Epac, in Synergy with cAMP-dependent Protein Kinase (PKA), Is Required for cAMP-mediated Mitogenesis

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Materials—Forskolin, isobutylmethylxanthine, and H89 were obtained from Calbiochem. TSH was obtained from Sigma. GSH-Agarose was from Amersham Biosciences. cAMP analogs for Epac (cE) and PKA (6-bZ) were obtained from Axxon/A Biol. Antibodies against HA (HA.11) and myc (9E10) serum albumin; BrdUrd, bromodeoxyuridine; PBS, phosphate-buffered saline.

Epac (exchange protein activated by cAMP) is a new cAMP-dependent, PKA-independent guanine nucleotide-exchange factor (GEF) for the small G-protein Rap (8, 9). Newly developed cAMP analogs (10) capable of discriminating between Epac and PKA are starting to unravel Epac’s role in diverse biological responses (11, 12). Epac’s N-terminal regulatory domain includes a DEP module (Disheveled, Egl10, Pleckstrin) responsible for its membrane localization (13) and a cAMP-binding domain (CBD) that directly binds cAMP (Kd ~ 4 μM) (13). The catalytic domain consists of a Ras exchange motif (REM), and the CDC25-like catalytic core, sufficient for GEF action (8). Biochemical (13–16) and crystallographic studies (17) unmasked a role for the regulatory domain in maintaining Epac in a basal autoinhibited state; deletion of its N terminus converted REM-cdc25-Epac to a constitutively active (cAMP-independent) Rap GEF. The addition of the N terminus in trans to the active C terminus was able to inhibit its GEF activity, and this inhibition could be relieved by cAMP. Mutation analysis indicated a constitutively active role for the N terminus sequence 321AAAAA325 (as in Epac1) as part of this inhibitory domain, and accordingly, disruption of this domain by conversion of this sequence to 321AAAAA325 (Ala) in the context of full-length Epac rendered it constitutively active. Thus, a model emerged where at low cAMP, the regulatory domain forms a rigid interaction with the C-terminal GEF, blocking the access of its substrate, Rap. Upon cAMP binding, a conformational change leads to the opening of the Rap-binding domain and activation. The DEP domain is not involved in cAMP-dependent Epac activation (14), and it was shown to be responsible for targeting Epac to a membrane compartment (13); its solvent accessibility in the Epac structure is consistent with a role as an anchoring device via protein-protein interaction.

Here we demonstrate using thyroid cells, a prototypical model for cAMP-mediated mitogenesis (18), that both cAMP effector pathways, i.e. Epac and PKA, are required for the mitogenic action of TSH. We have discovered that Epac and PKA act synergistically in order for cAMP to elicit an efficient proliferative response. These results are consistent with previous reports indicating that Rap1b potentiates cAMP stimulation of DNA synthesis in thyroid in a manner dependent on its GTP-bound and PKA phosphorylated state (19, 20).

EXPERIMENTAL PROCEDURES

CAMP stimulates proliferation in many cell types. For many years, cAMP-dependent protein kinase (PKA) represented the only known cAMP effector. PKA, however, does not fully mimic the action of cAMP, indicating the existence of a PKA-independent component. Since cAMP-mediated activation of the G-protein Rap1 and its phosphorylation by PKA are strictly required for the effects of cAMP on mitogenesis, we hypothesized that the Rap1 activator Epac might represent the PKA-independent factor. Here we report that Epac acts synergistically with PKA in cAMP-mediated mitogenesis. We have generated a new dominant negative Epac mutant that revealed that activation of Epac is required for thyroid-stimulating hormone or cAMP stimulation of DNA synthesis. We demonstrate that Epac’s action on cAMP-mediated activation of Rap1 and cAMP-mediated mitogenesis depends on the subcellular localization of Epac via its DEP domain. Disruption of the DEP-dependent subcellular targeting of Epac abolished cAMP-Epac-mediated Rap1 activation and thyroid-stimulating hormone-mediated cell proliferation, indicating that an Epac-Rap-PKA signaling unit is critical for the mitogenic action of cAMP.

