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REVIEW

EPAC proteins transduce diverse cellular actions of cAMP

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It has now been over 10 years since efforts to completely understand the signalling actions of cAMP (3′-5′-cyclic adenosine monophosphate) led to the discovery of exchange protein directly activated by cAMP (EPAC) proteins. In the current review we will highlight important advances in the understanding of EPAC structure and function and demonstrate that EPAC proteins mediate multiple actions of cAMP in cells, revealing future targets for pharmaceutical intervention. It has been known for some time that drugs that elevate intracellular cAMP levels have proven therapeutic benefit for diseases ranging from depression to inflammation. The challenge now is to determine which of these positive actions of cAMP involve activation of EPAC-regulated signal transduction pathways. EPACs are specific guanine nucleotide exchange factors for the Ras GTPase homologues, Rap1 and Rap2, which they activate independently of the classical routes for cAMP signalling, cyclic nucleotide-gated ion channels and protein kinase A. Rather, EPAC activation is triggered by internal conformational changes induced by direct interaction with cAMP. Leading from this has been the development of EPAC-specific agonists, which has helped to delineate numerous cellular actions of cAMP that rely on subsequent activation of EPAC. These include regulation of exocytosis and the control of cell adhesion, growth, division and differentiation. Recent work also implicates EPAC in the regulation of anti-inflammatory signalling in the vascular endothelium, namely negative regulation of pro-inflammatory cytokine signalling and positive support of barrier function. Further elucidation of these important signalling mechanisms will no doubt support the development of the next generation of anti-inflammatory drugs.
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Abbreviations: 8-pCPT-2′OMe–cAMP, 8- (4- chlorophenylthio)- 2′- O- methyladenosine- 3′, 5′-cyclic monophosphate; Sp-CAMPS, adenosine- 3′, 5′-cyclic monophosphorothioate, Sp-isomer

Introduction

3′-5′-cyclic adenosine monophosphate (cAMP) is a central second messenger that regulates key cellular responses, including central metabolic events, cardiac and smooth muscle contraction, secretory processes, cell growth, survival and differentiation, as well as inflammatory responses. Interaction of hormones and neurotransmitters with G-protein-coupled receptors promotes activation of the heterotrimeric G-protein, Gsα, stimulates the activation of one or more isoforms of adenylyl cyclase. This leads to the synthesis of cAMP, and levels are further regulated through hydrolysis to 5′AMP by a large family of cAMP phosphodiesterases (PDEs) (Houslay, 1998). Given these pleiotropic actions of cAMP it is not surprising that pharmaceutical manipulation of cAMP levels in cells has proven therapeutic benefit in a wide range of human disease. For example, cAMP-specific PDE inhibitors have broad therapeutic potential, including anti-inflammatory and antidepressant properties (Houslay *et al.*, 2005). Investigations into the mechanisms of cAMP signalling therefore have far reaching implications in the understanding and treatment of human disease.

A vital step towards a fuller understanding of the mechanisms of cAMP signalling came a decade ago with the discovery of the exchange proteins activated by cAMP (EPACs).

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EPACs are specific guanine nucleotide exchange factors (GEFs) for the Ras GTPase homologues, Rap1 and Rap2, which they activate independently of the classical route of cAMP signal transduction, through protein kinase A (PKA) (Kawasaki *et al.*, 1998; de Rooij *et al.*, 1998). Rap GTPases cycle between inactive GDP- and active GTP-bound forms. The two known EPAC isoforms, EPAC1 and EPAC2, have GEF activity that converts Rap proteins to the active form, after which GTPase-activating proteins complete the cycle by converting Rap to the inactive form (Bos, 2003). Cyclic AMP-binding sites in EPAC proteins facilitate their direct activation by cAMP, thereby relieving auto-inhibitory influences of the cAMP-binding domain towards the catalytic GEF domain.

A large body of work is now revealing that EPAC proteins mediate many of the critical actions of cAMP in the human body. These functions are varied and range from maintenance of proper circadian pacemaker (O'Neill *et al.*, 2008) and memory (Gelinas *et al.*, 2008; Ouyang *et al.*, 2008) functions to wound healing (Yokoyama *et al.*, 2008) and nerve regeneration (Murray and Shewan, 2008). Selective activation or inhibition of EPAC-regulated signalling events will therefore be the goal of cAMP-directed drug research in the future. The exciting outcome of this may be the specific modulation of EPAC-regulated signalling events leading to targeted therapeutic intervention with reduced side effects. This review therefore aims to highlight ongoing work into the diverse functions of EPAC proteins in humans, perhaps revealing putative sites for future drug research.

Structural basis of EPAC regulation

The EPAC proteins are each made up of an N-terminal regulatory region and a C-terminal catalytic GEF region, with each region able to function independently in terms of cAMP binding and GEF activity respectively. Several lines of evidence suggest that the EPACs populate multiple conformations in dynamic equilibrium ranging from a closed, auto-inhibited state in which the regulatory region sterically blocks access to the GEF's active site, to a dramatically different, open and catalytically active state in which the regulatory region binds to a different face of the GEF. In the absence of cAMP the proteins favour the closed auto-inhibited conformation, while cAMP binding shifts the equilibrium towards the active conformation. X-ray crystallography has provided highly informative snapshots of the open (Rehmann *et al.*, 2008) and closed (Rehmann *et al.*, 2006) conformations of EPAC2 while NMR spectroscopy has been used to explore how ligand binding affects the dynamics of the critical conformational switch in EPAC1 (Harper *et al.*, 2007; Mazhab-Jafari *et al.*, 2007; Das *et al.*, 2008). Lower resolution techniques have also been applied to the problem (Yu *et al.*, 2006; Brock *et al.*, 2007), but their limitations, that is, the requirement for an adequate prior model, are revealed in the light of the structure of the EPAC2·Sp-CAMPS·RAP1B complex (Rehmann *et al.*, 2008).

The catalytic regions of both EPAC1 and 2 are constructed from a Ras exchange motif (REM) and a CDC25 homology domain (Figure 1A) arranged as in the Ras-GEF Sos. Unlike Sos, the EPACs possess an intervening domain that adopts a ubiquitin fold, which has been proposed to be a functional Ras association domain in EPAC2 (Li *et al.*, 2006). However, the observation that this Ras association activity is cAMP-dependent is difficult to reconcile with the available structures in which access to the putative Ras association domain is unaffected by cAMP binding. GEF activity is achieved by an extensive association between the helical hairpin, ionic latch (IL) loop and central core of the CDC25 homology domain and the nucleotide-binding site of Rap1 (Rehmann *et al.*, 2008). The helical hairpin enters the binding site and distorts switch I and II regions, decreasing the number of favourable contacts that can be made to the bound nucleotide, facilitating its release. Selectivity for RAP1B over Ras seems to be determined in a coordinated fashion over the totality of the contact surface, rather than by any particular key residues.

The regulatory region of EPAC1 contains two recognized domains, a Dishevelled-Egl-10-Pleckstrin (DEP) domain and a cyclic mononucleotide-binding (CNB) domain as well as a, likely natively disordered, N-terminal extension. EPAC2 shares this organization, but possesses an additional CNB domain N-terminal to the DEP domain. DEP domains are typically membrane localization domains through either protein–protein or protein–membrane interactions, and EPACs' DEP domains seem to be no exception (de Rooij *et al.*, 2000; Qiao *et al.*, 2002). EPAC2's N-terminal CNB domain binds cAMP with a 20-fold lower affinity than the conserved CNB and does not affect activation of EPAC2 by cAMP (Rehmann *et al.*, 2003). The apparently intimate association between the two CNB domains seen in the cAMP-free EPAC2 crystal structure is therefore likely to be rather weak. The critical domain for the regulation of EPAC activity by cAMP is the conserved CNB domain immediately N-terminal to the catalytic domain (Figure 1A).

