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## A Rab4-like GTPase in *Dictyostelium discoideum* colocalizes with V-H<sup>+</sup>-ATPases in reticular membranes of the contractile vacuole complex and in lysosomes

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### SUMMARY

In the course of screening a cDNA library for *ras*-related *Dictyostelium discoideum* genes, we cloned a 0.7 kb cDNA (*rabD*) encoding a putative protein that was 70% identical at the amino acid level to human Rab4. Rab4 is a small  $M_r$  GTPase, which belongs to the Ras superfamily and functions to regulate endocytosis in mammalian cells. Southern blot analysis indicated that the *rabD* cDNA was encoded by a single copy gene while Northern blot analysis revealed that the *rabD* gene was expressed at relatively constant levels during growth and differentiation. Affinity-purified antibodies were prepared against a RabD fusion protein expressed in bacteria; the antibodies recognized a single 23 kDa polypeptide on western blots of cell extracts. Density gradient fractionation revealed that the RabD antigen co-distributed primarily with buoyant membranes rich in vacuolar proton pumps (V-H<sup>+</sup>-ATPases) and, to a lesser extent, with lysosomes. This result was confirmed by

examining cell lines expressing an epitope-tagged version of RabD. Magnetically purified early endocytic vesicles and post-lysosomal vacuoles reacted more weakly with anti-RabD antibodies than did lysosomes. Other organelles were negative for RabD. Double-label indirect immunofluorescence microscopy revealed that RabD and the 100 kDa V-H<sup>+</sup>-ATPase subunit colocalized in a fine reticular network throughout the cytoplasm. This network was reminiscent of spongiforms, the tubular elements of the contractile vacuole system. Immunoelectron microscopy confirmed the presence of RabD in lysosome fractions and in the membranes rich in V-H<sup>+</sup>-ATPase. We conclude that a Rab4-like GTPase in *D. discoideum* is principally associated with the spongiforms of contractile vacuole complex.

Key words: Rab GTPases, V-H<sup>+</sup>-ATPase, lysosomes, contractile vacuole system

### INTRODUCTION

The regulation of vesicle traffic is an important process necessary to ensure the biogenesis and functional maintenance of endomembrane organelles. Biochemical and genetic studies have identified a large number of proteins that participate in and direct such membrane flow (Rothman and Orci, 1992; Pryer et al., 1992). Discrete stages in this process include the production of vesicles from donor compartments, the transport and uncoating of these vesicles, and the fusion of these vesicles with acceptor membranes. The Rab family of small molecular weight Ras-like GTPases (Bourne et al., 1990, 1991) is involved in these processes (Valencia et al., 1991; Pfeffer, 1992; Goud and McCaffrey, 1992) and over 30 members of the Rab family (also called the Ypt1/Sec4 family) have been identified. Rab proteins localize to specific intracellular compartments, so each of the many steps in vesicle trafficking along the secretory and endosomal pathway may be regulated

by one or more of the Rab proteins. For instance, Rab1 associates with ER and Golgi membranes, and regulates vesicle transport between them (Tisdale et al., 1992; Pryer et al., 1992), while Sec4 associates with post-Golgi secretory vesicles and is required for the last step in exocytosis (Pryer et al., 1992). Rab4 (van der Sluijs et al., 1991), Rab5 (Chavrier et al., 1990), Rab7 (Chavrier et al., 1990) and Rab9 (Lombardi et al., 1993) associate with different subcompartments along the endocytic pathway. Rab4 (van der Sluijs et al., 1992) and Rab5 (Bucci et al., 1992) regulate early stages of endocytosis while Rab7 (Wichmann et al., 1992) and Rab9 (Lombardi et al., 1993) function to regulate membrane flow to and from late endosomes.

Because it is amenable to molecular genetic approaches, the simple eukaryote, *Dictyostelium discoideum*, has proven to be a useful system in which to investigate the molecular mechanisms regulating protein and vesicular trafficking along the biosynthetic and endosomal pathways leading to lysosomes

(reviewed by Cardelli, 1993). Also, the organism when starved undergoes a relatively simple developmental cycle culminating in the formation of a fruiting body consisting of only a few cell types (Loomis, 1982). During development, changes occur in the expression of a number of genes encoding lysosomal proteins and in the structure and function of lysosomes. Therefore, the lysosomal system can be studied from a number of different perspectives.

