Journal of Cellular Biochemistry 67:367–377 (1997)

Inducible Expression of a Mutant Form of MEK1 in Swiss 3T3 Cells

Li-Jyun Syu,[1,2,3] Kun-Liang Guan,[3] and Alan R. Saltiel[1,2]*

[1]Department of Cell Biology, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Co., Ann Arbor, Michigan 48105
[2]Department of Physiology, University of Michigan Medical School, Ann Arbor, Michigan 48109
[3]Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109

Abstract We conditionally overexpressed a MEK1 mutant that contains triple mutations in the regulatory and kinase domains, and investigated its effects on the MAP kinase cascade in Swiss 3T3 cells. Expression of the mutant produced a 60% blockade in MAP kinase activity. However, only a modest blockade in DNA synthesis was observed, without any reductions in the phosphorylation of two proteins known to be substrates of MAP kinase. Moreover, the overexpression of MEK1(3A) failed to block endogenous MEK1 activation, although MEK1(3A) formed complexes with both c-Raf and B-Raf as well as p42/p44 MAPK. These results suggest that there may be multiple biochemical inputs into the MEK/MAPK pathway. J. Cell. Biochem. 67:367–377, 1997. © 1997 Wiley-Liss, Inc.

Key words: MAP kinase; MEK; proliferation; dominant negative mutant

Growth factors, hormones, and cytokines are thought to regulate cellular growth and differentiation through a variety of signaling pathways that involve protein phosphorylation via the activation of protein kinases. One of the best characterized of these kinases is the enzyme known as MAP kinase, which is activated by a cascade of phosphorylations. MAP kinase activation results from its direct phosphorylation by MAP kinase kinase or MEK, a dual specificity kinase that phosphorylates MAP kinase on tyrosine and threonine residues in a conserved TEY region [Payne et al., 1991]. MEK, in turn, is regulated by phosphorylation catalyzed by one or more upstream protein kinases, including the product of the raf protooncogene [Howe et al., 1992; Kyriakis et al., 1992; Vaillancourt et al., 1994]. Additionally, other growth factor-stimulated kinases exist that can phosphorylate and activate MEK [Lange-Carter et al., 1993; Nebreda et al., 1993; Haystead et al., 1994; Pang et al., 1995; Reuter et al., 1995; Salmeron et al., 1996]. Although the precise details by which MEK is activated still remain to be determined, the raf kinase is thought to activate MEK by phosphorylation on serines 218 and 222 [Alessi et al., 1994; Zheng and Guan, 1994]. Mutation of these two sites to aspartic or glutamic acid can increase the catalytic activity of the enzyme [Huang and Erikson, 1994; Mansour et al., 1994; Huang et al., 1995]. Moreover, mutation of these two sites to alanine can block the phosphorylation induced by raf, and this mutant can produce a dominant negative phenotype when overexpressed in some cell types [Cowley et al., 1994; Seger et al., 1994]. In order to evaluate the role of these phosphorylation sites in more detail, we have expressed mutant forms of MEK under an inducible promoter. These studies suggest that numerous pathways may exist for the activation of MEK by growth factors.

Abbreviations: EGF, epidermal growth factor; PDGF, Platelet-derived growth factor; CS, calf serum; Tc, tetracycline; tTA, tetracycline-controlled transactivator; *tetO*, tetracycline operator.

*Correspondence to: Alan R. Saltiel, Department of Cell Biology, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Co., Ann Arbor, MI 48105.

Received 12 February 1997; Accepted 14 July 1997

© 1997 Wiley-Liss, Inc.

MATERIALS AND METHODS

Materials

All tissue culture reagents were purchased from GIBCO-BRL (Gaithersburg, MD). αp44^{mapk}, αp42^{mapk}, αMEK1, and αMEK2 antibodies for immunoprecipitation were purchased from Santa Cruz (Santa Cruz, CA). αSOS,

αpp90[rsk], αMEK1, αMEK2, αC-Raf, and αB-Raf for immunoblotting were from Santa Cruz, while αFLAG M2 antibody was from IBI (New Haven, CT). Protein A/G-Sepharose were purchased from Santa Cruz. Growth factors were from Intergen (Purchase, NY). [γ-^{32}P]ATP (3,000 Ci/mmol) for kinase reactions was from DuPont NEN (Boston, MA).

Generation of the Tetracycline-Inducible Cell Line

MEK1 (K97A, S218A, S222A, simplified as (3A)), tagged with Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG-tag) in the carboxyl terminus, was constructed by PCR and subcloning. Regions involved in PCR reactions were sequenced to assure no additional mutations. The full-length cDNA was cloned into pUHD10-3, and placed downstream of the *tetO* promoter in the *tet* operator (*tetO*) [Gossen and Bujard, 1992]. This gene is expressed when the transactivator, encoded by pUHD 15-1, activates the promoter.

Swiss 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 2 mM glutamine and 10% calf serum. Swiss 3T3 cells constitutively expressing tetracycline-controlled transactivator (tTA) (founder cells) were transfectants of linearized pUHD15-1, encoding the tTA gene. A stable founder line (Swiss 3T3-tTA) was selected for the highest induction of transactivator expression, shown in the reporter assay with pUHG16-3, in the absence of tetracycline (Tc) (Sigma, St. Louis, MO) in the culture. This founder line was used for construction of second-stage stable cells expressing a dominant negative mutant of MEK1(3A).

The plasmid pUHD10-3/MEK1(3A) was transfected into the Swiss 3T3-tTA founder line. Clones were selected and expanded against 200 μg/ml of hygromycin (Boehringer Mannheim, Indianapolis, IN) and 400 μg/ml of G418 (GIBCO-BRL) in the presence of 2 μg/ml of tetracycline in culture medium. Resistant clones were screened for conditional expression of MEK1(3A) in the absence of tetracycline for 48 h, using αFLAG immunoblotting. A cell line whose growth rate was not affected from plasmid integration, which expressed the highest level of MEK1(3A) in tetracycline-free medium, but without leaky expression in medium containing 2 μg/ml tetracycline, was selected for further study. This stable cell line was maintained in medium containing 400 μg/ml G418, 200 μg/ml hygromycin, and 2 μg/ml tetracycline. At the time of conditional overexpression of MEK1(3A), the cultured cells were washed twice with phosphate-buffered saline and grown in tetracycline-free medium.

