The RAP1 Guanine Nucleotide Exchange Factor Epac2 Couples Cyclic AMP and Ras Signals at the Plasma Membrane*

Yu Li, Sirisha Asuri, John F. Rebhun, Ariel F. Castro, Nivanka C. Paranavitana, and Lawrence A. Quilliam

From the Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, and Walther Cancer Institute, Indianapolis, Indiana 46202

Epac-1 and -2 (exchange proteins directly activated by cyclic AMP) are guanine-nucleotide exchange factors for the GTPases Rap1 and -2. Epac2 but not Epac1 was found to possess a RA (Ras association) domain similar to that found in the Ras effector Rap-GDS. This domain specifically bound Ras-GTP, enabling oncogenic Ras to translocate Epac2 from the cytosol to the plasma membrane. Consequently, a small pool of plasma membrane-bound Rap1 was activated at the expense of bulk Rap1 located on intracellular organelles. Whereas translocation of Epac2 was not mimicked by challenge with epidermal growth factor alone, costimulation with organelles. Whereas translocation of Epac2 was not mimicked by activated at the expense of bulk Rap1 located on intracellular organelles. Whereas translocation of Epac2 was not mimicked by challenge with epidermal growth factor alone, costimulation with forskolin, prostaglandin E2, or an Epac-selective cyclic AMP analog-activated at the expense of bulk Rap1 located on intracellular organelles. Whereas translocation of Epac2 was not mimicked by challenge with epidermal growth factor alone, costimulation with forskolin, prostaglandin E2, or an Epac-selective cyclic AMP analog-induced rapid relocation of GP-Epac2 but not -Epac1 to the plasma membrane in a Ras-dependent manner. Deletion of the cyclic AMP-binding domain overcame the need for nucleotide, suggesting that this domain normally masked the RA domain in the resting GEF. Thus, Epac2 can respond to costimulation by agonists that jointly elevate Ras-GTP and cyclic AMP levels, activating a specific pool of Rap1 at the plasma membrane. Therefore, despite its previous description as a Ras antagonist or independently functioning GTPase, Rap1/Krev-1 may additionally act downstream of Ras in cells that express the cyclic AMP-regulated GEF, Epac2.

Extracellular stimuli promote a large variety of biological events via Ras family proteins. In resting cells, Ras exists in an inactive GDP-bound conformation. Upon stimulation by extracellular ligands, guanine nucleotide exchange factors (GEFs)3 are activated that promote the dissociation of GDP, enabling the more abundant GTP to bind. Association with GTP induces conformational changes enabling Ras to bind and activate downstream effectors such as Raf, phosphatidylinositol 3-kinase, or RalGDS, thus transducing cellular signals. The bound GTP is hydrolyzed back to GDP by the intrinsic GTPase activity of Ras to turn off the signal, a process that is greatly accelerated by GTPase-activating proteins (1, 2).

Ras targets share a common Ras association (RA) domain that interacts with its effector-binding loop. The RA domain was first found in Rap-GDS, a GEF specific for the Ras protein Rap1 (3, 4) and since identified in many other effectors including phosphorylaminositol 3-kinase, AF6, Nore1, and phospholipase C-ε (5–8). The RA domain of Rap-GDS has similar structure to the Ras-binding domain of Raf-1 despite their low sequence similarity (9, 10). Like the Ras-binding domain of Raf-1, the Rap-GDS RA domain binds specifically to Ras-GTP, but not to Ras-GDP. The interaction of activated Ras and Rap-GDS leads to activation of Raf, thus establishing a small GTPase cascade in which one Ras protein regulates another via the recruitment of a GEF for the downstream GTPase (11, 12).

The founding members of the Ras family, H-, K-, and N-Ras, are critical regulators of cell proliferation and differentiation (13). Much less is known about the ~25 Ras family members that include R-Ras, Raf, Ral, Rheb, Rit, and Rap proteins (2). Rap1A was described in 1989 as an antagonist of Ras (Krev-1) that, upon overexpression, could revert the transformed phenotype of cells harboring an oncogenic K-Ras mutation (14). This was thought to be because of its ability to compete with Ras effectors such as c-Raf1 (1). Subsequently it was reported that Rap1A and -B might activate B-Raf, so mimicking Ras in other cell types (reviewed in Ref. 15). More recent studies have revealed that Rap1 also functions independently of Ras to regulate integrins and cell adhesion (reviewed in Refs. 16–18). However, because Rap1 had been reported to localize to endosomes, secretory granules, or the Golgi apparatus (19–22), it was unclear how it might regulate the activities of Raf, which needs to be recruited to the plasma membrane to be activated (23, 24), or integrins that also reside at the cell surface. A study by Matsuda and colleagues (25) suggested that Rap1 might migrate toward the plasma membrane upon activation, whereas others suggested that internalization of receptors is required for Rap1 regulation (21, 26).

