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PD 098059 Is a Specific Inhibitor of the Activation of Mitogen-activated Protein Kinase Kinase *in Vitro* and *in Vivo**

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PD 098059 has been shown previously to inhibit the dephosphorylated form of mitogen-activated protein kinase kinase-1 (MAPKK1) and a mutant MAPKK1(S217E,S221E), which has low levels of constitutive activity (Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 7686–7689). Here we report that PD 098059 does not inhibit Raf-activated MAPKK1 but that it prevents the activation of MAPKK1 by Raf or MEK kinase *in vitro* at concentrations (IC_{50} = 2–7 μM) similar to those concentrations that inhibit dephosphorylated MAPKK1 or MAPKK1(S217E,S221E). PD 098059 inhibited the activation of MAPKK2 by Raf with a much higher IC_{50} value (50 μM) and did not inhibit the phosphorylation of other Raf or MEK kinase substrates, indicating that it exerts its effect by binding to the inactive form of MAPKK1. PD 098059 also acts as a specific inhibitor of the activation of MAPKK in Swiss 3T3 cells, suppressing by 80–90% its activation by a variety of agonists. The high degree of specificity of PD 098059 *in vitro* and *in vivo* is indicated by its failure to inhibit 18 protein Ser/Thr kinases (including two other MAPKK homologues) *in vitro* by its failure to inhibit the *in vivo* activation of MAPKK and MAP kinase homologues that participate in stress and interleukin-1-stimulated kinase cascades in KB and PC12 cells, and by lack of inhibition of the activation of p70 S6 kinase by insulin or epidermal growth factor in Swiss 3T3 cells. PD 098059 (50 μM) inhibited the activation of $p42^{MAPK}$ and isoforms of MAP kinase-activated protein kinase-1 in Swiss 3T3 cells, but the extent of inhibition depended on how potently c-Raf and MAPKK were activated by any particular agonist and demonstrated the enormous amplification potential of this kinase cascade. PD 098059 not only failed to inhibit the activation of Raf by platelet-derived growth factor, serum, insulin, and phorbol esters in Swiss 3T3 cells but actually enhanced Raf activity. The rate of activation of Raf by platelet-derived growth factor was increased 3-fold, and the subsequent inactivation that occurred after 10 min was prevented. These results indicate that the activation of Raf is suppressed and that its inactivation is accelerated by a downstream component(s) of the MAP kinase pathway.

Stimulation of cells with growth factors and cytokines, or exposure to cellular stresses, activates several signal transduction pathways that have specific physiological roles. These include at least three in which a mitogen-activated protein kinase (MAPK)[1] homologue is involved. In one pathway, cell stimulation leads to the sequential activation of $p21^{ras}$ and the protein kinases c-Raf, MAP kinase kinase-1 and -2 (MAPKK1, MAPKK2), and p42 and p44 MAP kinases ($p42^{MAPK}$, $p44^{MAPK}$). These MAPKs phosphorylate a variety of proteins *in vivo* including MAP kinase-activated protein (MAPKAP) kinases 1α and 1β (also known as Rsk-1 and Rsk-2 (1)). The sustained activation of $p42/p44^{MAPK}$ is not only required, but it is sufficient to induce the proliferation or differentiation of several cells (2).

In order to dissect MAPK pathways and to elucidate their physiological roles, one approach has been to generate dominant negative mutants and overexpress them in cells. For example, dominant negative forms of $p21^{ras}$, c-Raf, and MAPKK1 all inhibit the activation of $p42/p44^{MAPK}$ and the growth factor-induced proliferation or differentiation of several cells (3). However, although dominant-negative mutants are useful, the generation of cell lines that stably express them is time consuming, and their expression may lead to erroneous conclusions. For example, overexpression of an inactive form of MAPKK1 that can be phosphorylated by Raf may not only prevent the activation of endogenous wild-type MAPKK1, but also the activation of other cellular substrates of Raf that might lie in distinct signaling pathways. The need to express a dominant negative mutant for many hours may also result in unwanted secondary effects. Similarly, the use of dominant negative mutants of Raf may affect Ras-dependent processes that are independent of Raf.

An alternative strategy is to identify small cell-permeant molecules that are specific inhibitors of particular protein kinases. An advantage of this approach is that the effects of these inhibitors can be investigated in any cell *in vivo*. Moreover, these inhibitors may have therapeutic potential as anti-cancer, or anti-inflammatory agents, or as immunosuppressants. Several such inhibitors have recently been described, including an inhibitor of the epidermal growth factor (EGF) receptor tyrosine kinase (4), which may be useful for treating human tumors that overexpress this receptor, and a specific inhibitor of the MAP kinase homologue termed reactivating kinase (RK) or p38 (5). The latter inhibitor prevents the synthesis of interleukin-1

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MEK, MAP kinase/ERK kinase; MAPKAP, MAP kinase-activated protein; EGF, epidermal growth factor; RK, reactivating kinase; IL, interleukin; NGF, nerve growth factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; GST, glutathione *S*-transferase; PDGF, platelet-derived growth factor; PP2A, protein phosphatase 2A; cdk, cyclin-dependent protein kinase; JNK, c-Jun kinase.

