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Molecules and Cells

Chemoattractant-Mediated Rap1 Activation Requires GPCR/G Proteins

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Rap1 is rapidly activated upon chemoattractant stimulation and plays an important role in cell adhesion and cytoskeletal reorganization during chemotaxis. Here, we demonstrate that G-protein coupled receptors and G-proteins are essential for chemoattractant-mediated Rap1 activation in *Dictyostelium*. The rapid Rap1 activation upon cAMP chemoattractant stimulation was absent in cells lacking chemoattractant cAMP receptors cAR1/cAR3 or a subunit of the heterotrimeric G-protein complex Gα2. Loss of guanylyl cyclases GCA/SGC or a cGMP-binding protein GbpC exhibited no effect on Rap1 activation kinetics. These results suggest that Rap1, a key regulator for the regulation of cytoskeletal reorganization during cell movement, is activated through the G-protein coupled receptors cAR1/cAR3 and Gα2 proteins in a way independent of the cGMP signaling pathway.

INTRODUCTION

The first step in chemotaxis is the binding of chemoattractants to cell surface G-protein coupled receptors (GPCRs). Upon ligand binding, GPCRs activate heterotrimeric G proteins and promote dissociation of the three subunits as Gα-GTP and a Gβγ dimmer. Both of the activated G-protein subunits subsequently regulate a diverse set of downstream effectors and help convert the extracellular signal into the cell interior (Jin et al., 2008; Kortholt and van Haastert, 2008; Ridley et al., 2003; Sasaki and Firtel, 2006).

Upon starvation, *Dictyostelium discoideum* undergoes a tightly regulated multicellular developmental process in which they secrete and chemotax toward cAMP, leading to the eventual formation of fruiting bodies (Chisholm and Firtel, 2004). The binding of chemoattractant cAMP to the surface receptors activates several signaling cascades that cause cells to crawl toward the source of cAMP (Chisholm and Firtel, 2004; Manahan et al., 2004). In *Dictyostelium*, four cAMP receptors (cAR) have been identified. Of the identified receptors, cAR1 has a high affinity for cAMP and is essential for early development and chemotaxis (Dormann et al., 2001). One G-protein β subunit and one Gγ subunit have been identified (Manahan et al., 2004). Of the 11 identified Gα subunits, Gα2, coupled to the cAR1 receptor, is most important for cAMP-mediated chemotaxis (Kumagai et al., 1989; Rietdorf et al., 1997).

Small GTPase Rap1 is involved in the control of diverse cellular processes, including integrin-mediated cell adhesion, cadherin-based cell-cell adhesion, and cell polarity in mammalian cells, as well as cell adhesion, phagocytosis, and cell migration in *Dictyostelium* (Bos, 2005; Jeon et al., 2007b; Kooistra et al., 2007; Lau and Haigis, 2009). Rap1 is the closest homologue of the small GTPase Ras and, like Ras, cycles between an inactive GDP-bound and an active GTP-bound form (Bos, 2005). Recently it has been reported that spatial and temporal regulation of Rap1 activity by Rap1 GTPase-activating proteins (GAPs), RapGAP1, RapGAPB, and RapGAP3 is required for proper cell migration, cell differentiation, and development (Jeon et al., 2007a; 2009; Parkinson et al., 2009). GbpD, which was previously identified as a putative cGMP-binding protein and contains a CDC25-homology domain, is known as a Rap1-specific guanine nucleotide exchange factor (GEF) (Kortholt et al., 2006).

Several studies in *Dictyostelium* have revealed important roles of Rap1 in cAMP-mediated chemotaxis. Rap1 is rapidly and transiently activated upon chemoattractant stimulation. The activated Rap1 regulates cell adhesion and helps establish cell polarity by locally modulating myosin II assembly and disassembly through the Rap1/Phg2 signaling pathway (Jeon et al., 2007a; 2007b). However, the molecular mechanism underlying Rap1 activation by chemoattractant stimulation is not yet clearly understood. Here, we demonstrate that GPCRs and G proteins are essential for chemoattractant-mediated Rap1 activation. Furthermore, our data suggest that Rap1 activation upon chemoattractant stimulation is independent of cGMP signaling.

