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Rap1-Mediated Activation of Extracellular Signal-Regulated Kinases by Cyclic AMP Is Dependent on the Mode of Rap1 Activation

Zhiping Wang, Tara J. Dillon, Viji Pokala, Snigdha Mishra, Kirstin Labudda, Brian Hunter, and Philip J. S. Stork*

The Vollum Institute, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97239-3098

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Like other small G proteins of the Ras superfamily, Rap1 is activated by distinct guanine nucleotide exchange factors (GEFs) in response to different signals to elicit cellular responses. Activation of Rap1 by cyclic AMP (cAMP) can occur via cAMP-dependent protein kinase A (PKA)-independent and PKA-dependent mechanisms. PKA-independent activation of Rap1 by cAMP is mediated by direct binding of cAMP to Rap1-guanine nucleotide exchange factors (Rap1-GEFs) Epac1 (*e*xchange *p*rotein directly *a*ctivated by *c*AMP 1) and Epac2 (Epac1 and Epac2 are also called cAMP-GEFI and -GEFII). The availability of cAMP analogues that selectively activate Epacs, but not PKA, provides a specific tool to activate Rap1. It has been argued that the inability of these analogues to regulate extracellular signal-regulated kinases (ERKs) signaling despite activating Rap1 provides evidence that Rap1 is incapable of regulating ERKs. We confirm that the PKA-independent activation of Rap1 by Epac1 activates a perinuclear pool of Rap1 and that this does not result in ERK activation. However, we demonstrate that this inability to regulate ERKs is not a property of Rap1 but is rather a property of Epacs themselves. The addition of a membrane-targeting motif to Epac1 (Epac-CAAX) relocalizes Epac1 from its normal perinuclear locale to the plasma membrane. In this new locale it is capable of activating ERKs in a Rap1- and cAMP-dependent manner. Rap1 activation by Epac-CAAX, but not wild-type Epac, triggers its association with B-Raf. Therefore, we propose that its intracellular localization prevents Epac1 from activating ERKs. C3G (*C*rk SH*3* domain *G*uanine nucleotide exchanger) is a Rap1 exchanger that is targeted to the plasma membrane upon activation. We show that C3G can be localized to the plasma membrane by cAMP/PKA, as can Rap1 when activated by cAMP/PKA. Using a small interfering RNA approach, we demonstrate that C3G is required for the activation of ERKs and Rap1 by cAMP/PKA. This activation requires the GTP-dependent association of Rap1 with B-Raf. These data demonstrate that B-Raf is a physiological target of Rap1, but its utilization as a Rap1 effector is GEF specific. We propose a model that specific GEFs activate distinct pools of Rap1 that are differentially coupled to downstream effectors.

Monomeric small GTP binding proteins such as Ras and Rap1 govern a wide variety of cellular functions, including cell growth and differentiation, cellular adhesion, cell movement, and intracellular trafficking, by activating distinct effector pathways (21, 55). Selective activation of G proteins is dictated both by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs function to promote the conversion of small G protein from the GDP-bound, inactive form into the GTP-bound, active form. GAPs, on the other hand, enhance the intrinsic GTPase activity of small G proteins to hydrolyze the bound GTP to GDP. Over the past few years, the number of known GEFs, including GEFs triggered by growth factors, hormones, integrins, antigens, and other stimuli, has grown considerably (21, 55). GEFs can be activated either directly by tyrosine kinases or by second messengers, including calcium, diacylglycerol, and cyclic AMP (cAMP). This redundancy ensures that Ras and Rap1 can be activated by diverse signals. The possibility that this redundancy also contributes to the selection of effector pathways has not been examined directly.

Both Rap1 and Ras proteins are directed to plasma membranes (PM) and endosomal membranes via CAAX motifs at their carboxyl termini that provide sites for lipid modification (21, 28). Targeting of specific Ras isoforms to distinct microdomains within the PM has been proposed to contribute to the distinct signaling capabilities of these Ras isoforms (65). The ability of cells to compartmentalize Ras signaling to endomembranes has received recent attention (8). Activation of Ras on Golgi membranes can be achieved via the Ras-GEF, RasGRP1, and is distinct from PM-directed Ras activation via Grb2/SOS (7, 16). The subcellular localization of Rap1 is also dynamically regulated (40). Rap1 is largely localized to intracellular vesicles (66) but has been visualized on the PM as well (9, 26). Recent studies have demonstrated that Rap1 is targeted to the PM upon growth factor activation (9, 37). However, visualization of active Rap1 by use of a chimeric fluorescent resonance energy transfer probe, Raichu-Rap, has suggested that active Rap1 localizes to intracellular vesicles following growth factor activation (42). Whether the distinct localization of different pools of Ras or Rap1 dictates its choice of downstream effectors has not yet been examined.

Rap1 was first identified as an antagonist of Ras-dependent transformation of fibroblasts (34). Rap1 and Ras show roughly 50% amino acid homology and can share a number of binding partners, including Ral-GDS, phosphoinositol-3 kinase, B-Raf, and Raf-1 (60). Whether these represent bona fide effectors of Rap1 depends on the partner examined. For example, the MAP kinase kinase kinase Raf-1 is expressed in all cells and

* Corresponding author. Mailing address: The Vollum Institute, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239-3098. Phone: (503) 494-5494. Fax: (503) 494-4976. E-mail: stork@ohsu.edu.

binds to GTP-loaded Rap1 but is not activated by this interaction. In contrast, B-Raf, another member of the Raf family, can be activated by both GTP-loaded Ras and Rap1 (48). Rap1 activation of ERKs has been reported for a number of B-Raf-expressing cells, including neurons and neuroendrocrine cells (60) such as PC12 cells, for which ERK activation can be triggered by multiple stimuli, including growth factors such as NGF (32, 47, 67) and hormones that are coupled to increased cAMP (61). For both NGF and cAMP, it is thought that Rap1 activation is mediated by the Rap1-GEF C3G (*C*rk SH*3* domain *G*uanine nucleotide exchanger) (32, 58, 67).

C3G was the first Rap1-GEF identified (27). C3G association with the family of Crk adaptor molecules allows C3G to be recruited via the SH2 domain of Crk to the PM where C3G is phosphorylated and activated (23, 49). In this manner, the recruitment of C3G/Crk to the PM enables receptor tyrosine kinases to activate Rap1 (29, 32, 66). C3G/Crk may also couple G-protein coupled receptors to Rap1 (52), including those that couple to cAMP-dependent protein kinase A (PKA) (58).

Recently, members of a family of membrane-associated Rap1-GEFs have been identified that are activated by the direct binding of cAMP. These Rap1-GEFs are called cAMP-GEFs, or Epacs (*ex*change *p*roteins directly *a*ctivated by *c*AMP), and are highly expressed in ovary, thyroid, kidney, adrenal, and brain cells (19, 33). Two Epacs have been identified. Epac1 contains a single cyclic nucleotide-binding domain and is widely expressed, whereas Epac2 contains two cyclic nucleotide-binding domains and is highly expressed in selected neuroendocrine and neuronal cells. The role of Epacs in Rap1 activation has been supported by studies using 8-CPT-2Me-cAMP, a cAMP analogue that retains a high affinity for Epacs but does not activate PKA (17). When Rap1 was activated by 8-CPT-2Me-cAMP, ERK activation was not observed (22), indicating that Epacs are unable to couple Rap1 to ERKs. 8-CPT-2Me-cAMP provides a unique tool to activate Rap1 selectively without affecting parallel signaling cascades. Because of this, the inability of 8-CPT-2Me-cAMP to regulate ERKs has been interpreted as evidence that Rap1 and ERKs are independently regulated (22).

The existence of two Rap1-GEFs (C3G and Epac) that are both targets of cAMP regulation raises an important biological question. Do GEFs function redundantly to augment the activation of Rap1 or do GEFs also contribute to the specificity of the effector pathways targeted by Rap1? In the present study, we examined Rap1 signaling in two neuroendocrine lines, PC12 cells and AtT20 cells. We demonstrated that the ability of activated Rap1 to activate ERK depends in part on which Rap1-GEF is utilized.

Both C3G and Epac1 activated Rap1 in PC12 cells and AtT20 cells, but only Rap1 that had been activated by C3G could activate ERKs. The coupling of C3G to Rap1 and ERKs was demonstrated using a novel interfering mutant as well as small interfering RNAs (siRNAs) with C3G. The inability of Epacs to activate ERKs appeared to be a consequence of its intracellular localization. Epac1 shows a distinct perinuclear expression pattern, whereas C3G is cytoplasmic in resting cells and recruited to the PM upon activation (54, 56). These distinct locales of C3G and Epac1 raise the possibility that each GEF activates a separate pool of Rap1 and that these pools may differentially couple to the ERK cascade. To address the hypothesis that the locale of Epac1 does not allow for Rap1-dependent ERK activation, we used the PM-targeting region of Ki-Ras, including the C-terminal CAAX motif, to relocalize Epac1 to the PM. Upon relocalization to the PM, Epac1 was able to couple endogenous Rap1 to ERKs. These studies suggest that GEFs not only function to activate Rap1 but also contribute to the choice of the downstream effector pathways.

MATERIALS AND METHODS

Reagents. Antibodies specific to phosphorylated ERK (pERK) that recognize phosphorylated ERK1 (pERK1) and ERK2 (pERK2) at residues threonine 202 and tyrosine 204, and antibodies specific to phosphorylated MEK (pMEK) that recognize phosphorylated MEK1 and MEK2 at residues serine 217 and 221, were purchased from New England Biolabs (Beverly, MA). Antibodies to Rap1, c-Myc (9E10), B-Raf (H145), C3G (C19), and Epac2 (A-7) and agarose-conjugated antibodies to Myc were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies to nuclear pore complex proteins (Mab414) were purchased from Covance (Princeton, NJ). Anti-Ras and anti-Epac1 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Flag (M2) antibody and agarose-coupled antibodies to the Flag epitope were purchased from Sigma-Aldrich (St. Louis, MO).

Forskolin, 3-isobutyl-1-methylxanthine (IBMX), *N*-(2-(*p*-bromocinnamylamino)-ethyl)-5-isoquinolinesulfonamide (H89), and myristylated PKI (myr-PKI) were purchased from Calbiochem (Riverside, CA). Fibroblast growth factor was purchased from Sigma-Aldrich (St. Louis, MO). 8-(4-chloro-phenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8-CPT-2Me-cAMP, also referred to in the text and figures as CPT-OMe) was purchased from Biolog Life Sciences (Bremen, Germany). PolyScreen polyvinylidene difluoride membrane and Chemiluminescence reagent were purchased from PerkinElmer Life Sciences (Boston, MA). Protran nitrocellulose membranes were from Schleicher & Schuell Bioscience (Keene, NH).

Cell culture conditions and treatments. AtT20 and COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% heat-inactivated fetal calf serum, penicillin-streptomycin, and L-glutamine at 37°C and 5% CO_2. PC12 cells were cultured in DMEM with 10% heat-inactivated horse serum and 5% heat-inactivated fetal calf serum plus penicillin-streptomycin and L-glutamine at 37°C and 5% CO_2. To generate stable cell populations, PC12 cells were transfected with Flag-Epac wild type (WT), Flag-Epac-CAAX, or Flag-Epac-SAAX and selected using 0.5 mg/ml G418 for 4 to 6 weeks in serum-containing medium, which was supplemented with PC12-conditioned medium during the first 3 weeks. Cells were maintained in serum-free DMEM for 16 h at 37°C and 5% CO_2 prior to treatments. Cells were treated with 10 μM Forskolin and 100 μM IBMX for 20 min unless otherwise indicated. myr-PKI was used at 5 μM and was added 20 min prior to Forskolin treatment. H89 was used at 10 μM unless otherwise indicated and was added 20 min prior to treatment. 8-CPT-OMe was added at 50 μM for 20 min, unless otherwise indicated. Acidic fibroblast growth factor was used at 50 ng/ml in the presence of 5 U/ml heparin for 20 min. Pervandate was used at 100 μM for 20 min.

Western blotting and immunoprecipitation. Cell lysates and Western blotting were prepared as described previously (63). Briefly, protein concentrations were quantified using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). For Western blotting, equivalent amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto either a polyvinylidene difluoride membrane for detection using a chemiluminescence reagent or Protran nitrocellulose membranes for detection using an Odyssey infrared imaging system (LI-COR, Inc.), and probed with antibodies according to the manufacturer's instructions. For coimmunoprecipitations, cells were lysed in radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 0.2% sodium dodecyl sulfate, 125 mM NaCl, 50 mM Tris [pH 8.0], 10% glycerol, 1 mM EDTA, 25 mM β-glycerophosphate, 25 mM NaF, 1 mM $NaVO_4$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). The lysates were subjected to 1 min of centrifugation at 4°C and 16,000 × *g*, followed by sonication for 1 min per condition. The lysates were spun again for 3.5 min at 4°C and 16,000 × *g*, and equal amounts of cell lysates (500 to 1,000 μg) were precipitated with agarose-coupled anti-Flag antibody for 4 h followed by two washes in radioimmunoprecipitation assay buffer. B-Raf associated with Flag-tagged Rap1 WT or Flag-tagged RapV12 was detected using anti-B-Raf antibody (H145). For detection of Flag-ERK2 (or Myc-ERK2), equal amounts of cell lysates per condition were immunoprecipitated with agarose-conjugated anti-Flag antibody (or agarose-coupled anti-Myc antibody) for 4 h at 4°C in lysis

buffer containing 50 mM Tris-Cl (pH 8.0), 10% glycerol, 1% Nonidet P-40, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 mM NaF, 0.1 μM aprotinin, and 1 mM sodium orthovanadate (Na_2VO_4). Anti-phospho-ERK1/2 was used to detect activated Flag- or Myc-ERK2. The results illustrated are from representative experiments repeated at least three times.