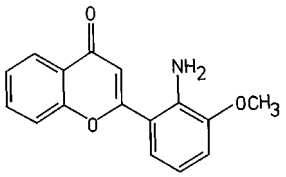

FIG. 1. **Structure of PD 098059.**

(IL-1) and tumor necrosis factor in monocytes induced by bacterial endotoxins and also blocks several actions of these cytokines on other cells (6). For these reasons, it may be efficaceous in the treatment of inflammatory diseases, such as rheumatoid arthritis. Another compound, rapamycin, prevents the IL-2-induced activation of p70 S6 kinase and the proliferation of T-cells (7, 8) and may therefore be useful as an immunosuppressant.

One of our laboratories recently described the first synthetic inhibitor of the MAP kinase pathway. This substance, PD 098059 (Fig. 1) was identified as a noncompetitive inhibitor of MAP kinase kinase (MAPKK) by screening a compound library using a MAP kinase cascade assay comprising unphosphorylated MAPKK1 (which possesses low basal activity) and unphosphorylated MAP kinase and monitoring phosphorylation of myelin basic protein. PD 098059 was subsequently demonstrated to inhibit the constitutively active mutant (MAPKK1-(S217E,S221E)) in which the serine residues phosphorylated by c-Raf had been mutated to glutamic acid (9). PD 098059 inhibited the activation of MAP kinase by growth factors *in vivo* and, consistent with a key role for this pathway in the proliferation of some cells and the differentiation of others, it reversed the transformed phenotype induced by Ras overexpression in KRNK and K-balb cells (9) or the nerve growth factor (NGF)-induced differentiation of PC12 cells (10). We now report that although PD 098059 inhibits MAPKK1(S217E, S221E), it surprisingly has no effect on MAPKK1 or MAPKK2 that have been activated by Raf. This remarkable finding has led us to demonstrate that PD 098059 blocks the activation of MAPKK1 by Raf or MEK kinase *in vitro* and that it acts *in vivo* as a highly specific inhibitor of the activation of MAPKK.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture reagents, EGF, and platelet-derived growth factor-BB (PDGF), rapamycin, and myelin basic protein were obtained from Life Technologies, Inc.; insulin was from Novo-Nordisk (Bagsvaerd, Denmark); 12-O-tetradecanoylphorbol 13-acetate (TPA) and human IL-1α were from Boehringer Mannheim; sodium arsenite and partially hydrolyzed and dephosphorylated casein were from Sigma; Protein G-Sepharose was from Pharmacia Biotech Inc. Polyclonal c-Raf antibody was raised in sheep against the C-terminal peptide CTLTTSPRLPVF and affinity-purified on peptide-AffiGel 15. $p42^{MAPK}$ antiserum (number 122) raised in rabbits against the C-terminal peptide EETARFQPGYRS was provided by Dr C. J. Marshall (Institute for Cancer Research, London). Affinity-purified polyclonal MAPKAP kinase-1α antibody raised in rabbits against the peptide QLVKGAMMATYSALNSSKPTPQLKPIESSILAQRRVRKLPSTTL was purchased from UBI (Lake Placid, NY), and affinity-purified polyclonal MAPKAP kinase-1β and p70 S6 kinase antibodies were raised in sheep against the peptides RNQSPVLEPVGRSTLAQRRGIKK and AGVFDILDQPEDAGSEDEL, respectively. c-Raf was coexpressed in Sf9 cells with oncogenic *ras* and the protein tyrosine kinase *lck* (11) and immunoprecipitated from the lysates as described (12) except that Protein A-Sepharose was replaced by Protein G-Sepharose. $p42^{MAPK}$ (13), MAPKK1 (11), and the catalytic domain of MEK kinase corresponding to residues 366–675 (14) were expressed in *Escherichia coli* as glutathione *S*-transferase fusion proteins and purified by affinity chromatography on glutathione-Sepharose (11). The MEK kinase construct was provided by Dr A. Ashworth (Institute for Cancer Research, London). MAPKK2 expressed in insect cells and partially purified was a gift from Drs K. Zeller and T. Sturgill (University of Virginia). The inactive and active forms of the MAPKK homologue, termed MKK4 (15), were partially purified by Mono S chromatography of extracts from unstimulated or osmotically shocked PC12 cells, respectively. The active form of RK kinase, the major MAPKK homologue that activates RK in KB cells and might be similar to MKK3 (15), was partially purified by Mono S chromatography of extracts from IL-1-stimulated KB cells. The catalytic subunit of protein phosphatase 2A (PP2A) from bovine heart was provided by Dr. R. MacKintosh (University of Dundee).

Protein Kinase Assays—c-Raf and MEK kinase were measured by their ability to activate MAPKK1 (or MAPKK2) in a 30-min coupled assay containing MAPKK1 (or MAPKK2) and its substrate p42 MAP kinase. One unit of c-Raf or MEK kinase activity was that amount which increased the activity of $p42^{MAPK}$ by 1 unit/min. MAPKK was assayed directly in the cell lysate by the activation of bacterially expressed $p42^{MAPK}$ (16). One unit of MAPKK was that amount which increased the activity of $p42^{MAPK}$ by 1 unit/min. The assays of c-Raf and MAPKK are quantitative and extremely sensitive and are detailed elsewhere (16).

