Differential Effects of cAMP on the MAP Kinase Cascade: Evidence for a cAMP-insensitive Step that Can Bypass Raf-1

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Because cAMP exerts opposite effects on cell proliferation in different cell types, we undertook to study its effect on the mitogen-activated protein kinase (MAPK) pathway in three cell lines (Rat-1, Swiss-3T3, and COS-7) chosen for their different mitogenic responses to cAMP. We measured the effect of cAMP on MAPK, MEK, and Raf-1 activities after stimulation by agonists acting through a tyrosine kinase receptor (epidermal growth factor) or a G protein-coupled receptor (lysophosphatidic acid). In Rat-1 cells we found that cAMP strongly inhibited all three activities (MAPK, MEK, and Raf-1), in good agreement with its effect on cell proliferation in these cells. In Swiss-3T3 and COS-7 cells, on the contrary, cAMP did not inhibit epidermal growth factor- and lysophosphatidic acid-induced stimulation of MAPK and MEK activities, and even stimulated MAPK activity slightly on its own. Again these results are in good agreement with the proliferative effect of cAMP in Swiss-3T3 cells. Raf-1 activity, on the other hand, was inhibited by cAMP in Swiss-3T3 and COS-7 as it was in Rat-1 cells. This result indicates that signaling pathways in Swiss-3T3 and COS-7 cells can activate MEK and MAPK in a Raf-1-independent and cAMP-insensitive manner. Our results add to growing evidence for the existence of Ras- and/or Raf-1-independent pathways leading to MEK and MAPK activation.

INTRODUCTION

Mitogen-activated protein kinases (MAPKs),¹ also known as extracellular signal-regulated kinases (ERKs), are a family of protein-serine/threonine kinases that become activated in response to growth factors, hormones, neurotransmitters, and other extracellular stimuli. These kinases exert mitogenic effects by phosphorylating a broad array of substrates (for review, see Marshall, 1994).

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¹ Abbreviations used: EGF, epidermal growth factor; ERKs, extracellular signal-regulated kinases; GST, glutathione S-transferase; HA, hemagglutinin; LPA, lysophosphatidic acid; MAPKs, mitogen-activated protein kinases; MAPKK, MAPK kinase; MBP, myelin basic protein; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulfonyl fluoride.

MAPKs are regulated by a kinase-mediated signaling cascade. A MAPK kinase (MAPKK) (Seger *et al.*, 1992), also known as MEK (Crews *et al.*, 1992), activates MAPKs by catalyzing MAPK phosphorylation on both threonine and tyrosine residues (Anderson *et al.*, 1990). MEK itself is activated by phosphorylation on serine residues by a MAPK kinase kinase (MKKK) (Alessi *et al.*, 1994; Zheng and Guan, 1994). Each phosphorylation in the cascade can be performed by two or more proteins, including two closely related MAPKs, p44^{mapk} (ERK1) and p42^{mapk} (ERK2) (Boulton *et al.*, 1991), two isoforms of MEK (MEK1 and MEK2) (Zheng and Guan, 1993a), and, at the MKKK level, three Raf kinase isoforms (Raf-1, Raf-A, and Raf-B) (Huleihel *et al.*, 1986; Sithanandam *et al.*, 1990; Stephens *et al.*, 1992) and a protein called MEKK (Lange-Carter *et al.*, 1993). This cascade of three consecutive protein kinases is highly conserved throughout eukaryotic evolution (reviewed in Neiman, 1993), and

similar kinase cascades transduce nonmitogenic signals such as stress or osmotic shock (Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994).

MAPKs can be activated by extracellular stimuli that act via tyrosine kinase receptors or G protein-coupled receptors. Activated receptor tyrosine kinases, such as the EGF and PDGF receptors, trigger activation of p21^{ras} by a well-established pathway involving the Grb2/SEM5 and SOS proteins (reviewed in McCormick, 1994). Activated p21^{ras} binds Raf-1 (Moodie *et al.*, 1993; van Aelst *et al.*, 1993; Vojtek *et al.*, 1993) and recruits it to the plasma membrane (Leevers *et al.*, 1994; Stokoe *et al.*, 1994), where it is activated to phosphorylate MEK. Less is known about how G protein-coupled receptors stimulate the MAPK pathway. We and others (Crespo *et al.*, 1994; Faure *et al.*, 1994) have shown that G protein $\beta\gamma$ subunits are involved in this response, and clear evidence implicates Ras and Raf as mediators of the MAPK response to these receptors in some cases (Cook *et al.*, 1993; Howe and Marshall, 1993; van Corven *et al.*, 1993; Winitz *et al.*, 1993). In addition, poorly characterized pathways independent of Ras and Raf can lead to MAPK activation in response to both receptor tyrosine kinases and G protein-coupled receptors (Burgering *et al.*, 1993a; Lange-Carter *et al.*, 1993).

