Role of *Dictyostelium* racE in Cytokinesis: Mutational Analysis and Localization Studies by Use of Green Fluorescent Protein

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Submitted November 20, 1996; Accepted February 3, 1997
Monitoring Editor: James A. Spudich

The small GTPase racE is essential for cytokinesis in *Dictyostelium* but its precise role in cell division is not known. To determine the molecular mechanism of racE function, we undertook a mutational analysis of racE. The exogenous expression of either wild-type racE or a constitutively active V20racE mutant effectively rescues the cytokinesis deficiency of racE null cells. In contrast, a constitutively inactive N25racE mutant fails to rescue the cytokinesis deficiency. Thus, cytokinesis requires only the activation of racE by GTP and not the inactivation of racE by hydrolysis of GTP. To determine the spatial distribution of racE, we created a fusion protein with GFP at the amino terminus of racE. Remarkably, GFP-racE fusion protein was fully competent to rescue the phenotype of racE null cells and, therefore, must reside in the same location as native racE. We found that GFP-racE localized to the plasma membrane of the cell throughout the entire cell cycle. Furthermore, constitutively active and inactive GFP-racE fusion proteins also localized to the plasma membrane. We mapped the domain required for plasma membrane localization to the carboxyl-terminal 40 amino acids of racE. This domain, however, is not sufficient to confer racE function onto a closely related GTPase. Taken together, these results suggest that racE functions at the cell cortex but it is not involved in determining the timing or placement of the contractile ring.

INTRODUCTION

During mitosis in animal cells, astral microtubules from the mitotic apparatus interact with the cell cortex to induce the formation of a contractile ring. This ring, rich in actin and myosin II, is responsible for the formation of the cleavage furrow which will ultimately divide the two daughter cells. The molecular mechanisms that control these events are not understood but there is a growing list of proteins known to be important for cytokinesis (Fishkind and Wang, 1995). Among these proteins, the rho family of small GTPases seem to play an important role during cytokinesis. Disturbing the function of cdc42 or rhoA disrupts cytokinesis in mammalian cells as well as in sea urchin and frog eggs (Kishi *et al.*, 1993; Mabuchi *et al.*, 1993; Dutartre *et al.*, 1996). Since these GTPases are known to be involved in the regulation of the actin

cytoskeleton (Zigmond, 1996), it is not surprising that they may control the actin-rich contractile ring. However, the precise role of these proteins during cytokinesis has not been elucidated. One problem in dissecting their function is that most cells contain multiple members of the rho, rac, and cdc42 subfamilies of small GTPases. These related proteins may have distinct yet overlapping functions *in vivo*. For example, mammalian cells contain at least three rho proteins: rhoA, rhoB, and rhoC (Maduale and Axel, 1985). Activated forms of all three rho proteins can induce the formation of stress fibers in 3T3 cells (Adamson *et al.*, 1992). However, although rhoA and rhoC are found mostly in the cytoplasm, rhoB is localized to endocytic vesicles. Thus, although mutant forms of cdc42 and rhoA affect cytokinesis, it is possible that they do so by affecting the normal function of another, as yet uncharacterized, related GTPase.

Most members of the rho family contain a polybasic domain adjacent to a carboxyl-terminal prenylation site that is presumed to allow them to interact with

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membranes. Indeed, the translocation of rac from the cytosol to the plasma membrane of human neutrophils correlated with the activation of NADPH oxidase (Quinn *et al.*, 1993). Similarly, rhoA was also found to translocate from the cytoplasm to the plasma membrane upon stimulation of SMDCK2 cells with phorbol ester or hepatocyte growth factor (Takaishi *et al.*, 1995). Thus, it is believed that rho proteins may move transiently to the plasma membrane to effect their function and are then released back to the cytosol. Since cytokinesis takes place at a very specific location within the cell to ensure proper segregation of the newly replicated daughter nuclei, it is tempting to speculate that rho GTPases may localize to the cleavage region to regulate the formation of the contractile ring.