$p42^{MAPK}$ was assayed by its ability to phosphorylate myelin basic protein (16) and MAPKAP kinase 1 α/β by the phosphorylation of a peptide related to the C terminus of ribosomal protein S6 [Gly-245,Gly-246]S6-(218–249) (1) (17). One unit of $p42^{MAPK}$ or MAPKAP kinase-1α/β was that amount which catalyzed the phosphorylation of 1 nmol of substrate peptide in 1 min. Protein kinase activities in immunoprecipitates were measured by adding the other assay components to the tubes containing the immunoprecipitated enzyme.

RK kinase from KB cells and MKK4 from PC12 cells were assayed by the activation of RK (18). RK itself (18), MAPKAP kinase-2 (18), p70 S6 kinase (19), protein kinase A (20), protein kinase Cα (21), 5′-AMP-activated protein kinase (22), cyclin A cyclin-dependent protein kinase-2 (23), phosphorylase kinase (24), glycogen synthase kinase-3 α/β (17), and myosin light chain kinase (25) were assayed as described previously. c-Jun kinase (JNK) from IL-1-stimulated KB cells was assayed using a GST-c-Jun-(1–191) fusion protein (26), a gift from Dr. R. Treisman (Imperial Cancer Research Fund, London).

Assay of c-Raf and MEK Kinase by the Phosphorylation of MAPKK and Other Proteins—c-Raf was assayed by the activation of MAPKK (16) except for the following modifications. The reaction (20 μl) contained. Buffer B (1.0 ml of 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.03% (w/v) Brij 35, 0.1% (v/v) 2-mercaptoethanol). MAPKK (1.4 μg), c-Raf immunoprecipitate (0.75 units of activity coupled to 5 μl of packed Protein G-Sepharose) or MEK kinase (0.75 units), 10 mM magnesium acetate, 0.1 mM [γ-^{32}P]ATP (~1 cpm/fmol). The assays were initiated with MgATP after incubating the other components for 5 min at 30 °C. After 30 min, the samples were denatured in SDS and electrophoresed on a 10% polyacrylamide gel, and the radioactivity in each band was quantitated using a PhosphorImager. The phosphorylation of myelin basic protein (0.2 mg/ml) and casein (0.5 mg/ml) was carried out in an identical manner, except that the concentration of c-Raf was increased 100-fold.

Cell Culture and Stimulation—Mouse Swiss 3T3 fibroblasts (27), human KB fibroblasts (5), and PC12 cells (18) were cultured to confluence. After stimulation with growth factors, IL-1, or arsenite for the times indicated, each 10-cm dish of cells was lysed in 0.4 ml of ice-cold Buffer A (20 mM Tris acetate, pH 7.5, 0.1% (by mass) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.1% (by volume) 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μg/ml leupeptin, 10 μg/ml aprotinin), and the lysates were frozen immediately in liquid nitrogen and stored at −80 °C until use. Protein concentrations were determined by the Bradford method (28).

Use of PD 098059—PD 098059 was dissolved in dimethyl sulfoxide (Me_2SO) to give a concentration of 50 mM, stored in aliquots at −80 °C, and diluted in aqueous buffers to <100 μM immediately prior to use. For studies *in vitro*, PD 098059 or the equivalent amount of Me_2SO carried over with the drug was incubated with protein kinases for 10 min before initiating the assays with MgATP. For studies *in vivo*, PD 098059 was added to the culture medium to give a final concentration of 50 μM, and 90 min later the cells were stimulated with growth factors (although similar results were obtained if the preincubation was only 30 min or as long as 16 h). Control incubations contained Me_2SO instead of PD 098059. The maximum concentration of Me_2SO in any experiment was 0.1% (v/v), which did not affect the activity or activation of any protein kinase examined.

Immunoprecipitation of Protein Kinases—Cell lysates were thawed and centrifuged for 2 min at 14,000 × *g*, and the supernatants were incubated for 90 min at 4 °C on a shaking platform with 5 μl of protein G-Sepharose conjugated to the appropriate antibody. The suspensions were centrifuged for 1 min at 14,000 × *g*, the supernatant was dis-

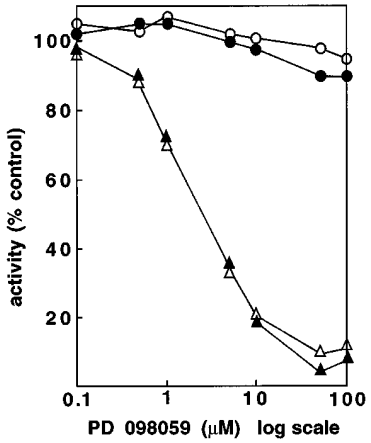

FIG. 2. **Effect of PD 098059 on the activity of different forms of MAPKK1.** MAPKK activity was assayed after preincubation for 10 min with the indicated concentrations of PD 098059, and the results are presented relative to control incubations in which the inhibitor was omitted. The concentrations of MAPKK1 in the assays were as follows: dephospho-MAPKK1, 0.3 μM; MAPKK1(S217E,S217E), 10 nM; Raf-activated MAPKK1, 0.2 nM. *Closed circles*, MAPKK assayed in lysates from Swiss 3T3 cells stimulated for 2 min with EGF; *open circles*, MAPKK1 maximally phosphorylated at Ser-217 and Ser-221 by c-Raf; *closed triangles*, dephosphorylated MAPKK1; *open triangles*, MAPKK1(S217E,S221E). MAPKK1 maximally phosphorylated at Ser-217 and Ser-221 has a 7000-fold higher activity than the dephosphorylated enzyme and 180-fold higher activity than MAPKK1(S217E,S221E). Each MAPKK preparation was diluted to give similar MAPKK activity in the assay in the absence of PD 098059. Similar results were obtained in three experiments.

