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Localization to the rhizoid tip implicates a *Fucus distichus* Rho family GTPase in a conserved cell polarity pathway

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Abstract Generation and expression of cell polarity in brown algal zygotes of the Fucales involve regulation of the actin cytoskeleton and localized secretion. We used degenerate PCR to isolate cDNAs that encode two small GTPases, FdRac1 and FdRab8, from zygotes of *Fucus distichus* (L.) Powell. Sequence analysis placed FdRac1 in the Rho family, which regulates actin, and FdRab8 in the Rab family, which regulates vesicle transport. As expected, bacterially expressed forms of both proteins bound GTP in vitro. When expressed in budding yeast, *FdRac1* showed some functional overlap with *CDC42*, the *Saccharomyces cerevisiae* Rho family gene required for yeast cell polarity. Immunolocalization revealed an asymmetric distribution of FdRac1 in polarized zygotes and embryos, with FdRac1 concentrated at or near the growing tip of the algal rhizoid. Our data support the hypothesis that FdRac1 regulates algal cell polarity, possibly via the actin cytoskeleton. Because brown algae belong to the heterokont group, which diverged from

other groups early in eukaryotic evolution, we argue that the Rho family function of regulating cell polarity is ancient and may extend throughout the eukaryotes.

Keywords Actin · Cell polarity · *Fucus* · Rho family · Small GTPase · Vesicle transport

Abbreviations AF: After fertilization · GST: Glutathione-S-transferase · MBP: Maltose-binding protein

Introduction

One of the fundamental problems in cell and developmental biology is understanding how cellular asymmetry, or polarity, is generated. Asymmetry within cells can define a functionally specialized region of the cell, supply positional information that affects cell fate, or serve other functions (Fowler and Quatrano 1997; Jürgens et al. 1997). There is growing evidence that certain mechanisms involved in establishing cell polarity are conserved in several eukaryotic species (Drubin and Nelson 1996). For example, spatially asymmetric activity and distribution of the actin cytoskeleton, and the conserved signaling molecules that regulate it, play a role in cell polarity in metazoans, fungi (Johnson 1999), amoebozoans (Chung et al. 2000) and higher plants (Yang 2002). Among these signaling molecules are small GTPases in the Ras superfamily, specifically ones in the Rho and Rab families, which regulate the actin cytoskeleton (Etienne-Manneville and Hall 2002) and vesicle transport (Lazar et al. 1997), respectively. These proteins are associated with cellular membranes, where they cycle between active (GTP-bound) and inactive (GDP-bound) forms, acting as switches to regulate downstream events (Sprang 1997).

We are interested in understanding the mechanisms by which cell polarity is generated, and how that polarity directs asymmetric developmental processes. For more than a century, furoid zygotes (those from

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intertidal brown algae of the genera *Fucus* and *Silvetia*) have provided a model for this problem. The zygotes, which are initially apolar in morphology, develop morphological polarity that can be directed by environmental cues (e.g., unidirectional light). During the 24 h following fertilization, zygotes first generate cellular asymmetries (e.g., localized accumulation of F-actin, a cortical Ca^{2+} gradient, directed transport of a population of Golgi-derived vesicles) with respect to the environmental cue, and then direct polarized growth of a rhizoid tip with respect to those cellular asymmetries. The initial cell division further partitions these asymmetries, generating a smaller, tip-growing rhizoid cell and a larger, chloroplast-containing thallus cell (reviewed in Fowler and Quatrano 1997; Kropf 1997; Robinson et al. 1999).

Several similarities between the fucoïd cell polarity pathway and those of other eukaryotes (e.g., the polarized budding of the yeast *Saccharomyces cerevisiae*) are apparent (Fowler and Quatrano 1997), despite the divergence of the heterokont group (to which *Fucus* and *Silvetia* belong) early in eukaryotic evolution (Baldauf 2003). For example, the ability to select an axis of polarity and to grow in a polarized fashion requires a dynamic actin cytoskeleton, as the actin inhibitors cytochalasin D and latrunculin B (Hable and Kropf 1998; Pu et al. 2000) and the actin stabilizer jasplakinolide (Hable et al. 2003) disrupt these processes. A localized accumulation of F-actin (an “actin patch”) in the spherical zygote that predicts the site of rhizoid growth has been detected (Alessa and Kropf 1999; Pu et al. 2000), although the timing with which this structure forms is controversial. This actin patch apparently transforms into a subapical ring that remains associated with the rhizoid tip as it grows (Alessa and Kropf 1999; Pu et al. 2000). This cytoskeletal structure has been hypothesized to delineate the growing and non-growing domains of the cell (Alessa and Kropf 1999), to restrict certain molecular components to the growing rhizoid tip (Pu et al. 2000), and to serve as a targeting site for localized secretion (Fowler and Quatrano 1997; Belanger and Quatrano 2000b).

Asymmetric transport and localized secretion of both adhesive material (Hable and Kropf 1998) and a population of Golgi-derived vesicles (Shaw and Quatrano 1996) is also associated with fucoïd cell polarity. A cell wall component, the sulfated polysaccharide F2, is locally deposited into the wall at the presumptive rhizoid growth site (reviewed in Fowler and Quatrano 1997; Kropf 1997). Brefeldin A treatments, which inhibit vesicle transport from the Golgi to the plasma membrane, also inhibit normal photopolarization in the spherical zygote, underscoring the importance of vesicle transport in generating cell polarity (Shaw and Quatrano 1996; Hable and Kropf 1998). During growth of the rhizoid, directed vesicle transport continues to be oriented toward the tip (Belanger and Quatrano 2000a), likely providing components that extend and strengthen the rhizoid wall (Bisgrove and Kropf 2001).

Given the similarities between the generation of cell polarity in fucoïd zygotes and other eukaryotes, we were interested in whether small GTPases of the Rho and Rab families were present in brown algae, and active in the generation or maintenance of cell polarity. We took a degenerate-PCR approach to identifying these genes from the brown alga *Fucus distichus*. Several lines of experimental evidence, most notably the asymmetric localization of a Rho family GTPase to the growing rhizoid tip, are consistent with our hypothesis that the identified genes encode molecular components that act in tip-growing *Fucus* zygotes to maintain polarity, and may aid tip growth itself.

Materials and methods

Isolation and sequence analysis of small GTPase-encoding clones

Degenerate primers corresponding to amino acids in the conserved G-1 (ACAGAAATTCGGNGAYGGNGC NKKNGGNAARAC) and G-3 (TARTCYTCYTGWCCWGCNGTRTC) loops of the Rho GTPase family (see Fig. S1 in the Electronic Supplementary Material) were designed to amplify a short fragment (≈ 170 bp) from Rho family genes. The mixture of ≈ 170 -bp products amplified from a *Fucus distichus* (L.) Powell cDNA library was cloned into the pCR II vector using a TA Cloning Kit (Invitrogen). Forty-four plasmids with inserts were recovered and sequenced to identify putative *F. distichus* genes encoding Ras superfamily GTPases. Plasmids with longer inserts, corresponding to the full-length *FdRac1* and *FdRab8* cDNAs, were isolated from a Lambda ZipLox cDNA library made from embryos at 24 h after fertilization (AF; gift of C. Taylor, UNC Chapel Hill; Goodner et al. 1995) using standard low-stringency radioactive screening methods (Ausubel et al. 1994). *FdRac1* clones were also isolated from a Lambda ZAP II cDNA library made from embryos at 18 h AF (also from C. Taylor; Belanger et al. 2003). Sequencing was done either manually or at the UNC DNA Sequencing Facility. Sequences were analyzed using the Wisconsin Package Version 10.3 (Accelrys Inc., San Diego, CA, USA).