Recently, using a genetic screen for *Dictyostelium* cytokinesis mutants, we identified a novel member of the rho family of GTPases, which we designated as racE (Larochelle *et al.*, 1996). This protein appears to have a specific role during cytokinesis since a null mutation in the *racE* gene does not perturb any cellular event except for cell division. Most important, although *Dictyostelium* cells express multiple forms of rac proteins (Bush *et al.*, 1993), none can compensate for the loss of racE. Thus, our *racE* null cells provide a useful system to determine the specific function during cytokinesis of a member of the rho family of GTPases without the functional overlap with other proteins. We report here the exogenous expression of racE mutant constructs which allowed us to test the requirement for GTP turnover in racE function during cytokinesis. Furthermore, we also report the determination of the intracellular localization of racE in live cells by expressing racE fused with GFP¹, a method that may be useful for such determinations of other small GTPases in other cell types. Through these studies we can begin to understand how cytokinesis and, more generally, the actin cytoskeleton, are modulated by the actions of small GTPases.

MATERIALS AND METHODS

Mutant and GFP Expression Constructs

Reverse transcription-polymerase chain reaction (PCR) was used to create cDNAs for the *Dictyostelium* *racE* and *racC* genes. These and all following constructs were cloned into the expression vector pTIKL-Bsr-Exp. This vector is based on the extrachromosomal vector pTIKL (Uyeda *et al.*, 1996). We replaced the G418 resistance gene of pTIKL with a blasticidin (*Bsr*) resistance gene (Sutouh, 1993). We also introduced an actin15 promoter expression cassette for expression of the various rac constructs. Two independent mutations in the *racE* cDNA were created by site-directed mutagenesis using PCR. These mutations resulted in the substitution of valine for glycine at the 20th amino acid position to create the V20racE (con-

stitutively active) mutant or the substitution of asparagine for threonine at the 25th amino acid position to create the N25racE (constitutively inactive) mutant. Although we have not formally tested these mutations here, they do occur in the highly conserved phosphate-binding loop L1 (Bourne *et al.*, 1991) and are based on similar mutations previously described in other members of the ras superfamily including ras, rho, rac, and cdc42 (Diekmann *et al.*, 1991; Ridley and Hall, 1992; Ridley *et al.*, 1992; Chung *et al.*, 1993; Kozma *et al.*, 1995). The active mutant is the result of reduced GTPase activity (Garrett *et al.*, 1989) and the inactive mutant is the result of an inability to exchange GTP for bound GDP (Feig and Cooper, 1988). A fusion protein was also designed, using PCR, to replace the short hypervariable carboxyl-terminal region of racC with the extended carboxyl-terminal region of racE (Larochelle *et al.*, 1996). This protein, named racC/E tail, contained amino acid residues 1–180 of racC and residues 184–223 of racE.

We also created a set of constructs in which the coding region for the mutant GFP S65T-GFP (herein referred to as GFP; Heim and Tsien, 1996) was fused behind the actin15 promoter and in front of the rac constructs described above. All of these constructs contained a spacer of 5 alanine residues between GFP and the rac coding region. Another construct contained the coding sequence for the C-terminal 40 amino acids of racE fused behind GFP. Finally, as a control for these experiments, a construct was engineered to express only the GFP and 5 alanine spacer coding region. The coding region of all constructs was sequenced by PCR sequencing (Promega Corp., Madison, WI) before transfection into *Dictyostelium* cells.

All constructs were transfected into racE null [strain 24EH6 (Larochelle *et al.*, 1996)] or wild-type DH1 (the parental strain for 24EH6) cells via electroporation. Briefly, 20 µg of DNA were electroporated into 8×10^6 cells, and cells were subsequently plated into five 96-well plates with HL5 media. After 24 h the media was replaced with fresh HL5 media containing 10 µg/ml blasticidin (Calbiochem, La Jolla, CA). After approximately 1 wk all independent transformed cell lines were grown on plates and analyzed for their ability to grow in suspension culture.

Western Blotting

To visualize the expression of the various rac constructs, Western blotting was used. Cell lysates were prepared according to Burns *et al.* (1995) and protein from 10^6 cells/lane was electrophoresed on a 10% SDS-polyacrylamide gel and subsequently transferred to Hybond-ECL (Amersham, Arlington Heights, IL). The blots were probed with either rabbit polyclonal antibodies generated to a racE-GST fusion protein (Figure 1a) or to GFP (Figure 1b; Clontech, Palo Alto, CA). A goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) was detected using an ECL kit (Amersham).

