The small GTPase Rap1 in cAMP signalling

Het kleine GTPase Rap1 in cAMP signalering

(met een samenvatting in het Nederlands)

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‘…’

-Clint Eastwood-

‘From coast to coast in 24 hours, I ain’t no fool!’

-Sgt. Bosco ‘Bad Attitude’ Barracus-

Aan mijn ouders

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Abbreviations

AC	adenylate cyclase
AF-6	acute lymphoblastic leukemia 1 fusion partner from chromosome 6
AKAP	A-kinase anchoring protein
ATF1	activating transcription factor 1
C	PKA catalytic subunit
C3G	Crk SH3-domain-binding guanine nucleotide releasing factor
CalDAG-GEF	calcium- and diacylglycerol-regulated GEFs
cAMP	cyclic adenosine 3',5'-monophosphate
CaM	calmodulin
CaMK	calmodulin-dependent protein kinase
CDC25	cell division cycle 25
CREB	cAMP responsive element binding protein
cGMP	cyclic guanosine 3',5' monophosphate
CNG-channel	cyclic nucleotide gated cation channel
cNMP	cyclic nucleotide monophosphate
CRE	cAMP response element
CREB	CRE binding protein
CREM	CRE modulator
Crk	chicken retrovirus kinase
DAG	diacylglycerol
DEP	Dishevelled, Egl-10, Pleckstrin
E6TP1	high-risk human papilloma viruses E6 oncoproteins targeted protein
EGF	epidermal growth factor
Epac	exchange protein activated directly by cAMP
ERK	extracellular signal regulated kinase
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GRF	guanine nucleotide releasing factor
GRP	guanine nucleotide releasing protein
GSK3	glycogen synthase kinase-3
GST	glutathion S-transferase
HA	haemagglutinin
ICAM-1	intercellular adhesion molecule
ICER	inducible cAMP early repressor
IL	interleukin
JNK	Jun kinase
kDa	kilo Dalton
KID	kinase inducible domain
LFA-1	leukocyte function-associated molecule-1
mantGDP	2',3'-bis(O)-N-methylantharanoloyl guanosine diphosphate
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
p130CAS	CRK-associated substrate-related protein
p70S6K	p70 ribosomal S6 kinase
PAK	p21 activated kinase
PBC	phosphate binding casette
PDE	phosphodiesterase
PI3K	phosphatidylinositol-3OH-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKI	protein kinase inhibitor
PLC	phospholipase C
PRS	peripheral recognition site
PDZ	PSD-95, Dlg, ZO-1/2
R	PKA regulatory subunit
RA	Ras association
Rap	Ras proximate
Ras	rat sarcoma
RBD	Ras binding domain
REM	Ras exchange motif
SDS-PAGE	sodium dodecyl sulfate-poly-acrylamide gelelectrophoresis
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SPA1	signal-induced proliferation-associated protein 1
SH	Src homology
Sos	son of sevenless
TCR	T cell receptor
TPA	2-O-tetradecanoylphorbol-13-acetate
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen 4

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General Introduction

Cyclic AMP

Regulation of cAMP levels

Cyclic adenosine 3',5'-monophosphate (cAMP) was the first identified second messenger (1). A large number of studies have elucidated the fundamental role of cAMP in the wide range of cellular responses to many hormones and neurotransmitters. The activity of two types of enzymes regulates the level of intracellular cAMP: adenylyl cyclases (ACs) and cyclic nucleotide phosphodiesterases (PDEs).

cAMP is generated from ATP by at least nine closely related AC isoforms (AC1-9) and two splice variants of AC8 (for a review see (2)). Each isoform consists of two hydrophobic domains (with six transmembrane spans) and two catalytic cytoplasmic domains (C1 and C2) (Fig. 1). Seven-transmembrane receptors stimulate the activity of AC through interaction with the $G\alpha_s$ subunit of heterotrimeric G proteins, whereas they inhibit AC activity through interaction with $G\alpha_i$ or $\beta\gamma$ subunits (for a review on G proteins see (3)). AC activity is subject to other signal transduction pathways as well. For instance, the intracellular Ca^{++} pool directly regulates AC activity (4), either positively (AC1 and AC8) or negatively (AC5 and AC6). Calcium also indirectly inhibits AC activity through the Ca^{++}-dependent phosphatase calcineurin (AC9 (5)) and CaMKinase II (AC3 (6)). Furthermore, PKC can directly phosphorylate and activate AC2 and AC5 (7), whereas phosphorylation by PKA may provide a negative feedback loop (AC5 and AC6 (8,9)). Finally, localization of ACs to specific sites in the cell creates very local microdomains of elevated cAMP levels (10,11).

cAMP is hydrolyzed by PDE1, PDE2, PDE3, PDE4, PDE7, and PDE8 phosphodiesterases (for a review see (12)). PDE activity is subject to several signalling pathways. The calcium pathway, through CaM, potently activates PDE1 (13). Activation of receptor tyrosine kinase receptors, e.g. the insulin receptor, induces PDE3 activity in a wortmannin sensitive manner, suggesting a role for PI3K (14). Furthermore, phosphorylation of PDE3 and PDE4 by PKA stimulates their activity, possibly providing a negative feedback loop for PKA signalling (15), whereas PDE4 is inhibited by ERK phosphorylation (16). Finally, due to PDE activity, cAMP is not freely diffusible in the cell, and specific localization of PDE activity regulates cAMP levels in discrete microdomains (10,11,17-19).

Targets of cAMP

Protein Kinase A

Protein Kinase A (PKA; also known as cAMP-dependent kinase, or A-kinase) was the first target of cAMP to be discovered (20). PKA is expressed in all animal cells, and until recently it has been thought to account for all the effects of cAMP. PKA is a serine/threonine kinase composed of two catalytic (C) subunits, which are kept in an inactive state when associated with two regulatory (R) subunits (21,22). Three different genes encode the catalytic subunits $C\alpha$, $C\beta$, and $C\gamma$, whereas four different genes express the regulatory subunits $RI\alpha$, $RI\beta$, $RII\alpha$, and $RII\beta$ (23-26). The heterotetrameric PKA holoenzyme exists in two forms, the type I holoenzyme, consisting of the C dimer associated with $RI\alpha$ and $RI\beta$ dimers, and the type II holoenzyme, consisting of the C dimer with the $RII\alpha$ and $RII\beta$ dimers. Each R subunit contains an N-terminal dimerization domain, two cAMP binding sites (A and B) and a 'hinge region'. The cAMP-A domain interacts directly with the C subunit and is essential for stable binding of the R subunit to the C subunit. In addition, two peripheral recognition sites (PRS1 and 2) on the C subunit are required for high affinity binding of C to R (27). The cAMP-B domain does not contact the C subunit, but functions to modulate access of cAMP to the cAMP-A domain. The hinge region contains an autoinhibitory sequence: a pseudo-substrate sequence (R-R-X-A-X) in the RI subunit, or a substrate-like sequence (R-R-X-S-X) in the RII subunit. These sequences directly contact the C subunits and prevent substrate binding (28). Binding of cAMP to the cAMP-B domain induces a conformational change, upon which the cAMP-A domain becomes accessible to cAMP. The cAMP-A domain consists of an eight-stranded β-barrel, and three helices, the A-, B-, and C-helix. The C-helix functions as a critical switch that changes conformation upon binding of either cAMP or the C-subunit, respectively (29). Binding of cAMP to the cAMP-A domain presumably results in folding

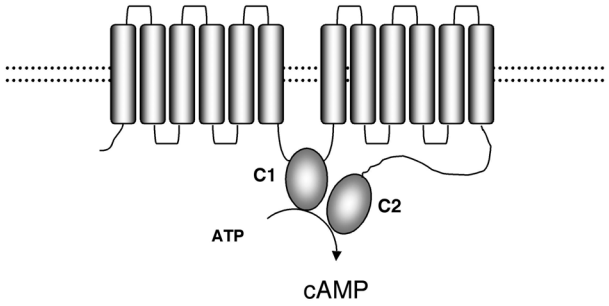

Figure 1. **Structure of adenylate cyclase.** Each AC isoform consists of two hydrophobic domains (with six transmembrane spans) and two cytoplasmic catalytic domains (C1 and C2). See text for details.

of the C-helix over the cAMP binding site, resulting in a conformational change which relieves the autoinhibitory contact between the regulatory and the catalytic domain. The catalytic domain dissociates, allowing phosphorylation of local substrates on serine sites within the canonical peptide sequence X-R-R-X-S-X (where X is any amino acid) (30).

In addition to R subunits, free C subunits are also inhibited in a cAMP-independent manner by binding to protein kinase inhibitors (PKIs), which contain a pseudosubstrate sequence (R-R-Q-A-I) (31,32).

Finally, PKA activity in the cell is spatially restricted by over 50 different A-kinase anchoring proteins (AKAPs). These nonenzymetic scaffolding proteins generally anchor RII-containing PKA holoenzymes, although some AKAPs may also bind RI holoenzymes (33). AKAPs localize inactive PKA holoenzymes to specific sites in the cell (for a review see (34)), allowing efficient cAMP signalling and appropriate substrate selection upon activation (35). For example, muscle-specific mAKAP binds both the PKA holoenzyme and the phosphodiesterase PDE4D3. Upon an increase in cAMP concentration, the C subunits dissociate and phosphorylate PDE4D3, resulting in an increase in PDE4D3 activity, thus providing a negative feedback loop (36).

A large number of proteins have been identified as substrates for PKA. A well studied target of PKA is the CREB family of transcription factors, which includes the cAMP responsive element (CRE)-binding protein (CREB), the cAMP responsive element modulator (CREM), and the activating transcription factor 1 (ATF1). These proteins directly bind as a dimer to CREs in various promoters (37). The CREB transactivation domain is bipartite, consisting of a constitutive (Q2 domain) and an inducible domain (KID, for kinase inducible domain) (38). Phosphorylation on a key acceptor site in the KID domain (S133 in CREB, S117 in CREM, and S63 in ATF1) by a number of possible kinases, including PKA, CaMKIV, and Rsk-2, recruits the transcriptional co-activator CBP and its paralogue p300 (39-44). Mutation of this phosphorylation site (S133A) disrupts the activity of CREB (39). Use of different internal promoters and differential splicing of CREB and CREM yields truncated proteins without the key phosphorylation site or transactivation domain and therefore may act as repressors, providing a negative feedback loop (45,46). For instance, elevated cAMP levels result in increased transcription from an alternative promoter in the CREM gene, yielding a truncated transcript which encodes a protein called Inducible cAMP Early Repressor (ICER). Subsequently, ICER binds to a cluster of four CREs in its own promoter and represses transcription, thus constituting a negative autoregulatory loop (47).

Cyclic nucleotide-gated cation channels and pacemaker channels

Direct binding of cAMP or cGMP modulates the activity of two types of closely related ion channels: cyclic nucleotide-gated cation channels (CNG-channels), and hyperpolarization-activated and cyclic nucleotide-gated channels (HCN channels, also known as pacemaker channels). CNG channels were originally identified in retina and olfactory

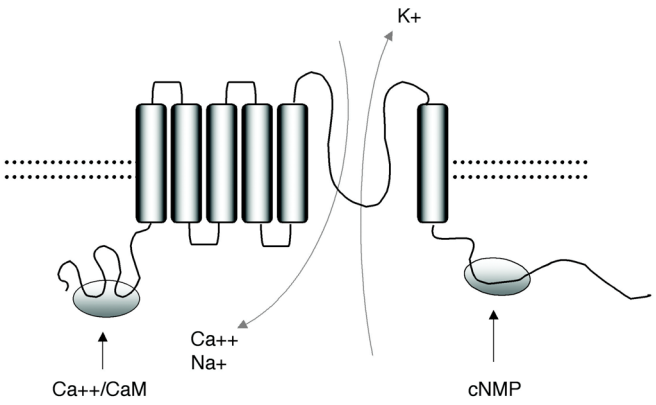

Figure 2. **Structure of CNG-channels and HCN-channels.** CNG- and HCN-channels contain six transmembrane domains, a Ca^{++}-CaM binding domain, and a cNMP binding domain which is capable of binding both cAMP and cGMP. Modified after (48).

tissues. However, recently, widespread expression of CNG channels has been demonstrated, particularly in the central nervous system, but also in heart, kidney, spleen, pancreas, lung, testis, muscle, liver, and keratinocytes (49). The functions of CNG-channels in most of these tissues are not well understood, but a common property may be the regulation of the calcium influx. The NCBI database contains six different human genes encoding CNG channels, four of which are α-subunits (CNGA1, 2, 3, and 4) and two of which are β-subunits (CNGB1 and CNGB3) (50). α-subunits can form active channels when expressed alone, whereas β-subunits do not form active channels when expressed alone, but can modulate the activity of the α-subunits. CNG-channels form hetero-oligomers, probably tetramers, composed of two α-subunits, and two β-subunits (for reviews see (48,50)). CNG-channels contain six transmembrane domains, a C-terminal cytoplasmic cNMP binding domain, a Ca^{++}/CaM binding region, and a putative pore region (Fig. 2). Binding of ligand, i.e. cAMP or cGMP, to the cNMP binding site increases conduction of cations. All known CNG-channels respond to these ligands, but generally lower concentrations of cGMP than cAMP are sufficient to open the channel (51,52). The sensitivity of CNG-channels for cGMP slowly increases upon repeated ligand stimulation. This increase of sensitivity is slowed down by phosphatase inhibitors and accelerated by purified type I phosphatase, suggesting that phosphorylation might modulate ligand sensitivity (53). Indeed, phorbol ester-induced phosphorylation by PKC of S577 and S579 in the cNMP binding domain of CNGA3 reduces the affinity for ligand two- to three-fold (54). Furthermore, ligand sensitivity of CNG-channels is negatively modulated by Ca^{++} binding proteins, possibly CaM (for a review see (55)).

HCN channels were originally identified in sino-atrial node cells (56-60), which coordinate pacemaker activity of the heart, in hippocampal pyramidal cells, and in photoreceptor cells (61-64). The HCN channel family consists of four members, HCN1-4. They are mainly expressed in brain and in heart ((65) and references therein). The structure of HCN channels strongly resembles the structure of CNG channels (Fig. 2), embodying six transmembrane domains (S1-6), a pore loop between S5 and S6, and a cNMP binding pocket in the C-terminus (65). Like CNG channels, HCN channels likely function as a tetramer, although it is unknown whether they form homo- or heterotetramers. Upon hyperpolarization, HCN channels open, resulting in Na^{+} inward current that slowly depolarizes the membrane until the threshold for a new action potential is reached. They determine the time elapsing between successive

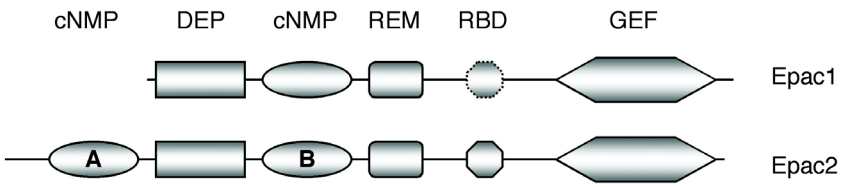

Figure 3. **Structure of Epac proteins.** See text for details. RBD of Epac1 is putative.

action potentials and therefore control firing frequency (65). Binding of cAMP or cGMP enhances channel activity (66-68), although the affinity is higher for cAMP than for cGMP (65). How cyclic nucleotides structurally increase channel activity remains to be established.

Epac

Epac (exchange protein directly activated by cAMP) proteins were first identified in a database search for genes encoding a combination of a cAMP binding domain and a CDC25 homology domain, which is a motif for Ras family guanine nucleotide exchange factors (GEFs) (69,70). The Epac family consists of two members, Epac1 and Epac2 (also called cAMP-GEF I and II), which both have GEF activity towards the Rap family of small GTPases (see below). Epac1 is widely expressed but is enriched in kidney, ovary, thyroid, brain, and skeletal muscle (70). Epac2 is mainly expressed in brain and adrenal gland. Epac2 is highly homologous to Epac1 (Fig. 3), but it contains an extra cAMP binding domain (cAMP-A domain), which has much lower affinity for cAMP than the second cAMP binding domain (cAMP-B domain) of Epac2 or the cAMP binding domain of Epac1 (71). The function of the cAMP-A domain is unclear, but it is not necessary for regulation of Epac2 by cAMP. Both proteins harbor a Dishevelled, Egl-10, Pleckstrin (DEP) domain, which is necessary for membrane localization (71-73). Furthermore, they contain a Ras exchange motif (REM) domain, an α-helix which likely plays a structural role and interacts with the GEF domain (74). Finally, Epac proteins contain a putative Ras association (RA) domain, which may be involved in proper localization of Epac proteins (Bos JL, 2003, in press). Recently, the crystal structure of the Epac2 regulatory domain, consisting of both the cAMP binding domains and the DEP domain, has been crystallized in the absence of cAMP (75). Comparison of this structure with the crystal structure of the regulatory subunit of PKA in the presence of cAMP revealed a unifying mechanism of how cAMP activates these proteins. The most important differences between the crystal structures are observed in the phosphate binding casette (PBC; a highly conserved structure in cNMP binding domains, which interacts with the phosphate-sugar region of cAMP) and the orientation of the hinge region which connects the cAMP binding pocket and the lid that covers the cAMP binding pocket (corresponding to the C-helix in the R subunit of PKA). In absence of cAMP, a conserved leucine residue in the PBC restricts the orientation of the hinge in Epac2. This leucine is positioned opposite of a phenylalanine residue in the hinge, thereby preventing the hinge from moving closer to the cAMP binding domain. Binding of cAMP, however, results in reorientation of the PBC, moving the leucine away from the phenylalanine in the hinge. Subsequently, the hinge is now able to move closer to the cAMP binding domain, allowing the lid to fold over the cAMP binding domain and to interact directly with cAMP, thus stabilizing the active state of the protein. In PKA the conformational change of the R subunit results in dissociation of the C subunit. Similarly, binding of cAMP to the cAMP binding domain of Epac may result in a conformational change which relieves inhibitory contacts between a short sequence (V-L-V-L-E; positioned just C-terminally of the lid) and the catalytic region, which is then exposed to its substrates.

