

# A Novel Ras-interacting Protein Required for Chemotaxis and Cyclic Adenosine Monophosphate Signal Relay in *Dictyostelium*

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We have identified a novel Ras-interacting protein from *Dictyostelium*, RIP3, whose function is required for both chemotaxis and the synthesis and relay of the cyclic AMP (cAMP) chemoattractant signal. *rip3* null cells are unable to aggregate and lack receptor activation of adenylyl cyclase but are able, in response to cAMP, to induce aggregation-stage, postaggregative, and cell-type-specific gene expression in suspension culture. In addition, *rip3* null cells are unable to properly polarize in a cAMP gradient and chemotaxis is highly impaired. We demonstrate that cAMP stimulation of guanylyl cyclase, which is required for chemotaxis, is reduced ~60% in *rip3* null cells. This reduced activation of guanylyl cyclase may account, in part, for the defect in chemotaxis. When cells are pulsed with cAMP for 5 h to mimic the endogenous cAMP oscillations that occur in wild-type strains, the cells will form aggregates, most of which, however, arrest at the mound stage. Unlike the response seen in wild-type strains, the *rip3* null cell aggregates that form under these experimental conditions are very small, which is probably due to the *rip3* null cell chemotaxis defect. Many of the phenotypes of the *rip3* null cell, including the inability to activate adenylyl cyclase in response to cAMP and defects in chemotaxis, are very similar to those of strains carrying a disruption of the gene encoding the putative Ras exchange factor AleA. We demonstrate that *aleA* null cells also exhibit a defect in cAMP-mediated activation of guanylyl cyclase similar to that of *rip3* null cells. A double-knockout mutant (*rip3/aleA* null cells) exhibits a further reduction in receptor activation of guanylyl cyclase, and these cells display almost no cell polarization or movement in cAMP gradients. As RIP3 preferentially interacts with an activated form of the *Dictyostelium* Ras protein RasG, which itself is important for cell movement, we propose that RIP3 and AleA are components of a Ras-regulated pathway involved in integrating chemotaxis and signal relay pathways that are essential for aggregation.

## INTRODUCTION

*Dictyostelium* is an excellent experimental system in which to examine the signaling pathways that control chemotaxis because of the availability of genetic and biochemical approaches as well as physiological assays to study mutant phenotypes (Firtel, 1995; Van Haastert, 1995; Chen *et al.*, 1996; Parent and Devreotes, 1996; Chung and Firtel, 1999). During the early phase of development, up to 10<sup>5</sup> cells chemotactically aggregate to form a multicellular organism in response to the chemoattractant cyclic AMP (cAMP) emitted from cells. This process requires the coordinated regu-

lation of pathways that control the activation of adenylyl cyclase and the relay of the cAMP signal, chemotaxis toward cAMP, the activation of guanylyl cyclase and the *Dictyostelium* homologue of mammalian Akt/PKB required for chemotaxis, and the activation of the expression of genes required for this process (including the receptor, G $\alpha$  subunit, and cell adhesion molecules) (Firtel, 1995; Van Haastert, 1995; Chen *et al.*, 1996; Parent and Devreotes, 1996; Meili *et al.*, 1999). Activation of *Dictyostelium* Akt/PKB requires the function of the PI3 kinases PI3K1 and PI3K2, which are related to mammalian p110 $\alpha$  PI3K (Zhou *et al.*, 1995; Meili *et al.*, 1999). All of these pathways are regulated through the cell surface serpentine cAMP receptor cAR1 and the coupled G protein containing the G $\alpha$ 2 subunit. Disruption of genes encoding the cAMP receptor, the sole G $\beta$  subunit, or the

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Gα2 subunit results in complete abrogation of all four pathways (Firtel, 1995; Van Haastert, 1995; Chen *et al.*, 1996; Parent and Devreotes, 1996; Meili *et al.*, 1999).

Activation of the cAMP receptor by extracellular cAMP results in stimulation of the activity of the adenylyl cyclase ACA, which has a molecular architecture similar to that of mammalian adenylyl cyclases (Pitt *et al.*, 1992). The activation is mediated by the Gβγ subunit and requires cytosolic proteins including CRAC, a pleckstrin homology domain containing protein that translocates to the plasma membrane in response to receptor activation, and Pianissimo (Lilly *et al.*, 1993; Insall *et al.*, 1994b; Lilly and Devreotes, 1995; Chen *et al.*, 1997). In addition, activation of adenylyl cyclase and cAMP accumulation requires the function of the putative Ras exchange factor (GEF) Aimless (AleA) and the MAPK ERK2 (Segall *et al.*, 1995; Insall *et al.*, 1996). The control of chemotaxis is even more complex; it requires the coordinated regulation of the actin cytoskeleton, the function of myosin II, and the unconventional myosins IB and IC (Schleicher and Noegel, 1992; Peterson *et al.*, 1995; Chen *et al.*, 1996; Wessels *et al.*, 1996; Uyeda and Titus, 1997; Zigmund *et al.*, 1997). One of the second messengers required for chemotaxis is cyclic GMP (cGMP), which is thought to function, in part, through a cGMP-dependent protein kinase to activate myosin II kinase (Dembinsky *et al.*, 1996; Van Haastert and Kuwayama, 1997). Guanylyl cyclase activity is very rapidly and transiently stimulated in response to chemoattractants and requires a MAPK pathway distinct from that containing the MAPK ERK2 (Van Haastert and Van Lookeren Campagne, 1984; Ma *et al.*, 1997; Van Haastert and Kuwayama, 1997). MEK1, the cloned component of this pathway, is also required for the reorganization of the actin cytoskeleton, which may be dependent on MEK1's role in regulating guanylyl cyclase activation (H. Ma and R.A. Firtel, unpublished data). Another signaling pathway needed for proper chemotaxis involves the *Dictyostelium* homologue of mammalian Akt/PKB that requires the function of the PI3 kinases PI3K1 and PI3K2, which are related to mammalian p110α PI3K (Meili *et al.*, 1999).

In addition to its control of the activation of adenylyl cyclase, the putative Ras GEF AleA is required for proper chemotaxis (Insall *et al.*, 1996). *aleA* null cells are unable to chemotax effectively or properly polarize in a chemoattractant gradient. The cells produce pseudopodia along the perimeter of the cell, in contrast to wild-type cells, in which a single, predominant pseudopod is extended at the leading edge in the direction of the chemoattractant signal. *Dictyostelium* has five known Ras proteins, two of which (RasD and RasG) are most closely related to their metazoan counterparts (Reymond *et al.*, 1984; Pawson *et al.*, 1985; Esch *et al.*, 1993). RasG is preferentially expressed during early growth and development, whereas RasD is maximally expressed during the multicellular stages. Disruption of *RasD* does not exhibit growth or aggregation-stage defects (R. Insall, unpublished data). Cells expressing constitutively active RasD exhibit aberrant morphogenesis after the multicellular aggregate is formed and no abnormal phenotypes during aggregation (Reymond *et al.*, 1986). *rasG* null cells, on the other hand, exhibit defects in cytokinesis and general cell movement (Tuxworth *et al.*, 1997). In contrast to the cell movement defect of *aleA* null cells, which is observed only during

chemotaxis, the *rasG* null cell movement defect is observed for randomly moving cells.

To identify proteins that may interact with different *Dictyostelium* Ras proteins, we undertook a two-hybrid screen using the activated form of mammalian Ha-Ras (Ha-Ras<sup>G12V</sup>) as the bait. The logic was that using mammalian Ha-Ras rather than each individual *Dictyostelium* Ras protein might identify a broad spectrum of interacting proteins whose function may regulate pathways similar to those regulated by mammalian Ras proteins. The initial two-hybrid screen identified three Ha-Ras-interacting proteins, RIP1, RIP2, and RIP3 (Lee *et al.*, 1997). RIP2 (RasGAP1) is related to mammalian IQGAPs and is required for proper cytokinesis during vegetative growth and for morphogenesis during multicellular development. This paper describes the analysis of RIP3, which we show preferentially interacts with the *Dictyostelium* RasG protein in a two-hybrid system. *rip3* null cells have phenotypes very similar to those of *aleA* null cells; they are unable to activate adenylyl cyclase in response to a cAMP signal and have an impaired ability to chemotax. A *rip3/aleA* double-knockout strain exhibits a complete impairment in the ability to chemotax in a cAMP gradient, suggesting that both genes may regulate Ras-dependent pathways essential for chemotaxis. Our results suggest that RIP3 is a component of the Ras regulatory network that is required for signal relay and proper chemotaxis. Our findings suggest that RIP3 functions with AleA and possibly RasG in coordinating two essential functions during aggregation: chemotaxis and signal relay.

## MATERIALS AND METHODS

### Cloning of RIP3 Genomic Clone

Part of the RIP3 genomic clone encoding the amino-terminal two-thirds of the RIP3 open reading frame (ORF) and ~2.0 kilobase (kb) of the 5' regulatory sequence were cloned by construction of a mini genomic library. This domain of the RIP3 gene was mapped to ~5 kb of an *NdeI/BglIII* fragment. *Dictyostelium* DNA was digested with these two restriction enzymes and run on an agarose gel. The region between 4 and 6 kb was excised and cloned into a Bluescript II vector constructed to carry *NdeI* and *BamHI* restriction sites. The ligated DNA was transformed into *Escherichia coli*, and colonies were screened using a PCR-amplified hybridization probe from nucleotides 1880–2210 of the RIP3 ORF. Positive clones were picked and analyzed by DNA sequencing to ensure that the 3' portion of the RIP3 gene was contained in the plasmids.

### Construction of the *rip3* Null Strain

To construct a *rip3* null strain, the Bsr selectable marker cassette was cloned into the *BamHI* restriction site at nucleotide 1492 of the ORF. The RIP3 vector carrying the Bsr cassette was digested with *SpeI* and *EcoRV* restriction endonucleases, and the DNA was electroporated into wild-type KAx-3 cells selecting for Bsr-resistant clones after selection for 5 d in Bsr-containing medium. Rapid transformants were plated clonally, and random clones were picked and screened by PCR and Southern blot hybridization to identify *Dictyostelium* clones in which the RIP3 gene was disrupted with the Bsr cassette. There is a one-to-one correlation between the *rip3* null aggregation-deficient phenotype and those clones carrying a *rip3* gene disruption. Several independently derived clones were analyzed for various developmental properties. After demonstrating that all have a similar developmental phenotype and the inability to chemotax, indicating that expression of the RIP3 gene complements the null phenotype, a single clone was used for all subsequent experiments.