In general, cAMP binds to the base binding site on one edge of the β-sheet sandwich of CNB domains orienting its cyclic phosphate group such that the conformation of an adjacent loop, known as the phosphate-binding cassette, is altered. As a result, the hinge helix that links the core CNB domain to the C-terminal lid is free to reorient, and the lid closes over the nucleotide-binding site making contact with the base (Rehmann *et al.*, 2007; Kornev *et al.*, 2008). The crystal structure of inactive EPAC2 revealed that, unlike other known CNBs, the lid of the EPACs' regulatory CNB domain forms part of a β-sheet (the switchboard) that is anchored to the REM of the catalytic region. This lid includes a VLVLE motif that is conserved across CNBs and typically forms a helix in the cAMP-bound conformation. The general mechanism by which EPACs are activated by cAMP had therefore been widely anticipated, with cAMP binding inducing the closing of the CNB domain's lid and reorientating the regulatory region away from the Rap1-binding site (Figure 1B). However, there is a surprising difference in detail revealed by the EPAC2·Sp-CAMPS·RAP1B complex – the lid's β-sheet conformation is not stripped back and reformed as an α-helix, but is instead retained inducing a ~180° rotation of the CNB domain from one face of the switchboard β-sheet to the other (Rehmann *et al.*, 2008). As well as inducing lid closure in the CNB domain, there is evidence that cAMP also affects the other major contact point with the regulatory region – the IL. Rather than causing a significant change in average structure,

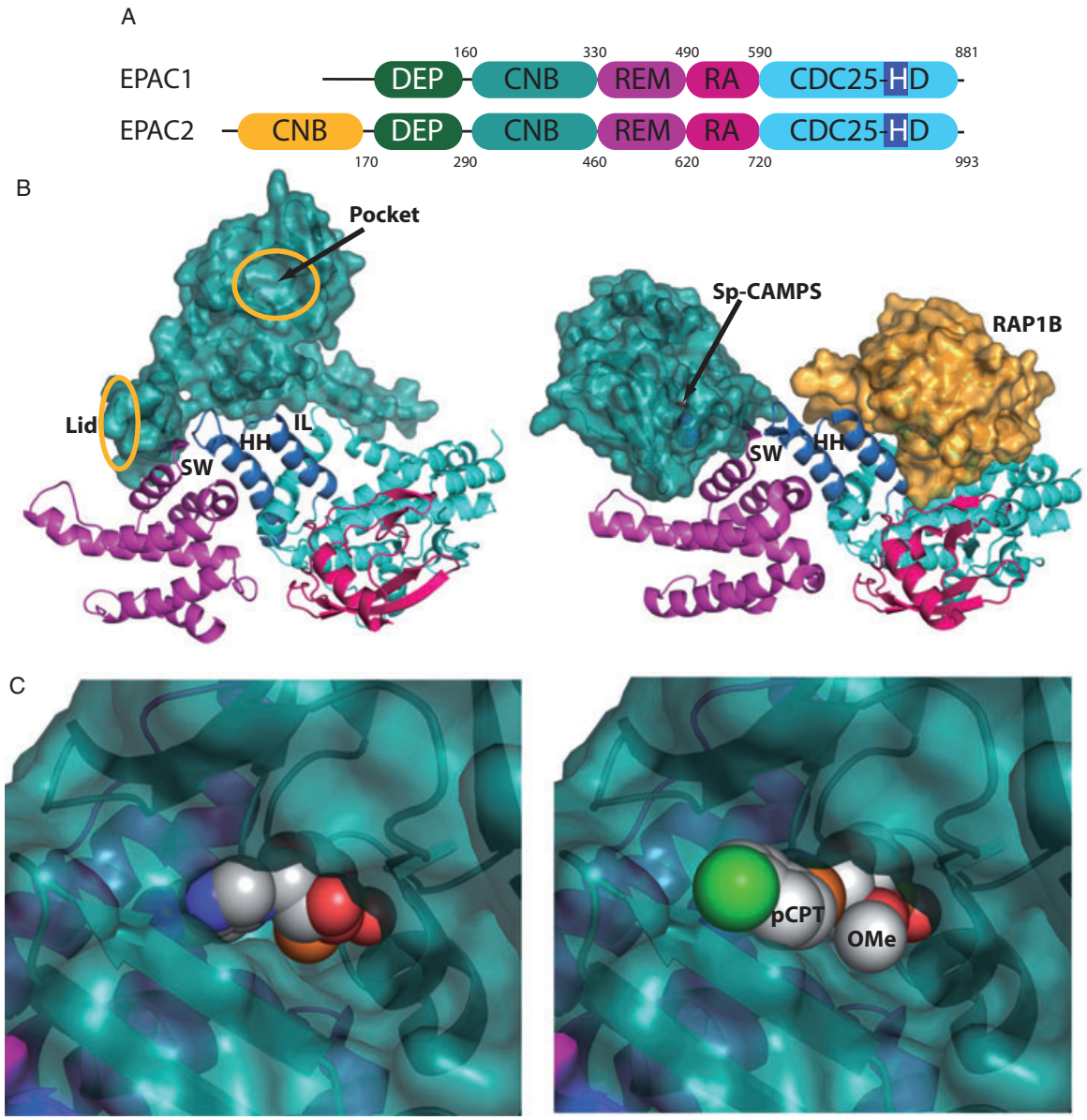

Figure 1 Structural basis of EPAC regulation. (A) Schematic diagram of EPAC domain organization. (B) Conformational changes upon cAMP binding. Cartoon representations of the regulatory CNB domain and catalytic regions of EPAC2 are shown in the inactive (PDB 2byv) and active (PDB 3cf6) conformations with the molecular surfaces of the CNB domain and bound RAP1B depicted. The yellow ovals indicate the two parts of the cAMP-binding site (the lid and the pocket) in the inactive conformation. (C) Close-up view of the cAMP-binding site with Sp-CAMPS bound & with 8-pCPT-2′OMe–cAMP docked in its place showing how the pCPT and OMe groups might be accommodated and contribute to tighter binding between the core and lid of the CNB domain. Images generated by using PyMOL [DeLano, W.L.The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA]. 8-pCPT-2′OMe–cAMP, 8- (4- chlorophenylthio)- 2′- O- methyladenosine- 3′, 5′-cyclic monophosphate; cAMP, 3′-5′-cyclic adenosine monophosphate; CDC25-HD, CDC25 homology domain; CNB, cyclic nucleotides binding; DEP, Dishevelled-Egl-10-Pleckstrin; EPAC, exchange protein activated by cAMP; HH, helix hairpin; IL, ionic latch; RA, Ras association; REM, Ras exchange motif; Sp-CAMPS, adenosine- 3′, 5′-cyclic monophosphorothioate, Sp-isomer; SW, switchboard.

it appears that cAMP loosens the structure around key IL residues making the closed, inactive conformation less favoured for entropic reasons (Das *et al.*, 2008).

Substantial progress has already been made in understanding the selectivity of cAMP analogues that are agonists and antagonists of PKA and the EPACs (Dao *et al.*, 2006; Poppe *et al.*, 2008). The EPAC2·Sp-CAMPS·RAP1B complex helps to explain why the commonly used, EPAC-selective analogue, 8-pCPT-2′OMe–cAMP (8- (4- chlorophenylthio)- 2′- O-methyladenosine- 3′, 5′-cyclic monophosphate) (Enserink *et al.*, 2002) is a super-activator of EPACs – the adenine 8 and ribose 2′ positions point towards the open mouth of the cAMP-binding pocket where the additional pCPT and methyl groups could be accommodated in a cleft between CNB domain and REM (Figure 1C), extending their interaction – and offers new opportunities for structure-guided agonist design. Further opportunities for therapeutic intervention in EPAC-mediated cAMP response lie in exploiting the, often cell type- and function-specific, protein–protein interactions that have been observed, for example with the PDZ domains of

Rim2 and Piccolo (Ozaki *et al.*, 2000; Fujimoto *et al.*, 2002), or the microtubule-associated protein (MAP) light chains (Magiera *et al.*, 2004; Gupta and Yarwood, 2005; Borland *et al.*, 2006).

Regulation of exocytosis through EPAC

The first observation linking EPAC to the regulation of exocytosis was the finding that EPAC2 interacts with Rim2, a target of the small GTPase Rab3, and mediates cAMP-induced, Ca^{2+}-dependent secretion of growth hormone from PC12 cells and the release of C-peptide from MIN6 cells, a highly differentiated and glucose-responsive pancreatic β-cell line (Ozaki *et al.*, 2000). The accumulated evidence now points towards EPAC as having a fundamental role in the regulation of exocytosis in a range of different cellular contexts. For example, EPAC activation, coupled to Ca^{2+} mobilization, has been implicated in promoting the secretory activity of pituitary melanotrophs (Sedej *et al.*, 2005), triggering fast exocytosis in rat chromaffin cells through the induction of CaV3 Ca^{2+} channels (Giancippoli *et al.*, 2006), facilitating exocytotic fusion between the outer acrosomal and plasma membranes in sperm cells (Branham *et al.*, 2006) and mediating apical exocytosis and exocytotic insertion of aquaporin-2, in response to arginine vasopressin, in the inner medullary collecting duct (Yip, 2006; Balasubramanian *et al.*, 2007). A number of excellent reviews detailing the role of EPAC in cAMP-promoted exocytosis have already been published (Holz, 2004; Seino and Shibasaki, 2005; Holz *et al.*, 2006).