carded, and the immunoprecipitates were washed twice with 1.0 ml of Buffer A containing 0.5 M NaCl and twice with Buffer B prior to assay.

c-Raf—Swiss 3T3 lysate (40–400 μg of protein) was immunoprecipitated using 1 μg of c-Raf antibody.

p42^{MAPK}—Swiss 3T3 lysates (10 μg of protein) were immunoprecipitated using 1 μl of p42^{MAPK} antiserum.

MAPKAP Kinase-1 α and β—The MAPKAP kinase-1α antibody does not immunoprecipitate MAPKAP kinase-1β, but the MAPKAP kinase-1β antibody immunoprecipitates both MAPKAP kinase-1β and MAPKAP kinase-1α. In order to assay each isoform of MAPKAP kinase-1, Swiss 3T3 cell lysate (20 μg of protein) was incubated for 90 min at 4 °C on a shaking platform with 5 μl of Protein G-Sepharose conjugated to 1 μg of MAPKAP-kinase 1α antibody. The suspension was centrifuged for 1 min at 14,000 × *g*, and the supernatant was added to 5 μl of Protein G-Sepharose conjugated to 2 μg of MAPKAP kinase-1β antibody. Under these conditions >90% of the MAPKAP kinase-1α and MAPKAP kinase-1β was immunoprecipitated sequentially from the lysates.

p70 ^{S6K}—This enzyme was immunoprecipitated from Swiss 3T3 lysates (60 μg of protein) using 2.5 μg of p70^{S6K} antibody.

RESULTS

Effect of PD 098059 on the Activity and Activation of MAP Kinase Kinase in Vitro—The dephosphorylated form of MAPKK1 has extremely low activity, which is increased 7000-fold after maximal phosphorylation with c-Raf. The activation of MAPKK1 results from the phosphorylation of Ser-217 and Ser-221, and mutating both residues to glutamate generates an enzyme (MAPKK(S217E,S221E)) that is 40-fold more active than dephosphorylated MAPKK but 180-fold less active than the phosphorylated enzyme (11).

PD 098059 was identified during a screen to identify inhibitors of dephospho-MAPKK and was subsequently found to inhibit MAPKK(S217E,S221E). PD 098059 inhibited both of these enzymes with an IC_{50} of 2 μM. Inhibition was essentially complete at 50 μM (Ref. 9; see Fig. 2) and unaffected by prior incubation of bacterially expressed dephospho-MAPKK1 with PP2A under conditions that completely inactivated the phosphorylated form of MAPKK1 (data not shown). However, to our surprise, PD 098059 did not inhibit MAPKK1 that had been

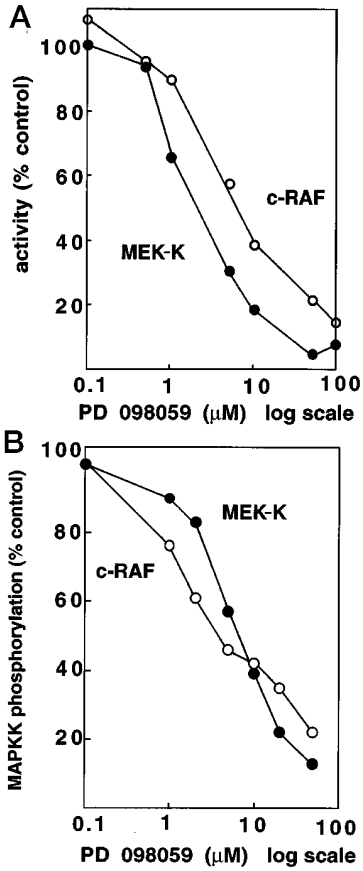

FIG. 3. **Effect of PD 098059 on the activation and phosphorylation of MAPKK1 by c-Raf and MEK kinase *in vitro*.** *A*, activation by c-Raf and MEK kinase. c-Raf and MEK kinase were diluted to achieve an equivalent reactivation of MAPKK1 in the absence of any inhibitor (100%). The MAPKK1 concentration in the assays was 0.3 μM. After a 10-min preincubation with the indicated concentration of PD 098059, c-Raf (*open circles*) and MEK kinase (*closed circles*) were assayed by the activation of MAPKK1. Similar results were obtained in three experiments. *B*, same as *A*, except that c-Raf or MEK kinase were assayed by the phosphorylation of MAPKK1 (see "Experimental Procedures"). Similar results were obtained in two experiments.

phosphorylated by c-Raf *in vitro* or MAPKK activity in cell lysates prepared from EGF-stimulated Swiss 3T3 cells (Fig. 2).