Assay of DNA Synthesis

Growth-arrested cells in 24-well plates were serum-starved for 24 h and then stimulated with growth factors for 19 h. Cells were labeled with 1 μCi/per well of [^{3}H] thymidine for additional 2 h. Cells were washed 3 times with ice-cold phosphate-buffered saline and harvested in 2% SDS, 10 mM Tris-Cl/1 mM EDTA. Ten percent trichloroacetic acid and 20 μg salmon testis DNA as carrier were added to precipitate cellular DNA. [^{3}H] thymidine incorporation in the acid-precipitable material was determined by liquid scintillation.

Assay of MAP Kinase Activity

Immunocomplex assay of MAP kinase activity was performed as described [Boulton et al., 1991]. Briefly, 70 to 90% confluent cultures of cells were serum starved overnight and then stimulated with growth factor. Cells were lysed in MIPA buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% deoxycholate, 1% Nonidet p-40, 50 mM NaF, 10 mM Na-pyrophosphate, 1 mM *para*-nitrophenylphosphate, 25 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonylflouride, and 1 mM benzamidine). Lysates were precleared and immunoprecipitated with αp44[mapk], αp42[mapk] antibodies for 2 h at 4°C. Immunocomplexes were bound to protein A/G-Sepharose for an additional 30 min and washed three times with lysis buffer and once with kinase reaction buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2 mM EGTA, and 1 mM DTT). Immunocomplexes were incubated in kinase buffer for 5 min at 30°C, prior to the initiation of the kinase reaction with 10 μg microtubule-associated protein-2 (MAP2) and 50 μM ATP containing 5 μCi [γ-^{32}P] ATP for a further 10 min at 30°C. Immune-complex reactions were stopped with SDS sample buffer and the samples were boiled for 5 min. ^{32}P-labeled MAP2 was resolved on 6% SDS gel and visualized by autoradiography. The amount of labeling was further quantitated by a phosphoimager (Molecular Dynamics, Sunnyvale, CA)

Association of MEK1 and Raf

Cells were lysed in modified p21 buffer [Catling et al., 1995]. Total proteins (1.5 mg) of

whole cell lysates were precleared and subjected to immunoprecipitation with αMEK1 or αFLAG preadsorbed protein A/G-Sepharose beads for 3 h at 4°C. Immunocomplexes were extensively washed and separated on 8% SDS-PAGE. The gel was transferred to nitrocellulose and probed with αC-Raf and αB-Raf. Immunoreactive proteins were visualized by Enhanced Chemiluminescent (ECL) detection.

Association of MEK1 and p42/p44 MAPK

Cells were lysed in buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 % Nonidet p-40, 10% glycerol, 1 mM DTT, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonylflouride, and 1 mM benzamidine). Total proteins (1 mg) of whole cell lysates were precleared and subjected to immunoprecipitation with αFLAG preadsorbed protein A/G-Sepharose beads for 1.5 h at 4°C. Immunocomplexes were extensively washed and separated on 10% SDS-PAGE. The gel was transferred to nitrocellulose and probed with $\alpha p42/p44^{mapk}$. Immunoreactive proteins were visualized by Enhanced Chemiluminescent (ECL) detection.

Image Processing

Immunoblots were quantified by computer-assisted video densitometry using the Bio Image system (Imaging Systems, Millipore Corp., Ann Arbor, MI). Autoradiographs were quantified using a phosphoimager (Molecular Dynamics).

RESULTS

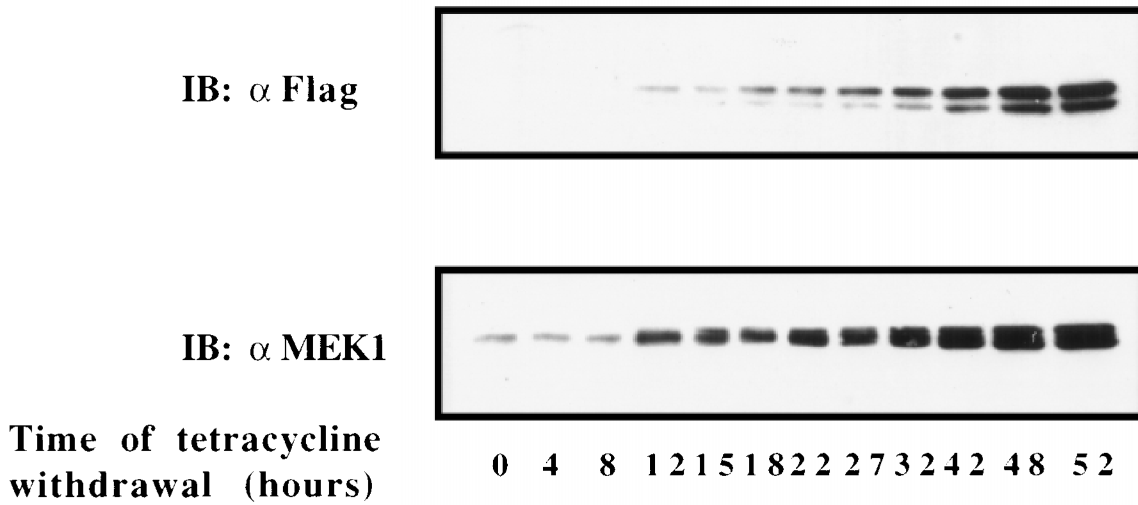
Stable Expression of a MEK1 Mutant Under an Inducible Promoter

