We have used a doubly disrupted rasC−/rasG− strain of Dictyostelium discoideum, which ectopically expresses the carA gene, to explore the relationship between the activation of RasC and RasG, the two proteins that are necessary for optimum cAMP signaling, and the activation of Rap1, a Ras subfamily protein, that is also activated by cAMP. The ectopic expression of carA restored early developmental gene expression to the rasC−/rasG− strain, rendering it suitable for an analysis of cAMP signal transduction. Because there was negligible signaling through both the cAMP chemotactic pathway and the adenyl cyclase activation pathway in the rasC−/rasG−/act15; carA strain, it is clear that RasG and RasC are the only two Ras subfamily proteins that directly control these pathways. The position of Rap1 in the signal transduction cascade was clarified by the finding that Rap1 activation was totally abolished in rasC−/rasG−/act15; carA and rasG− cells but only slightly reduced in rasC− cells. Rap1 activation, therefore, occurs downstream of the Ras proteins and predominately, if not exclusively, downstream of RasG. The finding that in vitro guanylyl cyclase activation is also abolished in the rasC−/rasG−/act15; carA strain identifies RasG/RasC as the presumptive monomeric GTPases required for this activation.

The Ras subfamily proteins are monomeric GTPases that function as molecular switches by cycling between the active GTP-bound and the inactive GDP-bound state (1). In mammalian cells, activated Ras proteins impact multiple cellular signal transduction pathways that include mitogen-activated protein kinase cascades, phosphatidylinositol 3-kinase (PI3K)3-regulated pathways and RalGDS-dependent activation of Ral (2). These Ras-mediated responses regulate a wide range of cellular processes, including proliferation, cytoskeletal function, and differentiation. The discovery of a large number of Ras subfamily homologues in mammals and in the model organisms Drosophila melanogaster, Caenorhabditis elegans, and Dictyostelium discoideum (3, 4) has raised important questions regarding the specific functions of individual Ras proteins. For example, the human Ras subfamily consists of 36 gene products that can be divided into several groups (5). The search for downstream effectors has revealed some specificity but also an enormous complexity of apparently overlapping functions (6). Thus, a major step in understanding the patterns of Ras signal transduction would be to identify the contributions made by each Ras subfamily member to a specific signaling pathway. This analysis is difficult, if there is cross-talk between the components of different pathways, or if a single input signal results in multiple responses. One useful tool in understanding Ras function is provided by examining genetically tractable organisms, such as Dictyostelium, that are disrupted in the ras gene of interest.

In the presence of nutrients, the cellular slime mold, Dictyostelium, grows as free-living, single-celled amoebae, but upon starvation these amoebae aggregate into a multicellular organism that progresses through a motile slug stage to form a spore mass or sorus supported by a stalk (7). The aggregation process is driven in response to cAMP, which is synthesized and secreted soon after the onset of starvation. cAMP binds to cell surface cAMP receptors (for example, the car1 receptor encoded by the carA gene), resulting in the dissociation and activation of a heterotrimeric G protein. This leads to chemotaxis to cAMP and to the activation of adenyl cyclase A (ACA) (8).

Fourteen Dictyostelium Ras subfamily proteins, with a high level of amino acid identity to the mammalian H, N, and K-Ras proteins, have been described (9). The rasC, rasS, rasG, and rasD genes have all been disrupted, and each of the resulting strains has a distinct phenotype, suggesting that each encoded product performs a different function (10–14). It has been previously shown that both RasC and RasG are activated in response to cAMP, suggesting roles for both RasC and RasG in cAMP signaling (15, 16). A role for RasC and RasG has been further indicated by the finding that rasC− cells fail to aggregate and rasG− cells are delayed in aggregation (10, 14). Furthermore, ACA activation is more impacted than chemotaxis in the rasC− cells, whereas chemotaxis is more impacted than ACA activation in the rasG− cells (10). However, the loss of RasG or RasC alone did not result in a total loss of either chemotaxis or ACA activation, suggesting three possibilities: some overlap of function between RasC and RasG; a requirement of both RasC and RasG for optimal chemotaxis and optimal ACA activation; or the existence of an additional pathway for cAMP signaling. The recent finding that Rap1 is activated in response to cAMP (17) provides a potential mechanism for the third possibility:
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EXPERIMENTAL PROCEDURES

Dictyostelium Strains—Generation of rasG\(^{-}\), rasC\(^{-}\), and rasC\(^{-}/\)rasG\(^{-}\) strains, all in the JH10 background, has been described previously (10). To generate rasC\(^{-}/\)rasG\(^{-}\) [act15]:carA strain, 20 \(\mu g\) of the PcAR1-B18 expression vector, which contains carA cDNA under the control of the constitutively active actin 15 promoter and a neomycin-resistance cassette (18), was electroporated into rasC\(^{-}/\)rasG\(^{-}\) cells as previously described (19). Clones containing the selectable marker were isolated 8–15 days following the application of the selection conditions and maintained in HL5 media supplemented with 10 \(\mu g/ml\) G418 (Invitrogen).

