Identification of a Novel Inhibitor of Mitogen-activated Protein Kinase Kinase*

(Received for publication, January 23, 1998, and in revised form, April 16, 1998)

Margaret F. Favata‡, Kurumi Y. Horiuchi§, Elizabeth J. Manos‡, Andrea J. Daulerio‡,
Deborah A. Stradley‡, Wendi S. Feeser‡, Drew E. Van Dyk‡, William J. Pitts‡,
Richard A. Earl‡, Frank Hobbs‡, Robert A. Copeland‡, Ronald L. Magolda‡,
Peggy A. Scherle‡, and James M. Trzaskos‡

From the ‡Inflammatory Diseases Research, *Chemical Enzymology and §Chemical and Physical Sciences, The DuPont
Merck Research Laboratories, Wilmington, Delaware 19880-0400

The compound U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) was identified as an inhibitor of AP-1 transactivation in a cell-based reporter assay. U0126 was also shown to inhibit endogenous promoters containing AP-1 response elements but did not affect genes lacking an AP-1 response element in their promoters. These effects of U0126 result from direct inhibition of the mitogen-activated protein kinase kinase family members, MEK-1 and MEK-2. Inhibition is selective for MEK-1 and -2, as U0126 shows little, if any, effect on the kinase activities of protein kinase C, Abl, Raf, MEKK, ERK, JNK, MKK-3, MKK-4/SEK, MKK-6, Cdk2, or Cdk4. Comparative kinetic analysis of U0126 and the MEK inhibitor PD98059 (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) demonstrates that U0126 and PD98059 are noncompetitive inhibitors with respect to both MEK substrates, ATP and ERK. We further demonstrate that the two compounds bind to ηN3-S218E/S222D MEK in a mutually exclusive fashion, suggesting that they may share a common or overlapping binding site(s). Quantitative evaluation of the steady state kinetics of MEK inhibition by these compounds reveals that U0126 has approximately 100-fold higher affinity for ηN3-S218E/S222D MEK than does PD98059.

We further tested the effects of these compounds on the activity of wild type MEK isolated after activation from stimulated cells. Surprisingly, we observe a significant diminution in affinity of both compounds for wild type MEK as compared with the ηN3-S218E/S222D mutant enzyme. These results suggest that the affinity of both compounds is mediated by subtle conformational differences between the two activated MEK forms. The MEK affinity of U0126, its selectivity for MEK over other kinases, and its cellular efficacy suggest that this compound will serve as a powerful tool for in vitro and cellular investigations of mitogen-activated protein kinase-mediated signal transduction.

Glucocorticoid hormones have been shown to act by binding to intracellular glucocorticoid receptors (GR). The glucocorticoid-GR complex once formed migrates to the nucleus and interacts with specific target sequences known as glucocorticoid response elements (GREs) in gene promoters resulting in enhanced transcriptional activity. This feature of steroid hormone/receptor interaction is shared with a family of other hormones and effector molecules and their receptors which characterizes the steroid/thyroid receptor superfamily of ligand-activated transcription factors (6–8).

Although steroids have diverse effects on metabolism, their clinical utility is derived from their potent anti-inflammatory and immune modulatory properties that result from inhibition of cytokine, adhesion molecule, and metalloproteinase gene expression (9–11). The ability of steroid hormones to suppress transcription of key inflammatory and immune response genes is mediated through a mechanism distinct from GRE binding, however. Antagonism of the transcription factors AP-1 and NF-κB, which are important regulators of immune response genes, has been demonstrated in numerous laboratories and is proposed to be the primary mechanism for the anti-inflammatory and immune suppressive effects of steroids (11). This functional antagonism or transrepression of transcription factors by the GR is due to a direct protein/protein interaction between the activated GR and the components of the AP-1 and NF-κB complexes. These findings have led to the notion that the anti-inflammatory and immune modulatory properties of steroids might be due solely to protein/protein interactions between the GR and various transcription factors, whereas the side effects of steroid hormones may be mediated through classical GR/GRE interactions (12). Indeed, if this were the case, we envisioned that an agent that could selectively activate the GR to provide for functional antagonism of AP-1 and NF-κB without affecting GRE activation would be an ideal anti-inflammatory agent. Therefore, we set out to identify agents that could discriminate these GR activities using reporter gene assays in transfected cells.

The abbreviations used are: GR, glucocorticoid receptors; GRE, glucocorticoid response element(s); MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAP kinase kinase; MEKK, MEK kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; DMEM, Dulbecco’s modified Eagle’s medium; PMA, phorbol 12-myristate 13-acetate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DTT, dithiothreitol; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; TRE, TPA response element; GST, glutathione S-transferase; FCS, fetal calf serum; TPA, 12-O-tetradecanoylphorbol-13-acetate; PCR, polymerase chain reaction; PHA, phytohemagglutinin; IL, interleukin; ATF, activating transcription factor; BSA, bovine serum albumin, CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Inflammatory Diseases Research, The DuPont Merck Research Laboratories, P. O. Box 80400, Wilmington, DE 19880-0400. Tel.: 302-695-7110; Fax: 302-695-9401; E-mail: James.M.Trzaskos@dupontmerck.com.
Herein, we report the results of these studies. Although we did not identify an agent that functionally antagonizes AP-1 gene transcription without affecting GRE transactivation through GR interaction, we did identify an agent that inhibited AP-1 independent of the GR. U0126 was identified as a cellular AP-1 antagonist. Mechanistically, U0126 did not prevent DNA binding by AP-1 rather it suppressed the up-regulation of c-Fos and c-Jun mRNA and protein levels in activated cells. Detailed investigation of the signaling cascades leading to c-Fos and c-Jun induction determined that U0126 was an inhibitor of the dual specificity kinase, MAP kinase (MEK). This inhibition appeared to be selective as U0126 did not affect the kinase activity of protein kinase C, Abl, Raf, MEKK, ERK, JNK, MKK3, MKK6, Cdk2, or Cdk4. Another compound, PD908059, has recently been reported to also function as a selective inhibitor of MEK activity in vitro and in cellular assays (13). In vitro steady state kinetic and equilibrium binding studies of both compounds with MEK reveal that their mode of inhibition is noncompetitive with respect to both substrates and that these two inhibitors share a common or overlapping binding site(s). Thus, both compounds are selective MAP kinase signaling cascade inhibitors and represent starting points for the identification and optimization of potent and pharmacologically useful MEK inhibitors.