The Epac-Rap pathway has been implicated in a number of physiological processes, including integrin-dependent cell adhesion to fibronectin (chapter 3), insulin secretion (76), activation of PLC and subsequent calcium release (77), calcium

release and subsequent hormone secretion (78,79), PI3K and PKB activation (80), and possibly p70S6K activation (chapter 4). The exact molecular mechanism of how the Epac-Rap pathway stimulates these processes awaits further analysis.

Ras family of small GTPases

The Ras superfamily of small GTPases presently consists of at least 13 families, comprising over 100 proteins which share extensive similarities in their effector domains. Ras-like small GTPases function as molecular switches, cycling between GDP- and GTP-bound forms. The switch is activated by guanine-nucleotide exchange factors (GEFs), which interact with the GTPase and induce release of the bound GDP, allowing the more abundant GTP to bind (74). Binding of GTP induces a conformational change in the switch 1 and switch 2 regions, allowing effector proteins to bind (74). This results in translocation from the cytoplasm to the cytoplasmic surface of membranes, subsequent activation of the effector protein, and induction of signalling cascades (81,82). The switch is turned off through hydrolysis of the GTP by the intrinsic GTPase activity, which is stimulated by GTPase activating proteins (GAPs) (83-85). The interaction between the GTPase and its effector is then abolished, resulting in termination of the signalling cascade.

Ras

Ras is the best studied member of the Ras superfamily and therefore the paradigm of small GTPases in signal transduction. They have been found to be mutated in 15% of all human tumors (86). These mutations all rendered a constitutively active, GTP-bound Ras. The three Ras proteins (H-Ras, K-Ras and N-Ras) are 95% identical and ubiquitously expressed, and localize to the plasma membrane (87). H-Ras$^{(-/-)}$ and N-Ras$^{(-/-)}$ knockout mice, as well as the H-Ras$^{(-/-)}$/N-Ras$^{(-/-)}$ double knockout mice are perfectly viable, whereas K-Ras$^{(-/-)}$ knockout mice die before birth, indicating that both H-Ras and N-Ras are dispensable for normal mouse growth, development, and fertility, while K-Ras is not only essential but also sufficient for normal mouse development (88).

Wild-type Ras is activated by a large variety of extracellular signals, particularly growth factors. Phosphorylated tyrosine residues on the cytoplasmic side of single-span transmembrane receptors recruit adaptor molecules like Shc and Grb2. In turn, these proteins bind and thereby translocate the RasGEF Sos to the plasma membrane, where it can activate Ras. Furthermore, seven-transmembrane receptors can activate Ras through a complex of Gα_i, Grb2 and mSos (89), and possibly via second messengers like calcium and diacyl glycerol (DAG), which recruit the RasGRF and RasGRP exchange factors to the membrane ((90,91); De Rooij, unpublished results). Ras GTPase activity is stimulated by p120-RasGAP, neurofibromin, and possibly members of the Gap1m family (92-94).

Several Ras effectors have been described, which all have been implicated in the effects of Ras on cell proliferation, morphology, and attachment. The best described Ras effectors are the members of the Raf family of serine/threonine kinases (A-Raf, B-Raf, and Raf1). Most attention has focused on Raf1, which contains an N-terminal Ras binding domain (RBD; amino acids 51-131) which is sufficient for Ras binding. Full activation of Raf1, however, is complex and requires phosphorylation and dephosphorylation of several residues (see below). Compared with Raf1, regulation of B-Raf seems less complex. Analysis of human tumors revealed that it can be fully activated by mutation of a single residue in the T-loop, which also overcomes the need for Ras-binding (95).

Another target of Ras is the RalGEF family, consisting of RalGDS, Rgl, Rlf, and Rgr (96-99), which all exhibit specific guanine nucleotide exchange activity towards Ral. RalGEFs likely play a role in Ras-induced cellular transformation, since Rlf-CAAX and Rsc stimulate serum-independent growth in NIH3T3 cells, and RalGDS cooperates with Raf1 in focus formation (99-101). Interestingly, whereas in murine cells the oncogenic properties of Ras seem to be mediated by the Raf pathway, in human cells they may be mediated by the RalGEF pathway (102). It should be noted however, that in this study a cell transformation assay was used in which both the SV40 large and small T antigens and

the catalytic subunit of telomerase (hTERT) were overexpressed; thereby the process of cell transformation might be oversimplified.

Interaction of Ras with the p110 catalytic subunit of PI3-kinase enhances its lipid kinase activity (103,104). However, whereas both endogenous and oncogenic Ras clearly activate the Raf- and RalGDS family members, the role of PI3K as an effector of Ras remains a matter of debate (105). Also, genetic data from *Drosophila* suggest that only activated Ras1 is able to activate PI3K, whereas endogenous Ras1 does not seem to be involved in PI3K regulation (106).

Rap

The family of Rap (Ras proximate) proteins was first identified in a screen for Ras homologous genes (107,108), and consists of Rap1A, Rap1B, Rap2A, and Rap2B. Much attention has focused on Rap1 after it was identified in a screen for cDNAs that could revert the morphology of K-Ras-transformed NIH3T3 cells (109). The hypothesis that Rap1 antagonizes Ras by trapping Ras effectors in an inactive complex through its highly homologous effector domain dominated the next decade, especially when it was found that Rap1 indeed could trap Raf1 in an inactive complex (110,111) and that overexpression of an activated Rap1 mutant could interfere with ERK signalling in fibroblasts (112,113). In contrast, Rap1 might also be able to activate Ras effectors (114) and induce cellular transformation of Swiss 3T3 fibroblasts (115). However, endogenous Rap1 is unlikely to play a role in ERK signalling ((116); chapter 2; also see below). Rather, Rap1 is likely to have a signalling function independent of Ras.

Recently, Rap1 has been implicated in integrin activation and cell adhesion ((117-126); chapter 3). Additionally, in *Drosophila* Rap1 is involved in positioning of adherens junctions, thus regulating cell-cell adhesion (127), although it remains possible that the effects observed in this study result from changes in cell-to-matrix adhesion.

Rap GTPases also regulate hormone secretion by pancreatic β-cells through activation of calcium channels that are sensitive to ryanodine (78,79). In addition, Rap2B may regulate calcium levels through activation of phospholipase C (PLCε), resulting in formation of DAG and inositol 3-phosphate (IP_3), which in turn activates IP_3-activated calcium channels (77,128).

In addition to Epac, Rap1 is activated by a number of other GEFs (Fig. 4). The first RapGEF to be identified was C3G (chicken retrovirus kinase (Crk) SH3-domain-binding guanine nucleotide releasing factor), which was initially isolated as a protein binding to the N-terminal SH3 domain of Crk (129,130). C3G has been shown to respond to B and T cell receptor activation (131), insulin and EGF in Chinese hamster ovary (CHO) cells stably expressing the insulin and EGF receptors (132), erythropoietin, and IL-3 (133). C3G constitutively associates with the SH3 domain of Grb2, CrkI and CrkII, and Crk-like (Crk-L)

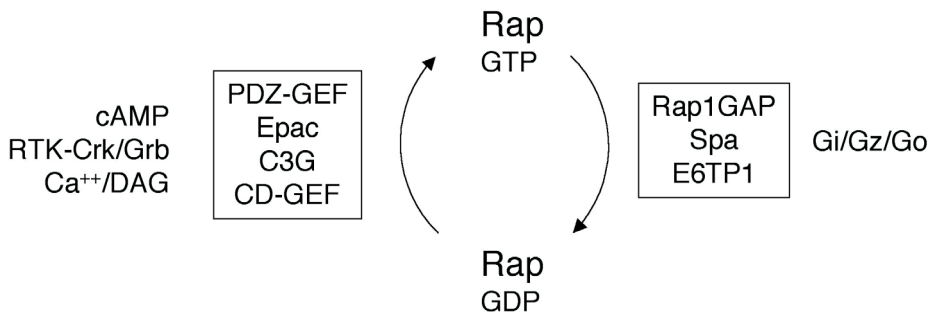

Figure 4. **Rap is regulated by common second messengers.** Tyrosine kinase signalling, cAMP, calcium (Ca^{++}), and diacylglycerol (DAG) all activate RapGEFs. Rap is downregulated by a number of RapGAPs, which may be directly regulated by Gα subunits of large G proteins.

proteins through its proline-rich sequences (130). The SH2 domain of Crk proteins in turn associates with phosphotyrosine proteins, resulting in membrane recruitment of the Crk-C3G module. This probably facilitates phosphorylation of Y504 in C3G, which may relieve the autoinhibitory effect of a domain outside the catalytic region (134). C3G-induced activation of Rap1 may be downregulated by the E3 ubiquitin ligase Cbl, possibly resulting in a decrease in cell adhesion (135). Activation of C3G results in activation of Rap1 but also R-Ras (136,137). C3G not only activates JNK through R-Ras (138), but has also genetically and biochemically been linked to the Ras-ERK pathway (133,139,140). C3G has been suggested to be important for cell adhesion, formation of focal adhesions, and cell migration (117,121,131,141). Indeed, C3G not only stimulates cell adhesion through Rap1, but it can also directly bind to the SH3 domain of p130 CAS (142). This in turn may activate R-Ras, resulting in phosphorylation of p130CAS and focal adhesion kinase, thus promoting focal adhesion formation (143).

The PDZ-GEF family consists of two family members (144-147) which both contain a PSD-95, Dlg, ZO-1/2 (PDZ) domain, a putative Ras association (RA) domain which may interact with active Rap1 or M-Ras (148-150), a Ras exchange motif (REM) and a GEF domain characteristic for GEFs for Ras-like small GTPases, and a cNMP domain, which, however, does not bind cAMP with a physiologically relevant affinity (151). It is unknown which signal activates PDZ-GEFs, but they have specific exchange activity towards Rap1 and Rap2.

The family of Ras guanine nucleotide releasing proteins (RasGRPs) (or CalDAG-GEFs, for calcium- and diacylglycerol-regulated GEFs) consists of four members which contain putative calcium and DAG binding domains and may be regulated by these second messengers. RasGRP2 (CalDAG-GEFI) has exchange activity towards Ras, RasGRP1 (CalDAG-GEFII) towards Rap1 and Ras, RasGRP3 (CalDAG-GEFIII) towards Rap, Ras, and R-Ras, and RasGRP4 (CalDAG-GEFIV) towards Ras (90,152-155).

Due to a conserved glutamine residue at position 61, Rap1 has very low intrinsic GTPase activity, and therefore downregulation of Rap1 critically depends on the activity of a number of RapGAPs. The first RapGAP to be identified was Rap1GAPI, and a longer transcript from the same gene was called Rap1GAPII (156,157). Their activity may be modulated by $G\alpha_i$, $G\alpha_o$, and $G\alpha_z$ subunits of large G proteins (156,158,159). Furthermore, members of the Spa family (Spa1, SPAR, SPAL, and E6TP1) also stimulate GTPase activity of Rap1, although it is largely unknown how their activity is regulated (160-163). Recently it has been suggested that Spa1 and Rap1GAPs may be regulated by the scaffolding protein AF-6, which simultaneously recruits Spa1/RapGAP and Rap1 through its PDZ domain and its RBD, respectively (164). The subsequent downregulation of Rap1 would thereby inhibit adhesion of cells to fibronectin.

Cross-talk

Cross-talk between the cAMP-pathways

Cells may deploy extensive cross-talk between cAMP signalling pathways. PKA can directly phosphorylate Rap1 (165-176) and Rap1GAP (177), although the consequences are uncertain. In thyroid cells, upon cAMP treatment, Epac activates a PI3K-PKB pathway, whereas PKA simultaneously inhibits PKB (80). Furthermore, PKA inhibits AC5 and AC6, whereas it stimulates PDE3 and PDE4 activity, all of which affect the availability of cAMP. Calcium may be a central player in crosstalk between the cAMP signalling pathways. Numerous studies report crosstalk between cAMP and calcium signalling pathways in diverse cell types, like endothelial cells and cardiac muscle cells (178-182) (Fig. 5). PKA, Epac, and CNG-channels all affect calcium levels in the cell. For instance, PKA can raise calcium levels by stimulating the IP3 receptor (IP_3R) calcium channels (183), upon which RapGEFs might be activated (184). Epac-Rap1 may also influence calcium fluxes through the ryanodine-sensitive calcium channels (RyR) (78,79) or IP_3R calcium channels (77) and possibly the sarco/endoplasmic reticulum Ca^{++}-ATPase (185,186). Subsequently, elevated calcium levels can affect cAMP levels (178,179). For instance, adenylate cyclases are either stimulated or inhibited in various ways by

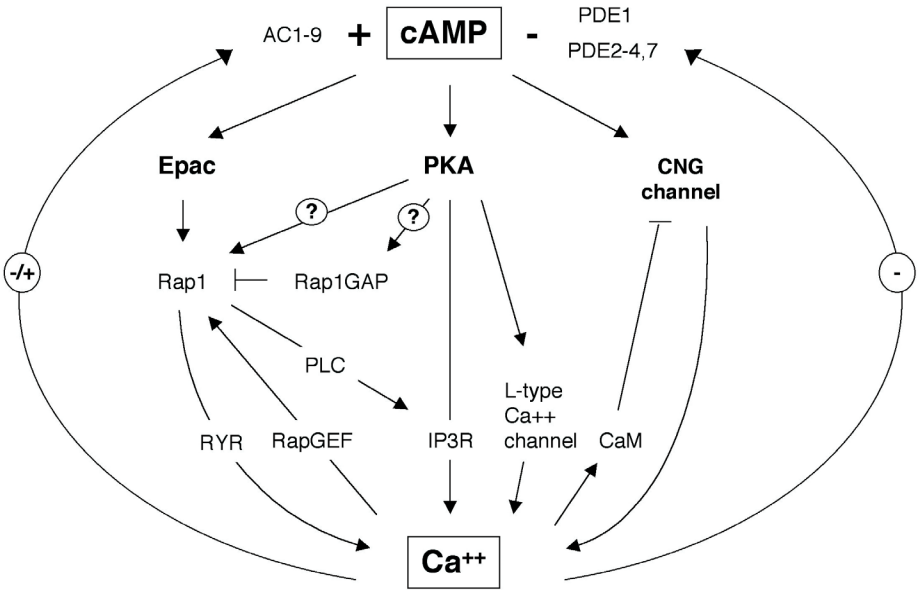

Figure 5. **Crosstalk between cAMP pathways, directly or via the second messenger calcium.** See text for details.

calcium, and PDE1 activity is enhanced by Ca^{++}-CaM. Since both calcium and cAMP are not freely diffusible in the cell (10,11,17-19), crosstalk between the Epac, PKA, and CNG channel signalling pathways is likely to depend on expression and specific localization of the involved proteins in microdomains in the cell; investigation of crosstalk in living cells remains a major challenge.

cAMP, PKA, and Rap1 in regulation of ERK

cAMP agonists can either stimulate or inhibit the ERK-1/2 pathway. It has been hypothesized that this mainly depends on which Raf isoforms are expressed in the cells (187).

In cells expressing Raf1, the Erk pathway would be inhibited by cAMP. In this model, cAMP would lead to activation of the small GTPase Rap1 through a pathway involving PKA and Src. Subsequently, Rap1 would form an inactive complex with Raf1, resulting in inhibition of ERK (188,189). However, activation of Src by PKA has recently been debated (190), and a number of studies have shown that activation of Rap1 is independent of PKA (69,70,116). Furthermore, growth factor activation of endogenous Rap1 does not correlate with inhibition of the ERK pathway (116), and activation of Rap1 with a cAMP analogue that specifically targets Epac but not PKA did not result in inhibition of ERK (chapter 2). Therefore, inhibition of the ERK pathway by cAMP is likely to be mediated by PKA and does not involve Rap1. Indeed, PKA phosphorylates Raf1 on multiple sites, resulting in Raf1 inhibition (Fig. 6). PKA also directly inhibits p21 activated kinase (PAK), which can phosphorylate the activating S338 residue in Raf1 (191).

Conversely, in cells expressing B-Raf, the ERK pathway would be activated by cAMP. This model also involves Rap1, because in contrast to the Rap1-Raf1 complex, the Rap1-B-Raf complex is active (114,192). Many reports support this model (140,148,156,189,193-209). However, recent studies indicate that endogenous Rap1 may not be involved in cAMP-induced ERK activation, but rather point towards a role for PKA and possibly Ras ((116,210-216); chapter 2). Which Ras-GEF would mediate the cAMP signal, and whether

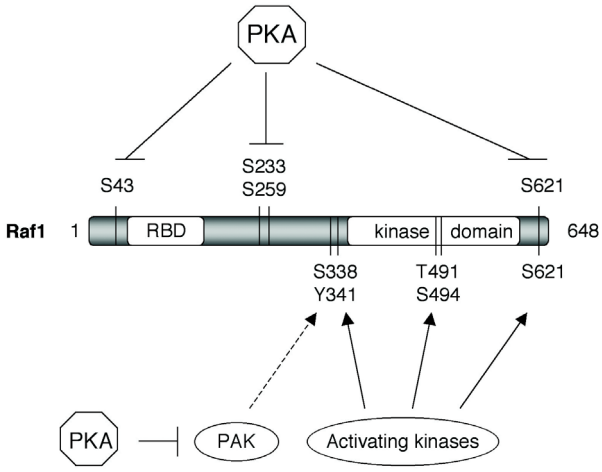

Figure 6. **Raf1 is activated by mitogens and inhibited by cAMP-PKA.** Mitogen stimulation results in phosphorylation of residues S338 (218) and Y341 (219), which synergize to stimulate kinase activity, and of T491, S494 (220), and possibly S621 (221), all of which may modulate kinase activity (222). The inhibitory residue S259 and possibly S233 are phosphorylated in resting state, and are hyperphosphorylated upon cAMP treatment (223-225). Dephosphorylation, possibly by PP2A (226), is essential for Raf1 activity. Phosphorylation of S259 interferes with multiple steps of Raf1 function, including membrane recruitment, Ras binding, phosphorylation by activating kinases, and coupling to substrates (225). Phosphorylation of S43 diminishes Ras binding (227), thereby inhibiting ERK signalling, but its physiological relevance is still a matter of debate (224,228). The same holds true for phosphorylation of S621, which has been claimed to be either activating or inhibiting (221).

Ras is not only required but actually also sufficient for ERK activation remains elusive. In addition, 14-3-3 proteins may also be involved in cAMP-activation of ERK by shielding B-Raf from inhibition by PKA (217). In conclusion, direct regulation of the ERK pathway by cAMP is likely to be independent of Rap1.