It is now evident that over 30 GEFs are responsible for activating various Ras family proteins and many of these activate Rap1 and -2 (2). These GEFs share a catalytic domain consisting of a CDC25 homology domain and a Ras exchange motif (see Fig. 1A). The diversity of additional regulatory domains results in many extracellular-generated signals converging on Rap1 activators. C3G, the first RapGEF identified, is regulated by interaction with Src homology 3 domain of the adapter protein Crk and is targeted to sites of protein-tyrosine kinase activity via the Crk Src homology 2 domain (2, 27). Several other Rap1 GEFs are regulated by diffusible second messengers. GRP2 and -3 are regulated by diacylglycerol and/or Ca2+ in a similar manner to protein kinase C (28, 29). The exchange proteins directly activated by cyclic AMP (Epacs or cAMP-GEFs) (28, 30) represent novel cAMP targets and help explain the protein kinase A (PKA)-independent effects of cAMP (31–35).

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1 Current address: Access Business Group, Ada, MI.
2 Current address: Dept. of Surgery, University of California, San Francisco, CA.
3 Current address: Methodist Research Institute, Indianapolis, IN.
4 To whom correspondence should be addressed: G3 Barnhill Drive, MS-4053, Indianapolis, IN 46202-5122. Tel: 317-274-8550; Fax: 317-274-4666; E-mail: lquilliam@lupel.edu.
5 The abbreviations used are: GEF, guanine nucleotide exchange factor; DsRed, red fluorescent protein from Discosoma sp. reef coral; GFP, green fluorescent protein; GST, glutathione S-transferase; IBMX, isobutyl 3-methylxanthine; 8-Me-cAMP, 8-(4-chloro-phenyl-thio)-2′-O-methyladenosine 3′,5′-cyclic monophosphate; RA, Ras association; PKA, protein kinase A; HA, hemagglutinin; PBS, phosphate-buffered saline; EGF, epidermal growth factor; CMV, cytomegalovirus; GTP-γ-S, guanosine 5′-3′-(O-thio) triphosphate.
Epac1 and Epac2 each contain a cyclic AMP-binding domain similar to that found in the PKA regulatory subunit. CAMP binds directly to Epacs and releases an autoinhibitory effect of the N terminus, leading to Rap1 activation (30). Epac2 also possesses a second, lower affinity cAMP-binding domain (36). Based on CDC25 homology domain alignment, four additional exchange factors belong to the Epac subfamily, namely PDZ-/RA-GEFs 1 and 2 that possess low affinity CAMP binding sites (37–40), MR-GEF (36, 41, 42), and a putative GEF, Link II (GenBank accession number NM016339) that lack CAMP-binding domains (Fig. 1A).

Previously, we and others identified RA domains in PDZ- and MR-GEF that bound to Rap1/2 or M-Ras (39, 41, 43). When we identified similar sequences in Epac1 and -2 we wished to determine which Ras protein(s) they bind and how this interaction might affect Rap1 activation. Here we report that Epac2 but not Epac1 binds specifically to H-, K-, and N-Ras-GTP through its RA domain. When co-expressed with constitutively active H-Ras, Epac2 translocates from cytosol to plasma membrane where Ras resides. Whereas this translocation reduces the total Rap1-GTP level in the cell, it activates a unique pool of Rap1 at the plasma membrane. Stimulation of cells with EGF alone did not induce Epac2 translocation but the GEF was rapidly recruited in the presence of growth factor plus cAMP in a Ras-dependent manner. Therefore Epac2 represents a node that assimilates inputs from both growth factor receptors that activate Ras and from G protein-coupled receptors that activate adenylyl cyclase to selectively activate a specific pool of Rap1 at the plasma membrane. Consequently, Rap1 can function as a downstream target of Ras as part of a novel GTPase cascade as well as its previously suggested roles as a Ras antagonist or independently functioning GTPase (1, 14, 16, 17).

**Materials and Methods**

**Cell Cultures and Transfections**—COS cells, 293T human embryonic kidney cells, and H1299 lung carcinoma cells (ATCC CRL-5803) were cultured in 5% CO2 at 37 °C in Dulbecco's modified Eagle's medium or RPMI 1640 media, supplemented with 10% fetal bovine serum (Equitech), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected using FuGENE 6 (Roche) as described by the manufacturer. H1299 cells stably expressing GFP-Epac2 were selected, ring-cloned, and maintained using 100 μg/ml Geneticin (Invitrogen); cells were further selected and cloned on coverslips, transiently transfected with pDsRed-K-RasVal-12 or -N-RasAsp-12 (or pFlag-CMV2-M-RasLeu-71, Fig. 4, E and F), and processed as above.