Phylogenetic trees were generated from amino acid sequences using the software package MrBayes v2.01 for Bayesian inference of phylogeny (Huelsenbeck and Ronquist 2001). Select amino acid sequences in the Rho and Rab families, spanning a variety of eukaryotes, were downloaded from GenBank, aligned with the *F. distichus* sequences, and adjusted manually. Sequences near the amino- and carboxy-terminal ends were omitted, as they could not be aligned with confidence. For phylogeny inference, the “jones” model was used, with gamma-distributed rate variation. Posterior probabilities, used as estimates of branch support, were calculated following a run of 200,000 generations, with the first 20,000 generations as the “burnin” period.

Protein expression, purification and biochemical assays

An *FdRac1* cDNA fragment, missing the first 12 nucleotides of the coding region, was cloned into the bacterial expression vector pPR997 (New England Biolabs) to create a fusion with maltose binding protein (MBP). An *FdRac1* cDNA fragment containing the full-length coding sequence was inserted into the bacterial expression vector pGEX-4T3 (Pharmacia), as a fusion with glutathione-S-transferase (GST). Similarly, an *FdRab8* cDNA fragment, missing the first six nucleotides of the coding region, was cloned into pGEX-4T2 (Pharmacia), as a fusion with GST, and into pPR997, as a fusion with MBP. Cloning junctions were confirmed by sequencing, and the constructs were transformed into UT5600, a protease-deficient *Escherichia coli* strain that facilitates protein purification. The MBP-FdRac1 and MBP-FdRab8 fusions were purified according to the manufacturer's instructions (New England Biolabs), except that a GTPase-specific lysis buffer [50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF)] was substituted for the manufacturer's lysis buffer. The GST-FdRac1 fusion was purified according to the methods of Self and Hall (1995), including cleavage of FdRac1 from the GST tag using thrombin. Following purification, the fusion proteins were dialyzed against high-salt buffer [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM MgCl₂, 0.1 mM DTT], and concentrated using a spin filter concentrator. Protein concentration was determined using the Bio-Rad Protein Assay.

GTP-binding activity for the purified proteins was confirmed using a filter-binding protocol from Self and Hall (1995). Briefly, 250 ng of purified protein was incubated in 40 µl assay buffer with 0.25 µl α-[³²P]GTP (Amersham; 370 MBq/ml, 111 TBq/mmol) for 15 min at room temperature, and then filtered through nitrocellulose. Cold GTPγS was added to 1 mM for competition experiments. Radioactivity was determined by scintillation counter. Assays were done in triplicate, and the mean and standard error for the scintillation counts were calculated.

Yeast cloning, expression and two-hybrid methods

To produce constitutively active and dominant-negative forms of FdRac1, site-directed mutagenesis by the "megaprimer" method was used (Sarkar and Sommer 1990). As was done to make other Rho family mutants (Etienne-Manneville and Hall 2002), we used mutagenic primers (TGGGTGACGTTGCGTTGGT and GGTTGGTAAAACTGCCTCC, respectively) to produce the constitutively active, GTP-bound *FdRac1-G12V* and the dominant negative, GDP-bound *FdRac1-T17N*. Sequences were confirmed following PCR and cloning. All three versions of *FdRac1* (wild-type, -*G12V*, and -*T17N*) were cloned into the *LEU2*-selectable, single-copy JAB-4 vector (gift of the Bloom lab, UNC-CH)

downstream of the *GAL1* promoter. Four independent transformants for each *FdRac1* construct were isolated for both a wild-type strain (YML28—*leu2 ura3*) and a mutant strain (M-YML10—*cde42-1^{ts} leu2 ura3 trp1 his4*; gifts of the Pringle lab, UNC-CH), and grown on media lacking leucine and containing either 2% glucose or 2% galactose/1% raffinose.

The yeast two-hybrid interaction trap (Gyuris et al. 1993) was used as described by Finley and Brent (1995). Briefly, full-length *FdRac1* was cloned into the pEG202 "bait" plasmid, in-frame with the LexA binding domain. Analogous constructs containing the yeast *CDC42*, *RHO1*, and *RAS2* genes were obtained, as were three constructs harboring yeast genes (*RDII*, *STE20*, and *BEM3*) in the pJG4-5 "prey" vector (Simon et al. 1995; Stevenson et al. 1995; all gifts of the Pringle lab, UNC-CH). Each combination of bait and prey vector was transformed into the EGY48 strain (*ura3 trp1 his3 leu2::pLEU2-LexAop6*), along with a *lacZ* reporter plasmid, pSH18-34. Three independent transformants from each combination were tested both for ability to complement the *leu2* mutation and ability to express β-galactosidase activity in the presence of galactose/raffinose, an inducer of bait protein expression. β-Galactosidase assays (Bartel and Fields 1995) were carried out after growth for 16 h at 30°C in liquid medium containing 2% galactose/1% raffinose.

Antibody preparation, western blotting, and immunolocalization

Crude polyclonal antisera were generated by subcutaneous injection of immunogens into NZW rabbits using a standard protocol (HRP, Denver, PA, USA). MBP-FdRac1 was the immunogen for anti-FdRac, whereas a BSA-conjugated peptide with sequence corresponding to the G-2 loop of FdRab8 (CQTFITTIGIDFKIKNIDLD) was the immunogen for anti-RabPeptide. Affinity-purified reagents were developed using the methodology described by Williams et al. (1995). Briefly, three affinity columns were prepared using three different ligands (GST-FdRac1, MBP-FdRab8, and whole bacterial lysate from an *E. coli* strain expressing MBP-lacZ) coupled to Actigel ALD (Sterogene). These columns were used to prepare more specific reagents from the crude antisera. For anti-FdRac, the crude antiserum was (1) diluted 1:1 into adsorption buffer [15 mM Hepes (pH 7.6), 50 mM NaCl, 5 mM MgCl₂], (2) adsorbed against bacterial lysate, (3) adsorbed to, and then eluted (using Actisept; Sterogene) from, GST-FdRac1, (4) dialyzed against adsorption buffer, (5) adsorbed against MBP-FdRab8, and (6) concentrated using a spin filter concentrator. A similar series of steps was used to prepare anti-RabPeptide, except that, in step 3, the antiserum was adsorbed to, and then eluted from, MBP-FdRab8, and in step 5, was adsorbed against GST-FdRac1. A negative-control antiserum was prepared by eluting antibodies bound to the bacterial

lysate column, following adsorption of the crude anti-RabPeptide serum.