Growth Curves

Following initial screening for growth in suspension using 24-well plates, selected clones were screened in shaking flasks as follows. Ten milliliters of media per flask were seeded with approximately 5×10^4 cells/ml and maintained on an orbital shaker at 225 rpm. At 24-h intervals aliquots were removed for counting.

Fixation and Microscopy

Cells transfected with the various GFP constructs were observed by either conventional epifluorescence microscopy or by scanning laser confocal microscopy. Live cells were held in a Dvorak-Stotler Controlled Environment Culture Chamber (Nicholson Precision Instruments Inc., Gaithersburg, MD) for microscopic observation. To increase the proportion of dividing cells during observation, the cultures were partially synchronized as follows. Cells were allowed to grow to saturation attached to Petri dishes and maintained under such conditions for approximately 12 h. Cells were resuspended gently and an aliquot was then transferred to a slide containing 150 µl of fresh media and

¹ Abbreviations used: DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein.

allowed to attach for 15 to 25 min. The media were subsequently removed and the slide was immersed in phosphate-buffered saline (PBS) containing 3.5% formaldehyde plus 0.05% Triton X-100. After 1 min the slide was transferred to PBS plus 3.5% formaldehyde for 1 h. Slides were washed twice in PBS and then incubated for 15 min in PBS plus 50 mM NH_4Cl to quench any free aldehyde groups remaining following fixation. This step was followed by a 10-min incubation in PBS plus 4,6-diamidino-2-phenylindole (DAPI; 0.1 $\mu\text{g}/\text{ml}$, Sigma Chemical Co.) to stain the nuclei. Finally, the cells were washed three times in PBS and then mounted under a coverslip using a 1:1 PBS/glycerol solution containing diazabicyclo[2.2.2]octane (10 mg/ml, Sigma Chemical Co.).

RESULTS

Exogenous Expression of racE Rescues the racE Null Phenotype

We have shown previously (Larochelle *et al.*, 1996) that *Dictyostelium* mutant cells which fail to express the small GTPase racE fail to grow in suspension culture due to a specific defect in cytokinesis. To better understand the role of racE during cytokinesis, we undertook a mutational analysis of this protein. As a first step we needed to show that the cytokinesis-deficient phenotype of racE null cells could be complemented by exogenous expression of racE protein. We therefore constructed an extra-chromosomal expression vector carrying the *racE* cDNA under the control of the actin15 promoter (see MATERIALS AND METHODS). Transfection of this plasmid into racE null cells resulted in the expression of racE protein at approximately wild-type levels (Figure 1A). These cells regained the ability to divide by cytokinesis and grew at wild-type rates in suspension cultures (Figure 2A, closed triangles). Therefore, the exogenous expression of racE rescues the cytokinesis deficiency of racE null cells. This result allowed us to explore the sequences of racE that are important for its function *in vivo*.

Cytokinesis Requires the Active (GTP-Bound) Form of racE

We were first interested in determining whether cytokinesis required the cycling of racE between the active (GTP-bound) and inactive (GDP-bound) forms. We created racE mutants containing mutations similar to those that have been shown in other small GTPases to maintain a constitutively active or inactive state (see MATERIALS AND METHODS). These mutants are designated as V20racE (constitutively active) and N25racE (constitutively inactive). Expression vectors carrying these mutations were introduced into racE null cells to express the mutant proteins (Figure 1A). As with the wild-type racE, the constitutively active mutant V20racE rescued the cytokinesis deficiency of racE null cells and allowed them to divide at wild-type rates in suspension culture (Figure 2A, open squares). In contrast, expression of the constitutively inactive mutant, N25racE, failed to rescue the racE null phenotype since cells expressing this mutant protein did not divide under the same conditions (Figure 2A, open diamonds).

The introduction of constitutively active or inactive rho proteins in other systems sometimes generates a dominant negative phenotype (Ridley *et al.*, 1992; Harden *et al.*, 1995). To test this possibility, we introduced racE, V20racE, and N25racE into *Dictyostelium* wild-type DH1 cells to determine whether these constructs created a mutant phenotype. None of these constructs altered the ability of DH1 cells to divide in suspension culture (Figure 2B). In addition, DH1 cells expressing the racE mutant constructs did not display any defects in growth on plates or development (our unpublished observations).