Cell Culture and Development—The JH10 strain was grown at 22 °C in HL5 medium (20) containing 50 \(\mu g/ml\) streptomycin sulfate (Sigma) and 100 \(\mu g/ml\) thymidine (Sigma). The various ras gene-disrupted strains were grown in the same medium without thymidine. In addition, rasC\(^{-}/\)rasG\(^{-}\) strain was grown in the presence of 10 \(\mu g/ml\) basicidin S (Calbiochem), and the rasC\(^{-}/\)rasG\(^{-}\) [act15]:carA strain was grown in the presence of 10 \(\mu g/ml\) basicidin S and 10 \(\mu g/ml\) G418 (Invitrogen). Because the rasG\(^{-}\), rasC\(^{-}/\)rasG\(^{-}\), and rasC\(^{-}/\)rasG\(^{-}\) [act15]:carA cells grow poorly in shakem, all strains were grown in Nunc tissue-culture plates.

To obtain CAMP-pulsed cells, wild-type and mutant vegetative cells were harvested, washed twice in KK2 (20 mM potassium phosphate, pH 6.1), and resuspended in KK2 to a final density of 5 \(\times\) 10\(^6\) cells/ml. 30 ml of cell suspension was shaken at 150 rpm for 1 h and then pulsed with 100 \(\mu l\) of CAMP to a final concentration of 50 nmol every 6 min for 5 h. To observe aggregation streaming, vegetative or 5-h CAMP-pulsed cells were washed twice in Bonner’s salts (10 mM NaCl, 10 mM KCl, 2 mM CaCl\(_2\)), seeded at ~4 \(\times\) 10\(^5\) cells/cm\(^2\) in Nunc tissue culture dishes submerged under Bonner’s salts, incubated at 22 °C, and photographed with a Leica DMLM inverted microscope (Leica Microsystems, Wetzlar, Germany), a Leica DFC350F camera (Leica Microsystems), and Openlab software (Improvision, Coventry, England).

CAMP Binding Assay—CAMP-pulsed cells were treated with 2 mM caffeine for 30 min, washed twice in ice-cold KK2, and resuspended to a density of 1.5 \(\times\) 10\(^7\) cells/ml. The binding of [\(\text{H}\)]CAMP to the cell surface was measured by the ammonium sulfate stabilization assay (21). Cells (1.5 \(\times\) 10\(^7\)) were incubated with 100 \(\mu l\) of KK2 containing 10 nm [\(\text{H}\)]CAMP (0.9 TBq/mmol, GE Healthcare, Little Chalfont, Buckinghamshire, England) and 5 mM dithiothreitol (Sigma) for 1 min at 0 °C. 1 ml of saturated ammonium sulfate was then added, followed immediately by the addition of 100 \(\mu l\) of bovine serum albumin (10 mg/ml, Sigma). After 5-min incubation at 0 °C, the samples were centrifuged at 8000 \(\times\) g for 2 min. The supernatant was removed, and the pellet was resuspended in 110 \(\mu l\) of 1 M acetic acid. 100 \(\mu l\) of the resuspended pellet was mixed with 4 ml of scintillation mixture (Ready Safe liquid scintillation cocktails, Beckman Coulter, Fullerton, CA), and the amount of bound [\(\text{H}\)]CAMP was measured using a Beckman LS6001C scintillation counter.

Chemotaxis Assays—For CAMP micropipet assays, cells were pulsed with CAMP as described above, washed twice with Bonner’s salts, and then seeded in Nunc tissue culture dishes at a cell density of ~5 \(\times\) 10\(^5\) cells/cm\(^2\) in Bonner’s salts. At \(t = 0\), an Eppendorf Femtotip Micropipet (Hamburg, Germany) filled with 100 \(\mu m\) CAMP was placed in the field of view, and cell movements toward the micropipet were monitored by time-lapse video microscopy using an Olympus IX-70 inverted microscope, a DAGE-MTI CCD-100 camera (Michigan City, IN), and Scion Image 4.0 software (Frederick, MD). Instantaneous velocities and chemotaxis indices were determined as described previously (22).