CAMP stimulates proliferation in several model systems (1). Particularly in endocrine cells, in vitro and in vivo studies demonstrated a role for cAMP in mitogenesis (2), a concept further supported by the identification of mutant receptors and G-proteins causally linking constitutive cAMP signaling with hyperproliferative states (3, 4). For many years, PKA2 represented the only known cAMP effector (5); however, although its activity is necessary, it is not sufficient for cAMP mitogenic action (6, 7). These studies indicated the existence of PKA independent effectors involved in cAMP-mediated proliferation.

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were from Covance. Antibodies against FLAG, F3I65, were from Sigma. Anti-Epac antibody (A5) was from Santa Cruz Biotechnology. Anti-phospho-CREB (Ser-133), clone 10E9, was from Upstate Biotechnology.

Cell Lines and Transfections—PCCL3, a normal TSH-dependent rat thyroid follicular cell line (21), was grown in Coon’s modified F-12 medium (Sigma) and supplemented with 5% FBS and the combination of four hormones: TSH (1 mIU/ml) (a-c) or the Epac specific analog, 8-pCPT-2′-O-Me-cAMP (c) (e). When indicated, cells were pretreated (20 min) with 15 μM PKA inhibitor H89 (b). Experiments were repeated three times, and shown are representative blots from independent lysates. WT, wild type. d, Epac expression levels in different cell lines tested (~80 μg of protein/lane). Epac1 expression (upper half) was normalized using an anti-EF1 antibody (lower half). CHO, Chinese hamster ovary. f, synergism between cAMP effector pathways in G1/S progression: 8-pCPT-2′-O-Me-cAMP dose responses performed in the presence of 10% FBS or 10% FBS plus 300 μM PKA-specific analog N°-benzoyladenosine-3′, 5′-cyclic monophosphate (6 μM). Results are expressed as mean ± S.E. (n = 3).

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Cell Lines and Transfections—PCCL3, a normal TSH-dependent rat thyroid follicular cell line (21), was grown in Coon’s modified F-12 medium (Sigma) and supplemented with 5% FBS and the combination of four hormones: TSH (1 μIU/ml; IU = international units), insulin (1 μg/ml), transferrin (5 μg/ml), and hydrocortisone (1 μM). HEK-293T cells were maintained in Dulbecco’s modified Eagle’s medium (Cambrex) supplemented with 10% FBS. Cells were kept at 37 °C in a 5% CO2, 95% humidified air environment. Transfections were performed using FuGENE transfection reagent (Roche Applied Science), adjusting the total amount of DNA plasmid to 0.5–1 μg/well as directed by the manufacturer. Additional transfections were performed with polyethyleneimine (Polysciences, Inc), with 6 μg of polyethyleneimine, 3 μg of DNA/well.

DNA Constructs—pCGN-HA-Rap and pCMV-myc-N-Epac constructs were already described. pSIREN red fluorescent protein was purchased from Clontech. pCMV-myc-ΔDEP-N-Epac was prepared by subcloning an EcoRI-BglII fragment from pGEX5X2-ΔDEP-Epac1. pCMV-myc-N-Epac ALA5 was prepared by subcloning a Sall-BglIII fragment from pMT2-HA-Epac1 ALA5. Both original plasmids were kindly provided by Dr. Bos. pFLAG-CMV2-Epac1 and R279E were kindly provided by Dr. Holz. pFLAG-CMV-ΔDEP-Epac1 R279E was generated by PCR using the following pair of primers: 5′-ggagggAAGCTTccgctgggaactcag-3′ (sense) and 5′-ggagggatttcatGATCTggtctgttggc-3′ (antisense). This fragment, containing the mutation, was replaced in pFLAG-CMV2-Epac1 with HindIII-BglII. pCMV-myc-ΔDEP-Epac was prepared by subcloning the fragment HindIII (filled-in with Klenow)/KpnI from pFLAG-CMV2-Epac1 into pCMV-myc Sall (filled-in with Klenow) KpnI.

Thymidine Incorporation Assays—Cells were plated into 96-well plates (10,000 cells/well). On the next day, cells were made quiescent by serum starvation in Dulbecco’s modified Eagle’s medium, 0.2% BSA for 20 h. Upon agonist stimulation (16 h), cells were labeled with [methyl-3H]thymidine (Amersham Biosciences; 1 μCi, 1 μCi/ml; 1 Ci = 37 GBq), and 24 h later, samples were collected by using a cell harvester. Filters were dried and analyzed by scintillation counting.

