

A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK

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cAMP is involved in a wide variety of cellular processes that were thought to be mediated by protein kinase A (PKA)¹. However, cAMP also directly regulates Epac1 and Epac2, guanine nucleotide-exchange factors (GEFs) for the small GTPases Rap1 and Rap2 (refs 2,3). Unfortunately, there is an absence of tools to discriminate between PKA- and Epac-mediated effects. Therefore, through rational drug design we have developed a novel cAMP analogue, 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8CPT-2Me-cAMP), which activates Epac, but not PKA, both *in vitro* and *in vivo*. Using this analogue, we tested the widespread model that Rap1 mediates cAMP-induced regulation of the extracellular signal-regulated kinase (ERK)^{4,5}. However, both in cell lines in which cAMP inhibits growth-factor-induced ERK activation and in which cAMP activates ERK, 8CPT-2Me-cAMP did not affect ERK activity. Moreover, in cell lines in which cAMP activates ERK, inhibition of PKA and Ras, but not Rap1, abolished cAMP-mediated ERK activation. We conclude that cAMP-induced regulation of ERK and activation of Rap1 are independent processes.

Rap1 and Rap2 are the closest known relatives of Ras. Interestingly, Rap1 was identified in a genome-wide screen for suppressors of Ras-mediated transformation⁶, suggesting that Rap1 may antagonize Ras-mediated signalling. This idea was supported by experiments showing that the introduction of constitutively active Rap1 results in the downregulation of growth-factor-induced ERK activation or subsequent ERK-mediated effects^{7,8}. Additionally, activated Rap1 can form an inactive complex with Raf1 (refs 9,10), which led to a model in which Rap1 interferes with Ras-mediated ERK activation by trapping Raf1 in an inactive complex. For instance, in cell lines where cAMP inhibits Ras-mediated ERK signalling¹¹, it was reported that Rap1 mediates this effect (ref. 4). Conversely, in cell lines where cAMP activates rather than inhibits ERK, Rap1 has also been implicated in the activation of ERK⁵. In this model, cAMP activates Rap1, which would then interact with B-Raf, a close relative of Raf1. In contrast to the Rap1–Raf1 complex, the Rap1–B-raf complex is active and promotes ERK activation^{5,12}. One unresolved issue concerning the interactions

between cAMP, Rap1 and ERK is the role of PKA. Although PKA is clearly required for cAMP-induced activation of ERK, cAMP-induced activation of Rap1 through Epac is clearly independent of PKA^{2,3}. This indicates that cAMP-mediated regulation of ERK and Rap1 are independent processes, and therefore the role of Rap1 in cAMP-induced ERK activation is questionable. Indeed, a number of groups have reported alternative mechanisms for cAMP-induced regulation of ERK that occur independently of Rap1 (refs 13–20).

To determine whether ERK is regulated by the cAMP–PKA pathway or the cAMP–Epac–Rap1 pathway, we generated a novel cAMP analogue that can activate Epac, but not PKA. First, we compared the amino-acid sequences of the Epac cAMP-binding domains with all other known cyclic-nucleotide-binding domains, including the cAMP domains of PKA, olfactory and pacemaker channels, and the bacterial catabolite gene activator protein. We noticed that the highly conserved glutamate residue that forms hydrogen bonds with the 2'-hydroxyl of the cAMP ribose group²¹ was absent in the cAMP-binding domain of Epac1 and in the high-affinity cAMP-binding-domain B of Epac2 (Fig. 1a). We hypothesized that this 2'-hydroxyl group, which is absolutely required for high-affinity binding of cAMP to the cAMP-binding domain of PKA, might not be required for efficient binding to and activation of Epac. On the basis of this assumption, we synthesized (synthesis method modified after ref. 22) and tested a large number of compounds, one of which, 8CPT-2Me-cAMP, was a very efficient activator of Epac1 *in vitro* (Fig. 1b). Half-maximal activation of Epac1 was detected at 2.2 μ M 8CPT-2Me-cAMP, compared with 30 μ M for cAMP (Fig. 1c). Interestingly, binding of 8CPT-2Me-cAMP to Epac1 results in a threefold higher maximal activity than that of cAMP, demonstrating that 8CPT-2Me-cAMP is a more potent allosteric regulator of Epac1 than cAMP. Currently, it is unclear why 8CPT-2Me-cAMP activates Epac more efficiently *in vitro* than cAMP, even at saturating conditions. This will be the subject of further investigations. In contrast, the ability of 8CPT-2Me-cAMP to activate the type-I and type-II holoenzymes of PKA was greatly impaired when compared with cAMP (Fig. 1d). These *in vitro* results indicate that 8CPT-2Me-cAMP may also function as a very potent compound to discriminate between the Epac and the PKA signalling pathways *in vivo*. Therefore, the effects of 8CPT-2Me-cAMP treatment was tested in NIH3T3-A14-Epac1 cells. We used Rap1 activity as an indicator of Epac activation and phosphorylation of the

