Journal of Cellular Biochemistry 70:29–37 (1998)

Cloning and Characterization of a *Dictyostelium* Gene Encoding a Small GTPase of the Rab11 Family

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Abstract Eukaryotic cells achieve complexity by compartmentalizing a subset of cellular functions into membrane-bound organelles. Maintaining this high level of cellular organization requires precise regulation of traffic between membranes. This task is accomplished, in part, by rab proteins. How these small GTPases regulate membrane traffic between cellular compartments is not clear. Here we report the characterization of a novel rab GTPase from the soil amoebae *Dictyostelium discoideum.* The predicted coding sequence of the new rab gene, *Dictyostelium* rab11b, encodes a protein of 25 kD containing all the structural hallmarks of a rab GTPase. Comparison of the sequence with the GenBank database and cladistic analysis demonstrated *Dictyostelium* rab11b to be a divergent member of the rab11 branch of rab proteins. Southern analysis revealed the presence of related genes in *Dictyostelium.* RNAse protection assays showed the *Dictyostelium* rab11b gene to be expressed at uniform levels throughout growth and development. Gene deletion experiments revealed that *Dictyostelium* rab11b was not essential for growth or development. Conceivably, the function of rab11b may be redundant with that of related genes in this organism. J. Cell. Biochem. 70:29–37, 1998.

Key words: small GTPase; membrane traffic; vesicles; transport

Members of the ras superfamily are monomeric GTPases ~ 25 kD in mass; these proteins regulate a wealth of cellular functions in eukaryotic cells. Members of one branch of the ras superfamily, the rab proteins, are thought to control specific steps in vesicular protein traffic in eukaryotic cells [Novick and Brennwald, 1993; Nuoffer and Balch, 1994; Pfeffer, 1992; Zerial and Stenmark, 1993]. Some of the original evidence for this idea stemmed from the behavior of rab mutants created in the yeast *Saccharomyces cerevisiae.* Yeast cells that harbor mutations in the rab genes sec4 and ypt1, exhibit a failure in secretion. More specifically, however, the defect exhibited by each of the two rab mutants resides in a discrete step in secretory vesicle transport: sec4 mutants are blocked in transport from the Golgi to the plasma membrane while ypt1 mutants fail in transport from the endoplasmic reticulum to the Golgi [Salminen and Novick, 1987; Segev et al., 1988].

Subsequent to their discovery in yeast, a host of rab proteins and genes have been identified in animal cells and plant cells. In addition to secretory routes, rab proteins also appear to function in the endocytic routes of vesicular traffic. For example, several rab proteins have been localized to different endocytic compartments in mammalian tissue culture cells, including early endosomes (rabs 4 and 5) and late endosomes (rab 7) [Chavrier et al., 1990; van der Sluijs et al., 1991]. Additional experimental evidence implicating rab proteins in endocytosis comes from several lines of evidence. These include: 1) deficiencies in endocytic compartments observed in cultured cells engineered to express mutant rab proteins [Buck et al., 1992; Gorvel et al., 1991; Lombardi et al., 1993; van der Sluijs et al., 1992] and 2) perturbation of endocytic routes in mutant yeast cells lacking specific rab proteins [Singer-Kruger et al., 1994]. Taken together, current evidence points to a function for rab proteins in discrete and specific steps of vesicular protein traffic in endocytosis and secretion. Recent evidence suggests that rab proteins may serve as a "timer" in the

Contract grant sponsor: NIH; Contract grant number: GM48625.

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Received 12 December 1997; Accepted 20 January 1998

control of membrane fusion in vesicular protein traffic [Rybin et al., 1996]. To date, however, the precise function of rab proteins in the regulation of vesicular traffic has not been defined.

Insight into this question may be found in the model system *Dictyostelium discoideum*. This eukaryotic microorganism traffics membrane similarly to more complex eukaryotes. For example, *Dictyostelium* cells rapidly internalize plasma membrane and also display extensive endocytosis of fluid phase markers [Thilo and Vogel, 1980]. Moreover, gene knockouts in *Dictyostelium* can yield informative phenotypes: elimination of the clathrin heavy chain gene has demonstrated important roles for clathrin during endocytosis, lysosomal enzyme transport, cytokinesis, and development [Niswonger and O'Halloran, 1997a,b; O'Halloran and Anderson, 1992; Ruscetti et al., 1994]. Similarly, studies of rab proteins in *Dictyostelium* could also yield new information. While several rab genes have been identified in *Dictyostelium* [Bush and Cardelli, 1994; Bush et al., 1993]; a mutant cell engineered to delete a rab gene has not yet been described. In this report we describe the characterization of a novel rab, *Dictyostelium* rab11b and the phenotype of *Dictyostelium* mutants engineered to eliminate rab11b expression.