MATERIALS AND METHODS

Materials and cell culture

We obtained anti-myc antibodies from Santa Cruz Biotechnology (USA) and glutathione-Sepharose beads from Amersham Biosciences (USA). The *car1/car3, gα2, gbpC*, and *gca/sgc* null strains were obtained from the DictyBase Stock Center, and myc-tagged Rap1 and RalGDS-YFP expression vectors were obtained from R. Firtel (University of California, USA). *Dictyostelium* strains were grown in HL5 axenic media or in asso-

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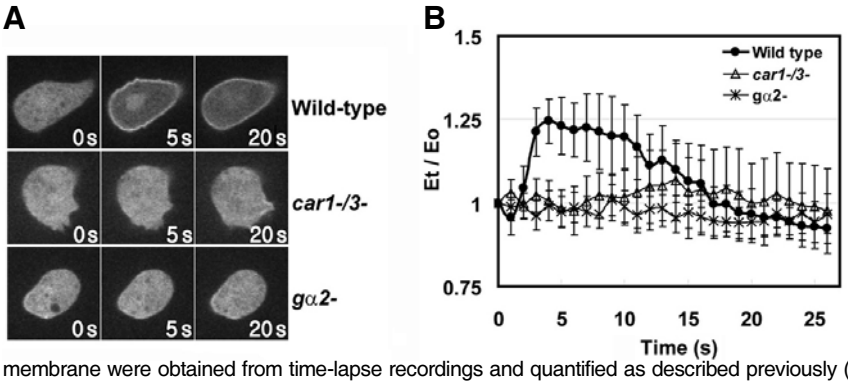

Fig. 1. Localization of RalGDS-YFP, a Rap1-GTP reporter, in cells lacking GPCR/G proteins. (A) Localization of activated Rap1 upon uniform cAMP stimulation. Translocation of RalGDS-YFP, a Rap1-GTP marker protein, to the cell cortex in response to uniform cAMP-chemoattractant stimulation in wild-type KAx-3 cells and in cells lacking GPCRs, cAR1/cAR3, or a subunit of a trimeric G protein Gα2 was imaged. The images pictured at the indicated times after stimulation were obtained from time-lapse recordings. (B) Translocation kinetics of RalGDS-YFP to the membrane were obtained from time-lapse recordings and quantified as described previously (Sasaki et al., 2004). The fluorescence intensity of membrane-localized YFP fusion protein was quantified as Et/Eo. The graph represents the mean of data from several cells in videos taken from at least three separate experiments. Error bars represent SD.

ciation with *Klebsiella aerogenes* at 22°C. The knock-out strains and transformants were maintained in 10 μg/ml blasticidin or 20 μg/ml G418.

Rap1 activation pull-down assay

The Rap1 activation assay was performed as described previously (Franke et al., 1997; Jeon et al., 2007b) with slight modifications. The Rap1-GTP-binding domain (RBD) of mammalian RalGDS was expressed in *Escherichia coli* as a GST fusion protein and purified using glutathione-coupled Sepharose beads, as described previously (Franke et al., 1997). The purified GST-RBD was used for detecting activated Rap1. Aliquots (300 μl) of aggregation-competent cells were stimulated with 15 μM cAMP and then lysed by mixing with an equal volume of lysis buffer at the indicated times. The lysates were cleared by centrifugation for 10 min followed by incubation with 10 μg GST-RBD on glutathione-Sepharose beads at 4°C for 30 min. The beads were washed and resuspended in 30 μl of 2 × sample buffer, followed by SDS-PAGE and Western blot analysis with an anti-myc antibody. For the control of input amount of total Rap1 in the assay, the same volume of aggregation-competent cells was centrifuged and resuspended in 300 μl 2 × sample buffer without any cAMP stimulation and incubation with glutathione-Sepharose beads, and then subjected to the following experiments.

