PD 098059 were added to the cell growth medium and incubation was continued for an additional 3 days. Cells were then removed from the wells by incubation with trypsin and enumerated with a Coulter Counter. For growth in soft agar, cells were seeded into 35-mm dishes at 5,000–10,000 cells per dish with growth medium containing 0.3% agar and desired concentrations of compound. After 7–10 days of growth, visible colonies were manually enumerated with the aid of a dissecting microscope.

RESULTS AND DISCUSSION

PD 098059 (Fig. 1) was identified by screening a compound library with a cascade assay that measured phosphorylation of MBP in the presence of GST-MEK1 and GST-MAPK fusion proteins. In this assay, the MAPK-catalyzed phosphorylation of MBP was dependent on activation by MEK. To determine whether PD 098059 inhibits MAPK directly or prevents activation of MAPK by inhibiting MEK, we utilized order-of-addition experiments. In the first protocol (Fig. 2A “Before” lanes), tubes initially contained GST-MEK1, buffer salts, and 10 μ M unlabeled ATP. PD 098059 was then added, followed by GST-MAPK, MBP, and [γ -³²P]ATP. In the second protocol (Fig. 2A, “After” lanes), tubes initially contained both GST-MEK1 and GST-MAPK, buffer salts, and 10 μ M unlabeled ATP. PD 098059 was then added, followed by MBP and [γ -³²P]ATP. PD 098059 was found to block MBP phosphorylation only when added to the assay prior to addition of GST-MAPK (Fig. 2A), suggesting that it prevented activation of MAPK. Addition of PD 098059 to the assay after GST-MAPK was activated by incubation with GST-MEK1 did not

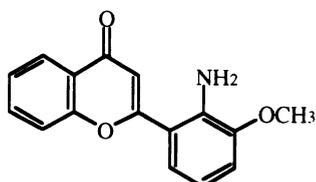


FIG. 1. Chemical structure of PD 098059.

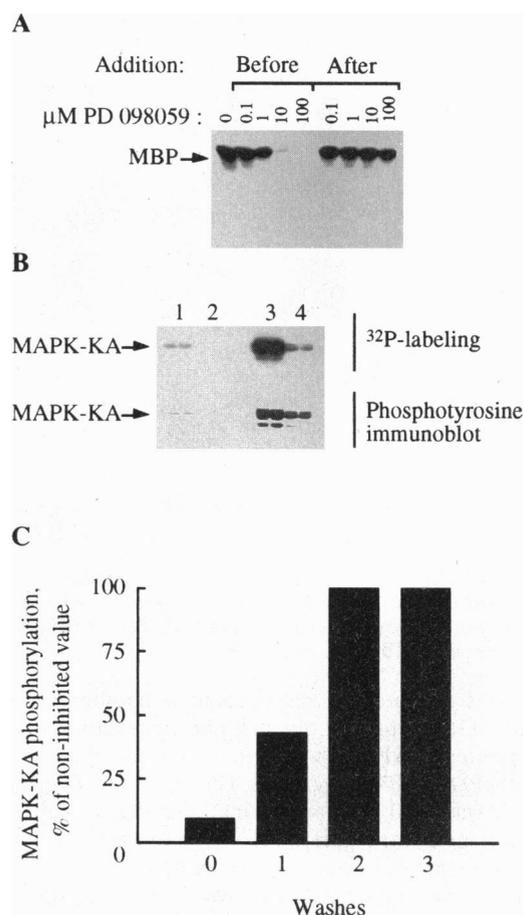


FIG. 2. *In vitro* MEK-inhibitory activity of PD 098059. (A) Phosphorylation of MBP in the presence of GST-MAPK and GST-MEK1. PD 098059 was added before or after addition of GST-MAPK to the tubes. (B) *In vitro* MEK assay. MAPK-KA was phosphorylated by MEK1 (lanes 1 and 2) or MEK-2E (lanes 3 and 4). For lanes 2 and 4, reaction mixtures also contained 50 μ M PD 098059. Samples were evaluated for ³²P content by autoradiography and for phosphotyrosine by immunoblot. Samples were run in duplicate. (C) Reversibility of PD 098059 inhibition. GST-MEK1 retained on glutathione beads was incubated with 30 μ M PD 098059 or vehicle (dimethyl sulfoxide, 1% final concentration). Aliquots were removed and washed the indicated number of times with 10 volumes of 50 mM Tris-HCl/10 mM MgCl₂/2 mM EGTA, pH 7.4. Ability to phosphorylate MAPK-KA was then assessed as above.