MATERIALS AND METHODS

Strains

Dictyostelium discoideum strain DH-1 lacking the *pyr*5-6 (UMP synthase) gene [Shi and Thornburg, 1993] was maintained at titers between 1×10^5 cells/ml and 4×10^6 cells/ml in HL5 medium supplemented with uracil at 40 µg/ml. Strains deleted for the rab11b gene, which contained the *pyr*5-6 marker, were maintained in HL-5 media.

Disruption of *Dictyostelium* rab11b by homologous recombination. A linear fragment of DNA was constructed that contained a 1.1 kb HindIII-EcoRI fragment of 5′ sequence and a 1 kb ClaI-EcoRI fragment of 3′ sequence of the *Dictyostelium* rab11b gene, separated by the 3.7 kb pyr5,6 gene. The linear fragment was electroporated into 8×10^6 *Dictyostelium* DH1 cells, which were then plated into five 96-well plates in FM minimal medium [Franke and Kessin, 1977]. The independent colonies of transformed lines were then expanded and the presence of the disrupted gene was assessed by Southern blot analysis.

Southern Analysis, RNAse Protection

For Southern analysis, 20 µg per lane of genomic DNA was digested with several different restriction enzymes, electrophoresed into a 1% agarose gel, blotted onto Hybond-N (Amersham, Arlington Heights, IL) and probed with a ^{32}P-labeled fragment of the gene. The bands were visualized on a Fujix Bas 1000 phosphorimager or by autoradiography using Kodak (X-OMAT AR) film. For RNAse protection assays, an RNA probe specific for *Dictyostelium* rab11b sequence was transcribed in vitro, and used to protect RNA isolated from growing or developing *Dictyostelium* cells from digestion with RNAse A as in [Gilman, 1993].

Genomic Cloning of rab11b

A 164 fragment of the *Dictyostelium* rab11b gene was identified using the primers: GGT/AG/AAT/CA/TG/CA/T/CGGT/ATTT/A/CGGT/AAAA corresponding to the amino acid sequence G D/N S G V G K, and the primer T/CT CTTGA/TTCA/G/TGCA/G/T GT A/G TC CCA complementary to the amino acid sequence WDTAGQE in a PCR amplification (30 cycles, 94°C, 1 min; 42°C 1 min; 72°C, 1 min). This PCR amplification product contained the sequence unique for the *Dictyostelium* rab11b. The entire gene was then cloned using an application of PCR that sidesteps the requirement of knowing sequence information at both ends of a desired fragment of DNA [Koonce, 1997]. In brief, we used Southern blot analysis to map convenient restriction sites that encompassed the rab11b gene. We then isolated 1.1 kb HindIII-EcoRI fragments of total genomic DNA and 0.8 kb HindIII-ClaI fragments from total genomic DNA and ligated these mixtures separately into pGEM plasmids. Each of the resulting ligation products were then used as a template for PCR. The 5′ end of the gene was amplified from the ligation mixture containing the HindIII-EcoRI fragment using the rab11b-specific primer TGTTGATAATTTTCCGACAC and the plasmid-specific primer TAAATCCACTGTGATATCTTATG; the 3′ end of the *Dictyostelium* rab 11b gene was amplified from the ligation mixture containing the HindIII-ClaI fragment with the rab11b specific primer AGTGTCGGAAAATTATCAAC and the plasmid-specific primer TAATACGACT-

Fig. 1. Sequence of the *Dictyostelium* rab11b gene. The open reading frame of the *Dictyostelium* rab11b gene is divided into three exons (bold capital letters) by two short introns (bold lower case letters). 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 nucleic acid sequence is numbered on the right. This sequence is available from GenBank under accession number AF037357.

CACTATAGGGCGA. PCR conditions were followed as described in [Koonce, 1997].

Assays

Development was examined as in [Niswonger, 1997a]. In brief, wild-type cells were harvested and washed twice with the starvation buffer PDF (11 mM K_2HPO_4, 13.2 mM KH_2PO_4,20 mM KCI, 1 mM $CaC1_2$, and 2.5 mM $MgSO_4$, pH 6.4). Cells were either plated on 1% Noble agar (Difco Laboratories, Inc., Detroit, MI) plates made with PDF, or on a 0.45 µm HA Millipore filter over two absorbent pads (Millipore Corporation, Bedford, MA).