***In vivo* Rap1 activation assay using RalGDS-YFP**

Log-phase vegetative cells expressing YFP-fusion RBD of RalGDS proteins were washed three times with Na/K phosphate buffer and shaken at a density of 5×10^6 cells/ml in Na/K phosphate buffer for 7 h to obtain cAMP-responsive, aggregation-competent cells. A small volume of the aggregation-competent cells (~100 μl) was placed on a 30-mm Petri dish, containing ~3 ml of Na/K phosphate buffer, with a hole covered by a glass coverslip, and the cells were allowed to adhere to the plate for 10 min. The cells were uniformly stimulated with cAMP by quickly pipetting 250 μl of 150 μM cAMP into the plate containing cells (Jeon et al., 2007b; Sasaki et al., 2004). The fluorescence images of RalGDS-YFP translocation to the membrane in response to uniform chemoattractant stimulation were taken at time-lapse intervals of 1 s for 1 min using an inverted microscope (IX71; Olympus, Japan) with a camera (DS-Fi1; Nikon, Japan). Membrane and cortical localization of YFP fusion proteins was quantified as described previously (Sasake et al., 2004). The frames were captured using NIS-elements software (Nikon) and analyzed using ImageJ software (National Institutes of Health, USA). The intensity of cortical YFP was measured and the level of cortical YFP was calculated by dividing the intensity before stimulation (Eo) by the intensity at each time point (Et).

RESULTS AND DISCUSSION

GPCRs/G proteins are required for chemoattractant-mediated Rap1 activation

In *Dictyostelium*, Rap1 is rapidly and transiently activated in response to cAMP chemoattractant stimulation and plays an important role in reorganizing cell cytoskeleton and cell-substratum attachment (Jeon et al., 2007a; J2007b). It has been well established that the initial response of the cells to extracellular cAMP chemoattractant stimulation is mediated by GPCRs and heterotrimeric G proteins (Chisholm and Firtel, 2004; Jin et al., 2008; Ridley et al., 2003). Therefore, it is postulated that GPCRs/G proteins are required for Rap1 activation in response to chemoattractant stimulation. However, the relationship between GPCRs/G proteins and chemoattractant-mediated Rap1 activation has not yet been determined. As part of our on-going work to understand the molecular mechanism underlying Rap1 activation upon chemoattractant stimulation, we determined first whether the GPCRs and the receptor-associated G proteins are required for Rap1 activation by measuring Rap1 activation kinetics in two mutant strains deficient in the cAMP receptors cAR1/cAR3 or Gα2.

In vivo Rap1 activation can be visualized using a YFP-tagged RBD of RalGDS (RalGDS-YFP), a Rap1-GTP reporter, which binds to Rap1-GTP and thus monitors Rap1-GTP levels and its location in cells (Jeon et al., 2007a; 2007b; Kang et al., 2002). We prepared *car1/car3* or *gα2* null cells expressing RalGDS-YFP and investigated localization and translocation of RalGDS-YFP to the cell membrane upon uniform chemoattractant stimulation. Before chemoattractant stimulation, RalGDS-YFP was mainly localized in the cytosol of the cells (Fig. 1A). Upon cAMP chemoattractant stimulation, wild-type cells displayed a rapid accumulation of RalGDS-YFP at the cell cortex with a peak at ~5 s followed by delocalization, as previously reported (Jeon et al., 2007b), suggesting that Rap1 is rapidly and transiently activated at the cell cortex in response to chemoattractant stimulation. In contrast, no such rapid and transient accumulation of RalGDS-YFP at the cell cortex was observed after chemoattractant stimulation in the two mutant strains, *car1/car3* null cells and *gα2* null cells (Figs. 1A and 1B). Although the mutant strains exhibited an even distribution of RalGDS-YFP in the cell cytosol before chemoattractant stimulation, as in wild-type cells, the level of RalGDS-YFP in the mutant cells at the cell cortex did not change upon chemoattractant stimulation, suggesting

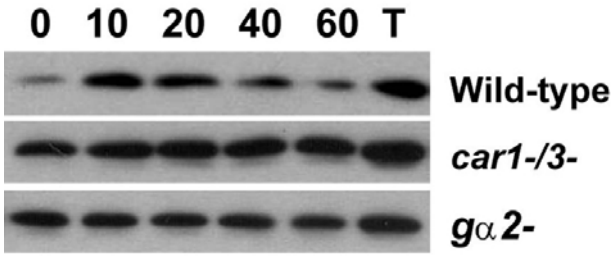

Fig. 2. Chemoattractant-mediated Rap1 activation in cells lacking GPCR/G proteins. (A) Aggregation-competent cells were treated with 15 μM cAMP for the indicated times. The activation level of myc-tagged Rap1 was measured by pull-down assay using GST-Rap1-GTP-binding domain proteins and an anti-myc antibody. Lanes labeled 'T' show the levels of total myc-Rap1 in the same volume of each lysate.

that there is no change in the Rap1-GTP level at the cell cortex in response to chemoattractant stimulation in the absence of the cAMP receptors cAR1/cAR3 or Gα2 proteins. These results were further confirmed by the following biochemical assay.