Conclusions

cAMP regulates many cellular processes through three different signalling pathways: the PKA, Epac, and CNG-channel pathways. The activity of each pathway may well be influenced by the other pathways, depending on expression and specific localization of the involved proteins in microdomains in the cell. cAMP activates Rap1 through Epac independently of PKA. Whereas PKA is absolutely required for regulation of the ERK pathway, Rap1 is clearly not. Rather, Rap1 is involved in cAMP-induced cell adhesion through modulation of integrin activity. Furthermore, cAMP may regulate a number of other processes through Epac and Rap GTPases as well, like calcium release, hormone secretion, and activation of p70S6K. It remains a major challenge to identify the Rap effector proteins which mediate these cAMP-induced, Epac-mediated cellular processes.

Scope of this thesis

We studied the role of the Epac-Rap pathway in cAMP signalling.

First, we developed an Epac-specific cAMP analogue, which only targets the Epac pathway and not the PKA pathway (chapter 2). We used this analogue to investigate the role of Rap1 in cAMP-regulation of ERK.

Next, we describe a novel function of cAMP: cell adhesion (chapter 3). We show that cAMP-induced cell adhesion is mediated by the Epac-Rap1 pathway, and is independent of PKA.

In chapter 4 we describe a function of the Epac pathway in cAMP-induced activation of p70S6K and its target ribosomal S6, which is likely to be independent of PKA.

Finally, the possible consequences of these findings are discussed in chapter 5.

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

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

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cAMP (1) is involved in a wide variety of cellular processes that were thought to be mediated by protein kinase A (PKA). However, cAMP directly regulates Epac1 and Epac2, guanine nucleotide exchange factors for the small GTPases Rap1 and Rap2 (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-transformation (6), 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 (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 signalling (11), it was reported that Rap1 mediates this effect (4). Conversely, in cell lines where cAMP activates rather than inhibits ERK, Rap1 has also been implicated in the activation of ERK (5). 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 Rap1 activation 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 (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 that forms hydrogen bonds with the 2'-hydroxyl of the ribose group of cAMP (21) 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 (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 observed at 2.2 µM 8CPT-2Me-cAMP, compared with 30 µM 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 effect 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 common PKA substrate CREB (cAMP-responsive element binding protein) (23) as an indicator for PKA activition. 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 µM 8CPT-2Me-cAMP already activates Rap1 (Fig. 1e, middle). However, 8CPT-2Me-cAMP did not induce phosphorylation of CREB, even at 100 µM (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 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 also 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 (7,8,10). 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 results 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*, it is apparently not sufficient for activation *in vivo*. The reason for this discrepancy is unclear, but it is 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

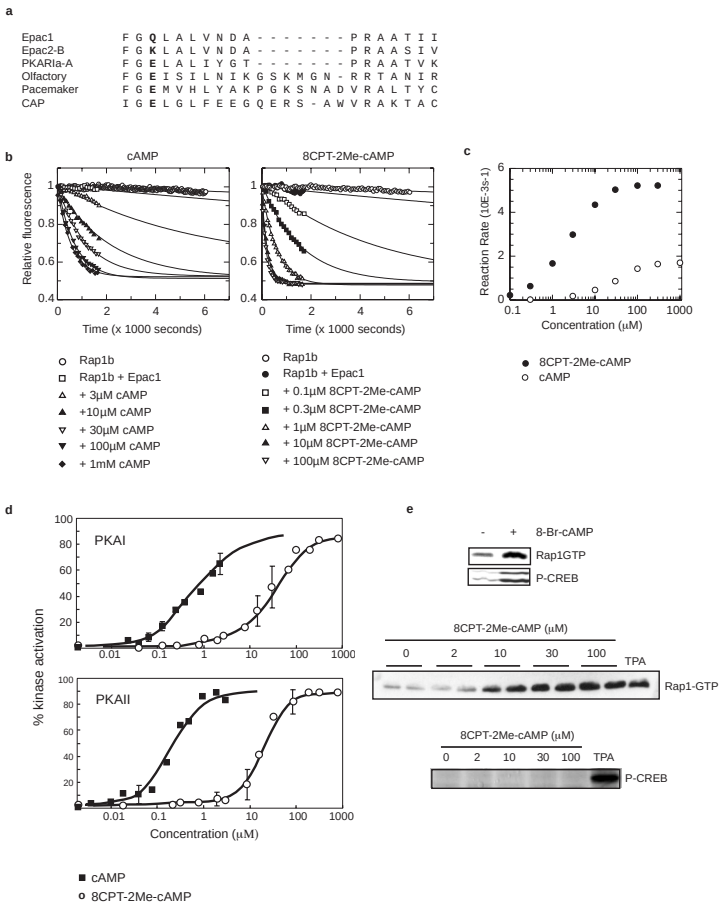

Figure 1. **Identification of an Epac-specific cAMP analogue. a,** Alignment of the cAMP binding domains of PKA, Epac, olfactory channels, pacemaker channels and the bacterial CAP protein. **b,** *In vitro* activation of Epac1. The left panel shows Rap1 loaded with fluorescent Mant-GDP in the presence of 100-fold excess of GTP and incubated with or without Epac1-ΔDEP in the presence of increasing concentrations of cAMP. The right panel shows Rap1 loaded with fluorescent Mant-GDP in the presence of 100-fold excess of GTP and incubated with or without Epac1-ΔDEP 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).

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 either 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.

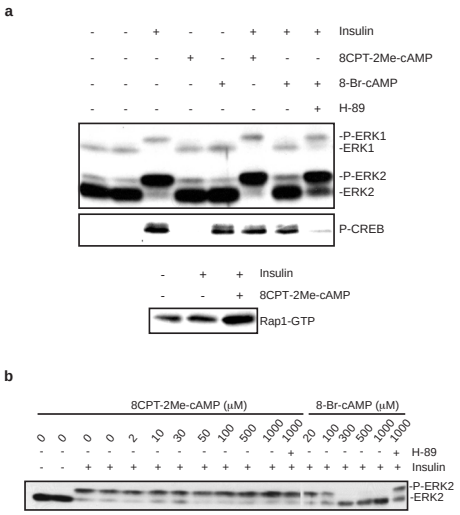

Figure 2. **ERK is inhibited by 8-Br-cAMP, but not by 8CPT-2Me-cAMP. a,** 8CPT-2Me-cAMP does not block insulin-induced ERK activation (top). NIH3T3-A14-Epac1 cells were either pretreated or not pretreated with the PKA inhibitor H-89 for 30 min, before treatment with 8-Br-cAMP or 8CPT-2Me-cAMP (100 μM) 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 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.

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-β_2M_3 cells, which express the β_2 adrenergic receptor (β_2-AR) and the M_3 muscarinic receptor (M_3-MR), thus providing a system with a physiological set-up that can be induced by extracellular stimuli. Stimulation of β_2-AR with isoproterenol activates ERK through the G_s heterotrimeric G-protein subunit, whereas stimulation of the M_3-MR with carbachol results in cAMP-independent ERK activation through the G_q G-protein subunit. In these cells, isoproterenol activated ERK and Rap1 (Fig. 4a). Ras is induced by isoproterenol, alhough the effect is weak (Fig. 4a). Isoproterenol-, forskolin-, or 8-Br-cAMP-induced activation of ERK is sensitive to H-89, whereas Rap1 activation 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 ((3) and J.M.E., J.d.R. and J.L.B., unpublished observations). Furthermore, activation of ERK with isoproterenol and carbachol 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. We next isolated clonal CHO-β_2M_3 cell lines that stably express haemagglutinin (HA)-tagged Rap1GAP1, 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. 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 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

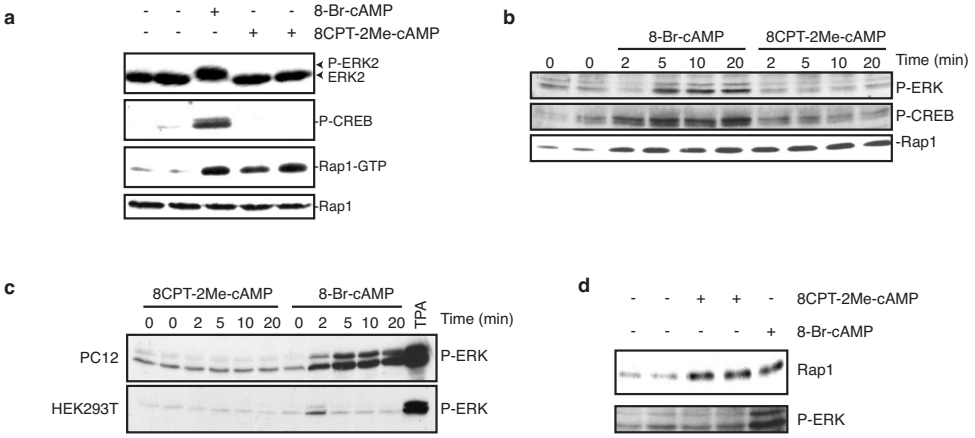

Figure 3. **cAMP-induced activation of ERK and Rap1 are distinct processes. a,** ERK is activated by 8-Br-cAMP, but not by 8CPT-2Me-cAMP, in CHO cells. Cells were treated with either 8-Br-cAMP or 100 µM 8CPT-2Me-cAMP for 15 min. Cells were lysed and assayed for GTP-bound Rap1. ERK phosphorylation was 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 µM) 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 µM 8CPT-2MecAMP 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-BrcAMP. Equal amounts of cell lysates were analysed for activation of Rap1 (top) and phosphorylation of ERK (bottom).

was unsurprising, considering the widely accepted role of PKA in cAMP-mediated regulation of ERK. Furthermore, our studies with Rap1GAP1-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 RalGEFs 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-cells (26). Furthermore, in genetic screens of lower eukaryotes, no elements of the Rap1 signalling pathway were found to affect the Ras-ERK pathway (27). Of course, indirect regulation of ERK by Rap1 is still possible. For instance, Rap1 has been shown to regulate integrin-mediated cell adhesion (28), an effect that can be mimicked by 8CPT-2Me-cAMP (29). 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 PKA (5). 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 the Rap1GAP1-overexpressing cell lines, an observation that is at odds with this model. Furthermore, PKA-induced phosphorylation of Rap1 at Ser179 has been suggested to be involved in the activation

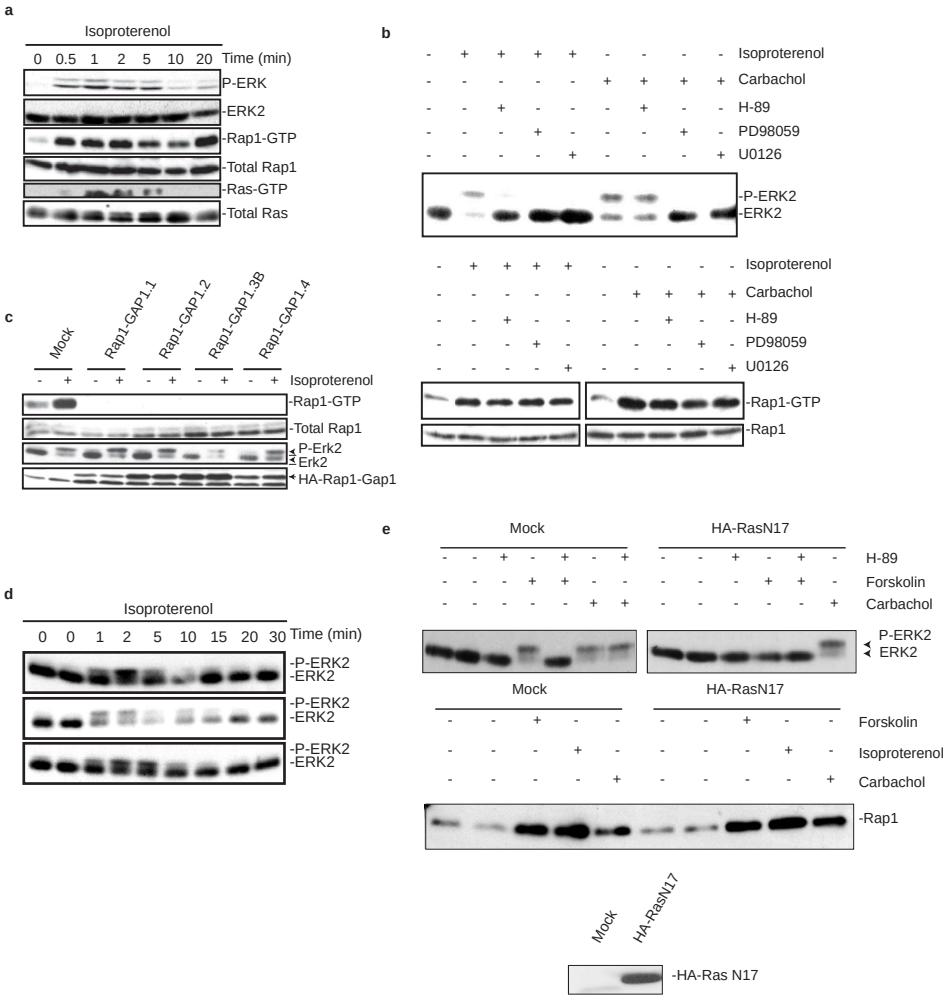

Figure 4. **cAMP-induced ERK activation is mediated by PKA and Ras. a,** Isoproterenol activates ERK, Rap1 and Ras in CHO-β_2M_3 cells. Cells were treated with isoproterenol for the indicated times and assayed for GTP-bound Rap1 and GTP-bound Ras. Activation of ERK was analysed by western blotting with a phosphospecific 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.

of Rap1 (5), although the precise function of this phosphorylation 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 phosphatase (18) and PKA-independent regulation of Ras in melanocytes (19). 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-p44/42 MAP Kinase (Thr202/Tyr204) and polyclonal phospho-CREB (Ser^{133}) (Cell Signalling, Beverly, MA, USA), Ras (Transduction Laboratories, Lexington, NY, USA), Rap1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The following inhibitors and stimuli were used at concentrations indicated, unless stated otherwise: H-89 (10 µM), PD98059 (50 µM) and U0126 (25 µM) (Biomol Research Laboratories Inc., Plymouth Meeting, PA, USA). Insulin (1 µg/ml), TPA (100 ng/ml), isoproterenol (10 µM) and carbamylcholine (carbachol, 100 µM) (Sigma-Aldrich, Steinheim, Germany). Forskolin (25 µM) (ICN, Costa Mesa, CA, USA), 8-Br-cAMP (1 mM) (Biolog Life Science Institute, Bremen, Germany).

Cells, plasmids, transfections
CHO cells stably expressing the β_2-adrenergic and the M_3-muscarinic receptor were maintained in αMEM with 10% foetal calf serum and 0.25 mg/ml hygromycin-B (Roche Diagnostics, Mannheim, Germany). Clonal cell lines were generated by calcium phosphate cotransfection of pBABE with either pMT2HA, pMT2HA-H-Ras^{S17N}, pMT2HA-Rap1GAP1 (28) and selection with puromycin 15 µg/ml. NIH3T3-A14 cells stably expressing Epac1 were grown in DMEM with 10% foetal calf serum and 2 µg/ml puromycin, Ovcar3 cells were maintained in RPMI with 10% foetal calf serum, HEK293T cells were maintained in DMEM with 10% foetal calf serum, PC12 cells in RPMI with 5% foetal calf serum and 10% donor horse serum. All cells except CHO cells were serum starved 16 hours prior to stimulation.

In vitro analyses
Cyclic AMP dependent protein kinase I and II were reconstituted from isolated subunits and assayed for kinase activity using 70 mM kemptide as substrate as described previously (30). Epac activation was measured as described (3) using 600 nM Rap1b loaded with the fluorescent nucleotide mantGDP in the presence of 100-fold excess GTP and in the absence or presence of 150 nM Epac1ΔDEP1. Increasing concentrations of cAMP or 8CPT-2Me-cAMP were added and single exponential curves were fit to the data to calculate reaction rates. Reactions were carried out in 96-well plates and measured in a Cary Eclipse from Varian Inc. using the manufacturers software.

In vivo analyses
Ras and Rap activation assays were performed as described previously (31). Phosphorylation of ERKs was either analysed by mobility shift on SDS-PAGE, which accompanies phosphorylation of these proteins, or western blotting using the phospho-specific antibody against ERK, where indicated.

Acknowledgements

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3

Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the β_2-adrenergic receptor

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Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the β_2-adrenergic receptor

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Cyclic AMP (cAMP) controls many cellular processes mainly through the activation of protein kinase A (PKA). However, more recently PKA-independent pathways have been established through the exchange protein directly activated by cAMP (Epac), a guanine nucleotide exchange factor for the small GTPases Rap1 and Rap2. In this report, we show that cAMP can induce integrin-mediated cell adhesion through Epac and Rap1. Indeed, when Ovcar3 cells were treated with cAMP, cells adhered more rapidly to fibronectin. This cAMP effect was insensitive to the PKA inhibitor H-89. A similar increase was observed when the cells were transfected with Epac. Both the cAMP effect and the Epac effect on cell adhesion were abolished by the expression of Rap1-GTPase activating protein, indicating the involvement of Rap1 in the signalling pathway. Importantly, a recently characterized cAMP analog, 8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate, which specifically activates Epac but not PKA, induced Rap-dependent cell adhesion. Finally, we demonstrate that external stimuli of cAMP signalling, i.e. isoproterenol, which activates the $G\alpha_s$-coupled β_2-adrenergic receptor can induce integrin-mediated cell adhesion through the Epac-Rap1 pathway. From these results we conclude that cAMP mediates receptor-induced integrin-mediated cell adhesion to fibronectin through the Epac-Rap1 signalling pathway.

cAMP is a common second messenger controlling many cellular processes. PKA is a general receptor for cAMP, resulting in the phosphorylation of a large variety of cellular targets. Specificity is regulated by AKAP proteins that target PKA to specific regions in the cell. A few years ago we discovered an additional cAMP target, Epac1 (exchange protein directly activated by cAMP). This protein, and its close relative Epac2 contain cAMP-binding domains very similar to the cAMP binding domains in the regulatory subunit of PKA, and are exchange factors for the small GTPases Rap1 and Rap2 (1-3).