Endocytosis of the fluid phase marker was measured as in [Ruscetti et al., 1994]. In brief, cells were incubated at 20°C with 2 mg/ml FITC-dextran (Sigma, St. Louis, MO). Endocytosis was stopped by chilling the cells at 0°C. Extracellular FITC-dextran was removed by washing extensively with 0°C HL-5, and the cells were lysed. Fluorescence was measured on a Beckman fluorimeter using excitation and emission wavelengths of 470 and 520, respectively.

RESULTS

Cloning of a Novel rab Gene, *Dictyostelium* rab11b

We identified members of the rab family from *Dictyostelium* with an approach based on the polymerase chain reaction (PCR). The PCR reaction used degenerate oligonucleotide primers corresponding to the amino acid sequences G D/N S G V G K and W D T A G Q E. These sequences correspond to highly conserved regions found in members of the ras superfamily and are separated by a more divergent region of approximately 40 amino acids [Valencia et al., 1991]. PCR reactions with these primers specifically amplified a single band of approximately 160 bp from genomic *Dictyostelium* DNA. The size of the 160 bp band corresponded well to the size for the DNA sequence predicted to be amplified by the two primers. After subcloning and sequencing the 160 bp PCR product, we determined that the band contained a heterogeneous DNA population that encoded multiple members of the ras superfamily of GTPases (data not shown).

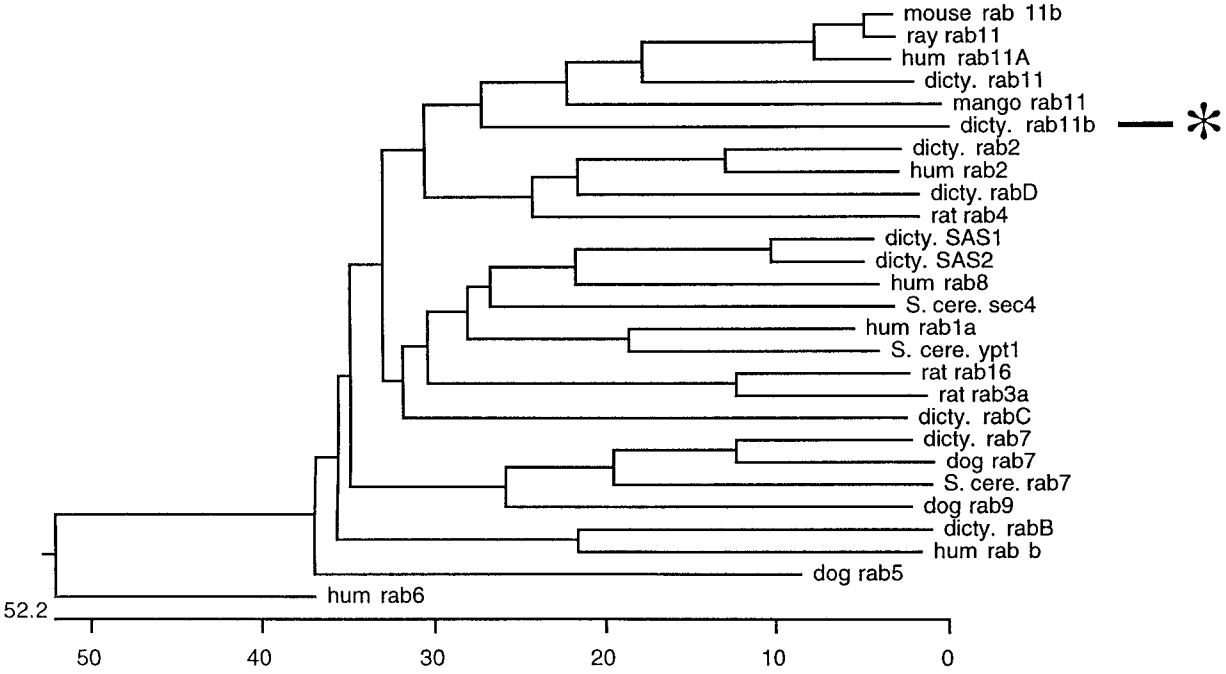

Fig. 2. Phylogenetic analysis of the rab family of small GTPases. This tree demonstrates the phylogenetic relationships among different members of the rab family. The *Dictyostelium* rab11b protein (asterisk) is most related to the rab11 branch of this family. Protein sequences were aligned with the Megalign program using the Clustal algorithm and a PAM250 table. The scale at the bottom indicates the number of substitutions between sequences.

