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cAMP antagonizes p21^{ras}-directed activation of extracellular signal-regulated kinase 2 and phosphorylation of mSos nucleotide exchange factor

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In fibroblasts, stimulation of receptor tyrosine kinases results in the activation of the extracellular signal-regulated kinase 2 (ERK2). The major signalling pathway employed by these receptors involves the activation of p21^{ras} and raf-1 kinase. Here we show that in NIH3T3 and rat-1 fibroblasts, elevation of the intracellular cAMP level results in the inhibition of ERK2 activation induced by PDGF, EGF and insulin treatment. Analysis of various signalling intermediates shows that cAMP interferes at a site downstream of p21^{ras}, but upstream of raf-1 kinase. Inhibition by cAMP depends on both the cAMP concentration and the absolute amount of p21^{ras} molecules bound to GTP, suggesting a mechanism of competitive inhibition. Also TPA-induced, p21^{ras}-independent, activation of raf-1 kinase and ERK2 is inhibited by cAMP. We have used the inhibitory effect of cAMP to investigate whether phosphorylation of mSos, a p21^{ras} nucleotide exchange factor, is dependent on the activity of the raf-1 kinase/ERK2 pathway. We found that phosphorylation of mSos, as monitored by a mobility shift, is delayed with respect to p21^{ras} and ERK2 activation and is inhibited by cAMP in a similar cell type- and concentration-dependent manner as the inactivation of ERK2. These results provide evidence for a model of p21^{ras}-directed signalling towards ERK2 that feeds back on mSos by regulating its phosphorylation status and that can be negatively modulated by protein kinase A and positively modulated by protein kinase C action.

Key words: cAMP/MAP kinase/raf-1 kinase/Ras

Introduction

The small GTPase p21^{ras} fulfils an important role in growth factor signal transduction. Microinjection of neutralizing p21^{ras} antibodies has demonstrated the requirement for p21^{ras} in a variety of cellular responses, including those induced by activators of receptor tyrosine kinases (Mulcahy *et al.*, 1985; Hagag *et al.*, 1986). Activation of p21^{ras} is regulated through binding of guanine nucleotides to p21^{ras} (for a review see Bourne *et al.*, 1990). Active p21^{ras} is bound to GTP whereas inactive p21^{ras} is bound to GDP. The ratio of GTP/GDP bound to p21^{ras} is regulated by nucleotide exchange and GTPase activity. Both nucleotide exchange and GTPase activity are catalysed by specific accessory proteins, respectively named exchange factors (mSos, p140^{GNRF}) and GTPase activating proteins (p120^{GAP}, neurofibromin). Activation of p21^{ras}, as measured by an increase in the level of p21^{ras}GTP, has now been shown to occur after treatment of cells with a variety of stimuli such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin (Downward *et al.*, 1990; Gibbs *et al.*, 1990; Satoh *et al.*, 1990a,b; Burgering *et al.*, 1991). In fibroblasts, activated receptor tyrosine kinases regulate p21^{ras} activity predominantly through an increase in nucleotide exchange (Buday and Downward, 1993b; Medema *et al.*, 1993). Activated receptor tyrosine kinases appear to increase exchange activity by stimulating a transient interaction between the receptor and the exchange factor mSos (Buday and Downward, 1993a; Chardin *et al.*, 1993; Egan *et al.*, 1993; Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993). This interaction is mediated by the adaptor protein Grb2, the mammalian homologue of *Caenorhabditis elegans* SEM-5 (Clark *et al.*, 1992; Lowenstein *et al.*, 1992). Grb2 not only binds to mSos and activated receptor tyrosine kinases but also to Shc (Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992). Shc may be involved in the regulation of p21^{ras} as well (Rozakis-Adcock *et al.*, 1992).

A detailed analysis of the biochemical signals that are directed by active p21^{ras} has become possible by the observation that p21^{ras} function is required for activation of the extracellular signal-regulated kinase 2 (ERK2) or p42$^{MAP\ kinase}$, by EGF, PDGF, NGF and insulin (De Vries-Smits *et al.*, 1992; Thomas *et al.*, 1992; Wood *et al.*, 1992). Activation of ERK2 has emerged as a central point in growth factor signal transduction. ERK2 is part of a signalling cascade involving several protein kinases/phosphatases. ERK2 can phosphorylate and activate other kinases (p90^{rsk}, Sturgill *et al.*, 1988; MAPKAP kinase 2, Stokoe *et al.*, 1992) and ERK2 itself is phosphorylated and activated by a MAP kinase kinase (MEK; Crews *et al.*, 1992), which in turn is phosphorylated and activated by yet another kinase. In signalling from activated receptor tyrosine kinases, this MEK kinase is presumably the raf-1 kinase (Howe *et al.*, 1992; Kyriakis *et al.*, 1992). How p21^{ras} activation propagates a signal to the raf-1 kinase is still unclear, but this may involve direct binding (Moodie *et al.*, 1993).

We have previously demonstrated that in rat-1 cells elevation of the intracellular level of cAMP results in the inhibition of growth factor-induced mitogenesis (Burgering *et al.*, 1989). Interestingly, expression of active, oncogenic p21^{ras}, as well as a growth factor-induced large increase in endogenous p21^{ras}GTP levels, could circumvent this cAMP-induced block in mitogenesis (Burgering *et al.*, 1989). On the basis of these results we started to investigate the effect of cAMP on p21^{ras}-mediated activation of ERK2. Here we show that an increase in the intracellular level of cAMP antagonizes the activation of ERK2 induced by activated receptor tyrosine kinases and that cAMP interferes through competitive inhibition at a site downstream of p21^{ras} but upstream of raf-1 and ERK2. Furthermore, we

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show that activation or inhibition of the raf-1 kinase/ERK2 pathway correlates with the phosphorylation status of mSos, a p21^{ras} exchange factor, suggesting that the raf-1 kinase/ERK2 pathway is involved in phosphorylating the mSos protein.

Results

Inhibition of growth factor-induced ERK2 activation by elevation of intracellular cAMP

To analyse the involvement of protein kinase A (PKA) activation in growth factor induced-activation of ERK2, rat-1 cells were treated with 8-bromo-cAMP and activation of ERK2 was determined by the appearance of a slower migrating form in gel electrophoresis due to phosphorylation of specific threonine and tyrosine residues (Leevers and Marshall, 1992). Mutation of these residues (Thr183 and Tyr185) results in a protein that can no longer be phosphorylated and lacks the mobility shift (Posada and Cooper, 1992). Treatment of cells with 8-bromo-cAMP did not result in activation of ERK2. This observation is in agreement with the findings of Lamy *et al.* (1993) who show that treatment with cAMP of dog thyrocytes, for which cAMP acts as a mitogen, does not lead to ERK2 activation. However, it was found that pretreatment of rat-1 cells for 10 min with 8-bromo-cAMP inhibited activation of ERK2 by subsequent treatment with growth factors, such as PDGF and EGF (Figure 1A, left panel). In NIH3T3 cells overexpressing the insulin receptor (A14 cells), pretreatment with 8-bromo-cAMP also inhibited insulin-induced activation of ERK2 (Figure 1A, middle panel). This effect of 8-bromo-cAMP appears to be cell type-specific, since pretreatment of PC12 cells did not result in the inhibition of EGF- or NGF-induced activation of ERK2 (Figure 1A, right panel). Also Swiss 3T3 cells were found to be refractory to the inhibitory effect of 8-bromo-cAMP (not shown).

To determine whether the effect of 8-bromo-cAMP was indeed mediated through elevation of intracellular cAMP levels, we tested the effect of other agents, known to elevate intracellular cAMP, on growth factor-induced activation of ERK2. Pretreating A14 cells with adenylate cyclase activators (prostaglandin E_1 or forskolin) in combination with a cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) also resulted in the inhibition of insulin-induced ERK2 activation (Figure 1B) and EGF- and PDGF-induced ERK2 activation (data not shown). This result indicates that the observed inhibition of 8-bromo-cAMP treatment on growth factor-induced ERK2 activation is due to the elevation of intracellular cAMP levels.