result in attenuated MBP phosphorylation, indicating that PD 098059 blocks the activity of MEK and not that of MAPK.

To further characterize the activity of PD 098059, we examined the ability of the compound to inhibit either basal MEK (GST-MEK1) or a partially activated MEK produced by mutation of serine at residues 218 and 222 to glutamate (GST-MEK-2E). Both fusion proteins catalyzed phosphorylation of a kinase-negative MAPK mutant construct, MAPK-KA (Fig. 2B). PD 098059 similarly inhibited the activity of both basal GST-MEK1 and GST-MEK-2E with an IC₅₀ of ≈ 10 μ M as measured by either incorporation of ³²P into MAPK-KA or evaluation of tyrosine phosphorylation by immunoblotting. MEK phosphorylates MAPK on both tyrosine and threonine residues, and these results indicate that PD 098059 can block both activities.

The inhibitory activity of PD 098059 is also reversible. GST-MEK1 bound to glutathione beads was fully inhibited by 30 μ M PD 098059 (Fig. 2C). After a single wash of the beads with buffer lacking PD 098059, $\approx 40\%$ of the kinase activity was regained, and full activity was recovered after two washes.

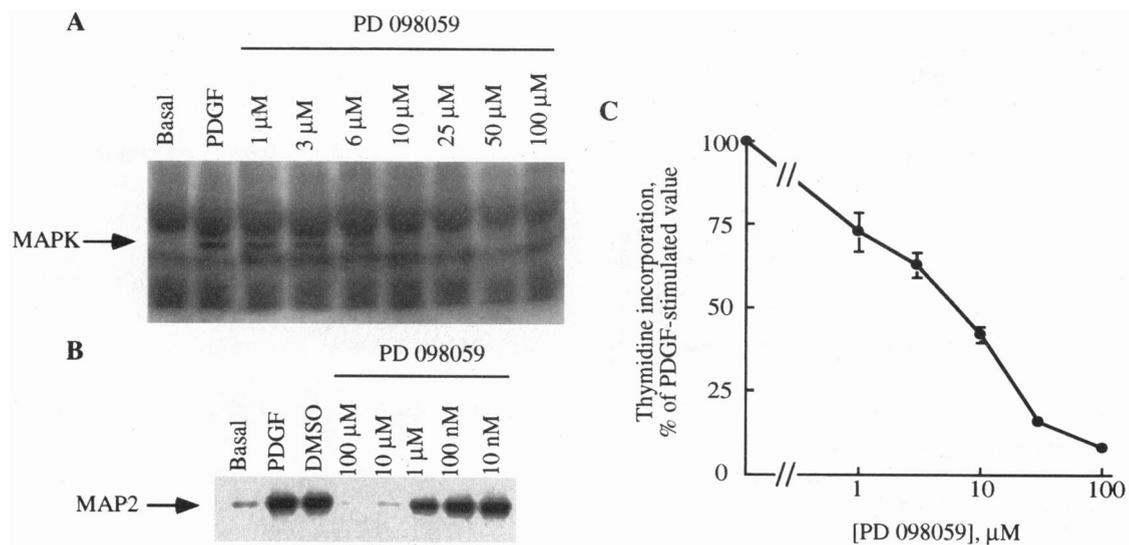


FIG. 3. Inhibitory activity of PD 098059 in cultured cells. (A) Phosphotyrosine immunoblot of MAPK immunoprecipitated from Swiss 3T3 cells. Cells were treated with indicated concentrations of PD 098059 for 30 min, followed by PDGF (100 ng/ml) for 5 min. (B) Phosphorylation of added MAP2 by lysates from 3T3 cells treated with PD 098059 and stimulated as above. (C) Effect of PD 098059 on PDGF-stimulated thymidine incorporation into 3T3 cells.