Since the phosphorylated form of MAPKK1 has a far higher activity than either MAPKK1(S217E,S221E) or dephospho-MAPKK1, these results suggested that PD 098059 might interact specifically with the inactive conformation of MAPKK and prompted us to investigate its effect on the activation of MAPKK1 *in vitro*. PD 098059 inhibited both the activation (Fig. 3*A*) and phosphorylation (Fig. 3*B*) of MAPKK1 *in vitro* by either c-Raf or MEK kinase with IC_{50} values of 4 ± 2 μM, similar to the IC_{50} for inhibition of dephospho-MAPKK1 or MAPKK1(S217E,S221E) (Fig. 2). Identical results were obtained using c-Raf expressed in insect Sf9 cells (Fig. 3) or immunoprecipitated from EGF-stimulated Swiss 3T3 cells (not shown). The activation of MAPKK2 by c-Raf was also inhibited by PD 098059 but over 10 times less potently (IC_{50} = 50 μM) than MAPKK1. Like Raf-activated MAPKK1, Raf-activated MAPKK2 was not inhibited by 50 μM PD 098059 (see Table II).

In contrast to the inhibition of MAPKK1 phosphorylation by c-Raf and MEK kinase, PD 098059 did not inhibit the (weak) phosphorylation of myelin basic protein and casein (29) by c-Raf, the autophosphorylation of c-Raf, or the activation of MKK4 by MEK kinase (data not shown).

PD 098059 Inhibits the Activation of MAPKK in Swiss 3T3 Cells—In Swiss 3T3 cells, many agonists activate the pathway

TABLE I

Effect of PD 098059 on stimulation of c-Raf, MAPKK, p42 MAPK, MAPKAP-K1α, and MAPKAP-K1β in Swiss 3T3 cells by different agonists

Cells were incubated for 90 min in the presence or absence of 50 μM PD 098059 and then stimulated for 2 min (c-Raf and MAPKK), 5 min (p42 MAPK), or 10 min (MAPKAP-K1) with 100 ng/ml EGF, 50 ng/ml PDGF, 0.4 μg/ml TPA, 10% serum, or 100 ng/ml insulin. The cells were lysed and assayed in triplicate for c-Raf, MAPKK, p42 MAPK, and MAPKAP-K1α/β activity, in the absence of PD 098059. The percentage conversion of MAPKK, p42 MAPK, and MAPKAP-K1α/β to the fully activated state was determined by dividing the kinase activity measured in the cell lysate by that obtained after incubating the lysates with either 1000 units/ml c-Raf (to maximally activate MAPKK), 1000 units/ml MAPKK (to maximally activate p42 MAPK), or 500 units/ml MAPK (to maximally activate MAPKAP-K1α/β). After full activation, the specific activities in the lysates were as follows: MAPKK, 35 ± 2.8 units/mg; p42 MAPK, 0.46 ± 0.05 units/mg; MAPKAP-K1α, 0.23 ± 0.03 units/mg; MAPKAP-K1β, 1.08 ± 0.09 units/mg. Similar results were obtained in three separate experiments. The standard errors presented are better than ±10%.

Stimulus	PD098059	c-Raf	Conversion to the fully activated state			
			MAPKK	p42 MAPK	MAPKAP-K1α	MAPKAP-K1β
		milliunits/mg	%	%	%	%
Control	−	1.5	<0.03	2.2	8.6	8.4
Control	+	3.0	<0.03	1.1	4.3	3.7
EGF	−	105	30	89	90	67
EGF	+	109	4.0	59	81	60
PDGF	−	6.0	8.0	48	86	47
PDGF	+	22	0.8	13	47	27
TPA	−	7.0	8.9	69	103	61
TPA	+	20	0.8	21	77	43
Serum	−	11	7.4	65	64	28
Serum	+	26	0.9	13	43	21
Insulin	−	2.5	0.3	13	34	23
Insulin	+	3.5	0.06	1.1	8.6	7..4

leading to the activation of p42/p44^{MAPK} and MAPKAP kinase-1α/β, but the extent of activation differs markedly from agonist to agonist. EGF is a more potent activator of c-Raf, MAPKK, and p42/p44^{MAPK} than PDGF, TPA, or serum, while insulin is the weakest activator of these enzymes (Table I). Incubation of Swiss 3T3 cells with PD 098059 (50 μM) suppressed by 80–90% the activation of MAPKK induced by each agonist, but the activation of c-Raf was enhanced 2–3-fold (Table I).

The effect of 50 μM PD 098059 on activation of the downstream targets of MAPKK, namely p42^{MAPK} and MAPKAP kinases 1α/β varied from agonist to agonist (Table I). PD 098059 (50 μM) prevented the activation of p42^{MAPK} and MAPKAP kinase-1α/β by insulin (the weakest activator of MAPKK) almost completely, but with PDGF, TPA, and serum (which are stronger activators of MAPKK) the activation of p42^{MAPK} and MAPKAP kinases-1α/β was only inhibited by 70−80% and 26–52%, respectively. After stimulation for 5 min with EGF (the strongest activator of MAPKK) the activation of p42^{MAPK} and MAPKAP kinase-1α/β was only inhibited by 33 and 13%, respectively (Table I). However, as the concentration of EGF was reduced, the extent of inhibition of p42^{MAPK} activation by 50 μM PD 098059 increased progressively. After stimulation for 10 min with 100 ng/ml EGF, the inhibition of p42^{MAPK} activation was only 8%, but at 0.1 ng/ml and 0.01 ng/ml EGF it was 89 and 100%, respectively (Fig. 4). PD 098059 cannot be used at higher concentrations because of its low solubility in aqueous solution. These results are considered further under "Discussion."