**Plasmid Constructs**—pcDNA3-Epac1-FLAG, -Epac2, and -Epac2-FLAG were kindly provided by Johannes Bos (Utrecht) and Jean de la Grange (New York University). Mark Philips (New York University). pcDNA3-Epac1-FLAG, -Epac2, and -Epac2-FLAG together with empty vector, or vector encoding HA-tagged Ras proteins. 24 h later, cells were serum starved overnight, and washed twice in ice-cold PBS. Cells were lysed in 1 ml of Rlf buffer (50 mM Tris-HCl, pH 7.4, 10% glycerol, 200 mM NaCl, 2.5 mM MgCl2, 1% IGEPA, 1 mM phenylmethylsulfonyl fluoride, 19 μg/ml aprotinin) and cell debris was removed by spinning at 18,000 × g at 4 °C for 10 min. The supernatant was pre-cleared with 30 μl of protein A/G-agarose beads (Santa Cruz Biotechnology). 800 μg of cleared lysate was then tumbled with 10 μg of HA antibody (Covance) for 30 min at 4 °C. 40 μl of protein A/G-agarose beads were added for an additional 30 min. The beads were washed 4 times with 1 ml of Rlf buffer and proteins were detected as above using anti-FLAG antibody. 10 μl of lysates were blotted to confirm equal expression levels of proteins from transfected plasmids.

**Immunofluorescence**—H1299 cells were seeded on glass coverslips and transfected with pEGFP-H-RasLeu-61 and/or pCMV2-FLAG-Epac2. 24 h later, cells were serum starved overnight, fixed in 4% paraformaldehyde in PBS for 10 min, and permeabilized in 0.2% Triton X-100 in PBS for 5 min. After blocking in PBS containing 0.2% bovine serum albumin, 2 mM MgCl2, 10% goat serum, 25 mM glycine, and 25 mM lysine for 30 min, cells were washed twice in PBS containing 0.2% bovine serum albumin and 2 mM MgCl2, and incubated with M2 antibody for 1 h, followed by two washes and a 1-h incubation with Alexa Fluor 594-conjugated goat anti-mouse IgG (Molecular Probes). After two final washes, coverslips were mounted onto slides using mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories). Confocal images were taken using a Zeiss LSM-510 confocal microscope equipped with laser lines at 364, 488, and 543 nm and PMT detectors. Images were transformed to tiff format and edited using Adobe Photoshop 5.5. For Fig. 5, H1299 cells were transfected with pEGFP-Epac2 or -Epac2ΔRA and stable transfectants were selected on G418. Individual colonies were ring cloned and those with modest green fluorescent protein (GFP) expression chosen. These cells were plated on coverslips, transiently transfected with pDsRed-K-RasVal-12 or -N-RasAsp-12 (or pFlag-CMV2-M-RasLeu-71, Fig. 4, E and F), and processed as above.

**Plasma Membrane Fractionation**—Sucrose gradient ultracentrifugation was performed as described (45) except 2.5 mM MgCl2 was included in buffers. Four confluent 100-mm plates of H1299 cells were used for each data point. Briefly, serum-starved cells were washed with ice-cold PBS, collected in 10 ml of 225 mM sucrose, 20 mM Hepes, pH 7.4, 1 mM EDTA, 2.5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 19 μg/ml aprotinin, and lysed by passing through a 26-gauge needle 10 times. The lysate was centrifuged twice at 10,000 × g at 4 °C for 10 min, and the pellet was resuspended in the above buffer and layered onto a 1.2 M sucrose cushion prior to ultracentrifugation, 100,000 × g at 4 °C for 10 min. The interface, or plasma membrane fraction, was pelleted and resuspended in Rlf buffer to solubilize membranes/Rap1 and 20 μg of plasma membrane fraction was tumbled for 4 h with 20 μg of GST-RalGDS-RA-bound to glutathione-agarose beads. The beads were washed 4 times and bound FLAG-Rap1-GTP was eluted and visualized by immunoblotting with anti-FLAG M2 antibody. Plasma membranes were confirmed to be enriched in integrin VLA2 and to lack detectable Golgi marker GM130 by immunoblotting using antibodies from Santa
Cruz Biotechnology,6 Endosomal contamination was determined by blotting with anti-EEA1 antibody (Transduction Laboratories).

Radioactive Assay to Measure Rap-GTP—Rap1-GTP levels were determined by 32P, labeling of guanine nucleotide pools, immunoprecipitation of Rap1, thin layer chromatography of Rap1-bound GDP and GTP, and detected using an Ambis β scanner essentially as described previously (44). Statistical significance was determined using an unpaired Student’s t test.

