A Novel Member of the *rho* Family of Small GTP-binding Proteins Is Specifically Required for Cytokinesis

Denis A. Larochelle, Kalpa K. Vithalani, and Arturo De Lozanne

Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

Abstract. Several members of the *rho/rac* family of small GTP-binding proteins are known to regulate the distribution of the actin cytoskeleton in various subcellular processes. We describe here a novel *rac* protein, *racE*, which is specifically required for cytokinesis, an actomyosin-mediated process. The *racE* gene was isolated in a molecular genetic screen devised to isolate genes required for cytokinesis in *Dictyostelium.* Phenotypic characterization of *racE* mutants revealed that *racE* is not essential for any other cell motility event, including phagocytosis, chemotaxis, capping, or development. Our data provide the first genetic evidence for the essential requirement of a *rho*-like protein, specifically in cytokinesis, and suggest a role for these proteins in coordinating cytokinesis with the mitotic events of the cell cycle.

The intimate association between mitosis and cytokinesis requires a means of coordination between these two processes to insure that the newly duplicated nuclei segregate properly with half of the cytoplasm into the daughter cells. Although much is known about these processes, the mechanism(s) by which they are coordinated remains unknown. The regulation of the mitotic cell cycle has been intensively studied over the last several years. Biochemical and genetic approaches have combined to identify many of the key proteins that control different aspects of the cell cycle. In addition, many of the structural proteins that compose the mitotic apparatus have been characterized. Similarly, much is understood about how cells achieve proper cytoplasmic division. In animal cells, this involves the formation of an equatorial contractile ring that consists largely of actin and myosin and constricts to divide the cell into two (Satterwhite and Pollard, 1992). However, it is not understood how these proteins localize to the equator of the cell at the appropriate time and in the correct orientation. From the work of Rappaport (1990), it is clear that the astral microtubules of the mitotic apparatus are intimately involved in determining the placement of the contractile ring. What is not clear is what kind of signals may be involved or how they may be transmitted by the mitotic apparatus to the cell cortex.

The *rho* family of ras-related small GTP-binding proteins (including *rho*, *cdc42*, and *rac* proteins) are known to have profound effects on the actin cytoskeleton (Hall, 1994). *Rho* proteins have been implicated in the regulation of cytokinesis in both sand dollar (Mabuchi et al., 1993) and *Xenopus* (Kishi et al., 1993) embryos. In both systems, the inhibition of *rho* activity by ADP ribosylation with *Clostridium botulinum* C3 exoenzyme prevented the formation and maintenance of the contractile ring. However, these experiments could not uniquely identify the specific *rho* protein that is required for cytokinesis. Similarly, in the yeast *Saccharomyces cerevisiae*, the protein *Cdc42p* is also important for cell division, although its function ranges from bud site selection to polarized growth and organization of the actin cytoskeleton (Adams et al., 1990; Johnson and Pringle, 1990). In mammalian cells, *rho* regulates the formation of stress fibers and focal adhesion plaques, whereas *rac* mediates the formation of lamellipodia. *Cdc42*, on the other hand, is involved in filopodia formation (Nobes and Hall, 1995). It is not clearly understood how these closely related proteins mediate such diverse effects.

We have taken a molecular genetic approach, using *Dictyostelium discoideum*, to identify additional components essential for the proper completion of cytokinesis. A screening protocol was designed to isolate cell lines containing mutations in genes absolutely required for cytokinesis (Vithalani et al., 1996). The validity of this screen was confirmed here by the isolation of two independent mutant cell lines containing a disruption in the gene encoding myosin II heavy chain, an essential component of the contractile ring. In addition, two independent cell lines suffered disruptions in the gene coding for a novel small GTP-binding protein, designated here as *racE.* Phenotypic characterization of these mutants revealed that *racE* is required only for cytokinesis. All other physiological processes mediated by the actin cytoskeleton, such as phagocytosis, receptor capping, cortical contraction, and chemotaxis appear normal in the *racE* mutant cells. Furthermore, these cells are able to complete the developmental life cy-

D.A. Larochelle and K.K. Vithalani have contributed equally to this study and should be considered co-first authors.

Please address all correspondence to Arturo De Lozanne, Department of Cell Biology, Box 3709, Duke University Medical Center, Durham, NC 27710. Tel: (919) 681-6851; Fax: (919) 681-7978.

© The Rockefeller University Press, 0021-9525/96/06/1321/9 $2.00

cle generating viable spores. Taken together, these results suggest that *racE* is specifically involved in the regulation of cytokinesis and may yield insights into how cytokinesis is so intimately tied to the regulation of the cell cycle. In addition, we are now in a position to begin dissecting the pathway through which *rho* proteins are able to modulate, in a profound manner, the actin cytoskeleton.

Materials and Methods

Restriction Enzyme–mediated Integration and Screening Protocols

The restriction enzyme–mediated integration (REMI)[1] mutagenesis protocol was based on that described by Kuspa and Loomis (1992) with the modifications indicated below. The screening protocol used to isolate cytokinesis mutants is similar to that described by Vithalani et al. (1996) with the following modifications. Electroporation was used to transfect 8 $\times$ 10^6 *Dictyostelium* DH1 cells with the plasmid pRHI30 (40 μg) in the presence of 150 U of the restriction enzyme DpnII. Before transfection, pRHI30 was linearized with the restriction enzyme BglII, which generates the same cohesive ends as DpnII. After electroporation, the cells were resuspended in FM minimal medium (Franke and Kessin, 1977) lacking uracil and distributed into 20 96-well plates. The medium in these plates was changed weekly until colonies appeared in the wells. To increase the probability that each well contained cells from a single clone, we discarded those plates that had >35 of 96 wells occupied. The individual colonies were then transferred in duplicate to 24-well plates. One 24-well plate was placed on an orbital shaker at 240 rpm, and the other plate was placed on a stationary shelf. After several days, duplicate wells were examined for colonies that displayed growth in the stationary plate but not in the shaking plate. Such colonies were picked and rescreened in the same manner. Positive clones from these two screens were then used to inoculate six-well plates and 50-ml flasks containing 10 ml of FM medium. The flasks were shaken at 240 rpm for several days and monitored for growth. After screening 7,500 independent clones, we found four cell lines that were incapable of growth in suspension culture. These cells were then grown on plates in large quantities for the isolation of genomic DNA.

Genomic DNA was digested with a number of restriction enzymes and analyzed by Southern blotting using the plasmid pRHI30 as the initial probe. Digests that generated single bands larger than pRHI30 were chosen for cloning. These were ligated and transformed into *Escherichia coli* strain DH5α and selected with ampicillin. The resulting plasmids contained pRHI30 with *Dictyostelium* flanking sequences. The flanking sequences were then isolated and used as a probe on Southern blots comparing mutant with wild-type (DH1) DNA. DNA sequencing was carried out by either the Sanger method or PCR sequencing with Taq polymerase (Promega Corp., Madison, WI).