A dose–response curve revealed a half-maximal value (IC_{50}) of ~0.2 mM for 8-bromo-cAMP inhibition of ERK2 activation (Figure 1C). This value is similar to the value determined previously for inhibition of growth factor-induced [^{3}H]thymidine incorporation (Burgering *et al.*, 1989).

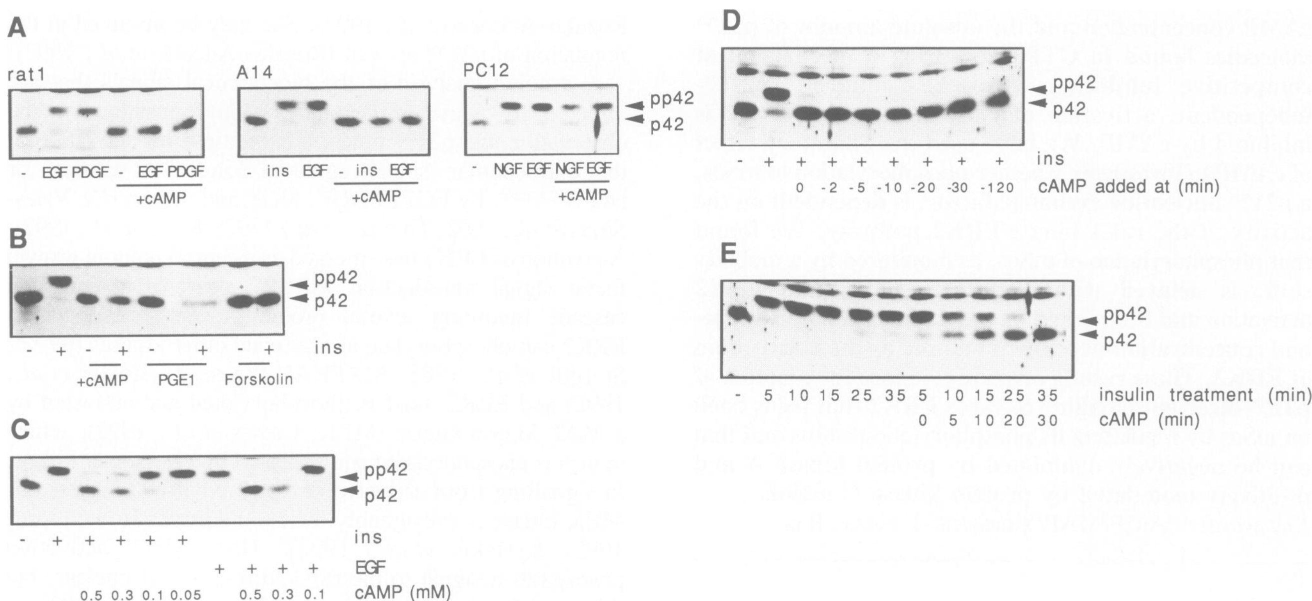

Fig. 1. 8-bromo-cAMP inhibits ERK2 activation by receptor tyrosine kinases. Different cell types (rat-1, A14 and PC12) were serum-arrested and either left untreated or treated with 0.5 mM 8-bromo-cAMP for 10 min prior to stimulation with growth factors as indicated. Growth factor stimulation was for 5 min. Final concentrations for growth factors were: EGF, 20 ng/ml; PDGF, 40 ng/ml; insulin, 1 μM; NGF, 20 ng/ml. Total protein lysate was prepared by scraping cells directly into SDS sample buffer. The activation of ERK2 was determined by the occurrence of a mobility shift. The position of the inactive ERK2 is indicated by p42 and the active (shifted) ERK2 is indicated by pp42. (**A**) 8-bromo-cAMP inhibits growth factor signal transduction towards ERK2 in A14 and rat-1 cells but not in PC12 cells (also in PC12 cells 8-bromo-cAMP by itself did not activate ERK2, not shown). (**B**) Effect of prostaglandin E_1 and forskolin, both in the presence of IBMX on insulin-induced activation of ERK2. A14 cells were serum arrested and either left untreated or pretreated (10 min) with either 8-bromo-cAMP (0.5 mM), prostaglandin E_1 (PGE_1, 30 μM) in combination with IBMX (100 μM) or forskolin (50 μM) in combination with IBMX followed by stimulation with insulin. (**C**) Effect of various concentrations of 8-bromo-cAMP on ERK2 inhibition. A14 cells were pretreated with various concentrations of 8-bromo-cAMP for 10 min prior to stimulation with EGF or insulin. (**D**) Time course analysis of 8-bromo-cAMP effect. A14 cells were pretreated with 8-bromo-cAMP for various times prior to stimulation with insulin. Time point 0 indicates that 8-bromo-cAMP and insulin are added simultaneously. (**E**) Reversion of ERK2 activation by 8-bromo-cAMP. A14 cells were stimulated with insulin for various time intervals (control time course) or stimulated with insulin for 5 min and subsequently treated with 8-bromo-cAMP for various time intervals. The time point 5 min insulin, 0 min cAMP indicates addition of insulin for 5 min followed by the addition of 8-bromo-cAMP. After mixing, the medium was immediately removed and the cells were washed with cold PBS and lysed in SDS sample buffer.

Next, we analysed the kinetics of 8-bromo-cAMP inhibition of ERK2 activation. Cells were pretreated for various time periods with 8-bromo-cAMP and subsequently stimulated with insulin for 5 min. Inhibition occurred almost immediately (between 0 and 2 min of cAMP treatment) and lasted for at least 2 h (Figure 1D). In addition, we tested whether 8-bromo-cAMP treatment could reverse growth factor-induced activation of ERK2. Cells were treated with insulin for 5 min to activate ERK2 fully, subsequently cells were treated with 8-bromo-cAMP for various time periods (Figure 1E). Activation of ERK2 was reversed by 8-bromo-cAMP treatment for 10–15 min. Together these results demonstrate that elevation of intracellular levels of cAMP, presumably through activation of the cAMP-dependent PKA, antagonizes ERK2 activation by receptor tyrosine kinases in a cell type-specific manner.

8-bromo-cAMP does not inhibit tyrosine kinase signalling at the receptor level

To determine at what level cAMP interferes with ERK2 activation by activated receptor tyrosine kinases, we analysed the effect of 8-bromo-cAMP treatment on various receptor-induced responses.

In the case of the receptor tyrosine kinases, ligand binding stimulates the intrinsic tyrosine kinase activity of the receptor, which results in autophosphorylation of specific residues within the receptor. Autophosphorylation is a prerequisite for further signalling, since kinase-negative receptors are functionally inactive (reviewed by Ullrich and Schlessinger, 1990). Therefore, we tested the effect of 8-bromo-cAMP treatment on insulin- and EGF-induced receptor autophosphorylation in A14 cells. Total protein of treated or untreated cells was separated by SDS–PAGE and immunoblotted with the anti-phosphotyrosine monoclonal antibody PY20 (Figure 2A). Ligand-induced autophosphorylation of the EGF receptor and the insulin receptor β-chain did not differ between untreated and 8-bromo-cAMP pretreated cells. In addition to the insulin receptor β-chain, insulin-dependent tyrosine phosphorylation of a protein with an approximate molecular weight of 185 kDa is observed. This protein has been identified in independent experiments as the insulin receptor substrate-1 (IRS-1) (Sun *et al.*, 1991). Tyrosine phosphorylation of IRS-1 is similarly not affected by pretreatment of cells with 8-bromo-cAMP.