### Activation of Adenylyl and Guanylyl Cyclases

Adenylyl cyclase assays were performed as previously described by Devreotes *et al.* (1987). Briefly, cells were starved with pulses of cAMP for 5 h and treated with caffeine and vigorous shaking for 30 min at room temperature. Caffeine inhibits adenylyl cyclase and is used to bring the cells to a basal level of enzyme activity. Cells were washed twice and resuspended at  $8 \times 10^7$  cells/ml in 5 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 6.2, and 2 mM MgSO<sub>4</sub>. Receptor-mediated activation was performed by stimulating cells with 10  $\mu$ M cAMP. At specific time points, cells were lysed and assayed for 2 min at room temperature. For guanine thiotriphosphate (GTP $\gamma$ S)-mediated activation, the cells were lysed in the presence or absence of 40  $\mu$ M GTP $\gamma$ S and 1  $\mu$ M cAMP, incubated on ice for 4 min, and assayed for 2 min.

To measure cGMP production in response to cAMP stimulation, cells were prepared, stimulated, and assayed as described previously (Van Haastert and Van der Heijden, 1983; Ma *et al.*, 1997). Samples (100  $\mu$ l) were taken at appropriate intervals and processed using the cGMP <sup>3</sup>H assay system (Amersham, Arlington Heights, IL) following the manufacturer's instructions. The assay of each strain was independently repeated at least three times. All mutant strains were assayed along with wild-type cells as a direct comparison and as a control that the activation response was normal. Results of a representative experiment are shown.

### Video Imaging and Chemotaxis Assays

The video imaging and chemotaxis assays were performed as previously described (Ma *et al.*, 1997; Meili *et al.*, 1999). Briefly, log-phase vegetative cells were washed three times with Na/K phosphate buffer and resuspended at a density of  $2 \times 10^6$  to  $3 \times 10^6$  cells/ml in Na/K phosphate buffer and pulsed with 30 nM cAMP for 5 h at 6-min intervals. Cells were washed and resuspended in Na/KPO<sub>4</sub> buffer. Cells were plated in Na/K phosphate buffer at a density of  $6 \times 10^4$  cells/cm<sup>2</sup> onto a plate with a hole covered by a 0.17-mm glass coverslip and allowed to adhere to the surface for ~30 min. With an Eppendorf Patchman micromanipulator, a glass capillary needle (Eppendorf Femtotip) filled with a 150  $\mu$ M cAMP solution was brought into the field of view of an inverted microscope. The response was recorded using time-lapse video and NIH Image software, and the images were recorded directly on a computer hard drive.

For phase-contrast video microscopy, log-phase cells were washed and plated on a 60-mm Petri dish containing a thin agar layer. Cells were recorded using a 4 $\times$  phase objective. The movies were recorded using a S-VHS time-lapse videotape recorded with a CCD72S video camera (DAGE MTI, Michigan City, IN) using a Nikon (Garden City, NY) Optiphot-2 microscope and a 4 $\times$  phase contrast lens. Individual frames were captured into an image-processing program (NIH Image) with the help of a SCION frame grabber board.

Visible light images of chemotaxing cells were taken with a Nikon Eclipse TE 300 inverted microscope equipped for differential interference contrast imaging with a Plan Fluor ELWD 20 $\times$ /0.45 or a Plan Fluor ELWD 40 $\times$ /0.60 lens. Individual frames were captured from a CCD72S video camera into an image-processing program (NIH Image) with the help of a SCION frame grabber board as described by Meili *et al.* (1999).

## RESULTS

### Identification of RIP3

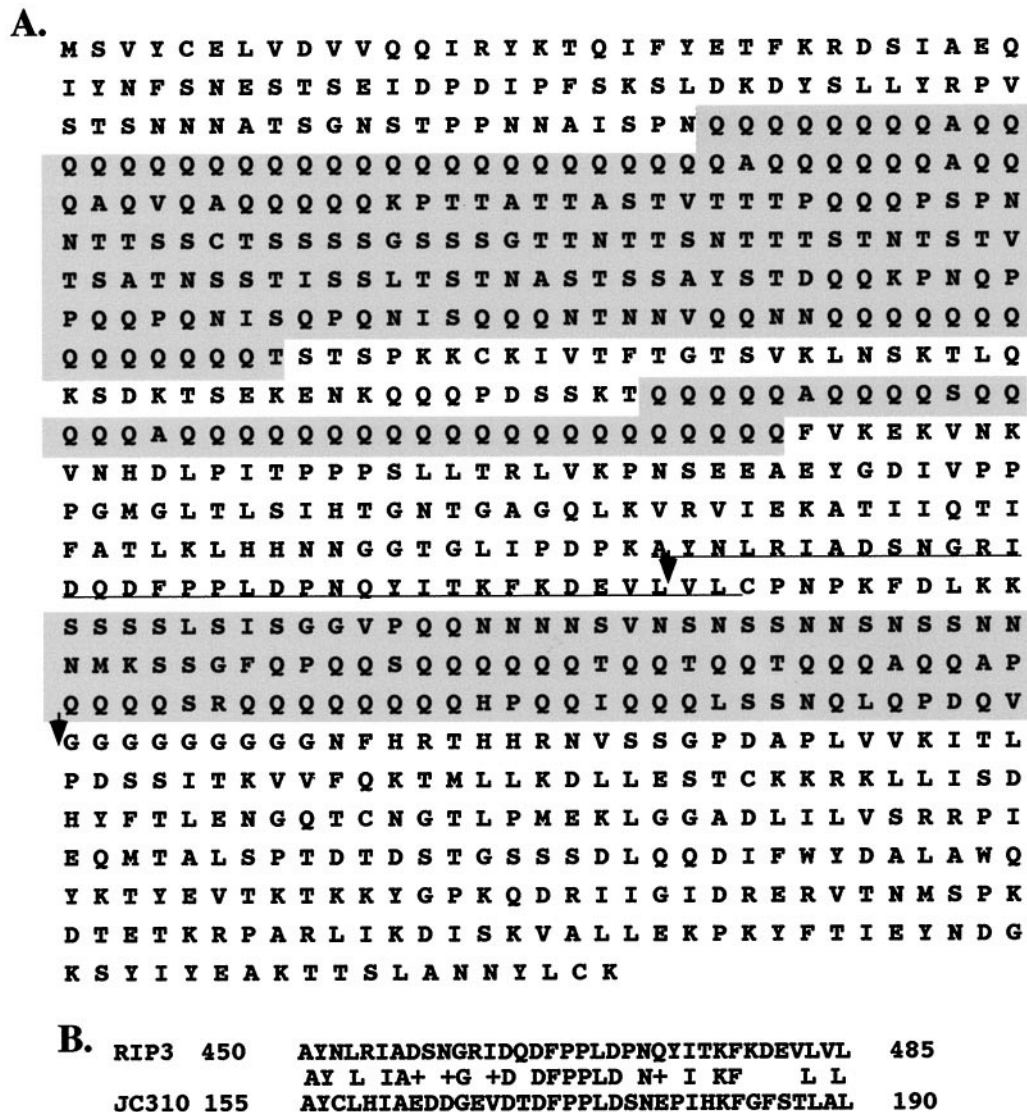
RIP3 was identified in a two-hybrid screen using an activated form of mammalian Ha-Ras (Ha-Ras<sup>G12V</sup>) as a bait (Lee *et al.*, 1997). Two inserts were identified of 1111 and 746 base pairs derived from the 3' end of the RIP3 gene (Figure

1A). (One insert was identified three times, the other once.) The insert from the yeast two-hybrid clone was used to screen a *Dictyostelium* cDNA library that yielded two partial cDNAs. The longest cDNA was used as a probe in a Southern blot analysis to map RIP3 to genomic DNA fragments to identify restriction sites to make a mini *Dictyostelium* genomic library. These results were then used to clone the remainder of the ORF and an ~2.0-kb upstream sequence containing the RIP3 promoter (see MATERIALS AND METHODS). Visual examination of the open reading frame derived from the genomic and cDNA clones shows polyglutamine stretches and other regions with highly reiterated polyamino acid sequences (Figure 1A). (The accession number for RIP3 is AF159241.) Although RIP3 has a higher fraction of such sequences than most *Dictyostelium* genes, such regions have been observed in numerous *Dictyostelium* genes and most are not thought to play a role in the function of the protein (Burki *et al.*, 1991; Mann and Firtel, 1991; Pitt *et al.*, 1992). A BLAST search did not suggest that RIP3 is highly homologous to known proteins. It did, however, identify a 36-amino acid nonsimple sequence region of RIP3 with homology to a region of a mammalian protein (clone JC310, GenBank number C38637) that was identified in a screen for mammalian proteins that suppress activated Ras function in the yeast *Saccharomyces cerevisiae* (Figure 1B; Colicelli *et al.*, 1991). As the two two-hybrid clones do not contain this region of homology, it cannot be the region that is responsible for the interaction with Ha-Ras in the two-hybrid screen.