PD 098059 Does Not Inhibit Other Protein Kinases in Vitro or in Vivo—PD 098059 did not inhibit 18 protein Ser/Thr kinases *in vitro* (Table II), including Raf-activated MAPKK1 or MAPKK2 as well as two other MAPKK homologues, MKK4 and RK kinase (see "Experimental Procedures"). PD 098059 did not inhibit the *in vivo* activation of MKK4 by osmotic shock in PC12 cells (data not shown), the activation of RK kinase by arsenite in KB cells (Fig. 5*B*), the activation of the MAP kinase homologues JNK (Fig. 5*A*) and RK (Fig. 5*B*), or the activation of MAPKAP kinase-2 in interleukin-1-stimulated or chemically stressed KB cells (Fig. 5*B*). PD 098059 also failed to inhibit the activation of p70 S6 kinase in EGF- or insulin-stimulated Swiss 3T3 cells (Fig. 5*C*).

Activation of c-Raf by PD 098059—The finding that PD 098059 enhanced both the basal activity of c-Raf and its activation by growth factors in Swiss 3T3 cells (Table I) prompted us to study this effect in greater detail. As shown in Fig. 6, PD 098059 caused a severalfold enhancement in the rate of activation of c-Raf by PDGF and largely prevented the precipitate drop in c-Raf activity that occurred after 10 min in the continued presence of PDGF. However, PD 098059 only increased slightly the maximal level of c-Raf activation attained after 10 min.

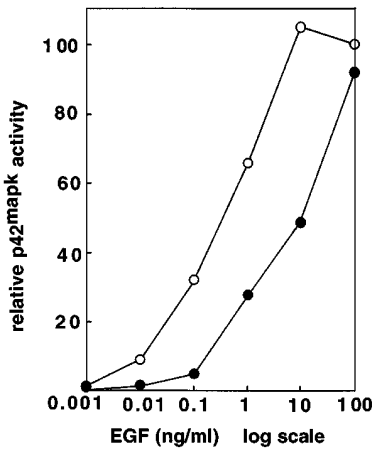

FIG. 4. **Effect of PD 098059 on the activation of p42^{MAPK} in Swiss 3T3 cells at different concentrations of EGF**. Cells were incubated for 90 min in the absence (*open circles*) or presence (*closed circles*) of 50 μM PD 098059 and stimulated for 10 min at the indicated concentrations of EGF. The cells were lysed, and p42^{MAPK} was immunoprecipitated and assayed as described under "Experimental Procedures." The p42^{MAPK} activity is plotted relative to the specific activity of p42^{MAPK} in the lysate after stimulation with 100 ng/ml EGF in the absence of PD 098059, which was 0.45 ± 0.02 units/mg.

DISCUSSION

Although PD 098059 inhibits forms of MAPKK1 with a low level of activity (dephospho-MAPKK1 and MAPKK1(S217E,S221E)), we demonstrate in this paper that it does not inhibit the phosphorylated forms of MAPKK1 but instead prevents the activation and phosphorylation of MAPKK1 *in vitro* (Fig. 3) and *in vivo* (Table I). These findings, coupled with the failure to inhibit the phosphorylation of other substrates of c-Raf and MEK kinase, and lack of competition with ATP (9), indicate that PD 098059 does not bind to the

TABLE II
Effect of PD 098059 on the activities of purified protein kinases in vitro

The protein kinases were incubated for 10 min at 30 °C in the presence or absence of 50 μM PD 098059 and then assayed as described under "Experimental Procedures." Activities are given ± S.E. for four separate determinations relative to control incubations in which the inhibitor was omitted. Myosin light chain kinase from avian gizzard and protein kinase Cα were provided by Dr. M. Ikebe (Case Western Reserve University) and Dr. P. Parker (Imperial Cancer Research Fund, London). Other protein kinases were from mammalian sources, and partially purified or homogeneous preparations were provided by other members of the MRC Protein Phosphorylation Unit and Department of Biochemistry at Dundee.

Protein kinase	Activity
	% control
MAP kinase kinase-1[a]	102 ± 1
MAP kinase kinase-2[a]	98 ± 3
p42 MAP kinase	101 ± 1
MAPKAP kinase-1	98 ± 1
RK kinase	88 ± 9
MKK4	87 ± 5
JNK	99 ± 5
RK	97 ± 6
MAPKAP kinase-2	109 ± 7
Protein kinase A	94 ± 3
Protein kinase Cα	100 ± 1
AMP-activated protein kinase	97 ± 3
Cyclin A/cdk2	95 ± 1
Phosphorylase kinase	99 ± 0
Glycogen synthase kinase-3α	87 ± 1
Glycogen synthase kinase-3β	93 ± 1
p70 S6 kinase	94 ± 2
Myosin light chain kinase	88 ± 2

[a] The MAPKK-1 and MAPKK-2 was activated by c-Raf *in vitro*.