BrdUrd Labeling—Cells were grown to 50% confluence on glass cover slips, transfected when appropriate for 24 h, and made quiescent by serum starvation in Coon’s, 0.2% BSA for 16 h. After agonist stimulation for 8 h, cells were labeled for 16 h with 100 μB BrdUrd (Sigma). At the end of the labeling period, cells were fixed in 4% paraformaldehyde (10 min, room temperature) and permeabilized with 0.5% Triton X-100 (20 min, room temperature). After washing, incorporated BrdUrd was detected by indirect immunofluorescence. Samples were incubated for 1 h at room temperature with sheep anti-BrdUrd antibody (Biodesign International; diluted 1:100 in PBS, 1% BSA) and the corresponding primary antibody (HA-11, 1:400; myc, 1:400 or FLAG, 1:5000) in the presence of DNase (Promega; 10 units/ml). After extensive washes in PBS, 1% BSA, samples were incubated for 1 h at room temperature with fluorescein isothiocyanate-conjugated goat-anti sheep (Sigma; dilution, 1:150 in PBS, 2% BSA) containing 0.2 μg/ml 4,6-diamidino-2-phenylindole (Sigma). After extensive washes in PBS, 0.1% Tween 20, samples were mounted in PermaFluor (Thermo) and viewed by epifluorescence (X60).

Rap Activation Assay Using RalGDS-RBD—Cells transfected with a plasmid expressing HA-Rap1b were lysed with a buffer
containing 200 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 10% glycerol, 25 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 2 μM aprotinin. Lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4 °C. 10 μg of bacterially expressed GST-RalGDS-RBD coupled to glutathione-Sepharose beads (Amersham Biosciences) were added to the supernatants and incubated at 4 °C for 60 min with agitation. Beads were washed four times in the same lysis buffer. After the final wash, Laemmli sample buffer was added to the samples. Proteins were fractionated in a 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane.

RESULTS

TSH-mediated Activation of Rap1b Is Independent of PKA—TSH, via cAMP, activates Rap1 in thyroid cells (20, 22–24); however, whether Epac activation of Rap1b is required for TSH-stimulation of DNA synthesis is not known. To address the mechanisms involved, the role of cAMP-dependent effector pathways, i.e. Epac and PKA, were investigated. TSH-dependent Rap1 activation shows a rapid onset followed by a sustained phase (Fig. 1a). PKA inhibition by H89 did not affect this profile (Fig. 1b), whereas PKA-dependent CREB phosphorylation was completely blocked. Consistent with these results, TSH activated Rap1-S179A, a phosphorylation-deficient mutant (Fig. 1c) (25). Thus, TSH-dependent activation of Rap1 is independent of PKA action.

Epac and PKA Act Synergistically in TSH-mediated Cell Proliferation—cAMP-dependent, PKA-independent activation of Rap1b suggested the involvement of Epac, a Rap GEF enriched in thyroid cells (9). Expression of Epac1 in PCCL3 thyroid cells was confirmed by Western blot (Fig. 1d). Its ability to activate Rap1 was assessed upon stimulation with 8-pMeOPT-2'-O-Me-cAMP, a novel cAMP analog able to discriminate between Epac and PKA (10). 8-pMeOPT-2'-O-Me-cAMP activated Rap1b in PCCL3 cells with a kinetic profile similar to TSH (Fig. 1e). Thus, in PCCL3 cells, Epac1 is present and fully functional. We hypothesized that Epac activation of Rap1b might represent the PKA-independent event in cAMP-stimulated mitogenesis. Since the mitogenic effects of cAMP are only manifested in the presence of a co-mitogen (i.e. FBS, IGF-1), we tested whether under these conditions, activators of each pathway, Epac and PKA, alone or in combination, could trigger a mitogenic response. Results presented in Fig. 1f indicate that although Epac-specific analog 8-pMeOPT-2'-O-Me-cAMP is not able to trigger a response, it significantly potentiates the action of the PKA-specific analog 6-Bz-cAMP (26). These results established that both cAMP effector pathways, Epac and PKA, coordinately collaborate to fully mimic TSH/cAMP proliferative response.