### RIP3 Is a RasG-interacting Protein

To examine whether RIP3 interacts with *Dictyostelium* Ras proteins, we performed a two-hybrid assay using wild-type and activated forms of the five known *Dictyostelium* Ras proteins and activated Ha-Ras. The *Dictyostelium* RasG and RasD proteins are very homologous to each other, having only three conserved amino acid sequence changes in the first 110 amino acids (S/T, D/E, Y/F) and the Ras proteins most homologous to human Ha-Ras (Figure 2A; *Dictyostelium* RasB is included in the comparison) (Reymond *et al.*, 1984; Pawson *et al.*, 1985; Esch *et al.*, 1993). *Dictyostelium* RasG and RasD and human Ha-Ras show weaker homology in the last 40% of the protein. As shown in Figure 2B, RIP3 preferentially interacts with the *Dictyostelium* Ras protein RasG<sup>Q61L</sup> and only very weakly interacts with RasD<sup>Q61L</sup>. RIP3 exhibits strong interaction with activated human Ha-Ras (Ha-Ras<sup>G12V</sup>), as expected from its isolation in a two-hybrid screen using activated Ha-Ras<sup>G12V</sup> as the bait. RIP3 does not interact with the dominant negative form of Ha-Ras (Ha-Ras<sup>G15A</sup>) or RasG (RasG<sup>S17N</sup>). In contrast, the domain of the *Dictyostelium* IQGAP-related gene DdRasGAP1 that interacts with Ha-Ras<sup>G12V</sup>, RasB<sup>Q61L</sup>, and RasD<sup>Q61L</sup> showed no interaction with RasG<sup>Q61L</sup> (S. Lee and R.A. Firtel, unpublished data). The *Dictyostelium* PI3 kinase PI3K1 (Zhou *et al.*, 1995) has a Ras-interacting domain that is related to the Ras-interacting domain of the mammalian family of p110 PI3 kinases (C. Ellsworth, S. Lee, T.B.K. Reddy, and R.A. Firtel, unpublished data). This domain interacts strongly with RasG<sup>Q61L</sup>, only weakly with RasD<sup>Q61L</sup>, and not at all with RasB<sup>Q61L</sup>. These control experiments indicate that our observation that RIP3 interacts with RasG and not any of





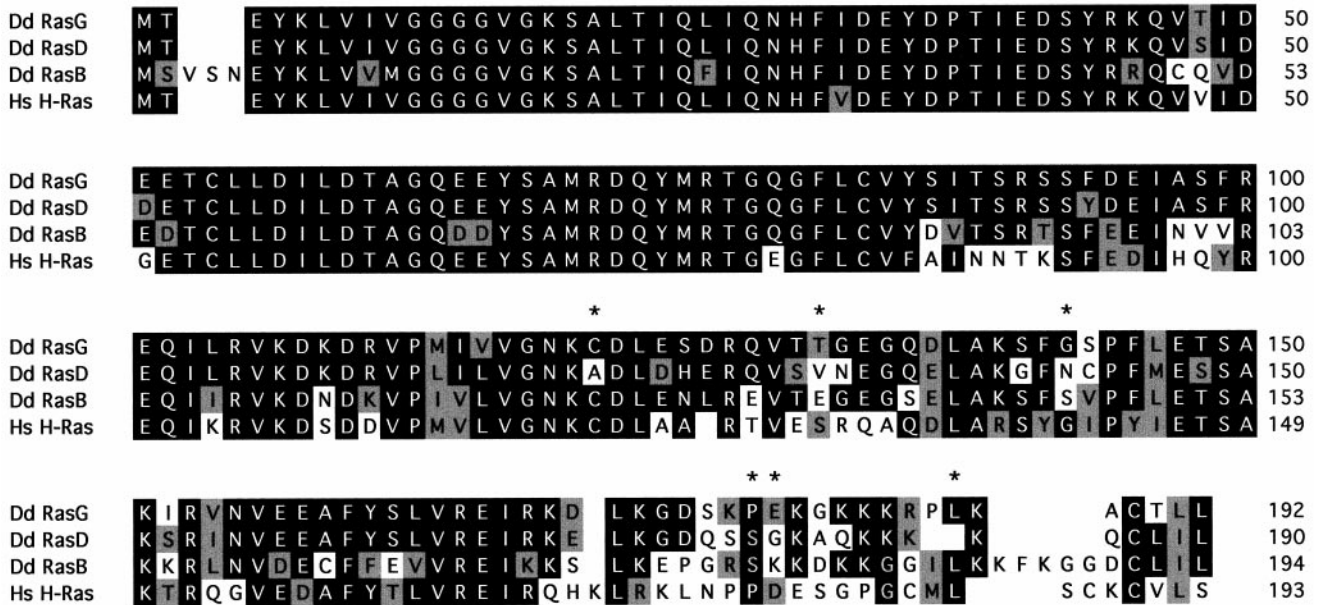
**Figure 1.** Sequence of RIP3. (A) The derived amino acid sequence of RIP3. The shaded areas contain a repetitive amino acid sequence that is often found in *Dictyostelium* ORFs and that is not thought to have an important function in the protein. RIP3 has a higher fraction of this sequence than other identified proteins. The domain that is homologous to the mammalian protein cloned, JC310, which was identified in a screen for mammalian genes that inhibit activated Ras function in yeast, is underlined. The two arrows mark the beginning of the ORFs of two different two-hybrid clones that were identified in our screen using activated mammalian Ha-Ras as bait. (B) Comparison of the amino acid homology between RIP3 and mammalian ORF JC310.

the other four *Dictyostelium* Ras proteins tested in these experiments is presumably not an expression artifact of the yeast two-hybrid system or the inability of the yeast two-hybrid system to demonstrate interaction of RasB and RasD with other *Dictyostelium* proteins. The difference in interaction between RasG and Ha-Ras with RIP3 compared with the lack of interaction with RasD was unexpected considering the level of homology of the proteins. Although we do not know which residues are responsible for these interaction differences in the two-hybrid system, there are six residues (marked with asterisks in Figure 2A)

in the terminal ~40% of the protein that are highly conserved between Ha-Ras and RasG but not RasD.

**RIP3 Is Developmentally Regulated and Required for Aggregation**

To examine the expression pattern of RIP3, a developmental RNA blot was made using RNA isolated from various stages in *Dictyostelium* development. As shown in Figure 3A, RIP3 is expressed at low levels in vegetative cells. The expression pattern is maximal during aggregation (4–8 h) and decreases thereafter.



	RIP3	Dd-IQGAP rel. RasGAP1	DdPI3K1
RasG(Q61L)	+++++	-	+++++
RasG(S17N)	-	-	-
RasD(Q61L)	-	+++	++
RasB(Q61L)	-	+++	-
RasC(Q61L)	-	-	-
RasS(Q61L)	-	-	-
Ha-Ras(G12V)	+++++	+++	+++
Ha-Ras(G15A)	-	-	-

Figure 2. Interaction of *Dictyostelium* RIP3 with different Ras proteins. (A) Amino acid sequence comparison of *Dictyostelium* RasG, RasD, and RasB and human Ha-Ras. The asterisks indicate positions of conservation between RasG and Ha-Ras but not RasD. (B) The carboxyl-terminal region of RIP3 that was identified and cloned in two-hybrid screens using mammalian Ha-Ras<sup>G12V</sup> as bait was used in two-hybrid assays to examine interaction of this domain with the activated form of the five identified *Dictyostelium* Ras proteins (RasG, RasD, RasB, RasC, and RasS). In addition, the dominant negative or nonactivatable form of RasG (RasG<sup>S17N</sup>) and interaction with Ha-Ras<sup>G12V</sup> and Ha-Ras<sup>G15A</sup> is shown. As controls, the interactions of the previously identified *Dictyostelium* IQGAP and the related gene RasGAPa as well as the Ras-interacting domain of *Dictyostelium* P110-related PI3 kinase DdPI3K1 are shown. The level of two-hybrid interactions was quantitated by the level of  $\beta$ -galactosidase production and the intensity of blue staining of yeast colonies.

To examine the function of RIP3, the gene was disrupted by homologous recombination as described in MATERIALS AND METHODS. Clones were picked randomly, and disruption of the RIP3 gene was confirmed by Southern blot analysis. There was a one-to-one correlation between the *rip3* null phenotype and disruption of the RIP3 gene by Southern blot analysis (our unpublished results). Wild-type cells form aggregates by 8 h, and by 13 h a tip forms (Figure 4A, a and b). The tip elongates to form a standing finger, which falls over, becoming a migrating slug or pseudoplasmodium (Figure 4Ac). As shown in Figure 4B, *rip3* null cells are unable to aggregate, producing only some rippling at 8 h (Figure 4Ba). At 24 h, there is some accumulation of cells into very loose, diffuse mounds (Figure 4Bb); however, the majority of the cells exhibit little sign of aggregation, and development does not proceed further. This phenotype is similar to that observed in *aleA* null cells (Insall *et al.*, 1996; our unpublished results). Expression of the RIP3 ORF from the cloned RIP3 promoter complements the *rip3* null phenotype (our unpublished results). Expression of the con-

struct in wild-type cells, which leads to a high overexpression of RIP3 transcripts, does not cause an observable aggregation or developmental phenotype (our unpublished results).

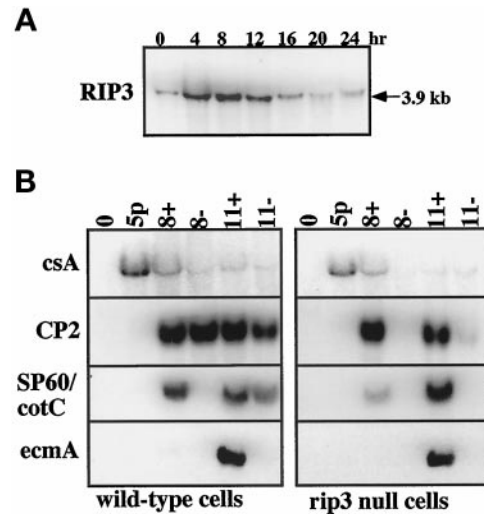
During aggregation, *Dictyostelium* cells respond to nanomolar oscillatory pulses of cAMP to induce the expression of genes required for this process (Gerisch, 1968; Noegel *et al.*, 1986; Mann and Firtel, 1987; Firtel, 1995). These genes include the cAMP receptor cAR1, the coupled  $G\alpha$  subunit  $G\alpha 2$ , and the cell adhesion molecule Contact Sites A (*csA*) (Noegel *et al.*, 1986; Mann *et al.*, 1988; Kumagai *et al.*, 1989; Saxe *et al.*, 1991). These genes are induced in wild-type strains during aggregation, with expression peaking at 4–8 h of development or in shaking culture in response to cAMP pulsing (Firtel, 1995; Ginsburg *et al.*, 1995). When *rip3* null cells are plated for development, no *csA* expression is detected (our unpublished results). We examined whether the inability of *rip3* null cells to aggregate might be caused by an inability of the cells to respond to cAMP and activate aggregation-stage gene expression or whether the cells can be induced if pulsed

with exogenous cAMP. As shown in Figure 3B, *csA* mRNA is normally expressed in *rip3* null cells in response to cAMP signaling, suggesting that the aggregation defect is not due to an inability to induce aggregation-stage gene expression.