Ligand-stimulated GFP-Epac2 Translocation—H1299 cells stably expressing GFP-Epac2 alone, or in addition to N-RasWT were seeded on glass-bottomed culture dishes (MatTek, MA). 48 h later, cells were serum starved overnight. Before stimulation, 50 mM Hepes, pH 7.5, was added to maintain the pH of the media in air. Cells were kept at 37 °C using a stage warmer, and stimulated with 50 ng/ml EGF, 50 μM forskolin, 0.2 mM isobutyl 3-methylxanthine (IBMX), 100 μM 8CPT-2Me-CAMP (BIOLOG), or 10 μM prostaglandin E2, alone or in combination as described in the legend to Fig. 7. Green fluorescence images were taken before and after stimulation using a Zeiss LSM-510 confocal microscopy. For PKA inhibition, cells were preincubated with 20 μM H89 (Sigma) for 30 min prior to stimulation.

RESULTS

Epac2 Specifically Binds to Activated H-Ras—RA domains in effector proteins are responsible for coupling them to upstream activation by their cognate GTPases (13). We previously demonstrated that the Rap-specific exchange factor, MR-GEF, binds activated M-Ras through its RA domain and regulates Rap1 activity (41). Similarly PDZ-GEF1 binds activated Rap1 through a RA domain interaction (39, 41). Because Ras also activates Ral by binding the RA domain of RalGDS (11, 12), we wished to address if Ras-mediated regulation of GEFs is a more global phenomenon.

Putative RA domains were identified in Epac1 and -2 (Fig. 1) as well as several other Ras family GEFs that included phospholipase C-e, Link II, and Graspl (2). Similarly to RalGDS family members, RA domains were located in the C termini of phospholipase C-e and Graspl but are found immediately N-terminal to the CDC25 homology (catalytic) domain in Epacs, Link II, MR-, and PDZ-GEFs (Fig. 1A). To establish if the RA-like sequences in Epac1 and Epac2 were functional, and if so, which GTP-bound Ras protein they bound to, FLAG-tagged Epac1 or -2 were expressed in COS cells. Lysates from cells overexpressing these GEFs were incubated with glutathione-agarose beads coupled to GST-fused Ras proteins, as indicated in Fig. 2A, that had been preloaded with GTPγS, a non-hydrolyzable analog of GTP. Any GEF bound to the beads was detected by Western blot with FLAG antibody. As shown in Fig. 2A, Epac2 specifically bound GTPγS-loaded H-Ras. In contrast, Epac1 failed to associate with any of nine Ras family GTPases.

The binding of Epac2 to Ras depended on the activation state of H-Ras in vitro because Epac2-containing lysates bound specifically to GTPγS-, but not GDP-loaded GST-H-Ras (Fig. 2B). Under these conditions Epac1 did not bind to either form of H-Ras. Having demonstrated that Epac2 specifically binds GTP-loaded H-Ras in vitro, we next addressed if this interaction also occurs in vivo and whether Epac2 also interacted with K- or N-Ras. Following coexpression of HA-tagged Ras mutants with Epac2, Ras proteins were immunoprecipitated with HA antibody, and Epac2 was found to co-precipitate with oncogenic Ras mutants, but not with the closely related M-RasLeu-71 that has an activating mutation equivalent to H-RasLeu-61 (Fig. 2C). Interestingly, K- and N-Ras bound more effectively to Epac2 than did H-Ras. Binding to activated H-Ras proteins containing Ser35, Gly37, or Cys40 mutations in their effector-binding loop was diminished (Fig. 2D). Collectively, these
data suggested that Epac2 specifically interacts with activated H-, K-, and N-Ras in vivo through an interaction with its effector loop.

Binding of Epac2 to Ras-GTP Is Dependent on the RA Domain of the GEF—To establish that association of Ras-GTP with Epac2 was mediated by the putative RA domain of the GEF rather than the Rap1-binding CDC25 homology domain, we deleted residues 650–689 that are predicted to form the Ras binding surface of the RA domain (9), and examined the ability of this Epac2RA mutant to bind activated H-Ras. GTPγS-loaded H-Ras failed to pull down Epac2RA in vitro under conditions that supported an interaction with full-length Epac2 (Fig. 2B). Furthermore, no Epac2RA was co-immunoprecipitated with the active mutant H-RasG12V, despite efficient coprecipitation of wild type Epac2 in vivo (Fig. 3A), indicating that the RA domain is required for the binding of Epac2 to activated H-Ras. Epac2RA retained its ability to activate Rap1 in vivo (Fig. 3B), demonstrating that partial deletion of the RA domain did not perturb the intrinsic catalytic activity of the exchange factor. Furthermore, forskolin stimulation of cells equally enhanced the abilities of Epac2 and Epac2RA to activate Rap1.7 Therefore, deletion of the RA domain also did not perturb the ability of the cyclic AMP-binding domain to inhibit GEF activity.