Of particular note are a growing number of studies that implicate EPAC2 in the control of insulin secretion from pancreatic β-cells (Shibasaki *et al.*, 2007). Incretin hormones, such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide, are known to potentiate glucose-induced insulin secretion through cAMP-dependent mechanisms in these cells (Holz, 2004). Incretin-promoted increases in intracellular cAMP levels lead to an EPAC-mediated mobilization of Ca^{2+} from intracellular Ca^{2+} stores (Holz, 2004). This, in turn, leads to the phenomenon of Ca^{2+}-induced Ca^{2+} release (Kang *et al.*, 2003; Hatakeyama *et al.*, 2007). Ca^{2+}-induced Ca^{2+} release activates mitochondrial dehydrogenases, thereby up-regulating glucose-dependent production of ATP. Consequently, there is an increase in the cytosolic ATP/ADP ratio leading to the closure of ATP-sensitive K^+ channels and membrane depolarization (Holz, 2004). This promotes an influx of Ca^{2+}, through voltage-dependent Ca^{2+} channels, stimulating exocytosis of insulin-containing secretory granules and promoting their fusion with the plasma membrane (Holz, 2004).

There are a number of possible mechanisms by which EPAC2 could potentially promote mobilization of intracellular Ca^{2+} (Holz *et al.*, 2006), including Rap-dependent stimulation of phospholipase Cε, leading to PIP2 hydrolysis and IP3 production as seen in cardiomyocytes (Oestreich *et al.*, 2007; Schmidt *et al.*, 2007). There is also some evidence suggesting that Rap1 may directly link EPAC signalling to Ca^{2+} mobilization, through direct protein–protein interactions with the SERCA Ca^{2+} ATPase in the endoplasmic reticulum (Lacabaratz-Porret *et al.*, 1998). Moreover, Ca^{2+} mobilization in pancreatic β-cells is sensitive to ryanodine (Kang *et al.*, 2005), which is particularly intriguing in light of a report suggesting that in cardiomyocytes EPAC1 exists in a protein complex involving the type 2 ryanodine receptor, a cAMP PDE and an PKA anchoring protein (Dodge-Kafka *et al.*, 2005). Whether such a macromolecular complex couples EPAC and ryanodine receptor activation in pancreatic β-cells remains to be determined; however, a functional link between EPAC and ryanodine receptor activation has been shown in cardiomyocytes (Pereira *et al.*, 2007). Here EPAC activation appears to stimulate CaMKII activity, leading to ryanodine receptor phosphorylation and a subsequent increase in Ca^{2+} spark frequency (Pereira *et al.*, 2007).

In addition to being involved these aspects of Ca^{2+}-dependent exocytosis, EPAC also appears to play a major role in the later stages of secretory granule release. Accordingly, direct activation of EPAC by 8-pCPT-2′OMe–cAMP has been shown to increase the number of exocytic sites in pancreatic β-cells (Kwan *et al.*, 2007a) and elevations in intracellular cAMP increase the density of insulin-containing granules underlying the plasma membrane, thereby facilitating glucose-induced membrane/granule fusion (Shibasaki *et al.*, 2007). These actions of cAMP seem to have both a PKA-dependent component, namely mobilization of slow-moving dense-core vesicles, and an EPAC-dependent component, involved in the mobilization of fast moving, small vesicles (Hatakeyama *et al.*, 2007). The ability of EPAC to potentiate rapid exocytosis may be partly due to the formation of complexes between EPAC2 and Rim2, a Rab3A GTPase-interacting protein that plays a central role in Ca^{2+}-dependent exocytosis, and Munc13-1, a diacylglycerol-binding protein essential for the priming stage of vesicle fusion (Kwan *et al.*, 2007b). Coupling of vesicle exocytosis to elevations in intracellular Ca^{2+} probably involves additional protein interactions between the EPAC2/Rim2 complex and the Ca^{2+} sensor protein, Piccolo, which forms a heterodimer with Rim2 in a Ca^{2+}-dependent manner (Fujimoto *et al.*, 2002).

Further priming of insulin secretory granules also appears to be assisted by protein interactions between EPAC2 and the nucleotide-binding fold-1 of the β-cell sulphonylurea receptor-1, SUR1 (Kang *et al.*, 2006). A model has been suggested whereby SUR1-mediated recruitment of EPAC2 to secretory granules leads to the stimulation of CIC-3 chloride channels, thereby generating an electromotive force that drives H^+ uptake through the v-type H^+-ATPase. This leads to priming of exocytosis through vesicle acidification. Future research into the role of EPAC in the control of insulin-granule exocytosis in pancreatic β-cells will cast light onto the mechanisms of action of incretin hormones in these cells. Moreover, because glucagon-like peptide-1 mimetics are currently being used for the treatment of type 2 diabetes, this type of research may lead to the development of small molecules that activate EPAC selectively and which might represent the next generation of anti-diabetic agents.

Regulation of cell proliferation by cAMP

A large number of studies have demonstrated the significance of cAMP in the control of cell division (Table 1). Indeed, cAMP can even promote reversion to the normal phenotype of certain transformed cells (Pastan *et al.*, 1975). Elevation of

Table 1 Mitogenic and anti-mitogenic effects of (3′-5′-cyclic adenosine monophosphate) cAMP-elevating agents

Cell system	*Stimulant*	*Effect on proliferation*	*References*
Rat parotid acinar cells	β-adrenergic	+	(Purushotham *et al.*, 1992)
Rat hepatocytes	Glucagon/β-adrenergic	+	(Thoresen *et al.*, 1990)
Rat brown adipocytes	β-adrenergic	+	(Cannon and Nedergaard, 1996)
Rat pancreatic β-cells	Glucose	+	(Rabinovitch *et al.*, 1980)
Murine mammary epithelial cells	Prostaglandin E1	+	(Burstein *et al.*, 1976)
Immature rat sertoli cells	Follicle-stimulating hormone	+	(Almiron and Chemes, 1988)
Swiss 3T3 fibroblasts	Prostaglandin E1	+	(Yamashita *et al.*, 1986)
Dog thyroid epithelial cells	Thyroid-stimulating hormone	+	(Roger and Dumont, 1984)
Rat arterial smooth muscle cells	Prostaglandin E1	–	(Nilsson and Olsson, 1984)
Human foreskin fibroblasts	Forskolin	–	(Heldin *et al.*, 1989)
Normal and neoplastic human B-cells	Forskolin	–	(Blomhoff *et al.*, 1987)
Transformed fibroblasts	8-bromo-cAMP	–	(Hordijk *et al.*, 1994)
Transformed NIH-3T3 fibroblasts	Constitutively active Gsα	–	(Chen and Iyengar, 1994)