The aggregation defects of *rip3* null cells were examined in more detail using time-lapse video microscopy (Ma *et al.*, 1997). In wild-type cells, aggregation centers and waves of cAMP are visualized as changes in the conformation of cells, resulting in lighter and darker regions within the field of cells. For wild-type cells, these patterns are first seen by 3 h, 40 min after plating (Figure 5A). Aggregation domains become defined shortly thereafter (Figure 5A). The initial stages of chemotaxis, as seen by the movement of cells toward the centers, are visible by 4 h, 20 min. Aggregates are formed by 6 h. The phase contrast patterns observed for *rip3* null cells are quite different. Wave patterns within the field of *rip3* null cells observed on the videotapes are very limited (our unpublished results), and the initial formation of aggregation centers is delayed until 6 h (Figure 5B). By 9 h, multiple aggregation centers are visible. In contrast to wild-type cells, the *rip3* null cell aggregation domains are quite small, suggesting an impairment in the ability to activate and relay the cAMP signaling pathway and/or to demonstrate cell shape changes in response to the cAMP. In addition, half of the *rip3* null cell aggregation domains form and then disperse after a few hours, and the same domains are not observed when the time lapse recordings are examined (our unpublished results). In addition, the cells do not aggregate and no true mounds are formed, even after 15 h. The domains are still observed at 21 h, although they are fewer and less organized. These data suggest that the inability to chemotax to form a mound could be caused by defects in chemotaxis and/or the response to cAMP signaling.

### *rip3* Null Cells Form Aggregates in Response to cAMP Signaling and Induce Cell-Type-Specific Gene Expression

Some aggregation-deficient strains that are impaired in the ability to aggregate are able to form multicellular organisms after the cells have been pulsed with 30 nM cAMP for 5 h to mimic the normal oscillatory pulses of cAMP that occur during aggregation (Devreotes *et al.*, 1987; Insall *et al.*, 1994b, 1996; Ma *et al.*, 1997). Figure 6B demonstrates that when *rip3* null cells are pulsed with cAMP and plated on a nonnutrient agar surface they form mounds, although mound formation is delayed compared with wild-type cells, which form mounds in 1.5 h under these conditions (Figure 6A). Wild-type cells proceed through development and form migrating slugs by 9 h and mature fruiting bodies by 15 h (Figure 6A; our unpublished results). The majority of *rip3* null mounds, however, arrest for 3–10 h before tip formation. By 30 h, less than half of these mounds have formed fruiting bodies (our unpublished results). Protein kinase (PKA) is required for multiple aggregation-stage pathways (Mann and Firtel, 1991; Schaap *et al.*, 1995; Mann *et al.*, 1997), and constitutive, high levels of expression of the catalytic subunit of cAMP-dependent PKA can bypass the inability of the aggregation-deficient null mutations in the MAPK ERK2 and the aggregation-stage adenylyl cyclase ACA to

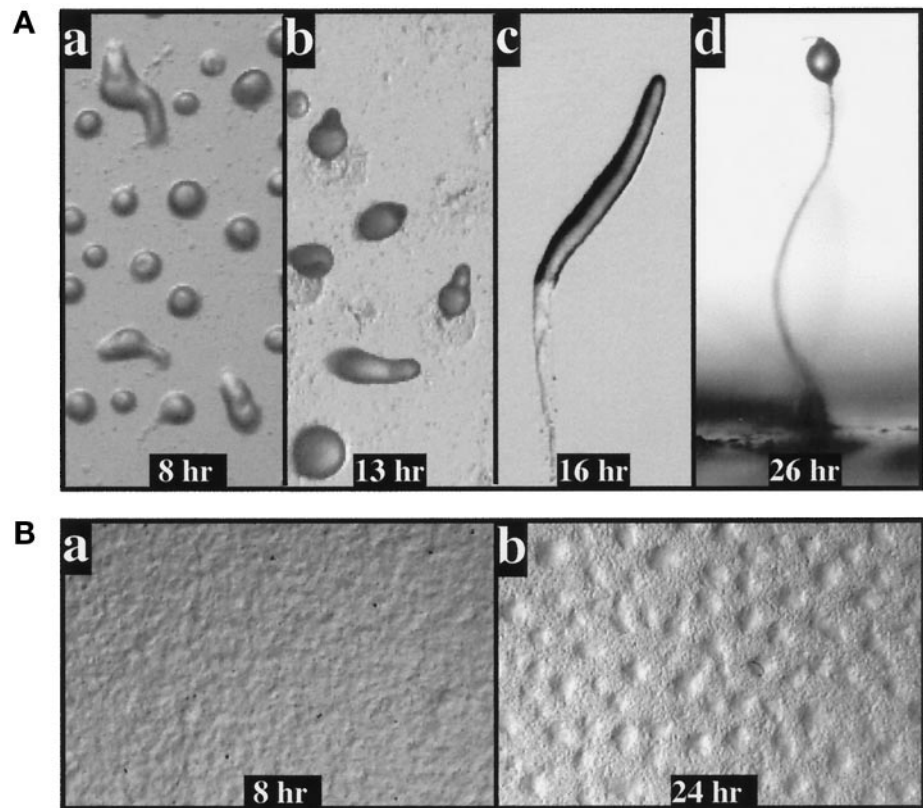


**Figure 3.** Developmental kinetics of RIP3 gene expression and the requirement of RIP3 for expression of aggregation and postaggregative genes. (A) RNA blot analysis of the expression pattern of RIP3 using RNA isolated at different stages of *Dictyostelium* development. Mound formation occurs at ~8 h and culmination initiates at ~18–20 h. (B) Expression of aggregation-stage, postaggregative, and cell-type-specific genes in wild-type and *rip3* null cells in suspension culture in response to cAMP. Cells were pulsed for 5 h with 30 nM cAMP (5p). Cells were given cAMP under slow-shake suspension conditions, which allow cell-cell interactions (120 rpm), to 300  $\mu$ M and were supplemented to 300  $\mu$ M every 2 h. 8+ and 11+ represent time points (hours) after the initiation of the experiment. The plus sign represents the addition of exogenous cAMP. Minus cultures (8- and 11-) did not receive exogenous cAMP. Cultures were split after 5 h of cAMP pulsing. *csA* (Contact Sites A) is a marker for aggregation-stage gene expression; CP2 is a marker for postaggregative gene expression. *SP60/CotC* is a prespore-specific gene; *ecmA* is a prestalk-specific gene.

form aggregates and undergo morphogenesis (Aubry *et al.*, 1997; Wang and Kuspa, 1997). *rip3* null cells constitutively expressing the catalytic subunit of cAMP-dependent PKA do not form aggregates when plated on non-nutrient agar under standard conditions, indicating that PKA is unable to suppress the *rip3* null phenotype (our unpublished results).

We investigated, by time-lapse video microscopy (as described in Figure 5), whether the aggregates formed by wild-type and *rip3* null cells after cAMP pulsing occur through the chemotactic aggregation of cells. Figure 6A shows that wild-type cells form aggregation domains, which are larger than those formed by the same strain when plated for development with previous exogenous pulsing with cAMP (compare with Figure 5A). These domains are observed within 40 min of plating and start to chemotax and form aggregates by 55 min. Large aggregates form in ~3 h. In contrast, *rip3* null cells form numerous microcenters similar to those observed for *mek1* null cells (Ma *et al.*, 1997), which leads to the formation of many small aggregates (Figure 6B). Closer analysis of the time-lapse videotapes reveals that the *rip3* null cells, like *mek1* null cells, coalesce to form the aggregates rather than chemotaxing like wild-type cells (our unpublished re-





**Figure 4.** Developmental phenotypes of wild-type and *rip3* null cells. (A) The developmental phenotypes of wild-type KAx-3 cells. Mound formation is visible at 8 h. At 13 h, most of the aggregates have formed a tip, and at 16 h, the cells have formed a migrating slug or pseudoplasmodium. Cells have culminated by 24–26 h. (B) Developmental phenotype of *rip3* null cells. At 8 h, the cells exhibit a small amount of rippling. At 24 h, very loose cellular associations are observed. Half or less of the cells are associated with the aggregates. Most cells show no sign of participation in aggregate formation.

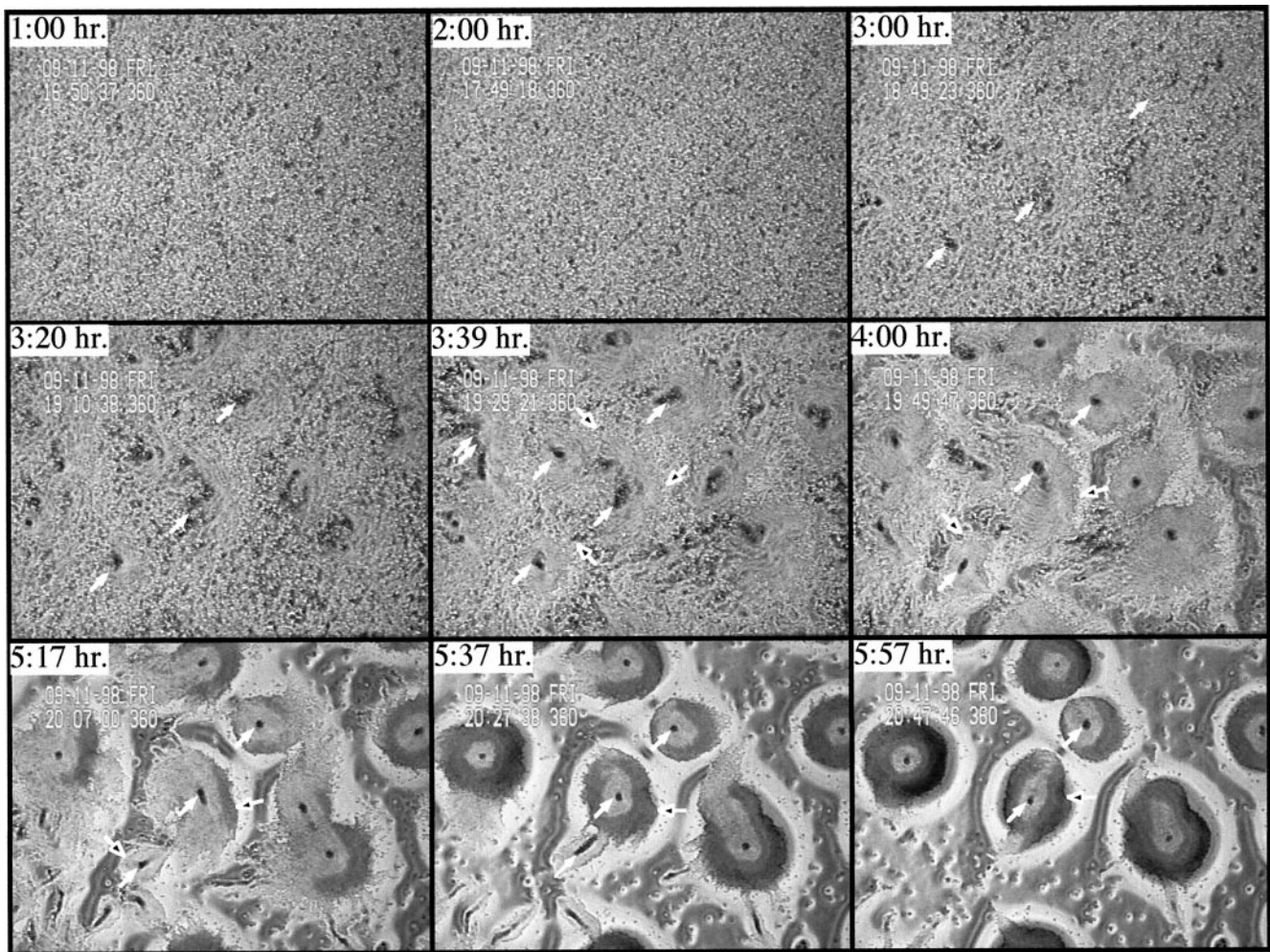
sults). The coalescence may be facilitated by cell-cell interactions mediated through cell adhesion molecules such as *csA* that are induced in response to cAMP pulses given before the cells are plated for development. These data suggest that *rip3* null cells exhibit a chemotaxis defect.