Because cAMP exerts opposite effects on cell proliferation in different cell types (Pastan *et al.*, 1975; Dumont *et al.*, 1989), it is reasonable to ask which elements of the MAPK pathway are targets for cAMP regulation. cAMP is known to inhibit MAPK activation in Rat-1 cells (Burgering *et al.*, 1993b; Cook and McCormick, 1993; Wu *et al.*, 1993), smooth muscle cells (Graves *et al.*, 1993), CHO cells and adipocytes (Sevetson *et al.*, 1993), and to stimulate the MAPK pathway in COS (Faure *et al.*, 1994) and PC12 cells (Frödin *et al.*, 1994). Accordingly, we investigated cAMP regulation of the MAPK cascade in three cell types: Rat-1 cells, in which cAMP inhibits cell proliferation, Swiss-3T3 cells, in which cAMP stimulates cell proliferation (Rozengurt *et al.*, 1981), and COS-7 cells, in which we have already shown that cAMP activates MAPK (Faure *et al.*, 1994). Our results indicate that in certain cells cAMP inhibits Ras-dependent stimulation of Raf kinase without inhibiting downstream components of the MAPK cascade.

MATERIALS AND METHODS

DNA Constructs

The plasmids used in this work are from the following origins: pHA-MAPK1, Chinese hamster cDNA encoding an HA-tagged p44^{mapk} (Meloche *et al.*, 1992), and pHA-MAPKK, Chinese hamster cDNA encoding a HA-tagged p45^{mapkk} (Pagès *et al.*, 1994), were gifts from J. Pouyssegur, (CNRS, Nice, France). pRaf-EE, cDNA encoding a Glu-Glu-tagged version of Raf-1 (Stokoe *et al.*, 1994), was a gift from D. Stokoe and J. Hancock (Onyx Pharmaceuticals, Richmond, CA). The plasmid pGEX-MAPK1, for expression in *Esch-*

erichia coli of MAPK1 as GST fusion, was constructed as follows. We made use of an *EcoRI* site present at the junction between the sequence encoding the HA epitope and the MAPK1 coding sequence in pHA-MAPK1. A 1.8-kb *EcoRI* fragment from pHA-MAPK1 was inserted in frame into pGEX-3X (Smith and Johnson, 1988) at the *EcoRI* site. Colonies containing plasmids with the insert in the right orientation were identified by the appearance of an ~70-kDa protein fusion (26 kDa for GST plus 44 kDa for MAPK1) after quick purification on glutathione-agarose beads as described (Smith and Johnson, 1988).

Cell Culture and DNA Transfection

COS-7 and Rat-1 cells were propagated in DMEM (1 g/l glucose) with 10% fetal calf serum. Swiss-3T3 cells were propagated in DMEM (1 g/l glucose) with 10% calf serum. COS-7 cells were transfected by the DEAE-dextran/chloroquine method as previously described (Conklin *et al.*, 1992). Cells were serum starved for 18 h in serum-free medium containing 0.1% bovine serum albumin before Raf-1, MEK, and MAPK assays.

Immunoblots

Total protein extracts were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with specific antibodies. Detection was performed with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (both from Amersham, Arlington Heights, IL). The mouse monoclonal anti-MEK1 and anti-MEK2 antibodies, the rabbit polyclonal anti-MEK1 and 2 antibody, and the mouse monoclonal anti-Raf-A antibody were obtained from Transduction Laboratories (Lexington, KY) and were used at 1 μ g/ml. The rabbit polyclonal anti-Raf-B antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and was used at 0.5 μ g/ml. The rabbit polyclonal anti-ERK1 (E1.2) and anti-ERK2 (E2.1) antisera (Cook *et al.*, 1993) were generous gifts from S. Cook (Onyx Pharmaceuticals) and were used at a 1/1500 dilution. The rabbit polyclonal anti-Raf-1 serum was a generous gift from R. Schatzman (Syntex Research, Palo Alto, CA).