Autophosphorylation of specific tyrosine residues within the receptor directs the association of SH2 domain-containing proteins with the receptor and the subsequent phosphorylation of these proteins. One of these proteins is Shc, a protein

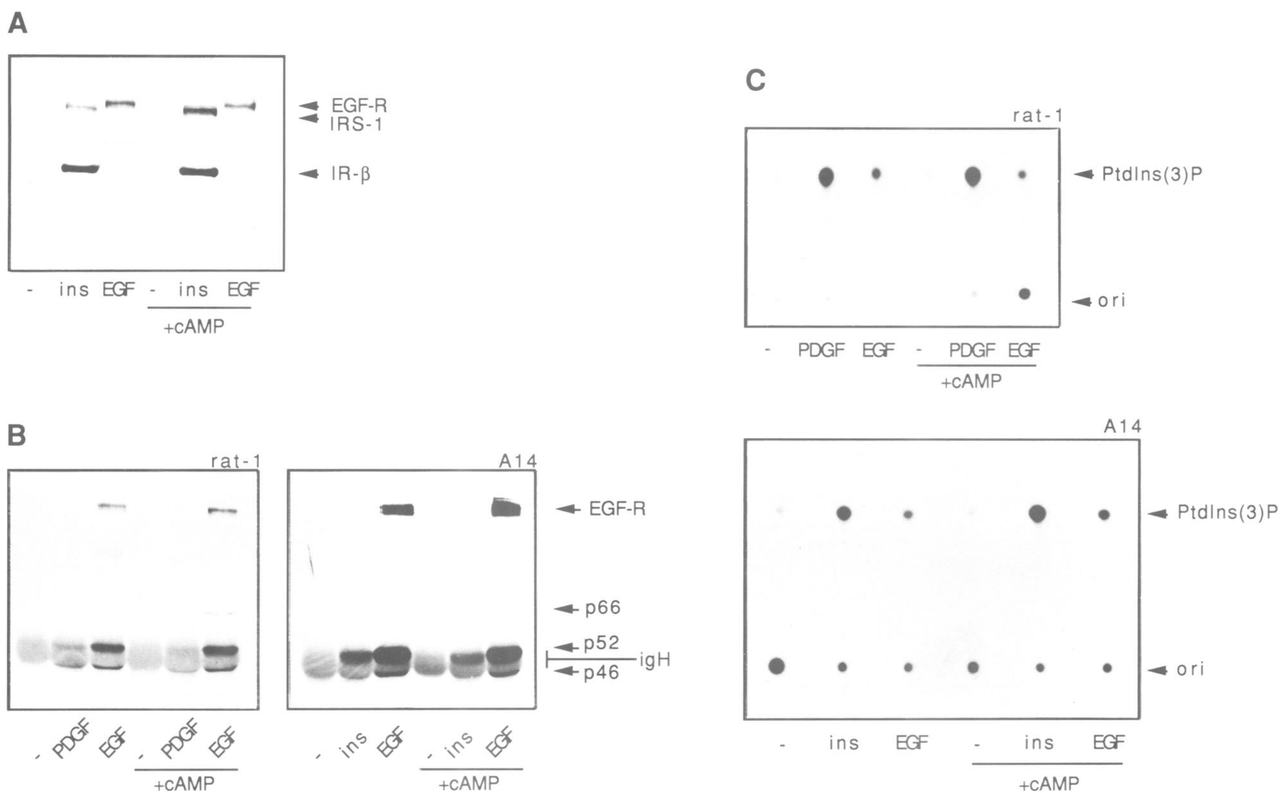

Fig. 2. 8-bromo-cAMP inhibits ERK2 activation by activated receptor tyrosine kinases at the post-receptor level. A14 or rat-1 cells were serum-starved and either left untreated or pretreated with 8-bromo-cAMP (0.5 mM) prior to stimulation with EGF (20 ng/ml), PDGF (40 ng/ml) or insulin (1 μM). (**A**) 8-bromo-cAMP does not affect ligand-induced receptor autophosphorylation on tyrosine. Total protein lysate of A14 cells was prepared by scraping cells into sample buffer. Fifty micrograms of total protein were subjected to gel electrophoresis and blotted onto nitrocellulose and probed for the presence of phosphotyrosine-containing proteins with monoclonal antibody PY20. The major tyrosine-phosphorylated proteins are indicated: IR-β is the β-chain of the insulin receptor; IRS-1 is insulin receptor substrate-1 and EGF-R is the EGF receptor. (**B**) 8-bromo-cAMP does not affect ligand-induced tyrosine phosphorylation of Shc. A14 and rat-1 cells were lysed and Shc was immunoprecipitated using a polyclonal antiserum directed against the SH2 domain of Shc. Ligand-induced tyrosine phosphorylation of Shc was detected by probing the immunoprecipitates with PY20. The different Shc isoforms are indicated (p46, p52 and p66). The tyrosine-phosphorylated EGF receptor (EGF-R) coprecipitating with Shc following treatment of cells with EGF is also indicated. (**C**) 8-bromo-cAMP does not affect ligand-induced activation of PI-3 kinase. Phosphotyrosine-containing proteins from A14 and rat-1 cells were immunoprecipitated with a polyclonal anti-phosphotyrosine serum and PI-3 kinase activity was determined by an *in vitro* kinase assay using phosphatidylinositol as a substrate. Autoradiographs of TLC plates of ^{32}P-labelled phosphatidylinositol 3-phosphate are shown.

that is implicated in receptor tyrosine kinase activation of p21^{ras} (Rozakis-Adcock *et al.*, 1992). Shc was immunoprecipitated from cells treated as indicated (Figure 2B) and analysed for tyrosine phosphorylation by immunoblotting. The extent of tyrosine phosphorylation of the different isoforms of Shc (p46, p52 and p66^{Shc}; Pelicci *et al.*, 1992) varies, p52^{Shc} being the predominantly phosphorylated form. Compared with EGF, both insulin and PDGF induced reproducibly lower levels of Shc tyrosine phosphorylation. More importantly, no differences were observed between growth factor-induced Shc tyrosine phosphorylation in untreated and 8-bromo-cAMP treated cells. EGF treatment also resulted in association of Shc with the activated EGF receptor. In contrast to the EGF-receptor, neither the activated insulin receptor β-chain (see also Pronk *et al.*, 1993) nor the PDGF receptor are detectable in Shc immunoprecipitates.

Besides Shc, the p85 subunit of PI-3 kinase associates with activated tyrosine kinase receptors, which results in the stimulation of PI-3 kinase activity (Cantley *et al.*, 1991). Therefore, the effect of 8-bromo-cAMP on growth factor-induced stimulation of PI-3 kinase activity was also analysed. Serum-starved rat-1 or A14 cells were stimulated as indicated and PI-3 kinase activity present in anti-phosphotyrosine immunoprecipitates was determined. As for Shc phosphorylation, the ability of the various growth factors to induce PI-3 kinase activity varied, PDGF being the most potent inducer. However, there was no apparent difference in growth factor-stimulated PI-3 kinase activity present in anti-phosphotyrosine precipitates of untreated or 8-bromo-cAMP pretreated cells.

These results strongly suggest that 8-bromo-cAMP antagonizes activated receptor tyrosine kinase-induced ERK2 activation at the post-receptor level.