Figure 1. Western blots demonstrating exogenous expression of racE and GFP-racE constructs. (A) Lysates were prepared from wild-type DH1 and racE null cells, electrophoresed on a 10% SDS-polyacrylamide gel, and probed with an antiracE antibody. The wild-type lane demonstrates the endogenous level of racE expression. The control lane refers to cells that were not transfected with vector for the exogenous expression of racE and demonstrates the lack of endogenous racE expression in the racE null cell line. The remaining lanes show the expression of the racE constructs as labeled at the top of each lane. (B) Lysates from racE null and wild-type DH1 cells were prepared and treated as described above and probed with a polyclonal antibody to GFP. Each lane is labeled at the top with the GFP-rac construct expressed by each respective cell line. Again, the control lanes refer to cells not transfected with exogenous plasmid DNA.

Figure 2. Growth curves of *racE* null and wild-type cell lines expressing exogenous wild-type and mutant *rac* proteins. *racE* null (A and C) and wild-type DH1 (B) cell lines were transfected with various *racE* constructs (A and B) or GFP-*rac* constructs (C) and subsequently assayed for their ability to grow in suspension culture as described in MATERIALS AND METHODS. The various constructs are as follows: A and B, control (nontransfected, filled circles), *racE* (filled triangles), V20*racE* (open squares), and N25*racE* (open diamonds); C, GFP-*racE* (filled triangles), GFP-V20*racE* (open squares), GFP-N25*racE* (open diamonds), GFP-*racE* C (open circles), GFP-*racE* C/E tail (filled diamonds), and GFP-*racE* tail (filled squares).

GFP-tagged *racE* Is Localized to the Plasma Membrane

To determine the site of action of *racE*, particularly in dividing cells, we attempted to localize *racE* by indirect immunofluorescence microscopy. Cells stained with a polyclonal antiserum against *racE* suggested a cortical distribution for *racE*; however, the intensity of the signal was weak (our unpublished observations). Consequently, we chose to use GFP as a fluorescent tag to determine the localization of *racE* both *in vivo* and *in vitro*. We designed a vector for the expression of a fusion protein containing GFP at the amino-terminal end of *racE* (see MATERIALS AND METHODS). Wild-type and *racE* null cells transfected with this vector expressed the fusion protein at levels similar to those of the endogenous *racE* protein (Figure 1B). Both cell lines expressing GFP-*racE* grew at wild-type rates in suspension culture and displayed no deleterious effect (Figure 2C and our unpublished observations). Since the GFP-*racE* fusion protein rescues the *racE* null phenotype, we conclude that the addition of GFP to the amino terminus of *racE* does not impair *racE* function. Consequently, it is likely that the localization of GFP-*racE* reflects the normal localization of *racE*.

Observations of both *racE* null and wild-type cells transfected with the GFP-*racE* expression vector demonstrated that GFP-*racE* localized primarily to the cortex of interphase cells (Figure 3C, *racE* null cells only). As a control we also expressed free GFP in the same cell lines and found that it was evenly distributed throughout the cytoplasm and excluded from a number of intracellular vesicles (Figure 3B). The cortical distribution of GFP-*racE* suggests that, like *ras* (Hancock *et al.*, 1989), *racE* associates with the plasma membrane.

To better understand the role of *racE* during cytokinesis, we determined the localization of GFP-*racE* in

dividing cells. Partially synchronized cells were fixed and stained with DAPI to identify mitotic cells. As can be seen in Figure 3D, the distribution of GFP-*racE* was not altered by the fixation protocol and was found primarily at the plasma membrane of interphase cells. Remarkably, this distribution remained constant in cells undergoing cytokinesis, as GFP-*racE* was uniformly localized throughout the plasma membrane and showed no particular distribution relative to the cleavage furrow (Figure 3E). Furthermore, the cell cycle distribution of GFP-*racE* was identical in wild-type DH1 or *racE* null cells expressing GFP-*racE* (our unpublished observations).