Production of bt-MAPK

p44^{mapk} used as a substrate for MEK assays was produced in *E. coli* (DH5 α) as a GST fusion expressed from the plasmid pGEX-MAPK1. LB medium (1.8 liters) containing 50 μ g/ml ampicillin was inoculated with 30 ml of a saturated preculture; expression was induced at OD₆₀₀ = 0.5 by adding 0.2 mM isopropyl B-D-thiogalactoside (IPTG). Three hours later the cells were harvested by centrifugation (10 min, 4°C, 4000 $\times$ g) and frozen in liquid nitrogen. Cells were lysed in 30 ml of STE buffer (50 mM Tris pH 7.2, 150 mM NaCl, 1 mM EDTA) plus protease inhibitors (0.1 mM PMSF, 1.5 μ M pepstatin, 2 μ g/ml leupeptin, and 4 μ g/ml aprotinin) and lysozyme (500 μ g/ml). After a 15-min incubation on ice, MgCl₂ (2.5 mM) and DNase I (50 μ g/ml) were added and the incubation was continued for another 30 min. Insoluble material was pelleted by centrifugation (15 min, 4°C, 27,000 $\times$ g). The GST fusion protein was purified using 2 ml of glutathione-agarose beads (Sigma) as described (Smith and Johnson, 1988) except that the purified fusion protein was dialyzed against ST buffer (50 mM Tris, pH 7.2, 150 mM NaCl). p44^{mapk} was cleaved off the GST fusion with Factor Xa (Pierce, Rockford, IL) at a 1/100 enzyme/substrate ratio in ST buffer plus 6 mM CaCl₂ for 2 h at 25°C. GST and uncleaved protein fusion were removed by binding to glutathione-agarose beads. The flow through containing p44^{mapk} was dialyzed against STEG buffer (50 mM Tris pH 7.2, 150 mM NaCl, 1 mM EDTA, 50% glycerol) and stored at -20°C. The protein concentration was measured by the Bradford method (Bradford, 1976) and the yield was about 2 mg of p44^{mapk} per liter of *E. coli* culture.

Production of Kinase-inactive MEK

Kinase-inactive MEK (MEKB) was produced in Sf9 cells infected with a recombinant baculovirus (a gift from S. Macdonald, Onyx Pharmaceuticals) expressing a Glu-Glu-tagged MEKB protein (Macdonald *et al.*, 1993). The recombinant protein was purified on a Glu-Glu monoclonal antibody affinity column as described (Macdonald *et al.*, 1993).

Measurement of $p42^{\text{mapk}}$ Activity

Cells were seeded in 50-mm plates and serum starved when they reached subconfluence. Eighteen hours later the cells were stimulated with various agonists as described in the figure legends, rinsed twice with 4 ml of Ca^{2+} - and Mg^{2+} -free ice-cold phosphate-buffered saline, and lysed in 0.6 ml of cold lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol [DTT], 40 mM $\text{Na}_2\text{P}_2\text{O}_7$, 200 μM Na_3VO_4 , 1% Triton-X100, 0.1 mM PMSF, 1.5 μM pepstatin, 2 $\mu\text{g}/\text{ml}$ leupeptin, and 4 $\mu\text{g}/\text{ml}$ aprotinin). After a 20-min incubation on ice the cells were scraped off with a rubber policeman, and the liquid was transferred into a 1.5-ml microtube and centrifuged (5 min, 4°C, 13,000 rpm) to pellet insoluble material. The supernatant was then transferred into a new microtube; protein concentration was determined using the Bio-Rad DC kit (Richmond, CA) to ensure that the amount of protein per sample stayed within a 10% margin. Endogenous $p42^{\text{mapk}}$ activity was measured essentially as described for $p44^{\text{HA-mapk}}$ (Faure *et al.*, 1994) except that $p42^{\text{mapk}}$ (ERK2) was immunoprecipitated using a rabbit polyclonal antibody ERK2 (C-14) from Santa Cruz Biotechnology (Santa Cruz, CA). Assays were performed in duplicate on 200 μl of cell lysate using 1 μg of antibody. Phosphorylation reactions were performed in 50 μl of kinase buffer (40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.5, 5 mM magnesium acetate, 2 mM DTT, 1 mM EGTA, and 200 μM Na_3VO_4) supplemented with myelin basic protein (MBP; 250 $\mu\text{g}/\text{ml}$) and $[\gamma^{32}\text{P}]\text{ATP}$ (50 μM , 2 $\mu\text{Ci}/\text{sample}$). The amount of radioactivity incorporated into MBP was determined by chromatography on P81 paper and scintillation counting as described (Faure *et al.*, 1994).

Measurement of MEK Activity

Endogenous MEK activity was measured by an immune-complex kinase assay performed on cell lysates prepared in the same way as for the $p42^{\text{mapk}}$ assay. MEK was immunoprecipitated using a rabbit polyclonal antibody directed against both MEK1 and MEK2, which was obtained from Transduction Laboratories. To confirm our results, the assay was also performed using the 12CA5 monoclonal antibody (Green *et al.*, 1982) to immunoprecipitate an HA-tagged MEK protein after transient transfection of the pHA-MEK plasmid into COS-7 cells. Assays were performed in duplicate on 200 μl of extract using 3 μg of antibody. Immune complexes were brought down with 30 μl (50% slurry) of Protein A-agarose beads (Life Technologies, Gaithersburg, MD). MEK activity was measured in 50 μl of kinase buffer (see above) supplemented with bacterially produced MAPK (40 $\mu\text{g}/\text{ml}$) and $[\gamma^{32}\text{P}]\text{ATP}$ (50 μM , 5 $\mu\text{Ci}/\text{sample}$). After a 20-min incubation at room temperature, the reaction was stopped by addition of 15 μl of 5 $\times$ SDS sample buffer and incubation at 95°C for 2 min. One-half (35 μl) of each sample was then subjected to SDS-PAGE (10% acrylamide); gels were dried and autoradiographed, and the radioactivity incorporated into bMAPK was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Two types of negative controls were performed: first, basal and EGF-stimulated samples were treated in the same way as the others but without anti-MEK antibody; second, a blank kinase reaction was performed without adding any immunoprecipitate to measure MAPK autophosphorylation. The value of the blank was subtracted from that of other samples; controls without anti-MEK antibody were not significantly different from the blank.