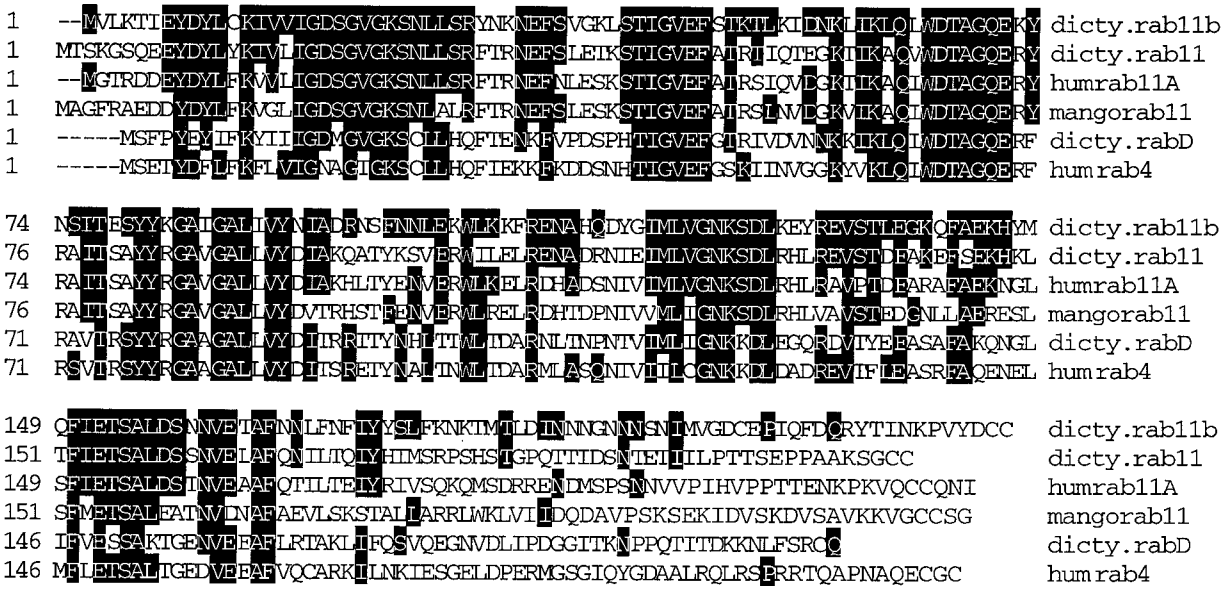

Fig. 3. Comparison of the *Dictyostelium* rab11b protein with other rab proteins. Residues that are identical between the *Dictyostelium* rab11b protein and other rab proteins are shown as white letters on a black background. Gaps inserted in the sequence for alignment are shown as dashes. dicty, *Dictyostelium;* hum, human. Sequence accession numbers: dicty. rab11b, GenBank AF037357; dicty. rab11, GenBank U02925; hum rab11a, GenBank M75153; mango rab11, EMBL accession Z71276; dicty. rabD, GenBank U02927; hum rab4, GenBank M28211.

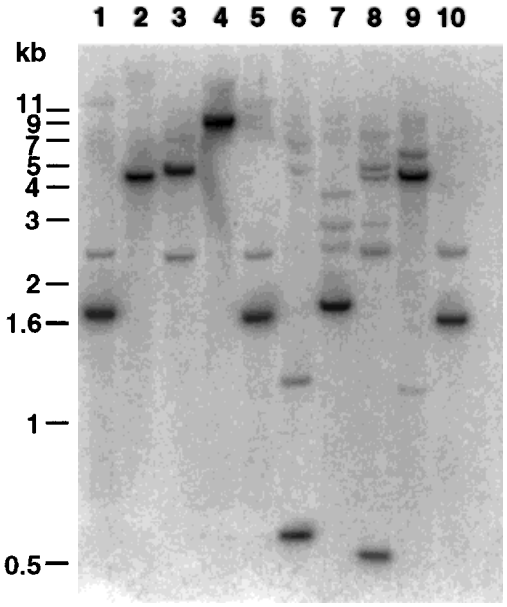

Fig. 4. Southern analysis of genomic DNA demonstrates genes related to *Dictyostelium* rab11b. Wild type (strain Ax2) genomic DNA was digested with: EcoRI (**lane 1**): PstI (**lane 2**); Bcl1 (**lane 3**); HindIII (**lane 4**); PstI/EcoRI (**lane 5**); Bcl1/EcoRI (**lane 6**); HindIII/EcoRI (**lane 7**); PstI/Bcl1 (**lane 8**); PstI/HindIII (**lane 9**); Bcl1/HindIII (**lane 10**). The gel was transferred to nitrocellulose and probed with a ^{32}P-labeled DNA fragment corresponding to the rab11b gene.