GFP-V20*racE* and GFP-N25*racE* Are Both Localized to the Plasma Membrane

Even though *racE* localized throughout the entire plasma membrane, it was possible that *racE* was activated selectively at a specific site on the plasma membrane during cell division. To test this hypothesis, we created expression vectors for the production of GFP-V20*racE* and GFP-N25*racE* fusion proteins. As described above, these were tested for their expression levels as well as their effects on growth in suspensions of both wild-type and *racE* null cells. Both constructs were expressed in both cell-type backgrounds (Figure 1B), and, consistent with the results obtained with our other *racE* constructs, GFP-V20*racE* rescued the *racE* null phenotype whereas GFP-N25*racE* did not (Figure 2C). Furthermore, neither construct produced a dominant negative phenotype when expressed in wild-type DH1 cells (our unpublished observation). Since the constitutively active GFP-V20*racE* mutant rescues the cytokinesis-deficient phenotype of *racE* null cells, we were particularly interested in determining whether this construct demonstrated a specific localization during mitosis or cytokinesis. Again, we found that GFP-V20*racE* was evenly distributed throughout the plasma membrane in interphase cells (Figure 4, A and E). Moreover, this distribution did not change in cells undergoing metaphase (Figure 4, B and F), anaphase (Figure 4, C and G), or cytokinesis (Figure 4, D and H). GFP-V20*racE* was neither concentrated nor depleted from the cleavage furrow of dividing cells. Similarly, GFP-N25*racE* also localized evenly throughout the plasma membrane of interphase *racE* null cells (Figure 5, A and D). However, because *racE* null cells expressing GFP-N25*racE* fail in cytokinesis, we did not determine its distribution in dividing *racE* null cells. Instead, observations of GFP-N25*racE* distribution in dividing cells were made using wild-type DH1 cells. The distribution of GFP-N25*racE* in these cells remained unchanged, localizing primarily to the plasma membrane in both interphase and dividing cells (Figure 5, B, C, E, and F).

Figure 3. Localization of GFP-racE in racE null cells. Confocal (A–C) and conventional (D–F) epifluorescence microscopy were used to visualize the expression of GFP-racE in live and fixed (respectively) racE null cells. (A) Control cell showing background level of autofluorescence. (B) Cell expressing GFP alone demonstrating a uniform cytoplasmic distribution and exclusion from vacuoles. (C) Live interphase cell expressing GFP-racE. (D) Fixed interphase cell expressing GFP-racE. (E) Fixed dividing cell expressing GFP-racE. (F) Same cell as in E stained with DAPI to visualize the nuclei. Bars, 10 μ m.

Localization of GFP-racE Is Mediated by the C Terminus of racE

Although all members of the rho family of small GTPases are very similar to each other, a salient feature of racE is the unique sequence near its carboxyl terminus (Larochelle *et al.*, 1996). In comparison to all other members of this family, the C-terminal domain of racE is extended by about 28 amino acids. Interestingly, the rab family of proteins also has an extended C-terminal domain, of about the same length, that has been shown to serve as a localization signal (Chavrier *et al.*, 1991). Therefore, we postulated that the C-terminal sequence of racE might also serve as a localization signal. To test this hypothesis, we first attempted to delete this region from the GFP-racE construct described above. This construct still contained the conserved prenylation CAAX box. Although cells transfected with this construct contained a mRNA of the expected size, they never produced any significant amount of the recombinant protein and failed to divide in suspension culture (our unpublished observa-

tions). These results suggested that the carboxyl-terminal tail of racE may be important for protein stability. We then engineered a GFP fusion protein containing the racE carboxyl terminus (Figure 6). This GFP-racE tail fusion protein was expressed at levels similar to those of our previous constructs (Figure 1B). As expected, this protein did not rescue the racE null phenotype (Figure 2C). Remarkably, this fusion protein had a cortical distribution identical to that of the full-length GFP-racE protein (Figure 7A), demonstrating that the racE C terminus is sufficient for targeting a protein to the plasma membrane.

The Function of racE Cannot Be Replaced by a Closely Related Protein

Currently, the protein most similar to *Dictyostelium* racE in the GenBank database is the *Dictyostelium* racC protein (Bush *et al.*, 1993; Larochelle *et al.*, 1996). Although racC shares 54% identical residues (68% overall conserved) with racE, it apparently cannot substi-

Figure 4. Localization of GFP-V20racE in *racE* null cells at different stages in the cell cycle. *racE* null cells expressing exogenous GFP-V20racE were fixed and stained with DAPI for microscopic observations by conventional epifluorescence microscopy. The various stages of the cell cycle shown are interphase (A and E), early metaphase (B and F), early anaphase (C and G), and cytokinesis (D and H). (A–D) Visualization of GFP. (E–H) Visualization of DAPI. Bar, 10 μ m.