Cell fractionation. Cells were grown on 10 cm^2 dishes to 90 to 100% confluence and serum starved for 18 h. 8-CPT-2Me-cAMP and Forskolin were added to the cells 20 min before lysis. H89 was added 20 min before adding the Forskolin for a total of 40 min of treatment. Cells were then placed on ice, the media was aspirated, and cells were scraped in ice-cold hypotonic lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml vanadate, 10 μg/ml trypsin inhibitor, 1 mM β-glycerolphosphate, and 10 mM Tris-HCl, pH 7.4). Cells were homogenized with 40 strokes in a Dounce-type homogenizer. Lysates were then spun at 800 × *g* for 5 min at 4°C to pellet the nuclear fraction (P1) and to isolate the supernatant (S1). The nuclear fraction was washed in hypotonic lysis buffer, resuspended in lysis buffer, and rocked at 4°C for 2 h to elute the perinuclear-nuclear proteins. The suspension was spun at 1,000 × *g* for 5 min at 4°C, the pellet containing noneluted nuclear material was discarded, and the supernatant (S2) was retained as perinuclear-nuclear proteins and assayed for protein content. The original supernatant (S1) was transferred to a new tube and spun at 20,000 × *g* for 30 min at 4°C to pellet the membrane fraction (P2), which was resuspended in lysis buffer. The remaining (S3) supernatant represented the cytosolic fraction, which was adjusted with 1× lysis buffer. P2 and S3 fractions were then rocked at 4°C for 2 h and then spun at 1,000 × *g* for 5 min at 4°C, and the supernatants from both fractions were assayed for protein content.

Plasmids and transfections. Epac-CAAX mutants were created as follows: the C-terminal 20 amino acids of Ki-Ras containing the Ki-CAAX motif (amino acids 169 to 188 of Ki-Ras) were subcloned into pcDNA3 to generate pcDNA3-CAAX; then, human Epac1 cDNA minus the stop codon was subcloned upstream of Ki-CAAX so that the 3′ end of Epac1 cDNA was fused to the Ki-Ras-CAAX motif to generate Epac1-CAAX. Finally, a Kosak sequence and an amino-terminal Myc epitope tag were then introduced into the 5′ end of Epac1-CAAX to generate Myc-Epac1-CAAX (Epac-CAAX). Epac-SAAX was constructed by PCR mutagenesis using Myc-EPAC-CAAX as a template and subcloned downstream of the Myc tag of Myc-Epac-CAAX to create Myc-Epac1-SAAX. Each of the Flag-Epac and green fluorescent protein (GFP)-Epac constructs utilized was created by in-frame replacement of the Myc tag with the Flag epitope or GFP cDNA, respectively, in the corresponding plasmids.

Myc-Epac-Ha-CAAX and Myc-Epac-Rap1b-CAAX were made from Myc-Epac-Ki-CAAX by replacing the Ki-Ras-CAAX from Myc-Epac-Ki-CAAX sequences with double-stranded oligonucleotides corresponding to the carboxy-terminal 24 amino acids of Ha-Ras (Ha-CAAX) and the carboxy-terminal 27 amino acids of Rap1b, respectively.

Rap1-AGE represents a triple-mutation Rap1: WT amino acid serine at position 17 for alanine (A), WT valine at position 29 for glycine (G), and WT lysine at position 117 for glutamic acid (E). Flag-Rap1-AGE was constructed by PCR mutagenesis, using Flag-Rap1b as a template, to introduce these mutations. The final PCR product was subcloned by use of EcoRI and XbaI restriction sites into vector pcDNA3 downstream of a Flag epitope to provide a carboxy-terminal Flag in frame with respect to the start methionine of Rap1b. All constructs were sequenced completely, confirming the predicted amino acids and frame transitions.

For transient transfection, 70 to 80% confluent AtT-20 or PC12 cells were cotransfected with the indicated cDNAs by use of Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The control vector, pcDNA3 (Invitrogen Corp.), was included in each set of transfections to assure that each plate received the same amount of transfected DNA. Following transfection, cells were allowed to recover in serum-containing media for 24 h.

Affinity assay for Rap1 and Ras activation. Active Rap1 was assayed using GST-Ral-GDS in an in vitro pulldown assay as previously described (24). Active Ras was assayed using a Ras activation assay kit and GST-Raf1-RBD in an in vitro pull down assay (Upstate Biotechnology) performed according to reference 24 and the manufacturer's instructions.

siRNA. Double-stranded RNA oligoribonucleotide GGACUUUGAUGU UGAAUGUTT (5′-3′), which corresponds to the positions 1250 to 1268 of mouse C3G (GenBank accession no. NM-054050), was purchased from Ambion Inc. (Austin, TX). Double-stranded RNA oligoribonucleotide NNGCGCGCU UUGUAGGAUUCA (5′-3′) was used as a control siRNA. PC12 cells were seeded on 100 mm plates and allowed to adhere overnight. At the time of transfection, PC12 cells were 50 to 60% confluent and the RNA duplexes were transfected into PC12 cells at a final concentration of 200 nM by use of Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Cells were kept for 72 h before Forskolin-IBMX treatment.

Fluorescence microscopy. PC12 cells were grown on poly-D-lysine (Sigma-Aldrich)-treated coverslips and transfected with GFP-Epac-WT, GFP-Epac-CAAX, and GFP-Epac-SAAX by use of Lipofectamine 2000 (Invitrogen Corp.) according to the manufacturer's instructions. Cells were fixed with 4% formaldehyde (Fisher Scientific, Hampton, NH) 24 h after transfection, permeabilized with 0.1% Triton (Sigma-Aldrich), and stained with Hoechst 33258 (Sigma-Aldrich). Slides were mounted using Elvanol. The location of the nuclear envelope was visualized by incubating permeabilized cells with Mab414 overnight at 4°C in 1% goat serum (Sigma-Aldrich) prior to Hoechst staining. Texas Red anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, CA) was used as a secondary reagent and applied for 1 h at room temperature. Images (60× lens objective) were acquired with an Applied Precision Deltavision image restoration system. Deconvolution using the iterative constrained algorithm of Sedat and Agard and additional image processing was performed on an SGI Octane workstation.

For detection of GFP-C3G, COS-1 cells were maintained in 5% CO_2 at 37°C in DMEM containing 10% fetal bovine serum and antibiotics. Cells to be observed by fluorescence microscopy were plated in 12-well plates (NuncTM) containing 18-mm glass coverslips (VWR Scientific). The glass coverslips were coated with poly-D-lysine (Sigma-Aldrich). The cells were grown overnight and then transfected using Lipofectamine (Invitogen) with GFP-C3G and CrkL. On the following day the cells were fixed with 4% formaldehyde (Fisher Scientific) and permeabilized with 0.1% Triton X (Sigma-Aldrich). Hoechst stain was used for the nucleus. The images were obtained using a Zeiss Axioplan 2 microscope with an enhanced green fluorescent protein filter. The TIFF images were acquired with Open Lab software and processed using Adobe Photoshop. All photomicrographs show representative cells from at least three independent experiments.

For detection of GFP-Ral-GDS, COS-1 cells were maintained in 5% CO_2 at 37°C in DMEM containing 10% fetal bovine serum and antibiotics. Cells to be observed by fluorescence microscopy were plated in Delta T culture dishes (Bioptech) with 0.17-mm thickness glass. The cells were grown overnight and then transfected using Lipofectamine (Invitrogen) with GFP-Ral-GDS cotransfected with Flag-Rap1 or Flag-RapV12. The cells were serum starved on the following day before stimulation with Forskolin (EMD Bioscience). Live cells were then examined using confocal microscopy. Images were acquired with the Applied Precision Deltavision image restoration system. Deconvolution using the iterative constrained algorithm of Sedat and Agard and additional image processing were performed on an SGI Octane workstation. The final TIFF images were processed with Adobe Photoshop. All photomicrographs show representative cells from at least three independent experiments.

RESULTS

PKA-dependent and -independent activation of Rap1 by cAMP. We examined the ability of cAMP to activate Rap1 in two cell types, mouse pituitary tumor cell line AtT20 and rat pheochromocytoma cell line PC12. PC12 cells have been widely used to examine NGF and cAMP signaling with respect to ERKs, for which a role of Rap1 has been proposed (5, 32, 47, 67). Rap1 activation by cAMP has not been directly studied in AtT20 cells; however, Rap1-GEF Epac2 has been implicated in cAMP-dependent exocytosis in these cells (50).

Both cell lines demonstrated Rap1 activation in response to Forskolin, a strong activator of adenylate cyclase. For these experiments, Forskolin was applied in the presence of the phosphodiesterase inhibitor IBMX (a set of conditions designated F/I). In PC12 cells, activation of Rap1 by F/I was blocked by the PKA inhibitor H89 at doses that are selective for PKA, indicating that Rap1 activation was PKA dependent (Fig. 1A). This was confirmed using myr-PKI, a selective peptide inhibitor of PKA (15) (Fig. 1A). In contrast, in AtT20 cells, the activation of Rap1 by F/I was not blocked by H89 or myr-PKI (Fig. 1B), indicating that Rap1 activation by cAMP in AtT20 cells was independent of the presence of

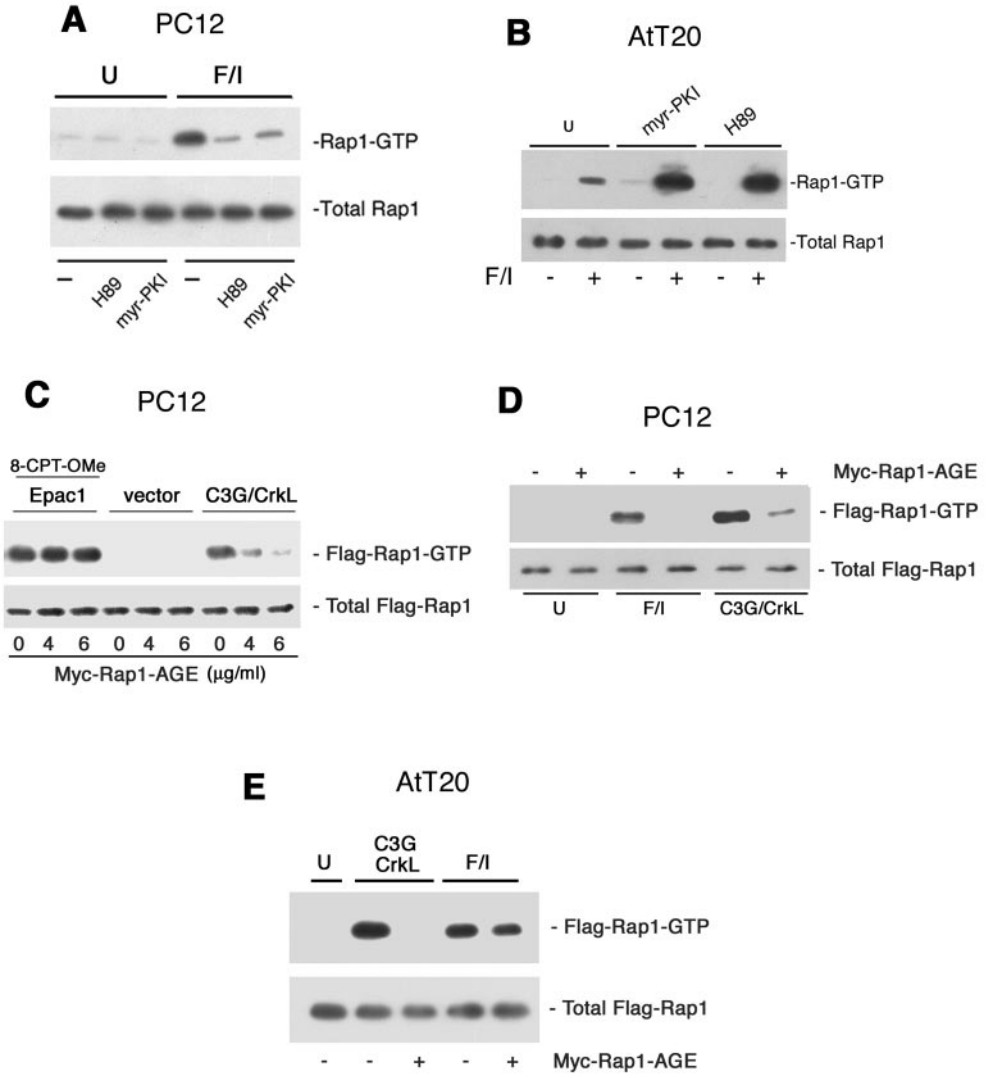

FIG. 1. Activation of Rap1 in a PKA-dependent and PKA-independent manner and its role in ERK activation. (A) PC12 cells were left untreated (U) or were treated with F/I and were pretreated with H89 (1 μM) or myr-PKI or left without pretreatment (−). Cell lysates were examined for activated Rap1 (Rap1-GTP) by GST-Ral-GDS assay as described previously. Total Rap1 protein levels are shown in the bottom panel. (B) AtT20 cells were left untreated (−) or treated with F/I (+) and were pretreated with H89 (1 μM) or myr-PKI or left without pretreatment (U). Cell lysates were examined for activated Flag-Rap1 (Flag-Rap1-GTP). Total Flag-Rap1 in cell extracts is indicated as a loading control. (C) PC12 cells were transfected with Flag-Rap1 and either Epac1, vector, or C3G/CrkL along with Myc-Rap1-AGE at 4 μg/ml (4) or 6 μg/ml (6) or no Myc-Rap1-AGE (0). Epac-transfected cells were stimulated with 8-CPT-2Me-cAMP (8-CPT-OMe). Cell lysates were examined for activated Flag-Rap1 (Flag-Rap1-GTP). Total Flag-Rap1 in cell extracts is indicated as a loading control. (D) PC12 cells were transfected with Flag-Rap1 with (+) or without (−) Myc-Rap1-AGE (6 μg/ml) and were left untreated (U) or stimulated with F/I, as indicated. In addition, cells were cotransfected with C3G and CrkL (C3G/CrkL) with (+) or without (−) Myc-Rap1-AGE. Cell lysates were examined for activated Flag-Rap1 (Flag-Rap1-GTP). Total Flag-Rap1 in cell extracts is indicated as a loading control. (E) AtT20 cells were transfected with Flag-Rap1, with (+) or without (−) Myc-Rap1-AGE, and were left untreated (U) or stimulated with F/I, as indicated. In addition, cells were cotransfected with C3G and CrkL (C3G/CrkL) with (+) or without (−) Myc-Rap1-AGE. Cell lysates were examined for activated Flag-Rap1 (Flag-Rap1-GTP). Total Flag-Rap1 in cell extracts is indicated as a loading control.