Rap1 is a GTPase of the Ras superfamily, which functions as a molecular "switch", cycling between inactive GDP- and active GTP-bound forms. Specific guanine nucleotide exchange factors are the "on switches" and GTPase-activating proteins (GAPs) are the "off switches" (for review see (4)). Rap1 was initially identified in a screen for proteins that can suppress the transformed phenotype of fibroblasts transformed by oncogenic K-Ras (5), providing a model in which Rap1 functions as an antagonist of Ras signalling mainly by trapping Ras effectors (Raf1) in an inactive complex. However, from numerous reports accumulated so far, it is evident that Rap1 signalling is important in itself, and independently of Ras regulates several important cellular processes (4). One of the most consistent findings is the involvement of Rap1 in integrin-mediated cell adhesion (6-12).

Integrins are heterodimeric cell adhesion molecules consisting of one of several different α chains and one of at least five different β chains. One of the first indications was that in 32D cells granulocyte colony stimulating factor-induced cell adhesion could be abolished by the introduction of Spa1, a GAP for Rap proteins (13). This finding was followed by three independent observations showing a role for Rap1 in the inside-out signalling to integrins. First, in Jurkat cells introduction of Rap1 induced integrin αLβ2 (LFA1)-mediated adhesion to the intercellular adhesion molecule. Importantly, adhesion induced by ligation of the T-cell receptor was inhibited by introduction of an interfering mutant of Rap1 (8). Second, also in Jurkat cells ligation of the adhesion molecule CD-31 induced activation of αLβ2, which was inhibited by blocking Rap1 signalling (9). Finally, in a macrophage cell line, complement-mediated phagocytosis, which requires activated αMβ2, was abolished by inhibition of Rap1 signalling (6). Other studies reached the same conclusion for integrins with a β1 chain, i.e. α5β1 (7) and for integrins with a β3 chain, i.e. αIIbβ3 (14). Recently, it was shown that in mice expressing active Rap1 in their T-cell compartment both the thymocytes and mature T cells exhibited increased integrin-mediated cell adhesion. In addition, these cells showed enhanced T cell receptor-mediated responses (12). From the above results we hypothesized that cAMP or signals that raise cAMP levels may regulate integrin-mediated cell adhesion through Epac and Rap1. We have tested this model and found that indeed cAMP was able to induce integrin-mediated cell adhesion to fibronectin. It has been reported previously that PKA was a key part in a signalling pathway activated by mAb 12G10, an antibody that can activate β1 integrins and induce integrin-mediated cell-cell and cell-substrate adhesion in human fibrosarcoma cells (15). To distinguish which of the two independent cAMP signalling pathways, mediated by either PKA or Epac, is involved in integrin regulation in Ovcar3 cells, we used a novel analogue of cAMP, 8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8CPT-2Me-cAMP) that specifically targets Epac and not PKA. We demonstrate that an Epac-Rap1 pathway mediates this effect independently of PKA. We also implicate physiological consequences of intracellular increases in cAMP *in vivo* by demonstrating that agonist stimulation of the β_2-adrenergic receptor is linked to increased cell adhesion via Rap1.

Results and discussion

To investigate whether cAMP could induce integrin-mediated cell adhesion, we used ovarian carcinoma cells (Ovcar3), since these cells express the β1 integrin chain in association with α chains 1-6 and α_v, with the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins mediating binding to fibronectin (16,17). Cytomegalovirus-luciferase-transfected cells were detached with trypsin and allowed to reexpress cell surface markers. The cells were seeded onto fibronectin-coated multi-well plates in the presence or absence of 8-Br-cAMP, and the amount of cells that adhered after a certain period of time was quantified. We observed that 8-Br-cAMP augmented cell adhesion and activated Rap1 in a concentration-dependent manner to fibronectin (EC50 ~ 0.2-0.5 mM) (Fig. 1 a and b). Rap1 was activated rapidly and remained active for at least 3 h. 8-Br-cAMP-induced adhesion was also observed using a different promoter (thymidine kinase [TK]-luciferase) driving luciferase expression, and a direct method of measuring adhesion by counting cells (unpublished data). Cell adhesion induced by cAMP was insensitive to the PKA inhibitor H-89 when cells were pretreated for a short time just before adhesion (Fig. 1c, Short). It has been reported that detachment of cells rapidly and transiently activates PKA, one of the well-established targets of cAMP (18), raising the possibility that if a potential PKA substrate with a sustained phosphorylation profile was involved, addition of H-89 at a later time (post-PKA activation) may falsely imply a PKA-independent mechanism. However, when cells were treated with H-89 before trypsinization and throughout the recovery period, we found that cAMP-induced adhesion was not blocked (Fig. 1c, Long), indicating that indeed PKA was not involved. As a control for H-89 activity we measured cAMP-induced phosphorylation of the direct PKA target CREB (19) and ERK, which is also PKA-dependent (Fig. 1d). Activation of Rap1, which is independent of PKA (1,3,20) was measured also. From these results we conclude that

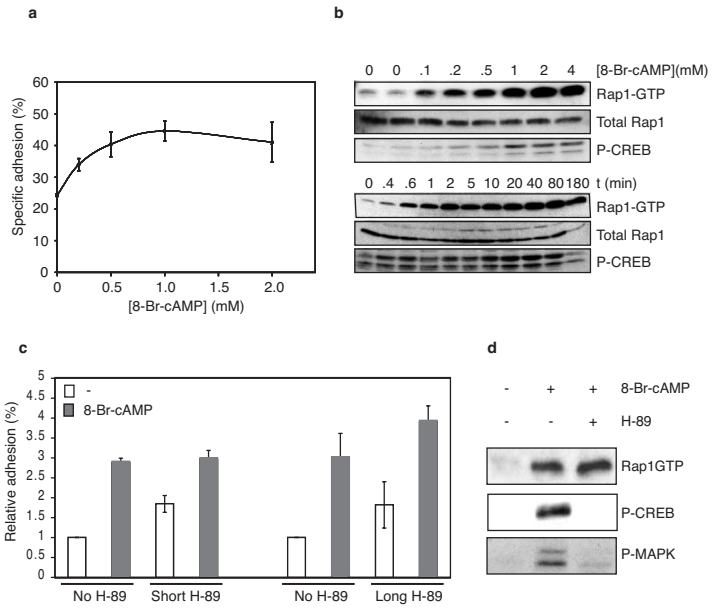

Figure 1. **cAMP induces cell adhesion to fibronectin in a PKA-independent manner. a,** Treatment with 8-Br-cAMP induces adhesion to fibronectin. Ovcar3 cells were transiently transfected with CMV-luciferase plasmid, and cells adhering to fibronectin (2 μg/ml) in the presence of increasing concentrations of 8-Br-cAMP were quantified as described in Materials and methods. **b,** 8-Br-cAMP induces Rap1 activation. (Top) Ovcar3 cells were treated with increasing concentrations 8-Br-cAMP for 15 min. Cells were lysed, and equal amounts of cell lysate were analyzed for activation of Rap1 (top blot) and CREB (bottom blot). Total levels of Rap1 in cell lysates are shown (middle blot). (Bottom) Ovcar3 cells were treated with 1 mM 8-Br-cAMP for the indicated times. Cells were lysed as above and analyzed for activation of Rap1 (top blot) and CREB (bottom blot). Total Rap1 levels are shown (middle blot). **c,** 8-Br-cAMP–induced adhesion is independent of PKA. Ovcar3 cells transiently transfected with CMV-luciferase plasmid were either preincubated at 37^{0}C for 30 min with the PKA inhibitor H-89 (10 μM) 30 min before seeding onto the wells (Short), or H-89 was added 30 min before trypsinization and during the recovery period (Long) and seeded onto wells with or without 8-Br-cAMP. Cells were allowed to adhere for 1 h, and nonadherent cells were removed. The percentage of adherent cells was quantified and plotted relative to unstimulated cells (range from 2–10%). The plot shown is representative of two (long pretreatment) and five (short pretreatment) experiments each in triplicate. Error bars represent SD. **d,** Activation of CREB and ERK but not Rap1 is blocked by H-89. Ovcar3 cells were pretreated with either H-89 or carrier for 30 min followed by stimulation with 8-Br-cAMP for 15 min. Cells were lysed, and equal amounts of cell lysates were incubated with precoupled GST-RalGDS-RBD, and activation of Rap1 was analyzed by immunoblotting using a Rap1 antibody. Phosphorylation of CREB and ERK was assayed by Western blotting using phospho-specific antibodies.

in Ovcar3 cells cAMP can induce cell adhesion to fibronectin independently of PKA.

Our finding that the induction of integrin-mediated cell adhesion by cAMP is independent of PKA suggested that Epac-Rap1 might be mediating this effect. To further test this idea, Ovcar3 cells were transiently transfected with Epac1. This resulted in an increase in basal adhesion to fibronectin, which was further increased by stimulation with 8-Br-cAMP (Fig. 2a), suggesting that Epac mediates cAMP-induced cell adhesion. This observation was further strengthened by the introduction of Rap1GAPII, an inhibitor of Rap1 (21), which attenuated Epac-induced cell adhesion (Fig. 2a). These results show that ectopic expression of Epac is sufficient to induce Rap1-dependent cell adhesion to fibronectin, which can be enhanced by additional stimulation with cAMP. It should be noted that although Rap1GAPII is more effective on Rap1 than on Rap2, we cannot exclude a role for Rap2 in this process.

To formally exclude the possibility that cAMP and Epac are on parallel pathways, both of which would be required for the induction of cell adhesion, we used a newly characterized analogue of cAMP, 8CPT-2Me-cAMP, which specifically activates Epac but not PKA even at high concentrations (20). As observed with 8-Br-cAMP, stimulation of Epac1-transfected cells with 8CPT-2Me-cAMP further increased cell adhesion to fibronectin (Fig. 2b),

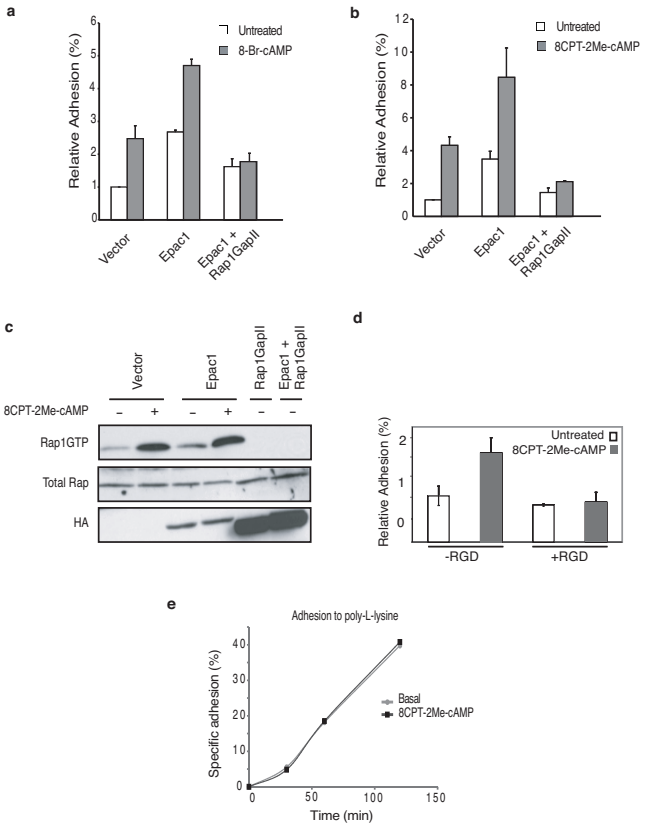

Figure 2. **Overexpression of Epac1 increases cAMP-induced cell adhesion, which is Rap1GAPII sensitive. a,** 8-Br-cAMP-Epac1–induced cell adhesion is blocked by Rap1GAPII. Ovcar3 cells were transiently transfected with TK-luciferase plasmid and either mock DNA (vector), HA-tagged Epac1, or HA-tagged Rap1GAPII where indicated. Cells were stimulated with 8-Br-cAMP, and adhesion of cells to fibronectin was quantified. The percentage of adherent cells was plotted relative to unstimulated, mock-transfected cells. Representative data performed in triplicate are shown, and error bars represent SD. The experiments were repeated at least four times with identical results. **b,** 8CPT-2Me-cAMP-Epac1–induced cell adhesion is blocked by Rap1GAPII. Ovcar3 cell were transiently transfected as above. Cells were stimulated with 8CPT-2Me-cAMP, and adhesion of cells to fibronectin was quantified. The percentage of adherent cells was plotted relative to unstimulated, mock-transfected cells. Representative data performed in triplicate are shown, and error bars represent SD. The experiments were repeated at least four times with identical results. **c,** 8CPT-2Me-cAMP-Epac1–induced Rap1 activation is blocked by Rap1GAPII. Cells were treated with 50 μM 8CPT-2Me-cAMP for 15 min, lysed, and GTP-bound Rap1 levels were determined as described in Materials and methods (top). Rap1 protein levels were equal (middle), and expression of transfected proteins was confirmed with an anti-HA antibody (bottom). **d,** A β1-integrin–blocking peptide containing the RGD sequence present in fibronectin inhibits 8CPT-2Me-cAMP–induced cell adhesion. Ovcar3 cells were pretreated for 20 min with RGD peptide (100 μM) where indicated and seeded in wells with or without 8CPT-2Me-cAMP. Cells were allowed to adhere for 1 h, and nonadherent cells were removed. The percentage of adherent cells was measured and plotted relative to unstimulated cells. Representative data from two experiments performed in triplicate are shown with error bars representing SD. **E,** 8CPT-2Me-cAMP does not increase the rate of cell adhesion to poly-L-lysine. Ovcar3 cells were transfected with CMV-luciferase and seeded onto poly-L-lysine–coated plates. At various time points, nonadherentcells were removed and adherent cells were quantified. A representative experiment in triplicate is shown.

and raised Rap1GTP levels (Fig. 2c). Expression of Rap1GAPII inhibited adhesion of cells to fibronectin and completely abolished Rap1GTP levels (Fig. 2 b and c), indicating that Rap1 is critically involved in cAMP-induced cell adhesion. We next investigated whether activation of endogenous Epac is sufficient to induce adhesion to fibronectin. Ovcar3 cells were treated with 8CPT-2Me-cAMP to activate endogenous Epac, which is abundantly expressed in ovary tissue (3). Indeed, 8CPT-2Me-cAMP significantly induced cell adhesion to fibronectin (Fig. 2d). To investigate whether cAMP-induced cell adhesion is indeed mediated by integrins, we pretreated Ovcar3 cells with the β1-integrin-binding arginine, glycine, aspartic acid (RGD) peptide. Peptides containing the RGD amino acid sequence

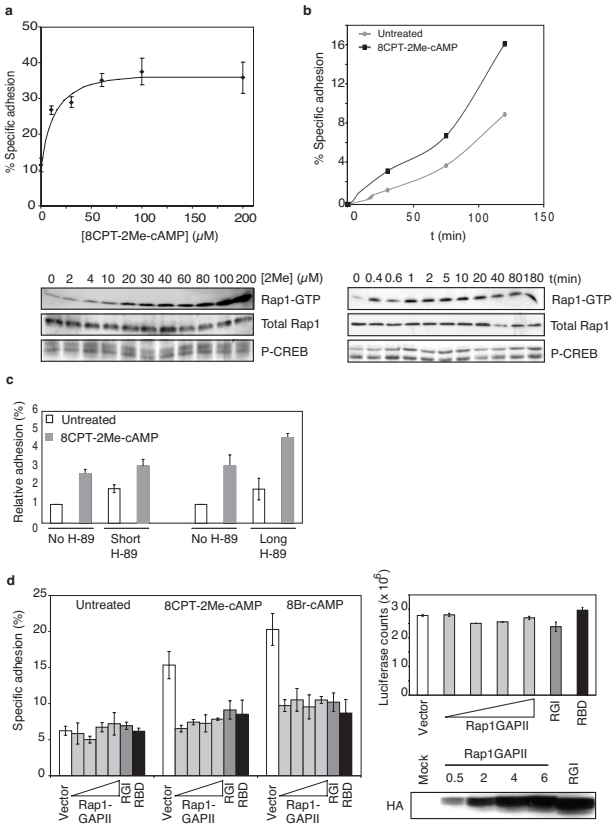

Figure 3. **8CPT-2Me-cAMP induces cell adhesion via Epac and Rap1. a,** 8CPT-2Me-cAMP stimulates cell adhesion. (Top) Ovcar3 cells were transiently transfected with CMVluciferase plasmid and treated with increasing concentrations of 8CPT-2MecAMP. Cells adhering to fibronectin (2 µg/ml) were quantified as described in Materials and methods. (Bottom) Ovcar3 cells were treated with increasing concentrations of 8CPT-2Me-cAMP for 15 min, and cells were lysed and analyzed for activation of Rap1 (top blot) and CREB (bottom blot). Total Rap1 levels are shown (middle blot). **b,** 8CPT-2MecAMP increases the rate of cell adhesion. (Top) Ovcar3 cells were transfected with TK-luciferase and seeded onto fibronectin-coated plates. At various time points, nonadherent cells were removed, and adherent cells were quantified. (Bottom) cells were treated with 60 µM 8CPT- 2Me-cAMP for the indicated times. Cells were lysed, and equal amounts of cell lysates were analyzed for activation of Rap1 (top blot) and CREB (bottom blot). Total levels of Rap1 in cell lysates are shown (middle blot). **c,** Ovcar3 cells were pretreated with H-89 as described in the legend to Fig. 1 C and seeded onto wells in the absence or presence of 8CPT-2Me-cAMP (100 µM). Cells were allowed to adhere for 1 h, and nonadherent cells were removed. The percentage of adherent cells was quantified and plotted relative to unstimulated cells (range from 2–10%). The plot shown is representative of two (long pretreatment) and five (short pretreatment) experiments, each in triplicate. Error bars represent SD. **d,** cAMP-induced adhesion to fibronectin is blocked by inhibitors of Rap1. (Left) Ovcar3 cells were transiently transfected with CMV-luciferase and either mock DNA, increasing concentrations of HA-Rap1GAP II (0.5, 1, 2, or 6 µg, respectively), HA-Rap1GAPI (6 µg), or HA-RBD of RalGDS (6 µg), respectively. Cells were treated with 8-Br-cAMP or 8CPT-2Me-cAMP, and adhesion to fibronectin (5 µg/ml) was determined and plotted relative to unstimulated, mock-transfected cells. Representative data from experiments performed in triplicate are shown with error bars representing SD. The experiments were repeated (Rap1GAPII, at least four times; Rap1GAPI and RBD, twice) with identical results. (Top right) Luciferase counts of total input cells per well in the above experiment are shown with error bars representing SD of triplicates. (Bottom left panel) Expression of HA-Rap1GAPs in the above experiment is shown.