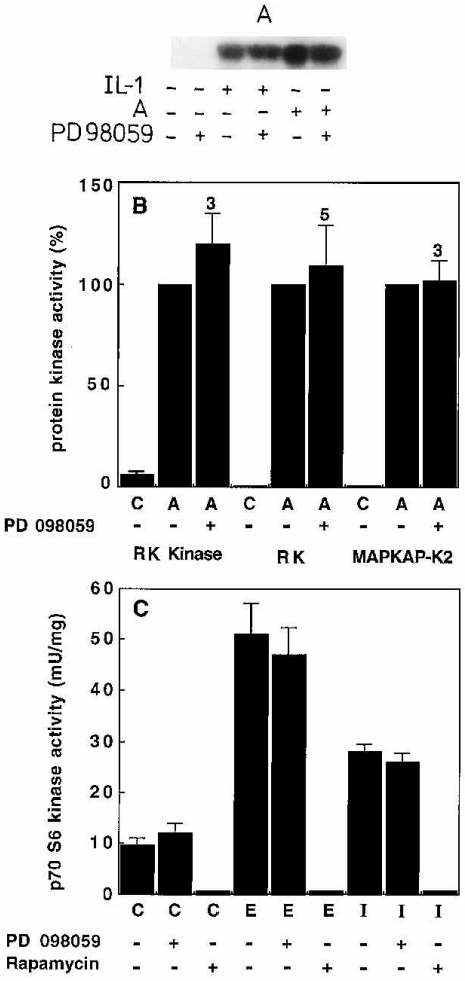

FIG. 5. **PD 098059 has no effect on the activation of JNK, RK kinase, RK, MAPKAP kinase-2, or p70^{S6K} *in vivo*.** *Panel A*, KB cells were incubated for 90 min in the absence (−) or presence (+) of 50 μM PD 098059 and then stimulated for 15 min with 20 ng/ml IL-1 or 0.5 mM sodium arsenite (*A*). The cells were lysed and assayed for JNK activity using GST-Jun-(1–191) as substrate. After phosphorylation, reactions were denatured in SDS, electrophoresed on a 10% polyacrylamide gel, and autoradiographed. Similar results were obtained in two experiments. *Panel B*, KB cells were stimulated in the absence (*C*) or presence (*A*) of arsenite and in the absence (−) and presence (+) of PD 098059 as in *panel A*. The cell lysates were chromatographed on Mono Q and assayed for RK activity or chromatographed on Mono S and assayed for RK kinase and MAPKAP kinase-2. The results are given ± S.E. for the number of experiments shown, as a percentage of the arsenite-stimulated activity in the absence of PD 098059. *Panel C*, Swiss 3T3 cells were incubated in the absence or presence of 50 μM PD 098059 as in *panel A* and then for 5 min in the presence (+) or absence (−) of 100 nM rapamycin prior to stimulation for 15 min with 100 ng/ml EGF (*E*), 100 ng/ml, insulin (*I*), or buffer (*C*, control). The cells were lysed and assayed for p70^{S6K} activity after immunoprecipitation. The results are given ± S.E. for three experiments.

active site of MAPKK1 but instead interacts at another site, thereby blocking access to activating enzymes. It will be interesting to find out whether PD 098059 binds to the activation loop of MAPKK1 in the vicinity of the phosphorylation sites, and our results raise the possibility that PD 098059 may mimic or displace an endogenous allosteric effector of these enzymes. Our observations explain the high degree of specificity of PD 098059, which only inhibits the activation of MAPKK2 weakly and does not affect the activities of 18 protein Ser/Thr kinases (Table II), four protein Tyr kinases, and phosphatidylinositol 3-kinase (9) that have so far been tested. Moreover PD 098059 does not prevent the *in vivo* activation of Raf (Table I, Fig. 6) or the activation of other MAPKK or MAPK homologues (Fig. 5, *A* and *B*), which lie in stress- and cytokine-activated signaling pathways, or the insulin-induced or EGF-induced activation of p70 S6 kinase (Fig. 5*C*).

Our results also demonstrate that inhibitors can be identified that differentiate between the active and inactive conformations of protein kinases. These observations could be of general significance and suggest that when screening for inhibitors of protein kinases, weakly active dephosphoenzymes or mutant enzymes should always be examined in parallel with the fully active phosphoenzyme. Indeed, inhibitors that interact with dephosphoenzymes and prevent their activation by upstream kinases may frequently turn out to be more specific inhibitors than compounds that block catalytic activity *per se*.

Although PD 098059 suppressed the activation of MAPKK in Swiss 3T3 cells by 80–90%, its effect on the activation of p42^{MAPK} and MAPKAP kinase 1α/β *in vivo* depended on the strength of activation of c-Raf and MAPKK by any agonist. PD 098059 (50 μM) prevented the activation of p42^{MAPK} and MAPKAP kinase-1α/β by insulin (Table I) or by low levels (0.01–0.1 ng/ml) of EGF (Fig. 4) almost completely, which were the weakest activators of these enzymes. PD 098059 (50 μM) partially suppressed the activation of p42^{MAPK} and MAPKAP kinase-1α/β by PDGF, TPA, or serum but had little effect on the activation of these enzymes at high concentrations of EGF (100 ng/ml), the most potent activator of c-Raf and MAPKK (Table I). Although PD 098059 (50 μM) suppressed by 85–90% the activation of MAPKK at high EGF, the activity remaining was still 50% of that observed after stimulation with PDGF, TPA, or serum and much higher than that observed after stimulation with insulin. This explains why the activation of p42^{MAPK} and MAPKAP kinase-1α/β at high EGF is hardly affected by 50 μM PD 098059 (Table I).