Disruption of racE by Homologous Recombination

The plasmid isolated from the BglII digestion of 24EH6 genomic DNA was used to recreate an identical mutation in wild-type cells by homologous recombination. This plasmid contains ~1.5 kb upstream and ~1.8 kb downstream of the insertion point of pRHI30 in the 24EH6 mutant. 20 μg of this plasmid was linearized with BglII and transfected (without enzyme) via electroporation into 8 $\times$ 10^6 *Dictyostelium* DH1 cells, which were then plated into five 96-well plates in FM medium lacking uracil. All independent transformed cell lines were grown on plates and analyzed for their ability to grow in suspension cultures. Two of these independent transformants were also subjected to Southern and Northern blot analyses.

Northern Blot Analysis

Total RNA from each cell line was isolated according to the method of Nellen et al. (1987). 20-μg aliquots were electrophoresed on formaldehyde/agarose gels. Electrophoresed RNA was then transferred to Hybond-N (Amersham, Arlington Heights, IL) and probed with either the *racE* or myosin II heavy chain gene.

1. *Abbreviations used in this paper*: REMI, restriction enzyme-mediated integration; SB, Sorensen's buffer.

Visualization of Actin, Myosin, and DNA in Fixed Cells

Agar-overlay immunofluorescence and staining were carried out according to the method of Fukui et al. (1987) to examine the distribution of myosin II and the number of nuclei in our cell lines. Briefly, cells were harvested from plates, allowed to attach to glass coverslips for 15 min, and washed in Sorensen's buffer (SB; 15 mM KH_2PO_4, 2 mM Na_2PO_4, pH 6.1). The amoebae were then overlaid with thin agarose M (Pharmacia Biotech, Uppsala, Sweden) sheets (0.17–0.25-mm thick), and excess buffer was carefully wicked away. The agar-overlaid coverslips were fixed in 1% formaldehyde in methanol at −20°C for 5 min, washed in TBS (50 mM Tris, 150 mM NaCl, pH 7.5), blocked with a 5% BSA solution for 30 min, and incubated with a polyclonal antibody raised against myosin II heavy chain for 45 min. Blocking and all subsequent steps were carried out at 37°C. After washing again in TBS, the cells were incubated for 45 min with a FITC-conjugated goat anti–rabbit IgG antibody (Molecular Probes, Inc., Eugene, OR). This antibody was preabsorbed against fixed and permeabilized *Dictyostelium* cells to remove background reactivity as described (Burns et al., 1995). The coverslips were washed a third time in TBS, stained with DAPI (1 μg/ml) for 10 min, and mounted onto glass slides for visualization by fluorescence microscopy.

To determine actin distribution, cells were harvested as described above, allowed to attach to coverslips, and subsequently fixed with 3.7% formaldehyde in 150 mM KCl, 5 mM $MgCl_2$, 20 mM K-phosphate, 10 mM EGTA, pH 6.1 at room temperature. Cells were then rinsed in TBS and incubated with rhodamine-phalloidin (1:100 dilution; Molecular Probes) in PBS containing 0.5% NP-40. The coverslips were rinsed, mounted, and visualized as described above.

Con A Capping, Cortical Contraction, and Phagocytosis Assays

To assay for capping of Con A receptors, cells were harvested from petri dishes, allowed to attach to glass coverslips for 15 min, and washed free of medium with SB. A 1 mg/ml solution of FITC–Con A (Sigma Chemical Co., St. Louis, MO) in SB was freshly prepared, and 100 μl of this solution was added to the cells for exactly 2 min at room temperature. The cells were once again washed in SB and allowed to incubate for an additional 5, 10, or 15 min. As indicated above, cells were fixed in formaldehyde/methanol, mounted on glass slides, and observed.

To assay for cortical contraction, cells were allowed to grow overnight in six-well plates or on glass coverslips, and were then exposed to medium containing 2 mM sodium azide. Cells were observed continuously at 200× for changes in cell morphology in response to the sodium azide treatment.

Phagocytosis assays were performed as described previously (Cohen et al., 1994).

Dictyostelium Development

Bacterial lawns of *E. coli* B/R cells were allowed to grow overnight at 37°C on SM/5 agar plates (Sussman, 1987). The lawns were then inoculated with the different *Dictyostelium* strains by adding 250-μl drops containing 5 $\times$ 10^6 *Dictyostelium* cells. The plates were incubated at 21°C and monitored continuously for the ability of the cells to phagocytose the bacteria and proceed with the *Dictyostelium* developmental program.

Results

Isolation and Recapitulation of Cytokinesis-deficient Mutants

To identify novel genes required for cytokinesis, we randomly mutagenized *Dictyostelium* cells by REMI (Kuspa and Loomis, 1992) and screened for cytokinesis-deficient cells. Our screen was based on the phenotype of *Dictyostelium* strains deficient for myosin II, a protein known to be essential for cytokinesis (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987). Because these mutant cells are unable to complete cytokinesis, they become large and multinucleate when grown in suspension culture. However, they are able to propagate on a solid substratum by "pinching-off" into smaller cells by a process known as

traction-mediated cytofission (Fukui et al., 1990). Accordingly, we screened mutagenized clones for those that failed to grow in suspension culture but were able to grow on a solid substrate. Four mutant cell lines with this phenotype were isolated out of a collection of 7,500 independent REMI transformants. When analyzed by Southern blot analysis with a specific probe for the *Dictyostelium* myosin II heavy chain (*mhcA*) gene, we found that two of the four mutant cell lines (28IF8 and 53PF1) contained a plasmid insertion within the *mhcA* locus (data not shown). This result established the screening protocol as a legitimate means of identifying cytokinesis mutants.

Southern blot analysis of a third cytokinesis mutant (24EH6) indicated that it contained a single copy of the transforming plasmid pRHI30 within an 8-kb BglII fragment (data not shown). Using this plasmid as a tag, we retrieved the flanking sequences by digesting genomic DNA from the 24EH6 mutant with BglII, self-ligating the DNA fragments, and introducing them into *E. coli* DH5α cells. A plasmid, p24EH6-BglII, which contained 1.5 kb of sequence upstream and 1.8 kb of sequence downstream from the insertion site of pRHI30, was recovered (Fig. 1). To confirm that we had cloned the genomic region affected by the plasmid insertion, a fragment of p24EH6-BglII (Fig. 1, probe A) was used as a probe in Southern blot analysis of wild-type (DH1) and mutant genomic DNA digested with BglII (Fig. 2). As predicted, this probe detected a 3.3-kb band in the wild-type or myosin II mutant DNA (Fig. 2, lanes *1* and *5*). In contrast, the same probe detected an ~8 kb band in the 24EH6 mutant DNA (Fig. 2, lane *2*). The difference in size between wild-type and mutant DNA corresponds to the size of the inserted pRHI30 plasmid (4.45 kb).

We subsequently determined that the fourth cytokinesis mutant in our collection, named 37TB1, also contained a disruption of the same genomic region as 24EH6. Southern blot analysis of 37TB1 mutant DNA probed with probe A from p24EH6-BglII revealed a disruption of the 3.3-kb BglII wild-type fragment (data not shown). Thus, our screen resulted in the isolation of four independent mutants containing disruptions in two different genes: the *mhcA* gene and a gene in the 24EH6 genomic region.