To collect zygotes for either protein extracts or immunolocalization, receptacles of *F. distichus* were collected at Newport, Oregon, and stored at 4°C. Gametes were released, using cold osmotic shock, into artificial seawater and grown at 16°C in either glass baking dishes or directly on slides according to published methods (Quatrano 1980). For western blotting, zygotes were harvested and extracts were prepared as described by Kropf et al. (1989), supplemented with a plant protease inhibitor cocktail (Sigma P9599). SDS-PAGE, western blotting, and detection with an alkaline phosphatase-conjugated secondary antibody, were performed according to standard methods (Ausubel et al. 1994). Immunolocalization was performed according to a protocol (on the web at <http://oregonstate.edu/~fowlerj/>) modified from that of Bouget et al. (1996). Detection of the primary antibody was with Alexa488 goat anti-rabbit secondary antibody (Molecular Probes). Samples were imaged by confocal laser scanning microscopy on a Leica DM IRBE with a 100×/NA1.4 objective, using TCS 4D software. Images were imported into NIH Image 1.63 for analysis and production of three-dimensional projections.

Results

Identification of *Fucus distichus* genes encoding GTP-binding proteins in the Rho and Rab families

To identify brown-algal genes in the Rho and Rab families, we PCR-amplified fragments using template DNA from an *F. distichus* cDNA library and degenerate primers corresponding to the conserved G-1 and G-3 GTP-binding domains of the Ras-related small GTPases (Sprang 1997). The inserts of 44 independently cloned PCR products were sequenced, revealing that 38 of the clones contained one of eight distinct Ras-related sequences: two members of the Ran family, five from the Rab family, and one from the Rho family (see Table S1 in the Electronic Supplementary Material). We focused on two: the single Rho family sequence (clone P9), and the Rab sequence (clone P1) that was most closely related to *Shizosaccharomyces pombe ypt2* and mammalian *Rab8* (both in the Rab VIII family; Pereira-Leal and Seabra 2001).

We used the two inserts as probes to isolate full-length cDNAs from two cDNA libraries, both derived from *Fucus* zygotes at different stages of development (18 h and 24 h after fertilization). Despite extensive, low-stringency screening using the *Fucus* Rho insert, the only cDNAs that were recovered corresponded to a single Rho family gene, which we named *FdRac1* (GenBank Acc. # AY438585). Together, the PCR cloning and low-stringency screening results suggest that only one Rho family GTPase, namely *FdRac1*, is expressed at readily detectable levels during zygotic development. The only long open reading frame in the transcript encoded a

predicted protein of 196 amino acids with a calculated molecular mass of 22 kDa (see Fig. S1a in the Electronic Supplementary Material). The *FdRac1* protein contained five conserved sequence elements (see Fig. S1a in the Electronic Supplementary Material), corresponding to the five conserved loops (G-1 through G-5) present in all Ras superfamily GTPases (Sprang 1997). *FdRac1* also contained a polybasic region and a C-terminal prenylation signal (the CaaX box), both required for the association of Rho family members with membranes (Seabra 1998). Surprisingly, *FdRac1* terminated not in the geranylgeranylated “CaaL” motif characteristic of most Rho family GTPases, but rather in “CVIS”, a motif that is predicted to be farnesylated. Sequence alignment and phylogenetic analysis confirmed that *FdRac1* belonged in the Rho family, most closely associated with the Rac subgroup, and not the Rho, Cdc42 or Rop groups (Fig. 1a). *FdRac1* was one of several Rac sequences in this subgroup from organisms that diverged from metazoans in the distant past (e.g., *Dictyostelium discoideum*, *Entamoeba histolytica*).

By screening the libraries with the Rab-related probe, we recovered four distinct clones corresponding to a transcript we designated *FdRab8* (GenBank Acc. # AY439005). The only long open reading frame in the transcript encoded a predicted protein of 205 amino acids with a calculated molecular mass of 23 kDa (see Fig. 1b in the Electronic Supplementary Material). This predicted protein also contained the five conserved sequence elements present in all Ras superfamily GTPases, and a C-terminal di-cysteine motif characteristic of other Rabs, which was a likely site of dual geranylgeranylation necessary for membrane association (Seabra 1998). Sequence alignment and phylogenetic analysis confirmed that *Fucus* *FdRab8* belonged in the RAB VIII subfamily (Pereira-Leal and Seabra 2001), which includes metazoan and fungal proteins known to regulate Golgi-to-plasma membrane transport (human *Rab8*, *S. pombe* *Ypt2*; Lazar et al. 1997), as well as those from more divergent species (*Dictyostelium* *Sas1/Sas2*, *Volvox carteri* *Ypt2*) (Fig. 1b).

To test whether the conserved sequences of *FdRac1* and *FdRab8* resulted in GTP-binding activity in both putative GTPases, we generated bacterially expressed proteins for use in in vitro binding assays. Both *FdRab8* (purified as a fusion with MBP) and *FdRac1* bound α -[³²P]GTP in filter-binding assays (Fig. 2). A purified MBP-lacZ control protein did not bind GTP. Furthermore, GTP-binding was inhibited in the presence of cold GTP- γ -S, a non-hydrolyzable GTP analog. Thus, both *FdRab8* and *FdRac1* specifically bound GTP, and are likely to function as GTPases in the *Fucus* zygote.

FdRac1 interacts with components of the *S. cerevisiae* Cdc42p signaling pathway in the two-hybrid assay

Due to the high amino acid conservation observed among *FdRac1* and other members of the Rho family,