Activation of Epac Is Required for TSH-mediated Mitogenesis—To assess whether Epac activation is involved in the physiological response of TSH, an Epac inhibitor is required. Since no specific pharmacological inhibitor is available, a dominant negative genetic approach was devised. A DEP domain present at Epac's N-terminal end (Fig. 2a), which
anchors it to a particulate fraction, is required for function (13). Although a DEP-binding partner is currently unknown, we reasoned that expression of a small fragment containing the DEP domain, but devoid of catalytic activity, might displace endogenous Epac from its normal intracellular compartment and block the ability of cAMP to activate endogenous Rap1b. Among the constructs tested, a ∼42-kDa fragment encompassing Epac1 DEP-CBD-REM (N-Epac) was the most stable and was therefore selected to assess its activity as dominant negative (Fig. 2a). We used HEK cells as an initial model system since cAMP did not activate Rap1 in these cells unless exogenous Epac is expressed (Figs. 1d and 2b), thus allowing us to specifically test the effect of N-Epac on cAMP-Epac activity. Co-expression of N-Epac inhibited in a dose-dependent manner the ability of Epac to activate Rap1b upon forskolin stimulation (Fig. 2b). N-Epac, however, did not affect the ability of forskolin to stimulate CREB phosphorylation (supplemental Fig. 1). Next, the ability of N-Epac to inhibit TSH action in PCCl3 thyroid cells was assessed. We used myc-N-Epac in transient transfection assays to assess its ability to interfere with TSH stimulation of G1/S progression. As shown in Fig. 2c, transfection of control myc-WT-Rap1b did not induce any proliferative response in these cells (20), but expression of N-Epac blocked TSH-mediated G1/S progression, demonstrating that Epac activation is critical for cAMP-mediated mitogenesis in thyroid cells.

Role of Epac’s DEP Domain on cAMP-mediated Mitogenesis—Several scenarios could explain N-Epac’s inhibitory action. Although cAMP (i.e. TSH) did not relieve the inhibition, it is possible that the presence of the VLVE inhibitory domain in N-Epac (15) could act in trans to block endogenous Epac. Hence, inactivation of the VLVE domain by alanine substitutions (Ala3) (15) should reverse N-Epac’s inhibitory action. However, N-Epac-Ala3 remained a potent inhibitor (Fig. 2d). These results are inconsistent with a direct inhibition of Epac by N-Epac; rather, they indicate a dominant negative action consistent with a DEP-mediated displacement of a critical Epac signaling complex by N-Epac.

To directly assess this possibility, we tested the effect of an N-Epac construct lacking the first 148 amino acids (ΔDEP-N-Epac, Fig. 2a). Despite expression levels similar to N-Epac, ΔDEP-N-Epac (Fig. 2d, inset) did not affect TSH-stimulated mitogenesis, indicating that the absence of the DEP abolished the dominant negative action of N-Epac. These results also rule out the possibility that the inhibitory action of N-Epac was mediated by its ability to titrate cAMP levels since the loss-of-function ΔDEP-N-Epac still binds cAMP (13, 27). Thus, N-Epac acts as a bona fide dominant negative, and its inhibition of cAMP-mediated mitogenesis underscores our proposal that Epac and PKA synergize in the transduction of cAMP mitogenic signaling.

These results indicate that proper Epac compartmentalization is required for transduction of the cAMP signal. If Epac 1–148 is required for Epac’s activity, then two predictions could be tested. An Epac construct missing this targeting domain should be unable to transduce the cAMP response, and the inhibitory action of R279E Epac, a mutation that inhibits cAMP binding (28), should be inactive in the absence of the DEP domain. These predictions were confirmed experimentally; ΔDEP-Epac cannot mediate Rap1 activation by forskolin (Fig. 2e), and the inhibitory effect of Epac-R279E on G1/S is lost when its DEP is deleted (Fig. 2f). Thus, these combined results demonstrate that Epac activity is required for cAMP-mediated G1/S entry, in ways that are dependent on its proper targeting via the N-terminal region containing the DEP domain.