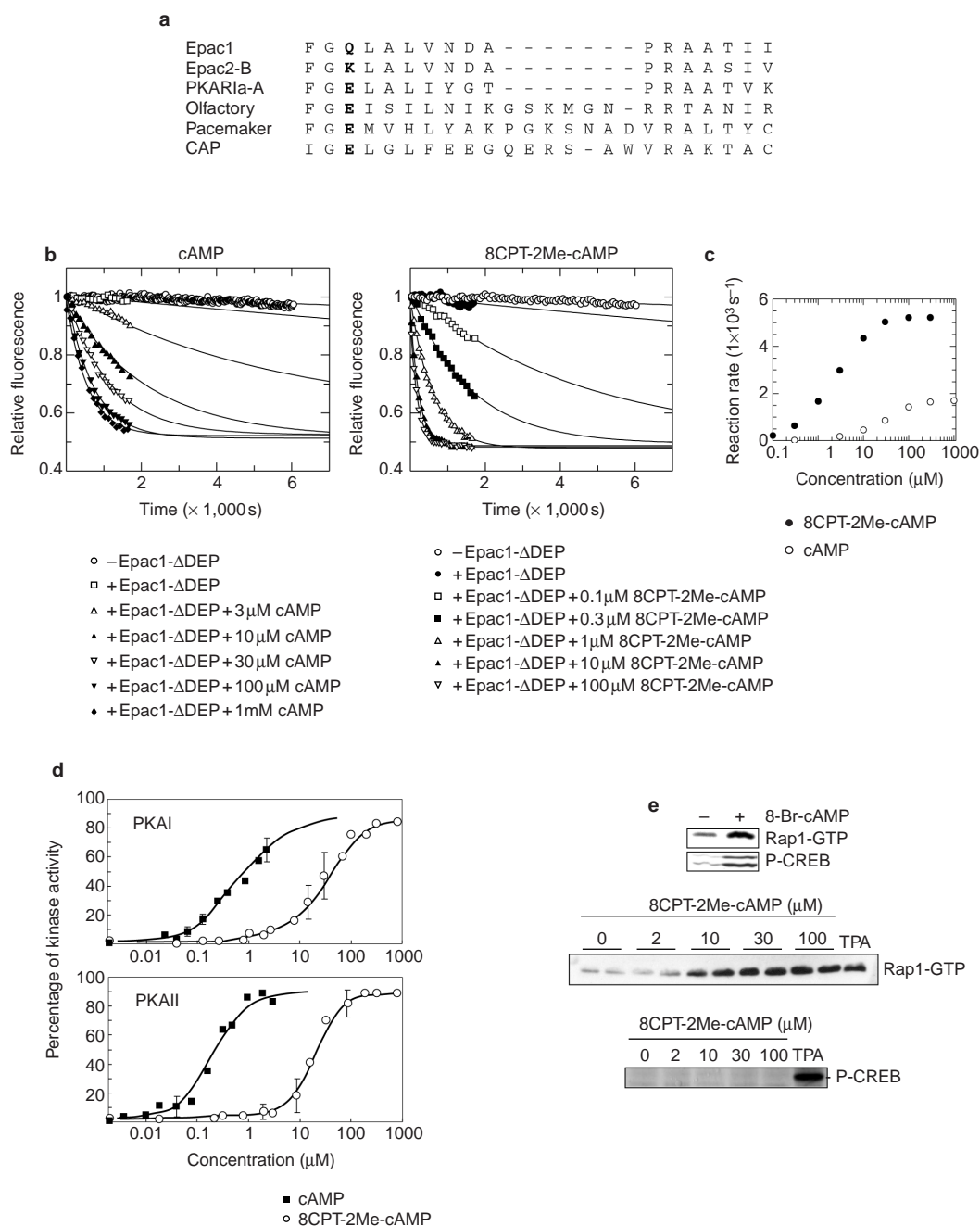


Figure 1 Identification of an Epac-specific cAMP analogue. **a**, Alignment of the cAMP-binding domains of PKA, Epac, olfactory channels, pacemaker channels and the bacterial CAP protein. **b**, *In vitro* activation of Epac1. The left panel shows Rap1 loaded with fluorescent Mant-GDP in the presence of a 100-fold excess of GTP and incubated with or without Epac1- Δ DEP in the presence of increasing concentrations of cAMP. The right panel shows Rap1 loaded with fluorescent Mant-GDP in the presence of a 100-fold excess of GTP and incubated with or without Epac1- Δ DEP in the presence of increasing concentrations of 8CPT-2Me-cAMP, as indicated. **c**, The *in vitro* reaction rates of Epac for cAMP and 8CPT-2Me-cAMP. **d**, *In vitro* PKA activity