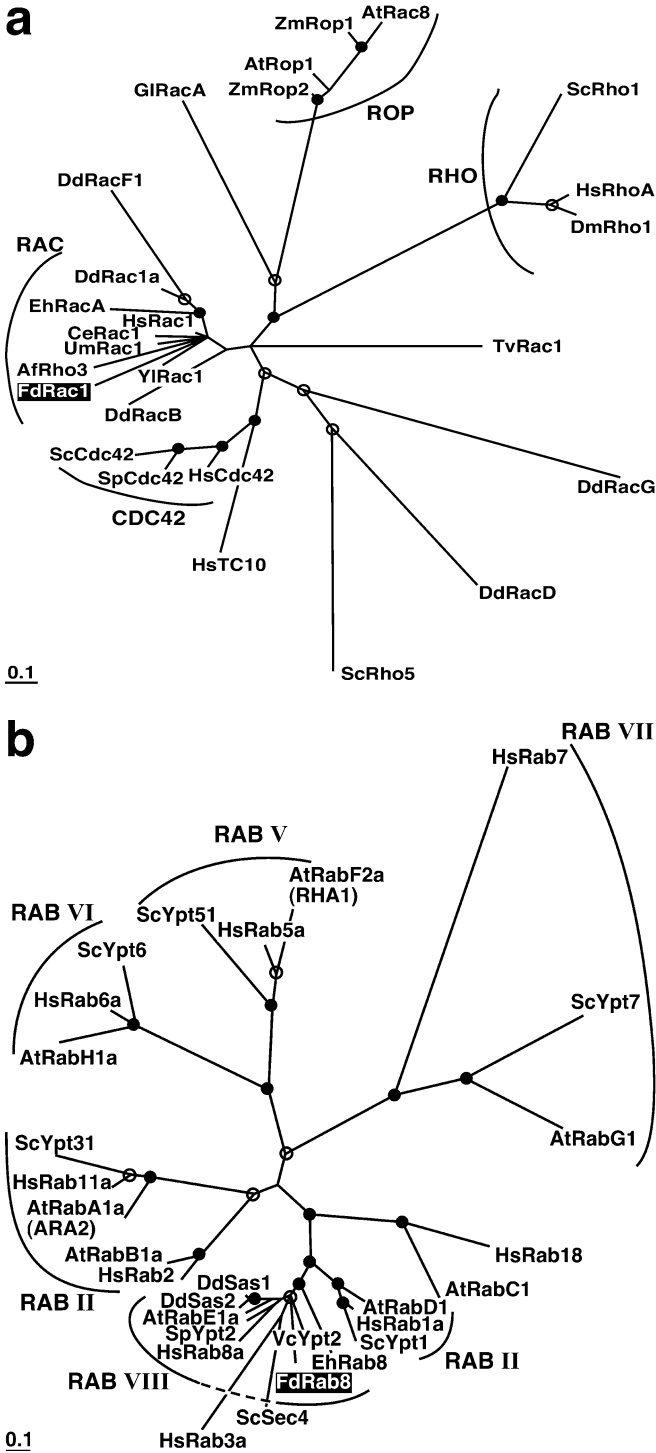


Fig. 1a, b Phylogenetic analysis, using Bayesian inference, places the *Fucus distichus* FdRac1 and FdRab8 proteins within the Rho and Rab GTPase families, respectively. **a** FdRac1 is within the Rac subgroup, although its position is not strongly supported. **b** Placement of FdRac8 in the Rab VIII subgroup is strongly supported. Analyses using additional Rho and Rab family sequences, as well as those with neighbor-joining algorithms, did not appreciably change the placement of the *Fucus* proteins (not shown). Tree nodes with strong support (>95% clade credibility) are marked with filled circles, and those with moderate support (>75% clade credibility), with open circles. Species designations: At, *Arabidopsis thaliana*; Af, *Aspergillus fumigatus*; Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Eh, *Entamoeba histolytica*; Fd, *Fucus distichus*; Gl, *Giardia lamblia*; Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Tv, *Trichomonas vaginalis*; Um, *Ustilago maydis*; Vc, *Volvox carteri*; Yl, *Yarrowia lipolytica*; Zm, *Zea mays*

We expressed *FdRac1* from an inducible promoter in transformed wild-type and *cdc42-1^{ts}* (temperature-sensitive) mutant yeast. Induction of *FdRac1* expression in

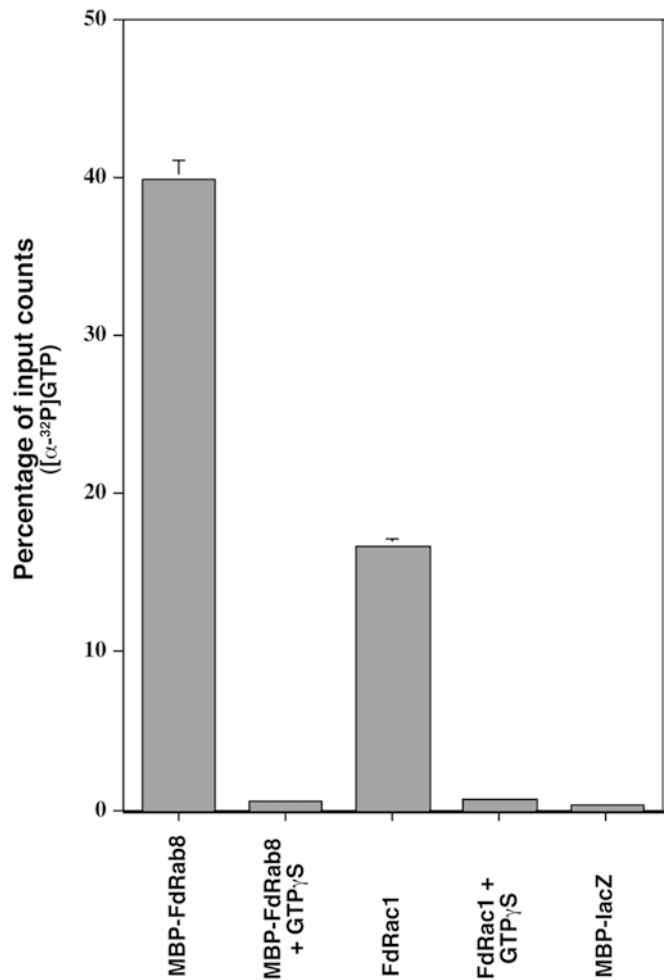


Fig. 2 Filter-binding assays demonstrate that FdRac1 and FdRab8 have GTP-binding activity. Bacterially expressed FdRab8 (fused to MBP) and FdRac1 both bind α -[³²P]GTP; binding is blocked by incubation with excess cold GTP_γS. The negative control, MBP-lacZ, did not bind GTP. Similar results were obtained in a second experiment

and the similarities between the generation of cell polarity in *Fucus* and *S. cerevisiae* (Fowler and Quatrano 1997), we investigated whether *FdRac1* could function in the yeast pathway that establishes cell polarity. Budding yeast lack a Rac subfamily GTPase; it is the Rho GTPase Cdc42p which is the key regulator of the yeast cell polarity pathway (Johnson 1999), and several other yeast proteins (e.g., Ste20p kinase; Simon et al. 1995) in this pathway are known to interact directly with Cdc42p.

yeast did not appreciably alter growth of wild-type yeast, nor did it rescue the slow-growth phenotype at a partially permissive temperature (35°C) for the *cdc42-1^{ts}* mutation (Fig. 3). We reasoned that differences between FdRac1 and Cdc42p could prevent FdRac1 from interacting with upstream activators of Cdc42p, thus leaving it in its GDP-bound form in *S. cerevisiae*. Therefore, we constructed both constitutively active (i.e., GTP-bound) and dominant-negative (i.e., GDP-bound) mutant versions of *FdRac1*, and expressed these in yeast, to test whether bypassing such upstream activators could affect yeast growth. Induction of *FdRac1-G12V*, the activated form of FdRac1, did confer a slight growth advantage to *cdc42-1^{ts}* cells, compared to vector-only controls, at the partially permissive temperature (Fig. 3). However, the mutant *Fucus* gene was not able to rescue either the *ts* mutant at the non-permissive temperature (37°C) or a strain with a deleted *cdc42* gene (data not shown).