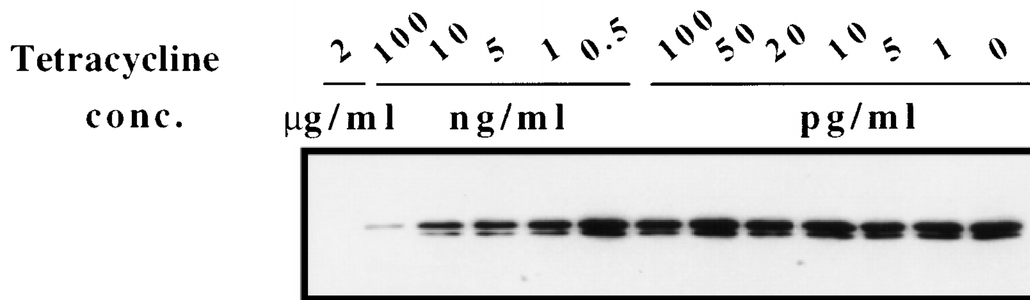
In order to evaluate the role of serines 218 and 222 of MEK1 in its regulation, a cDNA was prepared with substitutions of serines 218 and 222 and lysine 97 for alanine (MEK1(3A)), and was cloned into the tetracycline inducible system. pUHD 10-1 expressing the tTA transactivator and pSV2 expressing neomycin resistant marker were cotransfected into Swiss 3T3 cells, and the best founder clone was identified by screening with a transient-expression assay. The founder cell line was further cotransfected with pUHD10-3/MEK1(3A) and TK-hygromycin. The highest conditionally expressing clone (Swiss 3T3-tTA-MEK1(3A)) was selected. In tetracycline-free media, transactivators are released from tetracycline inhibition, and bind to the promoter upstream of MEK1(3A) to induce expression. To establish conditions for maximal expression of MEK1(3A), the time course of expression (Fig. 1a) in tetracycline-free medium and sensitivity to the concentration of tetracycline (Fig. 1b) were examined. Cells were plated in the presence of 2 μg/ml of tetracycline. Tetracycline was withdrawn from the medium at different time points. Cells were then maintained in tetracycline-free medium and harvested at the same level of confluence. MEK1(3A) expression was measured by αFLAG Western blot (Fig. 1a). MEK1(3A) expression was detected 12 h after tetracycline withdrawal. The expression was increased twofold by 22 h, another twofold at 32 h, and another fourfold at 48 h. Immunoblotting with αMEK1 revealed that the expression attained at 12 h after tetracycline withdrawal is 8 times that of endogenous wild type MEK1. After 48 h, expression of the mutant is 120-fold that of endogenous MEK1. Cells were cultured in the presence of different concentrations of tetracycline for 48 h and the expression of MEK (3A) was evaluated by αFLAG immunoblotting. Tetracycline (2 μg/ml) completely prevented the expression of MEK1(3A) (Fig. 1b). These data indicated that cells could be maintained in media containing 2 μg per ml tetracycline to ensure a tight control on MEK1(3A) expression.

To ensure that tetracycline itself does not affect MAP kinase activity, the Swiss 3T3-tTA founder line was grown in the presence of different concentrations of tetracycline for 48 h. Neither basal nor EGF-stimulated MAP kinase activity was affected by up to 2 μg/ml of tetracycline (Fig. 1c).

Overexpression of MEK1(3A) Only Partially Blocks MAP Kinase Activities

To determine the effect of overexpression of MEK1(3A) on the MAP kinase pathway, cells were cultured in the presence or absence of 2 μg/ml tetracycline (Fig. 2). Cells were then treated with or without 100 ng/ml EGF for 5 min. $p44^{mapk}$ and $p42^{mapk}$ activity was measured by separate immunocomplex kinase assays. In +Tc cells, treatment with EGF cause a 20.5-fold increase in $p44^{mapk}$ activity and 27.2-fold increase in $p42^{mapk}$ activity. In −Tc cells, fold stimulation for $p44^{mapk}$ and $p42^{mapk}$ activity was 9.7 and 11.9, respectively. Thus, despite a 100-fold overexpression of MEK1(3A), the acti-





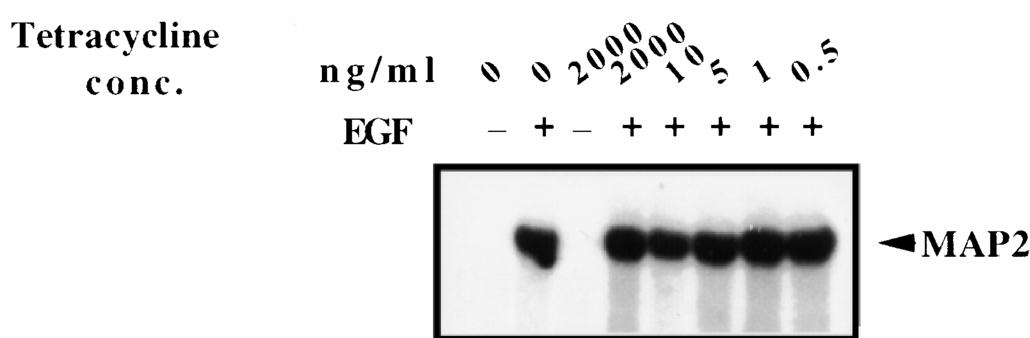


Fig. 1. Kinetics of inducible expression of MEK1(3A). **a:** Time course of expression. Swiss 3T3-tTA MEK1(3A) overexpressing cells were grown in tetracycline-free medium for the indicated times. Fifteen micrograms of total protein for each sample was separated in 10% SDS-PAGE, transferred to nitrocellulose, probed with αFLAG or αMEK1 antibodies, and visualized by Enhanced Chemiluminescent detection. **b:** Effect of tetracycline concentration on inducible expression. MEK1(3A) expressing cells were exposed to the indicated concentrations of tetracycline for 48 h. Fifteen micrograms protein was subjected to SDS-PAGE as described above, and probed with αFLAG antibodies. **c:** Effect of tetracycline concentration on MAP kinase activity. Swiss 3T3-tTA founder cells were cultured in the presence of the indicated concentrations of tetracycline for 48 h, serum starved, and stimulated with 100 ng/ml EGF for 5 min. MAP kinase was immunoprecipitated and assayed by ^{32}P phosphorylation of MAP2.

vation of both p44^{mapk} and p42^{mapk} activity was maintained at 40–45% that seen in uninduced cells. To ensure that this observation was not unique to this method of assay, similar results were obtained by examining the phosphorylation of p44^{mapk} and p42^{mapk} using an electrophoretic mobility shift assay (data not shown).