Measurement of Raf-1 Activity

Endogenous Raf-1 activity was measured by an immune-complex kinase assay performed on cell lysates prepared in the same way as for the $p42^{\text{mapk}}$ assay, except that 100-mm plates were used and cells were lysed in 0.8 ml of lysis buffer. Raf-1 protein was immunoprecipitated with a rabbit polyclonal antibody directed against Raf-1 (C-12) from Santa Cruz Biotechnology. To confirm our results, the assay was also performed using the Glu-Glu monoclonal antibody (Grussenmeyer *et al.*, 1985) to immunoprecipitate a Glu-Glu-tagged Raf-1 protein after transient transfection of the pRaf-EE plasmid into COS-7 cells. Assays were performed in duplicate on 400 μl of cell lysate using 1.5 μg of antibody. Immune complexes were brought down with 30 μl (50% slurry) of Protein A-agarose beads (Life Technologies). Raf-1 activity was measured in 50 μl of Raf kinase buffer (40 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM MgCl_2 , 2 mM DTT, and 1 mM EGTA) supplemented with kinase-inactive MEK-B protein (40 $\mu\text{g}/\text{ml}$) and $[\gamma^{32}\text{P}]\text{ATP}$ (50 μM , 5 $\mu\text{Ci}/\text{sample}$). After a 30-min incubation at 37°C the reaction was stopped by addition of 15 μl of 5 $\times$ SDS sample buffer and incubation at 95°C for 2 min. One-half (35 μl) of each sample was then subjected to SDS-PAGE (10% acrylamide) and subsequently treated as described for the MEK assay (see above). The same negative controls were also performed (mock I. P. and blank sample).

RESULTS

In serum-starved Rat-1, Swiss-3T3, and COS-7 cells, we studied effects of cAMP on stimulation of the MAPK pathway by EGF, which acts via a receptor tyrosine kinase, and LPA, which acts via a G protein-coupled receptor. We investigated three levels of the pathway, including MAPK itself ($p42^{\text{mapk}}$), MEK (MEK1 and 2), and Raf-1. Intracellular cAMP was elevated by treating cells with forskolin before stimulating MAPK with EGF or LPA.

Effect of cAMP on $p42^{\text{mapk}}$ (ERK2) Activity

We measured endogenous $p42^{\text{mapk}}$ by an immune-complex kinase assay. Both EGF and LPA activated $p42^{\text{mapk}}$ in all three cell lines (Figure 1), although fold-stimulation was lower in COS-7 cells (EGF, 4.5-fold; LPA, 4.8-fold) than in Rat-1 (EGF, 14.7-fold; LPA, 7.8-fold) and Swiss-3T3 (EGF, 12.9-fold; LPA, 7.7-fold) cells. The lower fold-response in COS-7 cells was associated with increased basal MAPK activity, perhaps because cells of this transformed line do not enter the G_0 phase upon serum starvation.

cAMP exerted dramatically different effects on the three cell lines. In agreement with previous reports (Burgering *et al.*, 1993b; Cook and McCormick, 1993; Wu *et al.*, 1993), cAMP markedly inhibited basal $p42^{\text{mapk}}$ activity and abolished the response to EGF or LPA (Figure 1). In contrast, forskolin by itself stimulated $p42^{\text{mapk}}$ activity 2.5-fold and failed to block EGF and LPA stimulation in both Swiss-3T3 and COS-7 cells. In accord with the known stimulatory effect of cAMP on mitogenesis in Swiss-3T3 cells (Rozengurt *et al.*, 1981), forskolin significantly increased the MAPK response to EGF or LPA. The stimulatory effect of cAMP on endogenous $p42^{\text{mapk}}$ activity in COS-7 cells

Figure 1. Effect of cAMP on p42^{mapk} activation. Serum-starved cells were either left untreated or pretreated for 10 min with 50 μ M forskolin, after which they were either left untreated (Basal), or stimulated with 50 ng/ml EGF (EGF) or 50 μ M LPA (LPA) for

was in agreement with our previous observation that elevated cAMP stimulated activity of a transiently expressed HA-tagged p44^{mapk} reporter protein (Faure *et al.*, 1994). In Rat-1 cells, cAMP also inhibited p44^{mapk} (ERK1) activity (our unpublished results); the other two cell lines do not express p44^{mapk} (see Figure 4, below).