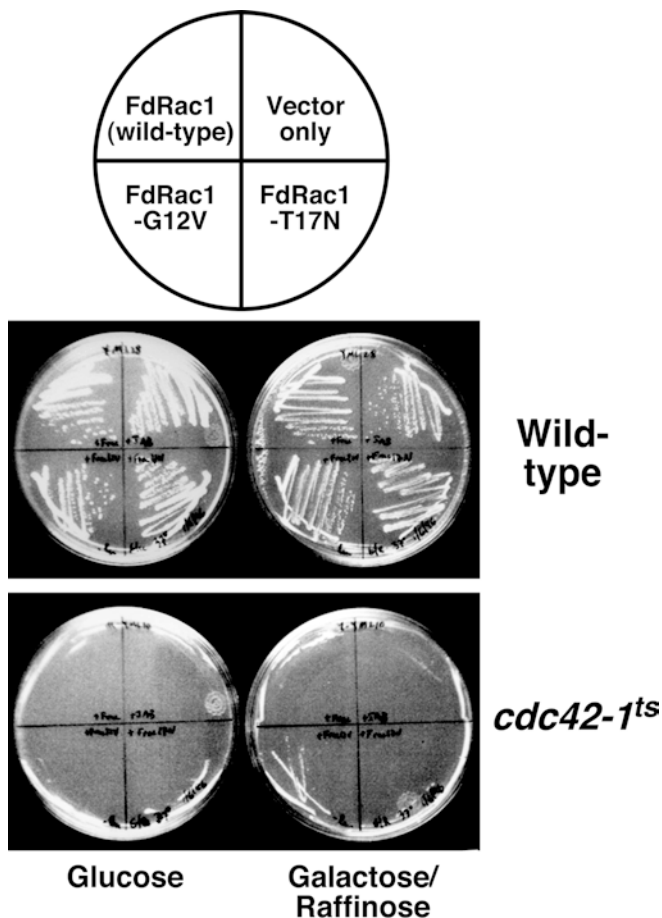


Fig. 3 The expression of a constitutively active mutant form of FdRac1 provides a growth advantage to a *cdc42-1^{ts}* budding yeast strain, when grown at a partially permissive temperature (35°C). A *GAL1* promoter construct (induced by growth on galactose/raffinose, repressed by growth on glucose) was used to express *FdRac1*, *FdRac1-G12V* (a constitutively active mutant), or *FdRac1-T17N* (a dominant negative mutant) in wild-type and mutant yeast. Only the *FdRac1-G12V* construct had an effect on growth of the mutant *cdc42-1^{ts}* strain, when compared to the vector-only control, and only in the presence of galactose/raffinose

Induction of the dominant negative *FdRac1-T17N* construct had no effect on either wild-type or mutant yeast.

Although *FdRac1* could not substitute for *CDC42* in *S. cerevisiae*, we also tested whether it was capable of interacting with certain components of the yeast polarity pathway. The LexA two-hybrid assay (Gyuris et al. 1993) has been used to characterize the interactions of Cdc42p with other yeast proteins in the polarity pathway (e.g., Simon et al. 1995). By subcloning *FdRac1* into a two-hybrid plasmid, we could express FdRac1 fused to the LexA DNA-binding domain (as a “bait”) to test it against known yeast *CDC42* interactors fused to an activation domain. If FdRac1 were able to bind to a Cdc42p interactor, it would bring the activation domain to the promoter of a reporter gene, in our case the β -galactosidase-encoding *lacZ* gene. We used the *S. cerevisiae* small GTPase genes *RHO1* and *RAS2* as control “baits”, for comparison of interaction specificities (Simon et al. 1995).

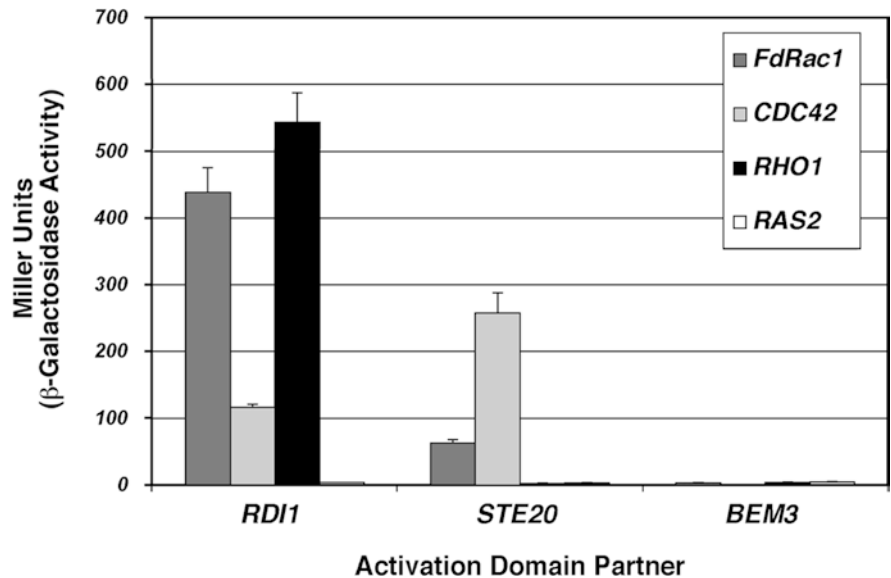
In the two-hybrid assay, *FdRac1* interacted specifically with the yeast genes *RDII* (which encodes Rho-GDI, a general negative regulator of Rho family GTPases; Olofsson 1999) and *STE20* (which encodes a protein kinase downstream of Cdc42p that does not interact with *RHO1*; Simon et al. 1995) (Fig. 4). The interaction exhibited by *FdRac1* and *RDII*, an interaction not shown by the *RAS2/RDII* pair, confirmed that FdRac1 has Rho family characteristics when expressed in yeast. Furthermore, the interaction between *FdRac1* and *STE20*, although weaker than that between *CDC42* and *STE20*, was clearly above the background levels displayed by the *RHO1/STE20* pair. *FdRac1* did not interact with the *CDC42* GTPase-activating protein *BEM3* (Fig. 4), a negative control, demonstrating that *FdRac1* did not induce expression of β -galactosidase activity on its own. In addition to activating the *lacZ* reporter, both of the *FdRac1* interactions turned on a *LEU2* reporter, in a second test confirming the interaction (data not shown).

FdRac1 is expressed during zygotic development

We raised polyclonal antisera against FdRac1 and FdRab8, using either a bacterially expressed fusion protein (MBP-FdRac1) or a peptide (FdRab8) as antigens. Antibodies against each protein were affinity-purified (see Materials and methods), and tested on Western blots against (i) *Fucus* protein extracts, (ii) purified proteins, and (iii) extracts from bacterial strains that expressed each of the proteins as a fusion (Fig. 5).

Purified MBP-FdRac1 antiserum (anti-FdRac) recognized a single major band at approximately 20 kDa in *Fucus* extracts, and purified FdRac1 running at the same size (Fig. 5a), in agreement with a predicted molecular weight of 22 kDa. Importantly, anti-FdRac did not recognize bacterially expressed GST-FdRab8, and it did bind to GST-FdRac1, as expected

Fig. 4 The two-hybrid assay, using a β -galactosidase reporter, detects interactions between *FdRac1* and genes encoding two components of the *Saccharomyces cerevisiae* Cdc42p signaling pathway, Rdi1p (RhoGDI) and Ste20p (a serine/threonine kinase). The *BEM3* construct serves as a negative control demonstrating that none of the constructs (*FdRac1*, *CDC42*, *RHO1*, *RAS2*) induces reporter activity alone. β -Galactosidase activities were measured as Miller units in three independent transformants of each strain (containing each pair of clones); the mean and standard error (bar) for each combination are shown



(Fig. 5b); furthermore, the MBP–*FdRac1* pre-immune serum did not recognize either of the bacterially expressed proteins (data not shown). Competition experiments confirmed that the anti-*FdRac* antiserum was specific: pre-incubation of anti-*FdRac* with an excess of bacterially expressed MBP–*FdRac1* protein greatly reduced the intensity of the ≈ 20 -kDa band in western blots of *Fucus* extracts, whereas pre-incubation with purified MBP–*FdRab8* control did not (data not shown).