EXPERIMENTAL PROCEDURES

**Methods**

**AP-1 Assay—**COS-7 cells were cultured at 5% CO2 at 37 °C in DMEM:F12 medium (Life Technologies, Inc.) plus 10% fetal bovine serum (HyClone Labs, Inc.), 0.1 mM minimum Eagle’s medium non-essential amino acids (Life Technologies, Inc.), 1 mM minimum Eagle’s medium sodium pyruvate solution (Life Technologies, Inc.), and t-glutamine 500 mg/liter. The cells were trypsinized and washed twice with Dulbecco’s phosphate-buffered saline without CaCl2 and MgCl2 and resuspended in Opti-MEM 1 medium (Life Technologies, Inc.). COS-7 cells (8 x 10^6 cells) were transiently transfected by the electroporation method (150 V, 500 microfarads, Bio-Rad electroporator) with either the AP-1 response element (2x TRE-luciferase) or the glucocorticoid response element (Pmam-neo-luciferase) plus human glucocorticoid receptor (GR) ligand (dexamethasone) to activate the TRE-luciferase reporter. The effect of U0126 on a promoter containing multiple inducible elements was examined in transient transfection assays using a human promotor-luciferase reporter construct, AP-1 luc. AP-1 luc was constructed by subcloning a 1.2-kilobase pair fragment (~1245 to +36) of the human AP-1 promoter 5’-untranslated region that contains both cAMP response elements and AP-1 response elements (TRE) (14) into the NheI and BglII sites of pGEM-3Z (Promega, Madison, WI) and kindly provided by Dr. Gwenn Wise (Du Pont Merck). COS-7 cells were transiently cotransfected with 20 µg of AP-1 luciferase and 2 µg of human GR as described above. The transfected cells were plated in 96-well plates and incubated for 24 h at 5% CO2, 37 °C before being treated with 10 ng/ml PMA or 1 mM N-2’-O- dibutyryl adenosine 3’5’ cyclic monophosphate (BudC-3’5’). After an additional 24-h incubation, the luciferase activity was measured as described above.

**Immunoprecipitations—**Jurkat cells were grown in RPMI 1640 medium (Life Technologies, Inc.) plus 10% fetal bovine serum (HyClone Labs, Inc.). Cells (1 x 10^7) were stimulated with 50 ng/ml PMA and 2 µg/ml PHA (phytohemagglutinin, Murex Biotechnology, San Diego, CA) at 37 °C. Drug-treated cells received U0126 at a final concentration of 10 µM in 0.1% Me2SO immediately prior to stimulation. Control cells received an equal amount of Me2SO. Cells were centrifuged at 1000 rpm, washed once with cold phosphate-buffered saline (PBS), resuspended in 1 ml of ice-cold RIPA buffer (1x PBS, 1% (w/v) Nonidet P-40, 0.25% (w/v) SDS, 0.1% (w/v) AP-2, 0.5% (v/v) phenyl-methylsulfonyl fluoride (PMSF)), and disrupted through a 21-gauge needle. Cellular debris was pelleted at 14,000 x g, and supernatant was mixed with 1 µg of the appropriate antibody (c-Raf-1(1c-12), MEK-1(1c-18), ERK-2(c-14)) from Santa Cruz Biotechnology, Inc. on a rocking platform at 4 °C. After 1 h, 40 µl of a protein-A agaroose slurry was added, and tubes were rocked for another 2 h. Agarose beads were pelleted by centrifugation at 1500 rpm for 5 min, washed 3 times with RIPA buffer, and then once with 20 µM Hapes, pH 7.0, buffer.

**Immune Complex Kinase Assays—**Immunoprecipitates were resuspended in 25 µl of kinase assay buffer (20 mM Hapes, pH 7.0, 5 mM 2-mercaptoethanol, 10 mM MgCl2, 0.1 mg/ml bovine serum albumin), containing 1 µg of His-MEK-1 (Santa Cruz Biotechnology), 5 µg of glyceraldehyde-3-phosphate dehydrogenase (GAPD) (Sigma, St. Louis, MO), or 3 µg of myelin basic protein (Upstate Biotechnology) for Raf, MEK, and ERK kinase assays, respectively. Kinase reactions were initiated by the addition of 10 µM ATP plus 10 µCi of [γ-32P]ATP (NEN Life Science Products) and incubated at 25 °C for 30 min. Reactions were terminated by the addition of Laemmli SDS sample buffer, boiled for 5 min, electrophoresed on a 10% Tris glycine gel (Novex), dried, and analyzed using a Molecular Dynamics PhosphorImager.

**Western Blot—**COS-7 cells were seeded in 60-mm dishes at 30,000 cells/cm2 in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) plus 10% fetal bovine serum (HyClone Labs, Inc.). Cells were pretreated with compound in MeSO (1% final concentration) for 15 min and then stimulated with 10 ng/ml PMA for 15 additional min. Cells were washed once with cold PBS, lysed with cold lysis buffer (10 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 50 µg/ml aprotinin, and 50 µg/ml leupeptin), scraped from the plate, and centrifuged. Supernatants were assayed for protein using Bio-Rad DC protein assay kit. Protein samples (20 µg) were analyzed by SDS-PAGE using the cold gel electrophoresis method on 10% Tris-glycine gel. Protein was electrotransferred to polyvinylidene difluoride membrane and probed with a polyclonal phosphospecific antibody against ERK (New England Biolabs). Detection of bands was carried out according to the manufacturer’s protocol and analyzed on Molecular Dynamics Personal Densitometer.

For the experiments to determine the effects of U0126 on Fos and Jun protein expression, 3T3-type fibroblasts were plated in 100-mm dishes in DMEM, 10% FCS, and when they reached confluency were serum-starved for 72 h in DMEM, 0.5% FCS. The cultures were then stimulated with 50 ng/ml TPA, 10% FCS in the presence or absence of compound for various lengths of time. Compounds were added to give a final concentration of 0.1% MeSO. At the end of the incubation period, nuclear extracts were prepared as follows. The cells were trypsinized, washed twice in cold PBS, and resuspended in 100 µl of Buffer 1 (10 mM Hapes, pH 7.9, 60 mM KC1, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT, and 1 mM PMSF). After 5 min on ice, the samples were centrifuged at 4,000 rpm for 5 min, and the pellet was resuspended in 100 µl of Buffer 2 (10 mM Hapes, pH 7.9, 60 mM KC1, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF). The samples were centrifuged at 4,000 rpm for 5 min, and the pellet was resuspended in 100 µl of Buffer 3 (250 mM Tris, pH 7.8, 50 mM KC1, 1 mM DTT, 1 mM PMSF). After three freeze/thaw cycles, the samples were centrifuged at 9,000 rpm for 15 min, and the supernatant was saved as the nuclear extract. Ten µg of this extract was analyzed by Western blot as described above using antibodies specific for c-Jun, c-Fos, and SP-1 (Santa Cruz Biotechnology).