tute for *racE* in cytokinesis since *racC* is expressed in *racE* null cells. We speculated whether the cytokinesis-specific function of *racE* might be provided by *racE*'s unique carboxyl-terminal region. We therefore designed GFP fusion proteins containing *racC* or a *racC*/

racE tail chimera and introduced these into *racE* null and wild-type cells (Figure 1B). Interestingly, both proteins had a distribution similar to that of GFP-*racE* in the plasma membrane of the cell (Figure 7, B and C). However, these proteins failed to rescue the cytokine-

Figure 5. Localization of GFP-N25racE in racE null and wild-type cells. Cells were fixed and stained with DAPI for microscopic observations by conventional epifluorescence microscopy. (A and D) Interphase racE null cell. (B and E) Interphase wild-type cell. (C and F) Dividing wild-type cell. (A–C) Visualization of GFP. (D–F) Visualization of DAPI. Bar, 10 μ m.

sis deficiency of racE null cells (Figure 2C), demonstrating that sequences unique to racE (in addition to its extended C terminus) are required for its function.

DISCUSSION

The important question of how cytokinesis is regulated in cells remains largely unanswered. Previously, we identified an absolute requirement for racE in cytokinesis (Larochelle *et al.*, 1996), but knew little about the specific role of racE in this event. The ability of exogenously expressed racE to rescue the cytokinesis-deficient phenotype allowed us to test the possibility that racE plays an important role in the timing of cytokinesis. The ability of exogenous racE and V20racE but not of N25racE to rescue the racE null phenotype suggests that racE must be activated by GTP to function in cytokinesis. Moreover, it is not necessary to convert racE from the GTP-bound to the GDP-bound state in order for cytokinesis to proceed normally. Since cell division can proceed normally in the presence of the GTP-bound constitutively active racE we conclude that racE cannot be the molecular

switch that governs the timing of contractile ring formation. Instead, we postulate that racE may play a permissive role in this process and the timing of cytokinesis is determined by other factors.

Figure 6. Construction of different GFP fusion proteins. The green fluorescent mutant protein S65T-GFP was placed at the amino terminus of all constructs. Between GFP and the different rac proteins there was a linker of five alanine residues. racE (open bar) is very similar to racC (hatched bar) sharing a total of 54% identical residues. However, racE has an extended carboxyl-terminal domain which is not found in racC or other rho proteins. The chimera GFP-racC/E tail contains this extended racE tail domain attached to residues 1–180 of racC. The same racE tail domain was also placed behind GFP.

Figure 7. Localization of GFP-racE tail, GFP-racC, and GFP-racC/E tail in racE null cells. Observations by confocal microscopy were made on racE null cells expressing these various rac constructs. (A) GFP-racE tail. (B) GFP-racC. (C) GFP-racC/E tail. Bar, 10 μ m.

The fact that the GDP-bound constitutively inactive mutant N25racE does not behave as a dominant negative when expressed in the wild-type DH1 background could be due to the levels of expression obtained. Relatively low levels of V20racE can rescue the null phenotype, suggesting that only a small amount of activated racE is required for cytokinesis (Figure 1 and personal communications). Consequently, much greater levels of the inactive mutant may be required for a dominant negative effect in the presence of wild-type racE. We were unable to overexpress racE mutants, suggesting that levels of racE may be tightly regulated in cells.

Interestingly, we demonstrated that fusing GFP to the amino terminus of racE does not appear to disturb its function. This GFP-racE chimera protein behaved identically to the native racE in its ability to function during cytokinesis. Consequently, the localization of GFP-racE based on the pattern of fluorescence distribution within a cell serves as a valid indicator of the intracellular residence of racE. This approach may be valuable to localize other members of the rho family of small GTP-binding proteins in *Dictyostelium* or other species.