Purified *FdRab8* antiserum (anti-RabPeptide) recognized purified MBP–*FdRab8* fusion protein, and a major band at the predicted size of 23 kDa in *Fucus* extracts (Fig. 5c). The anti-RabPeptide antiserum also recognized GST–*FdRab8* in induced bacterial cultures. However, despite the affinity-purification procedure and the lack of identity between the primary sequences of the *FdRab8* peptide antigen and the *FdRac1* protein, anti-RabPeptide antiserum also recognized bacterially expressed GST–*FdRac1* (Fig. 5d). Thus, the anti-RabPeptide antiserum was not specific for *FdRab8*. The antiserum may recognize a number of *Fucus* small GTPases, perhaps via a conserved structural element not dependent on primary sequence identity. We used the anti-RabPeptide antibody as a control, to demonstrate the specificity of the anti-*FdRac* pattern in immunolocalization experiments (see below).

Because we were interested in determining whether the level of *FdRac1* protein varied during the zygotic cell cycle, we made protein extracts from synchronized populations of zygotes at several time points after fertilization and probed them with anti-*FdRac* (Fig. 5e). Interestingly, although *FdRac1* was detectable throughout the 24 h after fertilization (AF), the level of protein was distinctly lower at the stage immediately after fertilization (2 h AF). By 9 h AF, just prior to the initial growth of the rhizoid tip, *FdRac1* protein levels had increased.

FdRac1 is distributed asymmetrically during polarized tip growth

To determine whether *FdRac1* was distributed asymmetrically in the zygote during polar growth, we used our anti-*FdRac* antibody in immunofluorescence experiments, and imaged whole-mount cells using confocal laser scanning microscopy. In zygotes undergoing rhizoid tip growth at 17 h AF, anti-*FdRac* signal localized preferentially at or near the growing tip, primarily in the cortical or sub-cortical regions (possibly associated with the plasma membrane; Fig. 6a,b,f). Using identical staining and imaging conditions, the epitope(s) detected by the anti-RabPeptide antibody appeared to be preferentially distributed to the rhizoid, but signal was present throughout the cytoplasm (Fig. 6g), with occasional zygotes also showing a tip-associated accumulation of signal (Fig. 6c). The signal distribution observed with anti-*FdRac* was a subset of the anti-RabPeptide signal, consistent with the ability of anti-RabPeptide to detect both *FdRab8* and *FdRac1* (and possibly additional small GTPases). Together with the generally symmetrical distribution exhibited by the chloroplast-localized fucoxanthin chlorophyll binding protein control (Apt et al. 1995) (Fig. 6e), the anti-RabPeptide results indicated that the asymmetric, cortex-enriched anti-*FdRac* localization pattern was not an artifact of the immunofluorescence or imaging procedures. As a negative control, we used an affinity-purified antiserum against whole bacterial lysate (see Materials and methods). Neither the negative control primary antiserum (Fig. 6d) nor the secondary antibody alone (data not shown) appreciably stained fixed zygotes.

As visualized using anti-*FdRac*, the cortex-associated fluorescence appeared punctate, detecting individual spots or groups of tightly clustered spots. The brightest patches of fluorescence were distributed within the rhi-

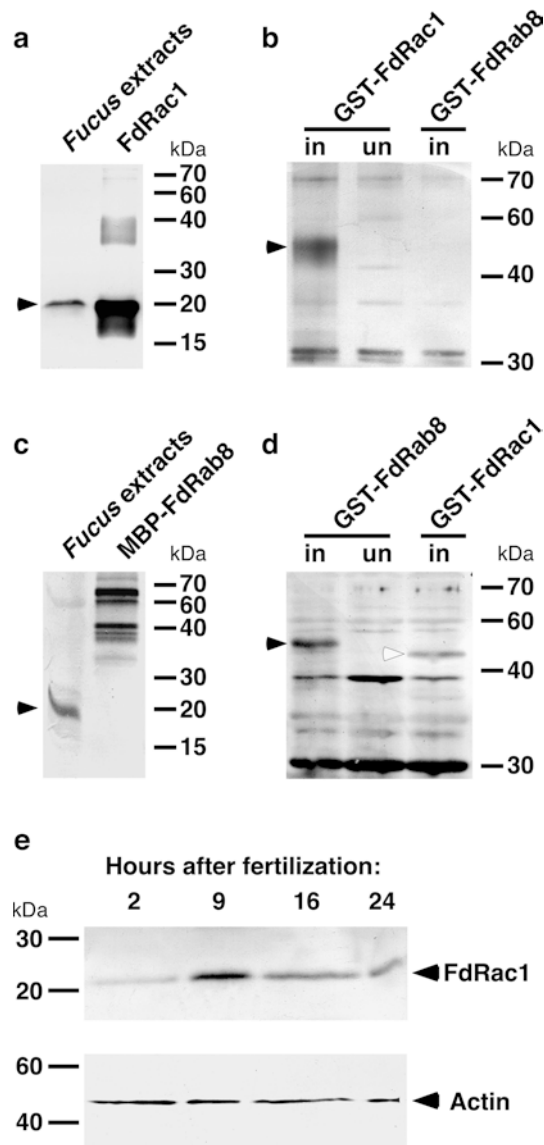


Fig. 5a–e Characterization of the anti-FdRac and anti-RabPeptide affinity-purified antibodies. Western blots were probed with either anti-FdRac1 (**a**, **b**) or anti-RabPeptide (**c**, **d**). anti-FdRac recognizes: **a** a single prominent band (arrow) in *Fucus* extracts prepared from embryos at 24 h AF (after fertilization), which runs at the same size as purified, bacterially expressed FdRac1; and **b** in bacterial extracts, a prominent band representing the GST–FdRac1 fusion protein (arrow) induced (*in*) by isopropyl β -D-thiogalactopyranoside (IPTG), which was not present in uninduced (*un*) cultures, or in a strain expressing the GST–FdRab8 fusion protein. anti-RabPeptide recognizes: **c** a single prominent band at \approx 22 kDa (arrow) in *Fucus* extracts 24 h AF, as well as purified, bacterially expressed MBP–FdRab8, primarily in its \approx 65-kDa full-length form, but also in multiple shorter, presumably degraded forms; and **d** in bacterial extracts, a band representing the GST–FdRab8 fusion protein (black arrow) induced by IPTG, which was not present in uninduced cultures. anti-RabPeptide also recognized the GST–FdRac1 fusion protein (white arrow). **e** FdRac1 is expressed during the early stages of embryonic development, with an increase in protein abundance occurring by 9 h AF. All lanes were loaded equally with 30 μ g of *Fucus* protein, as confirmed by probing with anti-actin. The morphological signs of rhizoid tip growth are first visible at \approx 10 h AF. By 16 h AF, the rhizoid has elongated, but not yet divided. By 24 h AF, the embryo has completed its first cell division

zoid at or near the tip apex (Fig. 6b,f); in certain zygotes, the brightest fluorescence resembled a subapical ring (Fig. 6a). Occasional bright spots and a faint signal were also associated with the entire rhizoid and thallus plasma membrane, and apparent in the rhizoid cytoplasm. The internal and sub-cortical punctate signals could represent an association of FdRac1 with vesicular membranes, a localization pattern also observed for a subset of other Rho family proteins (Ridley 2001).