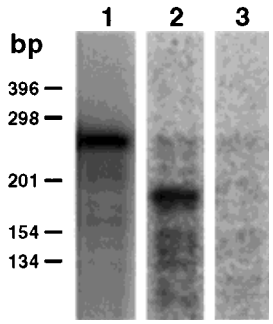

Fig. 5. Expression of the *Dictyostelium* rab11b gene. An RNAse protection assay was used to assess the presence of an RNA specific for *Dictyostelium* rab11b. Shown is an autoradiograph of an agarose gel containing: **Lane 1:** An in vitro transcribed 275 bp probe hybridized with wild-type *Dictyostelium* RNA, before ribonuclease digestion. The ^{32}P-labeled probe consisted of 174 bp of rab11b sequence (*Dictyostelium* rab11b sequence 209–383) and approximately 100 bp of irrelevant sequence derived from plasmid. **Lane 2:** The in vitro transcribed probe hybridized with wild-type *Dictyostelium* RNA, after ribonuclease digestion. 174 bp of the probe was protected from RNAse A digestion, whereas 100 bp derived from the irrelevant plasmid-derived sequence was digested by RNAse A. **Lane 3:** A control reaction of the in vitro transcribed probe hybidized with a tRNA, after digestion with ribonuclease A.

Preliminary sequence analysis of one clone derived from the PCR product suggested that it encoded a new member of the rab family (data not shown). In order to isolate the remaining portion of this new rab gene, we used a PCR-based approach to amplify the entire gene and flanking regions. After using Southern analysis to map restriction sites surrounding the gene, we size-selected appropriate restriction fragments from genomic *Dictyostelium* DNA. Using these mixtures of DNA fragments as templates, and oligos specific for the new gene, we amplified a specific 1.1 kb HindIII-EcoRI fragment containing the 5′ region of the new gene and a 0.8 kb HindIII-ClaI fragment containing the 3′ region of the gene. From these two DNA fragments, approximately 1,200 bp coding and flanking sequence was sequenced in entirety (Fig. 1). Analysis of the nucleotide sequence allowed us to define probable exon and intron boundaries of the new rab gene. Based on a richness in AT-nucleotide content (greater than 90%) and the presence of canonical splice acceptor and donor sites, we identified two probable introns in the gene as well as the probable initiation methionine.

Predicted Amino Acid Sequence of rab11b

The predicted exons for the new rab gene yielded an open reading frame (Fig. 1) that encoded an 25 kD protein. Examination of the amino acid sequence revealed conserved motifs common to all rab proteins [Valencia, 1991]. These four conserved domains, which coordinate the binding and hydrolysis of GTP, included: 1) $GX_4GK(S/T)$, 2) WDTAGQE, 3) N/T (K/Q)XD, and 4) SAK/L. A pair of cysteines at the carboxyl-terminus, a motif required for prenylation of rab proteins, was also identified. Cladistic analysis of the protein encoded by the new gene confirmed that it was a member of the rab family, and, in particular, could be grouped with members of the rab11/ypt3 branch (Fig. 2). Extensive homology with rab11 sequences was also found when the amino acid sequence of *Dictyostelium* rab11b was compared directly with other rab11 proteins. Excluding the c-terminal domain, a hypervariable domain in all rab proteins [Chavrier et al., 1991], the new rab sequence had the highest overall homology with members from the rab11 family of genes. The new gene shared 58% identity with human rab11 and 60% identity with *Dictyostelium* rab11, but exhibited lower homology with proteins on the adjacent branch of the rab proteins (45% identity with human rab4 and 44% iden-