Western Blot Analyses and PKB Phosphorylation Assays—Cells were pelleted by centrifugation and lysed in 1\(\times\) Laemmli SDS-PAGE loading buffer (6× buffer: 350 mM Tris-Cl, pH 6.8, 10% SDS, 600 mM dithiothreitol, 0.012% w/v bromphenol blue, 30% glycerol) by boiling for 5 min (23). 10 \(\mu g\) of protein was then fractionated by SDS-PAGE. After electrophoresis, the proteins were transferred electrotherapeutically onto Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences), which were then blocked with nonfat milk (23) and probed with the appropriate antibody. Antibody binding was detected by enhanced chemiluminescence (ECL, GE Healthcare). PKB phosphorylation was determined as described previously (14). Briefly, CAMP-pulsed cells were washed twice in KK2, resuspended to 5 \(\times\) 10\(^7\) cells/ml in KK2, and then stimulated by addition of CAMP to a final concentration of 100 nm. Aliquots of 100 \(\mu l\) were removed at intervals, before and after stimulation, and mixed with 20 \(\mu l\) of 6\(\times\) SDS-PAGE loading buffer. 10 \(\mu g\) of protein was fractionated by SDS-PAGE and then subjected to Western blot analysis using a phosphothreonine-specific antibody (Cat#9381, Cell Signaling Technologies, Danvers, MA). Equal sample loading was verified by staining a duplicate gel with Coomassie Blue. To assess equal PKB expression levels in all strains, Western blots were also analyzed using a PKB-specific antibody. The protein concentration was determined using the protein assay from Bio-Rad.
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Northern Blot Analyses—For Northern blot analyses, cells were pulsed with cAMP as described above, and total RNA was extracted at intervals using guanidinium isothiocyanate (24). 15-µg aliquots were size-fractionated on 1.25% agarose-formaldehyde gels, blotted onto Hybond-N+ membrane (Amersham Biosciences), and probed with DNA fragments from plasmids carrying the specific genes of interest, as previously described (25).

Adenylyl Cyclase Assay—In vitro adenylyl cyclase activity was determined as described previously (14), except that cell suspensions were frozen on dry ice and thawed, and then vortexed in the presence of glass beads (<106 µm, Sigma). cAMP was recovered by sequential chromatography through Dowex and Alumina columns, and the eluted [32P]cAMP was measured using a Beckman LS6000IC scintillation counter.

cGMP Production and Guanylyl Cyclase Activation—To measure cGMP production, cells were pulsed with cAMP as described above, washed twice with KK2, and resuspended to a density of 1 x 10^8 cells/ml in KK2 containing 2 mM caffeine. The cells were stimulated by addition of 100 µM of 3.5% perchloric acid, followed by the addition of 50 µL of 50% saturated KHCO3. cGMP levels were then measured using a [3H]cGMP assay kit (GE Healthcare, TRK500).

In vitro guanylyl cyclase assay was determined as described previously (26). Briefly, cAMP-pulsed cells were washed and resuspended to a density of 2 x 10^8 cells/ml in KK2. One volume of cell suspension was mixed with one volume of 10 mM Tris-Cl, pH 8, 1.5 mM EGTA, 500 mM sucrose in the presence or absence of 200 µM GTPγS, and immediately lysed by pushing the cells through a 5 µm Isopore Membrane Filter (Millipore, Ireland) placed directly between a 1-ml syringe and a B-D 26G1/2 needle (BD Biosciences). 150 µL of cell lysate was mixed with an equal volume of assay mix (10 mM Tris-Cl, pH 8, 250 mM sucrose, 40 mM dithiothreitol, 1 mM GTP, 5 mM MgCl2), and the mixture was incubated at 22 °C for 60 s. Aliquots (100 µL) were mixed with 100 µL of 3.5% perchloric acid, followed by addition of 50 µL of 50% saturated KHCO3. cGMP levels were then measured using a [3H]cGMP assay kit (GE Healthcare, TRK500).

RasG, RasC, and Rap1 Activation Assays—The Ras-binding domain (RBD) of Byr2 was expressed in Escherichia coli as a GST fusion protein and purified as described previously (15). The purified GST-Byr2-RBD was used for the detection of activated proteins. After cAMP pulsing, cells were harvested by centrifugation and resuspended to a density of 2 x 10^7 cells/ml in KK2 containing 1 mM caffeine. After 30 min, aliquots (2 ml) of cell suspension were stimulated by addition of cAMP to a final concentration of 15 µM. Cell suspensions (350 µL) were lysed at the indicated times by mixing with an equal volume of 2 x lysis buffer (20 mM sodium phosphate, pH 7.2, 2% Triton X-100, 20% glycerol, 300 mM NaCl, 20 mM MgCl2, 2 mM EDTA, 2 mM Na2VO4, 10 mM NaF, containing two tablets of protease inhibitor mixture (Roche Diagnostics) per 50-ml buffer). The lysates were centrifuged for 10 min, and the protein concentrations of the supernatants were determined using the protein assay from Bio-Rad. 400 µg of protein was incubated with 100 µg of GST-Byr2-RBD on glutathione-Sepharose beads (GE Healthcare Bio-sciences AB, Uppsala, Sweden) at 4 °C for 1 h.