Ras-GTP Binding Translocates Epac2 from Cytosol to Plasma Membrane—Subcellular localization is one important mechanism whereby association with Ras can regulate downstream effectors such as Raf (23, 24) and RalGDS (11, 46). To determine whether binding to activated H-Ras altered the subcellular distribution of Epac2, we directly visualized the two molecules using confocal fluorescence microscopy. Although H-Ras can reside on both plasma and Golgi membranes (47), we found that GFP-tagged RasG12V was predominantly restricted to the plasma membrane of H1299 lung carcinoma cells (Fig. 4A) making this a convenient cell line to monitor Ras-induced subcellular relocalization of Epac2. H1299 cells transfected with FLAG-Rap1 showed predominantly cytosolic staining (Fig. 4B). However, upon coexpression with GFP-H-RasG12V (Fig. 4C, D), the GEF was predominantly redistributed to the plasma membrane (Fig. 4D) where it colocalized with Ras (Fig. 4C). Similar findings were subsequently observed upon coexpression of Epac2 with activated K- and N-Ras (Fig. 5) but not M-RasG12V (Fig. 4F). This suggested that activated Ras translocates Epac2 from cytosol to plasma membrane. This observation was supported by subcellular fractionation analysis; more Epac2 was detected in the plasma membrane fraction in the presence of H-RasG12V (Fig. 4G).

To prove that translocation of Epac2 was because of its association with Ras, rather than a secondary effect of oncogenic Ras expression, we transfected H1299 cells with the Epac2RA mutant. Similarly to wild type Epac2 (Fig. 4B), Epac2RA was found in the cytosol of H1299 cells. However, upon coexpression with activated K- or N-Ras, Epac2RA failed to translocate to the plasma membrane (Fig. 5, F and H), presumably because of its inability to bind to Ras. Some association with intracellular structures was observed but this may be because of misfolding and accumulation of overexpressed GFP and DsRed fusion proteins in aggresomes (48).
Ras Regulates Rap1 via Epac2

FIGURE 4. H-RasLeu-61 targets Epac2 to the plasma membrane. H1299 cells were cultured on coverslips and transfected with plasmids encoding GFP-tagged H-RasLeu-61 and/or FLAG-Epac2. GFP was detected at 505–530 nm and the FLAG epitope was detected by indirect immunofluorescence using confocal microscopy. A, GFP-H-RasLeu-61, expressed alone was mostly localized to the plasma membrane. B, FLAG-Epac2 expressed alone resided in the cytosol. In panels C and D, cells were transfected with both constructs. Membrane localization of H-RasLeu-61 is shown in panel C and colocalization of Epac2 is shown in D. Panels E and F show failure of FLAG-M-RasLeu-61 (arrow) to translocate GFP-Epac2 to the plasma membrane. G, more Epac2 was found in the plasma membrane fraction upon coexpression with H-RasLeu-61. H1299 cells were transfected with empty pCMV2-FLAG vector or pCMV2-FLAG-Epac2 alone, or together with pCGN-H-RasLeu-61. A plasma membrane (PM) fraction was collected by sucrose gradient centrifugation and FLAG-Epac2 levels were compared with those in the total cell homogenate by immunoblotting. A nonspecific band migrated just below Epac2 in the whole cell homogenate. Data are representative of four experiments.

FIGURE 5. The RA domain of Epac2 is required for K- and N-Ras-induced membrane targeting. H1299 cells stably expressing GFP-Epac2 or GFP-Epac2DeltaRA were transiently co-transfected with plasmids encoding Ds-Red fluorescent N-RasRap1-12 or K-RasVal-12 fusion proteins as indicated above the figures. Subcellular localization of Ds-Red proteins (upper panels) or GFP-Epac2 mutant (lower panels) was determined by confocal microscopy as described in the legend to Fig. 4. Unlike GFP-Epac2 (panels E and G), GFP-Epac2DeltaRA failed to localize with Ras proteins on the cell membrane (panels F and H). Images representative of three experiments.