**RPS5—Luciferase Assay—**The effect of U0126 on a promoter containing multiple inducible elements was examined in transient transfection assays using a human promotor-luciferase reporter construct, RPS5-luc. RPS5-luc was constructed by subcloning a 1.2-kilobase pair fragment (~1245 to +36) of the human RPS5 promoter 5’-untranslated region that contains both cAMP response elements and AP-1 response elements (TRE) (14) into the NheI and BglII sites of pGL2-basic (Promega, Madison, WI) and kindly provided by Dr. Gwen Wise (Du Pont Merck). COS-7 cells were transiently cotransfected with 20 µg of RPS5-luciferase and 2 µg of human GR as described above. The transfected cells were plated in 96-well plates and incubated for 24 h at 5% CO2, 37 °C before being treated with 10 ng/ml PMA or 1 mM N-2’-O-dibutyryl adenosine 3’5’ cyclic monophosphate (BudC-3’5’). After an additional 24-h incubation, the luciferase activity was measured as described above.
Identification of a Novel MEK Inhibitor

Equilibrium Binding Measurements—To determine whether U0126 and PD98059 could displace one another from MEK, we performed displacement experiments with [3H]-labeled U0126 by equilibrium dialysis (18). Dialysis was performed with a 10-kDa cut-off Slide-A-Lyzer dialysis cassettes (Pierce). The cassette contained 0.5 ml of a 1 μM [3H]-U0126 solution in 200 mM glycine-0.1 M NaCl, pH 7.3. Displacement experiments were then performed as described above. The dialysis buffer was 0.1 M NaCl and 0.1 mg/ml dialysis casette (see above). Mixtures were dialyzed at room temperature for 4 h against 100 ml of 0.2 μM [3H]U0126 and varying concentrations of PD98059, in the same buffer system. A dialysis time of 4 h was chosen because experiments in which [3H]U0126 was dialyzed against buffer only established that this amount of time was sufficient to reach equilibrium. At the end of dialysis the amount of [3H]U0126 inside and outside the dialysis cassette was quantified by scintillation counting. Control experiments were performed in the same fashion with BSA, but not MEK, present in the dialysis cassette. Only low levels of compound binding to BSA were detected, and the MEK data were corrected for this nonspecific effect.

Northern Analysis—An immortalized 3T3-type cell line derived from mouse embryonic fibroblasts was used for these studies (17). The cells were plated at 100,000 cells/cm² in DMEM, 5% FCS, and when they reached confluency were starved for 48 h in DMEM, 0.5% FCS. The cultures were then stimulated with 100 ng/ml TPA, 10% FCS in the presence or absence of compound for 8 h. Compounds were added to give a final concentration of 0.1% MeSO. Total RNA was isolated using RNA-Zol® (Tel-Test Inc., Friendswood, TX), and Northern blots were performed as described (17) using 10 μg of RNA. The blots were hybridized with a digoxigenin-labeled cDNA probe (5′-ATF,CGATCTCGAGTCAAAGAGGGATATAAATCTAG-3′) representing the murine jun promoter. Single-stranded oligonucleotides were 5′-end-labeled with γ-[32P]ATP using T4 kinase, purified over a Chromaspin-10 column (CLONTECH) to remove unincorporated γ-[32P]ATP, and annealed. For each reaction, 1 × 10⁶ cpm (approximately 5 nCi) of radiolabeled oligonucleotide was incubated with 15 ng BSA in a final volume of 25 μl of 1× binding buffer for 25 min at 16°C. The binding buffer contained 12 μM Hepes, pH 7.9, 20 mM KCl, 1 mM MgCl₂, 0.5 mM DI TT, 0.5 mM PMSF, 12% glycerol, and 50 nM poly(dI-dC). Compounds were added to a final concentration of 10 μM. Blots were hybridized with a mixture of radiolabeled oligonucleotide in a 0.4% final 35S-90% phosgenated BSA solution. All reactions were analyzed by polyacrylamide gel electrophoresis using 5% gels in 0.5× TBE buffer.

Assay for Tyrosine Aminotransferase—FUS BDS. I rat hepatoma cells (20) kindly provided by Gary Firestone (University of California, Berkeley) were seeded at 50,000 cells/cm² in 96-well microtiter plates and incubated overnight at 5% CO₂, 37°C in Ham's F12:DME nutrient mix, 10% charcoal-dextran-treated FCS (HyClone Laboratories), 10 μM Hepes, 1 mM sodium pyruvate, 1x nonessential amino acids, and 50 μg/ml gentamycin (Life Technologies, Inc.). After 24 h, compounds were added in MeSO-media and incubated for an additional 24 h. The media were aspirated from the wells, and the cells were washed with PBS (without calcium and magnesium) before lysis with 25 μl/well 20 mM CHAPS in 0.1 M KHPO₄ for 15 min. The cell lysates were centrifuged at 3,000 rpm for 15 min. Tyrosine aminotransferase activity was performed on the cell lysates as described (21) with the following modifications to accommodate analysis in a 96-well plate format. Lysates (15 μl) were transferred to a 96-well plate to which 220 μl/well tyrosine aminotransferase reagent was added (150 mM KPO₄, 5 mg/ml bovine serum albumin, 1.2 mM pyridoxal-5′-phosphate, 1 mM 0.2 μM KPO₄, 0.1 μM KCl, 0.1 μM MgCl₂, 0.1 μM MnCl₂, 0.1 μM CaCl₂) added to all wells except blanks and gently mixed. The plates were covered with foil and incubated at room temperature for 5 h. NaOH (10 N) was added to all wells (15 μl/well) except blanks and mixed thoroughly. α-Ketoglutarate (0.5 mM in 0.2 mM KPO₄, pH 7.3) was added to the blank wells (8.0 μl/well) and mixed. The tyrosine aminotransferase activity was measured at 340 nm using a Molecular Devices UVmax kinetic plate reader.
Identification of a Novel MEK Inhibitor