The values are the means of two experiments.

### TABLE 1

| Strain | cAMP bound* (nmol/10^7 cells) |
|--------|-----------------------------|
| JH10   | 4.3 ± 0.4                   |
| rasG⁻/rasG⁻ | 0.8 ± 0.2                |
| rasG⁻/rasG⁻/act15⁻/carA⁻ | 6.6 ± 0.5            |

The beads were harvested by centrifugation and washed three times in 1 x lysis buffer. 50 µL of 1 x SDS gel loading buffer was then added to the pelleted beads, and the suspension was boiled for 5 min. Samples were subjected to SDS-PAGE and Western blot analysis with anti-RasG, RasC, or Rap1 antibodies.

### RESULTS

Restoration of Early Developmental Gene Expression in rasC⁻/rasG⁻ Strain by the Ectopic Expression of carA—Characterization of a rasC⁻/rasG⁻ double mutant by Northern blot analysis had revealed that expression of the early developmentally genes, carA, gpaB, and csaA, was dramatically reduced, with the effect on carA expression being the most pronounced (10). Thus, any signaling phenotype observed in this strain could be due to insufficient levels of essential developmentally expressed signaling proteins, such as carA, Goc2, and ACA. Because carA expression was the most defective, we expressed the gene encoding carA (carA⁻/carA) from the constitutively active act15 promoter in the rasC⁻/rasG⁻ strain, in an attempt to provide sufficient CAR1 to allow at least some cAMP signaling. As anticipated, carA was now clearly expressed in vegetative cells of this strain, whereas, in contrast it was undetectable in vegetative JH10 and rasC⁻/rasG⁻ cells. Surprisingly gpaB and csaA were also expressed in vegetative rasC⁻/rasG⁻/act15⁻/carA⁻ cells at much greater levels than in JH10 or rasC⁻/rasG⁻ cells. Even more surprisingly, the developmental expression of carA, gpaB, and carA⁻/carA was restored by the ectopic expression of carA (Fig. 1, A and C). Thus, the presence of carA in vegetative cells at the onset of development is a positive regulator of early gene expression and is able to offset the absence of RasG and RasC, normally required for this induction. Because early gene expression was restored in the rasC⁻/rasG⁻/act15⁻/carA⁻ strain, it enabled an investigation of the possible roles of other Ras proteins in cAMP-induced signal transduction.

To demonstrate that the restored carA expression in the rasC⁻/rasG⁻/act15⁻/carA⁻ strain produced functional carA, we determined the binding of cAMP to the cell surface of...
cAMP-pulsed cells. The results, shown in Table 1, indicated that cAMP binding was reduced, as expected, in the rasC−/rasG− relative to JH10, but restored to greater than wild-type levels in the rasC−/rasG−/act15::carA cells. This result demonstrated that the overexpression of carA indicated in Fig. 1 resulted in functional protein being localized on the surface of the rasC−/rasG−/act15::carA cells.

As was the case with the original rasG− cells (12), the new rasG− strains grew poorly in shaken suspension and were multinucleate (data not shown). The rasC−/rasG− and rasC−/rasG−/act15::carA cells exhibited no growth whatsoever in shaken suspension and grew less efficiently than rasG− cells on a plastic surfaces (data not shown). These results indicate that RasC is capable of performing some of the RasG functions in vegetative cells. In addition, it was shown previously that, although rasC− cell grew normally, they exhibited deficiencies in some vegetative cell functions, indicating an important role for RasC in vegetative cells (27).

Developmental Phenotype—When grown in association with bacteria, the rasC−/rasG−/act15::carA cells did not form aggregates on bacterial lawns (data not shown). In addition, aggregation streams were not observed when cells were plated in plastic dishes under non-nutrient buffer, although small cell clumps were formed after prolonged incubation (Fig. 2C), results similar to those observed previously for AX2/rasC− (14) and JH10/rasC− cells (10). The rasC−/rasG− cells did not form small clumps under these conditions (Fig. 2B). The formation of aggregation streams by the control JH10 cells was observed 10 h after plating, and typical tight aggregates formed by 12 h (Fig. 2A). When pulsed with cAMP for 5 h before plating in plastic dishes, rasC−/rasG−/act15::carA exhibited the same phenotype as the non-pulsed controls (data not shown). Thus, cells containing neither RasG nor RasC are incapable of aggregation under any of the experimental conditions tested, and this defect is not due to the inability to express the early developmental genes (Fig. 1C). These results show that aggregation is totally blocked in the absence of both RasC and RasG. To determine the role played by these proteins in the cAMP relay and chemotaxis signal pathways, these downstream responses to cAMP were further explored.