PKA. Interestingly, both H89 and myr-PKI enhanced Rap1 activation by cAMP in AtT20 cells, suggesting that PKA was limiting Rap1 activation by cAMP in those cells. This result does not necessarily rule out the presence of a PKA-dependent component of Rap1 activation by cAMP in AtT20 cells. Indeed, it is possible that the large increase in Rap1 activation seen in the presence of H89 might obscure the detection of an H89-sensitive component of Rap1 activation. Taken together, these data indicate that the mode of Rap1 activation by cAMP is cell type specific.

PKA-dependent activation of Rap1 and ERKs is mediated by Rap1-GEF C3G in PC12 cells. It has been suggested that cAMP can activate Rap1 via one of two mechanisms. PKA-independent activation of Rap1 by cAMP is thought to proceed via the direct activation of members of a family of cAMP-responsive Rap1-GEFs called Epacs, or cAMP-GEFs

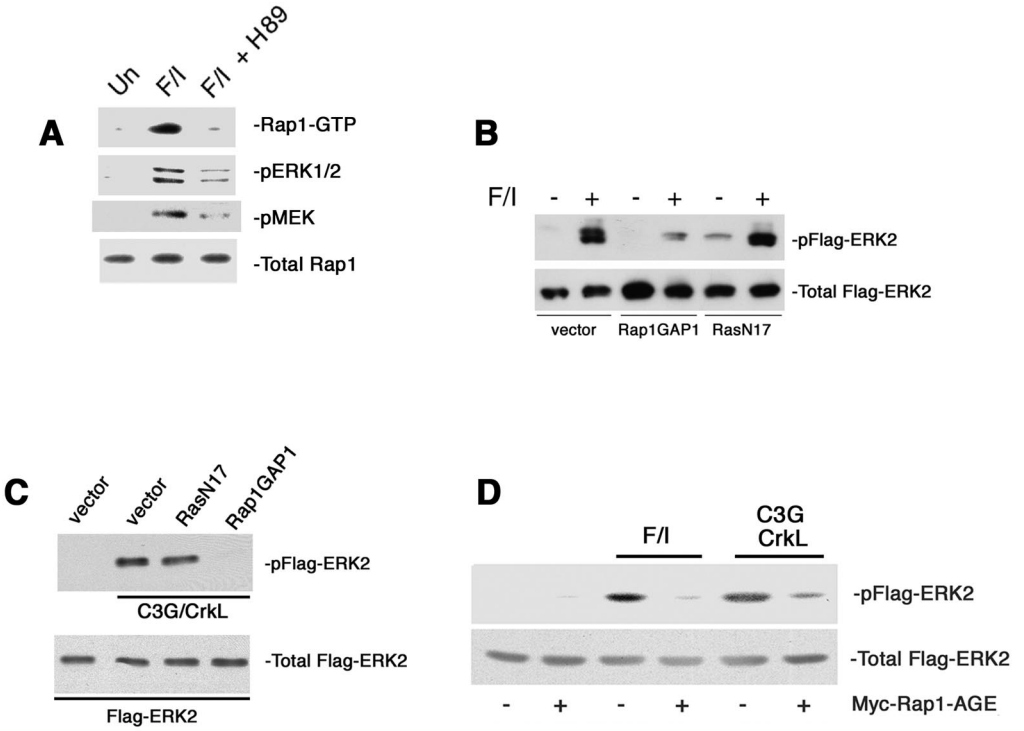

FIG. 2. Activation of PKA, Rap1, and C3G is required for the phosphorylation of ERK by Forskolin in PC12 cells. (A) Cells were left untreated (Un), treated with F/I, or pretreated with H89 prior to F/I treatment (F/I + H89). Cell lysates were examined for activated Rap1 (Rap1-GTP). Phosphorylation of ERK1/2 was visualized by Western blotting utilizing a phospho-ERK antibody (pERK1/2). Phosphorylation of MEK was visualized by Western blotting utilizing a phospho-MEK antibody (pMEK). Total Rap1 protein levels are shown in the bottom panel. (B) PC12 cells were transfected with Flag-ERK2 and vector, Rap1GAP1, or RasN17 as indicated and left untreated (−) or treated with F/I (+). Flag-ERK2 was immunoprecipitated from cell lysates and analyzed by immunoblotting with phospho-ERK antibodies (pFlag-ERK2). The levels of total Flag-ERK2 are indicated as a loading control. (C) Rap1 is required for the phosphorylation of ERK by C3G/CrkL in PC12 cells. PC12 cells were transfected with Flag-ERK2 and vector plus C3G/CrkL, Rap1GAP1 plus C3G/CrkL, or RasN17 plus C3G/CrkL, as indicated. Flag-ERK2 was immunoprecipitated from cell lysates and analyzed by immunoblotting with phospho-ERK antibodies (pFlag-ERK2). The levels of total Flag-ERK2 are indicated as a loading control. (D) PC12 cells were transfected with Flag-ERK2, with (+) or without (−) Myc-Rap1-AGE, and left untreated or stimulated with F/I, as indicated. In addition, cells were cotransfected with C3G and CrkL (C3G/CrkL) with (+) or without (−) Myc-Rap1-AGE. Flag-ERK2 was immunoprecipitated from cell lysates and analyzed by immunoblotting with phospho-ERK antibodies (pFlag-ERK2). The levels of total Flag-ERK2 are indicated as a loading control.

(19, 33). In contrast, PKA-dependent activation of Rap1 has been less well appreciated (22), although a role for C3G has been proposed (58).

To assess the involvement of C3G in PKA-dependent Rap1 activation in PC12 cells, we utilized newly developed mutants that interfere with C3G function. The Rap1 mutant Rap1-AGE was recently identified in a screening for mutants that bound tightly and preferentially to C3G and selectively inhibit C3G-mediated Rap1 activation (59). Initial experiments confirmed that Myc-Rap1-AGE inhibited Rap1 activation by C3G but not by Epac1 (Fig. 1C). For these experiments C3G was cotransfected with CrkL (C3G/CrkL), which induces the membrane association of C3G and the selective activation of Rap1 (31). Expression of Myc-Rap1-AGE in PC12 cells also blocked Rap1 activation by F/I (Fig. 1D). In contrast, in AtT20 cells, Rap1 activation by F/I was only modestly reduced by Rap1-AGE (Fig. 1E). Taken together, these results suggest that C3G mediates all Rap1 activation by cAMP in PC12 cells but only a small component of cAMP's activation of Rap1 in AtT20 cells. We predict that this component is H89 sensitive but cannot show this directly in whole-cell lysates, owing to the presence of a larger component of F/I's activation of Rap1 that is enhanced by H89 pretreatment, as shown in Fig. 1B.

Whether Rap1 can mediate cAMP-dependent activation of ERKs has remained controversial (22, 60, 63). In PC12 cells, activation of Rap1, MEK, and ERKs was H89 sensitive (Fig. 2A). In these cells, activation of ERK1/2 by F/I was blocked by Rap1GAP1, a selective GTPase-activating protein for Rap1 (11), but not by RasN17, an interfering mutant of Ras (Fig. 2B), demonstrating a requirement for Rap1. Transfection of C3G/CrkL also resulted in the robust activation of ERKs in PC12 cells. This activation was also blocked by Rap1GAP1 but not by RasN17 (Fig. 2C). Taken together, these data indicate that activation of ERKs by both F/I and C3G/CrkL requires Rap1 in these cells.

To test the possibility that C3G mediated activation of ERKs by cAMP in PC12 cells, we employed two approaches: selective interference with C3G via Rap1-AGE and the use of siRNA. The activation of ERKs by F/I was blocked by the expression of Myc-Rap1-AGE (Fig. 2D). Myc-Rap1-AGE also blocked the activation of ERKs by C3G/CrkL (Fig. 2D). A definitive role for C3G in the activation of Rap1 and ERKs by cAMP in these

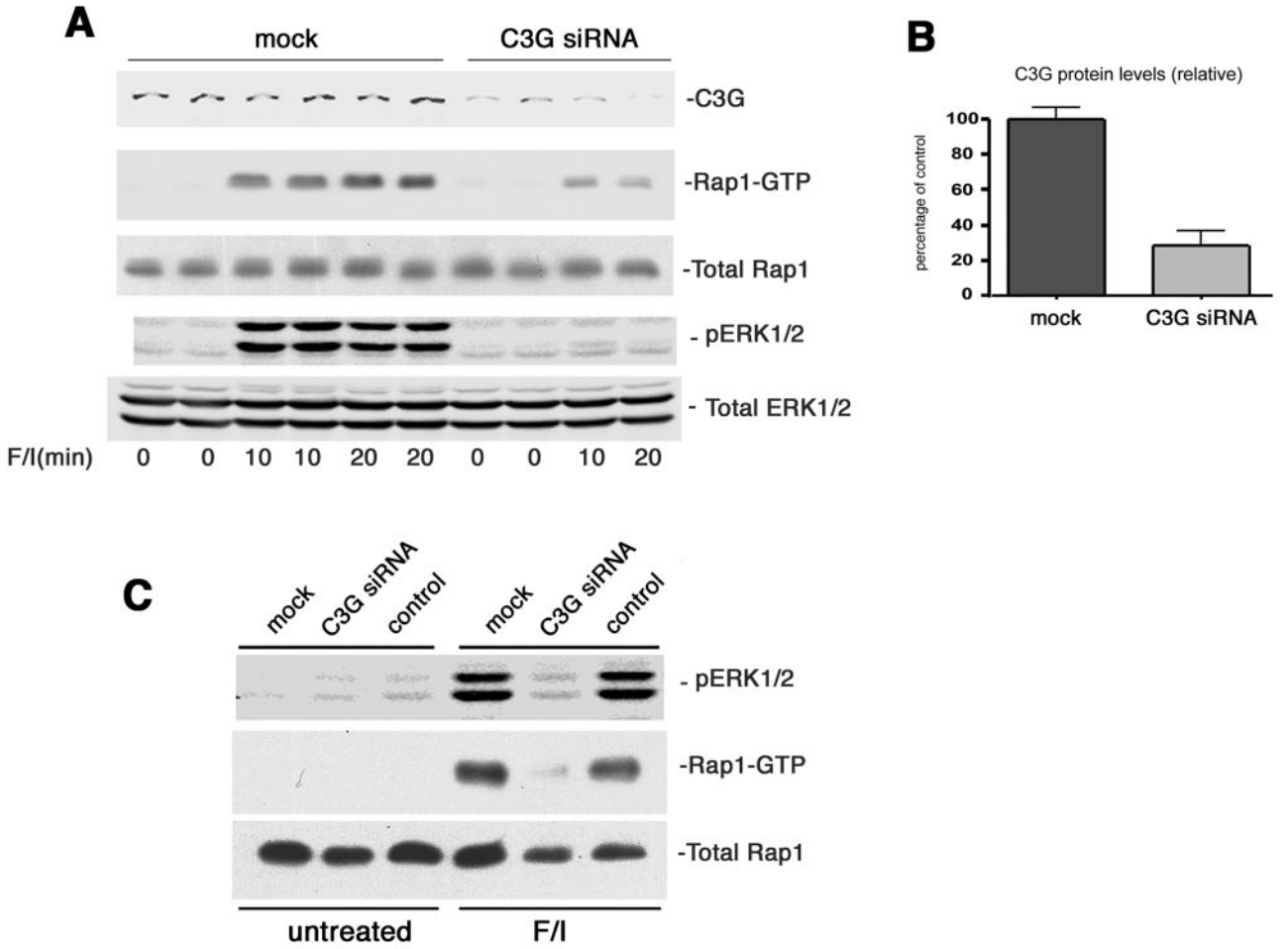

FIG. 3. Transfection of C3G siRNA blocks activation of Rap1 and ERKs by Forskolin in PC12 cells. (A) PC12 cells were transfected with C3G siRNA or mock transfected (mock) for 72 h and stimulated with F/I for 0, 10, or 20 min, as indicated. The expression of C3G was analyzed by immunoblotting with anti-C3G antibodies (C3G). Active Rap1 within cell lysates is shown (Rap1-GTP). Total Rap1 in cell extracts is indicated as a loading control. Activation of ERKs was analyzed by immunoblotting with phospho-ERK specific antibodies (pERK1/2). Total ERK1/2 from cell lysates was used as a loading control. (B) Quantitation of the efficacy of siRNA knockdown is presented as percentage of control ($n = 3$ [mean ± standard error of the mean]). (C) PC12 cells were transfected with C3G siRNA or control siRNA for 72 h or left untransfected (mock) and were stimulated with F/I or left untreated, as indicated. Activation of ERKs was analyzed by immunoblotting with phospho-ERK-specific antibodies (pERK1/2). Active Rap1 within cell lysates is shown (Rap1-GTP). Total Rap1 in cell extracts is indicated as a loading control.

cells was provided by siRNA. Transfection of PC12 cells with siRNAs designed for C3G resulted in a roughly 70% reduction in C3G expression compared to control results (Fig. 3A and B). In these cells, the abilities of F/I to activate both Rap1 as well as ERK were significantly reduced compared to the control results (Fig. 3A). In parallel experiments, unrelated double-stranded RNA oligonucleotides served as a negative control (Fig. 3C). Taken together, these results establish that C3G is required for cAMP/PKA-dependent activation of Rap1 and ERKs in PC12 cells.

C3G/CrkL is recruited and activated at the PM following growth factor stimulation (31, 56). To examine the distribution of C3G following treatment with F/I, we transfected GFP-C3G (along with CrkL) into COS cells. C3G was largely cytoplasmic and perinuclear in untreated cells (Fig. 4A, left panel) but was also detectable at the PM upon stimulation with F/I (Fig. 4A, middle panel). This membrane localization by F/I was blocked by pretreatment with H89 (Fig. 4A, right panel). Similar results were seen in PC12 cells (Fig. 4B).