The biosynthetic pathway has been well characterized in *D. discoideum*. Most but not all of its lysosomal hydrolases are synthesized as glycosylated, membrane-associated precursor polypeptides (Mierendorf et al., 1985; Cardelli et al., 1986; Bush and Cardelli, 1989). Although the carbohydrate side-chains on these proteins are sulfated and phosphorylated in the Golgi complex (Mierendorf et al., 1985; Freeze, 1986), these moieties are probably not required for their sorting to lysosomes (Cardelli et al., 1990a; Freeze et al., 1990). The initial proteolytic cleavage of acid hydrolase precursors occurs in a late Golgi or endosomal compartment (Cardelli et al., 1990b; Wood and Kaplan, 1988); proteolysis may be required for proper sorting of the hydrolases (Richardson et al., 1988). Acidic endosomal/lysosomal compartments are required for complete processing but a low pH is not required for efficient sorting (Cardelli et al., 1989).

The endocytic pathway in *D. discoideum* has also been recently characterized. Fluid-phase markers rapidly enter the cell via clathrin-coated vesicles (O'Halloran and Anderson, 1992; Ruscetti et al., 1994). These small vesicles enter larger vacuoles that are acidified and contain acid hydrolases (Rodriguez-Paris et al., unpublished data; Padh et al., 1993; Aubry et al., 1993; Cardelli et al., 1989). Fluid-phase components then enter larger post-lysosomal compartments of neutral pH from which they are egested (Padh et al., 1993; Aubry et al., 1993). It is not clear where the lysosomal biosynthetic and endocytic pathways merge but this junction is positioned before dense lysosomes (Cardelli et al., 1990b; Richardson et al., 1988). Finally, genetic evidence also supports the physical connection between these two pathways (Ebert et al., 1989); that is, a certain mutant is defective in endocytosis and missorts lysosomal hydrolases and the clathrin heavy chain is required for sorting and endocytosis (Ruscetti et al., 1994).

At least five *D. discoideum* genes belong to the *ras* superfamily including two *ras*-like genes (Reymond et al., 1984; Robbins et al., 1989), two *rab*-like genes (Kimmel et al., 1993; Saxe and Kimmel, 1990) and one *rap*-like gene (Robbins et al., 1990). Using an oligonucleotide probe to screen a cDNA library, we recently isolated 18 new genes belonging to the *rho* (Bush et al., 1993a), *ras* (Daniel et al., 1993), *ran* (Bush and Cardelli, 1993) and *rab* (Bush et al., 1993b) families that code for GTPases. In the Rab family, we have identified potential proteins very homologous to mammalian Rab1 (Bush et al., 1993a), Rab2 (this report), Rab4 (this report), Rab7 (Bush and Cardelli, 1994) and Rab11 (Bush and Cardelli, 1994), as well as three new members (Bush et al., 1993a; Bush and Cardelli, 1994).

To assess the function of a particular Rab protein, it must be localized to a known intracellular compartment. In this study, we present data indicating that while a Rab4-like protein (RabD) is enriched in the endocytic vacuoles of *D. discoideum*, most of the protein is associated with the tubular membranes (termed spongiomes) of the contractile vacuole complex. RabD

was closely colocalized with the V-H<sup>+</sup>-ATPase previously identified in both lysosomes and spongiome membranes (Rodriguez-Paris et al., 1993; Nolte et al., 1993; Heuser et al., 1993; Nolte and Steck, 1994).

## MATERIALS AND METHODS

### Organism

The *D. discoideum* wild-type strain Ax4 was grown axenically in TM broth in shaking water baths at 120 rpm at 21°C or on lawns of *Klebsiella aerogenes* spread on SM agar plates at 21°C (Free and Loomis, 1974). Developmental conditions were as previously described (Schatzle et al., 1991).