Materials

Synthesis of U0126 (1,4-Diamino-2,3-dicyano-1,4-bis-(2-aminophenylthio)butadiene, U0125, and U0124—U0124, U0125, and U0126 (22) were prepared by the addition of a thiol to tetracyanoethane as shown in Scheme 1. Tetracyanoethane (23) (6.5 g, 50 mmol) was dissolved in 20 ml of reagent grade acetone. In a separate flask ortho-aminobenzene-thiol (25 g, 20 mmol) was dissolved in 50 ml of degassed 10% sodium hydroxide. The tetracyanoethane solution was added in a single portion to the sodium hydroxide solution, and the reaction mixture was vigorously stirred for 2 h, during which time an oil separated. The reaction mixture was allowed to stand for 1 h, and the solid which formed was filtered. The crude product was triturated with ethanol (2 × 50 ml) and recrystallized twice from ethanol (300 ml) and dried under vacuum, m.p. 163–165 °C. 1H NMR (CD3OD) d 7.48, d, J = 7 Hz, 2H; 7.35, t, J = 7 Hz, 2H; 6.94, d, J = 7 Hz, 2H, 6.70, t, J = 7 Hz, 2H; 3.70, q, J = 7 Hz, 2H (EtOH), 1.28, t, J = 7 Hz, 2H (EtOH). The proton NMRs for each compound were indicative of a symmetric structure. The configuration of the butadiene in U0126 was confirmed by single crystal x-ray analysis (see Scheme 1).

All other materials were sourced as indicated above or were of the best grade commercially available.

RESULTS

Identification of a Novel Inhibitor of AP-1-driven Gene Activation—We screened a total of 40,000 compounds for their ability to functionally antagonize AP-1-driven gene activation without activating a GRE-driven gene. Our efforts identified U0126 whose structure is shown in Fig. 1. U0126 suppressed AP-1-mediated gene activation in transient transfection assays (Fig. 2) as described under “Methods” with an IC50 = 0.96 ± 0.16 μM (n = 12). In these same assays, U0126 was without effect on a constitutively expressed cotransfected gene, RSV-βGal, or constitutively expressed luciferase driven off the cdc2 kinase promoter (data not shown). A further test of U0126 specificity was performed in a number of other cell types using either transfected or endogenous promoters. The results are summarized in Table I. U0126 had no effect on cAMP-dependent response element-driven luciferase expression from the RP5′ promoter and was also without effect on the GRE-driven tyrosine aminotransferase gene. In contrast, U0126 did inhibit TPA-dependent TRE-driven luciferase expression in the RP5′ reporter construct along with MMP-1, a gene known to be highly dependent upon AP-1 for its inducible activity by multiple stimuli (24).

U0126 Inhibits c-Fos and c-Jun Induction in TPA-stimulated Cells—To determine the mechanism of U0126 inhibition of AP-1-mediated gene activation, we initially studied the ability of U0126 to inhibit directly AP-1 DNA binding. U0126 did not inhibit binding of an AP-1 peptide (18) to a TRE-containing oligonucleotide probe by electrophoretic mobility shift analysis (data not shown). Based upon these findings, our next series of studies focused on the ability of U0126 to affect components that make up the AP-1 transcription factor complex. In fibroblasts treated with TPA/serum, U0126 suppressed the up-regulation of c-Fos and c-Jun proteins by 50–80%, as detected by Western analysis (Fig. 3A). This effect was mirrored in Northern blot analysis which showed that U0126 blocked c-Fos and c-Jun mRNA up-regulation (Fig. 3B). Similar effects were observed in Jurkat cells stimulated with PHA/PMA (data not shown). In contrast, dexamethasone and U0124, an inactive U0126 analog, had no effect on c-Fos and c-Jun protein or mRNA levels. Induction of the Fos family member, FosB, and the zinc finger-containing transcription factor, Egr-1, was also inhibited by U0126 (data not shown). Treatment with 10 μM U0126 did not affect the protein levels of the constitutively expressed transcription factors SP-1 (Fig. 3A) or JunD and Fra-1 (data not shown). These studies suggested that the decreased AP-1 activity observed in cells treated with U0126 resulted from a decrease in Fos and Jun protein levels.

Ras Signaling and the MAP Kinase Cascade Provide the Target for U0126 Action—The ability to inhibit TPA up-regulation of c-Fos and c-Jun protein suggested that U0126 blocked signaling events generated at the cell surface leading to AP-1 induction. One signaling pathway known to be involved in...
TPA-mediated AP-1 up-regulation is the Ras pathway. Activation of the Ras pathway by TPA in fibroblasts or PHA/PMA in Jurkat cells was also consistent with our data on U0126 blockade of AP-1 up-regulation in these two cell types.

To address the effects of U0126 on members of the Ras signaling cascade, immune complex kinase assays were performed on cell lysates from Jurkat cells treated with PMA/PHA as described under “Methods.” The results of these studies showed that ERK activity was decreased in cells treated with U0126, whereas Raf and MEK activities were unaffected by the compound (data not shown). The effect was reversible as pretreatment of cells with compound followed by washout prior to activation showed no effect on ERK activity (data not shown). These results suggested that U0126 was either directly affecting ERK catalytic activity or was affecting the ability of MEK to activate ERK as part of the MAP kinase signaling cascade. Therefore, we evaluated the ability of U0126 to inhibit each member of the MAP kinase cascade directly in vitro kinase assays using enzymes isolated by immunoprecipitation from PHA/PMA-stimulated Jurkat cells.

As seen in Fig. 1, U0126 did not inhibit ERK or Raf kinase activity when added directly to kinase assays in vitro but did inhibit MEK enzymic activity in a concentration-dependent manner. These data demonstrate that U0126 is an inhibitor of MEK. Similar MEK inhibition was observed with U0125 although the potency was approximately 10-fold less than U0126. U0124 did not inhibit MEK at concentrations up to 100 μM. As expected, U0126 was able to inhibit MEK-dependent intracellular ERK phosphorylation in a concentration-dependent man-

**TABLE I**

| Activity                  | Cell type | Stimulus     | U0126 (IC₅₀) |
|---------------------------|-----------|--------------|--------------|
| **Reporter assays**       |           |              |              |
| AP-1-luciferase           | COS-7     | PMA          | 1.0 μM       |
| RSV-βgal                  | COS-7     | Inactive     |              |
| cdk-luciferase            | COS-7     | Inactive     |              |
| RPS-β-luciferase          | COS-7     | PMA          | 58% at 10 μM |
| **Endogenous genes**      |           |              |              |
| c-AMP                     | Hepatoma  | Inactive     |              |
| TAT protein               | T cell    | PMA/PHA      | 1.4 μM       |
| MMP mRNA                  | Fibroblast| TPA          | 2.0 μM       |
| c-Jun                     |           |              |              |
| c-Fos                     |           |              |              |
| SP1                       |           |              |              |

*Expressed as percent inhibition at 10 μM. Assays were performed as described under “Experimental Procedures.”