Overexpression of MEK1(3A) Does Not Substantially Block the Phosphorylation of SOS and Rsk

Activation of the MAPK pathway results in the phosphorylation of the ras nucleotide exchange protein, SOS, uncoupling the Grb2-SOS complex and terminating the activation of p21^{ras} [Waters et al., 1995; Chen et al., 1996]. This retrophosphorylation event was investigated in the MEK1(3A) overexpressing cells by examining the ability of a growth factor (EGF) to alter the electrophoretic mobility of SOS. Whole cell lysates were prepared from EGF-stimulated cells before and after induction of MEK1(3A) by tetracycline withdrawal. Lysates were immunoblotted with αSOS. As shown in Figure 3a, the electrophoretic mobility of SOS in control cells was markedly shifted following 5 min of EGF treatment, and reached a maximum by 10 min. This shift could be completely blocked by preincubation of cells with the specific MEK inhibitor PD098059 [Dudley et al., 1995]. In cells induced to express MEK1(3A) by tetracycline withdrawal, EGF still produced a shift in the mobility of SOS.

Activation of the MAP kinase pathway leads to a stimulation of the pp90^{rsk}, due to the direct phosphorylation of the enzyme by MAP kinase [Sturgill et al., 1988; Blenis, 1993]. To investigate whether MEK1(3A) expression would alter the phosphorylation of this protein, the mutant was induced by tetracycline withdrawal, and lysates were subjected to SDS-PAGE and immunoblotting with anti-pp90^{rsk} antibodies.

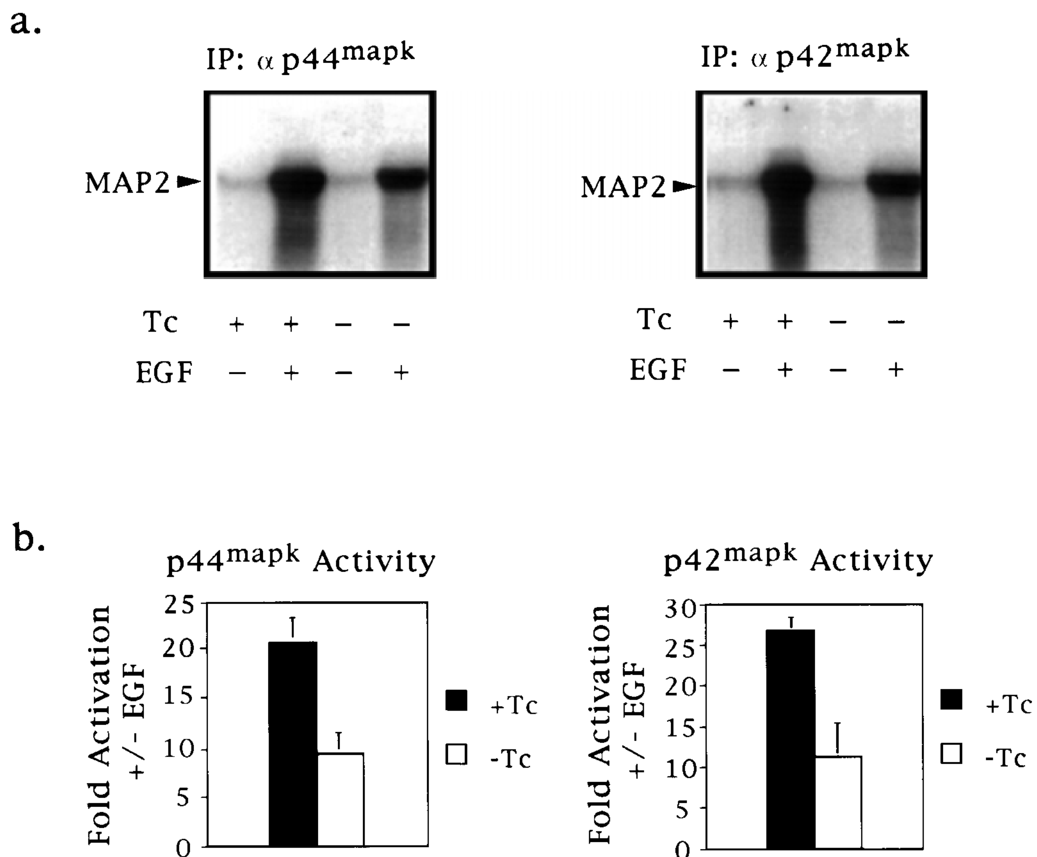
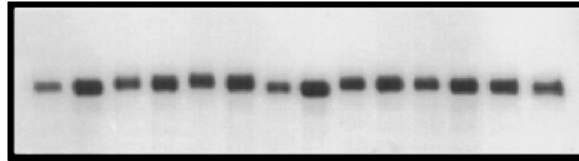


Fig. 2. Effect of MEK1(3A) expression on the kinase activities of immunoprecipitated p44^{mapk} and p42^{mapk}. **a:** Cells were cultured in the presence or absence of tetracycline for 48 h, serum-starved, and stimulated with 100 ng/ml EGF for 5 min. The activities of p44^{mapk} or p42^{mapk} were assayed in immunocomplexes. The data are representative of three separate experiments. **b:** Data are depicted as fold activation over background and are given as mean ± S.E. from three separate experiments.

a. SOS

EGF(min)	0		5		10		10		30		60		120	
PD098059	–	–	–	–	–	–	+	+	–	–	–	–	–	–
Tc	+	–	+	–	+	–	+	–	+	–	+	–	+	–



b. pp90 rsk

EGF(min)	0		5		10		10		30		60		120	
PD098059	–	–	–	–	–	–	+	+	–	–	–	–	–	–
Tc	+	–	+	–	+	–	+	–	+	–	+	–	+	–

Fig. 3. Effect of MEK1(3A) expression on growth factor-induced mobility shift of SOS, or $pp90^{rsk}$. **a:** Mobility shift of SOS. Cells were induced to express MEK1(3A) by tetracycline withdrawal and stimulated with 100 ng/ml EGF for different lengths of time. In samples with PD098059 treatment, 30 μM of PD098059 was added 60 min prior to EGF stimulation. Twenty micrograms of total protein was separated on 4–12% gradient SDS-PAGE, followed by immunoblotting with αSOS antibodies. **b:** Mobility shift of $pp90^{rsk}$. Samples were processed the same as in a except $\alpha pp90^{rsk}$ antibodies were used for probing.