PD 098059 (50 μM) does not inhibit the *in vivo* activation of MAPK and MAPKAP kinase-1 completely when cells are stimulated with high concentrations of agonists that are potent

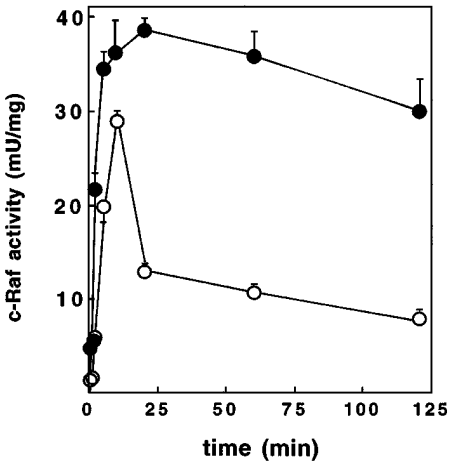

FIG. 6. **Effect of PD 098059 on the activation and inactivation of c-Raf by PDGF in Swiss 3T3 cells**. Cells were incubated for 90 min in the presence (*closed circles*) or absence (*open circles*) of PD 098059 and then stimulated for the times indicated with 50 ng/ml PDGF. The cells were lysed and assayed in triplicate for c-Raf activity after immunoprecipitating the enzyme from the lysates. The results are given ± S.E. for three experiments.

activators of MAPKK, and its low solubility in aqueous solution precludes its use at higher concentrations. An effect of PD 098059 on some biological processes may therefore only be revealed at low agonist concentrations or by using cell lines with low numbers of growth factor receptors. For example, we have noticed that PD 098059 inhibits the NGF-induced differentiation of some PC12 cells (10) but not others,[2] and this may reflect the different numbers of NGF receptors (and hence the strength of activation of MAPKK by NGF) in these cell lines. Indeed, in the PC12 cell line where PD 098059 failed to inhibit differentiation, the activation of MAPK by NGF was only suppressed by 50% after 15 min, despite an 80% inhibition of MAPKK by PD 098059 after 5 min.[2] It has been established that the sustained activation of MAPK is not only required, but is sufficient to induce the differentiation of PC12 cells (2, 3).

The results presented in Table I emphasize how little activation of MAPKK is needed to produce significant activation of $p42^{MAPK}$ and especially of MAPKAP kinase $1\alpha/\beta$. For example, either insulin in the absence of PD 098059, or PDGF, TPA, and serum in the presence of PD 098059, caused only a 0.3–0.9% conversion of MAPKK to the activated form, yet this was sufficient to cause 13–21% conversion of $p42^{MAPK}$ to the activated form and 34–77% conversion of MAPKAP kinase 1α to the activated form. PD 098059 would appear to be particularly useful for studying the role of the MAP kinase pathway in the biological actions of insulin, since it essentially abolished the activation of MAPK and MAPKAP kinase-1 by insulin in Swiss 3T3 (Table I) and L6 cells (30).

An unexpected observation was that PD 098059 enhanced the basal activity of c-Raf and its activation by growth factors in Swiss 3T3 (Table I) and L6 cells (data not shown) and prevented the inactivation of c-Raf in Swiss 3T3 cells that occurred after stimulation with PDGF for 10 min (Fig. 6). This result suggests that the rate of activation of c-Raf is suppressed and that its rate of inactivation is enhanced by a component of the kinase cascade downstream of Raf. We have also observed that PD 098059 blocks the hyperphosphorylation of c-Raf induced by PDGF in Swiss 3T3 cells and by IGF-1 in L6 cells (data not shown), suggesting that a kinase downstream of c-Raf may be responsible for hyperphosphorylation. These results are also consistent with the increasing evidence that growth factor-induced hyperphosphorylation of c-Raf does not correlate with activation (31, 32). Furthermore, Ueki *et al.* (33) reported that the overexpression of MAPK in Chinese hamster ovary cells attenuated the activation of c-Raf and enhanced its hyperphosphorylation by insulin. Although the hyperphosphorylation of c-Raf might contribute to its inactivation, other explanations are possible. For example, activation of MAPK by a variety of growth factors causes hyperphosphorylation of the GTP/GDP exchange factor (Sos), which catalyzes the activation of Ras (34, 35). Moreover, hyperphosphorylation of Sos in stimulated cells results in its dissociation from GRB2 and hence to the inactivation of Ras (34, 35). Treatment of L6 cells with PD 098059 inhibits the hyperphosphorylation of Sos following insulin stimulation (30). Therefore, PD 098059 by inhibiting the activation of MAPK and hence the hyperphosphorylation of Sos in growth factor-stimulated cells may prevent the dissociation of Sos from GRB2 and thus block the inactivation of Ras that would result in a sustained activation of c-Raf (Fig. 6).

[2] S. Traverse and P. Cohen, unpublished observations.

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