**Fig. 4.** U0126 inhibits MEK but not Raf or ERK in in vitro kinase assays. Raf (A), ERK (B), and MEK (C) were immunoprecipitated from PMA/PHA-treated Jurkat cells that had not received any drug treatment. U0126 at the indicated concentrations was then added directly to the immune complex kinase assay. The phosphatase PP2A (2A) (Upstate Biotechnology) was used as a positive control and was added at 1 μg/reaction. Gel quantitation by PhosphorImager analysis is shown in the histogram.
Identification of a Novel MEK Inhibitor

For both wild type and ΔN3-S218E/S222D MEK it was apparent that U0126 represents a much more potent inhibitor compared with PD098059. The IC₅₀ exhibited by U0126 for the ΔN3-S218E/S222D MEK (70 nM) was of similar magnitude to the total enzyme concentration used in the assay (10 nM). For this reason we were concerned that the inhibitor was acting as a tight binding inhibitor and, as such, would not be amenable to classical Michaelis-Menten kinetic analysis (25). To investigate this further we varied the enzyme concentration from 2 to 100 nM and measured the apparent IC₅₀ of U0126. As expected, the IC₅₀ increased with increasing enzyme concentration. In decreasing the enzyme concentration from 10 to 2 nM MEK, however, we did not see a significant drop in the measured IC₅₀ value; below 2 nM MEK the signal was too weak to measure reaction velocities. Tight binding inhibition is usually associated with a slow onset of inhibition and/or a slow dissociation of the enzyme-inhibitor complex (25). We therefore looked for a time dependence of inhibition with U0126 in two separate experiments. In the first experiment we initiated the reaction with enzyme, rather than with ATP, and looked for curvature in the reaction progress curves, which would indicate a time-dependent onset of inhibition. In the second experiment we premixed the enzyme and U0126 at high concentrations and, after varying incubation times, initiated the reaction by dilution into a reaction solution containing both substrates. In this second experiment, a slow off-rate for the enzyme-inhibitor complex would be manifested in a lag phase or upwardly curvature of progress curve (25). For both sets of experiments we observed linear progress curves, indicating a lack of any time dependence for U0126 inhibition. From these results we concluded that, despite the low value for its IC₅₀, U0126 did not

**TABLE II**

| Protein kinase                  | IC₅₀ (µM) |
|--------------------------------|----------|
| MEK1                           | 0.072 ± 0.02 (n = 6) |
| MEK2                           | 0.058 ± 0.02 (n = 2) |
| ERK1/2 (p44/p42 MAP kinase)     | >50      |
| MKK3/p38 MAP kinase*            | 38 ± 9 (n = 5)  |
| MKK4                           | >100     |
| MKK6/p38 MAP kinase*            | 20 ± 7 (n = 3)  |
| Protein kinase C               | >10      |
| Abl                            | >100     |
| cdk2                          | >10      |
| cdk4                          | >10      |

*Inhibition measured in a coupled kinase assay as described under "Methods."
respectively, on the steady state kinetics of concentration of enzyme used in the assays. This inhibitor (Table III) have, however, been corrected for the detailed in Ref. 25.

The convergent nature of these nested lines clearly rules out inhibition with respect to both substrates of the MEK reaction. In all cases the convergence point is clearly distant from the origin. This pattern is consistent with noncompetitive or mixed inhibition with respect to either substrate. In all forms of lines that converge on or near the x-axis, to the left of the origin. This pattern is consistent with noncompetitive or mixed inhibition with respect to both substrates of the MEK reaction. The convergent nature of these nested lines clearly rules out uncompetitive inhibition with respect to either substrate. In all cases the convergence point is clearly distant from the x-axis (i.e. 1/[S] = 0), thus ruling out a competitive mode of inhibition with respect to substrate.

Mixed or noncompetitive inhibition is best described by the following velocity Equation 2 (25).

\[ v = \frac{V_{\text{app}}^\text{max} [S]}{K_{\text{app}}^\text{max} + [S]} \]

where \( V_{\text{app}}^\text{max} \) and \( K_{\text{app}}^\text{max} \) are the apparent values of \( V_{\text{max}} \) and \( K_m \) at each individual inhibitor concentration. The double-reciprocal plots shown in Figs. 7 and 8 all display the same pattern, a nest of lines that converge on or near the x-axis, to the left of the origin. This pattern is consistent with noncompetitive or mixed inhibition with respect to both substrates of the MEK reaction. The convergent nature of these nested lines clearly rules out uncompetitive inhibition with respect to either substrate. In all cases the convergence point is clearly distant from the y-axis (i.e. 1/[S] = 0), thus ruling out a competitive mode of inhibition with respect to substrate.

Mixed or noncompetitive inhibition is best described by the following velocity Equation 2 (25).

\[ v = \frac{V_{\text{max}}[S]}{K_{\text{m}} + [S] \left( 1 + \frac{[I]}{K_I} \right)} \]

where \( K_I \) represents the dissociation constant of the inhibitor from the free enzyme (MEK) and \( \alpha K_I \) represents the dissociation constant of the inhibitor from the enzyme-substrate binary complex (MEK-ERK or MEK-ATP). For each of the data sets illustrated in Figs. 7 and 8, we have globally fit the entire data set to Equation 2, using the software package Enzyme Kinetics (Ronald Viola, Akron, OH). The results of these fits are presented in Table III. For each inhibitor, the data presented in Table III indicate only minor differences in affinity of the inhibitor for the free enzyme and the enzyme-substrate binary complexes. Thus, U0126 displays identical affinity for the free enzyme and the MEK-ATP complex and only about a 2-fold reduction in affinity for the MEK-ERK complex. In the case of PD098059, the inhibitor displays similar affinity for the two enzyme-substrate binary complexes and about a 3–5-fold decrease in affinity for the free enzyme.