To examine Rap1 activation kinetics in response to chemoattractant stimulation, we employed a pull-down assay using the RBD of RalGDS and anti-myc antibodies. Myc-tagged Rap1 was expressed in all strains used in these experiments and enabled us to measure Rap1-GTP levels. Consistent with the results obtained in experiments examining RalGDS-YFP localization, wild-type cells showed a rapid activation of Rap1 with a peak at 5-10 s in response to chemoattractant stimulation and then deactivation to the basal level within 40 s (Fig. 2). In contrast, the mutant strains, *car1/car3* null cells or *gα2* null cells exhibited a high basal level of Rap1-GTP before stimulation, compared to wild-type cells, and the level did not change upon chemoattractant stimulation (Fig. 2). It is worth noting that a lack of Rap1 rapid activation in *car1/car3* null cells and *gα2* null cells upon chemoattractant stimulation might be due to reduced availability of inactive Rap1, as an elevated basal level of activated Rap1 was found in the mutant strains (Fig. 2). In the pull-down Rap1 activation assay, the same number of cells was used to prepare total Rap1 in the T lanes and each activated Rap1 at the indicated time points (Fig. 2), but the control sample of input total Rap1 in the T lanes was diluted 10 times more than those used in the pull-down assay for detecting activated Rap1. Approximately less than 10% of the total Rap1 in *car1/3* null cells and *gα2* null cells appeared to exit as the Rap1-GTP form before stimulation, because the band intensity at the zero time point in the mutant strains was similar to that in the T lanes, implying that an elevated basal level of Rap1-GTP in the mutant strains was not a major reason for the disappearance of the rapid activation of Rap1 after stimulation. Taken together, these results suggest that the cAMP receptors cAR1/ cAR3 and a component of the heterotrimeric G-protein complex Gα2 are required for rapid and transient Rap1 activation in response to cAMP chemoattractant stimulation.

Spatial and temporal regulation of Rap1 activity is required for proper cell migration in *Dictyostelium* (Jeon et al., 2007a; 2007b; 2009). Misregulation of Rap1 activity in cells overexpressing constitutively active Rap1 or *rapGAP1* null cells leads to a severe chemotaxis defect and a delayed developmental phenotype during multicellular development (Jeon et al., 2007a; 2007b). Our present data demonstrate that a rapid and transient Rap1 activation is absent in cells lacking the cAMP receptors cAR1/cAR3 or a component of the heterotrimeric G-protein complex Gα2 in response to chemoattractant stimulation, indicating that GPCRs, cAR1/cAR3, and Gα2 proteins are essential components in the process of chemoattractant-mediated Rap1 activation. However, it is unlikely that the GPCRs, cAR1/cAR3, and Gα2 proteins are required for Rap1 activation in unstimulated cells, as there was an elevated basal level of activated Rap1 in the mutant strains before chemoattractant stimulation (Fig. 2). These results suggest that Rap1 can be initially activated without GPCRs or Gα2 and, additionally, that the cAMP receptor or G protein complex may play some role in the downregulation of Rap1 activity. In agreement with our results, several *Dictyostelium* studies have shown that other Ras proteins such as RasC and RasG play important roles in cAMP-mediated chemotaxis (Bolourani et al., 2008; Kolsch et al., 2008; Kortholt and van Haastert, 2008). Upon cAMP stimulation, both RasC and RasG are rapidly activated and their activation is absent in mutant strains lacking cAR1/cAR3, Gβ, or Gα2 (Kae et al., 2004). Interestingly, a high basal level of activated RasC and RasG is found in the mutant strains before chemoattractant stimulation, and a negative regulatory role for the intact G-protein-coupled receptor complex in Ras activation has been suggested (Kae et al., 2004).