To determine whether distribution of FdRac1 changed during embryonic development, we stained and imaged samples at 9, 13, 26 and 42 h AF. At 9 h AF, before emergence of the rhizoid tip, we never saw strong asymmetric accumulation of anti-FdRac signal (Fig. 7a,b). However, zygotes in the initial stages of rhizoid tip growth (13 h AF, Fig. 7c,d) displayed a pattern similar to that seen in zygotes at 17 h AF. The anti-FdRac signal also localized to the growing rhizoid tip in two-celled embryos (26 h AF; data not shown) and in eight-celled embryos (42 h AF, Fig. 7e). For comparison, anti-RabPeptide signal was not noticeably enriched at the rhizoid tip at 42 h AF (Fig. 7f). The strong, more symmetrically distributed anti-FdRac signal in the thallus cells 42 h AF (Fig. 7e) raises the possibility that FdRac1, like other Rho GTPases (Etienne-Manneville and Hall 2002; Yang 2002), has roles in signaling unrelated to cell polarity (e.g., cell cycle control, hormone response).

Discussion

Mechanistic models that account for the polarized development of the fucoid zygote and embryo have been proposed (Fowler and Quatrano 1997; Kropf 1997; Robinson et al. 1999). To identify specific candidate molecular components of these postulated mechanisms, with the goal of testing and refining the models, we targeted signaling proteins in the small GTPase superfamily. In the models, cell polarity is generated or maintained by regulating the actin cytoskeleton; in certain models, targeted secretion has also been assigned a role (Belanger and Quatrano 2000b). We have presented evidence supporting the conclusion that our two clones, *FdRac1* and *FdRab8*, encode small GTPases involved in these cellular processes.

First, the proteins' predicted amino acid sequences contain the conserved elements present in members of the small GTPase superfamily. Second, construction of phylogenetic trees places FdRac1 within the Rho family, which is associated with regulation of actin, and assigns FdRab8 to the Rab family, which is associated with vesicle transport. In the Rho family, FdRac1 is excluded from the strongly supported and distinct Rop, Rho and Cdc42 subgroups, and is instead most closely associated with members of the Rac subgroup. In the Rab family, the position of FdRab8 within the Rab VIII subgroup (defined by Pereira-Leal and Seabra 2001) is strongly supported. Members of the Rab VIII subgroup that

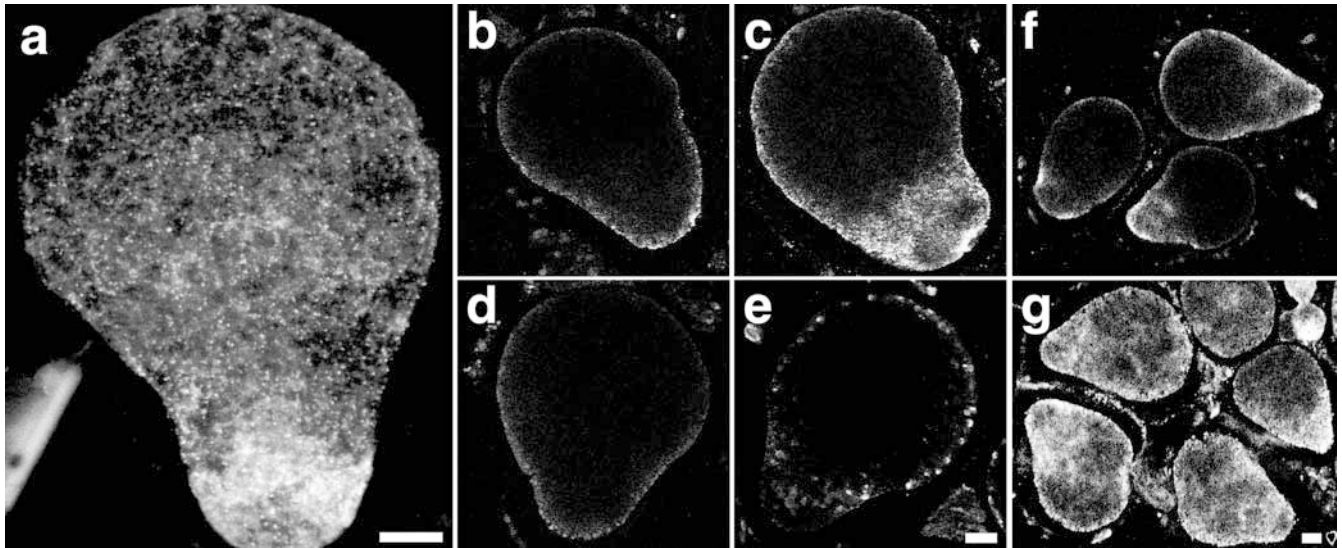


Fig. 6a–g Confocal imaging of anti-FdRac1 signal demonstrates asymmetric distribution of FdRac1 in the tip-growing *F. distichus* rhizoid at 17 h AF. **a, b, f** Stained with anti-FdRac1; **c, g** stained with anti-RabPeptide; **d** stained with a negative control “bacterial lysate” antiserum. **e** Stained with antiserum against fucoxanthin chlorophyll-binding protein, a chloroplast-localized epitope. All of the images except **a** are single confocal sections bisecting the rhizoid tip; **a** is a projection of a series of confocal sections collected to image the half of the zygote nearest the microscope objective. Bars = 10 μ m

have been functionally characterized participate in Golgi to plasma membrane transport (e.g., mammalian Rab8; Huber et al. 1993). Although the internal, punctate anti-RabPeptide immunolocalization signal from *Fucus* zygotes (Fig. 6c) is consistent with a hypothesized role for FdRab8 in vesicle transport, the lack of complete specificity of this antibody means that these data should be viewed with caution. Third, biochemical assays that demonstrate GTP-binding activity for FdRac1 and FdRab8 are also consistent with a regulatory function for both proteins.

We have additional evidence that supports a role for FdRac1 in the regulation of *Fucus* cell polarity. Two sets of experiments in budding yeast indicate that FdRac1 has conserved characteristics of the Rho family GTPases, in some respects similar to those of the budding yeast Cdc42p protein, a central determinant of yeast cell polarity (Johnson 1999). First, an *FdRac1-G12V* mutant construct, predicted to encode a constitutively active form of FdRac1, can partially compensate for the growth defect caused by the *cdc42-1^{ts}* mutation. However, *FdRac1-G12V* is unable to rescue either the *ts* mutant at the fully restrictive temperature or a deletion of the *CDC42* gene. Furthermore, the growth of wild-type and mutant yeast strains is unaffected by induced expression of either the wild-type *FdRac1* or the dominant negative *FdRac1-T17N*. Thus, in this stringent functional assay, FdRac1 has detectable, although only minor, functional similarities to yeast Cdc42p. A second set of experiments, using the yeast two-hybrid system, demonstrates that *FdRac1* has the capacity to interact,

across species boundaries, with the yeast *RDII* and *STE20* genes, which encode components of the Cdc42p signaling pathway. In this assay, the only budding yeast Rho family GTPase with which *STE20* interacts is *CDC42* (Simon et al. 1995). Thus, the interaction of *STE20* and *FdRac1* suggests that certain structural elements, which differentiate among Rho family GTPases, have been conserved in both FdRac1 and Cdc42p; this would imply that these elements, and possibly the mechanisms that utilize them, predate the ancient divergence of yeast and brown algae, and have been conserved over evolution. Taken together, the experiments in yeast indicate that, in vivo, FdRac1 possesses conserved characteristics of Rho family GTPases, which could allow it to function in a conserved signaling pathway that regulates cell polarity in *F. distichus*.