The Presence of Either RasG or RasC Is Essential for ACA Activation—The activation of ACA by signaling through the heterotrimeric G protein is essential during aggregation to provide the extracellular cAMP to relay the signal through the population (8). GTPγS activates ACA by uncoupling the Gα subunits from the Gβγ subunits without the requirement for receptor occupation by cAMP (8, 28). ACA activity in the absence of GTPγS was reduced in the lysates of cAMP-pulsed rasC−/rasG− cells (Fig. 3), suggesting that these cells expressed less ACA, a result consistent with the defect in early gene expression (Fig. 1). In contrast, lysates prepared from cAMP-pulsed rasC−/rasG−/act15::carA cells, had wild-type levels of ACA activity. When stimulated with GTPγS, there was no increase in ACA activity in either rasC−/rasG− or in rasC−/rasG−/act15::carA cells (Fig. 3). This result indicates that rasC−/rasG−/act15::carA cells are incapable of cAMP-induced signal relay and is in marked contrast to those observed previously for cells with single ras gene disruptions, which exhibit some ACA activation (10, 14). Thus, signal transduction from the heterotrimeric G protein to ACA must involve either RasC or RasG.

The Presence of Either RasG or RasC Is Essential for Chemotaxis, PKB Phosphorylation, and Guanylyl Cyclase Activation—To investigate the requirement of RasC and RasG for chemotaxis, cells that had been pulsed for 5 h with cAMP were subjected to a spatial gradient created by diffusion of cAMP from the tip of a micropipet. Under these conditions, chemotaxis-competent cells respond by migrating toward the tip (29). Chemotactic indices for the rasC−/rasG− and the rasC−/rasG−/act15::carA cells revealed the virtual absence of chemotaxis in these two strains (Table 2). Thus, cAMP-induced chemotaxis toward cAMP requires signal transduction through either RasC or RasG. The results for the double ras gene deletion strains are in marked contrast to
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TABLE 2
Chemotaxis analysis of Dictyostelium cells in a spatial gradient of cAMP

| Strain            | Number of cells | Instantaneous velocity | Chemotaxis indexa |
|-------------------|-----------------|------------------------|-------------------|
| JH10              | 20              | 11.18 ± 1.51           | 0.62 ± 0.12       |
| rasG              | 21              | 5.98 ± 1.73            | 0.45 ± 0.14       |
| rasC              | 22              | 12.97 ± 3.99           | 0.71 ± 0.15       |
| rasC/rasG         | 20              | 6.60 ± 3.33            | 0.05 ± 0.02       |
| rasC/rasG/act15   | 20              | 5.91 ± 3.41            | 0.04 ± 0.01       |

a Chemotaxis index was calculated as the net distance traveled toward the source of chemoattractant divided by the total distance traveled in that time period.

FIGURE 4. PKB phosphorylation in response to cAMP. Extracts from JH10, rasC/rasG, and rasC/rasG/act15 strains that had been pulse-chased with cAMP for 5 h were prepared at the indicated times after a single 100 nM cAMP stimulus and subjected to Western blot analysis using a phosphothreonine-specific antibody.

FIGURE 5. cGMP production in response to cAMP and in vitro GTPγS stimulation of guanylyl cyclase activity. A, JH10 ( ), rasC/rasG ( ), and rasC/rasG/act15 ( ) cells were pulsed with cAMP for 5 h with cAMP, washed, and then stimulated with 100 nM cAMP. Samples were taken at the indicated times and assayed for cGMP accumulation (see “Experimental Procedures”). The means and standard deviations for three independent experiments are shown. B, JH10, rasC/rasG, and rasC/rasG/act15 ( ) cells were pulsed with cAMP for 5 h, lysates were then assayed for guanylyl cyclase activity in the absence (gray bar) or the presence of GTPγS (black bar). The means and standard deviations for three independent experiments are shown.

those obtained previously for the single ras gene deletion strains, which both exhibited appreciable chemotaxis (see Table 2 for comparative values). The calculated average instantaneous velocities of the rasC/rasG- and rasC-/+ rasG-/+act15:carA cells were lower than those for the wild type, although the values clearly indicate that both strains are still capable of random motility (Table 2).

It has been suggested that PI3K-mediated signaling pathway is important for Dictyostelium chemotaxis (30–34). cAMP stimulation of aggregation competent cells leads to an increase in phosphatidylinositol 3,4,5-triphosphate at the leading edge of chemotaxing cells following by translocation and activation of PH domain containing protein such as PKB to the site of PI3K activity (32, 35, 36). To test the role of RasC and RasG in PI3K activation we monitored cAMP-stimulated phosphatidylinositol 3,4,5-triphosphate increase in phosphatidylinositol 3,4,5-triphosphate at the leading edge of chemotaxing cells followed by translocation of PKB, leading edge of chemotaxing cells followed by translocation and activation of PKB, leading edge of chemotaxing cells followed by translocation and activation of PKB, leading edge of chemotaxing cells followed by translocation and activation of PKB, leading edge of chemotaxing cells followed by translocation and activation of PKB.