of either type-I holoenzyme (top) or type-II holoenzyme (bottom) at increasing concentrations of cAMP or 8CPT-2Me-cAMP. **e**, 8CPT-2Me-cAMP activates Rap1, but not PKA, *in vivo*. NIH3T3-A14-Epac1 cells were treated with 8-Br-cAMP for 15 min (top). Cells were lysed and assayed for GTP-bound Rap1. Phosphorylation of CREB in corresponding cell lysates was analysed using a phospho-specific CREB antibody. NIH3T3-A14-Epac1 cells were treated in duplicate for 15 min with increasing concentrations of 8CPT-2Me-cAMP. Cell lysates were then analysed for activation of Rap1 (middle) and phosphorylation of CREB (bottom).

common PKA substrate CREB (cAMP-responsive element binding protein)²³ as an indicator of PKA activation. Importantly, whereas 8-Br-cAMP induced both the activation of Rap1 and the phosphorylation of CREB (Fig. 1e, top), 8CPT-2Me-cAMP induced the activation of Rap1 only. Serial dilution experiments demonstrated

that 10 μ M 8CPT-2Me-cAMP already activates Rap1 (Fig. 1e, middle). However, 8CPT-2Me-cAMP did not induce phosphorylation of CREB, even at 100 μ M, (Fig. 1e, bottom). Taken together, we conclude that 8CPT-2Me-cAMP is a highly specific and efficient activator of Rap1, as well as a very useful tool to discriminate

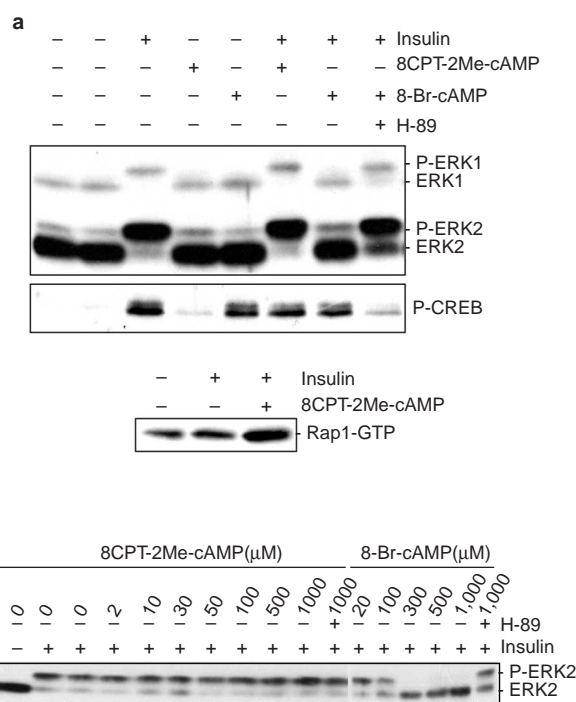


Figure 2 ERK is inhibited by 8-Br-cAMP, but not by 8CPT-2Me-cAMP.
a, 8CPT-2Me-cAMP does not block insulin-induced ERK activation (top). NIH3T3-A14-Epac1 cells were either pretreated or not pretreated with the PKA inhibitor H-89 for 30 min, before treatment with 8-Br-cAMP or 8CPT-2Me-cAMP (both at 100 μM) for 15 min. Subsequently, cells were stimulated with insulin for 5 min. Cells were lysed and phosphorylation of ERK was assayed by mobility shift through western blotting with an ERK1/2 antibody. As a control for PKA activation, CREB phosphorylation was analysed using a phospho-CREB antibody (middle). As a control for 8CPT-2Me-cAMP, cells were pretreated with 100 μM 8CPT-2Me-cAMP for 15 min before insulin treatment for 5 min (bottom). Cells were lysed and assayed for GTP-bound Rap1.
b, 8CPT-2Me-cAMP does not block activation of ERK at high concentrations. Where indicated, cells were pretreated with H-89 for 30 min before treatment with increasing concentrations of 8CPT-2Me-cAMP or 8-Br-cAMP for 15 min. Subsequently, cells were stimulated with insulin for 5 min. Cells were lysed and ERK phosphorylation was assayed by mobility shift through western blotting with an ERK1/2 antibody.

between the PKA- and Epac-Rap-mediated signalling pathways. Although we have not tested the effect of 8CPT-2Me-cAMP on olfactory and pacemaker channels, we predict that these proteins will not be affected, as both channels contain the conserved glutamate in their cAMP-binding domains.