We examined whether *rip3* null cells express postaggregative genes, which are induced at mound formation in wild-type cells, and cell-type-specific genes in response to cAMP signaling (Firtel, 1995). Postaggregative genes such as *CP2* are expressed at the mound stage or in cells in suspension that have been previously pulsed for 5 h with 30 nM cAMP to express aggregation-stage genes and then induced with continuous micromolar levels of cAMP (Pears *et al.*, 1985; Datta *et al.*, 1986). This leads to the induction of cell-type-specific (e.g., *ecmA* [prestalk-specific] and *SP60/CotC* [prespore-specific]) genes, which requires cell-cell contact in addition to exogenous, high, continuous levels of cAMP (Mehdy and Firtel, 1985; Jermyn *et al.*, 1987). The morphogen differentiation-inducing factor is also required for maximal prestalk gene expression and is supplied endogenously (Jermyn *et al.*, 1987; Williams *et al.*, 1987). *CP2* and the cell-type-specific genes *ecmA* (prestalk-specific) and *SP60/CotC* (prespore-specific) are not induced in *rip3* null cells plated for development (our unpublished results), as might be expected for cells with an aggregation defect. In suspension culture, *CP2* is induced in wild-type cells with or without exogenous cAMP (Figure 3B). Under assay conditions (slow-shake culture) that permit cell-cell contacts to form, there is sufficient endogenous cAMP produced for *CP2* gene ex-

pression in wild-type cells. However, *rip3* null cells induce *CP2* in suspension culture only in the presence of exogenous cAMP (Figure 3B). The absence of the expression of *CP2* in *rip3* null cells under these assay conditions suggests that *rip3* null cells may be defective in cAMP production. The cell-type-specific genes *ecmA* and *SP60/CotC* are induced by both *rip3* null and wild-type cells and require exogenous cAMP, as previously demonstrated for wild-type cells (Mehdy and Firtel, 1985; Jermyn *et al.*, 1987; Dynes *et al.*, 1994). The combined results indicate that *rip3* null cells are not defective in cAMP-induced gene expression but are defective in cAMP production.

#### *rip3* Null Cells Are Defective in Receptor Activation of Adenylyl and Guanylyl Cyclases

The developmental defects of *rip3* null cells suggest that they are impaired in the ability to activate adenylyl cyclase and relay the cAMP signal. To examine this possibility, receptor- and G protein-mediated activation of adenylyl cyclase were measured in wild-type, *rip3*, and *aleA* null cells. *aleA* null cells exhibit phenotypes (Insall *et al.*, 1996) similar to those of the *rip3* null cells described here. Cells were pulsed with cAMP for 5 h to maximally express receptors and G proteins. The pulsed cells were stimulated *in vivo* with cAMP, lysed at specific time points, and assayed for adenylyl cyclase activity, as described previously (Devreotes *et al.*, 1987). As depicted in Figure 7A, wild-type cells exhibit a 7.4-fold stimulation of adenylyl cyclase activity, which peaks at the 60-s time



**Figure 5.** Phase contrast video microscopic analysis of aggregate formation in wild-type and *rip3* null cells. (A) Phase contrast video microscopy of wild-type cells is depicted as described previously. Briefly, cells are plated as a monolayer on nonnutritive agar and examined by phase contrast video microscopy using a 4 $\times$  objective. The solid white arrows point to some of the aggregation centers. The open white arrows point to the outer regions of individual aggregation domains. Only a few of these are marked. (B) *rip3* null cells. A similar analysis was performed on *rip3* null cells.

point, consistent with previous results (Roos and Gerisch, 1976). In agreement with previously published results, *aleA* null cells exhibit very little or no activation (Insall *et al.*, 1996). Similarly, *rip3* null cells display very low levels of activity.

Activation of adenylyl cyclase is mediated by the G $\beta\gamma$  subunits and can be activated in wild-type cells that are lysed in the presence of GTP $\gamma$ S (Wu *et al.*, 1995). This activation does not require the coupled G $\alpha$ 2 subunit, as GTP $\gamma$ S activation occurs in *ga2* null cells, presumably because lysis of cells in the presence of GTP $\gamma$ S releases G $\beta\gamma$  from other heterotrimeric G proteins. Cells pulsed with cAMP for 5 h were lysed in the absence or presence of GTP $\gamma$ S and assayed for adenylyl cyclase activity. In wild-type cells, GTP $\gamma$ S induces the activity of adenylyl cyclase 31.5-fold compared with basal activity (Figure 7B). As we observed with the *in vivo* stimulation, *rip3* and *aleA* null cells exhibit greatly reduced adenylyl cyclase activity

in the presence of GTP $\gamma$ S (Figure 7B). The inefficiency of GTP $\gamma$ S stimulation of adenylyl cyclase activity in *rip3* null cells is unexpected, as it suggests that RIP3 function is required for function of the G protein (see DISCUSSION). The activity of adenylyl cyclase can be stimulated by MnSO<sub>4</sub>, which gives a measurement of the unregulated activity of the enzyme. Wild-type, *rip3* null, and *aleA* null cells show a similar level of MnSO<sub>4</sub>-stimulated activity, suggesting that the total adenylyl cyclase enzymatic activity is similar in the three strains (Figure 7B). These results were confirmed by Western blot analysis (our unpublished results).

Many of the developmental phenotypes of *rip3* null cells are similar to those of *aleA* null cells (Insall *et al.*, 1996). To examine this similarity in more detail, we created a *rip3/aleA* double-knockout strain. Adenylyl cyclase assays in this strain were done in parallel with those in the wild-type, *rip3*, and *aleA* null strains. Interestingly, the