In Dictyostelium, cGMP production has also been shown to be associated with the chemotactic response (37, 38). We therefore examined cGMP production in response to cAMP in the ras mutant strains. In the JH10 parental cell line, there was a burst of cGMP production 10 s after the application of cAMP, which then returned to the basal level after ~30 s. However, this response was not detectable in rasC-/+ rasG-/+act15:carA cells or rasC-/+ rasG-/+act15:carA cells (Fig. 5A), consistent with the requirement of either RasC or RasG for the activation of guanylyl cyclases. It had been shown previously that there was still appreciable cGMP production in response to cAMP in the single ras gene deletion strains (10).

GTPγS stimulates in vitro guanylyl cyclase activity (39), and an involvement of a monomeric GTPase has been proposed (40). We therefore investigated the possibility that either RasC or RasG might be this presumptive protein, by examining the GTPγS stimulation of guanylyl cyclase activity in the various ras mutant strains. GTPγS stimulated the guanylyl cyclase activity of JH10 lysates ~8-fold, but stimulation was not detectable in either the rasC-/+ rasG-/+act15:carA cell lysates (Fig. 5B). These results are consistent with the conclusion that all cAMP signal transduction responsible for guanylyl cyclase activation is mediated through pathways involving either RasG or RasC. The guanylyl cyclase activity of rasC-/+ rasG-/+act15:carA cell lysates was stimulated ~5.5-fold and that of rasG-/+act15:carA cell lysates ~2.5-fold (Fig. 5B), consistent with the idea that RasG is more important than RasC for guanylyl cyclase activation (10).

Rap1 Activation—To understand the relationship between Rap1 activation on the one hand and RasG and RasC activation...
on the other, Rap1 activation was determined in the various ras null strains. Strains were pulsed with cAMP for 5 h and then stimulated with cAMP. Cells were lysed, and the level of GTP-bound Rap1 was determined by an affinity "pulldown" assay (15). There was a high basal level of activated Rap1 in JH10 cells, but there was an appreciable further activation in response to cAMP (17), which was similar to that for JH10 strains. These data suggest that signaling through RasG is essential for Rap1 activation, although signaling through RasC may play a part.

**RasG and RasC Activation**—To investigate possible cross-talk between the RasG- and RasC-mediated signal transduction pathways, we determined whether either RasC or RasG was differentially activated in the rasG− and rasC− mutant strains. RasG was rapidly activated in rasC− cells with a peak of activation at ~10 s, which was similar to that for JH10 cells, suggesting no compensation. However, the subsequent loss of activated RasG was less pronounced (Fig. 6B) suggesting an alteration in the adaptation component of the signaling process. In rasG− cells, the activation of RasC was enhanced (Fig. 6B), suggesting a compensatory role for RasC in these cells.

**DISCUSSION**

During *Dictyostelium* aggregation, cAMP induces both the activation of ACA, and chemotaxis (Fig. 7). Both processes require the cAMP receptor (cAR1) and the associated heterotrimeric G protein (Gαoβγ) (41), and we have previously shown that RasC and RasG act downstream of Gαoβγ and upstream of the other known components (10, 14, 15). The available evidence suggests that signaling through RasC is more important for the activation of ACA, whereas signaling through RasG is more important for chemotaxis (10). However, neither ACA activation nor chemotaxis was completely eliminated in either the rasC− or the rasG− strains. Because the defects in the single null strains were not simply additive, we favored the hypothesis that there was some overlap of function (10). However, there are at least two other possibilities: that both RasC and RasG are required for both optimal chemotaxis and optimal ACA activation, or that an additional Ras protein, such as Rap1, recently shown to be activated in response to cAMP (17), is also important at this point in the signal transduction pathway.

The finding that early gene expression is markedly repressed in the rasC−/rasG− strain indicates an important role for RasC and RasG in response to starvation that induces early gene expression. Expression of *carA* gene from the constitutive *actin* 15 promoter markedly elevates the levels of *carA* expression in vegetative cells. This observed expression clearly restores the developmentally regulated expression of *carA* itself and that of...
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other developmental genes, that was suppressed in the rasC⁻/rasG⁻ strain. The mechanism by which early gene expression is repressed in the rasC⁻/rasG⁻ strain and the mechanism by which such expression is restored in the rasC⁻/rasG⁻/act15:carA strain were not pursued in this study, which was designed specifically to look at the role of Ras proteins in the signal transduction pathway.