One of the proposed functions of Rap1 is to antagonize Ras-mediated activation of ERK by binding to and inhibiting Raf1 (refs 7,8,10). Recently, cAMP-induced inactivation of ERK has also been suggested to be mediated by Rap1 (ref. 4). We tested this model using 8CPT-2Me-cAMP in NIH-3T3-A14 cells stably expressing Epac1, which should boost the activation of endogenous Rap1. In these cells, 8-Br-cAMP efficiently activated Rap1 (Fig. 1e) and inhibits insulin-induced activation of ERK (Fig. 2a, top). This cAMP-induced inactivation of ERK is rescued by the PKA inhibitor, H-89, confirming previous data suggesting that PKA mediates this effect. Although 8CPT-2Me-cAMP efficiently activates Rap1 in these cells (Fig. 1e), no effect on insulin-induced ERK activation was detected, even at concentrations of 1 mM (Fig. 2b). Although these high concentrations of 8CPT-2Me-cAMP can activate PKA *in vitro*, they are apparently insufficient for activation *in vivo*. The reason for this discrepancy is unclear, but it has been generally observed that activation of PKA *in vivo* requires much higher concentrations of

cAMP analogues than is required *in vitro* (S.O.D., unpublished observations and review manuscript in preparation). In contrast with 8CPT-2Me-cAMP, 8-Br-cAMP efficiently inhibited ERK activation at a concentration of 300 μM. These results show that cAMP-induced activation of Rap1 is not sufficient to inhibit growth-factor-induced activation of ERK.

In cell lines in which cAMP activates ERK, Rap1 has been proposed to mediate this effect by binding to and activating B-Raf. To determine whether Rap1 is indeed involved in cAMP-dependent activation of ERK, we incubated Chinese hamster ovary (CHO) cells with 8-Br-cAMP or 8CPT-2Me-cAMP. Although both analogues efficiently activated Rap1, only 8-Br-cAMP activated ERK and induced phosphorylation of CREB (Fig. 3a). Similar responses were also detected in OVCAR3 cells treated with 8-Br-cAMP or 8CPT-2Me-cAMP (Fig. 3b). Furthermore, 8-Br-cAMP, but not 8CPT-2Me-cAMP, activated ERK in PC12 cells and HEK293T cells (Fig. 3c), whereas Rap1 was readily activated by 8CPT-2Me-cAMP (Fig. 3d). Taken together, we conclude that cAMP-induced activation of Rap1 is not sufficient to activate ERK.

Next, we investigated whether activation of Rap1 is still required for cAMP-induced activation of ERK in cell lines in which cAMP activates ERK. For these experiments, we used CHO-β₂M₃ cells, which express the β₂ adrenergic receptor (β₂-AR) and the M₃ muscarinic receptor (M₃-MR), thus providing a system with a physiological set-up that can be induced by extracellular stimuli. Stimulation of β₂-AR with isoproterenol activates ERK through the G_s heterotrimeric G-protein subunit and cAMP, whereas stimulation of M₃-MR with carbachol results in cAMP-independent ERK activation through the G_i G-protein subunit. In these cells, isoproterenol activated ERK and Rap1 (Fig. 4a). Ras is induced by isoproterenol, although the effect is weak (Fig. 4a). Isoproterenol-, forskolin- or 8-Br-cAMP-induced activation of ERK is sensitive to H-89, whereas activation of Rap1 is insensitive to H-89 (Fig. 4b and data not shown). As a control, carbachol-induced activation of ERK was insensitive to H-89 (Fig. 4b). Furthermore, in all other cell lines tested, cAMP-dependent activation of Rap1 was insensitive to H-89 and Rap1 was readily activated by 8CPT-2Me-cAMP (ref. 3 and J.M.E., J.d.R. and J.L.B., unpublished observations). Furthermore, activation of ERK with isoproterenol and carbachol, but not with Rap1, was sensitive to the MEK inhibitors, PD98059 and U0126, demonstrating that activation of ERK is MEK-dependent (Fig. 4b). Thus, in these cells, cAMP-induced ERK activation, but not Rap1 activation, is mediated by PKA. Next, we isolated clonal CHO-β₂M₃ cell lines that stably express haemagglutinin (HA)-tagged Rap1-GAP1, a GTPase-activating protein for Rap1. In these cell lines, Rap1 could no longer be activated by isoproterenol, forskolin or 8-Br-cAMP, whereas ERK activation was unaffected (Fig. 4c and not shown). Furthermore, isoproterenol and forskolin induced ERK activation with normal kinetics (Fig. 4d). From these results, we conclude that Rap1 is not involved in cAMP-induced ERK activation.