Interestingly, expression of MEK1(3A) was without significant effect on the ability of EGF to induce a decrease in the electrophoretic mobility of $pp90^{rsk}$ (Fig. 3b).

Expression of MEK1(3A) Results in a Modest Inhibition of DNA Synthesis

To evaluate the impact of MEK1(3A) overexpression, we investigated the effect of EGF, PDGF, or calf serum on rates of DNA synthesis (Fig. 4). Expression of MEK1(3A) by tetracycline withdrawal had no impact whatsoever on the mitogenic signal upon stimulation with 10% calf serum stimulation. In cells stimulated with EGF or PDGF, a modest reduction in DNA synthesis was observed in cells induced to overexpress MEK1(3A). In contrast, treatment of both control and MEK1(3A) expressing cells with the MEK inhibitor resulted in the complete inhibition of DNA synthesis, suggesting that the MAP kinase pathway is absolutely required for S phase entry.

Overexpression of MEK(3A) Does Not Block Endogenous MEK1 Activity

All the data presented above suggest that despite significant overexpression in Swiss 3T3 cells, MEK1(3A) does not effectively block MAP

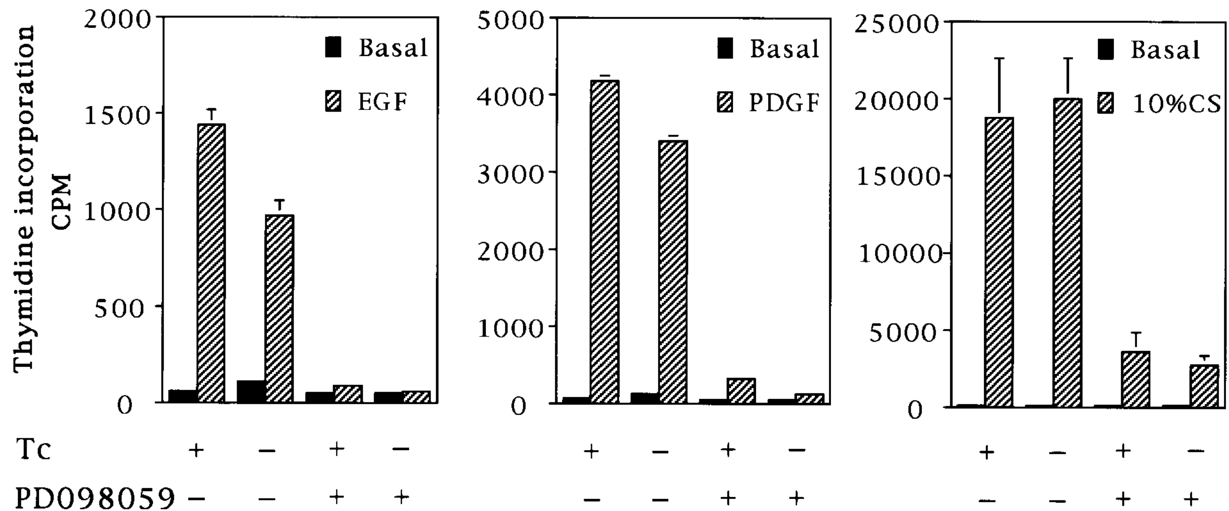


Fig. 4. Effect of MEK1(3A) expression on growth factor-stimulated DNA synthesis. Cells were cultured in the presence or absence of tetracycline for 48 h prior to growth arrested by serum starvation for 24 h. Cells reentered cell cycle by stimulation with various growth factors. Samples with pretreatment of MEK inhibitor PD098059 were included. Results of thymidine incorporation are expressed as cpm/10^5 cells, and the data are shown as the mean ± S.E. performed in triplicate.

kinase activation. Although there is a 55–60% inhibition of total MAPK activity, there is no significant impact of this reduction on substrate phosphorylation or DNA synthesis, despite the absolute dependence of these effects on MEK, as illustrated by the complete blockade achieved with the MEK inhibitor. These data indicate that mutations of these raf phosphorylation sites do not produce a true dominant negative phenotype. To explore this possibility in more detail, we next examined whether MEK1(3A) expression could block the activation of endogenous MEK1. MEK activity was assayed by phosphorylation of a catalytically inert GST-$p44^{mapk}$(K71R) fusion protein (Fig. 5). In order to evaluate the activity of endogenous MEK, lysates were precipitated first with anti-FLAG, to remove MEK1(3A). This precipitation quantitatively depleted all of the transfected mutant MEK from the cell lysates. This precipitated enzyme was devoid of any kinase activity, as expected. The resulting depleted supernatants were then evaluated for MEK activity, using an immunoprecipitation assay. Treatment of control cells with EGF produced a substantial increase in the activity of MEK1 detected in these depleted lysates. MEK2 activity was not detected. Immunoblotting revealed that MEK2 expression was only a fraction of that compared to MEK1. Interestingly, induction of MEK1(3A) by tetracycline withdrawal had no effect whatsoever on the activation of endogenous MEK1.

MEK1(3A) Can Interact With C-Raf and B-Raf as Well as With p42 and p44 MAPK

Phosphorylation site mutations in MEK and other kinases are presumed to interact with their upstream activators to form stable complexes and block the phosphorylation of the endogenous enzyme. This physical interaction between kinases and their substrates is thought to be transient, with a high off rate. In the case of MEK1, no interaction was observed between wild type MEK1 and raf in the yeast two hybrid system, although raf did interact with a MEK construct in which ser^{218} and ser^{222} had been mutated to alanine (MEK1(2A)) [Wu et al., 1996]. To confirm that MEK1(3A) could interact with raf, we examined these interactions in cells induced to express the mutant by tetracycline withdrawal (Fig. 6a). Control cells were immunoprecipitated with αMEK1. MEK1(3A)-expressing cells were immunoprecipitated with αFLAG, and immunoprecipitates were subjected to immunoblotting with αC-Raf and αB-Raf. Stable complexes between wild type MEK1 and C-Raf were not detected, while an interaction between MEK1(3A) and C-Raf was observed. Both forms of MEK interacted with B-Raf. Thus, the failure of MEK1(3A) to block endogenous MEK apparently did not result from an inability to complex with either C-Raf or B-Raf.