Direct visualization of the activated state of Rap1-GTP can be achieved using GFP-Ral-GDS, which, like Gst-Ral-GDS, binds Rap1 in a GTP-dependent manner (9). For these studies COS cells were used and Rap1 was cotransfected with Rap1 and GFP-Ral-GDS. These cells do not express Epacs (54). Untreated cells show fluorescent staining throughout the cytoplasm and perinuclear region due to the localization of the reporter in the absence of binding to Rap1 (Fig. 4C, left panel). Upon cAMP stimulation, a fluorescent signal could be detected at the PM (Fig. 4C, middle panel). Cells transfected with RapV12 and GFP-Ral-GDS showed fluorescent patches at the PM without treatment (Fig. 4C, right panel), indicating that F/I promotes C3G and Rap1-GTP accumulation at the PM.

Epac-mediated activation of Rap1 in AtT20 cells. The ability of cAMP to activate Rap1 in AtT20 cells in the presence of

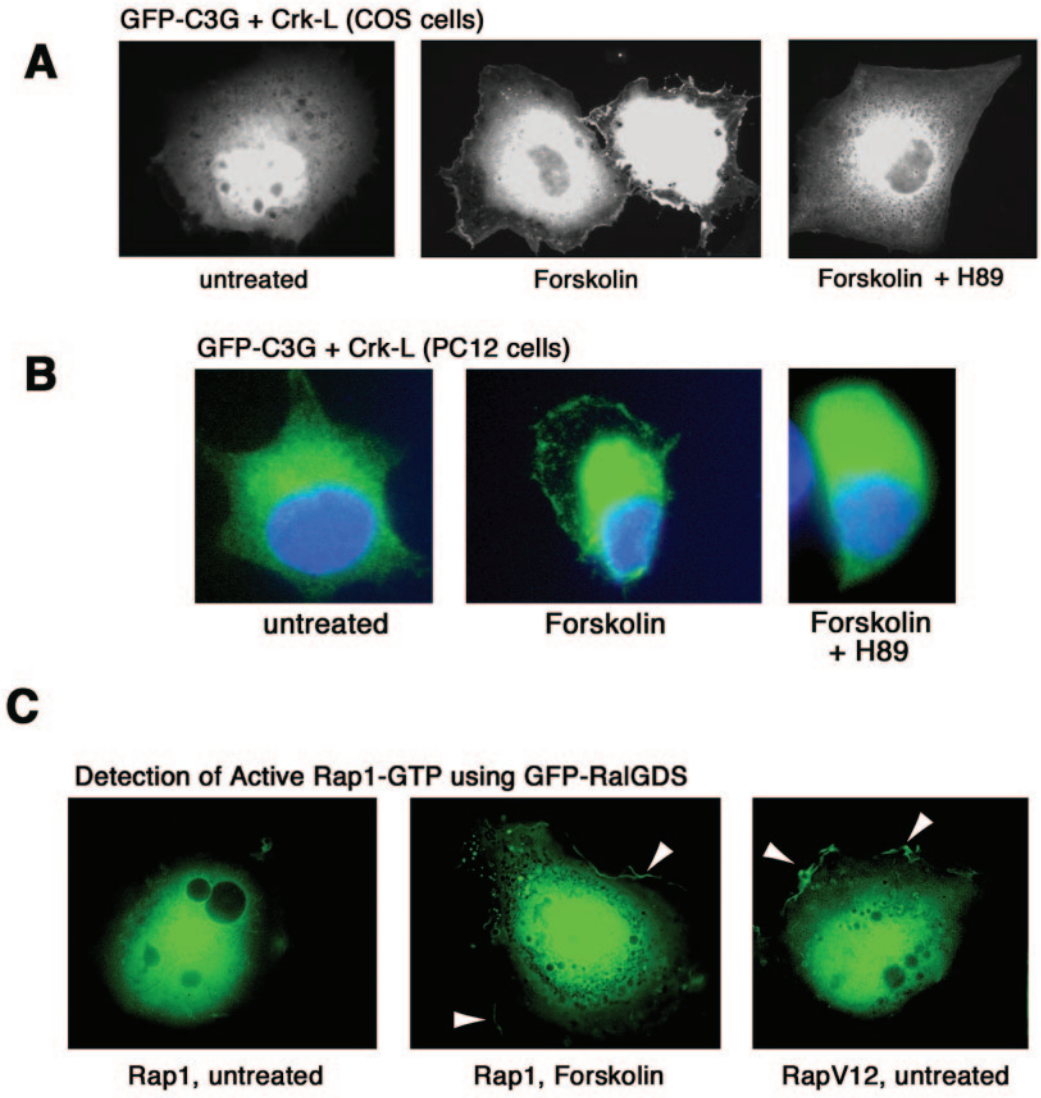

FIG. 4. Localization of C3G and activation of Rap1 at the PM by Forskolin. (A) C3G localization at the PM is triggered by PKA in COS cells. COS cells were transfected with GFP-C3G with CrkL and were left untreated or were treated with Forskolin or Forskolin plus H89, as indicated. (B) C3G localization at the PM is triggered by PKA in PC12 cells. PC12 cells were transfected with GFP-C3G with CrkL and were left untreated or were treated with Forskolin or Forskolin plus H89. (C) Activated Rap1-GTP can be detected at the PM upon cAMP treatment. COS cells were transfected with GFP-Ral-GDS and Rap1 or RapV12 and were left untreated or treated with Forskolin, as indicated. Arrows identify membrane patches that represent areas of GFP-Ral-GDS binding to Rap1-GTP (middle panel) and RapV12-GTP (right panel).

PKA inhibitors (Fig. 1B) suggests that Epacs may be the target of cAMP activation of Rap1 in these cells. Therefore, we compared the expression of Epac1 to that of Epac2 proteins in both PC12 and AtT20 cells. Both Epac1 and Epac2 were highly expressed in AtT20 cells, whereas PC12 cells displayed very low levels of Epac1 and undetectable levels of Epac2 (Fig. 5A). Both cells expressed C3G (Fig. 5B).

Specific activation of Epacs can be achieved with the cAMP analog 8-CPT-2Me-cAMP (17, 22). 8-CPT-2Me-cAMP activated Rap1 in AtT20 cells to levels similar to that achieved with F/I plus H89 (Fig. 5C), consistent with the expression of endogenous Epac within AtT20 cells. This demonstrates that endogenous Epacs are capable of activating endogenous Rap1 in AtT20 cells and that 8-CPT-2Me-cAMP provides a useful tool to activate Epacs independently of any potential negative effects of PKA.

Because AtT20 cells express both C3G and Epac, it is possible that both can participate in cAMP's activation of Rap1. This possibility is supported by the ability of the C3G-selective mutant Rap-AGE to partially block F/I's activation of Rap1 in these cells (Fig. 1E). We propose that distinct subcellular pools of Rap1 are activated by cAMP that can be distinguished by their PKA dependence. To address this biochemically, we undertook a rapid cell fractionation procedure to minimize loss of GTP binding to Rap1 during the procedure. This permitted the crude fractionation of AtT20 cells in membrane, cytosolic, and nuclear fractions. Rap1 was detected in both perinuclear

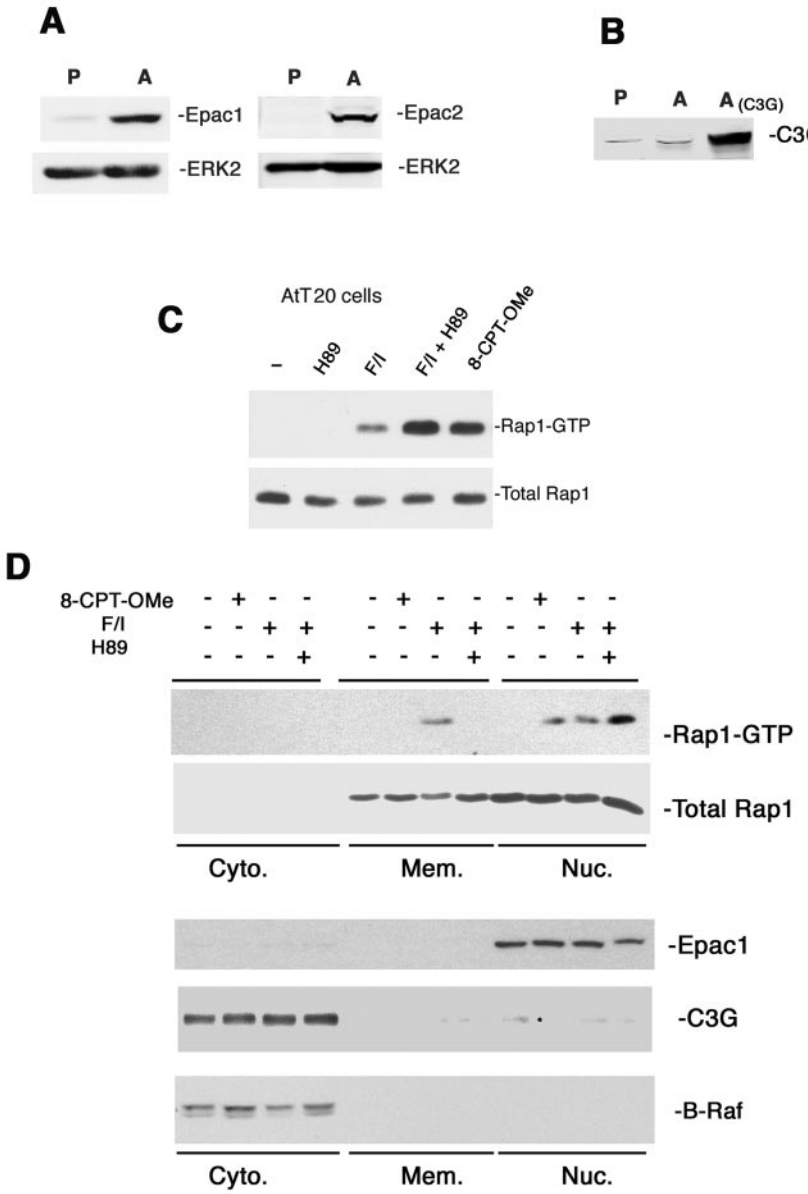

FIG. 5. Epac and C3G localize to distinct cellular compartments and correlate with distinct pools of Rap1 activation. (A) Epac proteins are present in AtT20 cells. Protein levels for Epac1 and Epac2 were examined in PC12 (P) and AtT20 (A) cell lysates by use of antibodies to Epac1 (upper left panel) and Epac2 (upper right panel). The levels of total ERK2 are indicated as a loading control. (B) C3G expression in PC12 cells and AtT20 cells. Endogenous C3G expression levels for PC12 cells (P) and AtT20 cells (A) were examined using antibodies to C3G. AtT20 cells that were transfected with C3G were used as a positive control. (C) 8-CPT-2Me-cAMP (8-CPT-OMe) activates Rap1 in AtT20 cells. AtT20 cells were left untreated (−) or treated with H89, F/I, F/I plus H89, or 8-CPT-2Me-cAMP (8-CPT-OMe), as indicated. Cell lysates were examined for activated Rap1 (Rap1-GTP). Total Rap1 within cell extracts is indicated as a loading control. (D) Rap activity and regulation within different cellular fractions. AtT20 cells were stimulated with either 8-CPT-2Me-cAMP (8-CPT-OMe) or F/I, with or without pretreatment with H89, as indicated. Cells were lysed, and cytosolic (Cyto.), membrane (Mem.), and perinuclear (Nuc.) fractions were isolated as described in Materials and Methods. Subcellular fractions (derived from equivalent numbers of cells) were assayed for activation of Rap1 (Rap-1-GTP). Total Rap1 is shown in the second panel. Endogenous Epac1, C3G, and B-Raf levels within each fraction are also shown (lower panels).

and membrane fractions and was activated by F/I in both fractions (Fig. 5D). However, the PKA dependence of that activation was different in each locale. In the perinuclear fraction, cAMP activation of Rap1 was not blocked by H89. In fact, inhibition of PKA appeared to enhance Rap1 activation in this fraction, as seen in whole-cell lysate results (Fig. 1B). Rap1 activation in this perinuclear fraction was also triggered by 8-CPT-2Me-cAMP, demonstrating Epac-dependent activation. Importantly, Epac1 was identified only in this fraction (Fig. 5D) in both the presence and absence of treatment. This suggests strongly that Rap1 activation by Epac1 is confined to the nuclear-perinuclear region. We were unable to confirm the perinuclear activation of Rap1 microscopically by use of GFP-Ral-GDS due to