MAPK is activated in response to a number of growth factors. PDGF-stimulated tyrosine phosphorylation of immunoprecipitated MAPK was inhibited by preincubation of 3T3 cells with PD 098059 (Fig. 3A). The apparent IC_{50} for this inhibition was $\approx 10 \mu$ M, indicating that PD 098059 readily crosses cell membranes and exerts inhibitory activity similar to that seen *in vitro*. To examine MAPK activity in whole cells, cells were preincubated with PD 098059 prior to stimulation

with PDGF. MAPK activity was then determined in cell lysates by assaying phosphorylation of added MAP2 (5). PD 098059 inhibited MAPK activation, reducing activity to basal levels (Fig. 3B). To evaluate effects of PD 098059 on DNA synthesis, we examined thymidine incorporation into 3T3 cells. In 3T3 cells stimulated with PDGF, PD 098059 inhibited thymidine incorporation with an IC_{50} of $\approx 7 \mu$ M (Fig. 3C). Together, these data show that PD 098059 inhibits PDGF-stimulated

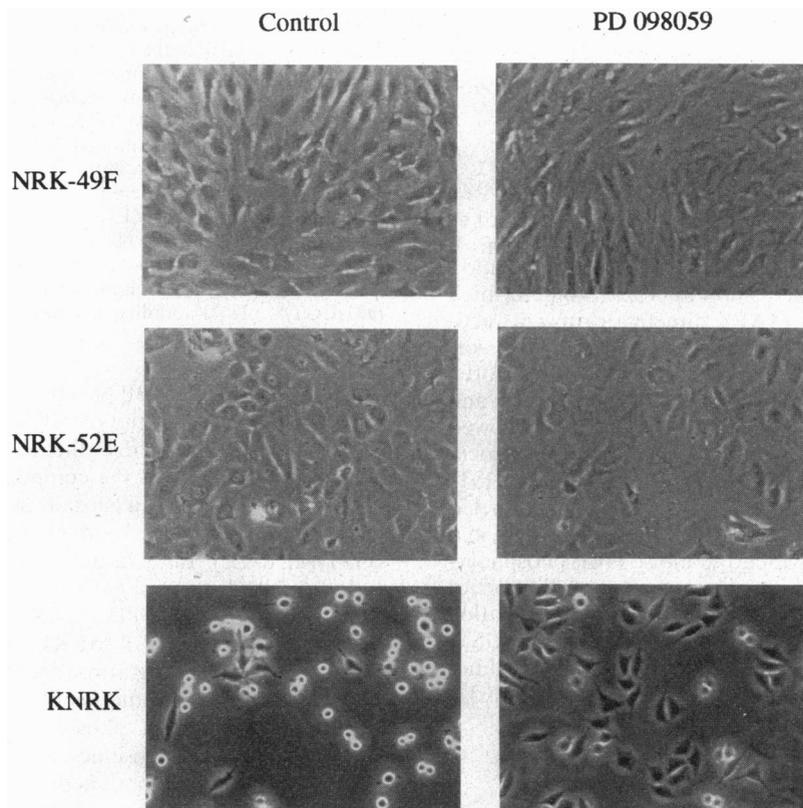


FIG. 4. Effect of PD 098059 on morphology of *ras*-transformed NRK rat kidney cells. KNRK, NRK-49F, and NRK-52E cells were seeded into multiwell plates and allowed to grow for 2 days in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. Then PD 098059 (50 μ M) or vehicle (dimethyl sulfoxide, 0.5% final concentration) was added and cells were photographed 48 hr later. Morphological changes of KNRK cells were apparent within 16 hr of treatment.