Both U0126 and PD098059 bind to free MEK and to the MEK-ERK and MEK-ATP complexes with minimal perturbations of their affinities due to substrate binding. Hence, both inhibitors can reasonably be described as noncompetitive. For all forms of the enzyme, however, U0126 displays significantly higher affinity than PD098059; this affinity difference ranges from 44-fold for the MEK-ERK complex to 357-fold for the free enzyme.

| Compound | MEK | MEK-ATP | MEK-ERK |
|----------|-----|---------|---------|
| U0126    | 0.041 ± 0.020 | 0.048 ± 0.003 | 0.109 ± 0.033 |
| PD098059 | 14.713 ± 2.338 | 3.062 ± 0.083 | 4.761 ± 1.147 |

Table III: Inhibitor constants (\( K_I \) or \( \alpha K_I \)) for U0126 and PD098059 for various forms of \( \Delta N3-S218E-S222D \) MEK.

Equilibrium Binding Studies—The affinity differences notwithstanding, the similarity in inhibition modality for U0126 and PD098059 begs the question of whether these inhibitors share a common binding site on the enzyme. To address this issue we have had U0126 synthesized with tritium incorporation at the 5-position (see Fig. 1) and used this radiolabeled form of the inhibitor to perform equilibrium binding displacement studies. For these studies, the preformed MEK-ATP complex was used, based on the data in Table III, to provide
maximal affinity of both compounds for the enzyme. \(^{[3H]}U0126\) was incubated with the MEK-ATP complex, and the ability of varying concentrations of PD098059 to displace the radiolabeled inhibitor was determined by equilibrium dialysis. The results of a typical displacement curve are illustrated in Fig. 9. The data clearly indicate that PD098059 is capable of displacing U0126 from the enzyme in a concentration-dependent fashion. Hence, the two inhibitors bind to MEK in a mutually exclusive manner. Similar displacement of \(^{[3H]}U0126\) by PD098059 was observed using free MEK in place of the MEK-ATP complex, but in this case a slightly higher concentration of PD098059 was required to effect half-maximal displacement (data not shown).

DISCUSSION

The mitogen-activated protein kinase (MAPK) signaling pathways are involved in cellular events such as growth, differentiation, and stress responses (26–32). These pathways are linear kinase cascades in that MAP kinase kinase kinase phosphorylates and activates MAP kinase kinase (MAPKKK) which phosphorylates and activates MAP kinase. To date, seven MAPK kinase homologs (MEK1, MEK2, MKK3, MKK4/SEK, MEK5, MKK6, and MKK7) and four MAPK families (ERK1/2, JNK, p38, ERK5) have been identified. The MAPK kinase family members are unique in that they are dual specificity kinases, phosphorylating MAPKs on both threonine and tyrosine. Specifically, MEK1 and MEK2 share 80% amino acid sequence identity and appear to be functionally redundant in cells (33). The same is true for ERK1 and ERK2 (34). Activation of these pathways regulates the activity of a number of substrates through phosphorylation. These substrates include transcription factors such as p62TCF (Elk-1), c-Myc, ATF2 and the AP-1 components, c-Fos and c-Jun (35).

We identified U0126 as an inhibitor of the MAPK cascade leading to ERK1 and ERK2 activation. U0126 is a potent and specific inhibitor of the dual specificity kinases MEK1 and MEK2 both in \textit{in vitro} enzymic assays as well as intracellularly where U0126 blocked phosphorylation and activation of ERK. Blockade of ERK activation would prevent downstream phosphorylation of a number of factors including p62TCF (Elk-1) preventing induction of c-Fos and c-Jun, components of the AP-1 complex. Discovery of U0126 was based upon blockage of AP-1 activation which relied upon this activation pathway.

We have demonstrated that U0126 is a potent (\(K_i\), 41–109 nm), noncompetitive inhibitor of \(\Delta N3\)-S218E/S222D MEK-1. Steady state kinetic analysis indicates that PD098059 likewise is a noncompetitive inhibitor of this enzyme. The noncompetitive nature of U0126 and PD098059 inhibition of MEK may be a pharmacological advantage for compounds of this type. Although undefined at present, the binding site for these compounds on MEK is clearly distinct from those for the two substrate molecules, ATP and ERK. Hence, such compounds may not suffer large diminution in efficacy in cellular systems due to high concentrations of substrate (i.e. ATP) or the existence of preformed binary enzyme-substrate complexes (i.e. MEK-ERK or MEK-ATP). The results of equilibrium binding studies indicate that the two compounds bind to \(\Delta N3\)-S218E/S222D MEK-1 in a mutually exclusive fashion. The simplest interpretation of this result is that the two compounds share a common binding site on the enzyme. Although we consider it less likely, we cannot rule out the possibility that the two compounds bind to different sites on the protein that are somehow in anti-cooperative communication with one another. Attempts are underway presently to clarify this issue further.

For both U0126 and PD098059 we observed a significant (at least 7-fold) reduction in affinity for wild type-activated MEK-1 as compared with the \(\Delta N3\)-S218E/S222D MEK-1 mutant. In fact, the diminution in affinity is so great for PD098059 that we were not able to achieve more than ~25% inhibition of wild type activated MEK-1 at the highest concentration tested for this compound (100 \(\mu\)M). This result is consistent with previous data for PD098059 reported by Dudley et al. (13) and by Alessi et al. (36). In these papers the authors demonstrated that PD098059 was an effective inhibitor (\(IC_{50}\) ~10 \(\mu\)M) of the ERK-phosphorylating activity of both nonactivated wild type (i.e. basal activity) and of a partially activated mutant MEK-GST fusion construct. In contrast, however, these authors found that PD098059 was incapable of inhibiting wild type MEK-1 that had been activated through Raf-mediated phosphorylation in stimulated Swiss 3T3 cells. From these data the authors suggested that PD098059 binds only to the inactive (i.e. nonphosphorylated) form of MEK-1, binding being mediated by the conformational change that attends activation of the enzyme. Our present data lead to a slightly different interpretation. The mutant MEK-1 that we have used (\(\Delta N3\)-S218E/S222D) has been shown to be fully activated and to stimulate AP-1-regulated gene transcription when expressed in mammalian cells (37). Hence any conformational transition that attends activation of MEK-1 must clearly be mimicked in the \(\Delta N3\)-S218E/S222D mutant. Both PD098059 and U0126 display potent binding to this activated MEK-1 form. Yet neither compound appears to bind as well to wild type MEK-1 that has been activated through Raf-mediated phosphorylation. We conclude, therefore, that there must be subtle conformational differences between \(\Delta N3\)-S218E/S222D MEK-1 and S218/S222-phosphorylated MEK-1 that mediate binding of U0126 and PD098059 to the former, but not the latter, enzyme form, despite the fact that both forms display high ERK-phosphorylating and subsequent AP-1-activating ability. The potency of PD098059 as an inhibitor of cellular AP-1-regulated gene transcription cannot be accounted for by its inhibitory potency toward wild type-activated MEK-1 (see above). This discrepancy was rationalized by Alessi et al. (36) by demonstrating that PD098059 blocked Raf-mediated activation (i.e. phosphorylation) of wild type MEK in cells, by direct binding to nonactivated MEK. Thus, despite the ability of this compound to inhibit activated and partially activated mutant forms of MEK-1 \textit{in vitro}, these authors concluded that the cellular effects of PD098059 were mainly due to binding of this compound to nonactivated MEK, leading to inhibition of MEK activation by Raf. This mechanism clearly does not apply for U0126, since MEK phosphorylation in stimulated Jurkat cells is unaffected by U0126 at concentrations that completely block downstream ERK phosphorylation (Fig. 4). Thus, whereas U0126 and PD098059 appear to bind to \(\Delta N3\)-S218E/S222D MEK-1 in similar fashions, and in fact may bind to a common site on the
enzyme, there remain some questions about the details of their individual modes of action in a cellular context.