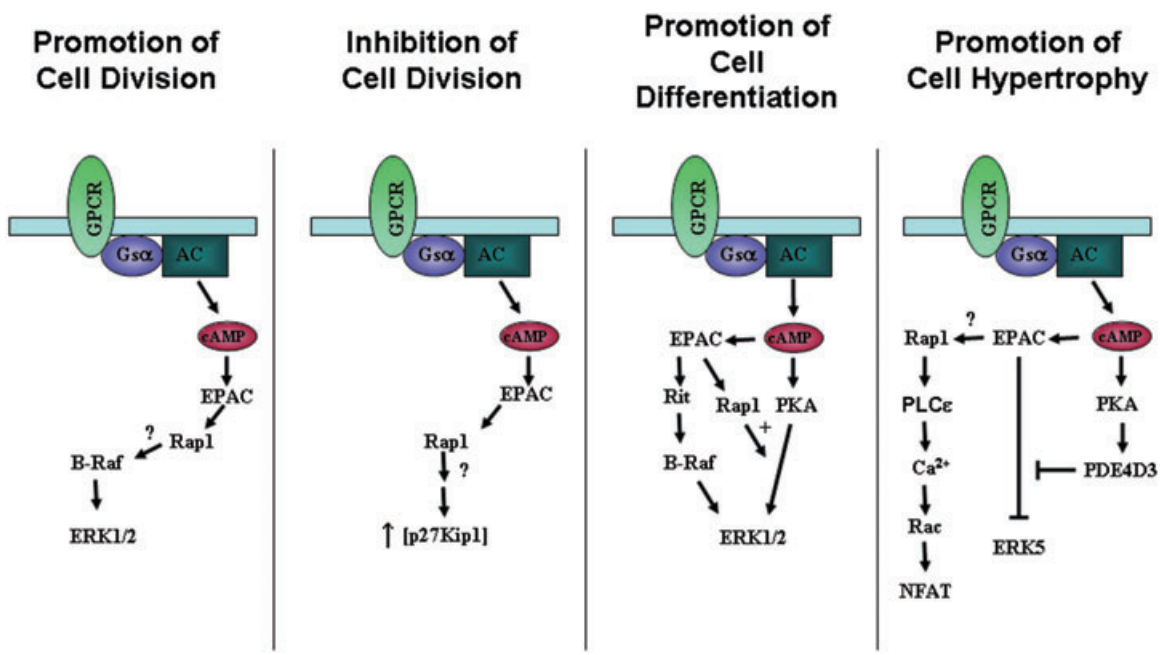

Figure 2 Control of cell fate by EPAC-activated intracellular signalling. The involvement of EPAC in intracellular signalling controlling cell division, cell differentiation and cell hypertrophy is shown. Generally activation of EPAC by cAMP leads to the activation of Rap1 GTPase, which, in turn, controls downstream signalling as indicated by black arrows. EPAC, exchange protein activated by cAMP; cAMP, 3′-5′-cyclic adenosine monophosphate; GPCR, G-protein-coupled receptor; PKA, protein kinase A; NFAT, nuclear factor of activated T-cell.

intracellular cAMP leads to the rapid activation of Rap1 (Stork and Schmitt, 2002), and it has been hypothesized that Rap1, like cAMP, can either stimulate or inhibit cell division, depending on the cellular context (Ribeiro-Neto *et al.*, 2002). This leads to the question whether EPAC is involved in the coupling of cAMP to pro- or anti-mitogenic signalling through Rap1. Indeed, intriguing recent work has suggested that direct activation of EPAC can inhibit the growth of pancreatic carcinoma cells (Lorenz *et al.*, 2008). Is EPAC therefore a valid target for future anti-cancer drugs? To address this we will examine the current evidence linking EPAC to the positive and negative regulation of cell proliferation. In addition we will also examine the role of EPAC in the related phenomena of cell adhesion, differentiation and hypertrophy. A summary of these mechanisms is presented in Figure 2.

Positive effects of EPAC on cell division

There is intriguing evidence that at least some of the actions of cAMP on the promotion of cell proliferation may involve EPAC. For example, over-expression of EPAC1 in COS-7 cells leads to an increase in multinuclear cell populations, suggesting that EPAC may play an important role in mitosis in these cells (Qiao *et al.*, 2002). Moreover, multiple pathways contribute to cAMP-stimulated mitogenesis in rat thyroid cells; however, it appears that only some of these are PKA-dependent (Cass *et al.*, 1999). Indeed, it has been recently suggested that EPAC acts synergistically with PKA in rat thyroid cells to enhance cAMP-mediated mitogenesis, but the mechanisms underlying this action of EPAC remain to be determined (Hochbaum *et al.*, 2007).

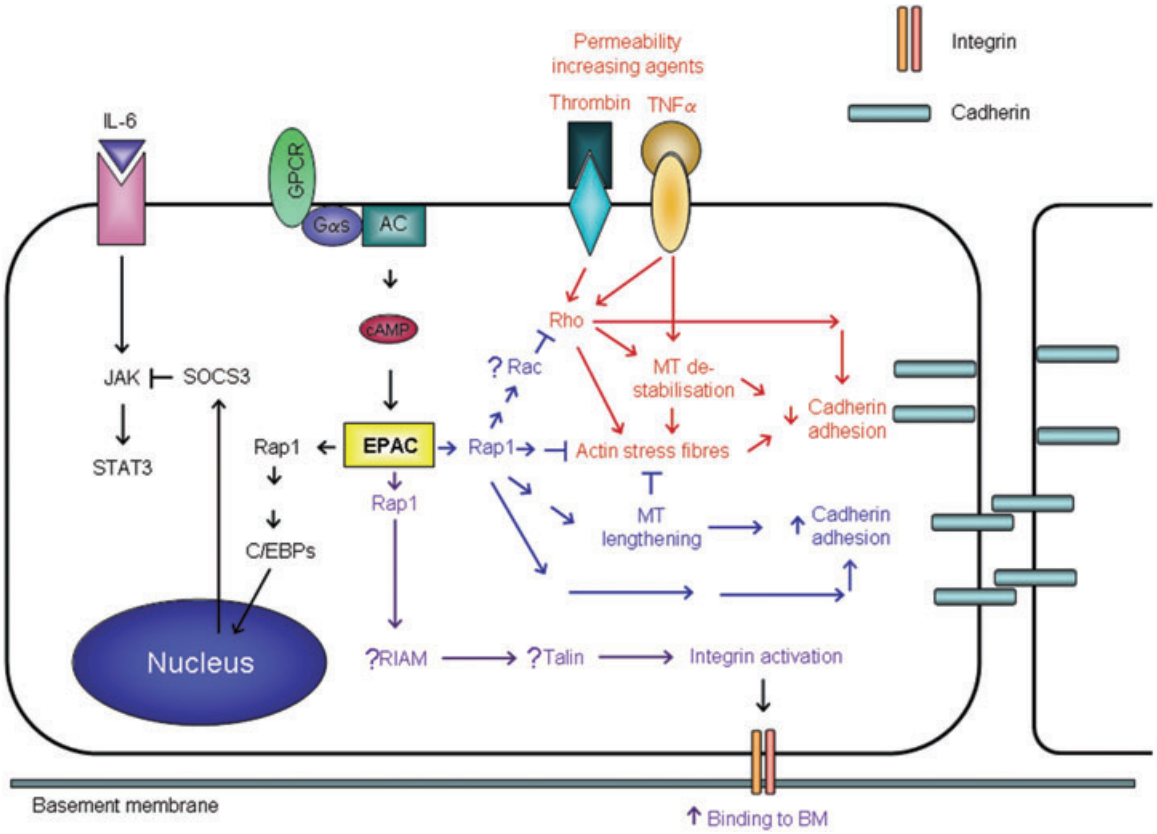

Figure 3 Anti-inflammatory effects of EPAC in VECs. EPAC mediates at least three anti-inflammatory signalling pathways in VECs. IL-6 signalling via STAT3 is inhibited by EPAC-dependent SOCS3 induction; this occurs via Rap1 and C/EBP transcription factors (black pathway). EPAC mediates activation of integrins involved in adhesion of VECs to the basement membrane; the signalling pathway between EPAC-activated Rap1 and integrin activation is not known, but may involve RIAM and talin, as reported for the αIIbβ3 platelet integrin (purple pathway). Agents such as thrombin and TNFα reduce adhesion of VECs to adjacent cells at adherens junctions via Rho, actin reorganization to reduce cortical ring and increase contractile stress fibres and destabilization of microtubules, resulting in reduced cadherin-mediated adhesion, increasing inter-endothelial gaps and thus increasing endothelial permeability (red pathway). In the presence of permeability-increasing agents, EPAC reduces endothelial permeability by inhibiting Rho activation and actin cytoskeleton reorganization, by promoting microtubule lengthening and by unidentified signalling pathways not requiring Rho or the cytoskeleton, all of which result in increased cadherin-mediated adhesion (blue pathway). C/EBP, CCAAT/enhancer-binding protein; EPAC, exchange protein activated by cAMP; GPCR, G-protein-coupled receptor; MT, microtubule; RIAM, Rap1-GTP-interacting adaptor molecule; SOCS3, suppressor of cytokine signalling 3; VECs, vascular endothelial cells.