Table 1. PD 098059 inhibition of cell growth in monolayer culture or soft agar

Cell type	IC ₅₀ , μ M	
	Soft agar	Monolayer
K-Balb	1.5	60
KNRK	2.0	40
<i>v-raf-3Y1</i>	4.5	50
SRA/3Y1	60	>100
EGFR/3T3	>100	>100
K562	60	>100

K-Balb and KNRK cells are *ras*-transformed BALB 3T3 mouse or normal rat kidney cells, respectively; *v-raf-3Y1* cells are rat embryo fibroblasts transformed with *v-raf*; SRA/3Y1 are Rous sarcoma virus-transformed rat embryo fibroblasts; EGFR/3T3 cells are an NIH 3T3 line stably transfected with DNA encoding human EGF receptors; K562 cells are a human erythroleukemia line transformed with *bcr-abl*. Data are expressed as the concentration of PD 098059 required to achieve 50% inhibition (IC₅₀) of cell growth in monolayer culture or soft agar.

tyrosine phosphorylation and activation of MAPK when applied to intact 3T3 cells and suggest that in this experimental setting, PDGF-stimulated thymidine incorporation is under control of the MAPK cascade.

We have examined a number of other kinase activities, both *in vitro* and in cultured cells, without noting significant inhibitory activity by PD 098059. These include Raf kinase, cAMP-dependent kinase, protein kinase C, v-Src, epidermal growth factor (EGF) receptor kinase, insulin receptor kinase, PDGF receptor kinase, and phosphatidylinositol 3-kinase. Further, PD 098059 does not inhibit the MAPK homologues JNK and P38 (D. R. Alessi and P. Cohen, personal communication). Kinetic analysis has indicated that PD 098059 does not compete for ATP binding or MAPK binding to MEK and most likely inhibits through an allosteric mechanism (D.T.D. and A.R.S., unpublished observations). This unusual mode presumably accounts for its high degree of selectivity for MEK inhibition.

Transformation of cells with *ras* can result in chronic activation of the MAPK pathway, and PD 098059 reduced basal levels of MAPK activity in *ras*-transformed BALB 3T3 cells (K-Balb) and rat kidney (KNRK) cells (data not shown). Interestingly, K-Balb and KNRK cells exhibited greater sensitivity to inhibition of colony formation than to growth in monolayer culture when exposed to continuous levels of PD 098059 (Table 1). Cells transformed with the viral *raf* oncogene (*v-raf-3Y1*) exhibited a similar pattern, consistent with the known ability of Raf to phosphorylate and activate MEK. In contrast, cells transformed with Src (SRA/3Y1), human EGF receptor (EGFR/3T3), or Bcr-Abl (K562) were relatively insensitive to PD 098059. Further, growth-inhibitory effects of PD 098059 were rapidly reversed when the compound was removed from the culture medium, indicating that PD 098059 acts in a cytostatic, rather than cytotoxic, manner (data not shown). PD 098059 also caused a dramatic change in cellular morphology of KNRK cells, from rounded and loosely attached to flatter and more spread out (Fig. 4). Nontransformed NRK-49F and NRK-52E cells, derived from similar initial conditions as KNRK, normally assume a flatter, spread-out appearance, and morphology of these cells was not appreciably altered with PD

098059 treatment. The *ras*-transformed K-Balb cells also underwent morphological changes similar to those observed in KNRK cells (data not shown). These results, combined with effects on colony formation in soft agar, suggest that PD 098059 acts to reverse the transformed phenotype obtained upon *ras* overexpression in KNRK and K-Balb cells.

These data reveal PD 098059 to be a selective inhibitor of the MAPK-activating enzyme MEK and, consequently, of the MAPK cascade. We also show that this pathway is crucial for maintenance of the *ras*-transformed phenotype in KNRK and K-Balb cells. The ability of PD 098059 to inhibit MEK when added to intact cells allows it to directly test proposed roles of the MAPK cascade in a variety of biological settings.

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