### Cloning cDNAs

A standard plaque lift (Sambrook et al., 1989) was used to screen a recombinant cDNA library constructed in  $\lambda$ gt11, using mRNA prepared from cells that had developed for 4 hours (Clontech). A <sup>32</sup>P end-labeled oligonucleotide (GAYACHGCHGGTCAAGAA; H= A or C or T, and Y= C or T) coding for the amino acids DTAGQE (conserved in all proteins belonging to the Ras superfamily) was used in the isolation of *rab4*-like cDNAs. The *rab2* clone was isolated using an oligonucleotide (ACYGATAAAKGGZTTYCAACCAGTH-CATGAT; H= A or C or T; Y= C or T; K= A or C; Z= A or T) coding for the amino acids T<sub>27</sub>DKRFQPVHD<sub>36</sub>, a stretch unique to Rab2 that partially overlaps the putative effector region (Zahraoui et al., 1989). Hybridizations, washing conditions and PCR sequencing of DNA were the same as previously described (Bush et al., 1993a).

### Northern and Southern blot analysis

For Southern blots, genomic DNA was digested with restriction enzymes and subjected to electrophoresis on a 0.8% agarose gel. DNA was transferred by capillary action to Gene-Screen membranes (NEN); hybridization with radiolabeled cDNAs and washing conditions were as described previously (Schatzle et al., 1992). For northern blot analysis, RNA was extracted from growing and developing cells, and separated on denaturing formaldehyde agarose gels as previously described (Schatzle et al., 1991). RNA was transferred to filters and hybridized with radiolabeled cDNA probes as described for Southern blots.

### Antibody production

The *rabD* cDNA was ligated directionally and 'in frame' from nucleotide 247 (first nucleotide for the Gly66 codon) to nucleotide 675 (end of the TAA stop codon) into the RSET bacteria fusion protein vector at the *Pst*I and *Eco*RI sites (Invitrogen). This vector construct was transformed into the *Escherichia coli* strain JM109, followed by induction of the synthesis of the RabD fusion protein. RabD was then purified from cells recovered from a 100 ml culture according to the manufacturer's instructions (Invitrogen). The purified protein was subjected to SDS-PAGE and gel slices containing the protein were used to immunize New Zealand male rabbits (Cocalico Biologicals). Specific antibodies were affinity-purified using a column containing the recombinant Rab4 protein coupled to CNBr-activated Sepharose (Pharmacia). The antibodies showed negligible cross-reactivity on western blots with other Rab proteins including Rab7, Rab11 and RabC (results not shown).

### Subcellular fractionation

For crude separation of membranes by differential centrifugation, 1.5×10<sup>9</sup> Ax4 cells were pelleted, resuspended in buffered 0.25 M sucrose, and broken with a Dounce homogenizer. Phase-contrast light microscopy indicated that greater than 95% of the cells were broken under these conditions. The homogenate was subjected to differential centrifugation to generate low- (5 minutes at 1,000 g), medium- (10

minutes at 10,000 g) and high-speed (60 minutes at 100,000 g) supernatants and pellets. The resulting pellets designated P1, P2 and P3, as well as the supernatants designated S1, S2 and S3, were subjected to SDS-PAGE and western blot analysis as described below.

For higher-resolution separation of membranes, growing cells (10<sup>9</sup> cells) were broken by homogenization and intracellular organelles were subjected to Percoll gradient or two different sucrose gradient fractionation schemes as previously described (Cardelli et al., 1989; Nolte et al., 1991). Marker enzymes,  $\alpha$ -mannosidase (lysosomes), alkaline phosphatase (contractile vacuoles) and  $\alpha$ -glucosidase (ER), were assayed as described (Ebert et al., 1989). Magnetic fractionation of cells fed colloidal iron was done as described (Rodriguez-Paris et al., 1993).

### Western blot analysis

Proteins separated by SDS-PAGE were transferred to nitrocellulose using a Hoefer Transfor Unit. Transfers typically took 3 hours at 600 mA in (25 Tris-HCl, 192 mM glycine, 20% methanol). Blots were incubated with primary antibodies (either a 1:100 dilution of affinity-purified rabbit anti-RabD antibodies or a 1:100 dilution of monoclonal mouse anti-100 kDa ATPase subunit antibodies) in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20, 0.1% gelatin (TBSTG), washed and then incubated in TBSTG with a 1:3000 dilution of anti-rabbit or anti-mouse goat antibodies coupled to alkaline phosphatase (Bio-Rad). Blots were developed using BCIP and NBT as substrates according to the manufacturer's instructions (Promega-Biotec). Relative amounts of RabD were estimated by comparison to a standard curve generated using known amounts (0.01-1.0  $\mu$ g) of the recombinant RabD antigen subjected to western blot analysis. Densitometric scans of photographic negatives of the blots indicated that the signal was linear over a 50-fold range of antigen concentration.