It is known that stimulation of cell growth through G-protein-coupled receptors or receptor-coupled tyrosine kinases normally involves the activation of protein kinase cascades. The first of these to be discovered involved the *ERK* gene products p42 (ERK2) and p44 (ERK1) MAP kinase (Her *et al.*, 1991; Pages *et al.*, 1995), which are activated simultaneously in response to signals transmitted sequentially through p21 Ras, p74 Raf and p45 MEK (Marshall, 1995). There is increasing evidence that cAMP can also activate ERK, and thereby promote cell proliferation, in a Ras-independent manner in certain cell types (Wang *et al.*, 2001; Fujita *et al.*, 2002). It has been suggested that this may be through a pathway involving the activation of B-Raf by Rap1 GTP (Fujita *et al.*, 2002; Gao *et al.*, 2006). Indeed, there have been recent reports that 8-pCPT-2′OMe–cAMP can promote modest phosphorylation of ERK in a number of different cell types, including SH-SY5Y neuroblastoma (Monaghan *et al.*, 2008), human umbilical vein endothelial cells (HUVEC) (Fang and Olah, 2007) and human melanoma cell lines (Gao *et al.*, 2006). However, this contradicts an earlier report demonstrating that 8-pCPT-2′OMe–cAMP failed to induce ERK phosphorylation in Chinese hamster ovary, HEK293T, OVCAR3 or PC12 cells (Enserink *et al.*, 2002). The involvement of EPAC in transducing signals to ERK therefore remains controversial, and there may be additional, uncharacterized determinants that facilitate the coupling of EPAC to ERK activation in certain cells.

One factor that may contribute to the effective coupling of EPAC to the ERK cascade is targeting of EPAC to discreet sub-cellular compartments in different cells. Cell type-specific intracellular targeting of EPAC may then lead to the activation of distinct, function-specific pools of Rap1, some of which may be linked to ERK-activation and cell cycle control. This idea is supported by three main observations. First, activation of EPAC1 in COS cells leads to specific activation of a pool of Rap1 in the perinuclear region (Ohba *et al.*, 2003; Borland *et al.*, 2006), whereas activation of EPAC2 is accompanied by its Ras-dependent translocation, thereby coupling cAMP to Rap1 activation solely at the plasma membrane (Li *et al.*, 2006). Second, effective membrane targeting of EPAC1, through its DEP domain, appears to be a prerequisite

for cAMP-stimulated, EPAC-dependent proliferation of rat thyroid follicular cells (Hochbaum *et al*., 2007). Finally, enforced relocalization of EPAC1 from its normal perinuclear location to the plasma membrane, through the addition of a CAAX box, then allows cAMP to activate ERK through Rap1 and B-Raf (Wang *et al*., 2006). Together these studies show that the correct intracellular targeting of EPAC may be critical in determining the response of cells to elevations in intracellular cAMP. Clearly, further work is required to unravel the potentially diverse and subtle mechanisms by which EPAC mediates the positive effects of cAMP on cell proliferation.

Negative effects of EPAC on cell division

The role of EPAC in anti-mitogenic signalling by cAMP also remains elusive. The mechanisms underlying the anti-proliferative effects of cAMP have revealed that increases in the levels of cell cycle inhibitor proteins, like p27Kip1, can cause a block in G1 in some cells, including leukaemic T lymphocyte cell lines such as Jurkat (Kato *et al*., 1994; van Oirschot *et al*., 2001). The inhibitory effects of cAMP on T lymphocyte proliferation are purportedly the result of increased PKA activity (Skalhegg *et al*., 1992; Bauman *et al*., 1994), which is required for inhibition of IL-2 production, an essential mitogenic hormone in T-cells (Cantrell and Smith, 1984; Anastassiou *et al*., 1992). However, consistent reports provide evidence for PKA-independent mechanisms for immunomodulatory and anti-proliferative effects of cAMP in these cells (Bryce *et al*., 1999; Staples *et al*., 2001). In this respect, over-expression of activated EPAC or activated Rap1 in Jurkat T-cells has been reported to inhibit IL-2 transcription and cell proliferation (Boussiotis *et al*., 1997; Boussiotis *et al*., 2000). Our recent findings contradict these reports, however, and demonstrate that 8-pCPT-2′OMe–cAMP failed to induce growth arrest or p27Kip1 in Jurkat T-cells (Fuld *et al*., 2005). This finding is supported by the work of Sebzda *et al*., who found that the expression of active Rap1 in transgenic T-cells provoked activation of integrins and cell adhesion, rather than inducing growth arrest per se (Sebzda *et al*., 2002).

The role of EPAC in anti-mitogenic signalling is therefore still open to question, and it remains to be seen to what extent the cAMP–EPAC–Rap1 pathway is involved in growth inhibitory signals and the up-regulation of anti-proliferative proteins like p27Kip1. Intriguingly, the principle EPAC effector in cells, Rap1, was originally identified as a protein that reverts the morphological phenotype of Ras-transformed cells (Kitayama *et al*., 1989). Given that cAMP can also promote the reversion of transformed cells (Pastan *et al*., 1975), it is tempting to speculate that this may involve the actions of EPAC and Rap1 as appears to occur in pancreatic cancer cells (Lorenz *et al*., 2008). Care should be taken in extrapolating these results to all cells however, because Rap1 has proven oncogenic potential in a number of human cell types, including papillary thyroid (De Falco *et al*., 2007), breast epithelium (Itoh *et al*., 2007) and hematopoietic cells (Lafuente *et al*., 2007). The involvement of EPAC in oncogenesis therefore remains to be determined, and the relationship may be complex.

EPAC and cell differentiation

In addition to its role as a mitogenic effector, cAMP has been shown to promote the differentiation of certain cell types. Most notably, cAMP stimulates specialized functions in thyroid cells (Pic *et al*., 1986), melanocytes (Chen *et al*., 1974), neuronal cell lines (Frodin *et al*., 1994; Hoffman *et al*., 1994), preadipocyte cell lines (Schmidt *et al*., 1990; Yarwood *et al*., 1998) and cultured brown adipocytes (Cannon and Nedergaard, 1996). Moreover, the requirement for normal differentiation and development of the *Drosophila* eye disc, ovary and embryo *in vivo* (Asha *et al*., 1999) leads to the speculation that Rap1 may also be involved in the transmission of cAMP-promoted, differentiative signals in these mammalian cell types. This idea is supported by a recent study showing that the eye-reducing and lethal effects of a dominant-negative mutant of Rap1 could be overcome by the over-expression of the *Drosophila* forms of EPAC or Rap1 (Dupuy *et al*., 2005). Further study of easily tractable, mammalian cell culture systems will allow us to unravel the mechanistic links between EPAC activation and cellular differentiation.

Although it is clear that the ERKs play a pivotal role in mitogenesis (Pages *et al*., 1993), accumulated data from several sources have suggested that the ERK cascade is an important transducer of differentiative signals. Very compelling evidence for the involvement of the ERK pathway in cellular differentiation came originally from the use of dominant-negative mutations of MEK, which inhibit differentiation of T-cells (Alberola-Ila *et al*., 1995) and PC12 cells (Cowley *et al*., 1994) and, later, a more direct antisense approach was used to demonstrate an obligatory requirement for ERK1 and 2 for the terminal differentiation of 3T3-L1 (Sale *et al*., 1995) and 3T3-F442A preadipocytes (Yarwood *et al*., 1999). Intriguingly, recent work on the adipose conversion of 3T3-L1 preadipocytes has demonstrated a dependence on EPAC activation, which appears to couple to low-level ERK activation in these cells (Petersen *et al*., 2008).

Research over a number of years into the differentiation of the PC12 pheochromocytoma cell line has placed this cell model as the paradigm of how the ERK cascade integrates cAMP signalling into a coordinated programme of cellular differentiation. Cyclic AMP induces activation of ERK in PC12 cells, although this is not sufficient to differentiate these cells (Frodin *et al*., 1994). This appears to be due to the transient nature of the cAMP stimulation, which is not sufficient to induce translocation of ERK to the nucleus (Yao *et al*., 1995). In contrast, differentiation induced by nerve growth factor (NGF) is characterized by prolonged activation and nuclear translocation of ERK (Traverse *et al*., 1992; Nguyen *et al*., 1993). Still, cAMP acts synergistically with NGF to potentiate PC12 differentiation (Yao *et al*., 1995). Therefore it appears that there is threshold duration and magnitude of ERK activation that must be achieved before a cell is committed to a pathway of differentiation.