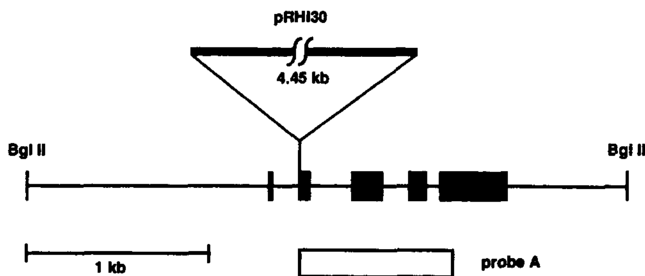

Figure 1. Map of the *Dictyostelium racE* gene and the plasmid insertion in the mutant 24EH6. The *racE* gene is encompassed within a 3.3-kb BglII genomic fragment in wild-type *Dictyostelium* cells (Fig. 2). The open reading frame is apportioned into five exons (*closed bars*). The *Dictyostelium* transformation plasmid pRHI30 (*broken bar*) was inserted at a DpnII site within the second exon of the *racE* gene as illustrated. This insertion results in the formation of an ~8-kb BglII genomic fragment in the 24EH6 mutant (see Fig. 2). An internal 0.84-kb fragment (probe A) was used as a probe in the Southern blot in Fig. 2.

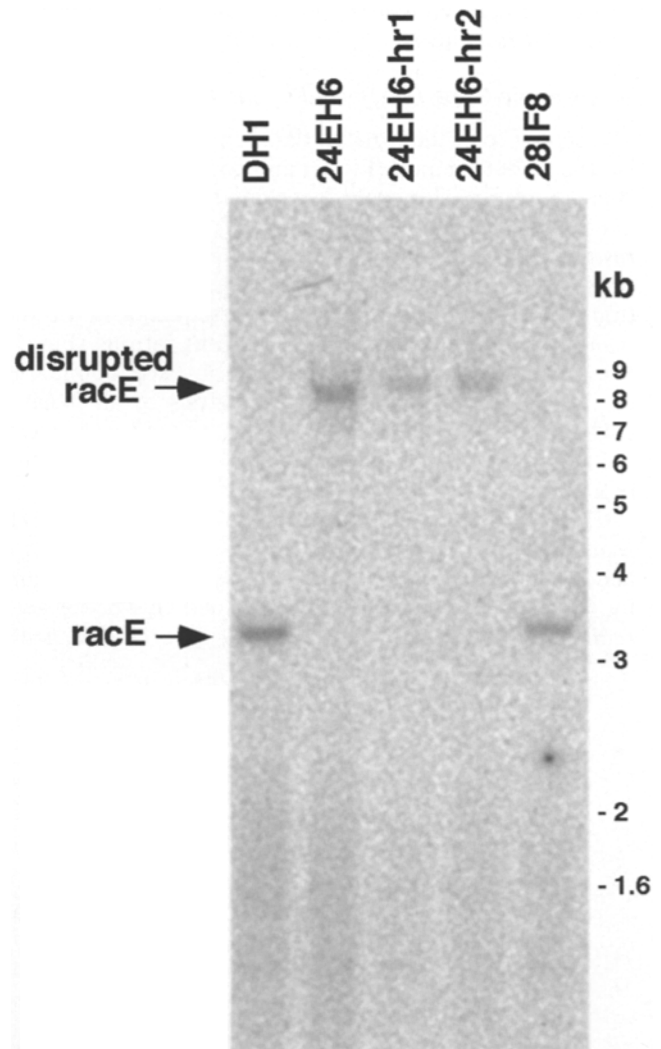

Figure 2. Disruption of the *Dictyostelium racE* gene by REMI and by homologous recombination. A Southern blot of BglII digests of genomic DNA isolated from wild-type (DH1) and several mutant cell lines was probed with the *racE* gene probe A described in Fig. 1. The *racE* REMI mutant (24EH6) and the mutants created by homologous recombination (24EH6-hr1 and 24EH6-hr2) contain a disruption of the *racE* locus as diagrammed in Fig. 1. Wild-type cells (DH1) and a myosin REMI mutant (28IF8) both contain an intact *racE* locus. Molecular weights in kilobases are indicated on the right.

To be certain that the phenotype of the 24EH6 mutant resulted from the disruption of the 24EH6 genomic region, and not to a secondary mutation occurring elsewhere in the genome, homologous recombination (which occurs at a high frequency in *Dictyostelium*; (De Lozanne and Spudich, 1987)) was used to recreate the 24EH6 mutation in wild-type cells. The 8-kb p24EH6-BglII plasmid rescued from the mutant cells (24EH6) was linearized with BglII and transfected into wild-type DH1 cells via electroporation. We then tested each transformed cell line for its ability to grow in suspension culture. We found that all of the transformants failed to grow under these conditions. Furthermore, we analyzed two of these mutants (24EH6-hr1 and 24EH6-hr2) by Southern blot analysis and confirmed

that they contained a disruption identical to that of 24EH6 (Fig. 2, lanes *3* and *4*).

A Novel rac Gene Is Affected by the REMI Mutation

The sequence of the isolated flanking regions of p24EH6-BglII was determined (Fig. 3) and compared to the GenBank database. This analysis revealed that the 24EH6 sequence encodes a protein that belongs to the *rho* family of *ras*-related GTP-binding proteins (Hall, 1994). The close similarity among these proteins allowed for the identification of an open reading frame in our sequence that is distributed over five exons with four short introns (Fig. 1). We subsequently confirmed the intron–exon boundaries of this novel gene by sequence analysis of an isolated cDNA clone (data not shown). The open reading frame of the *racE* gene extends over 672 bp and encodes a protein of 223 amino acids.

Cladistic analysis of the protein encoded by the 24EH6 sequence indicated that it is more closely related to the *rac* and *cdc42* subfamilies of proteins than to the *rho* subfamily (Fig. 4). Therefore, we have named this novel gene *racE*, after the nomenclature of the different *rac* genes that have been previously isolated from *Dictyostelium* (*rac1A*, *rac1B*, *rac1C*, *racA*, *racB*, *racC*, and *racD*; Bush et al., 1993). Alignment of the *racE* protein sequence with other members of the *rho* family (Fig. 5) demonstrates the high degree of similarity among these proteins. These proteins share the four conserved GTP-binding domains that are found in all other small GTP-binding proteins (Gilman, 1987; Bourne et al., 1991). These regions are the phosphate-binding loop L1, $G(X)_4GKS/T$ (amino acids 18–25); the region that interacts with the gamma phosphate, WDTAGQE (amino acids 64–70); the guanine specificity region, N/TKXD (amino acids 123–126); and the highly conserved SAK/L sequence (amino acids 164–166). In addition, the *racE* protein also ends in the conserved prenylation motif or CAAX box. The most divergent region of the *racE* protein is the region immediately preceding the COOH-terminal CAAX box. This region is much longer in the *racE* protein than in the other members of the *rho* family.