### Immunofluorescence microscopy

Cells were pipetted onto coverslips and fixed by one of three methods. To visualize lysosomal  $\alpha$ -mannosidase and RabD (method one), cells were fixed in a solution of 3.7% formaldehyde in phosphate buffered saline (PBS) at room temperature for 1 hour, washed in PBS, and then stained with primary, followed by secondary, antibodies to detect both proteins as described (Bush and Cardelli, 1989). To visualize optimally RabD (method 2), cells were fixed with 2% formaldehyde for 10 minutes, washed in PBS and then stained with antibodies. To visualize the 100 kDa ATPase subunit and RabD simultaneously (method three), cells were fixed in 2% formaldehyde in PBS contain one-third strength TM growth medium (Free and Loomis, 1974) and 0.1% DMSO for 5 minutes at room temperature, followed by incu-

bation for 5 minutes at -20°C in 100% methanol containing 1% formaldehyde. This latter method is almost identical to the method described by Fok et al. (1993) with the only difference being that cells were fixed without using an agar overlay. The ATPase antigen was only visualized using method three. Staining with antibodies was also done as previously described (Bush and Cardelli, 1989). Anti-RabD antibodies were added at a 1:20 dilution while anti-V-H<sup>+</sup>-ATPase antibodies were at a 1:50 dilution. Secondary antibodies were used at a 1:100 dilution. Cells were photographed using an Olympus model BH-2 fluorescence microscope and Kodak T-MAX 400 speed film.

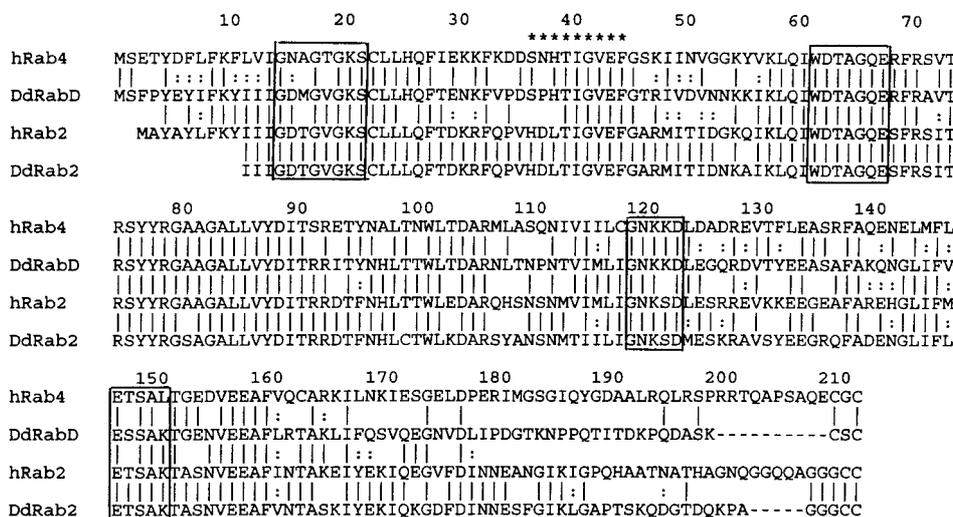
### Immunocytochemistry and electron microscopy

Preparations were washed in sodium cacodylate buffer, fixed as pellets and embedded in Lowicryl K4M as described (Nolte et al., 1993). Sections were reacted with affinity-purified antibodies against RabD and/or supernatants containing monoclonal antibodies to band 1, the 100 kDa subunit of the V-H<sup>+</sup>-ATPase (Fok et al., 1994). Secondary antibodies linked to 10 or 20 nm colloidal gold particles were used for detection. The immunodecorated sections were counterstained with uranyl acetate and viewed as described (Nolte et al., 1993).