Effect of cAMP on MEK Activity

Because MEK is the kinase immediately upstream from MAPK, it is reasonable to expect that regulation by EGF, LPA, and cAMP would show similar patterns for the two kinases. Measurements of MEK activity confirmed this expectation (compare MEK activities in Figure 2 with MAPK activities in Figure 1) in each of the three cell lines, although subtle differences were also apparent. Thus EGF and LPA increased activities of both kinases in all three cell lines, although LPA elevated MEK activity only for a short time in Rat-1 and Swiss-3T3 cells (5 min versus 10 min, Figure 2, A and B versus D and E), while elevations of p42^{mapk} were easily detectable at 10 min (Figure 1). Even at the earlier time, the effects of LPA on MEK were considerably smaller (representing only 20%) than those of EGF in Rat1 and Swiss-3T3 cells. LPA activated p42^{mapk} in these two cell lines ~50% as well as EGF, whether p42^{mapk} activity was measured at 10 min (Figure 1) or 5 min (our unpublished results). Although the MAPK/MEK discrepancy raises the possibility that LPA may elevate p42^{mapk} activity by a mechanism distinct from (and in addition to) activating MEK, it is possible that the observed small elevations in MEK activity are sufficient to activate p42^{mapk} fully.

Elevated intracellular cAMP exerted opposite effects on MEK activities in Rat-1 versus COS-7 cells, in direct accord with its effects on MAPK activities in the same cells (Figure 1). We cannot determine whether or not the elevation of p42^{mapk} induced by cAMP is mediated by MEK, because cAMP's effects on basal MEK activity were quite marginal in Swiss-3T3 and COS-7 cells (Figure 2). cAMP dramatically inhibited (> 90%) EGF- and LPA-stimulated elevations of MEK in Rat-1 cells. It also produced a real but quite modest inhibition

Figure 1 cont. another 10 min. p42^{mapk} activity was then assayed as described in MATERIALS AND METHODS and is expressed as the amount of ³²P radioactivity incorporated into MBP. The figure shows a single representative experiment. At least two independent experiments were performed with each cell line and condition. Fold-stimulations over basal (mean $\pm$ SD) were as follows. Rat-1 cells: EGF, 14.2 $\pm$ 0.8; LPA, 6.1 $\pm$ 2.5; Forskolin, 0.15 $\pm$ 0.1; EGF + Forskolin, 1.4 $\pm$ 1.0; LPA + Forskolin, 0.35 $\pm$ 0.1. Swiss-3T3 cells: EGF, 15.6 $\pm$ 3.7; LPA, 8.2 $\pm$ 0.6; Forskolin, 2.8 $\pm$ 0.2; EGF + Forskolin, 18.4 $\pm$ 5.4; LPA + Forskolin, 10 $\pm$ 0.6. COS-7 cells: EGF, 5.1 $\pm$ 0.8; LPA, 5.1 $\pm$ 0.4; Forskolin, 2.7 $\pm$ 0.2; EGF + Forskolin, 5.15 $\pm$ 0.1; LPA + Forskolin, 5.3 $\pm$ 0.7.

Figure 2. Effect of cAMP on MEK activation. Serum-starved cells were treated in the same way as in Figure 1. MEK activity was measured by an immune-complex kinase assay using bt-MAPK1 as a substrate (see MATERIALS AND METHODS). Radioactivity incorporated into bt-MAPK (A–E) was measured after SDS-PAGE by PhosphorImager analysis, and is expressed in arbitrary units representing the number of pixels contained in the image of each ^{32}P -labeled band. For Rat-1 (A and D) and Swiss-3T3 (B and E) cells, measurements performed 10 and 5 min after stimulation are shown. The mean $\pm$ SD of duplicate samples is shown in panels A, B, and C; single measurements are shown in panels D and E. Panel F shows a representative autoradiogram of the 10-min time point (10') shown for each cell line and for the 5-min time point (5') for Rat-1 and Swiss-3T3 cells. –, basal; 0, blank (no immunoprecipitate); Mock I.P., immunoprecipitation performed without antibody. The exposure times for each gel were as follows: RAT-1 5', 8 h; RAT-1 10', 18 h; SW-3T3 5', 8 h; SW-3T3 10', 18 h; COS-7 10', 24 h.

(10–30%) of MEK responses to EGF and LPA in COS-7 cells, although this effect was not reflected at the level of p42^{mapk} (Figure 1). A possible explanation for this inhibition is presented below.

MEK activity was measured by an immune-complex kinase assay. To insure the validity of these results, we wanted to be sure that the polyclonal antibody we used could immunoprecipitate both MEK isoforms and that the amounts of MEK protein immunoprecipitated were not lower in LPA-treated samples. The

antibody used in the MEK assays is described by the manufacturer as being able to recognize both MEK1 and MEK2; this was confirmed for the cell lines we used (see Figure 4, below). Proteins immunoprecipitated from the samples used for MEK assays were analyzed by Western blotting using antibodies specific for MEK1 and MEK2 (our unpublished results). For the three cell lines studied, the antibody we used immunoprecipitates both MEK1 and MEK2; amounts of immunoprecipitated MEK1 and MEK2 did not vary

significantly in samples from different cells or different treatments. Thus the weak stimulation of MEK activity by LPA in Rat-1 and Swiss-3T3 cells is not caused by inefficient immunoprecipitation. In COS-7 cells we also measured MEK activity using a HA-tagged MEK1 construct and obtained similar results.