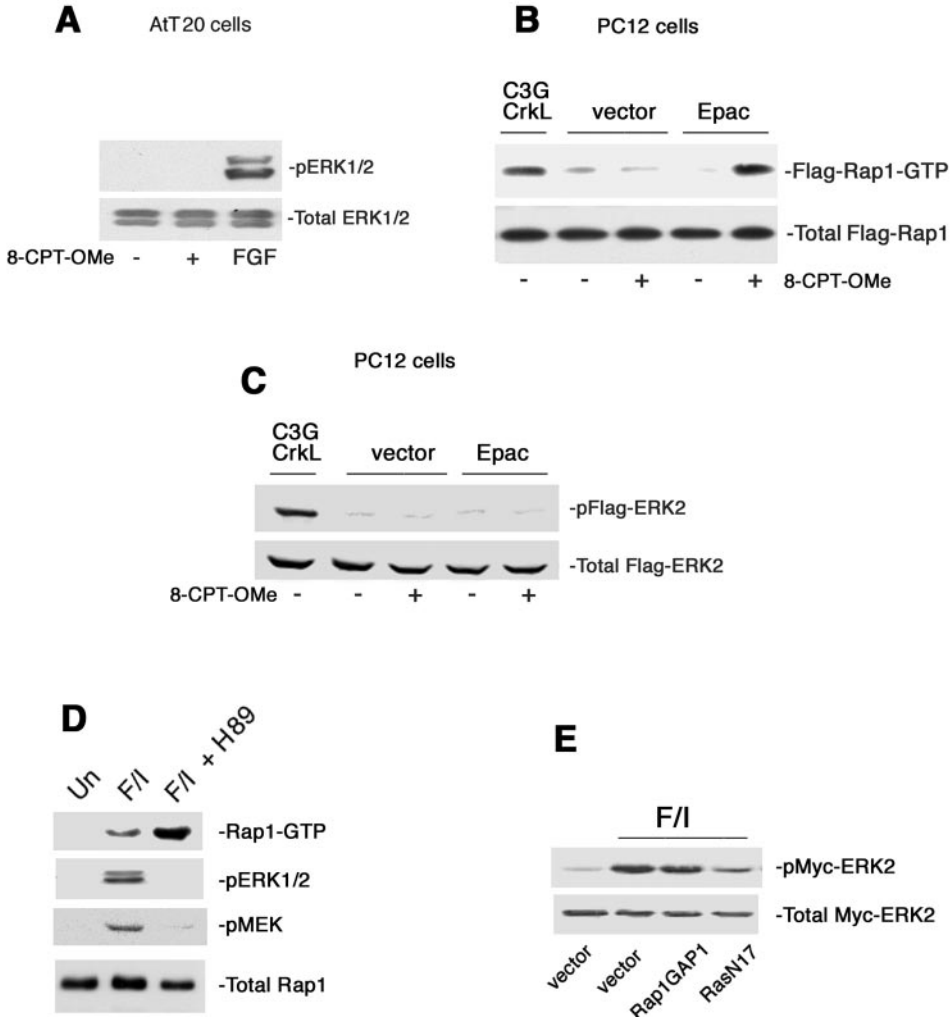

FIG. 6. PKA-independent Rap1 activation is mediated by Epacs in AtT20 cells and does not lead to ERK activation. (A) 8-CPT-2Me-cAMP does not activate ERKs in AtT20 cells. AtT20 cells were left untreated (−) or treated with 8-CPT-2Me-cAMP (8-CPT-OMe) (+), as indicated. Fibroblast growth factor (FGF) served as a positive control. Total cell lysates were examined for the phosphorylation of ERK1/2 by Western blotting (pERK1/2, upper panel). Total ERK1/2 is shown as a loading control. (B) Expression of Epac1 is required for 8-CPT-2Me-cAMP to activate Rap1 in PC12 cells. PC12 cells were transfected with Flag-Rap1 and either WT Epac1, vector, or C3G/CrkL and treated with 8-CPT-2Me-cAMP (8-CPT-OMe) (+) or left untreated (−), as indicated. Cell lysates were examined for activated Flag-Rap1 (Flag-Rap1-GTP). Total Flag-Rap1 is shown as a loading control. (C) Activation of transfected Epac1 in PC12 cells does not result in ERK activation. PC12 cells were transfected with Flag-ERK2 and either WT Epac1, vector, or C3G/CrkL and treated with 8-CPT-2Me-cAMP (8-CPT-OMe) (+) or left untreated (−) as indicated. Flag-ERK2 was immunoprecipitated from cell lysates and analyzed by Western blotting using phospho-ERK antibodies (pFlag-ERK2). The levels of total Flag-ERK2 are indicated as a loading control. (D) ERK is activated by PKA in AtT20 cells and participates in cAMP activation of ERKs. (A) AtT20 cells were left untreated (Un), treated with F/I alone for 20 min, or pretreated with H89 (F/I + H89). Cell lysates were examined for activated Rap1 (Rap1-GTP). Phosphorylation of ERK1/2 was visualized by Western blotting utilizing a phospho-ERK antibody (pERK1/2). Phosphorylation of MEK was visualized by Western blotting utilizing a phospho-MEK antibody (pMEK). Total Rap1 protein levels are shown in the bottom panel as a loading control. (E) Ras is required for the phosphorylation of ERK by F/I in AtT20 cells. AtT20 cells transfected with Myc-ERK2 were cotransfected with vector, Rap1GAP, or RasN17 as indicated. Cells were left untreated or treated with F/I and lysed. Myc-ERK2 was immunoprecipitated from cell lysates and analyzed by Western blotting using phospho-ERK antibodies (pMyc-ERK2). Total Myc-ERK2 levels are shown.

the high level of background staining (detectable in Fig. 4C, left panel) that may have obscured detection of Rap1-GTP within the perinuclear region.

In contrast, Rap1 activation by F/I in the membrane fraction was H89 sensitive (Fig. 5D), confirming the existence of a pool of Rap1 that could be distinguished biochemically from Epac1-dependent nuclear pool. This H89-sensitive pool was not apparent when unfractionated AtT20 cells were examined (Fig. 1B), presumably because it was overwhelmed by the presence of a larger pool of Rap1 whose activation was enhanced by H89.

These data demonstrate the existence of distinct pools of Rap1 that can be selectively activated by either PKA or Epac. We suggest that the PKA-dependent activation of Rap1 at the membrane occurs via C3G and may represent the component of Rap1 whose activation by F/I was blocked by Rap-AGE (Fig. 1E, F/I lanes). C3G was detected predominantly in the

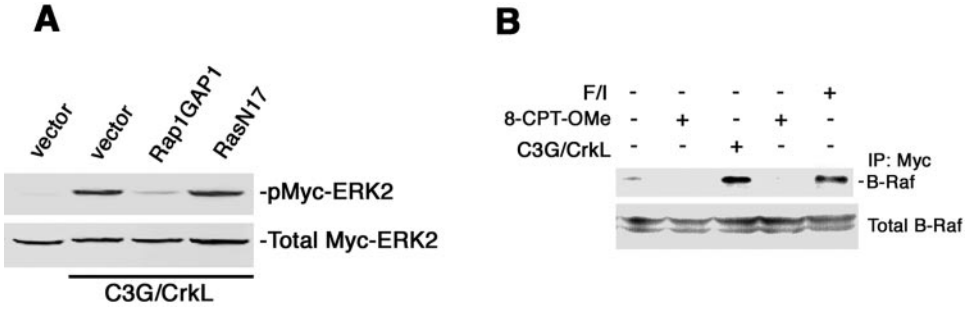

FIG. 7. Transfection of C3G/CrkL triggers Rap1/B-Raf association and ERK activation in AtT20 cells. (A) C3G/CrkL-mediated ERK activation was blocked by Rap1GAP1. Cells were transfected Myc-ERK2, and with vector alone or with C3G/CrkL, in the presence of vector, Rap1GAP1, or RasN17, as indicated. Myc-ERK2 was immunoprecipitated from cell lysates and analyzed by Western blotting using phospho-ERK antibodies (pMyc-ERK2). Total Myc-ERK2 levels are shown. (B) Forskolin/IBMX, but not 8-CPT-2Me-cAMP, promotes Rap1/B-Raf association in AtT20 cells. AtT20 cells were transfected with Myc-Rap1, and either cotransfected with C3G/CrkL or treated with F/I or 8-CPT-2Me-cAMP (8-CPT-OMe), as indicated. Cell lysates were immunoprecipitated (IP) with Myc antibodies to recover Myc-Rap1. Association of Myc-Rap1 with endogenous B-Raf was examined by Western blotting following Myc IP (B-Raf). Total B-Raf is shown as a loading control.

cytoplasmic fractions (Fig. 5D). By overloading membrane samples, low levels of C3G were detected at the PM (data not shown).

Epac-mediated activation of Rap1 is not involved in ERK signaling in AtT20 cells. It has been proposed that the PKA-independent activation of Rap1 by cAMP via Epacs is incapable of activating ERKs (22). Therefore, we examined whether activation of Epacs in AtT20 cells could couple Rap1 to ERKs. Although Rap1 was activated by 8-CPT-2Me-cAMP, this activation did not result in ERK phosphorylation (Fig. 6A).

In contrast to AtT20 cells, 8-CPT-2Me-cAMP was unable to activate Rap1 in PC12 cells (Fig. 6B). The inability of 8-CPT-2Me-cAMP to activate Rap1in PC12 cells appeared to be due to the limited expression of endogenous Epacs in these cells, as transfection of Epac1 into PC12 cells enabled 8-CPT-2Me-cAMP to activate Rap1 (Fig. 6B). Similarly, in PC12 cells transfected with WT Epac1, the activation of Rap1 by 8-CPT-2Me-cAMP did not couple to ERK activation (Fig. 6C). These results confirm that, as previously reported (22), Rap1 activated by Epacs is incapable of activating ERKs.

Although Epac-mediated activation of Rap1 does not lead to ERK activation in AtT20 cells, cAMP/PKA can activate ERKs in these cells (Fig. 6D). As in PC12 cells (Fig. 2A), activation of both MEK and ERK is blocked by H89 (Fig. 6D), confirming that Epacs are not mediating this activation of ERKs in AtT20 cells. To determine whether this activation of ERKs required Rap1 or Ras, we introduced RasN17 or Rap1GAP into AtT20 cells and examined the activation of Flag-ERK by F/I. Surprisingly, the activation of ERKs was only modestly blocked by Rap1GAP1 but was largely blocked by RasN17 (Fig. 6E). Indeed, cAMP can activate Ras in AtT20 cells and this activation is blocked by H89, suggesting that in AtT20 cells, cAMP activates Ras in a PKA-dependent manner (data not shown). Taken together, these data suggest that Ras plays a major role, and Rap1 a minor role, in the activation of ERKs by cAMP/PKA in AtT20 cells. Ras activation by cAMP has been reported, and PKA-independent pathways have also been proposed (4, 14, 51).

Activation of C3G permits the selective activation of Rap1 without activating Ras. To directly examine the ability of C3G to couple Rap1 to ERKs in AtT20 cells, we activated C3G independently of cAMP by cotransfecting C3G and CrkL into these cells (31). In similarity to our observations of PC12 cells (Fig. 1 and 2), the transfection of C3G/CrkL into AtT20 cells resulted in robust activation of ERKs that was blocked by Rap1GAP1 but not by RasN17 (Fig. 7A), indicating that Rap1 activation by C3G/CrkL was necessary to activate ERKs in these cells. This activation of ERKs by C3G/CrkL was not blocked by H89, consistent with the requirement of C3G downstream of PKA (data not shown).

Rap1 activation of ERKs has been proposed to be mediated by the selective recruitment of the MAP kinase kinase kinase B-Raf to GTP-loaded Rap1 (60, 63). In AtT20 cells, the transfection of C3G/CrkL can induce the association of endogenous B-Raf with Rap1 (Fig. 7B). Rap1 activated by 8-CPT-2Me-cAMP was unable to induce this association (Fig. 7B). This suggests that C3G and Epac activate pools of Rap1 that can be distinguished biochemically as well as spatially within the cell. The observations that Forskolin induced Rap1/B-Raf association and that interfering with C3G blocked a portion of Forskolin's activation of ERKs suggest C3G is likely capable of coupling Rap1 to B-Raf/ERKs in AtT20, although this represents a minor pathway by which cAMP activates ERKs in these cells compared to that mediated by Ras.

Relocalization of Epac1 allows Rap1 to activate ERKs. The differential coupling of C3G and Epacs to ERKs raises the possibility that distinct subcellular pools of Rap1 can couple to ERKs. In contrast to C3G, Epac1 shows a perinuclear localization (54). GFP-tagged Epac1 (GFP-Epac WT) was predominantly localized within the perinuclear region, in similarity to endogenous Epac1 results (37, 54) (Fig. 8A), and colocalized with nuclear pore complex proteins, as detected by indirect immunofluorescence (Fig. 8B and C). This localization of GFP-Epac WT was unchanged upon stimulation with F/I (data not shown), consistent with biochemical studies (Fig. 5D) and recent reports (10, 37). To examine the possibility that pools of Rap1 activated by Epac are not appropriately localized to couple to ERKs, we directed Epac1 to the PM. This was achieved by fusing the membrane-targeting motif of Ki-Ras (28), including amino acids 169 to 188 of Ki-Ras comprising the polybasic region and the CAAX domain (referred to here as -CAAX), to the C terminus of WT Epac1 (Epac-CAAX). To compare the localization of the Epac-

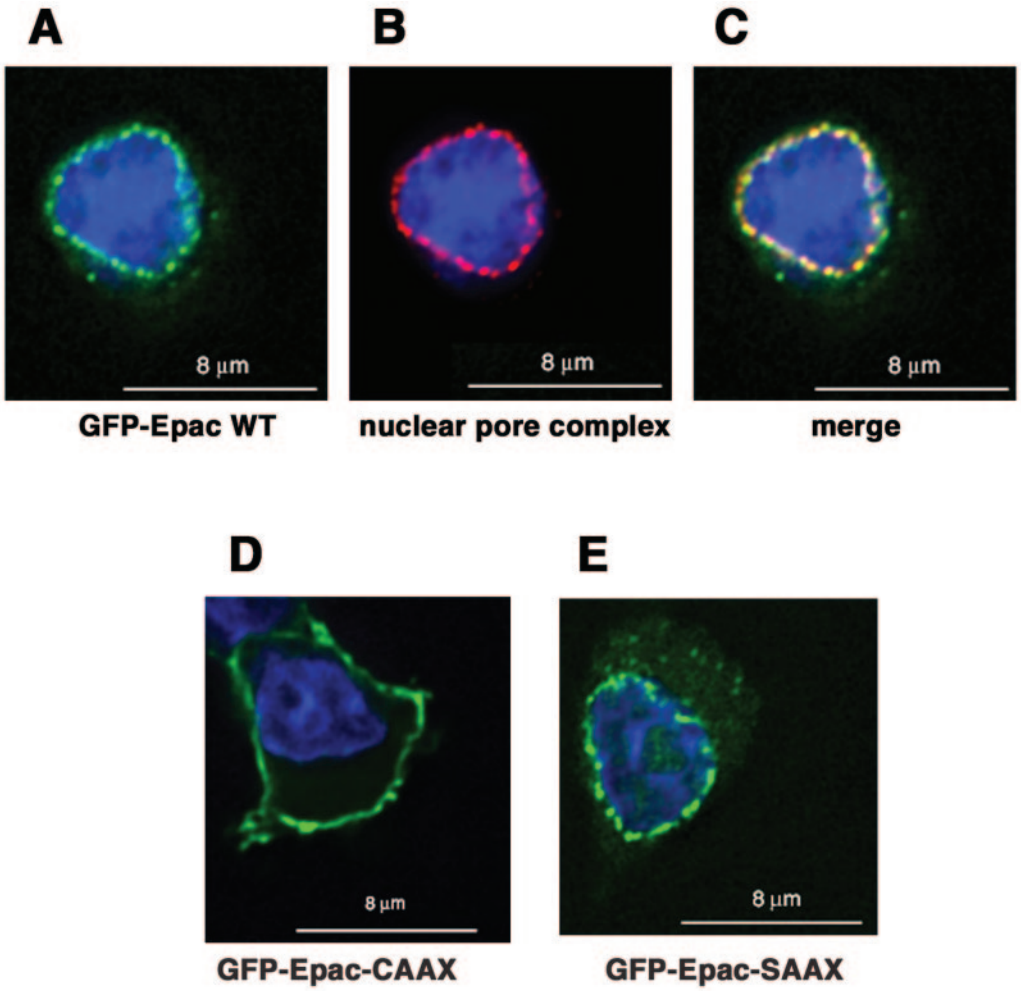

FIG. 8. Epac1 is localized to the perinuclear region. PC12 cells were transfected with GFP-Epac-WT. (A) The localization of GFP-Epac-WT is shown using epifluorescence. (B) The localization of the nuclear pore complex is shown using immunofluorescence using the antibody Mab414. (C) A merged image of GFP-Epac-WT and the nuclear pore complex shown in panels A and B is shown. (D) The addition of the Ki-Ras CAAX domain relocalizes Epac1 to the PM. PC12 cells were transfected with GFP-Epac-CAAX, and the localization of GFP-Epac-CAAX is shown using epifluorescence. (E) PC12 cells were transfected with GFP-Epac-SAAX and the localization of GFP-Epac-SAAX is shown using epifluorescence. Fixed cells were stained with Hoechst 33258 and analyzed using Image Restoration microscopy.