In mammalian cells, several positive and negative Rap1 activity regulatory mechanisms by various heterotrimeric G proteins have been suggested. As a negative control mechanism for Rap1 activation, activated α-subunits of heterotrimeric G proteins directly associate with a GAP protein for Rap1, resulting in modulation of Rap1 activity (Bos and Zwartkruis, 1999; Jordan et al., 1999; Mochizuki et al., 1999). Some GPCRs are linked to G proteins, which activate adenylyl cyclase or phospholipase C, leading to the production of intracellular second messengers such as cAMP, calcium, and diacylglycerol. These second messengers then activate GEFs for Rap1 (Bos and Zwartkruis, 1999; Bos et al., 2001; Jordan et al., 1999; Mochizuki et al., 1999). We do not know the exact mechanism for the activation of Rap1 via GPCRs and heterotrimeric G proteins in response to cAMP stimulation in *Dictyostelium*. In the *D. discoideum* genome, there are nine open-reading frames with a GAP domain for Rap1 and 25 genes encoding putative Ras-GEFs (Jeon et al., 2007a; Kortholt and van Haastert, 2008; Wilkins et al., 2005). Further studies are in progress to determine the interaction of Gα with Rap1GAP proteins and to characterize putative Ras-GEFs or GAP proteins for Rap1.

Chemoattractant-mediated Rap1 activation is independent of cGMP signaling pathway

Myosin II filaments are formed at the cortex in the back and at the lateral sides of moving cells. Many studies have shown that cGMP is a key regulator of myosin formation (Bosgraaf and van Haastert, 2006; Ridley et al., 2003; Veltman and Van Haastert, 2006). Cells deficient in forming cGMP have impaired recruitment of myosin II to the cytoskeleton. cGMP is rapidly produced in response to extracellular cAMP stimulation by the enzymatic activity of two guanylyl cyclases, membrane-bound guanylyl cyclase (GCA) and soluble guanylyl cyclase (SGC) (Bosgraaf and van Haastert, 2006; Veltman and Van Haastert, 2006). GbpC is a large multidomain protein containing a RasGEF domain and two cGMP-binding domains. GbpC is only one downstream target of cGMP identified so far and is involved in cGMP-mediated chemotaxis in *Dictyostelium* (Bosgraaf et al., 2005; Goldberg et al., 2002). Recent studies have demonstrated that Rap1 is involved in the control of myosin II assembly during *Dictyostelium* chemotaxis. GbpD, a Rap1-specific GEF protein, contains cGMP-binding domains (Kortholt et al.,

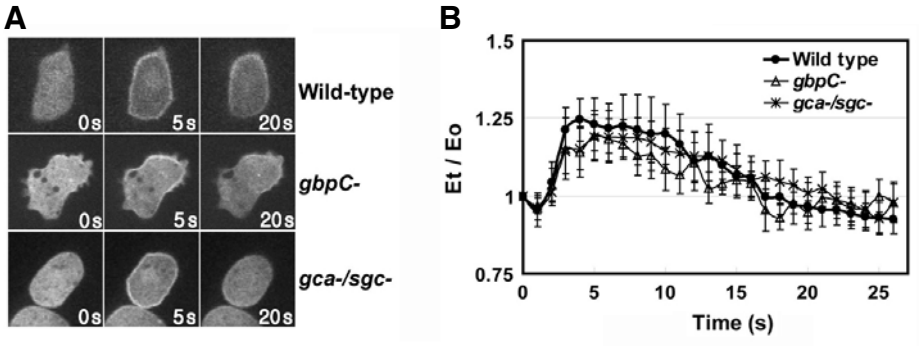

Fig. 3. Localization of RalGDS-YFP, a Rap1-GTP reporter, in cGMP signaling mutants. (A) Localization of activated Rap1 upon uniform cAMP stimulation. Translocation of RalGDS-YFP, a marker protein for Rap1-GTP, to the cell cortex in response to uniform cAMP-chemoattractant stimulation in wild-type KAx-3 cells and in *gbpC* null cells or *gca/sgc* null cells was imaged. The images were obtained from time-lapse recordings at the indicated times after stimulation. (B) Quantification and translocation kinetics of RalGDS-YFP to the membrane in cGMP signaling mutants were performed as described in the Fig. 1 legend.