8-bromo-cAMP does not inhibit growth factor-induced p21^{ras} activation

We have previously demonstrated that activation of p21^{ras} mediates ERK2 activation by insulin and PDGF treatment in A14 and rat-1 cells (De Vries-Smits *et al.*, 1992). Therefore, we analysed the effect of 8-bromo-cAMP on p21^{ras} activation. Cells were labelled *in vivo* with [^{32}P]orthophosphate, treated as indicated in Figure 3A and lysed in a buffer containing 1% Triton X-114 as detergent (Gutierrez *et al.*, 1989). After a phase split, separating the aqueous and the detergent phase, p21^{ras} was collected by immunoprecipitation from the detergent phase using the monoclonal antibody Y13-259. Bound nucleotides were eluted and separated by thin layer chromatography. ERK2 was immunoprecipitated from the aqueous phase, to control for the inhibitory effect of 8-bromo-cAMP on ERK2 activation. No inhibitory effect of 8-bromo-cAMP on growth factor-induced increase of p21^{ras}GTP levels was observed after 5 min of growth factor stimulation. Also the kinetics

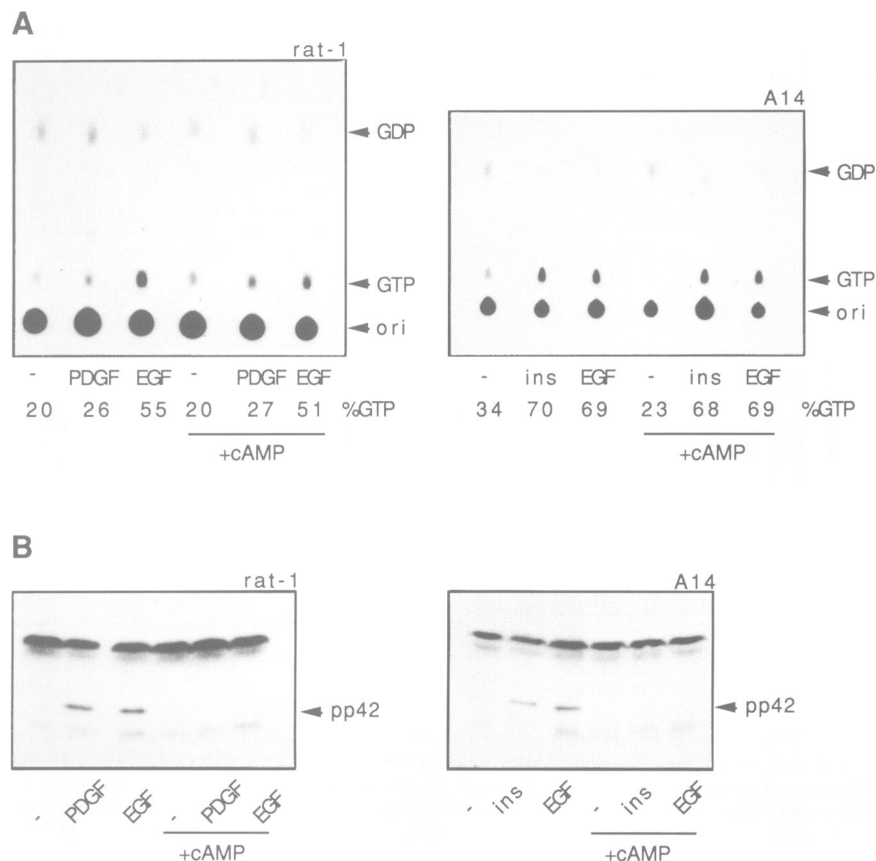

Fig. 3. 8-bromo-cAMP does not inhibit ligand-induced activation of p21^{ras}. Serum-starved A14 and rat-1 cells were labelled *in vivo* with [^{32}P]orthophosphate and stimulated as indicated. Cells were lysed and p21^{ras} and ERK2 were immunoprecipitated from the same lysate, i.e. p21^{ras} from the detergent phase and ERK2 from the aqueous phase after Triton X-114 phase split. (**A**) Nucleotides bound to p21^{ras} were eluted and separated by ascending chromatography. Autoradiographs of TLCs are shown, the positions of the cold GTP and GDP standards are indicated; ori is the origin of application. (**B**) ERK2 immunoprecipitates were separated on a 10% polyacrylamide gel, followed by autoradiography. The position of ERK2 is indicated.

of p21^{ras} activation (up to 20 min) did not differ between untreated and 8-bromo-cAMP pretreated cells (data not shown). As expected, phosphorylation of ERK2 was completely inhibited (Figure 3B). A small decrease in uninduced levels of p21^{ras}GTP, following 8-bromo-cAMP treatment, could be observed in A14 cells. This is similar to the effect reported by Buday and Downward (1993b). These results indicate that cAMP interferes in ERK2 activation at a site downstream of p21^{ras}.

8-bromo-cAMP inhibits receptor tyrosine kinase-induced raf-1 activation

raf-1 kinase activation is induced by p21^{ras} and may mediate ERK2 activation (Howe *et al.*, 1992; Kyriakis *et al.*, 1992). Therefore, as the next possible intermediate in the route towards ERK2 we analysed growth factor-induced raf-1 kinase activation in the absence or presence of 8-bromo-cAMP. Again, cells were left untreated or pretreated with 8-bromo-cAMP prior to growth factor stimulation. After growth factor stimulation cells were lysed and the activation of raf-1 kinase was determined by the presence of raf-1 kinase with reduced electrophoretic mobility, due to phosphorylation (Figure 4A), or by an *in vitro* kinase assay of immunoprecipitated raf-1 kinase using MEK as a substrate (Figure 4B). Both approaches show that growth factor-induced activation of raf-1 kinase was inhibited by 8-bromo-cAMP in the same manner as ERK2. This suggests that the site of inhibition for 8-bromo-cAMP is at the level of raf-1 kinase or at a site upstream of raf-1 kinase.

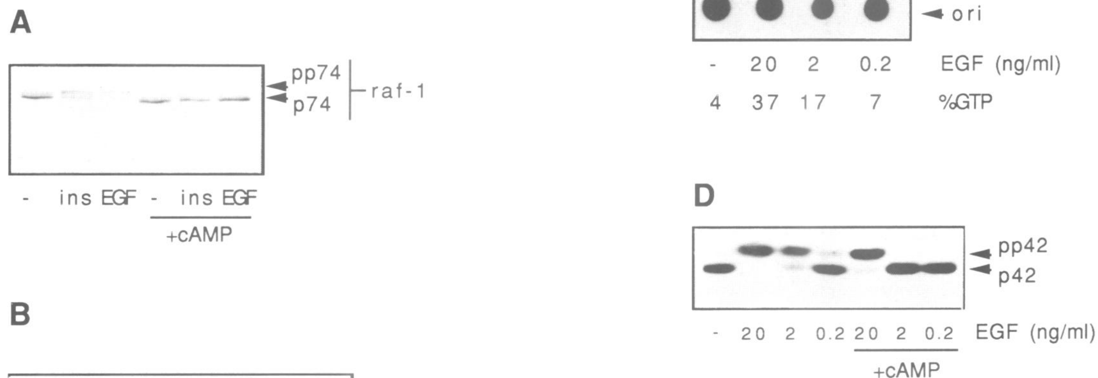

Fig. 4. 8-bromo-cAMP inhibits the activation of raf-1 kinase. A14 cells were serum-starved and either left untreated or pretreated with 8-bromo-cAMP (0.5 mM) prior to stimulation with EGF (20 ng/ml), insulin (1 μM) or TPA (100 ng/ml). raf-1 was immunoprecipitated using a polyclonal raf-1 antiserum (kindly provided by Johan Van Lint, University of Leuven). (**A**) 8-bromo-cAMP inhibits the raf-1 mobility shift. raf-1 immunoprecipitates were separated on a 10% SDS–polyacrylamide gel and probed with polyclonal antiserum directed against the SP63 peptide (see Materials and methods). (**B**) 8-bromo-cAMP inhibits ligand-induced raf-1 kinase activity. raf-1 immunoprecipitates were incubated with baculovirus-produced MEK protein (kindly provided by Susan Macdonald, Onyx Pharmaceuticals) in the presence of [γ-^{32}P]ATP. The reaction mixture was separated on a 10% polyacrylamide gel followed by autoradiography. The position of MEK is indicated.