motif bind to β1 integrins and have been shown to block fibronectin binding in ovarian carcinoma cells (16). As expected, 8CPT-2Me-cAMP-induced attachment to fibronectin was abolished (Fig. 2d). 8CPT-2Me-cAMP did not increase the integrin-independent adhesion of Ovcar3 cells to poly-L-lysine (Fig. 2e). From these results we conclude that activation of endogenous Epac induces integrin-mediated cell adhesion to fibronectin. 8CPT-2Me-cAMP enhanced cell adhesion to fibronectin and induced Rap1 activation at comparable concentrations (EC50, ~ 30 µM) (Fig. 3a). In a time-course analysis, we noted that increased adhesion was already observed after 30 min, which correlated with a rapid and sustained Rap1 activation (Fig. 3b). As expected, the induction of adhesion and activation of Rap1 were insensitive to the PKA inhibitor H-89 (Fig. 3c). However, even low levels of Rap1GAPII completely inhibited cAMP-induced adhesion of Ovcar3 cells to fibronectin (Fig. 3d, left

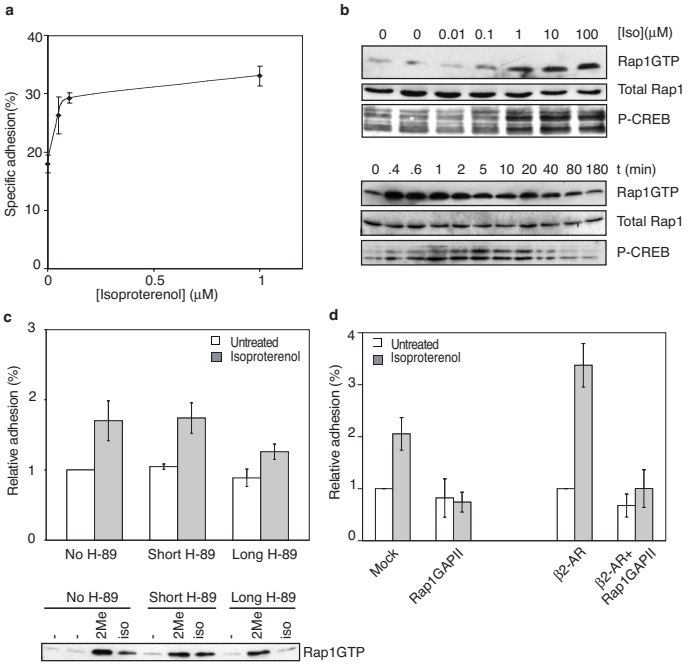

Figure 4. **Stimulation of the β2-AR with isoproterenol induces cell adhesion. a,** Isoproterenol induces adhesion to fibronectin. Ovcar3 cells transiently transfected with CMVluciferase plasmid were treated with increasing concentrations of the β2-AR agonist isoproterenol, and cells adhering to fibronectin (2 μg/ml) were quantified as described in Materials and methods. **b,** Isoproterenol induces activation of Rap1 and CREB. (Top) Ovcar3 cells were treated with increasing concentrations of isoproterenol for 5 min. Cells were lysed, and equal amounts of cell lysate were analyzed for activation of Rap1 (top) and CREB (bottom). Total levels of Rap1 in cell lysates are shown (middle blot). (Bottom) Cells were treated with 10 μM of isoproterenol for the indicated times. Cells were lysed, and equal amounts of cell lysate were analyzed for activation of Rap1 (top blot) and CREB (bottom blot). Total levels of Rap1 in cell lysates are shown (middle blot). **c,** Isoproterenol-induced adhesion to fibronectin is independent of PKA. (Top) Ovcar3 cells were pretreated with H-89 as described in the legend to Fig. 1 c and seeded onto wells in the absence or presence of isoproterenol (100 μM). Cells were allowed to adhere for 1 h, and nonadherent cells were removed. The percentage of adherent cells was quantified and plotted relative to unstimulated cells (range from 2–10%). The plot shown is representative of two (long pretreatment) and four (short pretreatment) experiments, each in triplicate. Error bars represent SD. (Bottom) Cells were pretreated with either DMSO or H-89 for 30 min before trypsinization and during the recovery period (DMSO and long H-89 treatment, respectively) or during the last 30 min of recovery (short H-89 treatment). Then cells were stimulated with either 50 μM 8CPT-2Me-cAMP for 10 min or isoproterenol for 2 min, respectively. Cells were centrifuged, cell pellets were lysed, and equal amounts of cell lysate were incubated with precoupled GST-RalGDS-RBD, and activation of Rap1 was analyzed on Western blot using a Rap1 antibody. **d,** Isoproterenol-induced adhesion to fibronectin is inhibited by Rap1GAPII. Ovcar3 cells were transfected with either mock DNA (Vector) or HA-Rap1GapII alone or in combination with a β2-AR expression vector where indicated. Adhesion of cells to fibronectin in the absence or presence of isoproterenol was quantified. The percentage of adherent cells was plotted relative to unstimulated, mock-transfected cells (range 5–15%). Summarizing data of four (for the left half of the plot) and two (for the right half of the plot) independent experiments performed in triplicate are shown with error bars representing SD.

plot). Furthermore, the Rap1-inhibitory proteins Rap1GAPI and Ras-binding domain (RBD) of Ral guanine nucleotide dissociation stimulator (RalGDS) (9) also inhibited adhesion to fibronectin (Fig. 3d, left plot). Transfection of cells with Rap1GAPs or RBD of RalGDS did not affect luciferase expression (Fig. 3d, right plot).

Our observations that cAMP analogs could induce adhesion of Ovcar3 cells to fibronectin prompted us to test whether cAMP-elevating receptors could also mirror the same effect, thereby linking an *in vivo* cAMP signalling system to integrin activation. The β_2-adrenergic receptor (β_2-AR) couples to $G\alpha_s$ type of heterotrimeric G proteins, resulting in elevation of intracellular cAMP levels and subsequent activation of PKA and Epac1-Rap1 signalling cascades (22,23). Ovcar3 cells endogenously express the β_2-AR and stimulation with isoproterenol, a ligand for the β_2-AR receptor, significantly increased adhesion to fibronectin (Fig. 4a). Treatment with isoproterenol also induced both activation of Rap1 and phosphorylation of CREB (Fig. 4b; EC50 for Rap1 activation and adhesion, ~0.05 μM). Isoproterenol-induced adhesion was insensitive to short pre-treatments with H-89 (Fig. 4c,

Short), but was partially inhibited when exposed very early to H-89 (Fig. 4c, Long). Therefore, we looked at activation of Rap1 under similar conditions. We observed that after early (Fig. 4c, Long) pretreatment with H-89, isoproterenol-induced Rap1 activation was clearly inhibited, whereas 8CPT-2Me-cAMP-induced Rap1 activation was not (Fig. 4c, bottom). Since both 8-Br-cAMP-induced and 8CPT-2Me-cAMP-induced adhesion were not blocked by H-89 (Fig. 1c and 3c), the effect of very early treatment of H-89 on β_2-AR signalling could likely be attributed to slow recovery and expression of the β_2-AR on the cell surface. This possibility is consistent with the observation that PKA is involved in vesicle fusion (24). Transient transfection of Ovcar3 cells with the β_2-AR receptor further enhanced the isoproterenol-induced adhesion to fibronectin, which was sensitive to the Rap1-inactivating protein, Rap1GAPII (Fig. 4d), showing a critical involvement of Rap1.

Our results demonstrate a clear connection between cell surface receptors that induce cAMP, cAMP signalling and integrin-mediated cell adhesion and show that this pathway is independent of PKA, but mediated by the cAMP-target Epac and the small GTPase Rap1. This conclusion is based on the observations that isoproterenol, which raises cAMP levels through activation of endogenous β_2-AR, is able to induce integrin-mediated cell adhesion to fibronectin in a Rap1-dependent, PKA-independent manner. Furthermore, importantly, a cAMP analogue that specifically activates Epac but not PKA is also able to induce cell adhesion. However, our results do not entirely exclude a role for PKA in this process. Both Rap1 and Rap1GAP are substrates for PKA (4), and thus PKA may modulate the effect of the Epac-Rap1 signalling pathway on the adhesion process.

This novel function of cAMP was found in Ovcar3, an ovarian carcinoma cell line that expresses the fibronectin-binding integrin $\alpha5\beta1$, and in NIH3T3 cells stably transfected with Epac1 (unpublished data). However, the effect may be more general and may include different cell types expressing Epac. Epac is particularly highly expressed in ovary, thyroid, kidney, adrenal gland and brain (1,25), and it is expected that the cAMP-Epac pathway leading to integrin activation may operate particularly in these tissues. In addition, Rap1 is implicated in the activation of a variety of integrins, including $\alpha L\beta2$ and $\alpha IIb\beta3$, and thus the effect may not be restricted to $\alpha5\beta1$ integrins. The regulation of integrin-mediated adhesion plays an important role in many cellular processes including cell migration, cell division, and reactions to mechanical stress, and cAMP may impinge on these processes by activation of the Epac-Rap1 pathway. In ovarian cancer cells, for instance, functional integrins and molecular events that regulate them are important for invasion into the sub-mesothelial ECM ((16,17,26); reviewed in (27)).

How Rap1 regulates integrin-mediated cell adhesion remains elusive. Integrin activity is regulated through various mechanisms, including cell surface expression (change of number), redistribution at the cell surface (change of avidity), and conformational changes (change of affinity) (12,27-30). Studies using activation-specific antibodies show that Rap1 regulates both avidity and affinity but not cell surface expression. For instance, in Jurkat cells Rap1 inhibits both the clustering and the increased affinity of $\alpha L\beta2$ (8,9,12), whereas in megakaryocytes Rap1 increases the affinity of $\alpha IIb\beta3$ (14). In addition, Rap1 is required for the direct activation of integrins by integrin-activating antibodies or manganese ions (11). Apparently, Rap1 modulates a process before integrin activation, for instance, the recruitment of an essential cofactor. Interestingly, it has recently been reported that Rap1 is essential in the formation of adherens junctions, though it is less clear whether the process involves integrin-mediated signalling (31).

Materials and Methods

Cells, plasmids, transfections

NIH-Ovcar3 (Ovcar3) cells were maintained at 37°C in RPMI 1640 supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum and 0.05% glutamine in the presence of penicillin and streptomycin. Haemagglutinin (HA)-tagged constructs of Epac1 and Rap1GAPII in the PMT2HA expression vector have been described previously (1,11). Transient transfection of Ovcar3 cells was performed using the FuGENE 6 transfection reagent (Roche Diagnostics Corporation) according to the manufacturer's procedures, using 6 µg total DNA including either a TK-luciferase plasmid (1 µg) or CMV-luciferase plasmid (0.2 µg) as indicated. Cells were serum-starved at least 16 h prior to stimulation.

Reagents

Western blotting of protein samples was carried out using polyvinylidene difluoride membranes. Antibodies against dually phosphorylated $p42/44^{MAPK}$ and phosphorylated CREB (directed against phosphorylated Ser^{133}) were obtained from Cell Signalling and antibodies against K-Rev/Rap1 and polyclonal anti-HA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The following inhibitors and stimuli were used at concentrations indicated: RGD peptide (100 µM) and H-89 (10 µM), obtained from Biomol Research Laboratories Inc. (Plymouth Meeting,). Isoproterenol (10 µM, unless indicated otherwise) was obtained from Sigma-Aldrich and 8-Br-cAMP (1 mM, unless indicated otherwise) and 8CPT-2Me-cAMP (100 µM, unless indicated otherwise) from Biolog Life Science Institute.

Adhesion Assay

24-well plates were coated overnight with fibronectin (Sigma-Aldrich; 1-5 µg/ml as indicated) in sodium bicarbonate buffer (Sigma-Aldrich). Poly-L-lysine was coated for 2 h at RT (0.1% w/v in water), washed twice with water, and dried overnight. Plates were washed in TSM buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) and blocked for 30-45 min at 37°C with 1% BSA/TSM. Transiently transfected Ovcar3 cells, serum starved 16 h before the adhesion assay, were detached by trypsinization. Cells were centrifuged at 1,500 rpm for 5 min and resuspended in serum-free RPMI containing 25 mM Hepes, 0.5% BSA and 1 g/L glucose to allow recovery of cell surface markers at 37°C for 1.5-2 h with gentle rotation in suspension. Cells were centrifuged, counted and resuspended at 3 x 10^5 cells/ml in serum-free RPMI with 0.5% BSA. The experiment was carried out in triplicates, and to each well 150 µl of cells was added to 150 µl of medium with or without stimulus. In studies with H-89 (10 µM), cells were either preincubated at 37°C for 30 min with the inhibitor before seeding the wells (short pretreatment) or H-89 was added before trypsinization, during the recovery period, and before seeding wells (long pretreatment). Cells were allowed to adhere for 1 hour at 37°C and non-adherent cells were removed by gently washing plates three times with warmed 0.5% BSA/TSM. Adherent cells were lysed in luciferase lysis buffer (15% glycerol, 25 mM Tris-phosphate pH 7.8, 1% Triton X-100, 8 mM $MgCl_2$, 1 mM DTT) at 4°C for 30 min, and units of luciferase activity were quantified with addition of equal volume of luciferase assay buffer (25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, pH7, 1 mM luciferin) using a luminometer (Lumat LB9507; Berthold technologies). Unseeded cells (150 µl) were lysed separately to determine luciferase counts in the total input cells. Specific adhesion (%) was determined (counts in cells bound/ counts in total input x 100) and plotted either directly or relative to the basal adhesion of HA vector-transfected cells. Error bars represent average deviation among experiments, and where representative experiments are depicted error bars represent standard deviation within each experiment. The expression of transfected constructs was confirmed by immunoblotting of total cell lysates.

Rap1 activation assay and phosphorylation of ERK and CREB

Rap1 activation assays were performed as described previously (32,33). Briefly, adherent cells (unless stated otherwise) were serum starved overnight, treated, and lysed in 750 µl lysis buffer (10% glycerol, 1% Nonidet P-40, 50 mM Tris-Cl pH7.5, 200 mM NaCl, 2 mM $MgCl_2$, 1 µM leupeptin, 0.1 µM aprotinin, 5 mM NaF, 1 mM $NaVO_3$). Lysates were clarified by centrifugation and 500 µl of lysate was incubated with GST-tagged RBD of RalGDS precoupled to glutathione beads to specifically pull down the GTP-bound forms of Rap1. Samples were incubated for 1 hr at 4°C while tumbling. Beads were washed four times in lysis buffer, and remaining fluid was removed with an insulin syringe. Proteins were eluted with Laemmli sample buffer and analyzed by SDS-PAGE and Western blotting using Rap1 antibodies (Santa Cruz Biotechnology, Santa Cruz Biotechnology, Inc). To 100 µl of clarified lysate 25 µl 5x Laemmli sample buffer was added, and phosphorylation of ERKs was analyzed by Western blotting using the phospho-specific antibody against $p42/44^{MAPK}$. Phosphorylation of CREB was analyzed by Western blotting using a phospho-specific antibody directed against phosphorylated Ser^{133}.

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Cyclic AMP induces activation of p70S6K through Epac

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Cyclic adenosine 3',5'-monophosphate (cAMP) controls a wide range of cellular responses to many hormones and neurotransmitters, mainly through protein kinase A (PKA). However, recently Epac, a novel cAMP receptor, was identified. Epac is a guanine nucleotide exchange factor for the Ras-like small GTPases Rap1 and 2. Here we show that cAMP, through Epac, regulates the p70/85 ribosomal S6 kinase (p70S6K). Treatment of Ovcar3 cells with 8-Br-cAMP resulted in phosphorylation of p70S6K as well as its downstream target ribosomal S6. Furthermore, the Epac-specific cAMP analogue 8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8CPT-2Me-cAMP), which only targets Epac but not PKA, also increased phosphorylation of p70S6K and S6. This effect was sensitive to the PI3K inhibitors wortmannin and LY294002, and to the mTOR inhibitor rapamycin. Cyclic AMP treatment of Ovcar3 cells neither resulted in increased phosphorylation of PKB, GSK3, and eIF4E-BP1, nor did it result in Rac activation. We conclude that cAMP activates p70S6K through Epac. Although Epac-induced activation of p70S6K requires PI3K- and mTOR activity, it might not activate the PI3K pathway.

cAMP was the first identified second messenger (1). A large number of studies have elucidated the fundamental role of cAMP in the wide range of cellular responses to many hormones and neurotransmitters. Protein Kinase A (PKA) was the first target of cAMP to be discovered (2), and was long thought to be the only mediator of cAMP signalling. However, widespread expression of cyclic nucleotide gated (CNG) channels has been demonstrated, which, like the closely related pacemaker channels, increase membrane conductance of cations like calcium upon binding of cAMP or cGMP (3-5). Furthermore, Epac, exchange protein directly activated by cAMP (also known as cAMP-GEF), has been identified, which is a widely expressed guanine nucleotide exchange factor (GEF) for the small Ras-like GTPases Rap1 and Rap2 (6,7).

Rap1 functions as a molecular 'switch' that cycles between an inactive GDP and an active GTP-bound conformation. GEFs 'switch on' the GTPases, whereas GTPase activating proteins (GAPs) function as 'off switches'. Rap1 received much attention since it was identified in a screen that could revert the K-Ras transformed phenotype of NIH3T3 cells (8). This resulted in a model in which Rap1 would function as an inhibitor of Ras by trapping Ras effectors in an inactive complex (9-14). However, recent studies have excluded a role for Rap1 in ERK signalling ((15); chapter 2). Rather, Rap1 has a function of its own. Indeed, Rap1 has been shown to regulate integrins and thereby cell adhesion ((16-22); chapter 3).