Effect of cAMP on Raf-1 Activity

Because Raf-1 kinase can directly activate MEK, and because cAMP inhibits Ras-dependent activation of Raf-1 in Rat-1 cells (Cook and McCormick, 1993; Wu *et al.*, 1993), we assessed the regulation of Raf-1 activity by forskolin, EGF, and LPA (Figure 3). In Rat-1 cells (Figure 3A) both EGF and LPA stimulated Raf-1 activity, although the stimulation by LPA was modest

(1.5-fold). Forskolin completely blocked stimulation of Raf-1 by both EGF and LPA. Confirming previous observations (Cook and McCormick, 1993; Wu *et al.*, 1993), these results suggest that inhibition of Raf-1 activation mediates cAMP's negative regulation of the MAPK pathway in Rat-1 cells.

In COS-7 cells (Figure 3C), both EGF and LPA strongly stimulated Raf-1 activity (37- and 17-fold, respectively). Surprisingly, forskolin dramatically inhibited activation of Raf-1 by both EGF and LPA (95% and 91% inhibition, respectively). For unknown reasons, the Raf-1 assay produced weak signals in Swiss-3T3 cells, especially in comparison to COS-7 cells. EGF, but not LPA, clearly stimulated Raf-1 activity in Swiss-3T3 cells; this stimulation, as in Rat-1 and COS-7

Figure 3. Effect of cAMP on Raf-1 activation. Serum-starved cells were treated in the same way as in Figures 1 and 2. Raf-1 activity was measured by an immune-complex kinase assay using kinase-inactive MEK protein (MEK-B) as a substrate (see MATERIALS AND METHODS). (A, B, and C) The amount of radioactivity incorporated into MEK-B was measured after SDS-PAGE by PhosphorImager analysis and is expressed as arbitrary units. For Rat-1 and COS-7 cells (A and C), the mean $\pm$ standard deviation of duplicate samples is shown; for Swiss-3T3 (B), a representative experiment is shown. (D) A representative gel is shown for each cell line. The lanes are labeled in the same way as in Figure 2, panel F. The exposure times for each gel were: Rat-1, 64 h; SW-3T3, 48 h; COS-7, 18 h.

cells, was blocked by forskolin (Figure 3B). The apparent potentiating effect of forskolin on LPA stimulated Raf-1 activity in Swiss-3T3 cells (Figure 3B) was not seen in other experiments and is probably the result of experimental variation.

cAMP inhibited Raf-1 activation by EGF in COS-7 and Swiss-3T3 cells, just as it did in Rat-1 cells. This parallel inhibition of Raf-1 contrasts sharply with the opposite effects of cAMP on activation of p42^{mapk} in COS-7 and Swiss-3T3 versus Rat-1 cells (Figure 1). This contrast indicates that in COS-7 and Swiss-3T3 cells, a stimulatory signal initiated by EGF is transmitted to MEK and MAPK through pathways that are independent of Raf-1 and insensitive to inhibition by cAMP. In COS-7 cells, LPA also appeared to stimulate p42^{mapk} by a cAMP-insensitive pathway that bypasses Raf-1. Finally, it is likely that the small inhibitory effect of cAMP on MEK activation observed in COS-7 cells (Figure 2C) resulted from the marked cAMP-induced reduction in Raf-1 activity, which presumably abrogated part of the stimulatory signal received by MEK.

Endogenous Raf-1 activities in Figure 3 were measured by an immune-complex assay after immunoprecipitation of Raf-1 protein with a specific antibody. In COS-7 cells we obtained similar results (our unpublished results) measuring the activity of a transiently expressed, epitope-tagged Raf-1 protein.

Expression Pattern of Kinase Isoforms

To ask whether different patterns of cAMP regulation of the MAPK pathway correlate with the presence or absence of a particular kinase isoform, we used antibodies directed against specific kinase isoforms to determine the expression of individual kinases in ex-

tracts of Rat-1, Swiss-3T3, and COS-7 cells (Figure 4). No such correlation was apparent. More specifically: of the three known Raf kinase isoforms (Huleihel *et al.*, 1986; Sithanandam *et al.*, 1990; Stephens *et al.*, 1992), Raf-1 (74 kDa) and Raf-A (68 kDa) were expressed at comparable levels in all three cell lines; of the two Raf-B isoforms, COS-7 cells expressed small amounts of the 95-kDa protein and none of the 68-kDa protein, whereas Rat-1 cells expressed the 68-kDa isoform and trace amounts of the 95-kDa isoform. Swiss-3T3 cells expressed large quantities of both isoforms, especially the one that migrates at 68-kDa.