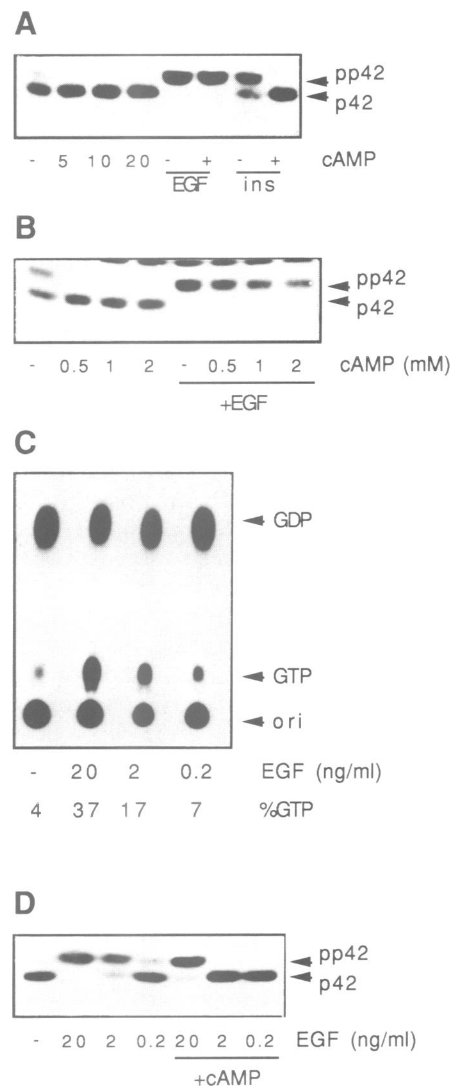

Fig. 5. Inhibition of 8-bromo-cAMP occurs through competitive inhibition. Serum-starved H13 cells (overexpressing normal H-*ras*) were either left untreated or pretreated with 8-bromo-cAMP (0.5 mM, unless stated otherwise) prior to stimulation with growth factors (insulin, 1 μM and EGF, 20 ng/ml, unless stated otherwise) as indicated. Total protein lysate was prepared by scraping cells into sample buffer. The activation of ERK2 was determined by the occurrence of a mobility shift. The position of the inactive ERK2 is indicated by p42 and the active (shifted) ERK2 is indicated by pp42. The GDP/GTP content of p21^{ras} was determined as described in the legend of Figure 3. (**A**) 8-bromo-cAMP inhibits basal and insulin-stimulated ERK2 activation, but not EGF-induced ERK2 activation in H13 cells. (**B**) Increasing 8-bromo-cAMP concentration does not restore the inhibitory effect on EGF-induced ERK2 activation. Cells were treated with different concentrations of 8-bromo-cAMP as indicated in the presence or absence of EGF and ERK2 activation was determined by mobility shift. (**C**) Activation of p21^{ras} is dependent on EGF concentration. Cells were treated with different concentrations of EGF as indicated. Shown is an autoradiograph of the TLC separation of ^{32}P-labelled GDP and GTP bound to p21^{ras}. The position of the cold GTP/GDP standards is indicated, ori is the origin of application. The level of p21^{ras}GTP, expressed as the percentage of total nucleotide bound to p21^{ras} (GTP + GDP), is given under each lane. (**D**) The inhibitory effect of 8-bromo-cAMP on ERK2 activation is dependent on the EGF concentration. Cells were treated with various concentrations of EGF as indicated and the activation of ERK2 was determined by the occurrence of a mobility shift.

Inhibition occurs through a mechanism of competitive inhibition

The observation that 8-bromo-cAMP inhibits ERK2 activation in a cell type-specific manner, may suggest the involvement of a cell type-specific factor, which may be present in limiting amounts. To gain further insight into the mechanism of inhibition by 8-bromo-cAMP, we analysed the effect of 8-bromo-cAMP in H13 cells, which overexpress normal p21^{ras} ~100-fold (Downward *et al.*, 1988). Also in these cells 8-bromo-cAMP by itself did not induce ERK2 activation (Figure 5A). Compared with the parental rat-1 cell line, an increase in basal ERK2 activity was observed in growing (not shown) and serum-arrested H13 cells (Figure 5A and B, left lanes). Since H13 cells are partially transformed cells (Burgering *et al.*, 1989), this increase is reminiscent of the observed increase in ERK2 activity in cells transformed by oncogenic p21^{ras} (Leevers and Marshall, 1992). This small increase in basal ERK2 activity is downregulated by 8-bromo-cAMP treatment. Insulin-induced ERK2 activity was also inhibited by the usual 8-bromo-cAMP pretreatment protocol, but surprisingly, EGF-induced ERK2 activation was not inhibited by pretreatment with 8-bromo-cAMP. Increasing the 8-bromo-cAMP concentration did not restore the inhibitory effect (Figure 5B). However, in these cells treatment with EGF at 20 ng/ml induces a much larger increase in p21^{ras}GTP levels compared with insulin treatment. The level of p21^{ras}GTP induced by EGF treatment is dependent on the concentration of EGF used (Osterop *et al.*, 1993). Upon treatment with decreasing concentrations of EGF, the induced level of p21^{ras}GTP reduces accordingly (Figure 5C). Thus, we tested the effect of 8-bromo-cAMP on ERK2 activation at different concentrations of EGF. As shown in Figure 5D pretreatment with 8-bromo-cAMP only inhibited induction of ERK2 activity by low concentrations of EGF ($\leq$2 ng/ml) and not by higher concentrations ($\geq$20 ng/ml). These results indicate a mechanism of competitive inhibition. Apparently, increasing the total number of p21^{ras}GTP molecules by higher EGF concentrations can overcome the inhibitory effect of 8-bromo-cAMP, demonstrating that inhibition is not only dependent on the cAMP concentration (Figure 1C), but on the p21^{ras}GTP concentration as well.

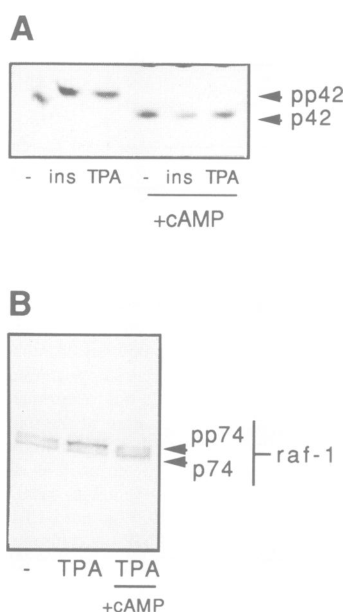

Fig. 6. 8-bromo-cAMP inhibits TPA-induced ERK2 and raf-1 kinase activation. A14 cells were serum-starved and either left untreated or pretreated with 8-bromo-cAMP (0.5 mM) prior to stimulation with insulin (1 μM) or TPA (100 ng/ml). (**A**) Inhibition of TPA-induced ERK2 activation. ERK2 activation is determined by a mobility shift. (**B**) Inhibition of TPA-induced raf-1 kinase activation. raf-1 kinase activation as monitored by the occurrence of a mobility shift. The inhibition by 8-bromo-cAMP of TPA-induced raf-1 kinase activity, as measured using MEK as substrate, is presented in Figure 4B, last two lanes.

Non-p21^{ras}-mediated ERK2 activation is also inhibited by 8-bromo-cAMP

In fibroblasts, activation of protein kinase C (PKC) appears not to be involved in p21^{ras}-mediated activation of ERK2 by receptor tyrosine kinases and activation of ERK2 by direct stimulation of PKC is not mediated by p21^{ras} (Medema *et al.*, 1991; De Vries-Smits *et al.*, 1992). We therefore wanted to know whether 8-bromo-cAMP pretreatment also affected PKC-induced activation of ERK2. Pretreatment of cells with 8-bromo-cAMP resulted in the inhibition of TPA-induced ERK2 activation (Figure 6A). 8-bromo-cAMP also inhibited raf-1 kinase activation, as measured by the occurrence of a mobility shift (Figure 6B) or by an *in vitro* kinase assay using MEK as a substrate (Figure 4B, last two lanes). This suggests that cAMP/PKA acts on a factor downstream of p21^{ras} in receptor tyrosine kinase signalling towards raf-1 kinase/ERK2 that is also involved in TPA/PKC signalling towards raf-1 kinase/ERK2.