As isoproterenol activated Ras (albeit modestly), we investigated whether Ras is involved in mediating cAMP-induced activation of ERK. In clonal CHO-β₂M₃ cell lines stably expressing the dominant-negative Ras mutant, Ras^{S17N}, induction of cAMP by either isoproterenol or forskolin resulted in normal activation of Rap1, but ERK activation was completely abolished (Fig. 4e). In contrast, carbachol-induced ERK activation was unaffected by Ras^{S17N}. From these results, we conclude that cAMP-induced ERK activation is mediated by PKA and Ras, but not by Rap1.

The cAMP analogue 8CPT-2Me-cAMP is an important tool for deciphering the functions of cAMP, as almost all commercially available cAMP analogues we have tested regulate both PKA and Epac (A.E.C. and J.d.R., manuscript in preparation). 8CPT-2Me-cAMP will allow us to measure the effects of activating the Epac-Rap1 pathway in a more physiological setting, that is, activation of endogenous Epac, Rap1 and Rap2. We have used 8CPT-2Me-cAMP to investigate the proposed role of Rap1 in cAMP-mediated regulation of ERK. Importantly, 8CPT-2Me-cAMP did

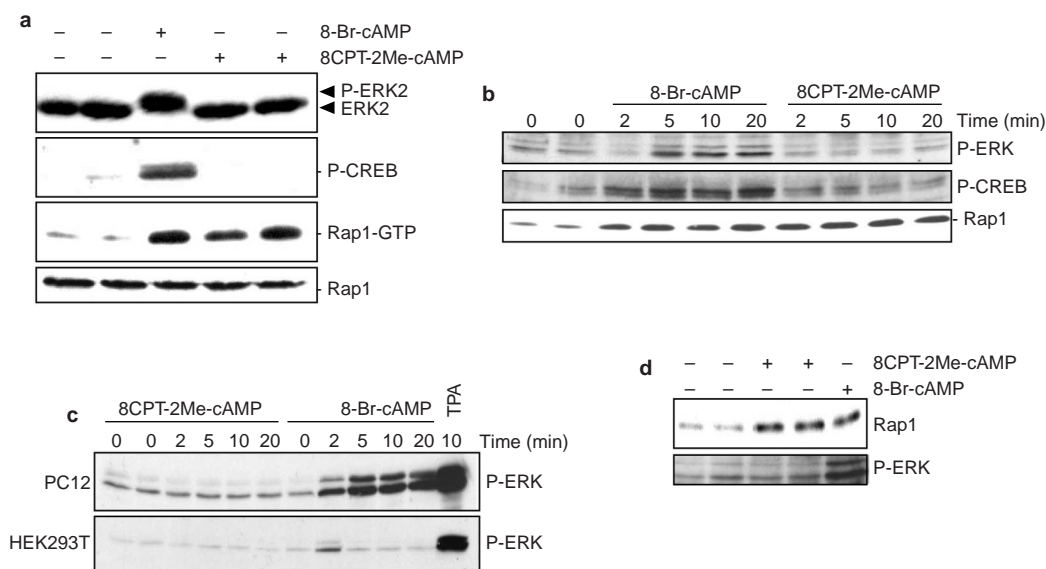


Figure 3 cAMP-induced activation of ERK and Rap1 are distinct processes. **a**, ERK is activated by 8-Br-cAMP, but not by 8CPT-2Me-cAMP, in CHO cells. Cells were treated with either 8-Br-cAMP or 100 μ M 8CPT-2Me-cAMP for 15 min. Cells were lysed and assayed for GTP-bound Rap1. ERK phosphorylation was assayed by mobility shift through western blotting with an ERK1/2 antibody. CREB phosphorylation was assayed by western blotting with a phospho-CREB antibody. **b**, ERK is activated by 8-Br-cAMP, but not by 8CPT-2Me-cAMP, in OVCAR3 cells. Cells were treated with 8-Br-cAMP or 8CPT-2Me-cAMP (30 μ M) for the indicated times. Equal amounts of cell lysate were assayed for GTP-bound Rap1. Phosphorylation of ERK