Thorner and co-workers have shown that high-affinity complex of MEK-MAPK requires neither the kinase catalytic core of MEK nor

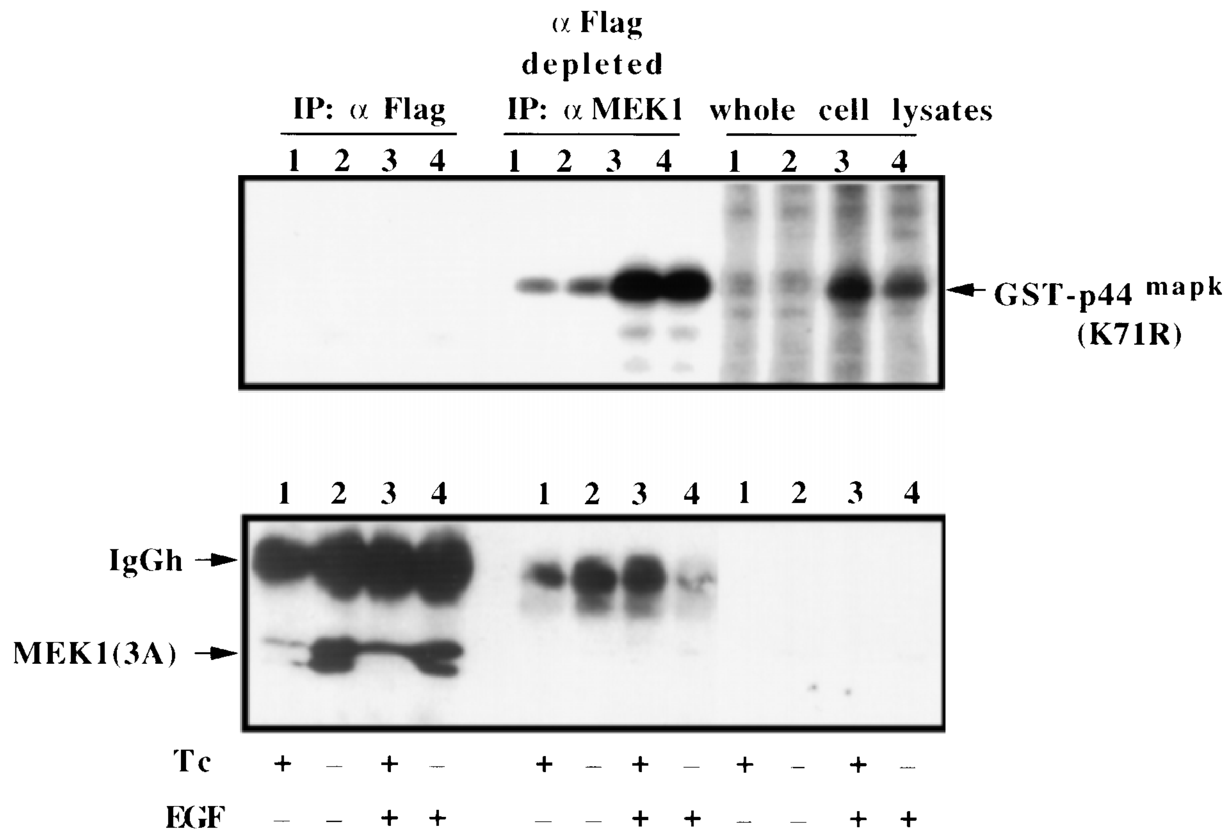


Fig. 5. Expression of MEK1(3A) has no effect on endogenous MEK1. Cells were cultured in the presence or absence of tetracycline for 48 h and serum-starved overnight. Cells were stimulated with 100 ng/ml EGF for 3 min. Lysates were subjected to sequential immunoprecipitations to separate MEK1(3A) and endogenous MEK1. Whole cell lysates, MEK1(3A) (in αFLAG immunoprecipitates) and endogenous MEK1 (in αMEK1 immunoprecipitates) were then examined for their ability to phosphorylate added GST-p44^{mapk} (K71R). The level of MEK1(3A) in each preparation were revealed by αFLAG immunoblotting (bottom).

phosphoacceptor regions of MAPK [Bardwell et al., 1996; Bardwell and Thorner, 1996]. To exclude the possibility that phosphorylation site mutations in MEK prevented its interaction with MAPK, we examined the MEK1(3A)-p42/p44 MAPK interaction in MEK1(3A)-expressing cells. MEK1(3A) was immunoprecipitated with αFLAG antibodies, and immunoprecipitates were subjected to immunoblotting with αp42/p44^{mapk} antibodies. As expected, MEK1(3A) and MAPK coprecipitated in these cells (Fig. 6b). Thus, the failure of MEK1(3A) to block endogenous MEK apparently did not result from an inability to complex with p42/p44 MAPK.

DISCUSSION

Numerous reports have indicated that the MAP kinase pathway is critically involved in the regulation of cellular proliferation, differentiation, and transformation. Expression of antisense C-Raf or a kinase-inactive mutant can interfere with the proliferation of NIH 3T3 cells. Similarly, a kinase defective mutant of MAP kinase was shown to interfere with the proliferation and transformation of fibroblasts [Pagès et al., 1993; Kortenjann et al., 1994]. Likewise, MEK1 mutants with alanine substitutions in the regulatory domain at serine 218 or serine 222 or in the kinase domain at lysine 97 also have shown a suppressing effect on MAP kinase activity, and have been reported to reduce the rate of proliferation in NIH 3T3 cells [Cowley et al., 1994; Seger et al., 1994]. Moreover, the blockade of growth factor-stimulated DNA synthesis by the MEK inhibitor PD98059 further supports an important role for this pathway in growth control [Dudley et al., 1995; Alessi et al., 1995]. However, mitogenesis can also be MAP kinase-independent in Swiss 3T3 cells and some other cell types [Wang et al., 1992; Casillas et