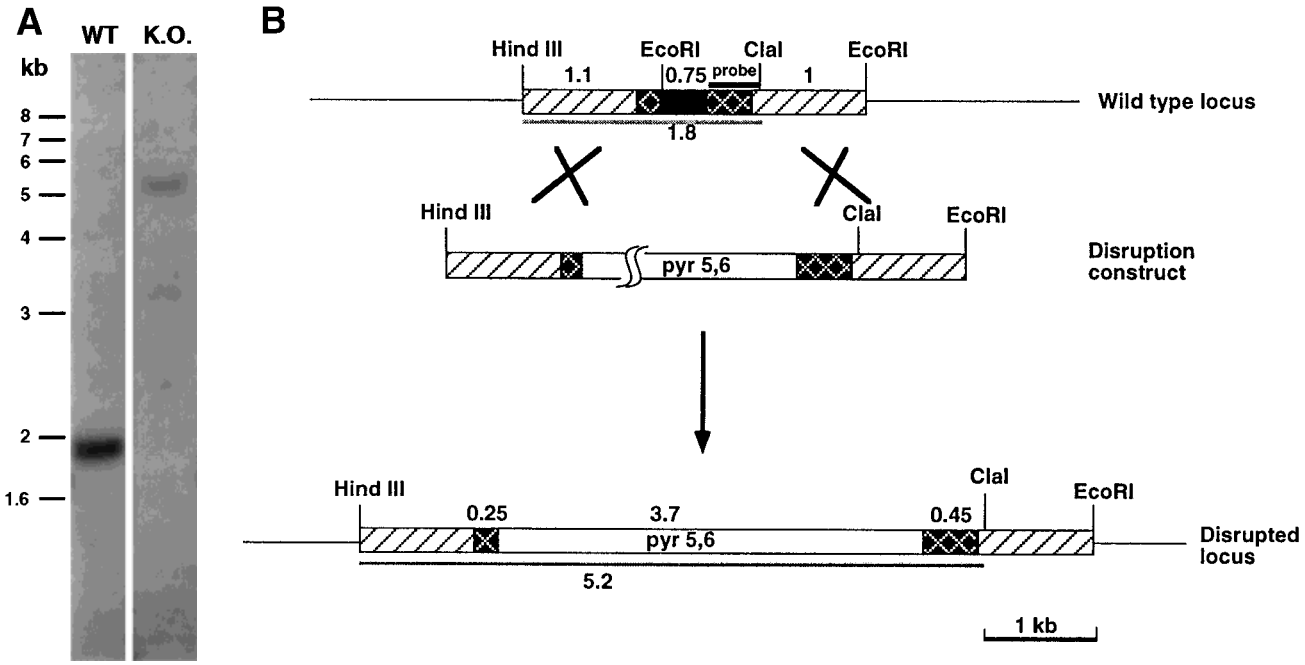

Fig. 6. Southern blot analysis of *Dictyostelium* rab11b deletion strain. **A:** Southern blot of 10 µg of DNA was digested with HindIII/ClaI, transferred to nitrocellulose and probed with a rab11b probe. A 1.8 kb band was detected in DNA isolated from wild-type cells (WT), whereas a 5.2 kb band was detected in the DNA isolated from the mutant strain (K.O.). This 5.2 kb band is the size predicted from the replacement of the middle of the rab11b gene by the pyr 5,6 cassette. **B:** Diagram showing restriction maps of rab11b gene (Wild-type locus); the DNA construct used for homologous recombination into the rab11b locus (Disruption construct); and the disrupted rab11b locus in the rab11b-minus mutants (Disrupted locus). Single-hatched boxes, noncoding flanking regions of rab11b gene; Double-hatched boxes, coding regions of rab11b gene; Black box, deleted region of rab11b gene; Open box, pyr 5,6 gene (selectable marker). Shown below the rab11b locus restriction maps are the ClaI-HindIII fragments detected by the rab11b probe.

tity with the *Dictyostelium* rab4-like gene, rabD; Fig. 3). Because the rab11/ypt3 branch already contains a rab11-like protein from *Dictyostelium*, called *Dictyostelium* rab11, we have named the new gene *Dictyostelium* rab11b.

Southern and RNase Protection Analysis of rab11b

Southern blot analysis was performed to examine the genomic structure of the new rab11 gene. In addition to the rab11b gene, analysis of blots of genomic DNA probed with the rab11b sequence revealed the presence of additional genes related to rab11b (Fig. 4). For example, Southern analysis of genomic DNA digested with EcoRI and HindIII revealed one major band that corresponds to the restriction map of the rab11b gene and three fainter bands that appeared to be derived from cross-hybridizing genes (Fig. 4, lane 7). Given its sequence similarity, at least one of the other cross-hybridizing bands probably corresponds to the second *Dictyostelium* rab11 gene.

To examine the expression of *Dictyostelium* rab11b, we used the rab11 probe on northern blots containing RNA from cells at different times of development. However, we did not detect a signal by northern analysis, suggesting that rab11b levels were low. Therefore, we used an RNAse protection assay as a more sensitive method to detect expression of rab11b mRNA. Analysis of RNA isolated from growing *Dictyostelium* cells revealed an RNA that was protected from RNAse A digestion by a rab11b probe (Fig. 5, lane 2). This protection was highly specific: 100 bp of the probe that was not homologous to rab11b was completely vulnerable to RNAse A digestion while the adjacent 174 bp of the probe corresponding to the *Dictyostelium* rab11b sequence remained intact (compare the size of the undigested probe in lane 2 with the RNAse-treated probe in lane 1). Additionally, the labeled probe was digested completely by RNAse A in the presence of an irrelevent RNA (lane 3). This RNAse protection assay was also used to examine the expression of rab11b in