and CREB was assayed using phospho-specific ERK and CREB antibodies. **c**, ERK is activated by 8-Br-cAMP, but not by 8CPT-2Me-cAMP, in PC12 and HEK293T cells. PC12 cells (top) or HEK293T cells (bottom) were treated with 30 μ M 8CPT-2Me-cAMP or 8-Br-cAMP for the indicated times, or with TPA for 10 min. Equal amounts of cells lysate were analysed for ERK phosphorylation by western blotting with a phospho-specific ERK antibody. **d**, As a control, 8CPT-2Me-cAMP readily activates Rap1. PC12 cells were treated in duplicate for 15 min with 8CPT-2Me-cAMP or 8-Br-cAMP. Equal amounts of cell lysates were analysed for activation of Rap1 (top) and phosphorylation of ERK (bottom).

not affect the regulation of ERK in a number of cell lines in which 8-Br-cAMP either induced ERK activation or inhibited growth-factor-induced activation of ERK. This result was unsurprising, considering the widely accepted role of PKA in cAMP-mediated regulation of ERK. Furthermore, our studies with Rap1-GAP1-overexpressing cells clearly show that Rap1 activity is completely dispensable for activation of ERK. These results are clearly at odds with a prominent role for Rap1 in the regulation of ERK^{4,5}. This role originates from the early finding that Rap1 suppresses Ras-mediated transformation and also from the high similarity between Ras and Rap1, particularly in the effector domain. Indeed, Rap1 has also been shown to interact with Ras effectors, such as Raf1, B-Raf and Ral-GEFs both *in vitro* and in overexpression systems^{9,24}. However, several studies have already indicated that Rap1 may not be involved in modulating ERK activity^{24,25}. For instance, activation of endogenous Rap1 by endothelin did not affect ERK activity and expression of activated Rap1 under the control of a T-cell-specific promoter in mice did not affect ERK activity in the subsequently isolated T cells²⁶. Furthermore, in genetic screens of lower eukaryotes, no elements of the Rap1 signalling pathway were found to affect the Ras-ERK pathway²⁷. Of course, indirect regulation of ERK by Rap1 is still possible. For instance, Rap1 has been shown to regulate integrin-mediated cell adhesion²⁸, an effect that can be mimicked by 8CPT-2Me-cAMP (S. Rangarajan, J.M.E. and J.L.B, manuscript in preparation). Indeed, activation of integrins results in 'outside-in' signalling that also affects ERK activity. Previously, it has been reported that the activation of Rap1 is mediated by PKA⁵. Our results do not formally exclude the possibility that two pathways exist to activate Rap1 — one mediated by PKA and one mediated by Epac — of which only the PKA-mediated Rap1 pathway may be involved in the activation of ERK. However, cAMP-induced activation of ERK is not abolished in Rap1-GAP1-overexpressing cell lines, an observation that is at odds with this model.

Furthermore, PKA-induced phosphorylation of Rap1 at Ser 179 has been suggested to be involved in the activation of Rap1 (ref. 5), although the precise function of this is still unclear.

If Rap1 is not involved in the regulation of ERK activity, what are the alternative mechanisms? For cAMP-induced inhibition of growth-factor-induced ERK activation, direct phosphorylation of Raf1 is the most attractive alternative. Recently, two studies showed that Raf1 is phosphorylated by PKA and thereby inactivated^{16,17}. Our results suggest that Ras is involved in the activation of ERK by cAMP, although how PKA activates Ras is unclear. Alternatively, as activation of Ras by cAMP is generally weak, Ras may cooperate with a target of PKA to induce ERK activity. Several additional possibilities have been described, such as the regulation of an ERK phosphatase¹⁸ and PKA-independent regulation of Ras in melanocytes¹⁹. Studies of these alternative pathways provide further support for our observations that cAMP-induced activation of Rap1 and cAMP-mediated regulation of ERK are independent processes. □

Methods

Reagents

Antibodies against the following proteins were used: polyclonal phospho-Thr 202/Tyr 204-p44/42 mitogen-activated protein kinase (MAPK) and polyclonal phospho-Ser 133-CREB (Cell Signalling, Beverly, MA), Ras (Transduction Laboratories, Lexington, NY) and Rap1 (Santa Cruz Biotechnology, Santa Cruz, CA). The following inhibitors and stimuli were used at concentrations indicated, unless stated otherwise: H-89 (10 μ M), PD98059 (50 μ M) and U0126 (25 μ M) were from Biomol Research Laboratories Inc., Plymouth Meeting, PA. Insulin (1 μ g ml⁻¹), 12-O-tetradecanoylphorbol-13-acetate (TPA; 100 ng ml⁻¹), isoproterenol (10 μ M) and carbamylcholine (carbachol, 100 μ M) were from Sigma, Steinheim, Germany. Forskolin (25 μ M) was from ICN, Costa Mesa, CA. 8-Br-cAMP (1 mM) was from Biolog Life Science Institute, Bremen, Germany.