Translocation of Epac2 to the Plasma Membrane Alters Its Substrate Pool—We demonstrated above that Epac2 binds specifically to activated Ras, and that this binding translocates the GEF from cytosol to plasma membrane. To address what effect this relocalization might have on Rap1 activation we measured Rap1-GTP levels. Following transfection of H1299 cells with Epac2, total Rap1-GTP levels were elevated as expected. However, upon co-expression of H-RasLeu-61 the activity of Rap1 was significantly reduced (Fig. 6A). Because the bulk of HA-tagged Rap1A protein was detected on perinuclear membranes with only a small fraction (see arrow on Fig. 6A) detectable at the cell periphery, C, plasma membrane-associated Rap1 is activated upon Epac2 re-localization. H1299 cells were transfected with FLAG-Rap1, together with empty vectors or vectors encoding Epac2-FLAG, or Epac2-FLAG plus HA-H-RasLeu-61. Plasma membrane (PM) fractions were prepared by sucrose gradient centrifugation. These and a fraction of solubilized total lysate were incubated with glutathione-agarose beads coupled with GST-fused RaLGDS-RA to pull-down Rap1-GTP. Despite reduced expression of Epac2 in the presence of H-Ras (right lane, fourth panel) H-Ras enhanced the ability of Epac2 to activate plasma membrane-associated Rap1 (upper panel) under conditions where total Rap1-GTP levels were reduced. It was necessary to immunoprecipitate Epac2 from lysate prior to detection by Western blot because of only modest expression. Data are representative of four experiments.

FIGURE 6. H-Ras-induced translocation of Epac2 to the plasma membrane regulates the activity of distinct Rap1 pools. A, H-Ras inhibits Epac2-induced Rap1 activation in whole cell lysates. H1299 cells were transfected with FLAG-tagged Rap1, together with empty vector or vector-encoding Epac2 in the absence or presence of H-RasLeu-61 as indicated. Rap1-GTP levels were measured as described in the legend to Fig. 3C. Pooled data from three independent experiments (mean ± S.D.) are shown. Epac2 expression was determined by Western blot, and quantified in arbitrary units. One representative blot is shown. Excess Epac2-encoding plasmid was transfected in the presence of Ras to offset the reduced expression of Epac2 that occurred under this condition, see right lanes of C and Fig. 4E. H-Ras-induced activation of Rap1 was measured by pull-down of Rap1-GTP from whole cell lysates. B, subcellular localization of Rap1 in H1299 cells. H1299 cells transfected with pCGN-Rap1 were fixed and stained with anti-HA. The bulk of the protein was perinuclear, with only a small fraction (arrow) detectable at the cell periphery. C, plasma membrane-associated Rap1 is activated upon Epac2 re-localization. H1299 cells were transfected with FLAG-Rap1, together with empty vectors or vectors encoding Epac2-FLAG, or Epac2-FLAG plus HA-H-RasLeu-61. Plasma membrane (PM) fractions were prepared by sucrose gradient centrifugation. These and a fraction of solubilized total lysate were incubated with glutathione-agarose beads coupled with GST-fused RaLGDS-RA to pull-down Rap1-GTP. Despite reduced expression of Epac2 in the presence of H-Ras (right lane, fourth panel) H-Ras enhanced the ability of Epac2 to activate plasma membrane-associated Rap1 (upper panel) under conditions where total Rap1-GTP levels were reduced. It was necessary to immunoprecipitate Epac2 from lysate prior to detection by Western blot because of only modest expression. Data are representative of four experiments.
Epac2 is Redistributed in Response to a Combination of Cyclic AMP Elevation and Growth Factor-induced Ras Activation. To address if the above translocation could occur in response to ligand-induced Ras activation, we established an H1299 cell line stably expressing a low level of N-RasWT. Following serum starvation, addition of EGF failed to promote significant redistribution of Epac2 (Fig. 7C). However, when co-challenged with the cAMP elevating agents forskolin and IBMX, GFP-Epac2 was rapidly (within 1 min) translocated to the plasma membrane (Fig. 7B). Challenge with forskolin/IBMX alone was without effect (Fig. 7D). These data suggest that cAMP may induce a conformational change in Epac2 that subsequently enables Ras binding to the GEF and its redistribution to the plasma membrane. Inhibition of PKA by preincubation with H89 did not affect translocation of Epac2 induced by forskolin/IBMX plus EGF (Fig. 7E). In addition, the Epac-selective cAMP analog, 8CPT-2Me-cAMP (49), acting together with EGF also induced Epac2 translocation (Fig. 7F), suggesting that this is a PKA-independent event. Co-challenge of cells with EGF and the cAMP-elevating hormone, prostaglandin E2 also promoted Epac2 translocation (Fig. 7G), suggesting that redistribution of Epac2 could occur under physiological conditions. Cyclic AMP and EGF-induced Epac2 translocation were also observed in the absence of exogenously expressed N-RasWT (compare Fig. 7, H and I), demonstrating that activation of endogenous Ras is sufficient to induce GEF redistribution. Interestingly, cell spreading (see arrows in Fig. 7, A and B) and ruffling, especially at the base of cells, was also observed in response to elevated cAMP/EGF stimulation, but not either agent alone. This is consistent with reports that Rap1 regulates integrin-mediated cell adhesion and spreading (16, 17).