Inhibition of receptor tyrosine kinase-induced mSos phosphorylation

In fibroblasts, activation of p21^{ras} by insulin and EGF occurs through enhanced nucleotide exchange (Buday and Downward, 1993b; Medema *et al.*, 1993). For EGF it has been shown that this is accompanied by complex formation between the EGF receptor, the Grb2 adaptor protein and the mSos nucleotide exchange factor (Buday and Downward, 1993a). Furthermore, upon EGF stimulation of HER14 cells the electrophoretic mobility of mSos reduces and a mobility shift is observed. This is due to phosphorylation, since treatment of this shifted form with phosphatases increases again its electrophoretic mobility (Rozakis-Adcock *et al.*, 1993). After treatment of A14 cells for different time periods with insulin we observed a decrease in the electrophoretic mobility of mSos as well (Figure 7A). Surprisingly, this shift in the mobility of mSos could not be detected during the earlier time points of insulin treatment, yet could be detected after 5 min and thereafter. Similar results were obtained with EGF (not shown). Since both EGF- and insulin-induced activation of p21^{ras} is already maximal at 2 min (Burgering *et al.*, 1991; Medema *et al.*, 1993; Osterop *et al.*, 1993), this finding suggests that mSos phosphorylation occurs independent of, or following, p21^{ras} activation. In analogy with yeast, where CDC25 phosphorylation appears to result from RAS activation (Gross *et al.*, 1992), we tested for the possibility that in fibroblasts mSos phosphorylation occurs as a consequence of p21^{ras} activation. Therefore, we analysed the effect of cAMP on growth factor-induced mSos phosphorylation. Both insulin- and EGF-induced mSos phosphorylation was blocked by 8-bromo-cAMP pretreatment (Figure 7B). To show that this inhibition was due to inactivation of the raf-1 kinase/ERK2 cascade we

analysed mSos phosphorylation after treatment of H13 cells with different concentrations of EGF. EGF at 20 ng/ml and at 2 ng/ml induced mSos phosphorylation, but as with the inhibition of ERK2 activation, mSos phosphorylation induced by 20 ng/ml EGF was not blocked by 8-bromo-cAMP, whereas mSos phosphorylation induced by 2 ng/ml EGF was blocked by 8-bromo-cAMP (Figure 7C). We also tested the effect of 8-bromo-cAMP in Swiss 3T3 cells, where 8-bromo-cAMP does not inhibit ERK2 activation. As shown in Figure 7C, EGF-induced mSos phosphorylation in Swiss 3T3 cells was not inhibited by 8-bromo-cAMP. It is also noteworthy that a variable amount of mSos appeared already phosphorylated in unstimulated H13 cells. Since there is a low level of constitutive activation of the raf-1 kinase/ERK2 pathway in the H13 cells (see Figure 5A and B, left lanes) this corroborates the idea that phosphorylation of mSos is due to kinase activity of this pathway. Taken together these data strongly suggest that the raf-1 kinase/ERK2 cascade is involved in phosphorylation of the mSos protein.

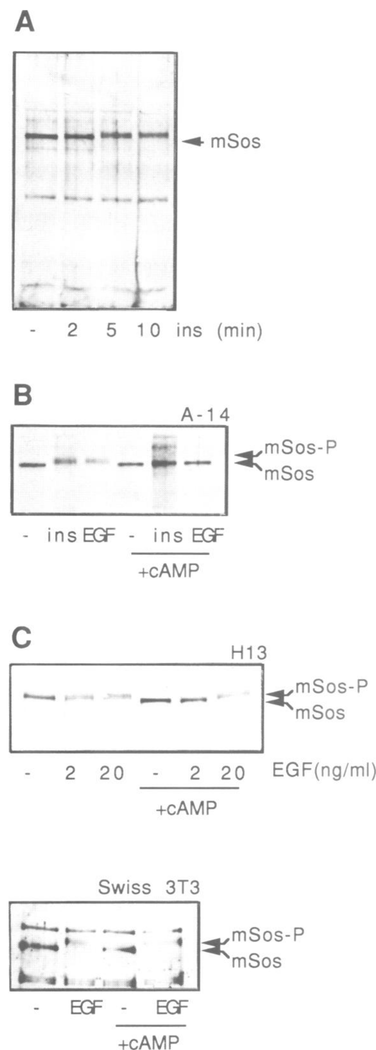

Fig. 7. mSos phosphorylated by the raf-1 kinase/ERK2 cascade. mSos protein was collected by incubating the lysates with GST–Grb2 fusion protein coupled to glutathione beads. Bound proteins were separated by SDS–PAGE and immunoblotted with an antiserum directed against the mammalian mSos protein. (**A**) Kinetics of insulin-induced mSos phosphorylation. Serum-starved A14 cells were treated with insulin (1 μM) for time periods indicated. (**B**) 8-bromo-cAMP inhibits EGF- and insulin-induced mSos phosphorylation. A14 cells were serum starved and treated as indicated. (**C**) 8-bromo-cAMP does not inhibit mSos phosphorylation in H13 cells stimulated with high concentrations of EGF and in Swiss 3T3 cells. Serum-starved H13 and Swiss 3T3 cells were treated with different concentrations of EGF as indicated (H13) or with 20 ng/ml EGF (Swiss 3T3).

Discussion

Inhibition of ERK2 activation by 8-bromo-cAMP

In this study we have investigated the effect of 8-bromo-cAMP on the regulation of ERK2 by growth factors that activate receptor tyrosine kinases. In fibroblasts 8-bromo-cAMP treatment inhibited ERK2 activation. The effect of 8-bromo-cAMP was due to an increase in intracellular cAMP levels, since other treatments that induce an increase in cAMP levels, e.g. forskolin/IBMX and PGE_1/IBMX, also inhibited ERK2 activation. Inhibition was cell type-specific, i.e. cAMP inhibited ERK2 activation in rat-1, NIH3T3 and cell lines derived herewith, but not in PC12 (Figure 1A) and Swiss 3T3 cells (not shown). Inhibition was concentration (IC_{50} ~0.2 mM) and time dependent (between 0 and 2 min after addition). Elevating intracellular cAMP could also revert activation of ERK2, even in the continuous presence of growth factor.

We have determined the site of interference of 8-bromo-cAMP by analysing different steps of the signalling cascade from the receptor towards ERK2. Receptor activation, Shc phosphorylation, PI-3 kinase activation and p21^{ras} activation were not affected by 8-bromo-cAMP treatment. Activation of raf-1 kinase, as measured by both a mobility shift due to phosphorylation and an *in vitro* kinase assay using MEK as a substrate, was inhibited by 8-bromo-cAMP treatment in a manner similar to ERK2. These results suggest that cAMP, presumably through activation of cAMP-dependent PKA, interferes with signalling towards ERK2 at a site downstream of p21^{ras}, but upstream of raf-1 kinase.

Not only ERK2 activation by activated receptor tyrosine kinases was blocked by 8-bromo-cAMP treatment, but also TPA-induced activation of ERK2. Considerable controversy exists over the role of PKC in p21^{ras}-mediated signalling. In the cell lines used in this study (rat-1, NIH3T3) a role for PKC upstream of p21^{ras} seems unlikely since the induction of ERK2 activity by TPA does not require functional p21^{ras} (De Vries-Smits *et al.*, 1992; B.M.Th. Burgering *et al.*, submitted) and TPA does not activate p21^{ras}, as measured by the nucleotide content of p21^{ras} after TPA stimulation (Medema *et al.*, 1991). Also downstream of p21^{ras}, a role for TPA-sensitive PKC in the pathway towards ERK2 activation seems unlikely, since in these cells inhibition of PKC activity does not block growth factor-induced ERK2 activation (De Vries-Smits *et al.*, 1992). Thus, by these criteria PKC-induced activation of ERK2 appears to be a pathway separate from the pathway involving p21^{ras}. Here we found that both TPA-induced raf-1 kinase and ERK2 activation were inhibited by 8-bromo-cAMP. These results indicate that in fibroblasts PKC fuels into the raf-1 kinase/ERK2 pathway at the level of raf-1 kinase activation. In keeping with this are recent results that suggest that PKC may directly activate the raf-1 kinase (Sözeri *et al.*, 1992; Kolch *et al.*, 1993).