In some cell systems cAMP inhibits cell proliferation, whereas in other cell types cAMP acts as a mitogen. For instance, in Wistar rat thyroid (WRT) cells, cAMP-elevating agents stimulate proliferation in PKA-dependent and PKA-independent pathways, involving p70/85 ribosomal S6 kinase (p70S6K) and phosphatidyl inositol 3-kinase (PI3K) (23,24). p70S6K is involved in cell cycle progression (25) and it regulates mitogen-induced translation of 5' TOP mRNAs, most likely through phosphorylation of the ribosomal protein S6 on multiple residues (26,27). Regulation of p70S6K is complex, and requires PI3K and PDK1 activity, as well as mammalian target of rapamycin (mTOR)

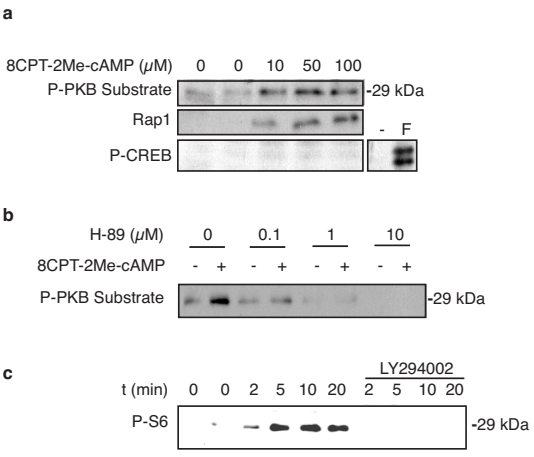

Figure 1. **Phosphorylation of a 30 kDa protein corresponding to S6 after treatment with 8CPT-2Me-cAMP. a,** treatment of Ovcar3 cells with 8CPT-2Me-cAMP induces phosphorylation of a 30 kDa protein. Cells were treated with increasing concentrations of 8CPT-2Me-cAMP for 10 min, lysed, and equal amounts of protein were analyzed for phosphorylation by Western blotting using an anti-phospho-PKB-substrate antibody (upper panel). Furthermore, equal amounts of cell lysate were checked for Rap1 activation (middle panel) and phosphorylation of CREB (lower panel). As a positive control for CREB phosphorylation, cells were treated with forskolin (F) for 10 min. **b,** phosphorylation of the 30 kDa protein is inhibited by H-89. Ovcar3 cells were pretreated for 30 min with increasing concentrations of H-89 as indicated, and treated with 8CPT-2Me-cAMP for 10 min where indicated. Cells were lysed and equal amounts of cell lysate were analyzed for phosphorylation using an anti-phospho-PKB-substrate antibody. **c,** 8CPT-2Me-cAMP induces PI3K-dependent phosphorylation of S6. Ovcar3 cells were pretreated with LY294002 for 30 min where indicated and subsequently treated with 8CPT-2Me-cAMP for the indicated times. Equal amounts of cell lysate were analyzed for phosphorylation of S6 using phospho-specific S6 antibodies.

activity (28). Recent studies have indicated that the tuberous sclerosis (TSC) gene products, TSC1 (hamartin) and TSC2 (tuberin), are regulated by PI3K and function as upstream regulators of mTOR and p70S6K (29-34). In parallel, mTOR also stimulates mRNA translation. It phosphorylates the eukaryotic initiation factor 4E binding protein 1 (eIF4E-BP1), which in resting state forms an inactive complex with eIF4E. Upon phosphorylation, eIF4E-BP1 dissociates, and eIF4E subsequently increases mRNA translation (35). In contrast to the acute phosphorylation of both p70S6K and eIF4E-BP1, the effects of mitogens on mTOR are small and controversial, and the exact mechanism of regulation of mTOR function has been questioned (35,36). In addition, activity of mTOR is regulated by carbon and nitrogen sources, which influence ATP levels in the cell, and therefore mTOR has been proposed to function as an ATP sensor (37). Cyclic AMP has been reported to stimulate p70S6K activity (23,24). Furthermore, Epac has been implicated in cAMP induced activation of PI3K and PKB, upstream regulators of p70S6K (38). Therefore, we investigated the possibility that Epac can mediate the stimulatory effects of cAMP on p70S6K. We show that treating Ovcar3 cells with either 8-Br-cAMP or 8CPT-2Me-cAMP, a cAMP analogue that specifically targets Epac and not PKA, results in phosphorylation of p70S6K and S6. Furthermore, we show that this effect of cAMP is dependent on activity of the PI3K and the mTOR pathways, since 8CPT-2Me-cAMP-induced p70S6K activation was completely blocked by the PI3K inhibitors LY294002 and wortmannin, and by the mTOR inhibitor rapamycin. However, no effect was observed of 8CPT-2Me-cAMP on the PI3K-targets PKB, GSK3, and Rac, nor phosphorylation of the mTOR target eIF4E-BP1, suggesting that although these pathways are critically required, 8CPT-2Me-cAMP activates p70S6K through a different yet unknown mechanism.

Results

Epac has been reported to be involved in activation of the PI3K and PKB pathway (38). Therefore, we searched for a role for PI3K in Epac-mediated cAMP signalling. We treated Ovcar3 cells with increasing concentrations of 8CPT-2Me-cAMP, which specifically targets Epac1 but not PKA (39), and probed whole cell lysates (WCL) on western blot with a phospho-PKB-substrate antibody. A protein of approximately 30 kDa became readily phosphorylated with similar stoichiometry as

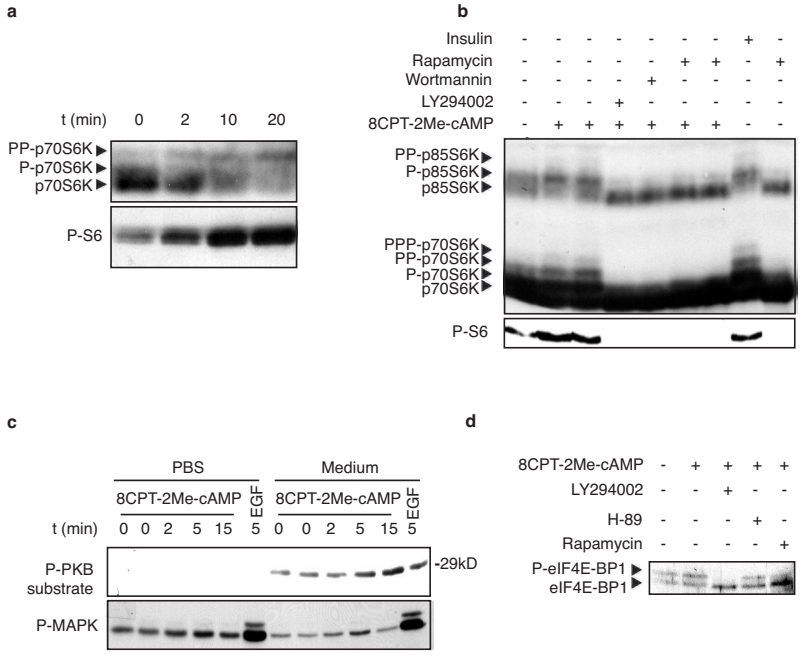

Figure 2. **cAMP induces phosphorylation of p70S6K through Epac1 and requires mTOR- and PI3K-activity. a,** 8-Br-cAMP activates p70S6K. Ovcar3 cells were treated with 1mM 8-Br-cAMP for the indicated times, lysed, and phosphorylation of p70S6K was analyzed on Western blot by shift (upper panel), or phosphorylation of S6 using phospho-specific S6 antibodies (lower panel). **b,** inhibition of mTOR and PI3K inhibits 8CPT-2Me-cAMP-induced p70/p85S6K phosphorylation. Ovcar3 cells were pretreated for 30 min with either rapamycin, wortmannin, or LY294002, respectively. Subsequently, cells were treated for 5 min with insulin or for 15 min with 8CPT-2Me-cAMP, lysed, and equal amounts of cell lysate were analyzed for phosphorylation of p70/p85S6K by shift on Western blot (upper panel), or phosphorylation of S6 ribosomal protein using phospho-specific antibodies (lower panel). **c,** amino acid starvation blocks 8CPT-2Me-cAMP-induced phosphorylation of the 30 kDa protein. Ovcar3 cells were incubated for 15 min with PBS ('PBS') or left on medium ('medium') before treatment with 8CPT-2Me-cAMP or EGF for the indicated times, respectively. Cells were lysed and equal amounts of cell lysate were analyzed on Western blot for phosphorylation of the 30 kDa protein or for phosphorylation of p42/44 MAPK using phospho-specific PKB-substrate antibodies or phospho-specific MAPK antibodies, respectively. **d,** 8CPT-2Me-cAMP does not induce eIF4EBP1 phosphorylation. Ovcar3 cells were pretreated with inhibitors where indicated and treated or not treated with 8CPT-2Me-cAMP for 15 min. Lysates were analyzed for eIF4EBP1 phosphorylation using eIF4EBP1 antibodies.

activation of Rap1 (Fig. 1a). Specificity of 8CPT-2Me-cAMP towards Epac was confirmed using a phospho-specific CREB antibody. It should be noted that phosphorylation of the 30 kDa protein was subject to variation, and seemed to depend on culture conditions and cell density, and possibly other factors, like starvation of cells for glucose or nitrogen (data not shown). Surprisingly, pretreatment of cells with the PKA inhibitor H-89 blocked 8CPT-2Me-cAMP-induced phosphorylation of the 30 kDa protein (Fig. 1b). However, 8CPT-2Me-cAMP even at high concentrations does not activate PKA ((39); and Fig. 1a), and H-89 not only inhibits PKA (IC50: 135 nM) but also efficiently inhibits other kinases with a potency similar to or greater than that for PKA. For example, p70S6K, MSK1, ROCK-II and PKB are inhibited by H-89 with IC50s of 80 nM, 120 nM, 270 nM and 2.6 µM, respectively (40). Therefore, a direct role for PKA in

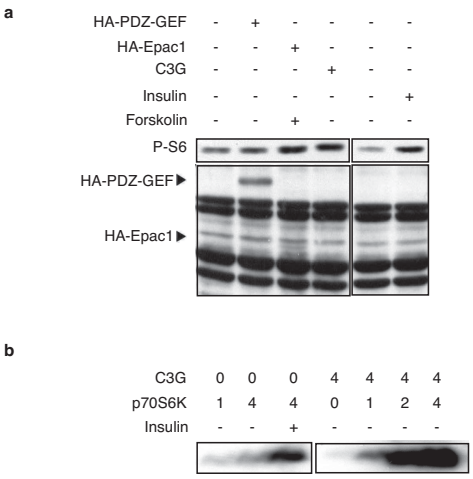

Figure 3**. Expression of Rap1 exchange factors induces S6 phosphorylation. a,** NIH3T3-A14 cells were transfected with exchange factors for Rap1 as indicated. 48 h after transfections cells were lysed and equal amounts of cell lysate were analyzed for S6 phosphorylation (upper panel). Expression of proteins was confirmed using HA antibodies. Expression of C3G could not be detected since it is not HA-tagged. **b,** NIH3T3 cells were transfected as indicated. After 48 h cells were lysed, and a kinase assay was performed using HA-immunoprecipitated p70S6K.

phosphorylation of the 30 kDa protein is unlikely. Since H-89 inhibits p70S6K even more efficiently than PKA, and the S6 ribosomal protein has a MW of approximately 30 kDa, we hypothesized the identity of the 30 kDa protein to be S6. To test this hypothesis we treated cells with 8CPT-2Me-cAMP and monitored S6 phosphorylation on Western blot. Indeed, S6 becomes readily phosphorylated upon 8CPT-2Me-cAMP treatment, which is completely blocked by the PI3K inhibitor LY294002 (Fig. 1c), confirming previous results that PI3K activity is necessary for full p70S6K activity (28). Taken together, cAMP induces phosphorylation of S6 through Epac and requires PI3K activity.

Activation of p70S6K follows phosphorylation of p70S6K on at least eight residues (41). Since cAMP treatment results in phosphorylation of ribosomal S6, we wanted to investigate p70S6K phosphorylation directly. Treatment of Ovcar3 cells with 8-Br-cAMP produced several shifted p70S6K protein bands on Western, indicating phosphorylation of p70S6K on multiple sites, and resulted in concomitant phosphorylation of the ribosomal S6 protein (Fig. 2a). Furthermore, treatment of cells with the Epac-specific cAMP-analogue 8CPT-2Me-cAMP induced multiple shifts of the p70 and p85 S6K isoforms, and phosphorylation of ribosomal S6 (Fig. 2b). Pretreating cells with the PI3K inhibitors wortmannin and LY294002 and the mTOR inhibitor rapamycin completely abrogated p70/p85S6K and ribosomal S6 phosphorylation, confirming previous studies showing that S6K activity requires activity of both the PI3K and the mTOR pathway (28). Since mTOR is required for activity of p70S6K, we wanted to investigate whether Rap1 is involved in regulation of mTOR. mTOR plays an important role in amino acid sensing (37,42). Therefore we starved Ovcar3 cells for amino acids by leaving them on PBS for 15 min. When cells were kept on medium, stimulation with either 8CPT-2Me-cAMP or EGF resulted in phosphorylation of the 30 kDa protein corresponding to ribosomal S6 (Fig. 2c, upper panel). However, when cells were stimulated in PBS, neither 8CPT-2Me-cAMP nor EGF was capable of inducing phosphorylation. In contrast, EGF treatment of cells in PBS induced MAPK phosphorylation just as well as in medium (Fig. 2c, lower panel). Thus, treatment of cells with 8CPT-2Me-cAMP does not bypass the requirement for mTOR-activity to induce full S6 phosphorylation, implying a link between cAMP and mTOR. Therefore, we investigated whether 8CPT-2Me-cAMP modulates mTOR activity directly. However, 8CPT-2Me-cAMP treatment of Ovcar3 cells did not result in enhanced phosphorylation of the mTOR target eIF4E-BP1, as visualized by shift on western blot, although pre-treatment of cells with LY294002 and rapamycin, and to a lesser extent H-89 resulted in decreased phosphorylation levels compared with untreated cells (Fig. 2d). Altogether, cAMP treatment results in p70/85S6K and ribosomal S6 phosphorylation through the Epac pathway, most likely involving the small GTPase Rap1. Furthermore, although the activity of PI3K and the mTOR pathways are required for S6

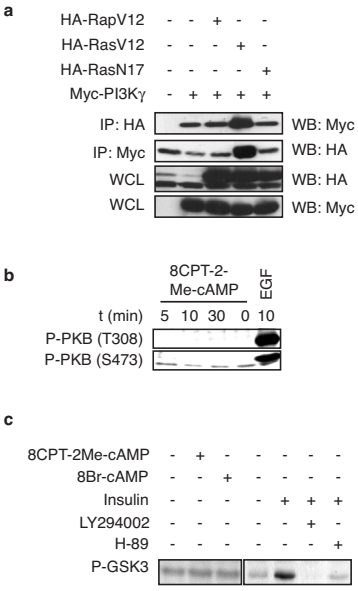

Figure 4. **Rap1 does not co-immunoprecipitate with PI3Kγ and does not induce phosphorylation of PKB and GSK3. a,** HA-Rap1bV12 does not co-immunoprecipitate with PI3Kγ. HEK293T cells were transfected as indicated. 48 hrs after transfection cells were lysed and either HA-tagged or Myc-tagged proteins were immunoprecipitated from equal amounts of cell lysate. Co-immunoprecipitation was checked on Western blot using antibodies against Myc or HA, respectively (upper two panels). Expression of proteins was confirmed using HA and Myc antibodies (lower two panels). **b,** 8CPT-2Me-cAMP does not induce phosphorylation of PKB. Ovcar3 cells were treated with either EGF or with 8CPT-2Me-cAMP for the indicated times. Cells were lysed and equal amounts of cell lysate were analyzed for phosphorylation of PKB using phospho-specific antibodies against T308 or S473, respectively. **c,** cAMP does not induce GSK3 phosphorylation. Ovcar3 cells were pretreated for 30 min with either LY294002 or H-89 where indicated. Subsequently, cells were stimulated for 15 min with either 8CPT-2Me-cAMP, 8-Br-cAMP, or insulin, respectively. Equal amounts of cell lysate were analyzed for phosphorylation of GSK3 using a phospho-specific GSK3 antibody.

phosphorylation, it does not directly activate mTOR.

In order to confirm that the Rap1-p70S6K pathway is functional in other cell systems as well, we transfected A14 cells (NIH3T3 cells overexpressing the human insulin receptor) with various RapGEFs. Transfection with PDZ-GEF gave a modest increase in S6 phosphorylation (Fig. 3a), whereas both transfection with Epac1 in conjunction with a 10 min forskolin treatment as well as transfection with C3G resulted in a more robust S6 phosphorylation. Furthermore, to directly assess p70S6K kinase activity we transfected A14 cells with increasing concentrations of p70S6K cDNA in absence or presence with equal amounts of C3G cDNA. As shown in Fig 3b, p70S6K is much more active when C3G is co-transfected, indicating that co-transfection of C3G boosts p70S6K activity. In conclusion, in NIH3T3 cells, transfection of RapGEFs results in an increase of p70S6K activity as well as S6 phosphorylation, indicating that activation of Rap1 results in activation of p70S6K.