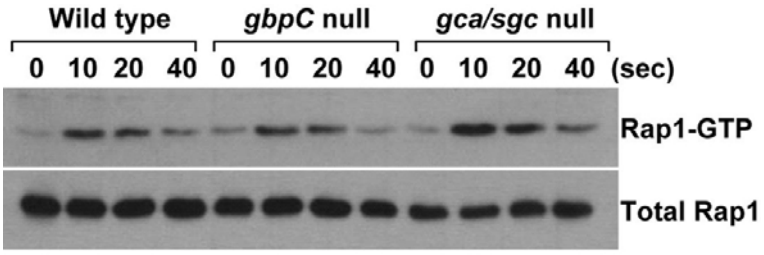

Fig. 4. Chemoattractant-mediated Rap1 activation in cGMP signaling mutants. Rap1 activation upon cAMP chemoattractant stimulation. After the cells were treated with cAMP for the indicated times, the activation level of myc-tagged Rap1 was measured by pull-down assay. Total Rap1 obtained from the samples before pulling down Rap1-GTP with GST-RBD was loaded as a loading control in the lower panel.

2006). These results raise a possibility that cGMP might be involved in the signaling pathway for Rap1 activation in response to chemoattractant stimulation. To test this possibility, we examined chemoattractant-mediated Rap1 activation kinetics in cGMP signaling mutants, *gca/sgc* null cells and *gbpC* null cells.

We first expressed RalGDS-YFP, a marker protein for Rap1-GTP, in cGMP-signaling mutant cells *gca-/sgc-* or *gbpC-*, and compared the translocation kinetics of the protein to the cell cortex to monitor the localization of the activated Rap1 in response to chemoattractant stimulation in live cells. It is noteworthy that when examining RalGDS-YFP localization we could not compare the exact ratio of the activated Rap1 at the cell cortex to that in the cytosol of the cells, because the expression level of RalGDS-YFP in the cells varied. As in wild-type cells, RalGDS-YFP was rapidly and transiently translocated to the cell cortex in the two cGMP-signaling mutants in response to uniform chemoattractant stimulation (Fig. 3), and no apparent difference was observed between wild-type cells and the mutants in the translocation kinetics of RalGDS-YFP upon chemoattractant stimulation (Fig. 3B), suggesting that Rap1 is rapidly activated in the absence of cGMP-producing proteins GCA/SGC or a cGMP-binding protein GbpC with similar activation kinetics to that in wild-type cells.

To compare the kinetics of Rap1 activation upon cAMP stimulation in cGMP-signaling mutants with those in wild-type cells, we measured Rap1-GTP levels in the mutants and wild-type cells after chemoattractant stimulation. As suggested in the RalGDS-YFP translocation experiments, no apparent difference in the level of activated Rap1 after chemoattractant stimulation was found between mutant strains and wild-type cells (Fig. 4). All strains showed almost identical rapid activation of Rap1 in response to cAMP stimulation with a peak at ~10 s and then the level decreased to the basal level within 40 s (Fig. 4), indicating that the cGMP-producing proteins GCA/SGC or the cGMP-binding protein GbpC are not essential for Rap1 activation upon cAMP stimulation.

Our data suggest that even though both Rap1 and cGMP are involved in regulating myosin assembly, cGMP is not linked to the Rap1 activation signaling pathway at the initial step of Rap1 activation in response to chemoattractant stimulation. Instead, cGMP and Rap1 may have their own distinct pathway to mediate myosin II assembly. Furthermore, our results show that loss of GbpC had no effect on chemoattractant-mediated Rap1 activation, suggesting that GbpC is unlikely to have GEF activity for Rap1. GbpC and GbpD have been identified as cGMP-binding proteins. These two proteins both contain cGMP binding domains and CDC25 homology domains. GbpC has a high affinity for cGMP and is involved in myosin II regulation (Bosgraaf et al., 2005). GbpD, homologous to the C-terminal half of GbpC (Kortholt et al., 2006), is a Rap1-specific GEF protein involved in substrate attachment and cell polarity. The *gbpC* null cell phenotypes, with defects in myosin II assembly, and a homologue to GbpD, a Rap1-specific GEF protein, suggest that GbpC might be an upstream regulator of Rap1 activation, but this postulate is excluded by our results. In support of our views, recent studies have shown that a RasGEF domain of GbpC has a GEF activity specific to an intramolecular Ras domain (van Egmond et al., 2008).

In summary, Rap1 is rapidly activated in response to chemoattractant stimulation via GPCRs cAR1/cAR3, and a heterotrimeric G-protein complex, as previously shown in other Ras proteins such as RasC and RasG. cGMP production by two guanylyl classes, GCA/sGC, or the only known cGMP-binding protein, GbpC, are dispensable to Rap1 activation by cAMP chemoattractant stimulation.

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