The experiments using the rasC⁻/rasG⁻/act15:carA mutant strain show conclusively that all chemotactic and signal relay responses to cAMP are mediated through either RasG or RasC, and the results negate the need for an additional Ras protein at this step of the pathway (Fig. 7). The partial chemotaxis and ACA activation observed in the single ras null mutants could be due to an overlap of RasC and RasG function, because there is close amino acid similarity between the two proteins. Thus, there could be an interaction of the one Ras protein with the effectors of the other. In the wild-type strain, each Ras protein would out-compete the other for its own effectors. However, in the single null strains, where one protein is missing, the competition would be removed, allowing an interaction of the remaining Ras protein with both sets of effectors. Consistent with this idea of a compensatory mechanism, is the finding that there is an increase in RasC activation in rasG⁻ cells (Fig. 6B), although there was no reciprocal increase in RasG activation in rasC⁻ cells (Fig. 6B).

It is also possible that there is a dual role for RasG and RasC in both ACA activation and in chemotaxis. This idea is attractive, because a requirement for the two Ras proteins for ACA activation could explain the enhancement in the cAMP signal relay as aggregation progresses. Thus, RasG might be responsible for initiating the process, but RasC might be important for the amplification of the signal that is needed to entrain the cells into aggregation streams. Similarly, if there is a dual role for RasG and RasC in chemotaxis, RasC might be required early in aggregation for a response to low concentrations of cAMP and RasG required later in aggregation for a response to higher concentrations of cAMP. This interpretation is consistent with the delayed aggregation by the rasG⁻ cells and the absence of aggregation of the rasC⁻ cells. However, because there is as yet no experimental support for these possibilities, they have not been included in our model (Fig. 7).

Rap1 overexpression leads to shape changes and enhanced cell adhesion of vegetative Dictyostelium cells (42, 43). In addition, the available evidence suggests that Rap1 is essential for vegetative cell viability (44). Activated Rap1 is capable of binding to the RBD of Phg2, and it has been suggested that this binding leads to an increase in cell adhesion (45) and plays a role in cell motility (17). It has also been shown that Rap1 is activated by the RasGEF activity of GbpD in vegetative cells (45). A potential role for Rap1 during aggregation has been recently suggested by the finding that the protein is activated in response to cAMP in aggregation competent cells (17). This activation was absent in rasC⁻/rasG⁻/act15:carA and rasG⁻ cells, indicating that it occurs downstream of signaling through RasG. Because we have no evidence for the exact role of Rap1 in this signal transduction pathway, we have simply placed it downstream of RasG and upstream of polarity/chemotaxis (Fig. 7). Rap1 activation in response to cAMP was slightly reduced in rasC⁻ cells, and we therefore can’t rule out the possibility that some signaling is mediated through RasC, but this has also not been considered in our model (Fig. 7). The levels of activated Rap1 that were present prior to the cAMP stimulation were also reduced in rasG⁻ cells but not reduced in rasC⁻ cells, indicating a role for RasG in regulating basal levels of Rap1 activation. Because GbpD is involved in Rap activation in vegetative cells (45), it might also be involved in the activation of Rap1 in chemotaxing cells in response to cAMP. It will be interesting to investigate the nature of the link between RasG and GbpD.

Because RasG interacts with the RBD of two of the Dictyostelium PI3Ks (PI3K1 and PI3K2), it was postulated that RasG is a regulator of PI3K activity (46). Initially it was suggested that the production of PI3P by the activity of PI3K plays a pivotal role in the chemotactic response to cAMP (35, 36, 47). However, recently there have been two reports indicating that strains with several deleted pi3k genes remain chemotactic, although they are less polar and do move more slowly in response to cAMP (30, 48). It would appear that PI3K is an important determinant for the establishment of polarity, which streamlines the chemotaxis process, but is not essential. In contrast, mutants that have disruptions in the genes encoding the enzymes responsible for cGMP production, gca and sgc, are only slightly chemotactic to cAMP (37). Taken together, these results indicate that the generation of cGMP plays a more important role in the chemotactic process than the production of inositol 1,4,5-trisphosphate. A comparison of the rates of chemotaxis of the various ras null strains relative to levels of PKB phosphorylation (as a measure of PI3K activity) and cGMP production is consistent with this interpretation. Thus, both chemotaxis and cGMP production were only reduced by a factor of two in rasG⁻ cells, whereas PKB activation was barely detectable in these cells. Furthermore, the rate of chemotaxis was very similar in rasC⁻ cells to that of the parental cell line, and cGMP production was only slightly reduced, whereas PKB phosphorylation was markedly reduced. In addition, an examination of the rasG⁻ cell morphology during chemotaxis revealed little polarity, even though these cells perform relatively efficient chemotaxis (10), consistent with the idea that cGMP pathway is more important for chemotaxis than the PI3K pathway.