Finally, our localization data provide a first test of the hypothesis that FdRac1 is distributed asymmetrically at the *Fucus* cell cortex to either establish and/or maintain cell polarity, as are a subset of other Rho family GTPases (Etienne-Manneville and Hall 2002). In both tip-growing zygotic and embryonic cells, we found that FdRac1 was preferentially associated with, or near, the plasma membrane at the rhizoid apex. This subcellular localization, along with the biochemical experiments and yeast expression studies, implies that FdRac1 is likely a regulator of algal cell polarity, possibly acting in the maintenance of tip growth. Tip growth requires localized coordination of processes that enable polarized cell and cell wall expansion. These processes include maintenance of a cortical Ca^{2+} gradient at the tip, targeted fusion of vesicles at the growth site, spatial control of the deposition and synthesis of cell wall components, and regulation of the dynamic actin cytoskeleton (Fowler and Quatrano 1997; Hepler et al. 2001). Pollen tubes are a well-characterized tip-growing cell type, and Rho GTPases of the Rop subfamily have a key role in their growth (Hepler et al. 2001; Yang 2002). Rop's role in controlling F-actin dynamics in the pollen tube is well established (Fu et al. 2001), and Rop has also been

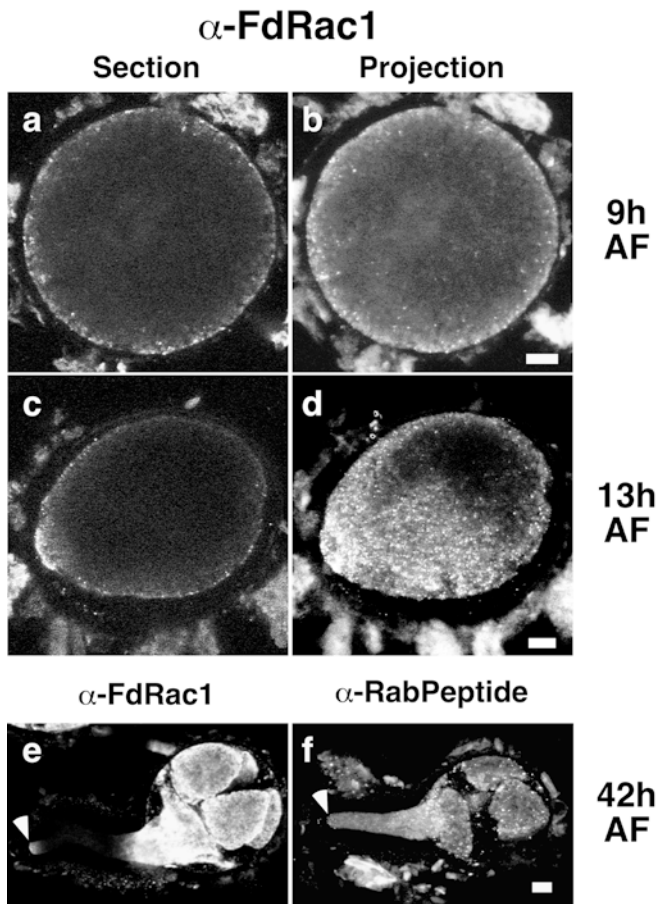


Fig. 7a-f Asymmetric localization of the anti-FdRac1 (α -FdRac1) signal is detectable throughout the period of active tip growth. No strong asymmetric signal is detectable at 9 h AF, prior to the emergence of the *F. distichus* rhizoid tip (a, b), but asymmetry is clearly detectable at 13 h AF, during the initial stages of rhizoid growth (c, d). Asymmetric localization of FdRac1 at or near the rhizoid tip (arrowhead) is also detected at later stages of embryonic development, including the eight-cell stage (e); at the same stage, the anti-RabPeptide (α -RabPeptide) signal does not show tip localization (f). a, c Single confocal sections bisecting the zygote; b, d projections from a series of confocal sections consisting of the six closest to the plane bisecting the zygote; e, f projections of a series of confocal sections collected to image the half of the zygote nearest the microscope objective. Bars = 10 μ m

linked to the regulation of the Ca^{2+} gradient (Yang 2002). The FdRac1 localization pattern mirrors that of F-actin in the tip-growing rhizoid (Alessa and Kropf 1999; Pu et al. 2000; Hable et al. 2003). Thus, like Rop in pollen tubes, FdRac1 appears to be at an appropriate location to regulate the dynamic polymerization of F-actin that is necessary for continued tip growth in fucoid cells (Hable et al. 2003), although other roles for FdRac1 are also possible (e.g., assisting in the maintenance of the cortical Ca^{2+} gradient).

However, we found no clear indication that FdRac1 localizes asymmetrically prior to the initial growth of the rhizoid tip. Although we occasionally observed spherical zygotes that had a weakly asymmetric anti-FdRac signal (i.e., small patches of localized, cortical signal), attempts

to correlate the orientation of these asymmetries with that of the presumptive rhizoid pole were inconclusive (unpublished observations). Thus, the functional relevance of these early anti-FdRac patches remains unclear, and we have no data to support a role for FdRac1 in regulating the accumulation of F-actin in spherical zygotes at the sperm entry point (Hable and Kropf 2000) or the presumptive rhizoid pole (Alessa and Kropf 1999). Nonetheless, we also cannot rule it out, given that our immunolocalization procedure could preclude robust detection of a slight polarization in FdRac1 distribution in spherical zygotes, or that FdRac1 could be locally activated to trigger F-actin accumulation, without requiring localized accumulation of FdRac1 itself. Thus, whereas a later role for FdRac1 in cell polarity (e.g., in maintaining tip growth) is supported, our data are equivocal regarding a role for FdRac1 in establishing cell polarity.

From an evolutionary perspective, our data reinforce the notion that the role of Rho family GTPases in cell polarity is an ancient one, conserved throughout even the basal branches of the eukaryotic phylogeny, and thus, throughout the eukaryotes. A role in cell polarity for the Rho family in fungi and metazoans is well established (Johnson 1999), and more recently has been shown in both the amoebozoan *Dictyostelium discoideum* (Chung et al. 2000) and in higher plants (Yang 2002). Our data from *Fucus* zygotes demonstrating a polarized localization of FdRac1, together with the evidence for some functional overlap with yeast Cdc42p, point to a role for FdRac1 in *Fucus* cell polarity. Because fucoid algae belong to the heterokont group of eukaryotes, which diverged early in eukaryotic evolution (Baldauf 2003), our data have revealed a new major group in which this role for the Rho family appears to have been retained. Definitive tests of FdRac1 and FdRab8 functions, and incorporation of these functions into models for development of polarity in fucoid zygotes, may have to await the development of tools that inhibit their activity in vivo.

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