MEK1 and MEK2 are the two main isoforms of MEK (Crews *et al.*, 1992; Zheng and Guan, 1993a). A third isoform MEK3 is similar to MEK1 except for a 26-residue deletion caused by alternative splicing (Zheng and Guan, 1993b); because the epitopes used to raise the anti-MEK1 antibody we used are shared with MEK3, MEK3 would probably be recognized by that antibody. Figure 4 shows that all three cell lines contained both MEK1 and MEK2. The antibody used for the immune complex kinase assays is directed against both MEK1 and MEK2; indeed this antibody detected doublet bands in extracts of Swiss-3T3 and Rat-1 cells (Figure 4, panel "MEK1&2"). The doublet was not prominent in COS-7 extracts, perhaps because these cells expressed MEK1 at a lower level than did the two other cell lines (Figure 4, panel "MEK1").

Two closely related MAP kinases have been described, called p42^{mapk} and p44^{mapk} (also called ERK2 and ERK1, respectively) (Boulton *et al.*, 1991). All three cell lines expressed p42^{mapk}, but only Rat-1 cells expressed p44^{mapk}. As far as we know the two isoforms have very similar functions and they usually follow

Figure 4. Expression pattern of components of the MAP kinase pathway in COS-7, Swiss-3T3, and Rat-1 cells. Total extracts from the three cell lines were analyzed by Western blot (see MATERIALS AND METHODS) with specific antibodies as indicated in the figure. C, COS-7; S, Swiss-3T3; R, Rat-1.

the same pattern of activation. The only known exception is that p42^{mapk}, but not p44^{mapk}, becomes activated in thrombin-induced platelet activation (Papkoff *et al.*, 1994). Here we have shown that for Rat-1 cells, which contain both p42^{mapk} and p44^{mapk}, the regulation pattern was identical for both isoforms (see above).

In summary, because all three cell lines express both MEK1 and MEK2, the weak stimulation of MEK by LPA in Rat-1 and Swiss-3T3 cells, relative to COS-7 cells, cannot be accounted for by differences in MEK isoforms. Similarly, Raf-B showed distinct patterns of expression in the three cell lines, but these did not agree with different responses of the cell lines to cAMP. In particular, expression of Raf-B did not correlate with the presence of a Raf-1-independent and cAMP-insensitive pathway.

DISCUSSION

We have assessed the effects of cAMP on responses of the MAPK pathway to EGF and LPA in three cell lines. In Rat-1 cells, cAMP inhibits mitogenesis, whereas in Swiss-3T3 cells it has a modest mitogenic effect on its own and synergizes with other mitogens (Rozengurt *et al.*, 1981). As expected, we found that cAMP effects on EGF-stimulated MAP kinase activities paralleled its effects on mitogenesis in both these cell lines. The

inhibitory effects of cAMP we observed in Rat-1 cells confirm previous reports (Burgering *et al.*, 1993; Cook and McCormick, 1993; Wu *et al.*, 1993b), which showed that cAMP blocks stimulation of the MAPK pathway by inhibiting stimulation of Raf-1. Two observations were novel. First, cAMP by itself produces a small stimulation of MAPK activity in Swiss-3T3 and COS-7 cells and fails to significantly inhibit EGF or LPA stimulation of MEK and MAPK. Second, cAMP inhibits growth factor activation of Raf-1 kinase in all three cell lines, including Swiss-3T3 and COS-7 cells, as well as Rat-1 cells. Taken together, these findings indicate that stimulation of the MAP kinase pathway in Swiss-3T3 and COS-7 cells can bypass Raf-1 altogether by using an unidentified step that is not sensitive to inhibition by cAMP (see model, Figure 5). A similar situation has been described in PC12 cells (Vaillancourt *et al.*, 1994), where cAMP inhibits both Raf-1 and Raf-B stimulation by growth factors without affecting activation of MEK or MAPK. These results contribute to an emerging picture in which multiple parallel and interconnected pathways lead to MAPK stimulation and eventually to mitogenesis.

Rat-1 Cells

The inhibitory effect of cAMP on mitogenesis in these cells parallels the ability of forskolin to block growth

Figure 5. Model of the regulation of the MAP kinase pathway by cAMP. A schematic representation of the regulation of the MAP kinase pathway by a tyrosine-coupled receptor (EGF) and a G protein-coupled receptor (LPA) is presented. The negative regulation of Raf-1 by cAMP is shown, as well as its stimulatory effect on MAPK through an unknown mechanism (dashed line) in COS-7 and Swiss-3T3 cells. The fact that in COS-7 and Swiss-3T3 cells the stimulatory signal can bypass the cAMP inhibition of Raf-1 is indicated by arrows feeding directly into the pathway at the level of MEK.

factor-induced increases in activities of Raf-1, MEK, and MAPK. Other laboratories have shown that cAMP acts at least in part by blocking the interaction between Ras and Raf-1 (Cook and McCormick, 1993; Wu *et al.*, 1993). This suggests that Raf-1 is the principal avenue for transmission of a mitogenic signal from the EGF and LPA receptors to MEK and MAPK in Rat-1 cells; if these two receptors trigger a mitogenic pathway independent of Raf-1 in these cells, that pathway is also inhibited by cAMP. Our results indicate that this is not the case in Swiss-3T3 and COS-7 cells.