MEK inhibition has been shown to differentially affect signaling events through a variety of cell surface receptors. Growth factor-mediated proliferation and chemotactic responses are blocked by PD098059 (24, 38). In addition, mitogenic effects of insulin on DNA synthesis and p90Rsk activation are also inhibited by PD098059, whereas insulin-mediated PHAS-I activation is not inhibited (39, 40). Interestingly, PD098059 did not affect glucose uptake, glycogen synthase activation, or lipogenesis in insulin-treated cells (39). Similar differential effects of MEK inhibition on heterotrimeric G-protein-coupled seven transmembrane receptor (7TM) signaling have appeared. For example, PD098059 blocks angiotensin II-induced ERK phosphorylation and thymidine incorporation into DNA in aortic smooth muscle cells but has no effect on AII-induced proliferation of smooth muscle cells (41).

Also, PD098059 inhibits ERK phosphorylation in myocytes exposed to phenylephrine but fails to block atrial natriuretic factor expression (42). In neutrophils, ERK activation occurs in response to the agonists N-formyl peptide, IL-8, C5a, and leukotriene B4, which is blocked by PD098059 (43). In addition, PD098059 blocks neutrophil chemotaxis in response to all agents but does not alter superoxide anion production. Similarly, U0126 blocks ERK activation in N-formyl peptide- and leukotriene B4-stimulated neutrophils but does not impair NADPH-oxidase activity or bacterial cell killing. Analogous differential effects of PD098059 have recently been shown in T cells stimulated with anti-CD3 monoclonal antibody in conjunction with either PMA or anti-CD28 monoclonal antibody (44). The MEK inhibitor blocked IL-2, tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor, interferon-γ, and IL-6 production but enhanced production of IL-4, IL-5, and IL-13. These findings obviate the need to ensure that ERK activation is indeed coupled to the cellular event or response attributed to MAP kinase pathway activation.

Another intriguing differential effect observed with MEK inhibition involves thrombin-induced arachidonic acid release in endothelial cells and platelets. PD098059 inhibits ERK activation in both platelets and endothelial cells in response to thrombin treatment. In platelets, this effect does not alter arachidonic acid release nor affect aggregation (45). In contrast, PD098059 blocks prostacyclin I2 release in thrombin-stimulated endothelial cells while not affecting von Willebrand factor secretion (46). Thus, blockade of thrombin signaling events in different cell types leads to dramatically different results upon arachidonic acid metabolism. Our own data support these findings. U0126 is unable to block arachidonic acid release or thromboxane synthesis in thrombin-stimulated platelets, whereas U0126 is able to block arachidonic acid release along with prostaglandin and leukotriene synthesis in keratinocytes stimulated with a variety of agents. Thus, the putative effector target, cytosolic phospholipase A2, is insensitive to MEK inhibition in platelets while showing sensitivity and a blunting of response in other cell types. These effects presumably reflect cytosolic phospholipase A2 activation by non-ERK mechanisms in platelets, whereas ERK is the activating kinase in other cell types (47).

The proximal involvement of Ras in the activation of the ERK pathway suggests that MEK inhibition might show efficacy in cancers where oncopgenic Ras is a determinant in the cancer phenotype. Indeed, PD098059 (36) as well as U0126 are able to impede the growth of Ras-transformed cells in soft agar, even though these compounds show minimal effects on cell growth under normal culture conditions. PD098059 has also been shown to reduce urokinase secretion controlled by growth factors such as epidermal growth factor, transforming growth factor-α, and fibroblast growth factor in an autocrine fashion in the squamous cell carcinoma cell lines UM-SCC-1 and MDA-TV-138 (48). In vitro invasiveness of UM-SCC-1 cells through an extracellular matrix-coated porous filter was blocked by PD098059, although the cellular proliferation rate was not affected. These results suggest that control of the tumor invasive phenotype by MEK inhibition may be a possibility.

Functional antagonism of AP-1 activity without activation of GR-mediated gene activation was the original intent of our screening effort. The notion that we could find a compound to interact with the GR and preferentially inhibit AP-1 was based upon the finding that steroids suppress gene transcription through this interaction. Although we were not successful in finding an agent with the ability to separate GR gene activation from gene suppression, identification of such compounds using other members of the steroid-thyroid receptor superfamily have been reported. By using the retinoic acid receptor as the vehicle to functionally antagonize AP-1-mediated gene activation, a number of compounds have been described that perform this function without activating retinoic acid response element-driven genes (49). U0126 and MEK inhibitors in general seem to accomplish the same result, although in a mechanistically distinct fashion.

Acknowledgments—We thank Dr. Michael Karin (University of California, San Diego) for providing AP-1 and glucoconjugate receptor constructs and reporter genes used in these studies. We also thank Dr. Sarah Cox and Katie Burton (DuPont Merck Pharmaceutical Co.) for performing the Cdk2 and Cdk4 kinase assays and Gwendolyn Wise for providing the RP5-luciferase construct.