To confirm that the above events were specifically because of EGF-induced Ras activation as opposed to other growth factor-induced signaling events, we first preincubated cells with the phosphatidylinositol 3-kinase inhibitor LY294002. This had no effect on the ability of EGF plus forskolin/IBMX to induce Epac2 translocation (Fig. 8A), suggesting that EGF does not recruit Epac2 via phosphatidylinositol 3-kinase activation. In contrast, when EGF-induced Ras activation was blocked by transient transfection of a dominant-inhibitory Ras17N mutant, Epac2 translocation to the plasma membrane was completely abrogated (Fig. 8B). Deletion of the Epac2 RA domain also prevented EGF translocation in response to EGF/cAMP (Fig. 8C). Furthermore, GFP-Epac1, which fails to bind to Ras-GTP, could not be recruited to the plasma membrane (Fig. 8D). Instead Epac1 localized to the nuclear membrane and punctate structures that might represent mitochondria as previously reported (50). Finally, deletion of the N-terminal cAMP binding region (encompassing the two cAMP-binding domains and DEP domain) of
Epac2 resulted in Epac2ΔCBR association with the plasma membrane even in the absence of stimuli (Fig. 8E). This localization was augmented by EGF stimulation but not by cAMP elevation (see loss of perinuclear staining in Fig. 8E), presumably because of increased Ras activation. These data suggest that, in addition to promoting Epac2 activation, the role of cAMP is to expose the RA domain, so enabling Ras-mediated recruitment of Epac2 to the plasma membrane.

**DISCUSSION**

In the absence of cAMP, Epac2 has been reported to exist in an inactive conformation such that access of Rap1 to its GEF domain is blocked by an intramolecular interaction between the high-affinity cAMP-binding domain and the catalytic region (36). When cAMP accumulates, nucleotide binding promotes a conformational change in Epac2 that uncouples the cAMP-binding domain from the catalytic region, enabling Rap1 activation (51, 52). We now report that growth factors that activate Ras can recruit Epac2 to the plasma membrane, so influencing the pool of Rap1 that is activated (summarized in Fig. 9). However, this translocation can only occur following cAMP-induced opening of the Epac2 molecule. Thus, Epac2 functions as a coordinator of growth factor and second messenger-induced signaling pathways.

Failure of EGF to promote translocation of Epac2 to the plasma membrane in the absence of cAMP elevation was likely because of the N terminus of the GEF blocking access of Ras to the RA domain in a similar manner to occluding the catalytic domain. We propose that the conformational change that occurs to provide Rap1 access to the catalytic domain also facilitates Ras-GTP binding to the RA domain. This is consistent with the constitutive membrane association of the Epac2ΔCBR mutant and its enhancement by EGF in the absence of cAMP (Fig. 8E). The ability of oncogenic Ras to relocate Epac2 in the absence of forskolin was likely because of opportunistic association of constitutively active Ras with a small fraction of Epac2 that adopts an open conformation in the absence of elevated adenyl cyclase activity, demonstrating how oncogenic Ras might promote a different biological outcome to its wild-type counterpart. That deletion of the RA domain disrupted Ras binding and Epac2 translocation but not intrinsic exchange activity is supportive of this domain being responsible for Ras-mediated regulation of Epac2. Epac2 has been implicated in the regulation of insulin secretion in pancreatic β cells (53). However, Ras has not previously been associated with insulin release. Existing reagents did not permit us to probe the endogenous Epac2-Ras interaction in insulinoma cells. It therefore remains to be determined what physiological events are regulated by the convergence of cAMP and Ras signaling pathways at the level of Epac2.

Surprisingly N- and K-Ras bound more effectively than H-Ras with Epac2 despite all three Ras proteins having identical effector-binding domains. However, N- and K-Ras have also been reported to bind the RA domain of PDZ-GEF (54), whereas H-Ras did not (41). This suggests that regions outside the effector binding loop of Ras may contribute to its downstream signaling specificity.

The RA domain of Epac2 is located between its Ras exchange motif and catalytic/CDC25 homology domain (Fig. 1A). This contrasts with Ras-GDS family members where the RA domain is located at the C terminus (1). Interestingly, a second H-Ras binding site on the Ras GEF, Sos1, has recently been identified. This site is found N-terminal to the CDC25 domain and encompasses the Ras exchange motif (55). Thus, although Sos1 lacks a RA domain, GT-Pase interaction with a region N-terminal to the CDC25 domain of GEFs may be a more general event. The crystal structure of Sos-Ras-GTP suggests that Ras stabilizes the active site of the GEF, a notion supported by the ability of Ras-GTP to increase Sos exchange activity in vitro (55). It will be interesting to determine whether H-Ras can also regulate the intrinsic exchange activity of Epac2 and to compare the structure of Epac2 with that of Sos1.