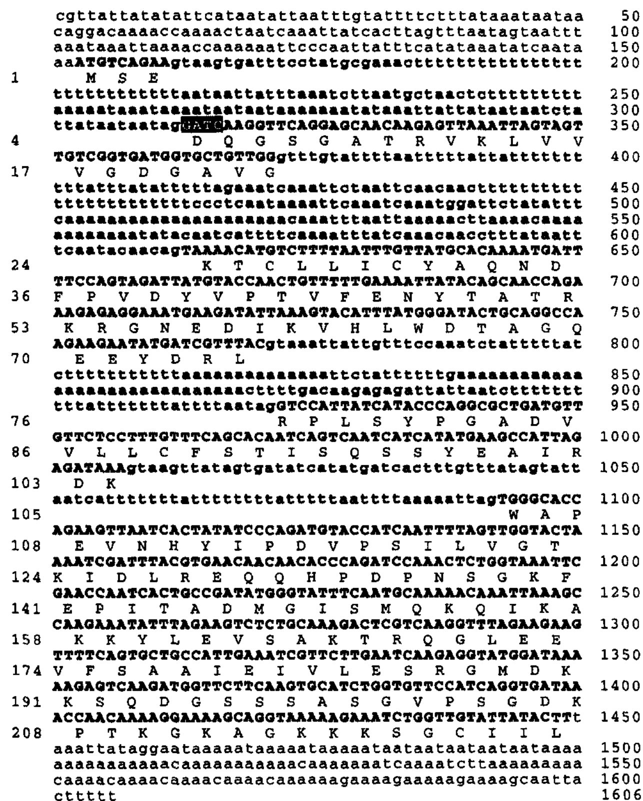

Figure 3. Sequence of the *Dictyostelium racE* gene. The open reading frame of the *racE* gene is divided into five exons (*bold capital letters*) by four short introns (*bold lower case letters*). The plasmid pRHI30 that disrupted this gene in the 24EH6 mutant was inserted at a DpnII site within the second exon (*white letters on black background*). The 5′ and 3′ flanking sequences are shown in lower case letters. The predicted amino acid sequence is shown below the respective coding sequence. The amino acid sequence is numbered on the left and the nucleic acid sequence on the right. This sequence is available from GenBank under accession number U41222.

The REMI Mutation Causes the Loss of racE Expression

To understand how the REMI-induced plasmid insertion within the second exon of the *racE* gene caused a mutant phenotype, we assessed the expression of *racE* mRNA by Northern blot analysis (Fig. 6). *racE* was expressed in DH1 cells (the parental wild-type strain), but not in the mutant 24EH6 cells. Furthermore, the two *racE* mutants created by homologous recombination (24EH6-hr1 and -hr2) also failed to express the *racE* gene. As a control, we examined the expression of the *racE* gene in the myosin II 28IF8 mutant isolated in the same screen. This mutant expressed *racE* at levels comparable to the wild-type cells. We also probed the same blot for *mhcA* gene expression. As predicted, all the cells expressed the *mhcA* gene except for the myosin II mutant 28IF8 cells. Thus, disruption of the *racE* gene results in the complete inactivation of *racE* expression.

Phenotypic Characterization of racE Mutant Cells

Our screening method was based on identifying cells that failed to divide in suspension culture. When grown either in suspension culture or on solid substrates, wild-type cells divide by cytokinesis and remain small and mono- or binucleate, with few cells accumulating more than two nuclei (Fig. 7, *A* and *B*). When grown on tissue culture plates, *racE* mutant cells were able to propagate by traction-mediated cytofission (Fukui et al., 1990) at rates comparable to wild-type cells (data not shown). The majority of *racE* cells grown on substrates were generally mono- or binucleate, with a small percentage of large and multinucleated cells (Fig. 7 *C*). When grown in suspension cultures, however, the *racE* mutant cells were no longer able to divide. Consequently, these cells became very large and accumulated many nuclei, as illustrated by the single cell shown in Fig. 7 *D*.

Since members of the *rho* family of proteins are known to be involved in the regulation of the actin cytoskeleton, it seemed possible that the loss of *racE* function would cause an abnormal actin distribution in the mutant cells. Accordingly, we examined the distribution of actin and