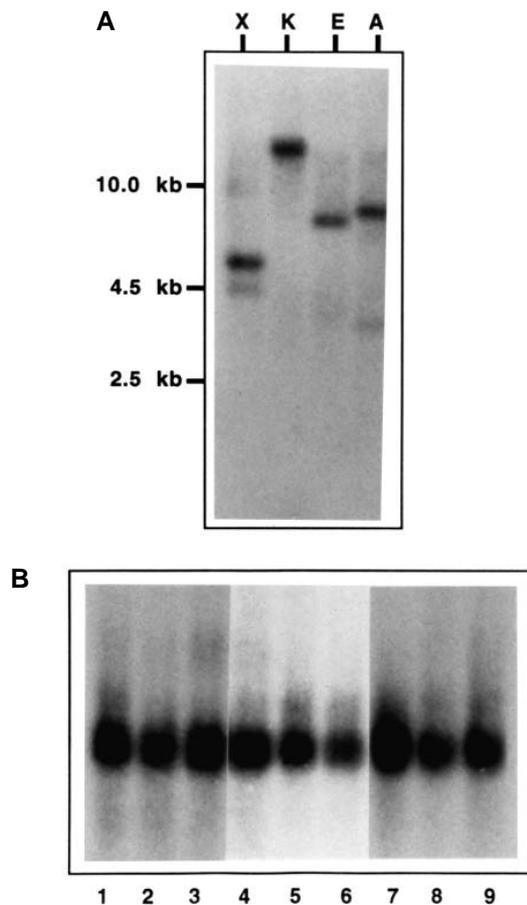
## RESULTS

### Cloning of a cDNA encoding a Rab4-like GTPase: sequence and gene expression studies

Using an oligonucleotide probe generated to a highly conserved region of Ras-like GTPases, we have cloned 18 new cDNAs encoding proteins belonging to the Rab, Rho, Ras and Ran families (Bush and Cardelli, 1993; Bush et al., 1993a,b; Daniels et al., 1993). The nucleotide sequence (695 nucleotides) of one of these clones (NBRF accession number U02927) contained one open reading frame (ORF) beginning with an ATG codon at nucleotide position 53 and ending with a TAA stop codon at nucleotide 671. The deduced amino acid sequence contained all four of the highly conserved regions (boxed in Fig. 1) found in the small GTPases. A search of the NBRF protein data base revealed that RabD had the greatest homology to human Rab4 (Zahraoui et al., 1989) and human Rab2 (Zahraoui et al., 1989): approximately 70% amino acid identity from position 10 to 170 and 57% amino acid identity over the entire protein (Fig. 1). However, the potential effector domain for RabD



**Fig. 1.** Alignment of the amino acid sequence of *Dictyostelium* RabD and Rab2 with human Rabs. DNA sequence information was entered into a Macintosh computer and analyzed using the MacVector computer software of IBI. The ORFs were subjected to conceptual translation and the putative amino acid sequence information was used to search the NBRF data base (release 31.0). The boxed areas are domains that are highly conserved and involved in GTP binding. Stars identify amino acids of the putative effector domains. Identical amino acids and similar amino acids are denoted with the symbols | and :, respectively.



**Fig. 2.** Southern blot and northern blot analysis of the *rabD* gene. For Southern blots (A), genomic DNA was cut with *AccI* (A), *EcoRI* (E), *XbaI* (X), and *KpnI* (K) and subjected to electrophoresis on 0.8% agarose gels. Following blotting to Gene-Screen (NEN), filters were hybridized with radiolabeled (nick-translated) cDNAs as previously described (Schatzle et al., 1992) and exposed to X-ray film. For northern blots (B), at various times during development (lane 5, 0 hours; lane 6, 2 hours; lane 7, 8 hours; lane 8, 14 hours; lane 9, 22 hours) or stages during growth (lanes 1 and 4,  $10^6$  cells/ml; lane 2,  $5 \times 10^6$  cells/ml; lane 3,  $2 \times 10^7$  cells/ml), RNA (10  $\mu$ g) was extracted from cells and subjected to electrophoresis on 1% denaturing agarose gels followed by transfer of RNA to Gene-Screen (Schatzle et al., 1991). Blots were hybridized with the radiolabeled *rab*-related cDNAs indicated. Total RNA was stained and quantified as previously described (Schatzle et al., 1991). In this developmental time course, cells aggregated by 10 hours and entered the culmination stage to form fruiting bodies by 19 hours.

(S37NHTIGVEF45) was nearly identical to the Rab4 effector region, differing only at amino acid position 38.