EPAC seems to play an important role in meditating the pro-differentiative action of cAMP. It has been demonstrated that in PC12 cells activation of EPAC converts cAMP from a proliferative to a differentiation-promoting signal (Kiermayer *et al*., 2005). This seems, at least in part, to occur through the ability of EPAC to extend the duration of PKA-dependent

ERK1/2 activation, leading to enhanced neurite outgrowth (Kiermayer *et al.*, 2005). The mechanisms for this potentiation of ERK activity remain to be determined, but it has been suggested that Rap1 contributes to the sustained activation of ERK promoted by NGF in PC12 cells (York *et al.*, 1998). Another candidate may be the novel small GTPase, Rit, which has been shown to enhance and sustain ERK activation following NGF stimulation of pheochromocytoma PC6 cells, a mechanism that probably involves the engagement of B-Raf (Shi and Andres, 2005; Shi *et al.*, 2006). Rit is also activated by cAMP through EPAC in PC6 cells in an apparently Rap1-independent manner, but this does not seem to be important for ERK activation by cAMP (Shi *et al.*, 2006). Rather, Rit activation by EPAC is required for CREB-dependent transcription, a process that promotes neurite outgrowth and terminal differentiation in these cells (Shi *et al.*, 2006).

The accumulated evidence from these cell culture systems points strongly towards a role for EPAC in mediating the positive effects of cAMP on cell differentiation. We hope the future development of EPAC knockout animals will throw further light on the role of EPAC in differentiation and development. In this respect, frustratingly little has so far been reported about the phenotype of the recent EPAC2 knockout mice (Shibasaki *et al.*, 2007). Intriguingly, it was reported, however, that these mice produced relatively few pancreatic β-cells (Shibasaki *et al.*, 2007), which suggests a role for EPAC2 in the development of this specialized cell type.

EPAC and cell hypertrophy

Hypertrophy is an increase in the size of cells in an organ or tissue and is distinguishable from hyperplasia, which occurs through cell division. For example, ventricular hypertrophy is the increase in size of the cells of the ventricles of the heart. Ventricular hypertrophy is the normal response to aerobic or anaerobic exercise, but can also be associated with pathological changes, such as those associated with hypertension. An increase in the expression of both EPAC1 and EPAC2 mRNAs has been noted during myocardial hypertrophy induced by chronic isoproterenol infusion (Ulucan *et al.*, 2007) In addition, EPAC1 mRNA is up-regulated during pressure overload-induced ventricular hypertrophy (Ulucan *et al.*, 2007). These observations suggest that EPAC proteins are involved in the pathophysiological processes leading to cardiac disease.

Further evidence for this link is provided by experiments that show that EPAC activation in cardiomyocytes leads to the activation of genes associated with cardiac hypertrophy, through a pathway involving the small GTPase Rac, and the calcium-regulated transcription factor nuclear factor of activated T-cells (NFAT) (Morel *et al.*, 2005). Calcium mobilization seems to be a critical determinant in linking EPAC activation to the hypertrophic response in cardiac myocytes (Morel *et al.*, 2005). This probably occurs through activation of phospholipase Cε, which has been shown to enhance intracellular calcium release in these cells following EPAC/Rap1 activation (Oestreich *et al.*, 2007). The link between calcium mobilization and Rac activation remains to be determined however, but this might involve a calcium-sensitive GEF analogous to RasGRP1, the calcium-regulated Ras-GEF (Keiper *et al.*, 2004). Another potential route is through the Rac-specific GEFs Tiam1 and Vav2, which are activated in endothelial cells by EPAC and Rap1 (Birukova *et al.*, 2008). Controversially, however, recent work points towards the hypertrophic effects of EPAC as being Rap1-independent and involving, rather, Ras, calcineurin and calcium/calmodulin-dependent protein kinase II (Metrich *et al.*, 2008). The involvement of Rap1 in these events therefore requires formal verification.

The potential role of EPAC in cardiac hypertrophy is further complicated by the recent observation that EPAC1 actually suppresses the activity of ERK5, a MAP kinase known to be hypertrophic in cardiomyocytes (Dodge-Kafka and Kapiloff, 2006). It should be noted however that in this study EPAC1 was found to be physically associated with the cAMP PDE4D3, in a complex containing ERK5 and the muscle-specific, PKA anchoring protein, mAKAP (Dodge-Kafka and Kapiloff, 2006). Accordingly, elevations in intracellular cAMP stimulate PDE4D3 activity through the activation of PKA (Dodge-Kafka and Kapiloff, 2006). This should reduce local cAMP concentrations around the mAKAP complex, which may limit the inhibitory effect of EPAC1 on ERK5 activation. The EPAC-containing, mAKAP complex may therefore represent a part of complex feedback mechanisms that govern the hypertrophic response of cardiomyocytes.

EPAC in cell adhesion and migration

A strong line of recent research points towards a role for EPAC as a key regulator of cell adhesion and migration. Central to the regulation of cell adhesion is the ability of EPAC and Rap1 to control the activity of cell surface integrins. The signalling mechanisms underpinning integrin activation are a matter of great interest, because the ability to control cell adhesion, and accordingly processes such as immune cell and tumour cell migration, would be of great therapeutic use in many diseases, including inflammatory conditions and cancer.

Integrins are a family of heterodimeric transmembrane proteins composed of α and β subunits and are involved in many cell adhesion processes [reviewed in (Pribila *et al.*, 2004)], often binding RGD motif-containing ligands. The ability of many integrins to bind ligand is dynamically regulated, in order to allow adhesion and de-adhesion to a substrate or another cell in response to external signals [reviewed in (Kinashi and Katagiri, 2005)]. Recently it has become clear that Rap1 plays a central role in integrin regulation; indeed, a defect in activation of Rap1 has been shown to be the basis of an inherited leucocyte adhesion deficiency disease [LAD-III; (Kinashi *et al.*, 2004)]. Rap1 activates integrins through changes in both affinity for ligand (Katagiri *et al.*, 2000; 2003; Bertoni *et al.*, 2002; Tohyama *et al.*, 2003; Fagerholm *et al.*, 2005; Han *et al.*, 2006; Lorenowicz *et al.*, 2006; Carmona *et al.*, 2008) and avidity (via clustering of integrins at the cell surface) (Sebzda *et al.*, 2002; Katagiri *et al.*, 2003; Carmona *et al.*, 2008). Although it is becoming clear that EPAC is not always responsible for Rap1-mediated integrin activation, it is important to understand Rap1 involvement in integrin activation in order to place the effects of EPAC in an appropriate context.

Due to its importance in lymphocyte transmigration and immunological synapse formation, much work has been

carried out on the role of Rap1 in activation of LFA-1 (Reedquist *et al.*, 2000; de Bruyn *et al.*, 2002; Liu *et al.*, 2002; Sebzda *et al.*, 2002; Shimonaka *et al.*, 2003; Katagiri *et al.*, 2004; Ghandour *et al.*, 2007), but many other integrins have also been shown to be activated by Rap1. These include VLA-4 [α4β1; (Reedquist *et al.*, 2000; Arai *et al.*, 2001; de Bruyn *et al.*, 2002; Liu *et al.*, 2002; Sebzda *et al.*, 2002; Shimonaka *et al.*, 2003)], the macrophage integrin Mac-1 [αMβ2; (Caron *et al.*, 2000)], the laminin receptor α5β1 (Sebzda *et al.*, 2002), the platelet integrin αIIbβ3 (Bertoni *et al.*, 2002; Han *et al.*, 2006) and the vitronectin and CD23 receptor αvβ3 (Gao *et al.*, 2006).

Use of 8-pCPT-2′OMe–cAMP has allowed the specific involvement of EPAC in integrin activation to be determined. Rangarajan *et al.* (2003) showed that integrin-mediated adhesion of the OvCar3 cell line to fibronectin was cAMP-dependent and PKA-independent, and that over-expression of EPAC1 in these cells increased both basal and cAMP-stimulated adhesion. An increase in adhesion was also found following treatment of the cells with 8-pCPT-2′OMe–cAMP, or with β2 adrenoceptor agonists. Although expression of EPAC proteins in leucocytes is not generally high (Tiwari *et al.*, 2004), EPAC does seem to be involved in the regulation of integrin activity in these cells. EPAC activation increased integrin-mediated adhesion of primary monocytes to HUVECs under flow (Lorenowicz *et al.*, 2006), which appears to be via β1 integrins as activation of β2 integrins could not be detected in these cells. EPAC is also involved in β2 integrin-dependent adhesion of human haematopoietic stem cells to ICAM-1 (Carmona *et al.*, 2008). As with Rap1, EPAC does not appear to regulate activation of all integrin types, as treatment of bladder carcinoma cells with 8-pCPT-2′OMe–cAMP increased adhesion via the α3β1, but not the α6β4, laminin receptor (Enserink *et al.*, 2004).