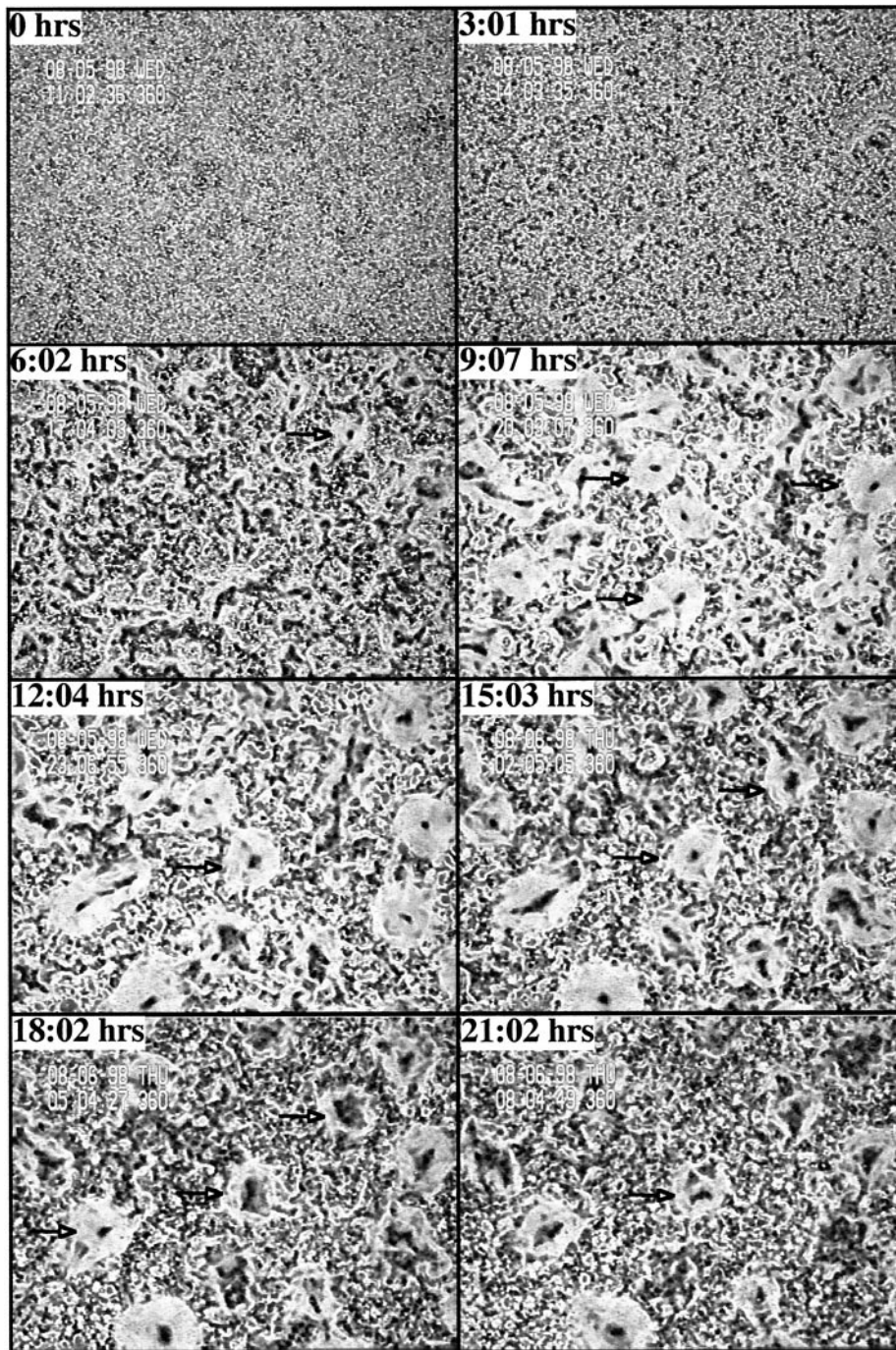


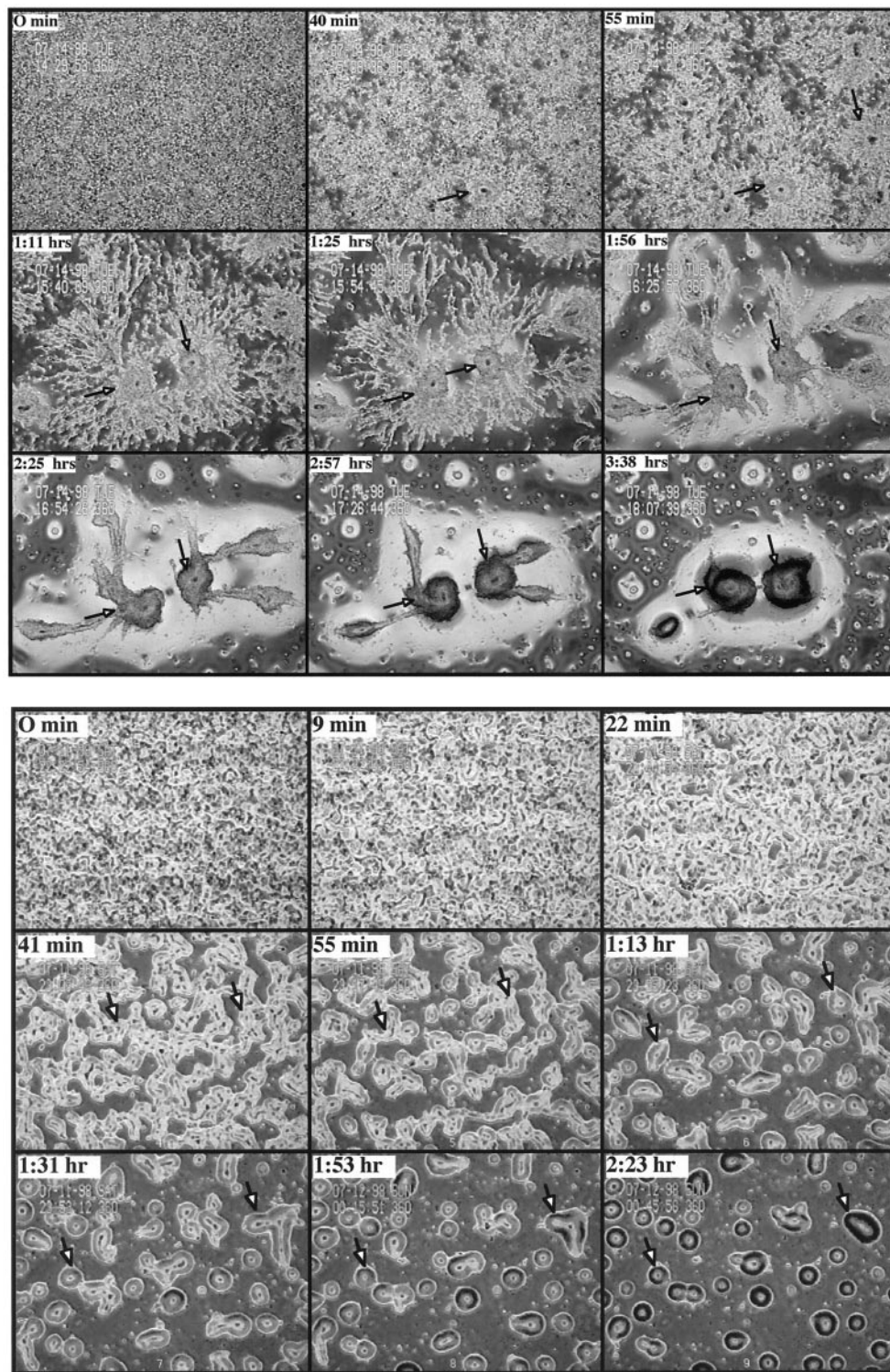
Figure 5B.

*rip3/aleA* double-knockout strain shows a slight adenylyl cyclase activity in response to cAMP stimulation (Figure 7B) and a slightly higher GTP $\gamma$ S-stimulated activity than either null strain (Figure 7B).

Receptor-mediated activation of guanylyl cyclase is required for chemotaxis (Van Haastert and Kuwayama, 1997). As described previously (Van Haastert and Van der Heijden,

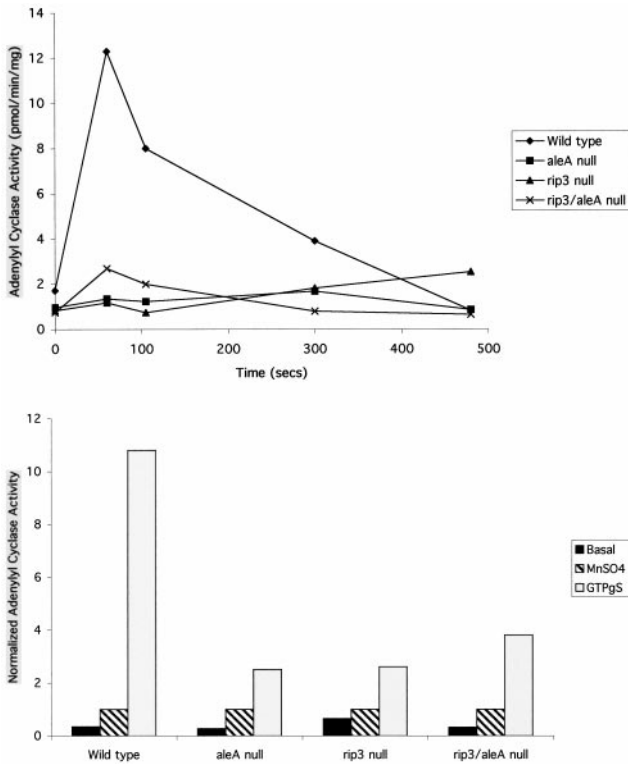
1983), cAMP stimulation of wild-type cells pulsed for 5 h (aggregation-competent cells) results in a rapid, transient activation of guanylyl cyclase (Figure 8). In contrast, *rip3* and *aleA* null cells show a significantly reduced cAMP-induced activation of guanylyl cyclase activation response that was ~40% that of the wild-type response in three separate experiments. When the *rip3/aleA* null strain was examined, the





**Figure 6.** Phase contrast video microscopic analysis of wild-type and *rip3* null cells aggregating after being pulsed for 5 h with cAMP. Wild-type (A) or *rip3* null (B) cells were pulsed for 5 h with 30 nM cAMP and plated on nonnutrient agar as described in Figure 5.



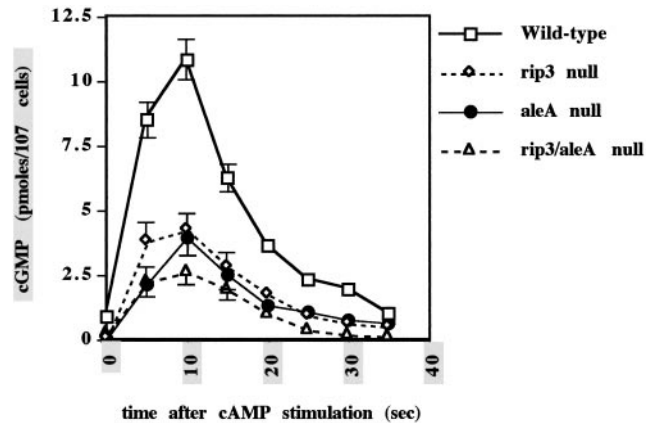


**Figure 7.** Adenylyl cyclase activation in wild-type and mutant cell lines. (A) Receptor-mediated stimulation of adenylyl cyclase activity. Differentiated cells were stimulated with 10  $\mu$ M cAMP, lysed at specific time points, and immediately assayed as described in MATERIALS AND METHODS. (B) GTP $\gamma$ S-mediated stimulation of adenylyl cyclase activity. Differentiated cells were lysed with or without GTP $\gamma$ S, or in the presence MnSO<sub>4</sub> as a measure of unregulated enzyme activity, incubated on ice for 4 min, and assayed as described in MATERIALS AND METHODS. The results are expressed as a ratio of the adenylyl cyclase activity to MnSO<sub>4</sub> activity. The absolute MnSO<sub>4</sub> activities are 4.0, 3.9, 2.5, and 2.5 pmol·min<sup>-1</sup>·mg<sup>-1</sup> for wild-type, *aleA* null, *rip3* null, and *rip3/aleA* null cells, respectively. The results presented are representative of at least two independent experiments.

level of stimulation was further reduced to ~25% that of wild-type cells in three separate experiments. These results indicate that *rip3* and *aleA* null cells are defective in the chemoattractant-mediated activation of guanylyl and adenylyl cyclases.