The ability to localize racE in dividing cells allowed us to explore the role of racE in the spatial regulation of cell division. From our localization studies, we learned that racE is likely to function at the plasma membrane. Surprisingly, the cortical distribution of racE did not change at any stage of the cell cycle. This result suggested two models for the involvement of racE in cytokinesis. One is that although most racE is uniformly distributed throughout the plasma membrane a subpopulation of racE may be selectively activated in a specific region of the cell. This local activation of racE could be part of a signaling mechanism that controls the placement of the contractile ring at the appropriate site. However, our experiments with the V20racE mutant deem this an unlikely possibility. Since V20racE localized uniformly throughout the plasma membrane in both interphase and dividing cells, activation of racE cannot be the sole determinant

in the placement of the contractile ring. If this were the case, we would have observed contraction throughout the cortex of cells expressing the V20racE mutation and not just at the equator of dividing cells. Consequently, we can rule out the possibility that selective activation of racE at the equator, or elsewhere, triggers cytokinesis. A second model postulates an indirect role for racE in contractile ring formation. The formation of a contractile ring requires both a signal from the mitotic apparatus as well as a cortex receptive to that signal (Schroeder, 1981; White and Borisy, 1983). It is possible that racE plays an integral role in preparing the cortex for that signal. Although this step could be coordinated with the cell cycle, it is the signal from the mitotic apparatus that ultimately determines the placement, as well as the timing, of contractile ring formation. Therefore, activating racE prematurely, as we have done, would have no effect on the ability of the cortex to respond to the signal leading to cytokinesis.

The uniform intracellular distribution of racE mainly to the plasma membrane of the cell proved to be unique among the members of the rho family of ras-related small GTPases. Although ras is known to localize primarily to the plasma membrane, rhoA and rhoC are concentrated primarily in the cytosol with only a small fraction in the plasma membrane (Adamson *et al.*, 1992); rhoB, on the other hand, associates with intracellular vesicles. In resting neutrophils, rac was also found to be primarily cytosolic, translocating to the plasma membrane only upon stimulation (Quinn *et al.*, 1993). Although cdc42 in yeast localizes to the plasma membrane, it is found primarily at a polarized region from which the bud generally emerges and is not evenly distributed throughout the plasma membrane (Ziman *et al.*, 1993). The distribution of racE, on the other hand, is markedly different. Although we detect some racE in the cytosol, when examined by confocal microscopy we find the vast majority to be in the cortex and presumably the plasma membrane. It is generally thought that these small GTPases are retained in the cytoplasm through

binding to specific cytosolic inhibitors (EDP dissociation inhibitors; Glomset and Farnsworth, 1994). The localization of both racE and racC to the plasma membrane suggests that these proteins are not bound to cytosolic GDI at appreciable levels in *Dictyostelium*.

What is the mechanism by which racE localizes to the plasma membrane? Based on the rules of prenylation of CAAX boxes (Moores *et al.*, 1991), it is likely that racE (which terminates in CIL) is geranyl geranylated at its carboxyl terminus. Moreover, adjacent to the CAAX box of racE is a polybasic domain consisting of 6 lysines spread over 11 residues. We postulate that the combination of prenylation with the polybasic domain is probably sufficient to hold racE at the plasma membrane as it has been shown for ras (Hancock *et al.*, 1991). Interestingly, we found that another *Dictyostelium* rac protein, racC, also localizes to the plasma membrane of the cell. Since racC also has a polybasic domain adjacent to its C-terminal prenylation group, it is likely to interact with the plasma membrane in the same manner as racE. Nonetheless, despite having the same distribution, it cannot fulfill the role of racE in cytokinesis. This result strongly suggests that racE must undergo highly specific protein interactions in the plasma membrane which allow cytokinesis to proceed. These interactions cannot be solely mediated by the unique extended carboxyl-terminal region of racE since this region is not sufficient for conferring cytokinesis function onto racC. Certainly, the carboxyl-terminal 40 amino acids of racE are sufficient for plasma membrane localization since GFP fused with these amino acids also localizes to the plasma membrane. This domain may also be important for the stability of racE protein and, consequently, we could not test the ability of racE minus this C-terminal domain to rescue the racE null phenotype. Future studies may reveal what domains of the racE protein are essential for the specific protein interactions that must occur during normal cytokinesis.

ACKNOWLEDGMENTS

We gratefully acknowledge Roger Tsien for his generous contribution of S65T-GFP. We thank Drs. Terry O'Halloran, Margaret Titus, Eunice Kwak, Maria Niswonger, and Kathy Riddelle for their critical reading of this manuscript. This work was supported by grants to A.D. from the American Cancer Society (JFRA-425) and National Institutes of Health (GM-48745).

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