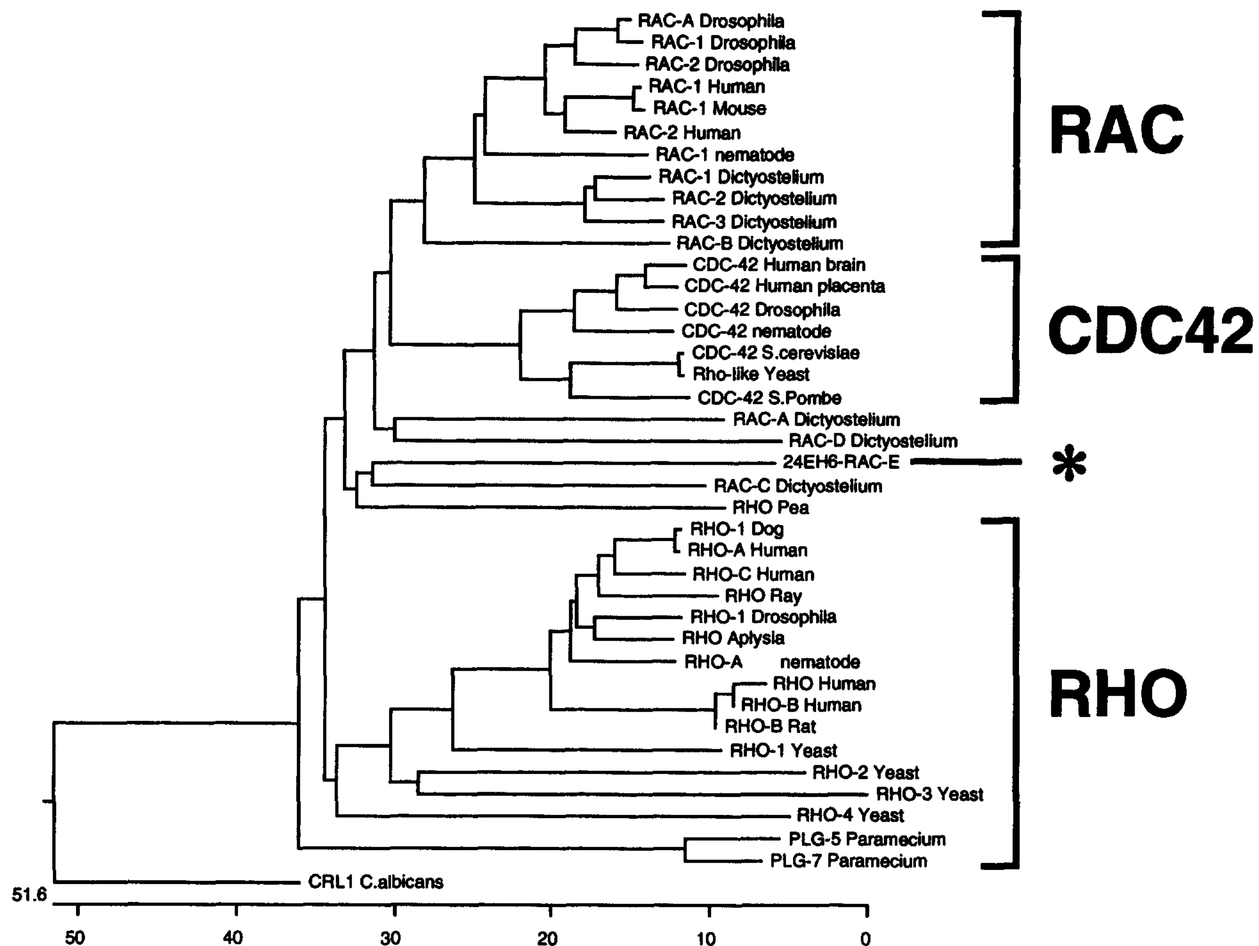

Figure 4. Phylogenetic analysis of the *rho* family of small GTP-binding proteins. This tree displays the phylogenetic relationships among different members of the *rho* family. The three known subfamilies, *rho*, *rac*, and *cdc42* are indicated. The *Dictyostelium racE* protein (*asterisk*) is most related to the *rac/cdc42* subfamilies, although it clearly has diverged to some extent from these subfamilies. The published sequences most similar to that of *racE* are those from *Dictyostelium racC* and from the pea "*rho.*" Sequences were aligned with the Megalign program using the Clustal algorithm and a PAM250 table. The scale at the bottom indicates the number of substitutions between sequences.

myosin II in *racE* mutant cultures grown on coverslips for a period of several days and compared them with parallel cultures of wild-type cells. We found that the mutant cells had the same actin-rich structures, such as filopodia, pseudopodia, and membrane ruffles, which wild-type cells have (Fig. 8, *A* and *D*). Similarly, myosin II was found mostly in the cortical region of the cells and had the punctate appearance characteristic of wild-type cells (Fig. 8, *B* and *E*). The formation of a contractile ring was never observed in these cells, but, because synchronization of these cell lines has not been successful, we cannot exclude the possibility that contractile rings are transiently formed in the *racE* mutants.

The disruption of the *racE* gene clearly affected cytokinesis and could potentially affect other functions mediated by the acto-myosin cytoskeleton. Therefore, the ability of the *racE* mutants to carry out some of these functions was tested. When wild-type cells are treated briefly with FITC-labeled Con A, they quickly concentrate their cross-linked Con A receptors into a polar cap in a process that requires myosin II (Fukui et al., 1990; Fig. 8 *C* in this paper). We found that the *racE* mutants had the same ability to cap their Con A membrane receptors (Fig. 8 *F*). Another known myosin II–dependent process is the cortical contraction of cells when treated with sodium azide (Pasternak et al., 1989). We found that both wild-type and *racE* mutant cells contracted quickly when treated with sodium azide, whereas the myosin II 28IF8 mutants did not (data not shown).

Phagocytosis is a third process that requires the actin cytoskeleton, in possible conjunction with the unconventional myosin I's (Jung et al., 1993). We have determined that racE is not essential for phagocytosis, since the *racE* mutants phagocytose to the same extent as wild-type cells (data not shown).

When starved of nutrients, *Dictyostelium* undergoes a well-defined yet simple developmental program that culminates in the formation of fruiting bodies containing spores. Myosin II has also been shown to be essential for the completion of this developmental program, demonstrating a role for the acto-myosin cytoskeleton in this process (De Lozanne and Spudich, 1987; Knecht and Loomis, 1988). To test for a possible role for the *racE* protein in development, 24EH6 cells were grown on bacterial lawns.

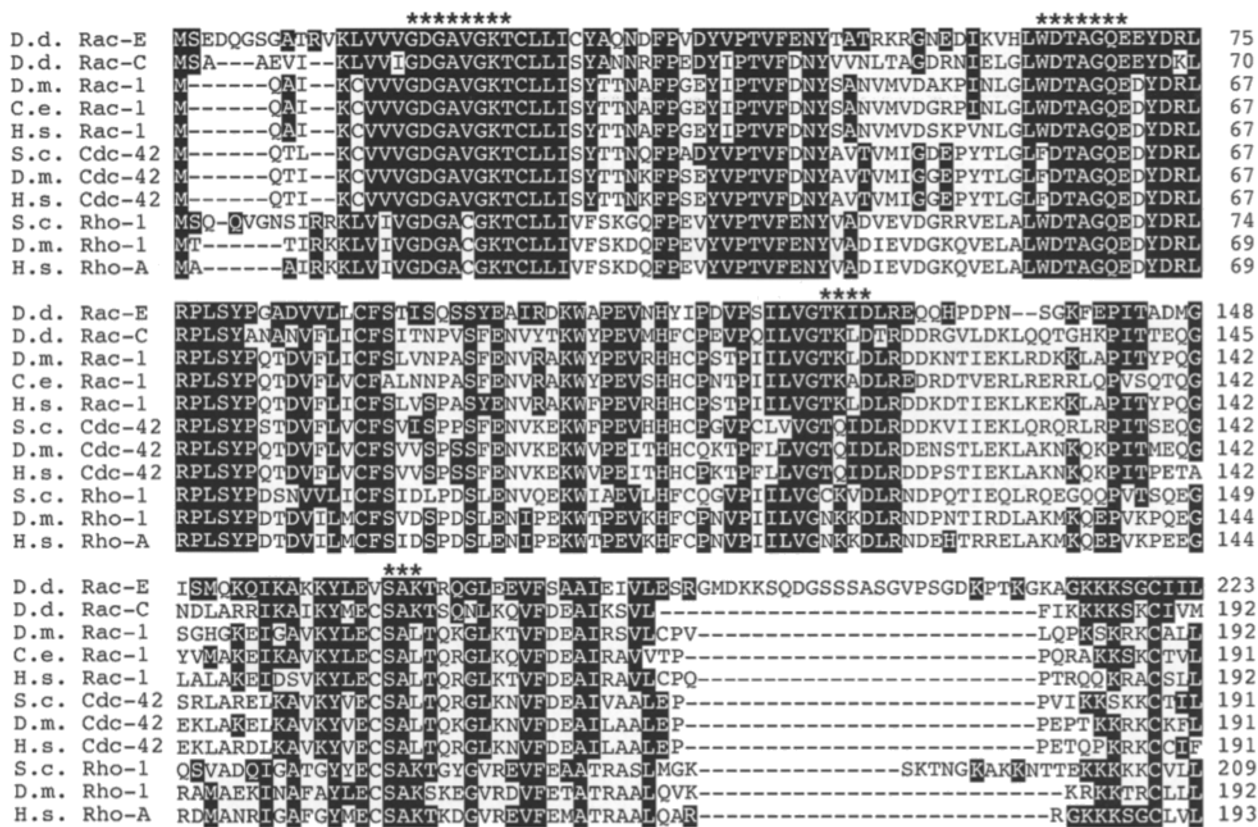

Figure 5. Comparison of the *Dictyostelium racE* protein with other members of the *rho* family. Residues that are identical between the *Dictyostelium racE* protein and the other members of the *rho* family are shown in white letters on a black background. Dashes indicate gaps inserted in the sequence for best alignment. The asterisks indicate the sequences known to be involved in GTP binding. *D.d.*, *Dictyostelium discoideum*; *D.m.*, *Drosophila melanogaster*; *C.e.*, *Caenorhabditis elegans*; *S.c.*, *Saccharomyces cerevisiae*; *H.s.*, *Homo sapiens.* GenBank accession numbers: D.d. *racE*, U41222; D.d. *racC*, L11593; D.m. *rac1*, L38309; C.e. *rac1*, L03711; H.s. *rac1*, M29871; S.c. *cdc42*, X51906; D.m. *cdc42*, U11824; H.s. *cdc42*, M35543; S.c. *rho1*, M15189; D.m. *rho1*, L38311; H.s. *rhoA*, X05026.