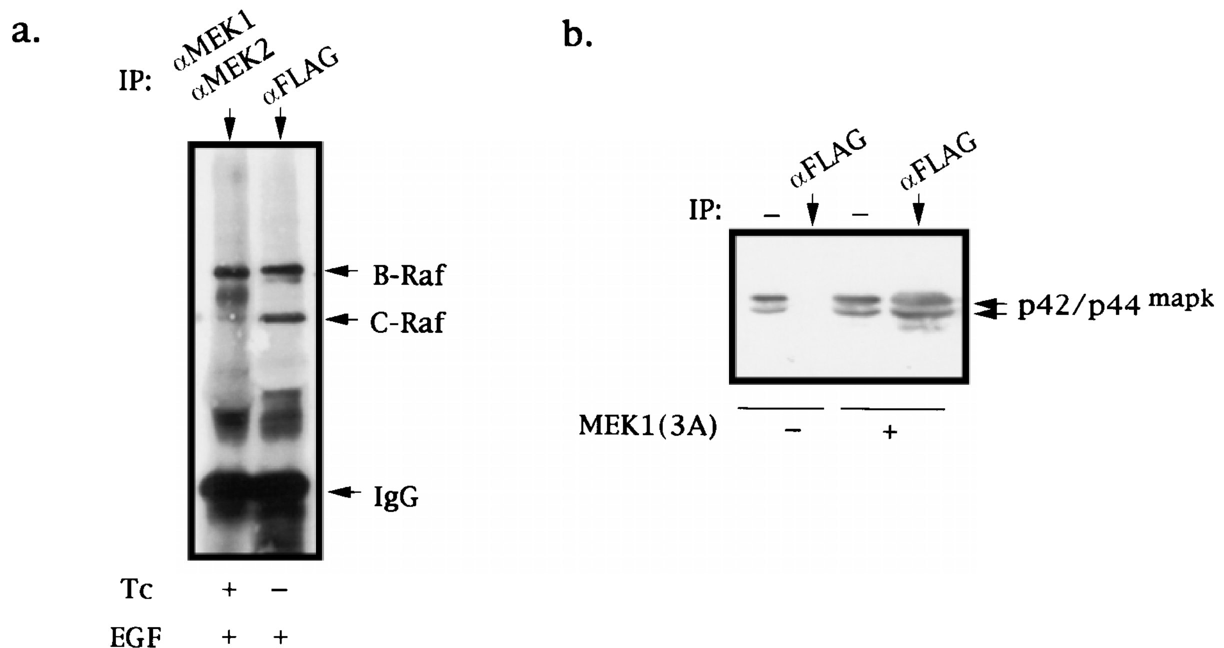


Fig. 6. Physical association of MEK1(3A) with Rafs and MAPK. **a:** Association with Rafs. Cells were cultured in the presence or absence of tetracycline for 48 h and serum-starved overnight. Cells were stimulated with 100 ng/ml EGF for 3 min and lysed in modified p21 buffer. Total proteins (1.5 mg) from MEK1(3A) expressing cells were immunoprecipitated with αFLAG preadsorbed A/G agarose beads, while proteins from controls cells were immunoprecipitated with αMEK1 and αMEK2 preadsorbed A/G agarose beads. Immunocomplexes were washed, resolved on 8% SDS-PAGE, transferred to nitrocellulose and probed with αC-Raf and αB-Raf antibodies. **b:** Association with p42/p44 MAPK. MEK1(3A) expressing cells were cultured in the absence of tetracycline for 48 h. Cells were then serum-starved overnight, stimulated with 100 ng/ml EGF for 3 min, and lysed in buffer as described in Materials and Methods. Total proteins (1 mg) were immunoprecipitated with αFLAG preadsorbed A/G agarose beads. Swiss 3T3-tTA founder cells were a control to exclude the possibility of nonspecific coprecipitation by αFLAG antibodies. Immunocomplexes were washed, resolved on 10% SDS-PAGE, transferred to nitrocellulose, and probed with αp42/p44^{mapk} antibodies.

al., 1993; Kortenjann and Shaw, 1995; Withers et al., 1995].

Studies described here utilized a MEK1 mutant with substitutions in the regulatory domain and the kinase domain, to render the kinase catalytically inactive, and incapable of phosphorylation by Raf. We utilized an inducible expression system to tightly control its expression in Swiss 3T3 cells. This construct encoded for an enzyme that was devoid of catalytic activity, and was expressed 100-fold over endogenous MEK1. However, induction of this mutant in Swiss 3T3 cells produced only a 60% blockade in MAP kinase activity, and only modestly affected growth factor-stimulated DNA synthesis. Moreover, despite the fact that MEK1(3A) formed stable complexes with both B-Raf and C-Raf as well as both p42 and p44 MAPK, the mutant had no influence on the activation of endogenous MEK1 by growth factors. We have also examined the MEK1(K97R) or p44 MAPK(K71R) overexpressing cells and have observed a similar phenotype. Taken together, these data suggest that endogenous MEK1 can be fully activated by Raf that is only fractionally active, or by other activators in Swiss 3T3 cells. However, the possibility does exist that the ineffectiveness of MEK1(3A) might result from its improper localization to the signaling complexes due to incorrect folding.

The existence of novel MEK kinases has been suggested in several cell types [Pang et al., 1995; Reuter et al., 1995]. In addition, an alternative pathway to EGF-induced p42^{mapk} phosphorylation has been suggested in Swiss 3T3 cells, where some studies indicate that mitogenesis can be dissociated from the MAP kinase cascade [Burgering et al., 1993; Withers et al., 1995]. Our data reinforced the idea of multiple pathways for the activation of MAP kinase and multiple activators upstream of MEKs.

REFERENCES

Alessi DR, Saito Y, Campbell DG, Cohen P, Sithanandam G, Rapp UR, Ashworth A, Marshall CR, Cowley S (1994): Identification of the sites in MAP kinase kinase-1 phosphorylated by $p74^{raf-1}$. EMBO J 7:1610–1619.

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR (1995): PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 270:27489–27494.

Bardwell L, Thorner J (1996): A conserved motif at the amino termini of MEKs might mediate high-affinity interaction with the cognate MAPKs. Trends Biochem Sci. 21:373–374.

Bardwell L, Cook JG, Chang EC, Cairns BR, Thorner J (1996): Signaling in the yeast pheromone response pathway: specific and high-affinity interaction of the mitogen-activated protein(MAP) kinases Kss1 and Fus3 with the upstream MAP kinase kinase Ste7. Mol Cell Biol 16:3637–3650.