Swiss-3T3 Cells

In keeping with the known effects of cAMP on mitogenesis in these cells, forskolin stimulated the MAP kinase pathway on its own and slightly increased the MAPK response to both EGF and LPA (see Figure 1). In contrast to its effect on MAPK activity, forskolin alone did not increase MEK activity in these cells; the mechanism by which cAMP stimulates MAPK activity is unknown. Although the activity of Raf-1 kinase appeared to be generally reduced in Swiss-3T3 cells, forskolin clearly blocked EGF stimulation of Raf-1 despite its inability to block EGF stimulation of MAP kinase. It is possible that the modest EGF-induced increase in Raf-1 activity observed in Swiss-3T3 cells indicates that a kinase distinct from Raf-1 plays a more important role in mediating the mitogenic response to EGF in these cells.

COS-7 Cells

As in Swiss-3T3 cells, forskolin did not inhibit EGF- or LPA-induced elevations of MAPK activity in COS-7 cells. Forskolin strongly inhibited elevations of Raf-1 activity in response to either EGF or LPA in COS-7 cells. The ability of cAMP to inhibit Raf-1 stimulation in all three cell lines is not necessarily surprising, because all three cell lines express the same Raf-1 gene and cAMP probably exerts its inhibitory effect by causing phosphorylation of Raf-1 by cAMP-dependent protein kinase (Cook and McCormick, 1993; Wu *et al.*, 1993). The failure of increased cellular cAMP to block growth factor-induced increases in MEK and MAP kinase activities indicates that growth factors in COS-7 cells, like Swiss-3T3, can trigger a pathway that is both independent of Raf-1 and insensitive to cAMP.

Other Possible Pathways

Although our results point to the existence of a Raf-independent, cAMP-insensitive avenue for MAP kinase activation in Swiss-3T3 or COS-7 cells, they do not indicate the mechanism by which mitogenic stimuli in these cells manage to bypass Raf-1. Part of the signal generated by the EGF receptor in COS cells can bypass Ras, as shown by the ability of EGF to stimu-

late a recombinant Raf-1 that is engineered to bind constitutively to the plasma membrane, even in the presence of a dominant-negative mutant Ras (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). The mechanism of this Ras-independent signal is not known, and its sensitivity to inhibition by cAMP has not been tested. It may or may not be relevant to the Raf-independent, cAMP-insensitive mechanism(s) utilized by EGF to stimulate the MAP kinase pathway in COS-7 and Swiss-3T3 cells.

Our proposal of a Raf-1-independent pathway assumes that Raf-1 activates MEK and that the extent of MEK activation directly and proportionally reflects the extent of Raf-1 activation. Our data, however, do not rule out the possibility that a low level of Raf-1 activity can fully activate MEK in Swiss-3T3 and COS-7 cells but not in Rat-1 cells, owing to, for example, a MEK-directed phosphatase with much greater activity in Rat-1 cells than in Swiss-3T3 or COS-7 cells. Indeed, the postulated Raf-independent, cAMP-insensitive pathway could itself regulate the mechanism responsible for lack of proportion between Raf-1 and MEK activities (e.g., by decreasing MEK dephosphorylation in Swiss-3T3 and COS-7 cells).

Expression patterns of Raf isoforms did not correlate with responses of mitogenic pathways to cAMP (see Figure 4), i.e., the presence of Raf-1-independent and cAMP-insensitive pathway(s) did not correlate with Raf-A and Raf-B expression. MEKK (Lange-Carter *et al.*, 1993) is an additional potential activator of MEK, and others will certainly be found in future studies. We suspect that MEKK is not the basis of a Raf-1-independent pathway, for two reasons: 1) although EGF and NGF lead to MEKK activation in PC12 cells, this effect is blocked by cAMP (Lange-Carter and Johnson, 1994); 2) In COS-7 cells, EGF does not activate an HA-tagged MEKK construct (Voyno-Yasenetskaya and Bourne, unpublished observation). Each of the many pathways leading to activation of MAPK will probably prove to contain specific elements and elements shared with other pathways. MAP kinase stimulation is likely to depend on Raf-1 in many of the cells in which cAMP inhibits mitogenesis. In cells where cAMP is mitogenic—including many endocrine target cells—one or more mechanisms will be discovered that allow stimulation of MAP kinase to bypass Raf-1.

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