### RIP3 Is Required for Proper Chemotaxis

To examine whether *rip3* null cells are impaired in the ability to chemotax toward cAMP, we used an assay in which cells chemotax on the glass coverslip toward cAMP that is emitted from a micropipet (Gerisch *et al.*, 1975; Meili *et al.*, 1999). As depicted in Figure 9A, wild-type cells become highly polarized and move toward the micropipet by extending pseudopodia in the direction of the micropipet (up the cAMP concentration gradient). Time-lapse video microscopy reveals that the vast majority of wild-type cells exhibit this highly polarized cell shape and produce very few pseu-



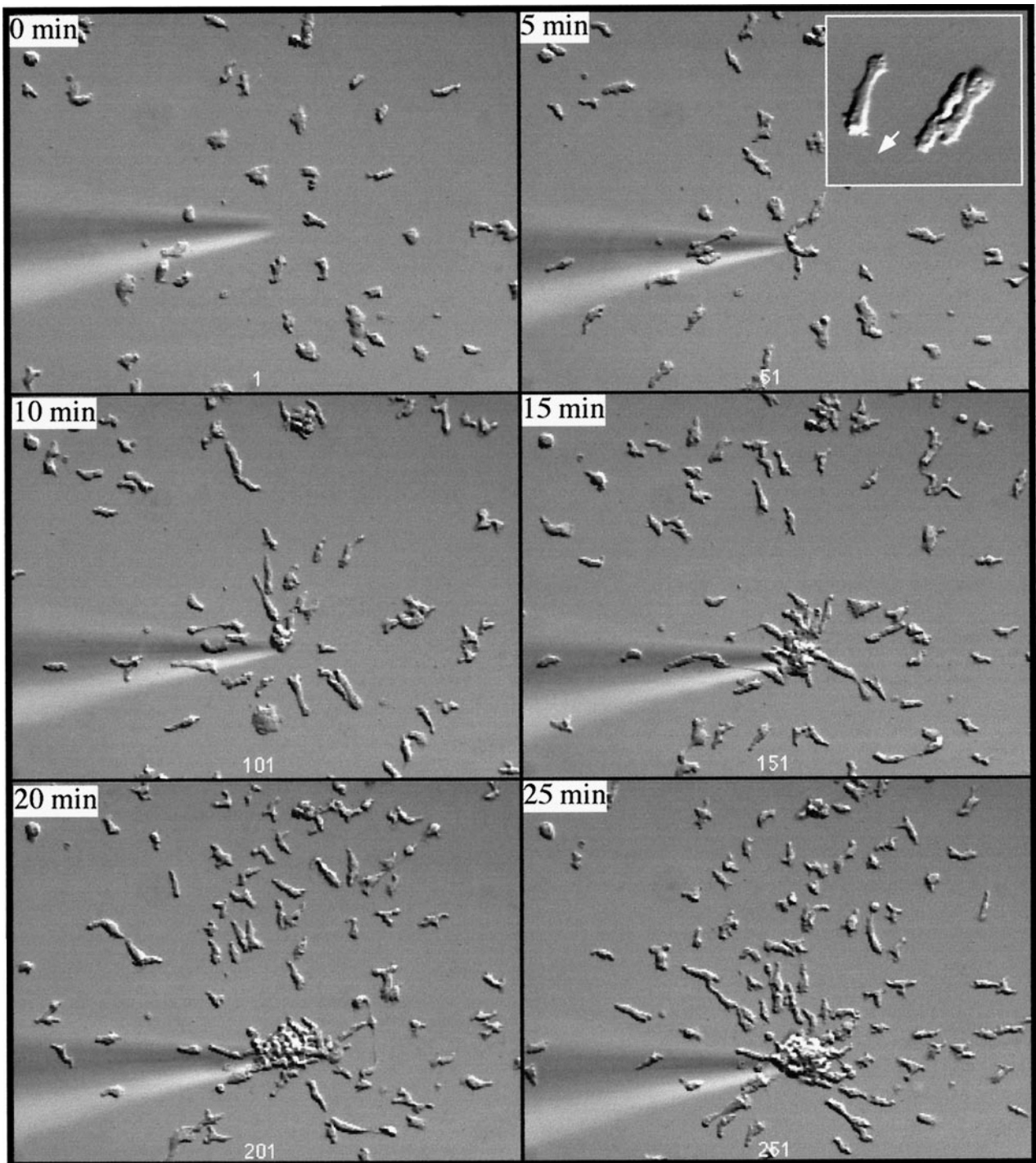
**Figure 8.** cAMP stimulation of guanylyl cyclase activity in wild-type, *rip3* null, *aleA* null, and *rip3/aleA* null strains. Cells were pulsed for 5 h with 30 nM cAMP and stimulated with cAMP, and measurements were taken for the quantitation of cGMP as described in MATERIALS AND METHODS. Unregulated activity of the enzyme was measured in the presence of 5 mM MnSO<sub>4</sub>. Each strain was analyzed a minimum of three times in association with wild-type cells. The results of representative experiments are shown. The differences between *rip3* and *aleA* null cells, wild-type cells and *rip3/aleA* null cells, and *rip3* and *aleA* null strains are reproducible.

dopodia in directions that are either perpendicular or oblique to the axis of the cAMP gradient (Figure 9A, inset). In contrast, *rip3* null cells are less polarized and migrate more slowly than wild-type cells (Figure 9B). Detailed analysis of the time-lapse video indicates that the cells produce numerous pseudopodia and filopodia at right or oblique angles to the direction of the cAMP gradient (Figure 9B, inset). These results indicate that *rip3* null cells are impaired in their ability to chemotax up a concentration gradient of chemoattractants, which is consistent with the aggregation-defective phenotypes described above.

*aleA* null cells exhibit aberrant chemotaxis toward cAMP (Insall *et al.*, 1996). To further examine potential genetic interactions of RIP3 and AleA in controlling cell movement, we performed a similar chemotaxis assay using the double-knockout cells. As shown in Figure 9C, these cells exhibit very little movement or polarization toward cAMP. When the *rip3/aleA* double-knockout cells are pulsed and plated for development, only a small fraction of the cells coalesce to form mound-like structures by 24 h (our unpublished results), and development does not proceed further.

### DISCUSSION

Chemotaxis requires coordinated changes in the actin and myosin cytoskeletons (see INTRODUCTION for references). In the case of *Dictyostelium*, these changes are mediated by chemoattractants such as cAMP and folic acid that function through discrete cell surface serpentine receptors coupled to heterotrimeric G proteins containing different G $\alpha$  protein subunits. Aggregation in *Dictyostelium* requires the integrated regulation of pathways controlling chemotaxis, activation of adenylyl and guanylyl cyclases, and relay of cAMP. These processes are mediated through the cAMP



**Figure 9.** Chemotaxis of wild-type, *rip3* null, and *rip3/aleA* null strains. Cells were pulsed for 5 h with 30 nM cAMP, plated on coverslips as described previously, and examined by differential interference contrast microscopy. The micropipet shown contains 150  $\mu$ M cAMP. Cells chemotax toward the cAMP gradient produced by diffusion of the cAMP from the micropipet. Insets in A and B depict the shapes of wild-type and *rip3* null cells, respectively. (A) Analysis of wild-type cells was done with a 20 $\times$  objective, whereas that of *rip3/aleA* null cells was done with a 40 $\times$  objective. (A) Wild-type cells. (B) *rip3* null cells. (C) *rip3/aleA* null cells.



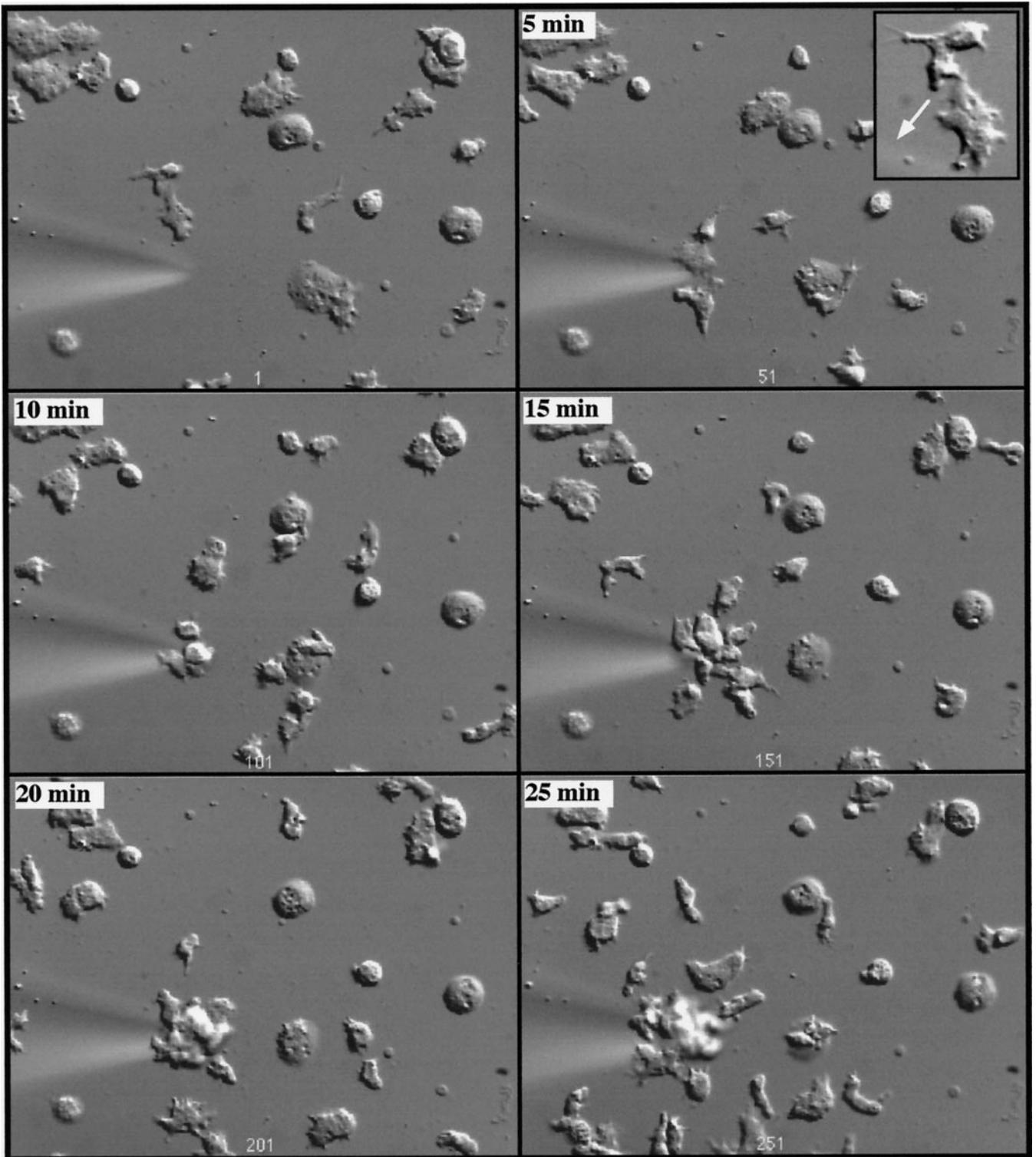


Figure 9B.