Mechanism of inhibition

Treatment of H13 cells, overexpressing normal p21^{ras}, resulted in differential inhibition of ERK2 activation by

8-bromo-cAMP. At high EGF concentration a relatively large amount of p21^{ras}GTP molecules is generated and ERK2 activation is not inhibited by 8-bromo-cAMP, whereas at lower EGF concentrations fewer p21^{ras}GTP molecules are generated and ERK2 activation is inhibited by 8-bromo-cAMP. Active p21^{ras} is thought to generate a downstream signal through interaction with an effector molecule of which the identity is as yet unknown. Considering this mechanism of downstream signalling the differential effect of 8-bromo-cAMP may be explained by two different models of competitive inhibition. First, cAMP induces binding between a molecule (X) and p21^{ras}GTP resulting in an inactive complex and thus X competes with the genuine p21^{ras} effector for binding to p21^{ras}GTP. Second, cAMP induces binding of a molecule (X) to the genuine p21^{ras} effector molecule and thus competes with p21^{ras}GTP for binding to its effector molecule. In the first model cAMP will increase the affinity of X for p21^{ras}GTP. Depending on the relative affinities of the p21^{ras} effector and X for p21^{ras}GTP a high level of p21^{ras}GTP will ensure that enough p21^{ras}GTP can interact with the genuine effector to activate downstream signalling. In the second case the large increase in p21^{ras}GTP in EGF treated H13 cells will drive the equilibrium from X/p21^{ras}–effector interaction towards p21^{ras}GTP/p21^{ras}–effector interaction. Which of these two possibilities accounts for the inhibitory effect of 8-bromo-cAMP remains to be determined.

A good candidate to mediate the effect of cAMP by a mechanism as suggested by the second possibility is the rap-1/Krev protein. Krev was initially discovered as a gene structurally related to the family of *ras* genes, which could reverse *ras* transformation when expressed at high levels (Kitayama *et al.*, 1989). Krev is identical to rap-1, the latter being cloned on the basis of p21^{ras} structural homology (Kawata *et al.*, 1988; Pizon *et al.*, 1988). Rap-1 and p21^{ras} are identical within the region of p21^{ras} genetically defined as the effector region. In addition rap-1GTP binds more tightly to p120^{GAP} than p21^{ras}GTP does (Frech *et al.*, 1990; Hata *et al.*, 1990). These results have led to the suggestion that rap-1 causes reversion of transformation through competition for the genuine effector of p21^{ras}. In this respect it is noteworthy that similar to rap-1 and Krev overexpression, cAMP treatment of *ras*-transformed cells also leads to a reversed phenotype (Pastan and Willingham, 1978).

Further analogy between the action of cAMP and rap-1 is suggested by transient transfection experiments of NIH3T3 cells, in which it was shown that rap-1 can inhibit src and p21^{ras}-induced c-*fos* activation but not c-*fos* induction by activated raf-1 kinase (Sakoda *et al.*, 1992). This result is in agreement with our finding that 8-bromo-cAMP interferes with the raf-1 kinase/ERK2 pathway at a site between p21^{ras} and raf-1 kinase. Yet, in Swiss 3T3 cells microinjection of recombinant rap-1 induces cellular DNA synthesis and thus, apparently does not antagonize p21^{ras} function in these cells (Yoshida *et al.*, 1992). This in turn is in agreement with our finding that in Swiss 3T3 cells cAMP does not inhibit ERK2 activation.

Recently, Cook *et al.* (1993) showed that expression of rap-1GTP (rap-1^{val12}) at physiological levels can indeed inhibit growth factor-induced activation of ERK2, without interfering with growth factor-stimulated increase in p21^{ras}-GTP. Nevertheless, in fibroblasts rap-1 is found to associate with the Golgi apparatus (Noda, 1993). This apparently contradicts a model of direct antagonism between rap-1 and p21^{ras}. However, rap-1 is an *in vitro* substrate for PKA and is phosphorylated *in vivo* after cAMP treatment and subcellular localization may be regulated by PKA (Lapetina *et al.*, 1989).

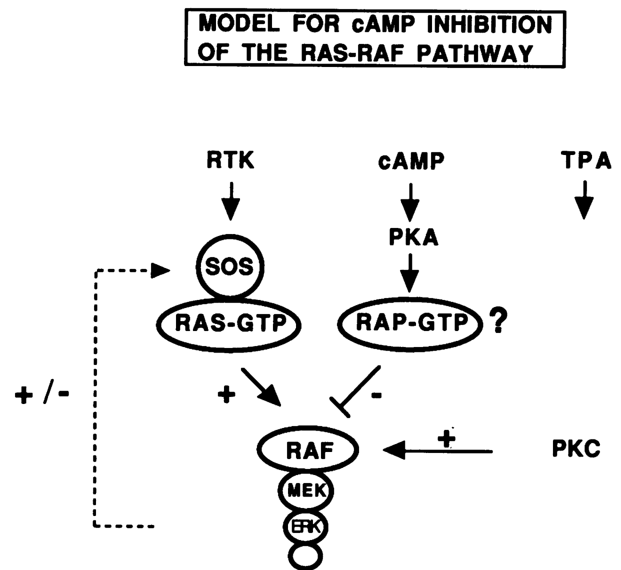

Fig. 8. A model for integrated growth factor signalling from receptor tyrosine kinases towards the raf-1 kinase/ERK2 cascade.

The results with 8-bromo-cAMP as described and discussed in this study suggest a model for ERK2 activation as depicted in Figure 8. The major pathway for activation of the raf-1 kinase/ERK2 cascade involves p21^{ras} activation. Both activation of PKA and PKC can modulate the activation of this kinase cascade. PKA acts as a negative regulator and PKC as a positive regulator. As far as can be determined precisely, the site of interaction of both PKA and PKC with the raf-1 kinase/ERK2 cascade maybe the same. In this respect it will be of interest to determine the effect of PKC stimulation on rap-1 function.

cAMP inhibits the phosphorylation of the mSos nucleotide exchange factor

After growth factor treatment of fibroblasts the electrophoretic mobility of mSos, a p21^{ras} nucleotide exchange factor, is reduced due to phosphorylation (Rozakis-Adcock *et al.*, 1993). The kinetics of mSos phosphorylation (Figure 7A) lagged behind the kinetics of p21^{ras} activation, as reported previously by Burgering *et al.* (1991). This observation may indicate that phosphorylation of mSos is directed by active p21^{ras}. This would be analogous to the regulation of CDC25, the yeast exchange factor for RAS (Gross *et al.*, 1992). In yeast, glucose starvation results in a RAS-mediated rise in intracellular cAMP. The cAMP produced activates cAMP-dependent kinase, which in turn phosphorylates CDC25. The phosphorylated form of CDC25 is predominantly localized in the cytosol, suggesting that phosphorylation of CDC25 reduces the interaction between RAS and CDC25.