When the bacteria were depleted, 24EH6 cells developed into mature fruiting bodies that were slightly smaller than wild-type fruiting bodies but contained viable spores (data not shown). Thus, these results show that the inactivation of the *racE* gene leads to the loss of a single actin- and myosin II–based function: cytokinesis.

Discussion

To date, few proteins are known to be essential for cytokinesis in animal cells. The first and best documented example is myosin II. From early microinjection studies in starfish eggs (Mabuchi and Okuno, 1977; Kiehart et al., 1982) and the analysis of mutants in *Dictyostelium* (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Pollenz et al., 1992; Chen et al., 1994) and *Drosophila* (Karess et al., 1991), it is clear that myosin II plays a central role in cytokinesis. In the present paper, we used a screening protocol (Vithalani et al., 1996) designed to identify novel gene products that are required for cytokinesis, based on the cytokinesis-deficient phenotype observed in *Dictyostelium* myosin II null cells. Using this method, we have isolated and characterized four cell lines that share this phenotype. Two of these cell lines contained disruptions in the myosin II heavy chain locus. Although these two mutants offer no new insights into the mechanism of cytokinesis, they do confirm the screening protocol as a valid one for isolating cytokinesis-deficient strains. The remaining two cell lines contained disruptions in a gene encoding a novel member of the *rho* family of small GTP-binding proteins, designated here as *racE.* That only two genes were disrupted in the four mutant cell lines described here might suggest that the screen has reached saturation for identifying genes required specifically for cytokinesis. We know that additional genes, however, such as the myosin II light chains (Pollenz et al., 1992; Chen et al., 1994) and profilin genes (Haugwitz et al., 1994), are also required for cytokinesis in *Dictyostelium.* Furthermore, a novel cytokinesis-deficient mutant has been isolated in an independent screen using a different plasmid, restriction enzyme, and *Dictyostelium* strain (Vithalani et al., 1996). Thus, it is likely that further screening, with variations in the choice of enzymes and plasmids used for REMI, may allow the isolation of additional genes. Any gene involved in cytokinesis that is also essential for the viability of *Dictyostelium* cells, however, would not be detected using this approach.

The superfamily of small GTP-binding proteins consist of the *ras* oncogene product and a growing number of ras-related proteins (Hall, 1990). These proteins are known to modulate a wide variety of cellular activities ranging from

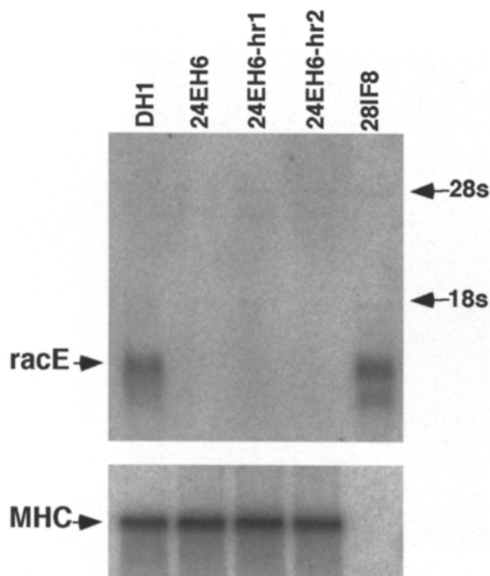

Figure 6. Disruption of *racE* leads to a loss of gene expression. Northern blot analysis of RNA isolated from wild-type (DH1), *racE* mutants (*24EH6*, *24EH6-hr1*, and *24EH6-hr2*), and myosin II mutant (*28IF8*) cell lines. The upper panel was probed for *racE* expression using probe A (Fig. 1). The bottom panel shows the same blot probed with the full-length myosin II heavy chain gene. The migration of ribosomal RNA is indicated on the right-hand side of the figure.

cell proliferation and differentiation (*ras* family) to intracellular vesicle transport (*rab* family) to regulation of the actin cytoskeleton (*rho* family). Precisely how this superfamily of proteins mediates such a wide variety of effects remains to be determined. It is evident that many cell types contain multiple forms of *rho* proteins. For example, Swiss 3T3 cells express at least one member each of the *cdc42*, *rac*, and *rho*$^-$ subfamilies (Nobes and Hall, 1995), and it is likely that a single cell type expresses more than one member of each of these subfamilies. Whether proteins within the same subfamily have distinct or overlapping functions is an open question. In *Dictyostelium*, seven different *rac* genes have been previously identified, although no function has been assigned to these proteins (Bush et al., 1993). *racE*, the eighth member of the *rac* subfamily to be identified in this organism, appears to be essential solely for cytokinesis. Since we could not detect any other defects in the *racE* mutants, we postulate that this protein may not be involved in other cellular functions. Of course, given the relatively large number of *rac* genes in *Dictyostelium*, it is possible that there may be some redundancy of function between them. However, if *rac* proteins are able to overlap in function, it is apparent that none of the other *rac* genes can compensate for the absence of *racE* during cytokinesis. It is tempting to speculate that the extended COOH terminus before the CAAX box of *racE* (the most strikingly different region of this protein when compared to the other *rac*, *rho*, and *cdc42* proteins) may be important in delineating the specificity of *racE* for cytokinesis. Members of the rab family of proteins are also known to have an extended COOH terminus, which has been found to be involved in the subcellular localization of these proteins (Chavrier et al., 1991). Similarly, this region of the *racE* protein may be responsible for targeting *racE* to the appropriate site in the cell during cytokinesis.

Although we have designated the novel gene described here as *racE*, it is only 49.5% identical to its nearest relative (*Dictyostelium racC*). At the same time, it is 47.2% identical to human *rhoA*. Though cladistic analysis has placed *racE* in the *rac*/*cdc42* branch, as opposed to the *rho* branch, it is almost equally divergent from both branches. It is possible that the *racE* protein defines a new branch of the *rho* family of small GTP-binding proteins, with a specific function during cytokinesis. Only through a search for homologous proteins in other species can this hypothesis be tested.