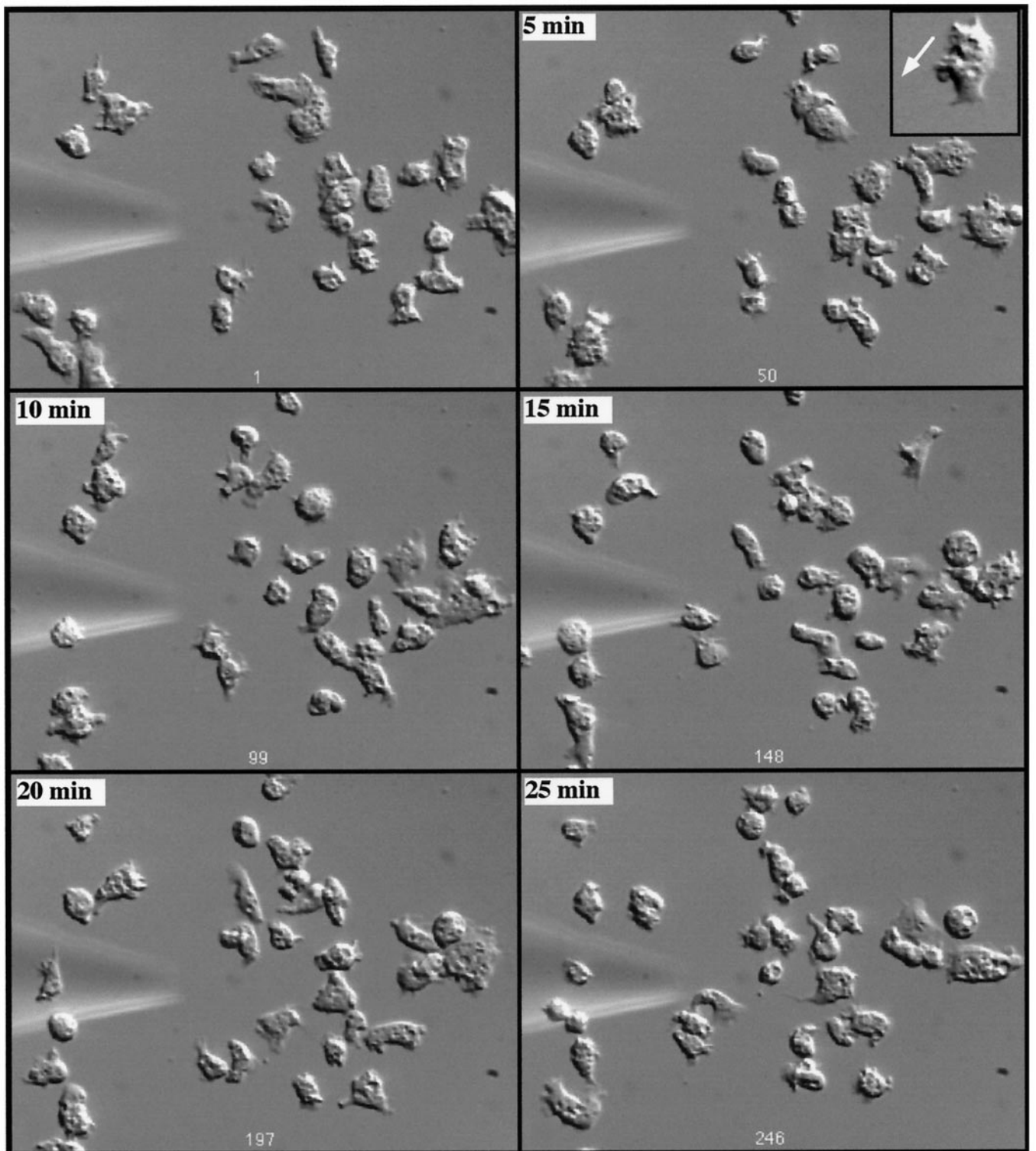


Figure 9C.



receptor and downstream signal transduction pathways that require the heterotrimeric G protein containing the  $G\alpha_2$  subunit.

Our analysis demonstrates that *rip3* null cells are impaired in the signal relay pathway and chemotaxis. *rip3* null cells show only a minimal level of cAMP-mediated activation of adenylyl cyclase in cells lysed at various times after stimulation. When cells are lysed in the presence or absence of  $GTP\gamma S$  to quantitate GTP-dependent stimulation of adenylyl cyclase activity, *rip3* and *aleA* null cells exhibit very little adenylyl cyclase activity compared with wild-type cells, although  $MnSO_4$ -stimulated activity is similar in all strains. The most simple genetic interpretation of these biochemical results suggests that RIP3 and AleA are required for  $G\beta\gamma$  stimulation of adenylyl cyclase. One possible function of RIP3 is to control the function of other components of the pathway that are required for  $G\beta\gamma$ -stimulated adenylyl cyclase activity. As the function of the pleckstrin homology domain-containing protein CRAC is essential for activation of adenylyl cyclase (Insall *et al.*, 1994a; Lilly and Devreotes, 1995; Parent *et al.*, 1998), one possible role of RIP3 is to potentiate one of these processes. Previous results and the results presented here indicate that *aimless* and *rip3* null cells exhibit similar defects in adenylyl cyclase activation. Moreover, of the numerous genes that are required for proper aggregation (Firtel, 1995; Chen *et al.*, 1996; Parent and Devreotes, 1996), only RIP3 and AleA are required for both chemotaxis and signal relay. It is possible that RIP3 and AleA function in a similar pathway, especially considering that RIP3 interacts in vitro with RasG and AleA is a putative Ras GEF.

*rip3* null cells do not express aggregation-stage or postaggregative and cell-type-specific genes when the cells are plated for development. These defects are probably due to the defect in the activation of cAMP and signal relay, as *rip3* null cells induce all three classes of genes in response to exogenous cAMP. PKA activity is required for multiple developmental pathways, including aggregation (Mann and Firtel, 1991; Reymond *et al.*, 1995; Schulkes and Schaap, 1995; Mann *et al.*, 1997). We demonstrate that constitutive expression of the PKA catalytic subunit does not complement the *rip3* null phenotype, indicating that *rip3* null cells have sufficient levels of endogenous PKA activity for these processes. This suggests that there is probably a low level of activation of adenylyl cyclase in vivo. Consistent with this possibility, we observe a low level of  $GTP\gamma S$ -stimulated activity in vitro and the formation of rudimentary aggregation domains, which requires a low level of cAMP relay, when *rip3* null cells are plated for development as visualized in the phase contrast time-lapse video imaging.

It is very striking that *rip3* null cells have a defect in chemotaxis. The cells are less polarized than wild-type cells in a cAMP gradient, being unable to properly elongate. Instead, *rip3* null cells extend pseudopodia laterally as well as in the direction of the cAMP signal. This defect is observed whether the micropipet is close to or farther away from cells, indicating that the defect occurs in response to different concentration gradients of cAMP. As with the regulation of adenylyl cyclase, *aleA* and *rip3* null strains exhibit similar chemotaxis defects. We have shown that the activation of guanylyl cyclase is reduced in *rip3* null cells. However, it is not clear that the reduced activation of guanylyl

cyclase is sufficient to cause the chemotaxis defects we observe. Although we have no direct evidence, our view is that RIP3 controls other pathways that are also required for chemotaxis and that it is the defect in these pathways or a combined defect in multiple pathways that leads to the chemotaxis defects. Our analysis of the *rip3/aleA* double knockout indicates that these cells exhibit an even greater impairment in the ability to chemotax and do not respond to cAMP gradients produced by cAMP diffusing from a micropipet. The stronger phenotype of the double knockout has two interpretations. The first is that the proteins function in two direct, parallel pathways leading to chemotaxis and that the combined partial impairment of both pathways results in a sufficient loss of function to render the cells unable to chemotax. The second interpretation is that RIP3 and AleA function on the same pathway but both null phenotypes are leaky with respect to their requirement for the pathway. The double knockout would result in more efficient blocking of the pathway. As AleA is a putative Ras exchange factor (GEF) and RIP3 has specific interactions with RasG, which is known to be required for proper cell movement, it is reasonable to postulate that AleA and RIP3 control a common pathway by either regulating or being regulated by the Ras protein, presumably RasG. However, as the *rasG* null phenotype is not the same as that of *rip3* null cells during aggregation, it is possible that RIP3 interacts genetically with another, not-yet-identified Ras protein or has a function that does not require interaction with RasG or another Ras protein. Although we expect Ras to be involved, we cannot distinguish between functions of AleA and RIP3 that do or do not require Ras and the phenotypes we observe.

One of our most striking observations is that this pathway that we expect involved Ras-mediated pathways is important for the activation of adenylyl cyclase and chemotaxis. It is not unexpected that such a coordination would be important to produce an integrated biological response. Although this coordination was expected to be mediated, at least partially, through chemoattractant activation of a common heterotrimeric G protein, the direct coupling of these pathways through pathways containing components that interact with Ras was unexpected. The phenotypes of AleA and RIP3 during chemotaxis are more severe than those of *rasG* null cells. We cannot exclude the possibility that another yet-to-be-identified Ras may be the key component of this pathway or that RIP3 may exert functions independent of possible interactions with RasG. It is possible that RIP3 could function as a scaffolding for various components of the chemotaxis and signal relay pathways and that RasG may coordinate or activate solely the chemotaxis component of the aggregation-stage pathways. RIP3 exhibits a restricted homology to a mammalian gene that was identified as a protein that represses the phenotype of activated Ras in yeast (Colicelli *et al.*, 1991). Unfortunately, this restricted homology does not shed any light on the function of either the mammalian gene or RIP3. It is possible that this homology is more an indicator of a Ras-interacting or other regulatory domain than the biological function of either gene. Identification of interacting proteins or second-site suppressors should further elucidate the pathway that requires RIP3 function.

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