REFERENCES

1. Baxter, J. D. (1976) Pharmacol. Ther. 2, 655–659
2. Parrillo, J. E., and Faucci, A. S. (1979) Annu. Rev. Pharmacol. Toxicol. 19, 179–201
3. Ringold, G. M. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 529–566
4. Cato, A. C. B., Ponta, H., and Herrlich, P. (1992) Prog. Nucleic Acid Res. Mol. Biol. 43, 395–444
5. Behrens, T. W., and Goodwin, J. S. (1989) in Arthritis and Allied Conditions (McCarty, D. J., ed) pp. 604–612, Lea & Febiger, Philadelphia
6. Gehring, U. (1987) Biochem. Sci. 12, 399–402
7. Fuller, P. J. (1991) FASEB J. 5, 3982–3999
8. Magelgerd, D. J., Thammabch, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 385–389
9. Brattsand, R., and Lindem, M. (1996) Aliment. Pharmacol. Ther. 10, 81–90
10. Schroen, D. J., and Brinckerhoff, C. E. (1996) Gene Expr. 6, 197–207
11. Cato, A. C. B., and Wade, E. (1996) BioEssays 18, 371–378
12. Herrlich, P., and Ponta, H. (1994) Trends Endocrinol. Metab. 5, 341–346
13. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7682–7689
14. Burt, D. W., Nakamura, N., Kelley, P., and Dzau, V. J. (1989) J. Biol. Chem. 264, 7357–7362
15. Carlson, B. A., Dubay, M. M., Sausville, E. A., Brizuela, L., and Worland, P. J. (1996) Cancer Res. 56, 2973–2978
16. Bell, J. E., and Bell, E. T. (1988) Proteins and Enzymes, pp. 378–399, Prentice-Hall, Englewood Cliffs, NJ
17. Hu, E., Mueller, E., Olivero, S., Papaianou, V. E., Johnson, R., and Spiegelman, B. M. (1994) EMBO J. 13, 3984–3103
18. Davies, L. G., Dibner, M. D., and Battey, J. F. (1986) Basic Methods in Molecular Biology, pp. 143–164, Elsevier Science Publishing Co., Inc., New York
19. O’Neil, K. T., Hoess, R. H., and Degrado, W. F. (1990) J. Am. Chem. Soc. 112, 7907–7922
20. Cook, P. W., Swanson, K. T., Edwards, C. P., and Firestone, G. L. (1988) Mol. Cell. Biol. 8, 1449–1459
21. Grammer, D. K., and Tomkins, G. M. (1990) Methods Enzymol. 17, 633–657
22. Middleton, W. J., Engelhardt, V. A., and Fisher, B. S. (1958) Methods Enzymol. 529–566
23. Middleton, W. J., Ferris, R. E., Little, E. L., and Krespan, C. G. (1958) Proc. Natl. Acad. Sci. U. S. A. 55, 197–207
24. Middleton, W. J., Heckert, R. E., Little, E. L., and Krespan, C. G. (1958) J. Am. Chem. Soc. 80, 2783–2788
25. Vincent, M. P., White, L. A., Schroen, D. J. L., Benhow, U., Brinckerhoff, C. E. (1996) Crit. Rev. Eucharyotic Gene Expr. 6, 391–411
26. Copeland, R. A. (1996) Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis, pp. 187–261, Wiley-VCH Publishers, Inc., New York
27. Handel, M. M., Siverson, S., Watts, K. C. W., Day, R. O., and Sutherland, R. L. (1993) Agents Actions 689, 219–223
28. Schneyder, B. Car, and J. M. Trzaskos, unpublished observations.
29. G. Fisher and J. M. Trzaskos, unpublished observations.
Identification of a Novel MEK Inhibitor

27. Malarkey, K., Belham, C. M., Paul, A., Graham, A., McLees, A., Scott, P. H., and Plevin, R. (1995) Biochem. J. 309 361–375
28. Hunter, T. (1995) Cell 80, 225–236
29. Katz, M. E., and McCormick, F. (1997) Curr. Opin. Genet. & Dev. 7, 75–79
30. Karin, M. (1991) Cell Biol. 3, 467–473
31. Kyriakis, J. M., and Avruch, J. (1996) J. Biol. Chem. 271, 24313–24316
32. Fanger, G. R., Gerwins, P., Widmann, C., Jarpe, M. B., and Johnson, G. L. (1997) Genet. Dev. 7, 67–74
33. Zhang, C. F., and Guan, K. L. (1995) J. Biol. Chem. 268, 11435–11439
34. Owaki, H., Makos, R., BouilHon, T. G., Cobb, M. H., and Geppert, T. D. (1992) Biochem. Biophys. Res. Commun. 182, 1416–1422
35. Davis, R. J. (1995) J. Biol. Chem. 268, 14553–14556
36. Alessi, D. R., Cucnerda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) J. Biol. Chem. 270, 27489–27494
37. Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F., and Ahn, N. G. (1994) Science 265, 966–970
38. Graf, K., Xi, X., Yang, D., Fleck, E., Hsueh, W. A., and Law, R. E. (1997) Hypertension 29, 334–339
39. Lazar, D. F., Wiese, R. J., Brady, M. J., Mastick, C. C., Waters, S. B., Yamauuchi, K., Pessin, J. E., Cuatrecases, P., and Saltiel, A. R. (1995) J. Biol. Chem. 270, 26801–26807
40. Azpiazu, I., Saltiel, A. R., DePaoli-Roach, A. A., and Lawrence, J. C., Jr. (1996) J. Biol. Chem. 271, 5033–5039
41. Servant, M. J., Glasson, E., and Meloche, S. (1996) J. Biol. Chem. 271, 16047–16052
42. Post, G. R., Goldstein, D., Thuersuf, D. J., Glemeltski, C. C., and Brown, J. H. (1996) J. Biol. Chem. 271, 8452–8457
43. Kuroki, M., and O’Flaherty, J. T. (1997) Biochem. Biophys. Res. Commun. 232, 474–477
44. Dumont, F. J., Staruch, J. M., Fischer, P., DaSilva, C., and Camacho, R. (1998) J. Immunol. 160, 2579–2589
45. Borsch-Haubold, A. G., Kramer, R. M., and Watson, S. P. (1996) Biochem. J. 318, 207–212
46. Wheeler-Jones, C. P., May, M. J., Houliston, R. A., and Pearson, J. D. (1996) FEBS Lett. 388, 180–184
47. Leslie, C. C. (1997) J. Biol. Chem. 272, 16709–16712
48. Simon, C., Juarez, J., Nicolson, G. L., and Boyd, D. (1996) Cancer Res. 56, 5369–5374
49. Chen, J.-Y., Pence, S., Ostrowski, J., Balaguer, P., Pons, M., Starrett, J. E., Reczek, P., Chambon, P., and Gronemeyer, H. (1995) EMBO J. 14, 1187–1197