Given that there is an absolute requirement for *racE* in cytokinesis, how might this protein be involved in the regulation of this process? *racE* may be responsible for the reorganization of actin filaments in the presumptive cleavage furrow at the onset of cytokinesis. As such, *racE* may be the signal that is carried by the mitotic apparatus and determines the placement of the contractile ring at the ap-

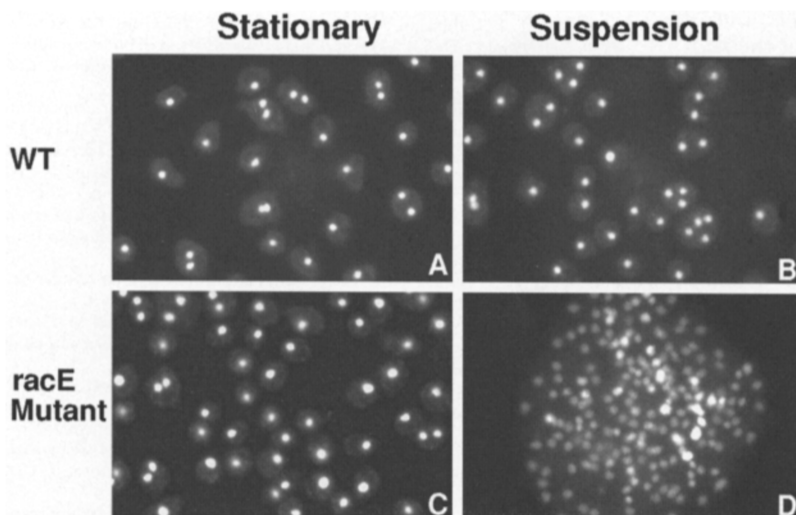

Figure 7. Dictyostelium racE mutant cells become large and multinucleate when grown in suspension culture. Nuclear staining was carried out on wild-type (DH1) (*A* and *B*) and *racE* mutant (24EH6) (*C* and *D*) cells grown on stationary tissue culture plates (*A* and *C*) or in suspension (*B* and *D*) for 3 d. Note that *D* shows a single *racE* mutant cell grown in suspension. All frames are shown at the same magnification.

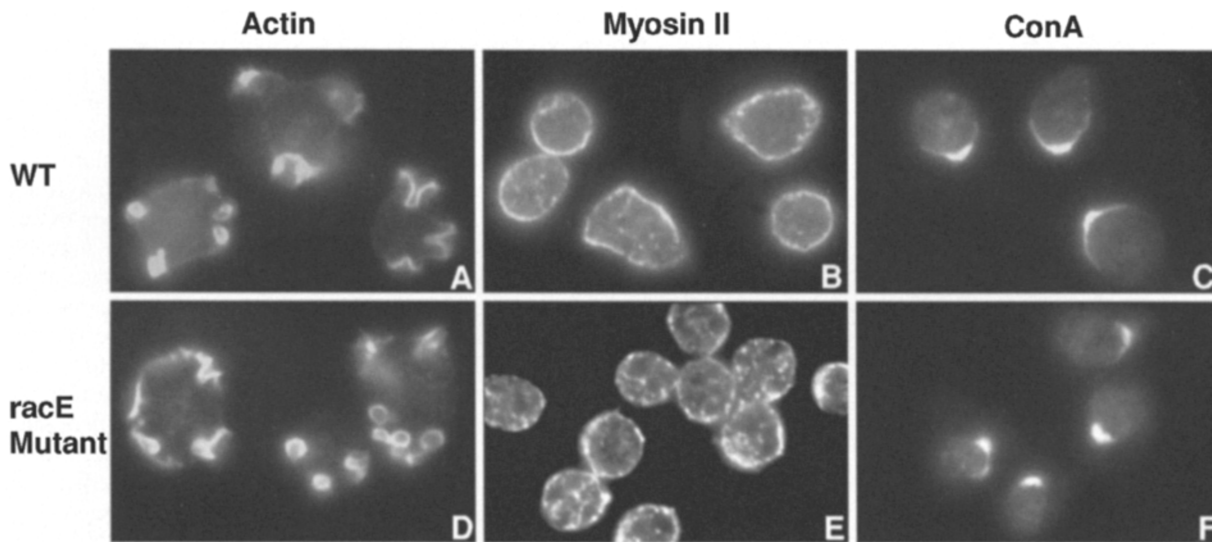

Figure 8. Actin and myosin distribution and Con A capping in wild-type and *racE* mutant cells. Wild-type (*A–C*) or *racE* mutant (*D–F*) cells were allowed to grow on coverslips and were then stained with rhodamine-phalloidin (*A* and *D*) or an anti–myosin II antibody (*B* and *E*) to determine the organization of these two cytoskeletal proteins. The cells were also challenged with FITC-conjugated Con A for 2 min (*C* and *F*) to observe their receptor-capping response.

propriate site. Alternatively, *racE* may be selectively activated in the presumptive furrow region by a different signal, again transmitted by the mitotic apparatus. This localized placement or activation of *racE* would then be responsible for the rearrangement of actin filaments into a contractile ring. It is unknown whether this rearrangement is brought about by the recruitment of preexisting actin filaments or the formation of actin nucleation sites. Most importantly, we don't know whether *racE* is required to be associated with the cleavage furrow at all, only transiently, or throughout the life of the contractile ring.

It is also possible that the involvement of *racE* in cytokinesis is not directly linked to the regulation of actin distribution. *Cdc42* and *rac* can both bind to $p65^{PAK}$ (Manser et al., 1994), a potential upstream activator of mitogen-activated protein kinase (MAPK) cascades. Activation of a kinase cascade by *racE* may be a requisite step in the formation and regulation of a contractile ring. Indeed, protein phosphorylation has been implicated in the regulation of cytokinesis in other studies (Satterwhite et al., 1992; Larochelle and Epel, 1993). Furthermore, members of the *rho* family have also been shown to influence the activity of other members of this family, thereby creating a cascade of events that ultimately lead to profound changes in cell morphology (Chant and Stowers, 1995). A similar cascade of GTP-binding proteins, coupled to one or more kinase cascades, may all be involved in regulating different aspects of cytokinesis.

The exquisite spatial and temporal regulatory mechanisms that orchestrate the formation of a contractile ring at precisely the right place and the right time of the cell cycle have yet to be defined. Actin and myosin II clearly provide the mechano-chemical force that is necessary to divide a cell into two; however, many other proteins must participate in the formation and regulation of the contractile ring. Our data suggest that the pathway through which the mitotic machinery communicates with the acto-myosin cytoskeleton to form a contractile ring may involve small GTP-binding proteins, possibly as part of a signal-transducing cascade. Understanding how these small GTP-binding proteins modulate the actin cytoskeleton is an essential step toward defining the molecular steps that control cytokinesis.

The authors gratefully acknowledge Robert Insall for providing the pRHI30 plasmid and the DH1 cells. We thank Dr. Harold Erickson, Dr. Daniel Kiehart, and Dr. Theresa O'Halloran for their critical reading of this manuscript. We also wish to thank members of the O'Halloran lab for useful comments throughout the period of this work. Finally, we thank Dana Zarnescu for her technical assistance.

This work was supported by grants to A. De Lozanne from the American Cancer Society (JFRA-425) and the National Institutes of Health (GM48745).

Received for publication 1 February 1996 and in revised form 25 March 1996.