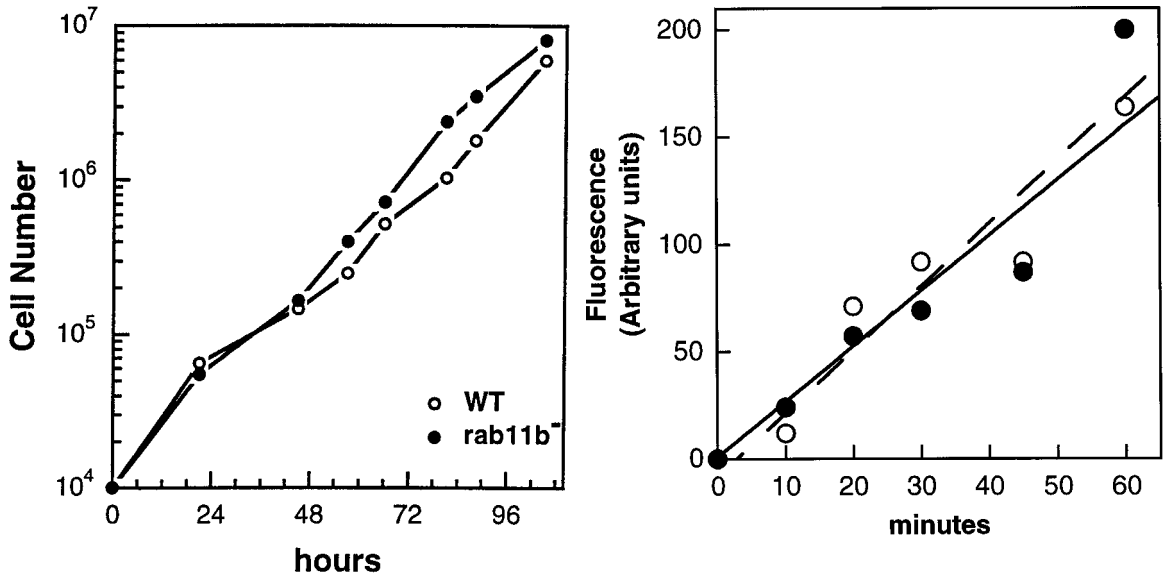

Fig. 7. **A:** Growth rates of wild-type and rab11b deletion strain. Cells were seeded at 1×10^4 cells per ml in HL-5 media. Cells were counted daily; the plot shows the number of cells vs. time. **B:** Endocytosis of fluid-phase marker by wild-type and rab11b deletion strain. Cells of both strains were incubated with the fluid-phase marker, FITC-dextran. At various time points, extracellular FITC-dextran was removed by washing and fluorescence of the internalized FITC-dextran was quantified. Closed circles, dashed line = rab11b-minus cells; open circles, solid line = wild-type cells.

developing *Dictyostelium* cells. The levels of rab11b expression were constant throughout 0–12 hours of development, demonstrating that expression of this gene was not developmentally regulated (data not shown).

Analysis of rab11 Null Mutants

To explore the physiological role of rab11b in *Dictyostelium* cells, we created rab11b null cells by specific disruption of the rab11b gene. For these experiments, we transformed cells with a disruption vector designed to replace approximately 150 bp of the rab11b gene with the 3.7 kb pyr 5,6 gene. DH-1 cells, a *Dictyostelium* cell line auxotrophic for pyr5,6 synthesis, were transformed with the vector and then cloned for single transformants. Southern blots confirmed a specific disruption of the rab11b locus in several cell lines (Fig. 6A). For example, a HindIII/ClaI digest probed with rab11b detected a 1.8 kb band in DNA isolated from wild-type cells and a 5.2 kb band in targeted cells. The larger 5.2 kb band is the size predicted from the targeting and replacement of the rab11b gene with the disruption vector (Fig. 6B).

We next analyzed the phenotype of the rab11b null mutants. When compared with the parental DH-1 cell line or other wild-type control cell lines, rab11b mutants grew with apparently normal rates (Fig. 7A). Rab11b mutant cells also looked indistinguishable from wild-type cells when examined by phase-contrast microscopy: both the size and number of membranes and membrane-bound organelles appeared normal (data not shown). The proposed role of rabs in membrane traffic prompted us to assess endocytic properties of the rab11b null mutants. We examined endocytosis by measuring the internalization of the fluid-phase marker FITC-dextran (Fig. 7B). We found that mutant cells internalized a volume of fluid-phase marker that was similar to the amount internalized by wild-type cells.

In addition to examining properties characteristic of growth, we examined properties characteristic of *Dictyostelium* development. When nutrients are plentiful, *Dictyostelium* grow as individual amoebae. Once food sources are depleted, a simple developmental program is initiated. Using secreted cAMP as a signal to find each other, around a million cells aggregate to form a mound of cells. Cells within the mound differentiate into either stalk cells or spore cells; subsequently the mound of cells transforms into multicellular fruiting body composed of an elongated stalk supporting a sorus of spore cells.