Southern blot analysis using the *rabD* cDNA as a probe under moderately stringent hybridization conditions revealed that *rabD* was apparently a single-copy gene (Fig. 2A). One or two additional radiolabeled fragments were detected, but these were faint and probably represent more distantly related *rab4*-like or *rab2*-like genes. A single species of mRNA was also detected on northern blots using the same radiolabeled cDNA as a probe (Fig. 2B). Steady-state levels of the *rabD* mRNA remained relatively constant (no more than a twofold

variation) throughout the 24 hour developmental pathway (lanes 5-9). The same was true in vegetative cells in culture at all phases of growth in axenic culture; for example, Fig. 2 shows cells in early log phase (lanes 1 and 2) and stationary phase (lane 3).

### Cloning a cDNA encoding a GTPase highly related to human Rab2

We tested for a *Dictyostelium* gene more closely related to mammalian *rab2* than to *rab4*. We used an oligonucleotide probe containing an ORF exactly matching the amino acids just upstream of and overlapping with the effector domain of Rab2 (TDKRFQPVHD) to clone a cDNA (NBRF accession number) that encoded a protein (Fig. 1) sharing greater than 85% sequence identity, between amino acid position 10 to position 170 and 78% identity from position 10 to the end of the protein, with human Rab2 (Zahraoui et al., 1989). *D. discoideum* Rab2 was only 60% identical with human Rab4 and was identical to human Rab2 in the putative effector region (HDLTIGVEF). It differed by three amino acids from the effector region of RabD and Rab4. Given the similarity between RabD and Rab4 in the putative effector region and the existence of a separate *rab2*-like cDNA, we suggest that RabD may function more like Rab4.

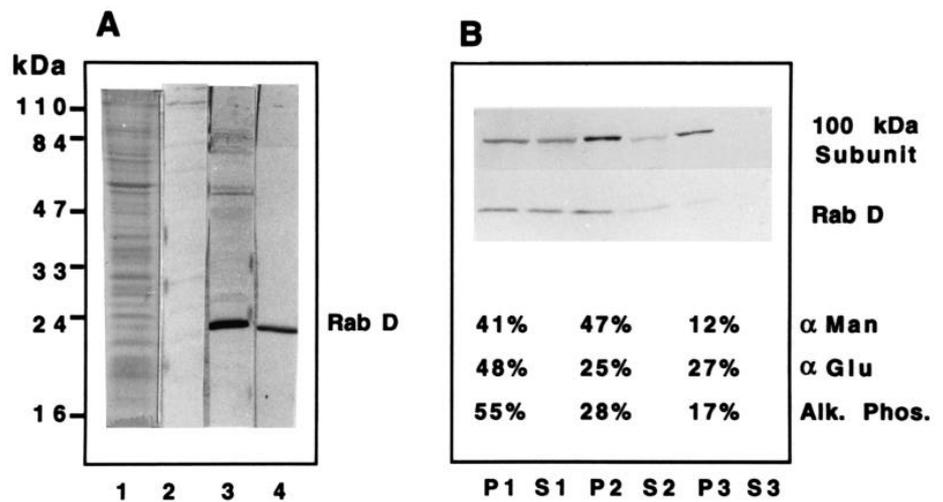
### Subcellular fractionation studies to localize RabD

RabD was expressed as a fusion protein in *E. coli* and the purified protein was injected into rabbits to generate polyclonal antisera. The unfractionated serum and affinity-purified antibodies were used to probe western blots of total membrane proteins. Lane 1 in Fig. 3A contains total cell proteins stained with Coomassie Blue. A single protein of molecular mass 23 kDa was recognized by affinity-purified antibodies (lane 4), while this protein and other higher molecular mass proteins reacted with the crude antisera (lane 3 in A). Preimmune serum did not recognize a 23 kDa protein (lane 2). The cDNA sequence for RabD contained an ORF that predicted a translation product of 23 kDa, in good agreement with the molecular mass of the major polypeptide recognized on western blots in Fig. 3. Finally, the RabD antibody did not cross-react to any appreciable extent with *D. discoideum* Rab7, Rab11 or RabC (results not shown).