References

Adams, A.E.M., D.I. Johnson, R.M. Longnecker, B.F. Sloat, and J.R. Pringle. 1990. *CDC42* and *CDC43*, two additional genes involved in budding and establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* 111:131–142.

Bourne, H.R., D.A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. *Nature (Lond.)*. 349:117–127.

Burns, C.G., M. Reedy, J. Heuser, and A. De Lozanne. 1995. Expression of light meromyosin in *Dictyostelium* blocks normal myosin II function. *J. Cell Biol.* 130:605–612.

Bush, J., K. Franek, and J. Cardelli. 1993. Cloning and characterization of seven novel *Dictyostelium discoideum rac*-related genes belonging to the *rho* family of GTPases. *Gene (Amst.)*. 136:61–68.

Chant, J., and L. Stowers. 1995. GTPase cascades choreographing cellular behavior: movement, morphogenesis, and more. *Cell.* 81:1–4.

Chavrier, P., J.-P. Gorvel, E. Stelzer, K. Simons, J. Gruenberg, and M. Zerial. 1991. Hypervariable C-terminal domain of rab proteins acts as a targeting signal. *Nature (Lond.)*. 353:769–772.

Chen, P., B.D. Ostrow, S.R. Tafuri, and R.L. Chisholm. 1994. Targeted disruption of the *Dictyostelium* RMLC gene produces cells defective in cytokinesis and development. *J. Cell Biol.* 127:1933–1944.

Cohen, C.J., R. Bacon, M. Clarke, K. Joiner, and I. Mellman. 1994. *Dictyostelium discoideum* mutants with conditional defects in phagocytosis. *J. Cell Biol.* 126:955–966.

De Lozanne, A., and J.A. Spudich. 1987. Disruption of the *Dictyostelium* myo-

sin heavy chain gene by homologous recombination. *Science (Wash. DC).* 236:1086–1091.

Franke, J., and R. Kessin. 1977. A defined minimal medium for axenic strains of *Dictyostelium discoideum. Proc. Natl. Acad. Sci. USA.* 74:2157–2161.

Fukui, Y., A. De Lozanne, and J.A. Spudich. 1990. Structure and function of the cytoskeleton of a *Dictyostelium* myosin-defective mutant. *J. Cell Biol.* 110:367–378.

Fukui, Y., S. Yumura, and T.K. Yumura. 1987. Agar-overlay immunofluorescence: high-resolution studies of cytoskeletal components and their changes during chemotaxis. *Methods Cell Biol.* 28:347–356.

Gilman, A.G. 1987. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* 56:615–649.

Hall, A. 1990. The cellular functions of small GTP-binding proteins. *Science (Wash. DC).* 249:635–640.

Hall, A. 1994. Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Annu. Rev. Cell Biol.* 10:31–54.

Haugwitz, M., A.A. Noegel, J. Karakesisoglou, and M. Schleicher. 1994. *Dictyostelium* amoebae that lack G-actin sequestering profilins show defects in F-actin content, cytokinesis and development. *Cell.* 79:303–314.

Johnson, D.I., and J.R. Pringle. 1990. Molecular characterization of *CDC42*, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. *J. Cell Biol.* 111:143–152.

Jung, G., Y. Fukui, B. Martin, and J.A. Hammer. 1993. Sequence, expression pattern, intracellular localization, and targeted disruption of the *Dictyostelium* myosin ID heavy chain isoform. *J. Biol. Chem.* 268:14981–14990.

Karess, R.E., X.J. Chang, K.A. Edwards, S. Kulkarni, I. Aguilera, and D.P. Kiehart. 1991. The regulatory light chain of nonmuscle myosin is encoded by spaghetti-squash, a gene required for cytokinesis in *Drosophila. Cell.* 65: 1177–1189.

Kiehart, D.P., I. Mabuchi, and S. Inoué. 1982. Evidence that myosin does not contribute to force production in chromosome movement. *J. Cell Biol.* 94: 165–178.

Kishi, K., T. Sasaki, S. Kuroda, T. Itoh, and Y. Takai. 1993. Regulation of cytoplasmic division of *Xenopus* embryo by *rho* p21 and its inhibitory GDP/GTP exchange protein (*rho* GDI). *J. Cell Biol.* 120:1187–1195.

Knecht, D.A., and W.F. Loomis. 1987. Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum. Science (Wash. DC).* 236:1081–1086.

Knecht, D.A., and W.F. Loomis. 1988. Developmental consequences of the lack of myosin heavy chain in *Dictyostelium discoideum. Dev. Biol.* 128:178–184.

Kuspa, A., and W.F. Loomis. 1992. Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. *Proc. Natl. Acad. Sci. USA.* 89:8803–8807.

Larochelle, D.A., and D. Epel. 1993. Myosin heavy chain dephosphorylation during cytokinesis in dividing sea urchin embryos. *Cell Motil. Cytoskeleton.* 25:369–380.

Mabuchi, I., Y. Hamaguchi, H. Fujimoto, N. Morii, M. Mishima, and S. Narumiya. 1993. A *rho*-like protein is involved in the organisation of the contractile ring in dividing sand dollar eggs. *Zygote.* 1:325–331.

Mabuchi, I., and M. Okuno. 1977. The effect of myosin antibody on the division of starfish blastomeres. *J. Cell Biol.* 74:251–263.

Manser, E., T. Leung, H. Salihuddin, Z. Zhao, and L. Lim. 1994. A brain serine/threonine protein kinase activated by cdc42 and rac1. *Nature (Lond.).* 367: 40–46.

Nellen, W., S. Datta, C. Reymond, A. Siversen, S. Mann, T. Crowley, and R.A. Firtel. 1987. Molecular biology in *Dictyostelium*: tools and applications. *Methods Cell Biol.* 28:67–100.

Nobes, C.D., and A. Hall. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell.* 81:53–62.

Pasternak, C., J.A. Spudich, and E.L. Elson. 1989. Capping of surface receptors and concomitant cortical tension are generated by conventional myosin. *Nature (Lond.).* 341:549–551.

Pollenz, R.S., T.L.L. Chen, L. Trivinoslagos, and R.L. Chisholm. 1992. The *Dictyostelium* essential light chain is required for myosin function. *Cell.* 69:951–962.

Rappaport, R. 1990. Role of the mitotic apparatus in furrow initiation. *Ann. N.Y. Acad. Sci.* 582:15–21.

Satterwhite, L.L., M.J. Lohka, K.L. Wilson, T.Y. Scherson, L.J. Cisek, J.L. Corden, and T.D. Pollard. 1992. Phosphorylation of myosin II regulatory light chain by cyclin p34cdc2: a mechanism for the timing of cytokinesis. *J. Cell Biol.* 118:595–605.

Satterwhite, L.L., and T.D. Pollard. 1992. Cytokinesis. *Curr. Opin. Cell Biol.* 4: 43–52.

Sussman, M. 1987. Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods Cell Biol.* 28:9–29.

Vithalani, K.K., J.D. Shoffner, and A. De Lozanne. 1996. Isolation and characterization of a novel cytokinesis-deficient mutant in *Dictyostelium discoideum. J. Cell. Biochem.* In press.