Cells, plasmids, transfections

CHO cells stably expressing the β_2 -adrenergic and the M₁-muscarinic receptor were maintained in α MEM containing 10% fetal calf serum (FCS) and 0.25 mg ml⁻¹ hygromycin-B (Roche Diagnostics,

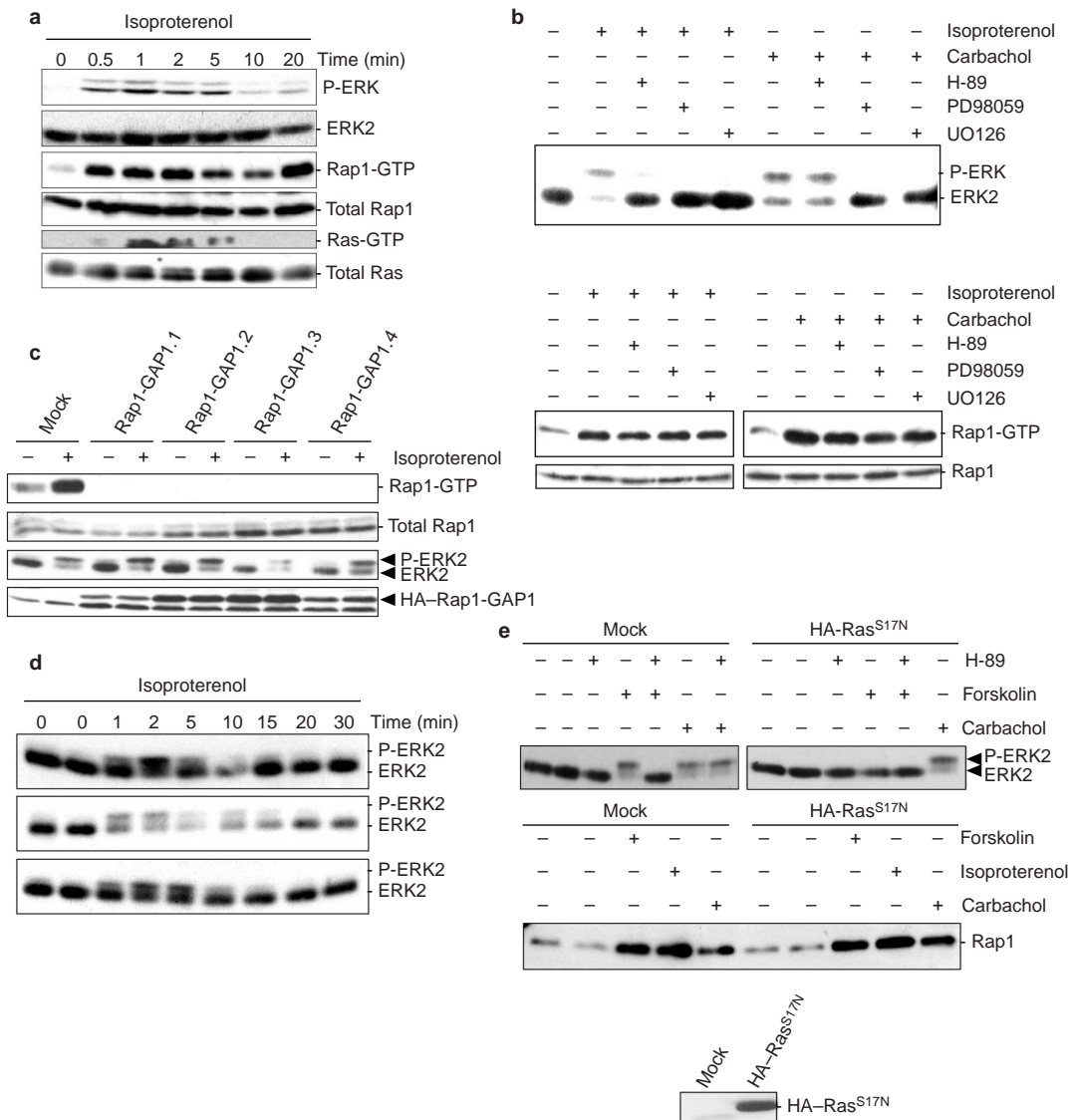


Figure 4 cAMP-induced ERK activation is mediated by PKA and Ras.