We examined the ability of the rab11b null mutants to accomplish these developmental tasks by removing their food source. Under all

developmental conditions, rab11b mutants streamed together to form mounds that were indistinguishable in size and appearance to those formed by wild-type cells. Subsequently the mounds of cells progressed to form fruiting bodies identical to those formed by wild-type cells (data not shown). Therefore, the developmental capabilities of the rab11b mutants were not impaired by the absence of rab11b protein.

DISCUSSION

To date, 11 rabs have been identified in *Dictyostelium* [Bush and Cardelli, 1994; Bush, et al., 1993]. Here we report the characterization of a twelfth member of this family, *Dictyostelium* rab11b. The predicted protein sequence of this new gene contained every hallmark of a small GTPase, and included all the domains necessary for GTP hydrolysis as well as the c-terminal motif required for lipid modification of the tail. Comparison with the GenBank database confirmed that the protein product of the new gene was most similar to rab11 genes cloned from widely divergent organisms, including mammalian, plant and fungal organisms. Interestingly, cladistic analysis showed rab11b to be an outlyer of the rab11 branch: the sequence of *Dictyostelium* rab11b was the most divergent of all known rab11 proteins. The extensive divergence of the *Dictyostelium* rab11b protein is probably not attributable to intraspecies' differences since another rab11-like gene from *Dictyostelium* is much more similar to the other members of the rab 11 family. Two divergent forms of rab11-like genes are also present in mammalian species: a second rab11b sequence identified recently in mouse differs considerably from previously identified mammalian rab11 proteins [Lai et al., 1994]. Conceivably, the presence of multiple rab11 genes may give eukaryotic cells the opportunity to regulate rab11 function in different ways.

What is the cellular function of rab11b? While the molecular mechanism for any rab protein is not understood in detail, rab proteins are thought to regulate the targeting and/or fusion of protein transport vesicles with their particular acceptor membranes. As a member of the rab family, rab11b likely also functions in the regulation of protein vesicle transport in *Dictyostelium* cells. The homology with other rab11 family members suggests more specific cellular functions for rab11b. Rab11 members have been localized to a variety of compartments in different cells, including constitutive and regulated secretory vesicles associated with the trans-Golgi network in neuroendocrine cells, synaptic vesicles in the marine ray, and the pericentriolar recycling compartment in cultured mammalian cells [Ullrich et al., 1996; Urbe et al., 1993; Volknandt et al., 1993]. Expression of mutant "dominant-negative" rab11 homologues perturbs recycling from a late recycling endosome in cultured cells, while rab11 isoforms ypt31p and ypt32p are required for Golgi function in yeast [Singer-Kruger, et al., 1994; Ullrich et al., 1996]. Taken together, these studies suggest that rab11 isoforms could generally regulate different trafficking compartments that are in equilibrium with the trans-Golgi network. *Dictyostelium* rab11b may also control traffic to similar compartments. The low level of expression of rab11b, unusual for a rab protein, suggests that rab11b is not involved in a general housekeeping aspect of membrane traffic such as constitutive vesicle transport through the Golgi stacks, where large numbers of vesicles would need sufficient amounts of protein to function. Rather, rab11b may function in a more specialized step of membrane traffic where few vesicles, and thus lower levels of rab protein, are required. For example, one specialized function employed only at a particular moment in the life of the cell is the fusion of the prespore vesicle with the plasma membrane during sporulation. This secretory vesicle is derived from the Golgi and stores proteins of the spore coat [West and Erdos, 1990]. Perhaps rab11b functions during this or another membrane fusion step that is needed only at a particular time and involves only a limited number of vesicles.

In an attempt to uncover the cellular role of rab11b directly, we deleted the gene encoding this rab. The resulting phenotype of rab11b null cells was not dramatically different from wild-type cells: the mutant cells functioned in growth, endocytosis and development similarly to wild-type cells. Morphogenesis also appeared normal; under appropriate conditions, rab11b null mutants constructed fruiting bodies that were indistinguishable from wild-type fruiting bodies. This indicates that the majority of complex processes associated with growth and starvation-induced development, such as endocytosis of nutrient media for growth, secretion of extracellular cAMP for chemotaxis, cell differentiation, and morphogenesis into a fruiting body

were intact in the mutant cells. Conceivably, in the absence of rab11b, another protein may be able to substitute for its function. As *Dictyostelium* rab11 is the most likely candidate to substitute for rab11b function, it will be important to study the phenotype of a double mutant cell line harboring a "knock-out" of both genes.

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