CAAX with WT Epac1, we created GFP fusion proteins for both Epac1 and Epac-CAAX (designated GFP-Epac-WT and GFP-Epac-CAAX, respectively).

The subcellular localization of Epac-CAAX was examined in PC12 cells. GFP-Epac-CAAX was targeted to the PM (Fig. 8D), demonstrating that the CAAX domain was dominant over targeting sequences within Epac itself. GFP-Epac-SAAX (in which the terminal cysteine was replaced with a serine) was incapable of localizing to the PM and showed a perinuclear staining result similar to that of GFP-Epac-WT (Fig. 8E), demonstrating that targeting to the PM required an intact CAAX domain. Expression of all three Epac constructs (Epac-WT, Epac-CAAX, and Epac-SAAX) in PC12 cells could activate Rap1 when cells were stimulated with the Epac-selective analog 8-CPT-2Me-cAMP (Fig. 9A, top panel), demonstrating that they all possessed catalytic activity.

To determine the consequence of Rap1 activation, we examined Rap1's ability to associate with B-Raf, as detected by in coimmunoprecipitation with Flag-Rap1 (Fig. 9A, third panel). As expected, endogenous B-Raf was recruited to constitutively GTP-loaded Flag-RapV12 (lane 2), and was recruited to WT Flag-Rap1 in response to stimulation by F/I (lane 4) or transfection of C3G/CrkL (lane 11). In the presence of both F/I and transfected C3G/CrkL, the recruitment of B-Raf to Flag-Rap1 was enhanced (lane 12). In contrast, neither WT Epac (lanes 5 and 6) nor Epac-SAAX (lanes 9 and 10) could induce the association of endogenous B-Raf with Flag-Rap1 in the presence or absence of 8-CPT-2Me-cAMP. However, Epac-CAAX was able to trigger Rap1/B-Raf association and this was dependent on stimulation with 8-CPT-2Me-cAMP (lanes 7 and 8). This indicates that the ability of Rap1 to activate ERKs is directly related to its ability to form complexes with B-Raf.

The ability of Epac chimeras to activate ERK was also tested. Neither Epac-WT nor Epac-SAAX was able to activate ERKs in either the presence or absence of 8-CPT-2Me-cAMP. In sharp contrast, the expression of Epac-CAAX resulted in

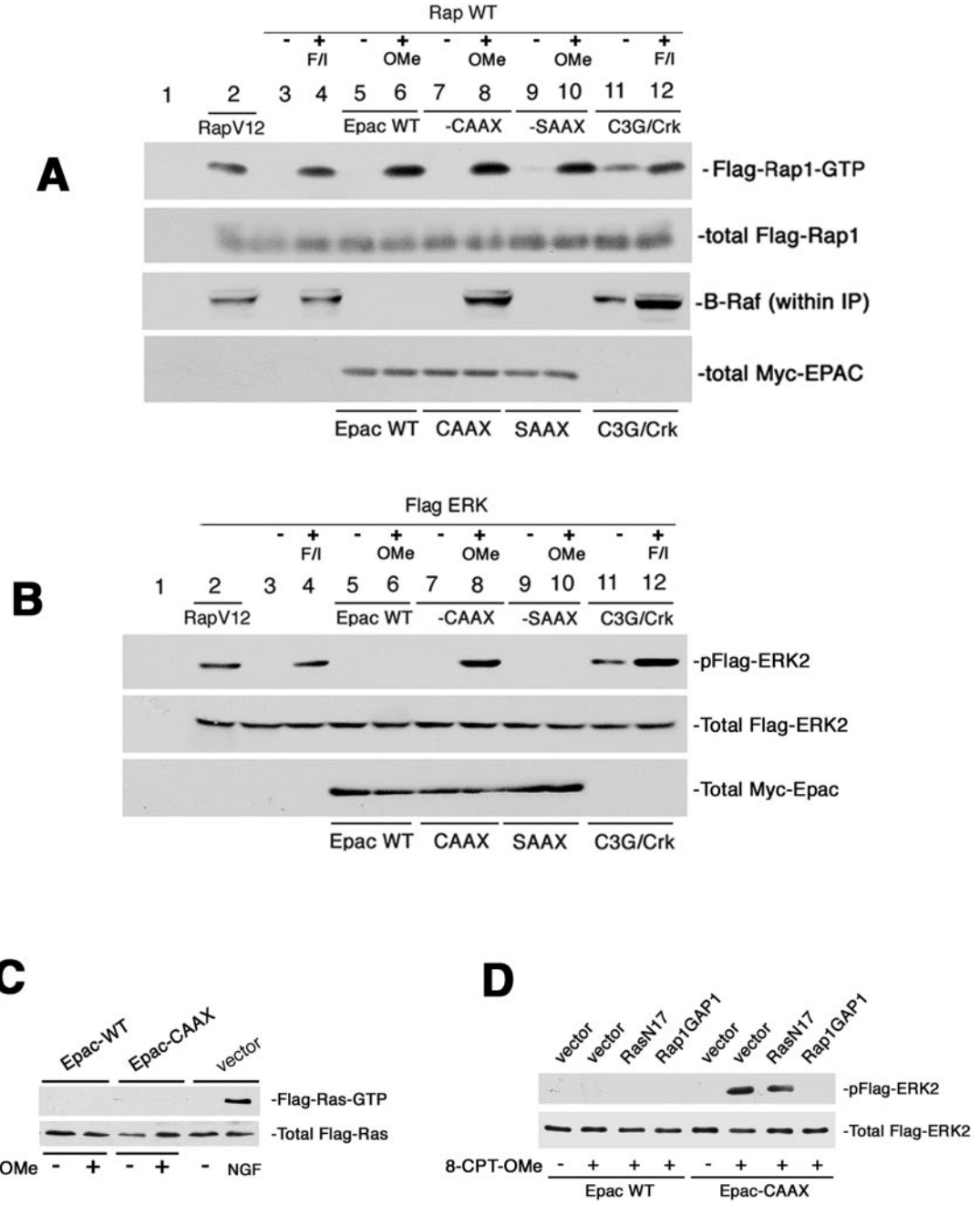

FIG. 9. Epac-CAAX, but not Epac WT or Epac-SAAX, promotes ERK activation and Rap1/B-Raf association. (A and B) Parallel experiments in PC12 cells were conducted to examine the selectivity of Rap1-GEFs for Rap1 activation and association with B-Raf, shown in panel A, and activation of ERKs, shown in panel B. For the experiments depicted in both panels A and B, cells were transfected with Myc-Epac WT (lanes 5 and 6), or Myc-Epac-CAAX (lanes 7 and 8), or Myc-Epac-SAAX (lanes 9 and 10). All cells transfected with Epac constructs were either treated with 8-CPT-2Me-cAMP (OMe; lanes 6, 8, and 10) or left untreated (lanes 5, 7, and 9). Cells were transfected with C3G/CrkL (lanes 11 and 12). Cells were treated with F/I in lanes 4 and 12. (A) All Epac constructs activate Rap1, but only Epac-CAAX can trigger Rap1 to associate with B-Raf. In addition to use of the cDNAs described above, cells were cotransfected with vector (lane 1), Flag-RapV12 (lane 2), or WT Flag-Rap1 (lanes 3 to 12) and treated as described above. Cell lysates were split and one fraction examined for Rap1 activation (Flag-Rap1-GTP). Total Flag-Rap1 is shown to indicate equal transfection and loading. The second fraction was immunoprecipitated (IP) with Flag antibody to recover Flag-Rap1, and association with endogenous B-Raf was examined by Western blotting [B-Raf (within IP)]. The expression levels of Myc-Epacs (WT, Epac-CAAX, and Epac-SAAX) are shown in the lower panel (Total Myc-Epac). (B) Only Epac-CAAX, but neither Epac WT nor Epac-SAAX, can activate ERKs. In addition to the cDNAs described above, cells were transfected with vector (lane 1), or Flag-ERK2 (lanes 2 to 12), and treated as described above. Flag-RapV12 (lane 2) was used a positive control. Flag-ERK2 was immunoprecipitated from cell lysates and analyzed by Western blotting using phospho-ERK antibodies (pFlag-ERK2). The levels of total Flag-ERK2 and total Myc-Epac proteins are shown. (C) Neither Epac nor Epac-CAAX can activate Ras. PC12 cells were transfected with Flag-Ras and either Epac-WT, Epac-CAAX, or vector. Cells were left untreated (−) or treated with 8-CPT-2Me-cAMP (8-CPT-OMe) (+), as indicated. NGF was used as a positive control. Cell lysates were examined for activated Flag-Ras (Flag-Ras-GTP). Total Flag Ras levels are shown to indicate equal transfection and loading (lower panel). (D) ERK activation by Epac-CAAX is Rap1 dependent. PC12 cells were transfected with Flag-ERK2 and Epac-CAAX in the presence of vector, Rap1GAP1, or RasN17, as indicated. Cells were left untreated (−) or treated with 8-CPT-2Me-cAMP (8-CPT-OMe) (+), as indicated. Flag-ERK2 was immunoprecipitated from cell lysates and analyzed by Western blotting using phospho-ERK antibodies (pFlag-ERK2). The levels of total Flag-ERK2 protein are shown.

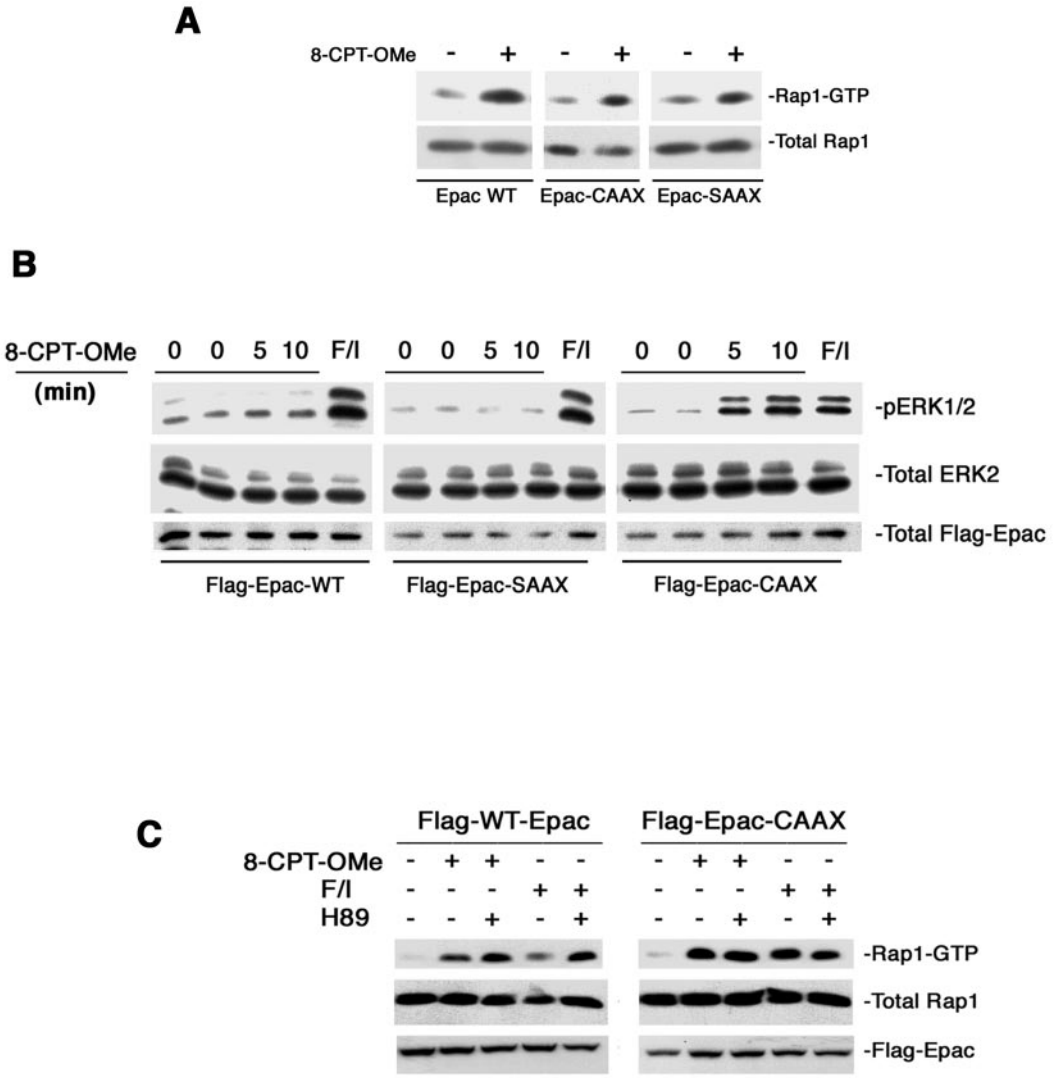

FIG. 10. Stable expression of Epac-CAAX in PC12 cells permits ERK activation by 8-CPT-2Me-cAMP. (A) Stably selected populations of Flag-Epac-WT, Flag-Epac-CAAX and Flag-Epac-SAAX-expressing PC12 cells show Rap1 activation by 8-CPT-2Me-cAMP. Cells were treated in the presence (+) or absence (−) of 8-CPT-2Me-cAMP (8-CPT-OMe), and lysates were examined for activation of Rap1 (Rap1-GTP). Total Rap1 levels are shown as a loading control. Flag-Epac-WT-, Flag-Epac-SAAX-, and Flag-Epac-CAAX-expressing PC12 cells are shown in the left, middle, and right panels, respectively. (B) PC12 cells stably expressing Flag-Epac-CAAX show ERK activation in response to 8-CPT-2Me-cAMP. Cells were treated with 8-CPT-2Me-cAMP (8-CPT-OMe) for the indicated times (min). F/I (1 μM) was used as a normalization control. Phosphorylation of ERK1/2 was visualized by Western blotting utilizing a phospho-ERK antibody (pERK1/2). Total ERK2 levels are shown as a loading control. The bottom panels show Flag-Epac expression for each transfectant. Flag-Epac-WT-, Flag-Epac-SAAX-, and Flag-Epac-CAAX-expressing PC12 cells are shown in the left, middle, and right panels, respectively. (C) Rap1 activation by cAMP in stable populations of Epac-expressing PC12 cells is not inhibited by PKA. Stable populations of Epac-CAAX- and Epac-WT-expressing PC12 cells were stimulated with 8-CPT-2Me-cAMP (8-CPT-OMe) or F/I with (+) or without (−) H89 pretreatment. Cell lysates were examined for activated Rap1 (Rap1-GTP, top panel). Total Rap1 was visualized by Western blotting (middle panel). Total cell lysates were examined for Flag-Epac expression (bottom panel).

the activation of ERKs. This activation of ERKs was dependent on the Epac agonist 8-CPT-2Me-cAMP (Fig. 9B). As a positive control, we showed that RapV12 could also activate ERKs (Fig. 9B). This activation was largely insensitive to the presence of H89 (data not shown).