Previously, we have used vaccinia virus expressing the dominant negative p21^{ras} mutant p21^{rasAsn17} to study p21^{ras} involvement in growth factor signal transduction (De Vries-Smits *et al.*, 1992). Since p21^{rasAsn17} is thought to inhibit

p21^ras activation by binding to mSos and thereby competing for the mSos–p21^ras interaction (Buday and Downward, 1993a), p21^rasAsn17 cannot be used to analyse p21^ras-directed effects on mSos. The effects of cAMP described in this study open the way to analyse p21^ras-directed events from a new perspective. Our results show that in mammalian cells, in contrast to yeast, cAMP elevation by itself did not result in phosphorylation of mSos, within the time course that activated receptor tyrosine kinases did phosphorylate mSos. Elevation of cAMP levels resulted in the inhibition of mSos phosphorylation induced by growth factors. This inhibition correlated with the inhibition of the raf-1 kinase/ERK2 pathway. In H13 cells 8-bromo-cAMP inhibited ERK2 activation and mSos phosphorylation induced by low concentration of EGF (2 ng/ml), whereas ERK2 activation and mSos phosphorylation were not inhibited by high concentration of EGF (20 ng/ml). In addition, in Swiss 3T3 cells 8-bromo-cAMP did not inhibit EGF-induced ERK2 activation and mSos phosphorylation. Thus, it is conceivable that one of the kinases within this cascade phosphorylates the mSos protein. The functional consequence of mSos phosphorylation remains to be determined, but the results in yeast may suggest that phosphorylation is involved in negative feedback. However, we observed no differences in the kinetics of p21^ras activation in the presence or absence of 8-bromo-cAMP. This may indicate that the effect of mSos phosphorylation on p21^ras activation is rather subtle, or that other regulatory mechanisms, not involving mSos, are functioning in p21^ras activation as well.

Materials and methods

General methods and cell culture

8-bromo-cAMP (Boehringer, Mannheim) was dissolved in 10 mM Tris (pH 7.0). Phosphate-free DMEM, prostaglandin E_1, forskolin, IBMX, insulin, PDGF BB homodimer and EGF were obtained from Sigma. [^{32}P]orthophosphate (3000 Ci/mmol) and enhanced chemiluminescence (ECL) was from Amersham and used according to the manufacturer's procedures. Monoclonal anti-phosphotyrosine antibody (PY20) was from ICN.

rat-1 and A14 cells were routinely grown in DMEM, 10% FCS and 0.05% glutamine. PC12 cells were grown in DMEM (high glucose), 10% FCS (heat inactivated) and 5% horse serum. For experiments subconfluent cultures of cells were grown in DMEM and 0.5% FCS [PC12 cells DMEM (high glucose) and 0.5% horse serum] for 18 h prior to stimulation with growth factors. Except for raf-1 experiments for which cells were serum-arrested for 48 h prior to stimulation with growth factors.

GTP/GDP binding to p21^ras

Serum-arrested cell cultures were labelled with 100 μCi/ml [^{32}P]-orthophosphate for 4 h. After growth factor stimulation, cells were lysed and processed as described (Burgering *et al.*, 1991). Mature p21^ras was separated from non-processed p21^ras by a Triton X-114 phase split as described by Gutierrez et al. (1989) and Burgering *et al.* (1991). p21^ras was precipitated from the detergent phase with monoclonal antibody Y13-259 [or as a control immunoprecipitation with a non-related antibody directed against SV40 large T (KT3)]. Bound nucleotides were eluted from immunoprecipitates and analysed by ascending TLC as described by Burgering *et al.* (1991) and after exposure to Kodak XAR film spots corresponding to GDP and GTP were cut out. The amount of radioactivity was determined by liquid scintillation counting.

Determination of ERK2 activation

Cells were starved for 24 h in 0.5% fetal calf serum and either left untreated or treated with growth factors as indicated. Cells were scraped into sample buffer and 50 μg of total protein was electrophoresed on a 10% SDS–polyacrylamide gel, blotted to nitrocellulose and incubated with an antiserum (anti-ERK2) directed against recombinant ERK2. Immune complexes were detected by HRP-conjugated second antibodies, followed by ECL.

Determination of PI-3 kinase activity

PI-3 kinase assay was performed essentially as described previously by Burgering *et al.* (1991). In brief, anti-phosphotyrosine immunoprecipitates collected on protein A–Sepharose were incubated in 30 mM HEPES (pH 7.5), 200 μM adenosine and 0.2 mg/ml sonicated phosphatidylinositol for 15 min at 25°C in a total volume of 50 μl. Adenosine was included to inhibit any contaminating PI-4 kinase activity (Whitman *et al.*, 1987). The reaction was started by adding 30 mM $MgCl_2$, 40 μM ATP and 10 μCi [γ-^{32}P]ATP (final concentrations) and incubation continued for another 25 min at 25°C. The reaction was terminated by the addition of 100 μl 1 M HCl and quick mixing. Lipids were extracted by addition of 200 μl chloroform–methanol (1:1). The organic phase was washed one additional time with methanol–1 M HCl (1:1). An aliquot of the organic phase of 50 μl was applied to a silica gel G plate and developed in chloroform–methanol–4 M NH_4OH (45:35:10).

Shc immunoprecipitation and immunoblotting

After treatment, cells were washed twice with ice-cold PBS–1 mM sodium orthovanadate and after lysis in RIPA (30 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μM PMSF, 0.1 μM aprotinin, 1 μM leupeptin and 1 mM sodium orthovanadate) the cell lysates were cleared by centrifugation. Shc immunoprecipitations were performed with a polyclonal rabbit antiserum, raised against a GST–Shc fusion protein as described earlier by Pronk *et al.* (1993). Immunoprecipitated proteins were separated by SDS–PAGE and transferred to nitrocellulose by electroblotting. Phosphotyrosine immunoblots were performed with PY20 and subsequent detection by incubation with goat-anti-mouse IgG linked to horseradish peroxidase followed by ECL. Immunoprecipitations were performed on equal amounts of protein.

raf-1 kinase activity determination

For determination of the mobility shift of raf-1 kinase, cells were directly scraped into sample buffer and 50 μg of total protein was analysed by electrophoresis through polyacrylamide gels followed by western blotting onto nitrocellulose. Western blots were probed with anti-raf-1 kinase polyclonal antiserum directed against a peptide of the C-terminal part of raf-1 (SP63 peptide; Carroll *et al.*, 1990). For *in vitro* kinase activity measurements, cells were washed with ice-cold PBS and lysed in buffer containing 20 mM Tris (pH 7.9), 137 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 μM PMSF, 0.1 μM aprotinin, 1 μM leupeptin, 1 mM sodium orthovanadate, 10 mM NaF and 1 mM sodium pyrophosphate. After lysis, insoluble material was removed by centrifugation and raf-1 kinase was immunoprecipitated. Immune complexes were collected with protein G–Sepharose and washed twice in lysis buffer. Kinase activity was assessed by incubating beads with a reaction mixture containing, 25 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, 1 μM ATP, 20 μCi [γ-^{32}P]ATP and 50 ng of purified MEK. Kinase reaction was performed for 30 min at 30°C. Labelled protein was analysed by SDS–PAGE and after electrophoresis proteins were blotted and exposed to film.

Analysis of mSos

Cells were lysed in lysis buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 0.1 μM aprotinin, 1 μM leupeptin and 1 mM sodium orthovanadate), and lysates were cleared by centrifugation. For *in vitro* binding assays 2.5 μg of GST–Grb2 protein were coupled to 5 μl glutathione–agarose (Sigma) in lysis buffer. Loaded beads were washed three times with lysis buffer before incubating them in cell lysates for 4 h at 4°C. Beads were spun down and washed four times with lysis buffer and once with 10 mM Tris–HCl pH 7.0 and 10 mM EDTA. Washed beads were boiled for 5 min in SDS sample buffer. The eluted proteins were fractionated by electrophoresis on 7.5% SDS–polyacrylamide gels and transferred to nitrocellulose by electroblotting. The filters were incubated with polyclonal antiserum directed against mSos (Chardin *et al.*, 1993). Antigen–antibody complexes were detected by incubation with goat-anti-rabbit IgG linked to horseradish peroxidase followed by ECL.

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