In addition to integrin activation, EPAC has been associated with polarization of migrating cells. In migrating cells, extension occurs at the leading edge, often in response to a chemotactic gradient, with retraction at the uropod resulting in cell movement in the direction of the leading edge. Lorenowicz *et al.* showed in U937 monocytic cells that treatment with 8-pCPT-2′OMe–cAMP of cells already adherent to fibronectin doubled the number of polarized cells and caused a redistribution of EPAC to the uropod (Lorenowicz *et al.*, 2006). This study also demonstrated an increase in cell migration towards a chemotactic signal after stimulation of EPAC. In non-haematopoietic cells, EPAC activation has been shown to increase migration of endothelial progenitor cells on fibronectin (Carmona *et al.*, 2008), while, conversely, EPAC activation inhibits migration of epithelial cells (Lyle *et al.*, 2008), although the latter appears to be independent of effects on integrin activation, because it could not be mimicked by enforced integrin activation by activating antibodies (Lyle *et al.*, 2008).

Signalling pathways in EPAC-mediated integrin activation

The most thorough investigation into signalling of Rap1-dependent integrin activation has been carried out by Han *et al.* (2006). It was previously known that conformational changes associated with integrin activation were the result of talin binding to integrin β subunits (Vinogradova *et al.*, 2002). This group recapitulated the αIIbβ3 (platelet) integrin activation pathway by transfecting the integrin and potential signalling pathway components into Chinese hamster ovary cells, demonstrating that activation of both protein kinase C and a RapGEF, by ligand-induced calcium and DAG production, promotes association of active Rap1 with Rap1-GTP-interacting adaptor molecule (RIAM) at the plasma membrane. This leads to the recruitment of talin into a RIAM-talin complex, and subsequent integrin activation. It is not yet clear whether this activation pathway is universal, or whether other integrins in other cell types are regulated via a different mechanism.

The Rap1 effector RAPL (regulator for cell adhesion and polarization enriched in lymphoid tissues) was identified by Katagiri *et al.* as a regulator of integrin activation in lymphoid cells, involved in both affinity and avidity changes in LFA-1, and is indispensable for LFA-1 redistribution and polarization after chemokine or T-cell receptor (TCR) stimulation of T cells (Katagiri *et al.*, 2003). A particular role for RAPL in Rap1-mediated cell migration has been suggested by Fujita *et al.*, who showed that RAPL associated with microtubules in vascular endothelial cells (VECs) and suggested a role for Rap1/RAPL in microtubule extension towards the leading edge of migrating cells (Fujita *et al.*, 2002). Although RAPL is clearly an important Rap1-regulated integrin activator, there are no data currently available addressing the issue of potential EPAC involvement in RAPL-mediated integrin activation.

An increasing body of evidence, however, links EPAC to the control of integrin activation via Rap1. Because EPAC has been shown to be able to mediate both affinity and avidity modulation of integrins (Carmona *et al.*, 2008), it is unlikely that different RapGEFs mediate different types of integrin activation; instead, it is probable that different RapGEFs are used to activate Rap1 in response to different extracellular signals. Thus, EPAC does not appear to be involved in regulation of LFA-1 activation in T cells, because such integrin activation occurs largely in response to TCR or chemokine stimulation (Katagiri *et al.*, 2000; Sebzda *et al.*, 2002; Ghandour *et al.*, 2007), which result in activation of CalDAG-GEFI rather than EPAC. It is also possible that sub-cellular localization determines whether EPAC stimulation results in integrin activation. It has become clear over the past several years that cAMP elevation in cells is not uniform, and that concentration gradients are important is determining which cAMP effectors become activated in the cell (Houslay and Milligan, 1997). EPAC is generally localized to a perinuclear region in unpolarized, non-migrating cells (Borland *et al.*, 2006), and it has been shown, at least in T cells, that integrin activation occurs in response to activation of a pool of Rap1 at the plasma membrane (Bivona *et al.*, 2004). Thus, CalDAG-GEFI, which is also found at the plasma membrane of these cells, rather than EPAC, is associated with LFA-1 activation.

Thus, it is clear that EPAC plays an important role in regulating integrins, both in inflammatory processes (such as macrophage adhesion to HUVECs (Lorenowicz *et al.*, 2006)) and in cells not directly involved in inflammation. However, given the generally anti-inflammatory effects of cAMP on

Table 2 Pro- and anti-inflammatory actions of EPAC

Cell type	*Effect*	*Details*	*References*
Alveolar macrophages	Anti-inflammatory	EPAC activation suppresses phagocytosis	(Aronoff *et al.*, 2005)
Peritoneal macrophages/alveolar macrophage	N/A	EPAC activation does not affect production of inflammatory cytokines	(Aronoff *et al.*, 2006)
RAW264.7 mouse macrophage line	Pro-inflammatory	EPAC signalling results in NF-κB activation	(Moon and Pyo, 2007)
RAW264.7 mouse macrophage line	Pro-inflammatory	β2AR or EPAC activation increases pro-inflammatory cytokine production	(Tan *et al.*, 2007)
J774A.1 mouse macrophage line	Anti-inflammatory	PGE_2 suppresses inflammatory cytokine production via EPAC-dependent mechanism	(Xu *et al.*, 2008)
Bone marrow-derived mouse dendritic cells	Anti-inflammatory	EPAC activation suppresses production of inflammatory chemokines	(Jing *et al.*, 2004)
Bone marrow-derived mouse dendritic cells	Anti-inflammatory	EPAC activation suppresses production of inflammatory cytokines and chemokines	(Aronoff *et al.*, 2006)

EPAC, exchange protein activated by cAMP; N/A, not applicable; NF-κB, nuclear factor kappa B; PGE, prostaglandin E.

cells, it is difficult to predict exactly how EPAC activation will affect overall cell adhesiveness and migratory capacity (and therefore inflammation) until more data are available, although it is likely that effects will be cell type-dependent.

Regulation of inflammation in vascular endothelium by EPAC

Recent research has implicated EPAC in regulation of inflammatory processes in VECs, including the regulation of endothelial cell–cell junction stability (important in controlling oedema) (Schmidt *et al.*, 2007) and down-regulation of IL-6-mediated inflammatory processes (Sands *et al.*, 2006). Whereas the role of EPAC in phagocytes during inflammation appears to be cell type-specific (Table 2), the involvement of EPAC in multiple anti-inflammatory processes in one cell type presents an intriguing model in which to study how distinct cellular processes may interact to present a coordinated programme of anti-inflammatory responsiveness (Figure 3).

A number of groups have recently implicated EPAC signalling in the regulation of endothelial cell–cell adhesion via the vascular endothelial (VE)-cadherin (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005; Kooistra *et al.*, 2005). VE-cadherin in adherens junctions (AJs) on lateral surfaces of adjacent VECs undergo homophilic adhesion, resulting in the formation of a tight barrier between cells (Vandenbroucke *et al.*, 2008). This limits exchange between the vasculature and the underlying tissue, and increased endothelial permeability as a result of junction disruption can cause oedema. EPAC has been shown to reduce VEC permeability as a result of redistribution of junctional molecules (such as VE-cadherin) to lateral surfaces where they can interact with binding partners on adjacent cells, and by inhibiting cytoskeletal reorganization, which is essential for increased endothelial permeability (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005; Kooistra *et al.*, 2005). Importantly, the main function of EPAC in regulating permeability appears to be in reversing increases in permeability caused by inflammatory mediators such as thrombin and TNFα (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005; Kooistra *et al.*, 2005).

Known mechanisms increasing endothelial barrier permeability include endothelial cell contraction through reorganization of the actin cytoskeleton (e.g. by thrombin) (Bogatcheva *et al.*, 2002; Vouret-Craviari *et al.*, 2003), microtubule-dependent AJ disassembly (e.g. by TNFα) (Petrache *et al.*, 2003) and changes in phosphorylation status of AJ proteins (Mehta and Malik, 2006), all leading to internalization of AJ proteins. Activation of the small GTPase Rho plays a pivotal role in integrating many of these permeability-increasing signals. EPAC has been implicated in both actin-dependent (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005; Kooistra *et al.*, 2005; Birukova *et al.*, 2007; Baumer *et al.*, 2008) and microtubule-dependent (Sehrawat *et al.*, 2008) stabilization of endothelial barriers, but has not yet been directly associated with signalling pathways leading to VE-cadherin or its associated catenin proteins, although it is known to regulate AJ protein redistribution to lateral membranes after internalization (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005; Kooistra *et al.*, 2005).