**DISCUSSION**

Although the precise mechanisms whereby cAMP stimulates mitogenesis are poorly understood, it is becoming evident that cAMP engages both PKA-dependent and PKA-independent events. Utilizing both cellular (20) and mouse models (19), we have previously demonstrated that Rap1b, a substrate for Epac and PKA, plays an essential role in cAMP-mediated cell proliferation. Our studies established a critical role for active and phosphorylated Rap1, indicating its potential function as integrator of both PKA-dependent and PKA-independent cAMP effector pathways.

However, prior to this report, it was unclear whether and how the PKA-independent component functions in the cAMP mitogenic response. In this study, we have established a role for Epac, acting synergistically with PKA, in cAMP-mediated cell proliferation. Moreover, we have demonstrated that Epac activity is strictly dependent on its proper compartmentalization, which is determined by its DEP domain.

In PCCl3, the PKA-independent effect of cAMP in mitogenesis appears to solely involve Epac activation of Rap1b. PKA-independent Epac activation of Rap1 by cAMP has been reported in other thyroid cells (20, 22–24, 29). Surprisingly, in primary dog thyrocyte, apart from Rap1 activation, Epac action played no apparent role in any aspect of cAMP biology, including mitogenesis (30). Although one could envision numerous scenarios for the lack of action of activated Epac on this model, we note that it has been difficult to unequivocally implicate Epac activation in any specific biological response to cAMP, mainly, contrary to PKA, due to lack of a specific Epac inhibitor. Until now, the experimental approaches have been restricted to the use of PKA- or Epac-selective analogs together with specific inhibitors of PKA. An important contribution of the present report, illustrated by the newly generated Epac inhibitor, N-Epac, is that cAMP-mediated mitogenesis depends on both Epac as well as PKA activation. In this way, the synergy between PKA and Epac in cAMP-mediated G1/S entry reflects the essential role of activated and phosphorylated Rap1b in this vital process (19, 20).

Moreover, we have demonstrated that Epac activity is strictly dependent on its proper compartmentalization mediated by its DEP domain. This was clearly shown by disrupting the DEP-dependent subcellular targeting of Epac, which abolished both cAMP-Epac-mediated Rap1 activation and TSH-stimulated cell proliferation. This argues that Epac, Rap, and PKA are ordered in a signaling complex that is critical for the mitogenic action of cAMP. The precise molecular composition of such a complex in thyroid cells is currently being investigated. Elegant experiments in cardiac myocytes revealed that Epac1 is present in a signaling complex containing minimally the phosphodies- terase PDE4D3, PKA, mAKAP, and the extracellular-regulated kinase ERK5 (31, 32). In this context, cAMP promotes a PKA-
independent activation of Rap1, which inhibits ERK5 activity and its inhibitory action on PDE, resulting in a negative feedback loop that controls cAMP levels. In this model, Epac interacts with the mAKAP complex via PDE4D3, but whether the DEP domain is involved was not addressed in those studies. As in cardiac myocytes, it is possible that the postulated Epac-binding partner involved in cAMP-mediated mitogenesis in thyroid cells may turn out to be a PDE or an AKAP-like protein. In any case, we predict that such a target shall display a stringent dependence on Epac’s DEP domain.

The DEP domain comprises a module of ~90–100 residues, originally identified in Disheveled, Egl-10, and Pleckstrin (33) and later found in other proteins involved in signal transduction, such as RGS and Epac (34). Structurally, they all share an α/β fold, with a conserved helical core and distinct β-sheets, that most likely play a role in binding specificity (27, 35). Biochemical (36) and structural studies (35) suggested a role for DEP as a membrane-anchoring device. Specifically, in Epac, cellular fractionation studies showed that the DEP domain served to localize it to the particulate fraction (13). Likewise, in contrast to N-Epac, we found that ∆DEP-N-Epac, ∆DEP-Epac, and ∆DEP-Epac-R279E, mutants devoid of dominant negative action, localize primarily to the soluble fraction and are enriched in the nuclear compartment (not shown). Thus, the absence of the DEP domain unMASKs a potential cryptic nuclear localization signal. These results raise the intriguing possibility that post-translational modifications affecting the interaction of DEP with its putative membrane target might be involved in regulating intracellular Epac localization and function. Although the identity of the elusive Epac’s DEP-binding partner is for the moment unknown, our results predict that it plays a critical role in cAMP-mediated mitogenesis, and possibly, other cellular processes regulated by cAMP.

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