Since cAMP-induced p70S6K activation requires PI3K activity, and PI3Kγ has previously been shown to bind H-Ras through its Ras Binding Domain (RBD) (43), and since Rap1 has an identical effector domain as Ras, we wanted to know whether Rap1 can also directly interact with this PI3K isoform. However, in transiently transfected HEK293T cells, only H-RasV12 co-immunoprecipitated with PI3Kγ, whereas Rap1V12 and, as a control, H-RasN17 did not (Fig. 4a). Furthermore, PKB and GSK-3, *in vivo* downstream targets of PI3K, did not get phosphorylated when Ovcar3 cells were treated with 8CPT-2Me-cAMP, whereas EGF treatment clearly resulted in phosphorylation of these proteins (Fig. 4b and 4C, respectively). Finally, Rac, a small GTPase which is also activated in a PI3K-dependent manner, and which previously has been reported to be activated by cAMP (44,45), was neither activated by 8CPT-2Me-cAMP nor by forskolin in Ovcar3 cells and Chinese Hamster Ovary E14 (CHO-E14) cells (Fig. 5a). Since cAMP-induced Rac activation could be transient,

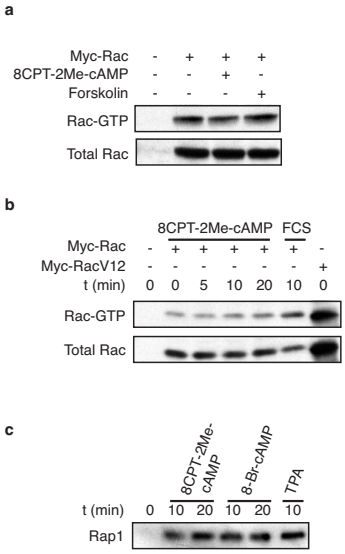

Figure 5. **cAMP does not activate Rac in Ovcar3 cells and in CHO-E14 cells. a,** cAMP does not activate Rac. CHO-E14 cells (upper panel) and Ovcar3 cells (lower panel) were transfected with Myc-tagged Rac where indicated. 24 h after transfection cells were serum starved, and 48 h after transfection cells were treated with either 8CPT-2Me-cAMP or forskolin for 15 min, lysed, and equal amounts of cell lysate were analyzed for Rac using Myc antibodies. **b,** in a time course, 8CPT-2Me-cAMP does not activate Rac. CHO-E14 cells (upper panel) and Ovcar3 cells (lower panel) were transfected with Myc-tagged Rac or Myc-tagged RacV12 where indicated. 24 h after transfection cells were serum starved, and 48 h after transfection cells were treated with 8CPT-2Me-cAMP for the indicated times or with 20% FCS ('FCS'), where indicated. Subsequently, cells were lysed and equal amounts of cell lysate were analyzed for Rac activation using Myc antibodies. **c,** Rap1 is activated by cAMP and TPA in CHO-E14 cells. Cells were serum starved for 24 h, treated with either 8CPT-2Me-cAMP, 8-Br-cAMP, or TPA for the indicated times. Subsequently, cells were lysed, and equal amounts of cell lysate were analyzed for Rap1 activation.

we also performed a time course of 8CPT-2Me-cAMP in Ovcar3 cells and CHO-E14 cells (Fig. 5b). As a control, Rap1 was readily activated upon treatment with either 8CPT-2Me-cAMP, 8-Br-cAMP, or TPA (Fig. 5c). In conclusion, although PI3K activity is required for cAMP-induced phosphorylation of p70S6K and ribosomal S6, Rap1 most likely does not directly activate PI3K upon cAMP treatment.

Discussion

Cyclic AMP directly regulates many cellular processes like for example glucose metabolism, long-term potentiation, cell cycle progression, cell adhesion, and hormone secretion. Cyclic AMP affects many of these processes through diverse cellular signalling pathways, like for instance the ERK pathway (46), the calcium pathway (47,48), and PI3K. Although PKA was long thought to account for all of these effects, recently Epac has attracted much attention. Epac, but also CNG channels, appear to mediate a number of the effects of cAMP. For example, Epac has been implicated in calcium signalling (47-51), hormone secretion (50,52), and cell adhesion (53). Furthermore, Epac may play a role in modulation of the PI3K-PKB pathway (38).

In this study, we analyzed the ability of cAMP to activate p70S6K. Both 8-Br-cAMP and the Epac-specific cAMP analogue 8CPT-2Me-cAMP increased phosphorylation of p70S6K and S6 in Ovcar3 cells, indicating that Epac mediates this effect. Although the activity of the PI3K and mTOR pathways were absolutely necessary for cAMP to activate p70S6K, they did not seem to be activated by cAMP, since neither the PI3K targets PKB, GSK3, and Rac, nor the mTOR target eIF4E-BP1 were affected by cAMP. It should be noted that there was variation in activation of p70S6K by cAMP. It is not clear what might be the cause of this, but culture conditions, cell density (54), days of cell culturing and thereby starvation of e.g. carbon or nitrogen (37), or the extent of cell adhesion and thereby focal adhesion kinase (FAK) activity (55) may all play a role. Furthermore, the effect of 8CPT-2Me-cAMP on S6 was only observed in Ovcar3 cells, and may therefore be very cell-type specific (data not shown).

Cyclic AMP has been reported to activate p70S6K in a number of cell types, including rat thyroid cells, rat pancreatic cells, and Swiss3T3 cells (24,56,57). Additionally, cyclic AMP may activate the PI3K-PKB pathway through Epac in human embryonic kidney (HEK) 293 cells (38). Our results indicate a role for Epac in cAMP-induced p70S6K activation. How exactly Epac mediates this effect remains unknown. Activation of Epac

did not seem to result in activation of the PI3K pathway or the mTOR pathway, although it cannot be excluded that activation of Epac results in activation of a specific, very localized PI3K, that only signals to p70S6K but not to PKB (58). Alternatively, although PI3K and mTOR pathways are clearly required, Epac may activate p70S6K through a different mechanism, for example by inactivating a phosphatase (59), or by activating integrins, which results in outside-in signaling (55).

The effect of cAMP on PKB seems to be the result of a balance between the inhibiting activity of the PKA pathway and the activating activity of the Epac pathway (38). There is considerable cross-talk between cAMP signalling pathways (47,48). Regulation of p70S6K by cAMP therefore may be complex and subject to several cAMP-regulated pathways.

Materials and methods

Cells, plasmids, transfections

NIH-Ovcar3 (Ovcar3) cells and HEK293T cells were maintained at 37°C in RPMI 1640, NIH3T3-A14 cells were maintained in DMEM. All media were supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum and 0.05% glutamine, in the presence of penicillin and streptomycin. Expression vector for C3G was a generous gift from dr. Michiyuki Matsuda (International Medical Center of Japan, Tokyo, Japan), expression vectors for Myc-tagged PI3Kγ and S6 cDNA were generously provided by dr. Phil Hawkins (The Babraham Institute, Babraham, Cambridge CB2 4AT, UK) and dr. George Thomas (Friedrich Miescher Institute, Basel, Switzerland), respectively. HA-tagged constructs of Epac1, PDZ-GEF, Rap1bV12, H-RasV12, H-RasN17 have been described previously (7,17,18,60). Transient transfection of HEK293T cells was performed using the FuGENE 6 transfection reagent (Roche Diagnostics Corporation) according to the manufacturer's procedures, using 5 µg total DNA. NIH3T3-A14 cells were transfected by the calcium phosphate method.

Reagents

The following inhibitors and stimuli were used at concentrations indicated: H-89 (10 µM), LY294002 (10 µM) and rapamycin (10 nM) were all from Biomol Research Laboratories Inc, Plymouth Meeting, PA, USA; wortmannin (100 nM), insulin (1 µg/ml) and EGF (20 ng/ml) were from Sigma; Forskolin (25 µM; ICN, Costa Mesa, CA, USA); 8-Br-cAMP (1 mM) and 8CPT-2Me-cAMP (100 µM, unless indicated otherwise) were from Biolog Life Science Institute, Bremen.

Antibodies

Western blotting of protein samples was carried out using polyvinylidene difluoride membranes. Antibodies against dually phosphorylated p42/44^{MAPK}, phospho-CREB, phospho-S6, phospho-PKB (S473 or T308), phospho-GSK3, and a phospho-PKB-substrate antibody were all obtained from Cell Signalling Technology. Antibodies against K-Rev/Rap1 and p70S6K were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Haemagglutinin (HA)-tagged proteins were immunoprecipitated and detected with monoclonal HA antibodies (12CA5), Myc-tagged proteins were immunoprecipitated and detected with monoclonal Myc antibodies (9E10).

Rap1 activation assay

Rap1 activation assays were performed as described previously (61,62). Briefly, cells were treated, lysed in 750 µl lysis buffer (10% glycerol, 1% Nonidet P-40, 50 mM Tris-Cl pH7.5, 200 mM NaCl, 2 mM $MgCl_2$, 1 µM leupeptin, 0.1 µM aprotinin, 5 mM NaF, 1 mM $NaVO_3$). Lysates were clarified by centrifugation and 500 µl of lysate was incubated with GST-tagged Ras Binding Domain of RalGDS pre-coupled to glutathione beads to specifically pull down the GTP-bound forms of Rap1. Samples were incubated for 1 hr at 4^{0}C while tumbling. Beads were washed four times in lysis buffer, remaining fluid was removed with an insulin syringe. Proteins were eluted with Laemmli sample buffer and analyzed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and Western blotting using Rap1 antibodies. To 100 µl of clarified lysate 25 µl of 5x Laemmli sample buffer was added and phosphorylation of proteins was analyzed by Western blotting using phospho-specific antibodies, phosphorylation of p70S6K and p85S6K isoforms were analyzed by shift on Western blot using p70S6K antibodies, which also recognize the p85S6K isoform.

Immunoprecipitation

HEK293T cells were lysed in lysis buffer containing 10% glycerol, 1% Nonidet P-40, 50 mM Tris-Cl pH7.5, 200 mM NaCl, 2 mM $MgCl_2$, 1 µM leupeptin, 0.1 µM aprotinin, 5 mM NaF, 1 mM $NaVO_3$ and transfected proteins were immunoprecipitated using antibodies against HA or Myc precoupled to protein G-sepharose beads for three hours at 4^{0}C. Beads were washed four times with lysis buffer and proteins were eluted with 1x laemmli sample buffer followed by SDS-PAGE and Western blotting.

Kinase assay

Cells transfected with cDNA encoding HA-tagged p70S6K were washed twice with ice-cold lysis buffer (NaCl 120 mM, Tris 50 mM pH7.5, EDTA 1mM, EGTA 6 mM, NaF 20 mM, benzamidine 1 µM, pyrophosphate (NaPPi) 15 µM, and para-nitrophenylphosphate (pNPP) 30 µM). Cells were lysed in lysis buffer supplemented with Nonidet P-40 1%. Cell lysates were precleared with pre-immune rabbit antibody precoupled to prot-A agarose beads for 15 min at 4^{0}C, and p70S6K was immunoprecipitated for 2.5 hrs at 4^{0}C using the HA antibody 12CA5 precoupled to prot-A agarose beads. Subsequently, beads were washed with ice-cold lysis buffer for three times and once with ice-cold dilution buffer (MOPS 20 mM, Triton X-100 0.2%, and $MgCl_2$ 10 mM). After resuspending beads in 5 µl dilution buffer supplemented with DTT 1 mM, and 5 µl reaction mix (ATP 34 µM, DTT 340 µM, MOPS (pH7.2) 17 mM, $MgCl_2$ 3.4 mM, pNPP 3.4 µM, PKI 0.25 µg, p70S6K Rsk substrate peptide (Santa Cruz Biotechnology) 4 µg, and γ-32P-ATP), they were incubated at 30^{0}C for 15 min and tumbled occasionally. Reaction was stopped by adding Laemmli sample buffer.

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5

Discussion

Cyclic AMP, in 1956 by Earl Sutherland the first second messenger to be discovered, regulates a wide variety of cellular processes in response to many neurotransmitters and hormones. Since the identification of PKA (1) it was long thought that all the effects of cAMP were solely executed through this kinase. Therefore it came as a surprise when an ion channel was discovered that was also regulated by direct binding of cAMP (2). Since then two families of cyclic nucleotide-regulated ion channels have been identified: cyclic nucleotide-gated ion channels (CNG channels), and hyperpolarization-activated and cyclic nucleotide-gated channels (HCN channels, also known as pacemaker channels) (3-5). More recently Epac, a third direct cAMP-target, has been discovered (6,7). Epac is an exchange protein for the small Ras-like GTPases Rap1 and Rap2. Cyclic nucleotide-regulated ion channels and Epac may participate in a number of cAMP-regulated cellular processes that were originally thought to be executed solely through PKA, like for example cell proliferation, cell adhesion, regulation of calcium levels, and hormone secretion.

Cyclic AMP and cell proliferation

A well-known effect of cAMP is regulation of proliferation. In some cell types cAMP inhibits proliferation, whereas in other cell types it stimulates proliferation. In cells in which cAMP inhibits proliferation, it also inhibits growth factor-induced ERK activation (8,9). Recently, this effect of cAMP has been claimed to be mediated by the small GTPase Rap1 (10,11), which can form an inactive complex with Raf1 (12,13). In this model, cAMP activates Src through PKA, upon which a complex of Cbl, Crk, and the Rap1-GEF C3G assembles and Rap1 is activated. However, PKA-mediated activation of Src has recently been debated (14). Furthermore, hormone-induced activation of endogenous Rap1 does not interfere with Ras signaling (15). Finally, a cAMP analogue that specifically activates the Epac1-Rap1 pathway but not the PKA pathway failed to inhibit growth factor-induced ERK signaling (chapter 2). Therefore it is unlikely that cAMP inhibits growth factor induced ERK signaling through Rap1. Indeed, a number of recent studies have shown that PKA completely abolishes growth factor-induced ERK activation either through direct phosphorylation of Raf1 (16-19), or through phosphorylating and thereby inhibiting PAK, an upstream activator of Raf1 (20).

How cAMP inhibits cell proliferation is not clear. Although phosphorylation of Raf1 by PKA slows down proliferation, it does not block the cell cycle (16). Therefore, cAMP probably also targets other cell cycle regulators. For instance, cAMP increases levels of the cell cycle inhibitor $p27^{Kip1}$ in macrophages (21). Furthermore, in addition to $p27^{Kip1}$ levels, cAMP also suppresses cyclin D3 levels in leukemic T cell lines (22). cAMP also activates the transcriptional repressor ICER, which has anti-tumorigenic and cell cycle inhibiting activity by inhibiting expression of c-fos and cyclin A ((23) and references therein). Since PKA substrate motifs and CREs are widespread, most likely many other ways of cAMP to control cell proliferation exist.

In certain cell lines cAMP stimulates cell proliferation and activates Erk. The fact that in one cell line cAMP stimulates Erk, whereas in others it inhibits Erk signaling, has been explained by expression of different Raf isoforms. In cells expressing Raf1, cAMP would inhibit Erk signaling by promoting formation of an inactive Rap1-Raf complex, whereas in cells expressing the B-Raf isoform, cAMP would activate Erk. In contrast to the Rap1-Raf1 complex, the Rap1-B-Raf complex is active (24), and therefore Rap1 has been suggested to be mediating cAMP-induced Erk activation (25,26). This model is supported by a large number of reports (27-46). However, recent studies indicate that endogenous Rap1 may not be involved in cAMP-induced ERK activation, but rather point towards a role for PKA and possibly Ras (15,47-53). Indeed, a cAMP analogue that specifically targets Epac-Rap1 but not PKA, did not result in Erk activation, and overexpression of dominant negative Ras^{S17N}, but not the Rap inhibitor Rap1GAPI blocked cAMP-induced Erk activation (chapter 2). Therefore, Rap1 is unlikely to be involved in Erk regulation. How cAMP would activate Erk is unknown. Several mechanisms have been suggested, including inactivation of an Erk phosphatase by PKA, and PKA-independent regulation of Ras (47,53), although it remains elusive which Ras-GEF would mediate the cAMP signal.

Another potential mediator of cAMP-stimulated cell proliferation is the PI3K pathway. PI3K, and its targets PKB and p70S6K are involved in regulation of cell proliferation and survival (54-56). One of the best studied model systems for the effect of cAMP on cell proliferation is the thyroid cell system. In these cells cAMP-elevating agents stimulate proliferation in PKA-dependent and PKA-independent pathways, involving PI3K and p70S6K (57,58). Since ectopic expression of PKA does not mimick cAMP-induced thyroid cell proliferation (59), other cAMP pathways must also play a role, and although the involvement of the Epac-Rap1 pathway remains unclear from these studies, cAMP efficiently activates Rap1 in thyroid cells (60). Whether Rap1 is indeed the missing link in cAMP-induced cell proliferation remains to be investigated. Interestingly, in Ovcar-3 cells cAMP may stimulate the activity of p70S6K through the Epac pathway, although the mechanism remains unknown (chapter 4). Recently, the tuberous sclerosis gene products TSC1 and TSC2 have been identified as upstream regulators of p70S6K (61-66). TSC1 and TSC2 function in a complex to repress p70S6K activity, and upon phosphorylation by PKB, TSC2 has been suggested to become poly-ubiquitinated and targeted for the proteasome. Epac has been reported to activate PI3K and PKB, possibly through Rap1 (67). Therefore, it would be tempting to speculate that Epac regulates p70S6K through a pathway involving Rap1, PI3K, PKB, and TSC2. However, no increase in PKB phosphorylation was observed after treatment with 8CTP-2Me-cAMP (chapter 4). Alternatively, cAMP may activate p70S6K in a PKB-independent manner. Indeed, recently, in thyroid cells cAMP has been reported to activate p70S6K through PI3K and PDK without activating PKB (68), although the role of the Epac pathway remains elusive. Furthermore, cAMP-Epac may activate p70S6K in a pathway involving integrins. The cAMP-Epac-Rap1 pathway increases cell adhesion by activating integrins (chapter 3), which in turn may result in 'outside-in' signalling, resulting in activation of p70S6K (69).

Cross-talk between the cAMP pathways may explain some of the effects of cAMP. For instance, in a recent report Rap1 has been suggested to cooperate with PKA in cAMP-induced DNA synthesis and thyroid cell proliferation (70,71) Furthermore, in HEK293 cells, cAMP activates PKB through a pathway involving Epac and PI3K, whereas PKA has an inhibiting effect on PKB (67).

In conclusion, whereas cAMP-mediated inhibition of cell proliferation and Erk signaling generally is executed only through PKA, in specific cell types Epac and PKA might act in concert to regulate cell proliferation through other signaling pathways, like for instance the PI3K pathway.