a, Isoproterenol activates ERK, Rap1 and Ras in CHO- β_2M_3 cells. Cells were treated with isoproterenol for the indicated times and assayed for GTP-bound Rap1 and GTP-bound Ras. Activation of ERK was analysed by western blotting with a phospho-specific ERK antibody. **b**, Isoproterenol-induced activation of ERK, but not Rap1, is blocked by inhibitors of PKA and MEK. CHO cells were pretreated for 30 min with H-89 or the MEK inhibitors, PD98059 or U0126. Cells were then treated with isoproterenol or carbachol for 5 min. Cell lysates were assayed for ERK2 phosphorylation by mobility shift, detected by western blotting (top) and Rap1 activation (bottom). **c**, Expression of Rap1-GAP1 blocks isoproterenol-induced activation of Rap1, but not ERK. Four HA-tagged Rap1-GAP1-expressing clonal CHO- β_2M_3 cell lines (Rap1-GAP1.1–1.4) and a mock-transfected clonal cell line were treated with isoproterenol for 5 min. Cell lysates were assayed for Rap1 activation and ERK

phosphorylation. Expression of Rap1-GAP1 was confirmed by western blotting with anti-HA antibodies. **d**, Kinetics of ERK activation are identical in untransfected cells (top), mock-transfected cells (middle) and Rap1-GAP1-expressing cells (bottom). Cells were treated with isoproterenol for the indicated times and the kinetics of ERK2 phosphorylation were assayed by mobility shift. Similar results were obtained in three additional Rap1-GAP1-expressing cell lines (data not shown). **e**, Dominant-negative Ras^{S17N} blocks forskolin-induced ERK activation. Mock-transfected cells and Ras^{S17N}-expressing cells (top) were pretreated with H-89 (30 min), forskolin (10 min) or carbachol (5 min), as indicated. ERK2 phosphorylation was assayed by mobility shift. Mock-transfected CHO- β_2M_3 cells and Ras^{S17N}-expressing cells were treated with forskolin (10 min), isoproterenol (5 min) or carbachol (5 min), as indicated. Cell lysates were assayed for Rap1 activation. Expression of Ras^{S17N} was detected by western blotting with anti-HA antibodies.

Mannheim, Germany). Clonal cell lines were generated by calcium phosphate cotransfection of pBABE with either pMT2HA, pMT2HA-H-Ras^{S17N}, pMT2HA-Rap1-GAP1 (ref. 28) and selection with 15 $\mu\text{g ml}^{-1}$ puromycin. NIH3T3-A14-Epac1 cells (NIH3T3-A14 cells²⁴ stably expressing Epac1) were grown in DMEM containing 10% (FCS) and 2 $\mu\text{g ml}^{-1}$ puromycin. OVCAR3 cells were maintained in RPMI containing 10% FCS. HEK293 cells were maintained in DMEM containing 10% FCS. PC12 cells were maintained in RPMI containing 5% FCS and 10% donor horse serum. With the exception of CHO cells, all cells were serum-starved 16 h before stimulation.

In vitro analyses

cAMP-dependent protein kinase I and II were reconstituted from isolated subunits and assayed for

kinase activity with 70 mM kemptide as substrate, as described previously²⁹. Activation of Epac was measured as described³ using 600 nM Rap1b loaded with the fluorescent nucleotide methyl-*o*-ntraniloyl-GDP in the presence of a 100-fold excess of GTP and in the absence or presence of 150 nM Epac1- Δ DEP1 (ref. 3). Increasing concentrations of cAMP or 8CPT-2ME-cAMP were added and single exponential curves were fitted to the data to calculate reaction rates. Reactions were carried out in 96-well plates and measured in a Cary Eclipse (Varian Inc., Middelburg, The Netherlands) using the manufacturer's software.

In vivo analyses

Ras and Rap activation assays were performed as described previously³⁰. Briefly, equal amounts of cell

lysates were incubated with either the minimal Ras-binding domain (RBD) of Ral-guanine nucleotide-dissociation stimulator (GDS) fused to glutathione S-transferase (GST) or the RBD of Raf1 fused to GST. These fusion proteins were precoupled to glutathione-agarose beads to specifically pull down activated GTP-bound forms of Rap1 and Ras, respectively. Samples were then analysed by western blotting using Rap1 and Ras antibodies. Phosphorylation of ERKs was either analysed by mobility shift on SDS-polyacrylamide gel electrophoresis (PAGE), which accompanies phosphorylation of these proteins, or western blotting using the phospho-specific antibody against ERK, where indicated.

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COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the paper on *Nature Cell Biology's* website (www.nature.com/naturecellbiology).