Blenis J (1993): Signal transduction via the MAP kinases: Proceed at your own RSK. Proc Natl Acad Sci USA 90:5889–5892.

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD (1991): ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65:663–675.

Burgering BMT, de Vries-Smits AMM, Medema RH, van Weeren P, Tertoolen LGJ, Bos JL (1993): Epidermal growth factor induces phosphorylation of extracellular signal-regulated kinase 2 via multiple pathways. Mol Cell Biol 13:7248–7256.

Casillas AM, Amaral K, Chegini-Farahani S, Nel AE (1993): Okadaic acid activates p42 mitogen-activated protein kinase(MAP kinase; ERK-2) in B-lymphocytes but inhibits rather than augments cellular proliferation: contrast with phorbol 12-myristate 13-acetate. Biochem J 290:545–550.

Catling AD, Schaeffer H-J, Reuter CWM, Reddy GR, Weber MJ (1995): A proline-rich sequence unique to MEK1 and MEK2 is required for Raf binding and regulates MEK function. Mol Cell Biol 15:5214–5225.

Chen D, Waters SB, Holt KH, Pessin JE (1996): SOS phosphorylation and disassociation of the Grb2-SOS complex by the ERK and JNK signaling pathways. J Biol Chem 271:6328–6332.

Cowley S, Paterson H, Kemp P, Marshall CJ (1994): Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 77:841–852.

Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR (1995): A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA 92:7686–7689.

Gossen M, Bujard H (1992): Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89:5547–5551.

Haystead CM, Gregory P, Shirazi A, Fadden P, Mosse C, Dent P, Haystead TA (1994): Insulin activates a novel adipocyte mitogen-activated protein kinase kinase kinase that shows rapid phasic kinetics and is distinct from c-Raf. J Biol Chem 269:12804–12808.

Howe LR, Leevers SJ, Gómez N, Nakielny S, Cohen P, Marshall CJ (1992): Activation of the MAP kinase pathway by the protein kinase Raf. Cell 71:335–342.

Huang W, Erikson RL (1994): Constitutive activation of MEK1 by mutation of serine phosphorylation sites. Proc Natl Acad Sci USA 91:8960–8963.

Huang W, Kessler DS, Erikson RL (1995): Biochemical and biological analysis of Mek1 phosphorylation site mutants. Mol Biol Cell 6:237–245.

Kortenjann M, Shaw PE (1995): Raf-1 kinase and ERK2 uncoupled from mitogenic signals in rat fibroblasts. Oncogene 11:2105–2112.

Kortenjann M, Thomae O, Shaw PE (1994): Inhibition of v-Raf dependent c-*fos* expression and transformation by a kinase-defective mutant of the mitogen-activated protein kinase Erk2. Mol Cell Biol 14:4815–4824.

Kyriakis JM, App H, Zhang X-F, Banerjee P, Brautigan DL, Rapp UR, Avruch J (1992): Raf-1 activates MAP kinase-kinase. Nature 358:417–421.

Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ, Johnson GL (1993): A divergence in the MAP kinase regulatory network defined by MEK kinase ans Raf. Science 260:315–319.

Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande Woude GF, Ahn NG (1994): Transformation of mammalian cells by constitutively active MAP kinase kinase. Science 265:966–970.

Nebreda AR, Hill C, Gomez N, Cohen P, Hunt T (1993): The protein kinase *mos* activates MAP kinase kinase in vitro and stimulates the MAP kinase pathway in mammalian somatic cells in vivo. FEBS Lett 333:183–187.

Pagès G, Lenormand P, L'Allemain G, Chambard J-C, Meloche S, Pouysségur J (1993): Mitogen-activated protein kinases $p42^{mapk}$ and $p44^{mapk}$ are required for fibroblast proliferation. Proc Natl Acad Sci USA 90:8319–8323.

Pang L, Zheng C-F, Guan K-L, Saltiel AR (1995): Nerve growth factor stimulates a novel protein kinase in PC-12 cells that phosphorylates and activates mitogen activated protein kinase kinase (MEK). Biochem J 307:513–519.

Payne DM, Rossomando AJ, Martino P, Erickson AK, Her J-H, Shananowitz J, Hunt DF, Weber MJ, Sturgill TW (1991): Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J 10:885–892.

Reuter CWM, Catling AD, Jelinek T, Weber MJ (1995): Biochemical analysis of MEK activation in NIH3T3 fibroblasts. J Biol Chem 270:7644–7655.

Salmeron A, Ahmad TB, Carlile GW, Pappin D, Narsimhan RP, Ley SC (1996): Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. EMBO J 15:817–826.

Seger R, Seger D, Reszka AA, Munar ES, Eldar-Finkelman H, Dobrowolska G, Jensen AM, Campbell JS, Fisher EH, Krebs EG (1994): Overexpression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH 3T3 cells. J Biol Chem 269:25699–25709.

Sturgill TW, Ray LB, Erikson E, Maller JL (1988): Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. Nature 334:715–718.

Vaillancourt RR, Gardner AM, Johnson GL (1994): B-Raf-dependent regulation of the MEK-1/mitogen-activated

protein kinase pathway in PC12 cells and regulation by cyclic AMP. Mol Cell Biol 14:6522–6530.
Wang LM, Keegan AD, Paul WE, Heidaran MA, Gutkind JS, Pierce JH (1992): IL-4 activates a distinct signal transduction cascade from IL-3 in factor-dependent myeloid cells. EMBO J 11:4899–4908.
Waters SB, Holt KH, Ross SE, Syu LJ, Guan KL, Saltiel AR, Koretzky GA, Pessin JE (1995): Desensitization of Ras activation by a feedback disassociation of the SOS-Grb2 complex. J Biol Chem 270:20883–20886.
Wither DJ, Bloom SR, Rozengurt E (1995): Dissociation of cAMP-stimulated mitogenesis from activation of the mitogen-activated protein kinase cascade in Swiss 3T3 cells. J Biol Chem 270:21411–21419.
Wu X, Noh SJ, Zhou G, Dixon JE, Guan K-L (1996): Selective activation of MEK1 but not MEK2 by A-raf from EGF stimulated Hela cells. J Biol Chem 271:3265–3271.
Zheng C-F, Guan K-L (1994): Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. EMBO J 13:1123–1131.