Because Ras proteins are post-translationally prenylated and associated with cellular membranes it is not surprising that regulation of their upstream activators should include membrane targeting. It is, however, surprising that GEF regulation involves recruitment by other Ras family proteins. In addition to the well-characterized Ras effector, RaLGDS, several Rap GEFs contain RA domains (Fig. 1) and association with M-Ras, Rap1, or Rap2 can influence their subcellular localization (31–35, 39, 41, 43). Although the majority of Rap1 is reported to be on intracellular membranes (Fig. 6B) (19–22), Rap1 is concentrated at the periphery of epithelial cells (56–58) where the effect of Epac2 relocation might have a much larger impact. A recent study demonstrated that Rap1 is recruited from an endosomal compartment and activated at the plasma membrane of fibroblasts and T cells (58). In addition, Rap1 is activated just under the plasma membrane in response to actin polymerization (59). Because Rap1 regulates the avidity and/or affinity of integrins for extracellular matrix proteins at the cell surface (16–18, 60), recruitment of Epac2 to this locale might greatly facilitate this Rap1 function. Whereas Bivona et al. (58) reported that Rap1 is only activated upon translocation to the plasma membrane, the fact that redirection of Epac2 to this locale by Ras reduced Rap1-GTP levels would argue that Rap1 can also be activated intracellularly. This is unlikely to be an artifact of exogenous Epac2 expression in the current study because the levels of Epac2 expression in H1299 cells were only modest (requiring immunoprecipitation with anti-FLAG antibody prior to detection in Fig. 6C). Another possibility was that the plasma membrane fraction was contaminated with other organelles. However, immunoblotting indicated that there was considerably less of the Golgi (GM130) or minimal endosomal (EEA1) markers in the plasma membrane fraction versus total cell lysate following sucrose gradient purification.

Despite considerable structural similarity, several differences in the regulation and function of Epac1 and -2 are emerging. As reported here, Epac1 lacks a functional RA domain and so, unlike Epac2, is insensitive to Ras regulation. This is likely because of the absence of key basic residues within the RA-like region of Epac1 compared with Epac2, RaLGDS etc. (see Fig. 1B) that are required for Ras interaction with RA domains (61, 62). Epac1 contains a DEP domain that contributes to physiological events are regulated by the convergence of cAMP and Ras signaling pathways at the level of Epac2. It therefore remains to be determined what physiological events are regulated by the convergence of cAMP and Ras signaling pathways at the level of Epac2. Surprisingly N- and K-Ras bound more effectively than H-Ras with Epac2 despite all three Ras proteins having identical effector-binding domains. However, N- and K-Ras have also been reported to bind the RA domain of PDZ-GEF (54), whereas H-Ras did not (41). This suggests that regions outside the effector binding loop of Ras may contribute to its downstream signaling specificity.

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nuclear membrane binding (Refs. 36 and 50, and Fig. 8C). We only observed (plasma) membrane association of Epac2 upon extreme overexpression or at high cell confluence,6 questioning the role of its putative DEP domain. Epac1 contains a functional mitochondria localization signal at its N terminus (50) that is not present in Epac2, consistent with the lack of punctate staining of tagged Epac2 in H1299 cells. Instead, the N terminus of Epac2 is required for Rim2 binding to modulate insulin secretion (63). Finally, Epac1 can dissociate from the nuclear membrane during metaphase and localize to the mitotic spindle and centrosomes (50), whereas GFP-Epac2 remained diffused in the H1299 cytosol during cell division.6 All these findings suggest that Epac1 and -2 play quite different roles in regulating Rap activity in response to cAMP. It is possible that some of the differential effects of cAMP on Rap1 activation reported in various cell lines (discussed in Ref. 15), might be explained by the two Epac isoforms having distinct subcellular localization and/or regulation by Rap.

Epac2 protein level was reduced upon coexpression of oncogenic Ras (see Figs. 4G and 6C). This is unlikely because of reduced transcription as oncogenic Ras typically stimulates gene expression from the CMV promoter. Because the expression levels of the mammalian Ras GEFs, Sos2 and GRF2, plus Saccharomyces cerevisiae cdc25p are controlled by ubiquitin–targeted degradation following Ras binding (64–66) it is possible that Epac2 meets a similar fate. GRF2 and cdc25p contain a destruction box (D box) similar to those found in cyclins (66). Interestingly, mutation of the cdc25 domain to disrupt Ras binding also eliminated ubiquitination of GRF2 (65). Degradation of the Rap GEF, PDZ-GEF1, following NEDD4-mediated ubiquitination also required Rap1 binding (38). Although no well conserved D box could be identified in Epac2, sequence alignment identified a KEN box (67) between residues 38125–38130.

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