Next, we examined the dependence on Rap1 of the activation of ERKs by Epac-CAAX. Neither Epac1 nor Epac-CAAX was able to activate Ras under any condition (Fig. 9C). The activation of ERKs by Epac-CAAX was blocked by Rap1GAP1, but not by RasN17 (Fig. 9D), indicating that membrane-localized Epac-CAAX activates ERKs in a Rap1-dependent and Ras-independent manner.

Taken together, the data demonstrate that a distinct pool of Rap1 can be activated by Epac-CAAX and that this pool is capable of activating ERKs. These results were confirmed in studies using populations of PC12 cells stably transfected with Epac WT, Epac-CAAX, and Epac-SAAX (Fig. 10). All three stable transfectants showed Rap1 activation by 8-CPT-2Me-

cAMP (Fig. 10A), but 8-CPT-2Me-cAMP activated ERKs only in cells expressing Epac-CAAX (Fig. 10B). As expected, F/I also stimulated Rap1 in both Epac WT- and Epac-CAAX-expressing cells and this was not blocked by H89, demonstrating that the expression of Epac WT or Epac-CAAX permitted PKA-independent activation of Rap1 by cAMP (Fig. 10C).

The ability of PM-targeted Epac to activate ERKs was also seen using CAAX domains derived from other small G proteins. Chimeras of Epac can be targeted to the PM when fused to other CAAX domains derived from Rap1b and Ha-Ras (GFP-Epac-Ha-Ras-CAAX and GFP-Epac-Rap1b-CAAX) (data not shown). For Rap1, it has been shown that perinuclear localization requires additional sequences (amino acids 85 to 89 in Rap1) that are not contained within this chimera (45). Like Epac-Ki-CAAX, both Epac-Rap1b-CAAX, and Epac-Ha-Ras-CAAX could activate ERKs in an 8-CPT-2Me-cAMP-dependent manner (data not shown).

These data show that only select pools of activated Rap1 can associate with B-Raf. The potential of Rap1 to form a complex with B-Raf and subsequently activate ERKs appears to be dependent on which Rap1-GEF is involved. C3G activation of Rap1 is capable of coupling Rap1 to ERKs, whereas Epac activation of Rap1 is not, unless it is redirected to the PM.

DISCUSSION

The large number of known Rap1-GEFs that have currently been identified greatly expands the range of extracellular signals capable of activating Rap1. Whether Rap1-GEFs also participate in the selection of Rap1 effectors is not known. We show here that C3G activation of Rap1 could couple Rap1 to ERK activation, while Rap1 activation by Epac1 could not. The ability of Epac to activate ERKs has been proposed in selected systems (25, 30, 36, 38). However, when an Epac-selective agonist was used to activate Rap1, regulation of ERKs was not seen (22). Using endogenous and transfected Epac, we confirmed that Epac-dependent activation of Rap1 could not activate ERKs in either PC12 or AtT20 cells. Importantly, activation of Rap1 by another selective Rap1-GEF, C3G, was able to activate ERKs in both cell types.

C3G is recruited to membranes via CrkL (23) and has been localized to the PM (43, 56), endosomes (64), and Golgi complex (56). In contrast, Epac1 is largely perinuclear in interphase cells (10, 54) and localizes with members of the nuclear pore complex protein family. We show here that the inability of Epac to activate ERKs could be overcome by relocalizing Epac1 to the PM and that this activation triggered by relocalized Epac required Rap1. Our data suggest that a PM pool of Rap1 is competent to activate the ERK cascade.

Studies done by Bivona and colleagues demonstrated that the PM is the principal platform from which Rap1 signaling emanates (9). Our results support their view in that cAMP/PKA activation of Rap1 led to an accumulation of active Rap1-GTP at the membrane, as detected microscopically and biochemically. However, cell fractionation also revealed a second (perinuclear) site of Rap1 activation by cAMP that was PKA independent and subject to activation by Epac agonists. The ability of Epac to activate this perinuclear pool of Rap1 without activating ERKs suggests strongly that perinuclear Rap1-GTP is incapable of coupling to ERK. The ability of Epac-CAAX to activate ERKs is consistent with the presence of a regulated pool of Rap1 that is capable of activating the ERK cascade from the PM. B-Raf, the Rap1 effector that couples Rap1 to ERKs, and other components of the ERK signaling cascade have also been shown to form large signaling complexes within the PM and endosomal compartments (44, 53, 64). Indeed, only those Rap1 molecules that were activated by Epac-CAAX or C3G/CrkL, but not by Epac or Epac-SAAX, formed a complex with B-Raf. Therefore, we propose that activation of selected pools of Rap1 that are associated with specific membrane domains is able to activate components of the ERK signaling cascade.

cAMP is capable of activating Rap1 in AtT20 cells via both PKA-independent and PKA-dependent mechanisms. The existence of a PKA-dependent pathway was revealed following cell fractionation and occurs within the membrane compartments. We propose that this PKA-dependent activation of Rap1 is mediated by C3G in AtT20 cells, in similarity to the results shown for PC12 cells. Recently, the ability of Epac2 to activate PM pools of Rap1 has been reported (37). In that study, recruitment of Epac2 via its association (RA) domain to Ras at the PM required activation of Ras by growth factors. Although AtT20 cells express Epac2, we did not examine endogenous Epac2 directly in this study and did not include Epac2 in our transfection experiments conducted with PC12 cells. Because cAMP can likely activate both Ras and Epac2 in AtT20 cells, this pathway may contribute to the Rap1 activation seen at the PM.

In addition, AtT20 cells display a PKA-independent activation of Rap1 via Epac that occurs within a perinuclear compartment. Surprisingly, it appears that Epac activation of Rap1 via cAMP is not merely PKA independent but is instead actually inhibited by PKA. This is best seen with whole-cell lysates, with which H89 enhances Rap1 activation by F/I to levels seen with 8-CPT-2Me-cAMP (Fig. 1B and 5C). We believe that this large increase in Rap1 activation by H89 obscures the H89-inhibited (PKA-dependent) component of Rap1 activation.

There are at least two possible mechanisms for this inhibitory action of PKA on Epac activation of Rap1. First, PKA might negatively regulate Epac via direct phosphorylation. Indeed, peptides within the amino terminus of mouse Epac1 (but not human Epac1) are capable of being phosphorylated by PKA in vitro (Q. Low and P. J. S. Stork, unpublished data), although the phosphorylation of Epac by PKA in vivo and its potential consequences are still under investigation. Second, PKA may negatively regulate Rap1 itself. Rap1 is a direct target for PKA phosphorylation (1, 2), and PKA-dependent phosphorylation of Rap1 at amino acid 179 has been shown to modulate Rap1 function (39), perhaps by altering the localization or stability of Rap1 (57). Indeed, the augmentation of cAMP's activation of Rap1 by H89 is diminished in a mutant of Rap1 that is resistant to PKA phosphorylation (Z. Wang and P. J. S. Stork, unpublished data). This might provide a mechanism for the reported antagonism of Epac by PKA in thyroid cells (41) and pancreatic β cells (35). Interestingly, this antagonistic action of PKA was not seen following PKA stimulation of Epac-CAAX, suggesting that PKA's inhibition of Epac activation of Rap1 was limited to the perinuclear pool of Rap1. We propose that the outcome of cAMP production (PKA versus Epac) must be highly regulated to maximize signaling

specificity. The recent discovery of the direct binding of Epac1 to the perinuclear mAKAP suggests that these two targets of cAMP are tightly integrated within the cell (20).

Another novel finding is that ERK activation by cAMP/PKA utilizes Ras. This action was seen only in AtT20 cells, not in PC12 cells, and was PKA dependent. The mechanisms by which cAMP activates Ras are not well established. Both PKA-independent (4, 14, 62) and PKA-dependent (3) activation of Ras have been reported. One candidate is Ras-GRF1, which is directly phosphorylated by PKA (46).

In this report, we show that the ability of Rap1 to couple to ERKs can be determined by the GEFs that activate it. Epac-mediated Rap1 activation does not permit Rap1 to couple to ERKs, whereas C3G-induced Rap1 activation results in ERK activation. This limitation of Epac/Rap1 with respect to activation of ERKs could be overcome by relocalizing Epac1 to the PM, suggesting that the location where Rap1 is activated determines the consequence of Rap1 activation. Importantly, the specificity of the Epac agonist 8-CPT-2Me-cAMP provides the unique ability to selectively activate Rap1. Therefore, the 8-CPT-2Me-cAMP-dependent activation of ERKs through Epac-CAAX demonstrates that the activation of selected pools of Rap1 alone is sufficient to recruit B-Raf and activate ERKs. These studies emphasize that distinct pools of Rap1 couple to distinct effector pathways. We propose that the extended family of Rap1-GEFs has evolved to selectively target Rap1 to distinct effector pathways, as determined, in large part, by the spatial distribution of Rap1-GEFs and effectors within the cell. Similar utilizations of GEFs have been proposed to dictate the choice of effectors for the small G protein Rac1 of the Rho superfamily (6, 12, 13, 18, 68). This study demonstrated that this function of GEFs in dictating effector utilization also exists for members of the Ras superfamily. It is likely that similar regulation dictates the choice of effector pathways by other small G proteins as well.