Cyclic AMP and cell adhesion

Several studies suggest a role for cAMP in cell adhesion. For instance, cAMP inhibits adhesion of neutrophils to human umbilical vein endothelial cells (HUVECs) (72,73). On the other hand, it stimulates adhesion of other cell types, like lymphocytes (74), thymocytes (75), and HT-1080 human fibrosarcoma cells (76). Thus far, all the positive and negative effects of cAMP on cell adhesion have been attributed to PKA. However, cAMP may also stimulate cell adhesion through the Epac-Rap1 pathway (chapter 3). Rap1 appears to be a key regulator of integrin activity (78-89). Many if not all integrins respond to Rap1 acivity, including the ICAM and VCAM receptors $\alpha L\beta 2$ and $\alpha M\beta 2$, the fibronectin receptors $\alpha 4\beta 1$ and $\alpha 5\beta 1$, and the fibrinogen receptor $\alpha IIb\beta 3$. How Rap1 activates integrins is unknown, and conflicting results have been reported. For instance, in mouse T cells, Rap1 has been suggested to affect clustering of LFA-1 but not its affinity (89). In contrast, in T lymphocytes Rap1 has been reported to modulate LFA-1 affinity (87), and in B lymphocytes stimulation of both integrin affinity and avidity by Rap1 has been reported (86). A recent study suggests that activation of Rap1 results in polarized cell-surface expression of proteins involved in cell adhesion and migration (88). Such a function of mammalian Rap1 resembles the function of Bud1, the closest homologue of Rap1 in budding yeast. Bud1 determines the location of the new bud site by recruiting cell-polarity factors CDC24 and CDC42 (90-92). However, activation of Rap1 by cAMP does not result in activation of Rac (chapter 4), and therefore the mechanism of cell polarization by Rap1 probably differs from the yeast mechanism. For example, Rap1 might regulate vesicle transport,

since a number of proteins that contain putative RA or RBD domains also contain domains that are found in proteins involved in vesicle sorting, like PH (Pleckstrin homology) domains, PX (phox) domains, and VPS9 (vacuolar protein sorting) domains (SMART database). For example, Sorting Nexin 27 harbours a putative Ras association domain and a putative PX domain, which may bind phosphatidylinositol (PI) lipids and thereby localize the protein to specific sites in the cell. In addition, Rap1 has been localized predominantly at intracellular membranes in the perinuclear region and at endocytic and exocytic vesicles (93). It is conceivable that Rap1 regulates integrin distribution on the cell surface by directing transport of molecules involved in cell adhesion from one side of the cell to the other side, thereby potentiating cell adhesion. It is also possible that Rap1 facilitates integrin connections with the cytoskeleton. For example, overexpression of Rap1 in *Dictyostelium discoideum*, induces changes in the cytoskeleton, resulting in cell spreading and flattening (94-96). Furthermore, Rap1 binds the junctional protein AF-6/Afadin (97), which localizes to adherens and tight junctions and is thought to connect cell surface adhesion molecules with the actin cytoskeleton. Interestingly, AF-6 can simultaneously bind Rap1-GTP and the Rap1GAP Spa1, thereby affecting cell adhesion (98). In *Drosophila*, Rap1 and Canoe, the *Drosophila* AF-6 homologue, both affect photoreceptor formation in the eye, suggesting that they could be in the same genetic pathway (99,100). Rap1 has also been shown to regulate localization of adherens junctions in *Drosophila*, possibly suggesting another link between Rap1 and Canoe (101). However, it is not clear from this study whether Rap1 regulates tight junction formation directly or indirectly, for instance via integrins.

Many other processes that require integrin functions may also be regulated by Rap1. For example, Rap1 has been implicated in agonist-induced phagocytosis of opsonized particles by macrophages (85), morphogenesis and cell migration in *Drosophila* (102), and transmigration of leukocytes through endothelial monolayers (88). Interestingly, DOCK4, a CDM family member that regulates intercellular junctions and is disrupted during tumorigenesis, has been shown to stimulate Rap1 activation (103).

Cyclic AMP has differential effects on many of the processes that require integrin activity. It remains to be established how cAMP exerts these effects, and what the exact contribution of the three cAMP signaling pathways is.

Cyclic AMP, calcium, and hormone secretion

Cyclic AMP is well known to affect calcium levels in the cell. cAMP directly binds CNG-channels and pacemaker channels, thereby increasing inward calcium conductivity (see for reviews (3-5)). Furthermore, PKA regulates calcium levels through direct phosphorylation of the IP_3R (for a review see (104)). Finally, a number of recent reports indicate the involvement of the Epac signaling pathway in regulation of calcium levels. For instance, Epac might activate PLCε through the small GTPase Rap2B (105). PLCε contains two putative Ras binding domains, one of which has been shown to bind Ras-GTP and Rap-GTP (106-108). Activation of PLCε results in formation of DAG and IP_3 and subsequent calcium release. Furthermore, Epac2 may somehow regulate the RYR-type calcium channels, resulting in calcium release and hormone secretion (109,110), but it may also be involved in hormone secretion through direct interaction with Rim2 (Rab3 interacting molecule) (111). The exact role of the Rap small GTPases remains elusive from these studies.

In conclusion, cAMP regulates a wide variety of cellular processes through three different cAMP signaling pathways: PKA, Epac, and ion channels. The contribution of each of these pathways in cAMP signaling and the exact physiological consequences for cellular processes remain to be established, but it is clear that it is not just PKA any more. Unraveling the cAMP signaling network may have important implications for treatment of human diseases like diabetis, thrombosis, tumorigenesis, and tumor invasion.

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SUMMARY

Background

All living creatures are built up of cells. The smallest life forms are made out of just one cell, like for example bacteria and yeast. In contrast, the human body consists of billions of cells. Every cell in the body has its own specific location and function. Cells have to cooperate tightly in order to make the body function properly. There needs to be a lot of communication between cells to coordinate all physiological processes. Therefore, cells secrete signal molecules called hormones. Some of these hormones travel via the bloodstream, and are recognized elsewhere in the body by specific receptors, which are often located on the outside of the cell membrane. Thousands of receptors may exist, each recognizing a specific molecule. A well-known example of a hormone that may travel a large distance is insulin, which is produced upon raised glucose levels in the blood by the pancreatic islets of Langerhans after a meal. Insulin is secreted into the blood and can therefore reach cells with an insulin receptor in the entire body. There are also hormones that do not travel far and only reach neighboring cells.

When a hormone is bound by a receptor, a range of intracellular reactions may be unleashed. For example, in the case of insulin liver cells start producing a protein that imports large amounts of glucose from the blood. Subsequently, the glucose is modified and stored by other proteins (enzymes). Other hormones can induce cell proliferation, or differentiation of a cell in order to make it fulfill a specialized function in the body.

Often cells instantaneously get a plethora of signals. It is very important that a cell interprets these signals properly. Therefore cells make use of networks of with each other communicating proteins. In that way the signals are integrated, resulting in a certain decision of the cell, ultimately with the goal to make the body function properly. The transmission of hormonal signals and the communication between the proteins in the cell is denoted *signal transduction*. The smallest mistakes in signal transduction can already result in a catastrophe for the body. Cancer is an example of awry communication between signal transduction proteins, resulting in wrong interpretation of signals and continuous cell division.

Replication of DNA, the genetic blueprint of a cell, is essential for a cell in order to divide. Binding of some hormones to their receptors may ultimately result in activation of the process of DNA replication. This information needs to be transferred from the receptor on the cell surface to the cell nucleus, where the DNA is stored. In doing so, some receptors make use of small, intracellular messenger molecules, called *second messengers*. Principally these can diffuse freely throughout the cell. Calcium and cAMP (cyclic Adenosine Mono Phosphate) are two examples of such second messengers.

Cyclic AMP, discovered in 1956, can bind and activate three types of proteins (described in chapter one). The first type is PKA (Protein Kinase A), in 1968 identified as the first cAMP-binding protein. During approximately twenty years it was thought that all cellular effects of cAMP were executed through this protein. Therefore it came as a surprise when two other proteins were found to be activated by cAMP, being cAMP-activated ion-channels (proteins that form a pore in the cell membrane through which ions can move), and Epac.

Epac (exchange protein directly activated by cAMP) is a so-called *guanine-nucleotide exchange factor* (abbreviated as GEF) for the protein Rap, which belongs to the family of Ras-like small GTPases. This is an important group of proteins involved in signal transduction. They function as a molecular switch, 'off' when they bind GDP, and 'on' when they bind GTP. The function of a GEF is to replace the GDP for GTP, thereby turning the switch on. The switch is turned off by proteins called GAPs (GTPase Activating Proteins), which promote processing of GTP into GDP.

Ras is the best studied small GTPase. Ras activity is for example often required for cell division. Small, specific changes ('mutations') in Ras-like GTPases can result in a state in which they are constantly in complex with GTP, so they are continuously switched on. That is also the case for Ras. Approximately 15% of all human tumors contain such a mutated, activated Ras. The function of Rap, however, is still elusive.

Scope

The most important goal of this thesis was to study the role of Epac and Rap in cellular processes that are regulated by cAMP. Since cAMP can activate three types of proteins, in chapter two the development is described of a novel, synthetic cAMP analogue, which can exclusively bind and activate Epac, but not PKA or ion channels. Using this analogue the role of Epac and Rap1 was studied in cAMP-mediated regulation of the protein Erk (Extracellular Signal Regulated Kinase), which is involved in for example cell proliferation. There is a lively discussion in literature about the role of Rap1 in this process. Using this analogue it became clear that Epac and Rap1 are not involved in this process, but that it is actually regulated by PKA, one of the two other cAMP-regulated proteins.

In chapter three a novel function of cAMP, Epac and Rap1 is described; stimulation of integrin-mediated cell adhesion. Adhesion of cells to their surroundings occurs via proteins called integrins. Cell adhesion is important for the integrity of the body, but also for fighting infections by blood cells, or formation of a small blood clot upon damage of a blood vessel. Malignant tumors barely adhere to their surroundings, allowing them to metastasize. In contrast, thrombosis is a disease in which blood platelets adhere too much, causing formations of blood clots in healthy vessels. Whether Rap1 plays a role in these processes is still unclear. It is also unclear how Rap1 increases cell adhesion.

The experiments described in chapter four suggest a role for cAMP, Epac, and possibly Rap1 in activation of the protein p70 S6 kinase (p70S6K), which in turn activates the protein S6. p70S6K and S6 are important for cell proliferation, and possibly also survival of cells. The mechanism of p70S6K activation by Epac and Rap1 remains unknown.

Conclusions

As discussed in chapter five, it is unlikely that cAMP regulates the protein Erk through Epac and Rap1; probably this effect is mediated by PKA. It remains possible that Epac and Rap1 play a role cell proliferation of specific cell types, for example by influencing the activity of p70S6K. Furthermore, a novel function of cAMP, Epac and Rap1 is described, being cell adhesion. Furthermore, the result from the development of a molecule that can very specifically activate a single protein, Epac, can contribute to more efficient drug design in the future.

SAMENVATTING

Achtergrond

Alle levende wezens zijn opgebouwd uit cellen. De kleinste levensvormen zijn opgebouwd uit slechts één cel, zoals bijvoorbeeld bacteriën en gist. Het menselijk lichaam echter bestaat uit miljarden cellen. Elke cel heeft zijn eigen specifieke plaats en functie in het lichaam. Cellen moeten nauwgezet samenwerken om het lichaam juist te laten functioneren. Er is veel communicatie nodig tussen cellen om alle fysiologische processen te coördineren. Daarom scheiden cellen signaalmoleculen uit, hormonen genaamd. Sommige van die hormonen reizen via de bloedbaan, en worden door cellen elders in het lichaam herkend via zogenaamde receptoren, die meestal aan de buitenkant van de cel zitten, op het celmembraan. Er bestaan wellicht duizenden soorten receptoren, die elk een heel specifiek molecuul herkennen. Een bekend voorbeeld van zulke ver reizende moleculen is het hormoon insuline, dat na een maaltijd als gevolg van een hoog bloedsuikergehalte gemaakt wordt door de eilandjes van Langerhans in de pancreas. Insuline wordt uitgescheiden in het bloed en kan op die manier in het hele lichaam cellen met een insuline-receptor bereiken. Andere hormonen reizen niet ver, en bereiken slechts receptoren op naburige cellen.

Als een hormoon een receptor bindt, wordt een scala aan cellulaire reacties onteketend. In het geval van insuline wordt bijvoorbeeld door levercellen een eiwit gemaakt dat extra veel glucose uit het bloed kan opnemen. Het glucose wordt vervolgens door andere eiwitten (enzymen) in de levercel gemodificeerd en opgeslagen. Andere hormonen kunnen ervoor zorgen dat een cel bijvoorbeeld gaat delen, of zich gaat specialiseren in een bepaald celtype.
Vaak krijgen cellen een hele waaier aan signalen tegelijk binnen. Het is erg belangrijk dat een cel al deze signalen op de juiste wijze interpreteert. Cellen maken daarom gebruik van netwerken van met elkaar communicerende eiwitten. Op die manier worden de signalen geïntegreerd, leidend tot een bepaald besluit van de cel, met als uiteindelijk doel het lichaam adequaat te laten functioneren. Het overbrengen van hormonale signalen, en de communicatie tussen de eiwitten in de cel, wordt aangeduid met de term *signaaltransductie*. Minieme foutjes in signaaltransductie kunnen al catastrofale gevolgen hebben voor het lichaam. Kanker is zo'n voorbeeld van foutieve communicatie tussen signaaltransductie-eiwitten in een cel, met als gevolg misinterpretatie van signalen en continue celdeling.

Verdubbeling van DNA, de genetische blauwdruk van de cel, is essentieel voordat een cel kan delen. Binding van sommige hormonen aan hun receptor resulteert uiteindelijk in activatie van het gehele proces van DNA-verdubbeling. Die informatie moet van de receptor naar de celkern overgedragen worden, waarin het DNA is opgeslagen. Sommige receptoren maken daarbij gebruik van kleine, intracellulaire boodschappermoleculen, *second messengers* genaamd. Deze kunnen in principe vrij door de cel diffunderen. Calcium en cAMP (cyclic Adenosine Mono Phosphate) zijn twee voorbeelden van zulke second messengers.

Cyclic AMP, ontdekt in 1956, kan aan drie soorten eiwitten binden en ze daardoor activeren (beschreven in hoofdstuk een). De eerste soort is PKA (Protein Kinase A), in 1968 het eerste cAMP-bindende eiwit dat geïdentificeerd werd. Gedurende ongeveer twintig jaar dacht men dat alle cellulaire effecten van cAMP door dit eiwit uitgevoerd werden. Tot ieders verrassing bleken er echter nog twee andere eiwitten geactiveerd te worden door cAMP, namelijk cAMP-geactiveerde ion-kanalen (eiwitten die een kleine porie in de celmembraan vormen om ionen door te laten), en Epac.

Epac (exchange protein directly activated by cAMP) is een zogenaamde *guanine-nucleotide uitwisselings factor* (afgekort als GEF, voor Guanine Nucleotide Exchange Factor) voor het eiwit Rap, dat behoort tot de familie van de zogenaamde kleine Ras-achtige GTPases. Dit is een belangrijke groep eiwitten betrokken bij signaaltransductie. Ze functioneren als een moleculaire schakelaar, en staan 'uit' als ze het molecuul GDP gebonden hebben, en 'aan' als ze GTP gebonden hebben. De functie van een GEF is het vervangen van GDP voor GTP, zodat de schakelaar aangezet wordt. De schakelaar kan worden uitgezet doordat een GTPase bindt aan een eiwit genaamd GAP (GTPase Activating Protein), dat omzetting van GTP naar GDP bevordert.

Ras is het best bestudeerde GTPase. Aanschakelen van Ras is vaak nodig voor celdeling. Kleine, specifieke veranderingen ('mutaties') in Ras-achtige GTPases kunnen er echter voor zorgen dat ze altijd GTP gebonden hebben, zodat de schakelaar altijd aanstaat. Dat is ook voor Ras het geval. Ongeveer 15 procent van alle humane tumoren hebben zo'n foutief Ras dat altijd aanstaat. De functie van Rap is echter nog onduidelijk.

Doelstelling

Het belangrijkste doel van deze promotiestudie was het achterhalen van de rol die Epac en Rap hebben in cellulaire processen die door cAMP beïnvloedt worden. Omdat cAMP drie soorten eiwitten kan activeren, wordt in hoofdstuk twee de ontwikkeling van een nieuw, synthetisch analoog van cAMP beschreven, dat exclusief Epac bindt en activeert, maar niet PKA of ion-kanalen. Met behulp van deze analoog werd de rol bestudeerd van Epac en Rap1 in cAMP-gestuurde regulatie van het belangrijke eiwit Erk (Extracellular Signal Regulated Kinase), dat betrokken is bij onder andere celdeling. In de literatuur woedt een levendige discussie over de rol die Rap zou hebben in dit proces. Mede dankzij het Epac-specifieke cAMP-analoog werd duidelijk dat Epac en Rap geen effect hierop hebben, maar dat het volledig afhankelijk is van een van de twee andere cAMP-gevoelige eiwitten, namelijk PKA.

In hoofdstuk drie wordt een nieuwe functie voor cAMP, Epac en Rap beschreven, namelijk stimulatie van integrine-afhankelijke celadhesie. Adhesie van cellen aan hun omgeving gebeurt via eiwitten die integrines genoemd worden. Celadhesie is belangrijk voor de integriteit van het lichaam, maar ook voor het bestrijden van bijvoorbeeld infecties door witte bloedcellen, of het vormen van een klein bloedpropje als een bloedvat beschadigd is. Kwaadaardige tumorcellen hebben nauwelijks nog adhesie, waardoor ze kunnen uitzaaien. Thrombose is een ziekte waarbij bloedplaatjes juist weer te goed hechten, en daardoor gezonde bloedvaten afsluiten. Of Rap een rol speelt in deze processes is onbekend. Hoe Rap integrines activeert en daarmee celadhesie stimuleert is ook nog onduidelijk.

De experimenten beschreven in hoofdstuk vier suggereren een rol voor cAMP, Epac, en Rap in activatie van het eiwit p70 S6 kinase (p70S6K), dat op zijn beurt het eiwit S6 kan activeren. p70S6K en S6 zijn erg belangrijk voor celdeling, en mogelijk ook voor het overleven van cellen. Hoe Epac en Rap p70S6K activeren is onbekend.

Conclusies

Zoals bediscussieerd in hoofdstuk vijf, is het onwaarschijnlijk dat cAMP het eiwit Erk reguleert via Epac en Rap1; waarschijnlijk gebeurt het via PKA. Wel blijft het mogelijk dat Epac en Rap een rol spelen in celdeling in specifieke soorten cellen, door bijvoorbeeld de activiteit van p70S6K te beïnvloeden. Verder is een nieuwe functie voor cAMP, Epac en Rap1 beschreven, namelijk cel adhesie. De resultaten uit het onderzoek naar de ontwikkeling van een molecuul dat heel specifiek één eiwit kan aanschakelen, namelijk Epac, kunnen een belangrijke bijdrage leveren aan de toekomstige wijze van ontwikkeling van farmaceutische middelen.

Curriculum vitae

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