Vascular endothelial-cadherin in AJs associates with α- and β-catenins via its C-terminal domain, and p120 catenin via its N-terminus (Lampugnani *et al.*, 1995); α- and β-catenins link VE-cadherin to the actin cytoskeleton (Lampugnani *et al.*, 1995), while p120 catenin appears to regulate cadherin adhesion by controlling signalling mechanisms (Kondapalli *et al.*, 2004; Yanagisawa *et al.*, 2004; Mehta and Malik, 2006). Maintenance of endothelial barrier function requires a cortical actin ring, and treatment of endothelial cells with permeability-increasing agents such as thrombin often results in actin reorganization to form contractile stress fibres (Vandenbroucke *et al.*, 2008); phosphorylation of myosin light chain by endothelial myosin light chain kinase, in a Ca^{2+}/calmodulin-dependent manner (Garcia *et al.*, 1995; Goeckeler and Wysolmerski, 1995) and potentiated by the Rho effector ROCK (Noda *et al.*, 1995; Yoshioka *et al.*, 2007), drives myosin-actin cross-bridge cycling leading to cell contraction. Because VE-cadherin is connected to the actin cytoskeleton via catenins, actin-mediated cell contraction physically pulls the cadherin out of contact with its binding partner on an adjacent cell and causes inter-endothelial gaps to appear, increasing permeability (Moy *et al.*, 1996).

A number of studies have implicated EPAC in reversal of thrombin-mediated increases in permeability, demonstrating that an increase in cAMP concentration, or direct activation of EPAC, stabilizes the cortical actin ring, allowing redistribution of VE-cadherin to AJs (Cullere *et al.*, 2005; Fukuhara

et al., 2005; Kooistra *et al*., 2005; Birukova *et al*., 2008). While EPAC has also been shown to inhibit activation of Rho (Cullere *et al*., 2005), possibly via Rac1 (Birukova *et al*., 2007; 2008), it is not clear whether this is the mechanism of cortical actin stabilization, or whether EPAC interacts with another signalling pathway. However, EPAC-induced increases in cortical actin are independent of its direct effects on VE-cadherin: cells in which VE-cadherin has been down-regulated from the cell surface by incubation in low calcium-containing medium still show increased cortical actin when EPAC is stimulated (Kooistra *et al*., 2005). Activation of EPAC also increases the length of microtubules, independently of its effects on VE-cadherin and Rap activation (Sehrawat *et al*., 2008). These investigators showed that an intact microtubule network was required for EPAC-dependent increases in cortical actin and barrier enhancement, and that 8-pCPT-2′OMe–cAMP treatment of cells could reverse TNFα- and TGFβ-mediated increases in endothelial cell permeability. Intriguingly, EPAC1 has been shown to interact with microtubules and MAPs (Magiera *et al*., 2004; Gupta and Yarwood, 2005), although whether these interactions are required for the regulation of cell contraction remains to be investigated.

One of the important questions regarding the effect of EPAC on endothelial cells is whether inhibiting endothelial permeability has any effect on endothelial transmigration by leucocytes. This remains an open question that two studies have so far attempted to answer: Cullere *et al*. saw no effect of EPAC activation in endothelial cells on transmigration of human neutrophils (Cullere *et al*., 2005), while Wittchen *et al*. found an inhibition of transmigration of (neutrophil-like) differentiated HL-60 cells (Wittchen *et al*., 2005). It is not yet clear whether these differences are due to differences in cells or experimental technique, or whether there truly is a difference in the effects of transmigration of primary neutrophils and cell lines. However, even if this issue is clarified, it will still not be clear whether any alterations in rates of transmigration are due specifically to the effects of EPAC on endothelial barrier permeability, or whether they are due to other effects of EPAC on endothelial cells.

EPAC and suppressor of cytokine signalling 3 induction

Recent research in our laboratory has identified an entirely novel anti-inflammatory function for EPAC in endothelial cells, involving induction of suppressor of cytokine signalling 3 (SOCS3) expression, resulting in inhibition of IL-6 signalling (Sands *et al*., 2006).

IL-6 is a pro-inflammatory cytokine that has been detected in atherosclerotic plaques (von der Thusen *et al*., 2003; Schieffer *et al*., 2004). Signalling by IL-6 occurs through the IL-6 receptor complex, composed of an IL-6-binding α chain (IL-6Rα) and gp130, which cannot bind IL-6, but rather interacts with IL-6Rα [reviewed in (Heinrich *et al*., 2003)]. The gp130 chain is widely expressed, including on vascular endothelium (Heinrich *et al*., 2003), and although IL-Rα is not expressed by HUVECs (Sands *et al*., 2006), IL-6 signalling can be achieved by binding of IL-6 to soluble IL-6Rα chain (released by a number of cell types, including activated macrophages at sites of inflammation), and binding of this complex to endothelial cell surface gp130 to allow signalling into the cell. On binding of the complex to gp130, receptor clustering takes place and the JAK–STAT signalling pathway is activated. Activated STAT3 homodimerizes and is translocated to the nucleus, where it acts as a transcription factor for induction of IL-6-responsive genes.

Clearly, regulation of such pro-inflammatory signalling is vital to prevent runaway inflammation. One of the most important mechanisms for down-regulating JAK-STAT signalling is via the SOCS family of proteins [reviewed in (Yoshimura *et al*., 2007)]. SOCS proteins are often induced directly by the JAK–STAT pathway, they then go on to inhibit, forming a classical negative feedback loop. SOCS3 inhibits gp130 signalling by binding to tyrosine-phosphorylated JAKs via the SOCS3 SH2 domain and preventing JAK-mediated recruitment and activation of STATs (Sasaki *et al*., 1999). While investigating the possible effects of cAMP on signalling of pro-inflammatory cytokines and using IL-6 signalling in HUVECs as a model, we discovered a PKA-independent, cAMP-mediated inhibition of IL-6 signalling: stimulation of HUVECs with a combination of forskolin and rolipram, or with a β2 adrenergic receptor agonist, reduced STAT3 phosphorylation in response to treatment with the IL-6/IL-6Rα *trans*-signalling complex (Sands *et al*., 2006). This inhibition did not require PKA, but rather was dependent on EPAC/Rap1 signalling, and was the result of EPAC-mediated induction of SOCS3 expression. Downstream signalling from EPAC/Rap1 leads to activation of CCAAT/enhancer-binding proteins (C/EBPs), which associate with the SOCS3 promoter via one or more C/EBP consensus binding sites and promote increased transcription of the gene (Yarwood *et al*., 2008). The intermediate signalling steps between Rap1 and C/EBP activation are not yet clear; there is evidence for an involvement of ERK in SOCS3 induction in HUVECs, because pretreatment of cells with the MEK inhibitor U0126 inhibits cAMP-mediated SOCS3 induction (Sands *et al*., 2006), but its exact role has not yet been clarified, and it may act in a pathway parallel to that involving EPAC.

Cyclic AMP-mediated induction of SOCS proteins is not a novel finding in itself, as treatment of cells with cAMP-stimulating agonists has been shown to induce SOCS3 in leucocytes (Gasperini *et al*., 2002) and in differentiated 3T3-L1 adipocytes (Fasshauer *et al*., 2002). However, this is the first demonstration of EPAC involvement in such an anti-inflammatory process, and one of the first reports of regulation of gene transcription by EPAC, and is thus an important advance in the EPAC field. The dual roles of EPAC in both down-regulating cytokine-mediated inflammation and maintaining the endothelial cell barrier suggest a central role for this GEF in controlling endothelial responses to inflammatory stimuli. Activation of EPAC both stabilizes the endothelial barrier (which can be compromised by such inflammatory mediators as TNFα) (Cullere *et al*., 2005; Fukuhara *et al*., 2005; Kooistra *et al*., 2005) and inhibits signalling through the IL-6–STAT3 pathway through induction of SOCS3 (Sands *et al*., 2006; Yarwood *et al*., 2008). EPAC has also been reported to activate integrins involved in VEC adhesion to basement membrane, further reducing endothelial permeability

(Netherton *et al.*, 2007). While no studies have looked at these anti-inflammatory processes in the same cells, it is entirely possible that they could be triggered by the same physiological G-protein-coupled receptor ligand triggering cAMP production; certainly, the biological outcomes of the pathways would be compatible.

Overall we hope that we have presented in this review an insight into the diverse actions of EPAC in controlling cell functions. Given that these roles impinge on the regulation of cellular mechanisms intimately involved in the manifestation of diseases, like cancer and inflammation, we predict that further research into the structure and function of EPAC proteins will lead to the development of novel therapeutics.

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Conflict of interest

None.

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