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REFERENCES

1. **Altschuler, D., and E. G. Lapetina.** 1993. Mutational analysis of the cAMP-dependent protein kinase-mediated phosphorylation site of Rap1b. J. Biol. Chem. **268:**7527–7531.
2. **Altschuler, D. L., S. N. Peterson, M. C. Ostrowski, and E. G. Lapetina.** 1995. Cyclic AMP-dependent activation of Rap1b. J. Biol. Chem. **270:**10373–10376.
3. **Ambrosini, A., S. Tininini, A. Barassi, G. Racagni, E. Sturani, and R. Zippel.** 2000. cAMP cascade leads to Ras activation in cortical neurons. Mol. Brain Res. **75:**54–60.
4. **Amsen, E. M., N. Pham, Y. Pak, and D. Rotin.** 2005. The guanine nucleotide exchange factor CNrasGEF regulates melanogenesis and cell survival in melanoma cells. J. Biol. Chem. **281:**121–128.
5. **Arevalo, J. C., H. Yano, K. K. Teng, and M. V. Chao.** 2004. A unique pathway for sustained neurotrophin signaling through an ankyrin-rich membrane-spanning protein. EMBO J. **23:**2358–2368.
6. **Arthur, W. T., L. A. Quilliam, and J. A. Cooper.** 2004. Rap1 promotes cell spreading by localizing Rac guanine nucleotide exchange factors. J. Cell Biol. **167:**111–122.
7. **Bivona, T. G., I. Perez De Castro, I. M. Ahearn, T. M. Grana, V. K. Chiu, P. J. Lockyer, P. J. Cullen, A. Pellicer, A. D. Cox, and M. R. Philips.** 2003. Phospholipase Cγ activates Ras on the Golgi apparatus by means of RasGRP1. Nature **424:**694–698.
8. **Bivona, T. G., and M. R. Philips.** 2003. Ras pathway signaling on endomembranes. Curr. Opin. Cell Biol. **15:**136–142.
9. **Bivona, T. G., H. H. Wiener, I. M. Ahearn, J. Silletti, V. K. Chiu, and M. R. Philips.** 2004. Rap1 up-regulation and activation on plasma membrane regulates T cell adhesion. J. Cell Biol. **164:**461–470.
10. **Borland, G., M. Gupta, M. M. Magiera, C. J. Rundell, S. Fuld, and S. J. Yarwood.** 2005. Microtubule-associated protein 1B-light chain 1 enhances activation of Rap1 by exchange protein activated by cyclic AMP but not intracellular targeting. Mol. Pharmacol. **69:**374–384.
11. **Brinkmann, T., O. Daumke, U. Herbrand, D. Kuhlmann, P. Stege, M. R. Ahmadian, and A. Wittinghofer.** 2002. Rap-specific GTPase activating protein follows an alternative mechanism. J. Biol. Chem. **277:**12525–12531.
12. **Buchsbaum, R. J., B. A. Connolly, and L. A. Feig.** 2002. Interaction of Rac exchange factors Tiam1 and Ras-GRF1 with a scaffold for the p38 mitogen-activated protein kinase cascade. Mol. Cell. Biol. **22:**4073–4085.
13. **Buchsbaum, R. J., B. A. Connolly, and L. A. Feig.** 2003. Regulation of p70 S6 kinase by complex formation between the Rac guanine nucleotide exchange factor (Rac-GEF) Tiam1 and the scaffold spinophilin. J. Biol. Chem. **278:**18833–18841.
14. **Busca, R., P. Abbe, F. Mantoux, E. Aberdam, C. Peyssonnaux, A. Eychene, J. P. Ortonne, and R. Ballotti.** 2000. Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. EMBO J. **19:**2900–2910.
15. **Cheng, H. C., B. E. Kemp, R. B. Pearson, A. J. Smith, L. Misconi, S. M. V. Patten, and D. A. Walsh.** 1986. A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. J. Biol. Chem. **261:**989–992.
16. **Chiu, V. K., T. Bivona, A. Hach, J. B. Sajous, J. Silletti, H. Wiener, R. L. Johnson, A. D. Cox, and M. R. Philips.** 2002. Ras signalling on the endoplasmic reticulum and the Golgi. Nat. Cell Biol. **4:**343–350.
17. **Christensen, A. E., F. Selheim, J. de Rooij, S. Dremier, F. Schwede, K. K. Dao, A. Martinez, C. Maenhaut, J. L. Bos, H. G. Genieser, and S. O. Doskeland.** 2003. cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. J. Biol. Chem. **278:**35394–35402.
18. **Connolly, B. A., J. Rice, L. A. Feig, and R. J. Buchsbaum.** 2005. Tiam1-IRSp53 complex formation directs specificity of Rac-mediated actin cytoskeleton regulation. Mol. Cell. Biol. **25:**4602–4614.
19. **de Rooij, J., F. J. Zwartkruis, M. H. Verheijen, R. H. Cool, S. M. Nijman, A. Wittinghofer, and J. L. Bos.** 1998. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature **396:**474–477.
20. **Dodge-Kafka, K. L., J. Soughayer, G. C. Pare, J. J. Carlisle Michel, L. K. Langeberg, M. S. Kapiloff, and J. D. Scott.** 2005. The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. Nature **437:**574–578.
21. **Ehrhardt, A., G. Ehrhardt, X. Guo, and J. Schrader.** 2002. Ras and relatives—job sharing and networking keep an old family together. Exp. Hematol. **30:**1089.
22. **Enserink, J. M., A. E. Christensen, J. De Rooij, M. Van Triest, F. Schwede, H. G. Genieser, S. O. Doskeland, J. L. Blank, and J. L. Bos.** 2002. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat. Cell Biol. **4:**901–906.
23. **Feller, S. M.** 2001. Crk family adaptors-signalling complex formation and biological roles. Oncogene **20:**6348–6371.
24. **Franke, B., J.-W. Akkerman, and J. L. Bos.** 1997. Rapid Ca^{2+}-mediated activation of rap1 in human platelets. EMBO J. **16:**252–259.
25. **Fujita, T., T. Meguro, R. Fukuyama, H. Nakamuta, and M. Koida.** 2002. New signaling pathway for parathyroid hormone and cyclic AMP action on extracellular-regulated kinase and cell proliferation in bone cells. Checkpoint of modulation by cyclic AMP. J. Biol. Chem. **277:**22191–22200.
26. **Gao, X., T. Satoh, Y. Liao, C. Song, C. D. Hu, K. Kariya Ki, and T. Kataoka.** 2001. Identification and characterization of RA-GEF-2, a Rap guanine nucleotide exchange factor that serves as a downstream target of M-Ras. J. Biol. Chem. **276:**42219–42225.
27. **Gotoh, T., S. Hattori, S. Nakamura, H. Kitayama, M. Noda, Y. Takai, K. Kaibuchi, H. Matsui, O. Hatase, H. Takahashi, et al.** 1995. Identification of Rap1 as a target for the Crk SH3 domain-binding guanine nucleotide-releasing factor C3G. Mol. Cell. Biol. **15:**6746–6753.
28. **Hancock, J. F.** 2003. Ras proteins: different signals from different locations. Nat. Rev. Mol. Cell Biol. **4:**373–384.
29. **Huang, E. J., and L. F. Reichardt.** 2003. TRK receptors: roles in neuronal signal transduction. Annu. Rev. Biochem. **72:**609–642.
30. **Iacovelli, L., L. Capobianco, L. Salvatore, M. Sallese, G. M. D'Ancona, and A. De Blasi.** 2001. Thyrotropin activates mitogen-activated protein kinase pathway in FRTL- 5 by a cAMP-dependent protein kinase A-independent mechanism. Mol. Pharmacol. **60:**924–933.
31. **Ichiba, T., Y. Kuraishi, O. Sakai, S. Nagata, J. Groffen, T. Kurata, S. Hattori, and M. Matsuda.** 1997. Enhancement of guanine-nucleotide exchange activity of C3G for Rap1 by the expression of Crk, CrkL, and Grb2. J. Biol. Chem. **272:**22215–22220.

32. **Kao, S., R. K. Jaiswal, W. Kolch, and G. E. Landreth.** 2001. Identification of the mechanisms regulating the differential activation of the MAPK cascade by epidermal growth factor and nerve growth factor in PC12 cells. J. Biol. Chem. **276:**18169–18177.
33. **Kawasaki, H., G. M. Springett, N. Mochizuki, S. Toki, M. Nakaya, M. Matsuda, D. E. Housman, and A. M. Graybiel.** 1998. A family of cAMP-binding proteins that directly activate Rap1. Science **282:**2275–2279.
34. **Kitayama, H., Y. Sugimoto, T. Matsuzaki, Y. Ikawa, and M. Noda.** 1989. A ras-related gene with transformation suppressor activity. Cell **56:**77–84.
35. **Kwon, G., K. L. Pappan, C. A. Marshall, J. E. Schaffer, and M. L. McDaniel.** 2004. cAMP dose-dependently prevents palmitate-induced apoptosis by both protein kinase A- and cAMP-guanine nucleotide exchange factor-dependent pathways in beta-cells. J. Biol. Chem. **279:**8938–8945.
36. **Laroche-Joubert, N., S. Marsy, S. Michelet, M. Imbert-Teboul, and A. Doucet.** 2002. Protein kinase A-independent activation of ERK and H, K-ATPase by cAMP in native kidney cells: role of Epac I. J. Biol. Chem. **277:**18598–18604.
37. **Li, Y., S. Asuri, J. F. Rebhun, A. F. Castro, N. C. Paranavitana, and L. A. Quilliam.** 18 November 2005, revision date. The Rap1 guanine nucleotide exchange factor, Epac2, couples cyclic AMP and Ras signals at the plasma membrane. J. Biol. Chem. [Online.] http://www.jbc.org/cgi/content/abstract/M508165200v1.
38. **Lin, S. L., N. N. Johnson-Farley, D. R. Lubinsky, and D. S. Cowen.** 2003. Coupling of neuronal 5-HT7 receptors to activation of extracellular-regulated kinase through a protein kinase A-independent pathway that can utilize Epac. J. Neurochem. **87:**1076–1085.
39. **Lou, L., J. Urbani, F. Ribeiro-Neto, and D. L. Altschuler.** 2002. cAMP inhibition of Akt is mediated by activated and phosphorylated Rap1b. J. Biol. Chem. **277:**32799–32806.
40. **Maridonneau-Parini, I., and J. de Gunzburg.** 1992. Association of rap1 and rap2 proteins with the specific granules of human neutrophils. Translocation to the plasma membrane during cell activation. J. Biol. Chem. **267:**6396–6402.
41. **Mei, F. C., J. Qiao, O. M. Tsygankova, J. L. Meinkoth, L. A. Quilliam, and X. Cheng.** 2002. Differential signaling of Cyclic AMP. Opposing effects of exchange protein directly activated by cAMP and cAMP-dependent protein kinase A on protein kinase B activation. J. Biol. Chem. **277:**11497–11504.
42. **Mochizuki, N., S. Yamashita, K. Kurokawa, Y. Ohba, T. Nagai, A. Miyawaki, and M. Matsuda.** 2001. Spatio-temporal images of growth-factor-induced activation of Ras and Rap1. Nature **411:**1065–1068.
43. **Moriguchi, T., H. Kawasaki, S. Matsuda, Y. Gotoh, and E. Nishida.** 1995. Evidence for multiple activators for stress-activated protein kinases/c-jun amino-terminal kinases.′. J. Biol. Chem. **270:**12969–12972.
44. **Morrison, D. K., and R. J. Davis.** 2003. Regulation of MAP kinase signaling modules by scaffold proteins in mammals. Annu. Rev. Cell Dev. Biol. **19:**91–118.
45. **Nomura, K., H. Kanemura, T. Satoh, and T. Kataoka.** 2004. Identification of a novel domain of Ras and Rap1 that directs their differential subcellular localizations. J. Biol. Chem. **279:**22664–22673.
46. **Norum, J. H., T. Methi, R. R. Mattingly, and F. O. Levy.** 2005. Endogenous expression and protein kinase A-dependent phosphorylation of the guanine nucleotide exchange factor Ras-GRF1 in human embryonic kidney 293 cells. FEBS J. **272:**2304–2316.
47. **Obara, Y., K. Labudda, T. J. Dillon, and P. J. Stork.** 2004. PKA phosphorylation of Src mediates Rap1 activation in NGF and cAMP signaling in PC12 cells. J. Cell Sci. **117:**6085–6094.
48. **Ohtsuka, T., K. Shimizu, B. Yamamori, S. Kuroda, and Y. Takai.** 1996. Activation of brain B-Raf protein kinase by Rap1B small GTP-binding protein. J. Biol. Chem. **271:**1258–1261.
49. **Okada, S., M. Matsuda, M. Anafi, T. Pawson, and J. E. Pessin.** 1998. Insulin regulates the dynamic balance between Ras and Rap1 signaling by coordinating the assembly states of the Grb2-SOS and CrkII-C3G complexes. EMBO J. **17:**2554–2565.
50. **Ozaki, N., T. Shibasaki, Y. Kashima, T. Miki, K. Takahashi, H. Ueno, Y. Sunaga, H. Yano, Y. Matsuura, T. Iwanaga, Y. Takai, and S. Seino.** 2000. cAMP-GEFII is a direct target of cAMP in regulated exocytosis. Nat. Cell Biol. **2:**805–811.
51. **Pham, N., I. Cheglakov, C. A. Koch, C. L. de Hoog, M. F. Moran, and D. Rotin.** 2000. The guanine nucleotide exchange factor CNrasGEF activates ras in response to cAMP and cGMP. Curr. Biol. **10:**555–558.
52. **Posern, G., U. R. Rapp, and S. M. Feller.** 2000. The Crk signaling pathway contributes to the bombesin-induced activation of the small GTPase Rap1 in Swiss 3T3 cells. Oncogene **19:**6361–6368.
53. **Pouyssegur, J., V. Volmat, and P. Lenormand.** 2002. Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. Biochem. Pharmacol. **64:**755–763.
54. **Qiao, J., F. C. Mei, V. L. Popov, L. A. Vergara, and X. Cheng.** 2002. Cell cycle-dependent subcellular localization of exchange factor directly activated by cAMP. J. Biol. Chem. **277:**26581–26586.
55. **Quilliam, L. A., J. F. Rebhun, and A. F. Castro.** 2002. A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases. Prog. Nucleic Acid Res. Mol. Biol. **71:**391–444.
56. **Radha, V., A. Rajanna, and G. Swarup.** 2004. Phosphorylated guanine nucleotide exchange factor C3G, induced by pervanadate and Src family kinases localizes to the Golgi and subcortical actin cytoskeleton. BMC Cell Biol. **5:**31.
57. **Rundell, C. J., C. E. Repellin, and S. J. Yarwood.** 2004. Protease inhibitors prevent the protein kinase A-dependent loss of Rap1 GTPase from the particulate fraction of COS1 cells. Biochem. Biophys. Res. Commun. **315:**1077–1081.
58. **Schmitt, J. M., and P. J. S. Stork.** 2002. PKA phosphorylation of Src mediates cAMP's inhibition of cell growth via Rap1. Mol. Cell **9:**85–94.
59. **Shi, S., M. Noda, and H. Kitayama.** 2004. Rap1 mutants with increased affinity for the guanine-nucleotide exchange factor C3G. Oncogene **23:**8711–8719.
60. **Stork, P. J. S.** 2003. Does Rap1 deserve a bad Rap? Trends Biochem. Sci. **28:**267–275.
61. **Stork, P. J. S., and J. M. Schmitt.** 2002. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol. **12:**258–266.
62. **Tsygankova, O. M., E. Kupperman, W. Wen, and J. L. Meinkoth.** 2000. Cyclic AMP activates Ras. Oncogene **19:**3609–3615.
63. **Vossler, M. R., H. Yao, R. D. York, M. G. Pan, C. S. Rim, and P. J. S. Stork.** 1997. cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell **89:**73–82.
64. **Wu, C., C. F. Lai, and W. C. Mobley.** 2001. Nerve growth factor activates persistent Rap1 signaling in endosomes. J. Neurosci. **21:**5406–5416.
65. **Yan, J., S. Roy, A. Apolloni, A. Lane, and J. F. Hancock.** 1998. Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase. J. Biol. Chem. **273:**24052–24056.
66. **York, R. D., D. C. Molliver, S. S. Grewal, P. E. Stenberg, E. W. McCleskey, and P. J. S. Stork.** 2000. Role of phosphoinositide 3-kinase and endocytosis in nerve growth factor-induced extracellular signal-regulated kinase activation via Ras and Rap1. Mol. Cell. Biol. **20:**8069–8083.
67. **York, R. D., H. Yao, T. Dillon, C. L. Ellig, S. P. Eckert, E. W. McCleskey, and P. J. S. Stork.** 1998. Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature **392:**622–625.
68. **Zhou, K., Y. Wang, J. L. Gorski, N. Nomura, J. Collard, and G. M. Bokoch.** 1998. Guanine nucleotide exchange factors regulate specificity of downstream signaling from Rac and Cdc42. J. Biol. Chem. **273:**16782–16786.