There is strong evidence for specificity among the RasGEFs proteins, that act upstream of Ras proteins in the cAMP signaling pathway (49). RasGEFA is specific for RasC and appears to be the only RasGEF capable of converting RasC to the GTP-bound form (Fig. 7). RasGEFR is specific for RasG, but because RasG is still partially activated in a gfpR⁻ strain, other RasGEFs (whose identities are currently unknown and, therefore, denoted as RasGEF-? in Fig. 7) can also activate RasG. Thus, the input to RasG is more complex than is the input to RasC, which may reflect the fact that RasG has multiple functions in the cell. A key question, still to be examined, is how the activation of the RasGEFs is linked to components of the cAMP receptor complex.
Downstream of RasG and RasC there is clearly a bifurcation of the signaling pathways (Fig. 7). The differential impact of RasG and RasC on ACA activation versus chemotaxis is almost certainly due to differences in the interaction of the Ras proteins with their downstream effectors. There is already evidence for differential affinity, because the RBDS of mammalian Ral1 and RalGDS and of Dictyostelium PI3K1 and PI3K2 bind more effectively to RasG than to RasC (15). The higher affinity of PI3K1 and PI3K2 for RasG is consistent for the proposed roles for PI3K and RasG in establishing polarity. A yeast two-hybrid analysis has shown that RIP3 also interacts strongly with RasG but does not interact with RasC (50), but the function of RIP3 remains an enigma, because there is a marked discrepancy between this biochemical and the available genetic data. The rip3− strain is defective in aggregation (50, 51), a phenotype that closely resembles that of the rasC− and gefA− strains, and very different from the delayed aggregation phenotype of rasG− cells. This genetic data, therefore, indicates a close connection between RIP3, RasGEFA, and RasG and provides no evidence for a connection between RIP3 and RasC. The failure of RIP3 to bind to RasC in yeast two-hybrid assays (50) is not simply an artifact, resulting from the failure of RasC to express properly in yeast, because RIP3 binds to RasG but does not bind to RasC in a RBD-RIP3 pulldown assay. A further complication is that RIP3 is a component of the TORC2 complex, which is necessary for optimum ACA activation and optimum chemotaxis. Understanding these signaling pathways will require a greater understanding of the role(s) of the TORC2 complex. In addition to these affinity considerations, a spatial separation of RasG and RasC may play an important role in the signal bifurcation, because ACA activation occurs at the trailing end of a migrating cell while PI3K stimulation of PKB occurs at the leading end. The study of this activation in live cells is currently hampered by the lack of specific reagents that can uniquely identify activated RasC and activated RasG. Creating such specific reagents will be an important step for future understanding.

Acknowledgments—We thank the Dicty Stock Center, Columbia University for providing the PcAR1-B18 plasmid used in this study, and we thank F. Jiang and Dr. R. Dottin (Hunter College, New York) for the PKB-specific antibody.

REFERENCES

1. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) Nature 349, 117–127
2. Campbell, S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998) Oncogene 17, 1395–1413
3. Reuther, G. W., and Der, C. J. (2000) Curr. Opin. Cell Biol. 12, 157–165
4. Wilkins, A., and Insall, R. H. (2001) Trends Genet. 17, 41–48
5. Mitin, N., Rossman, K. L., and Der, C. J. (2005) Curr. Biol. 15, R563–R574
6. Rodriguez-Viciana, P., Sabatier, C., and McCormick, F. (2004) Mol. Cell. Biol. 24, 4943–4954
7. Kessin, R. H. (2001) Dictyostelium: Evolution, Cell Biology, and the Development of Multicellularity, Developmental and Cell Biology Series, pp. 1–8, Cambridge University Press, Cambridge, UK

H. Kae and G. Weeks, unpublished observations.
Role of Ras in Chemotaxis and cAMP Relay

Weeks, G., Wittinghofer, A., and Van Haastert, P. J. (2006) *J. Biol. Chem.* 281, 23367–23376

46. Funamoto, S., Meili, R., Lee, S., Parry, L., and Firtel, R. A. (2002) *Cell* 109, 611–623

47. Funamoto, S., Milan, K., Meili, R., and Firtel, R. A. (2001) *J. Cell Biol.* 153, 795–810

48. Hoeller, O., and Kay, R. R. (2007) *Curr. Biol.* 17, 813–817

49. Kae, H., Kortholt, A., Rehmann, H., Insall, R. H., Van Haastert, P. J., Spiegelman, G. B., and Weeks, G. (2007) *EMBO Rep.* 8, 477–482

50. Lee, S., Parent, C. A., Insall, R., and Firtel, R. A. (1999) *Mol. Biol. Cell* 10, 2829–2845

51. Lee, S., Comer, F. I., Sasaki, A., McLeod, I. X., Duong, Y., Okumura, K., Yates Iii, J. R., Parent, C. A., and Firtel, R. A. (2005) *Mol. Biol. Cell* 16, 4572–4583