To begin to localize RabD, membranes and cytosol were fractionated by serial differential centrifugation of cell homogenates and subjected to SDS-PAGE and western blot analysis (Fig. 3B). All of the RabD was membrane associated; that is, there was no detectable antigen in the high-speed supernatant S3. Most of the antigen was found in the P1 pellet (3,000 g, 5 minutes) and the P2 pellet (10,000 g for 10 minutes). The antigen remained associated even after treatment with sodium carbonate, suggesting that it is attached to membranes via a lipid anchor (not shown). Interestingly, the distribution of RabD more closely paralleled the distribution of lysosomal  $\alpha$ -mannosidase than the ER or contractile vacuole bladder markers,  $\alpha$ -glucosidase II and alkaline phosphatase, respectively (Fig. 3B). Similarly, antibodies to the 100 kDa subunit of the V-H<sup>+</sup>-ATPase also reacted strongly with both the P1 and P2 membranes; no soluble 100 kDa antigen was detected in the cytosolic fraction (Fig. 3B).

To determine more accurately the intracellular location of

**Fig. 3.** Characterization of RabD antibodies and distribution of RabD in crude membranes. (A) Cells ( $5 \times 10^6$  c/ml) were harvested by centrifugation and resuspended in Laemmli buffer. Proteins (100  $\mu$ g) were resolved by SDS-PAGE (12% gels) and either blotted to nitrocellulose (lanes 2-4) or stained with Coomassie Blue (lane 1). Western blot strips were incubated with preimmune serum (lane 2), RabD antiserum (lane 3) or affinity-purified RabD antibodies (lane 4). (B)  $10^9$  cells were harvested by centrifugation and disrupted with a Dounce homogenizer. The suspension was subjected to three successive centrifugation runs to generate three pellets, P1 (1,000 *g* for 5 minutes), P2 (10,000 *g* for 10 minutes) and P3 (100,000 *g* for 45 minutes), and their corresponding supernatants. An equivalent percentage of each fraction was subjected to western blot analysis using the affinity-purified RabD antibodies or monoclonal antibodies to the 100 kDa V-H<sup>+</sup>-ATPase subunit. The percentages below the blots represent the pelleted fraction of total enzyme activity ( $\alpha$ -mannosidase, lysosomal marker;  $\alpha$ -glucosidase, ER marker; alkaline phosphatase, contractile vacuole bladder marker).

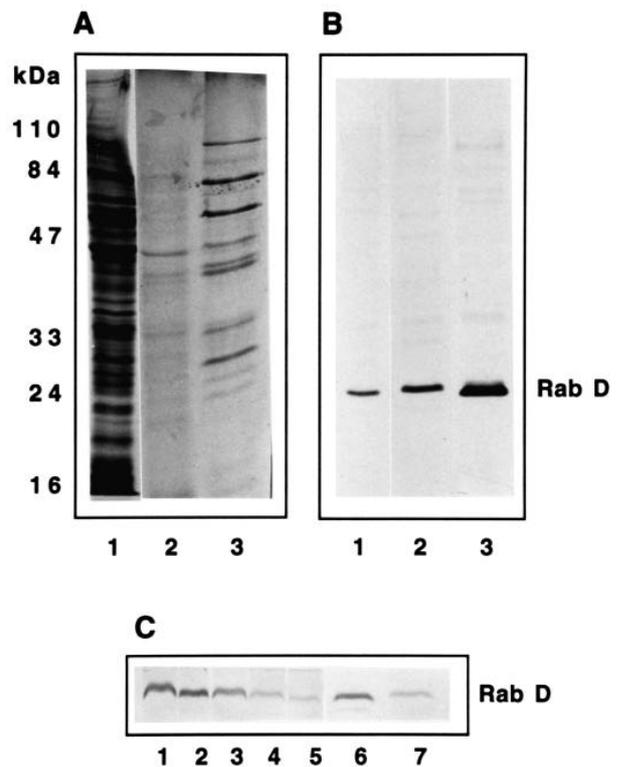


supernatants. An equivalent percentage of each fraction was subjected to western blot analysis using the affinity-purified RabD antibodies or monoclonal antibodies to the 100 kDa V-H<sup>+</sup>-ATPase subunit. The percentages below the blots represent the pelleted fraction of total enzyme activity ( $\alpha$ -mannosidase, lysosomal marker;  $\alpha$ -glucosidase, ER marker; alkaline phosphatase, contractile vacuole bladder marker).

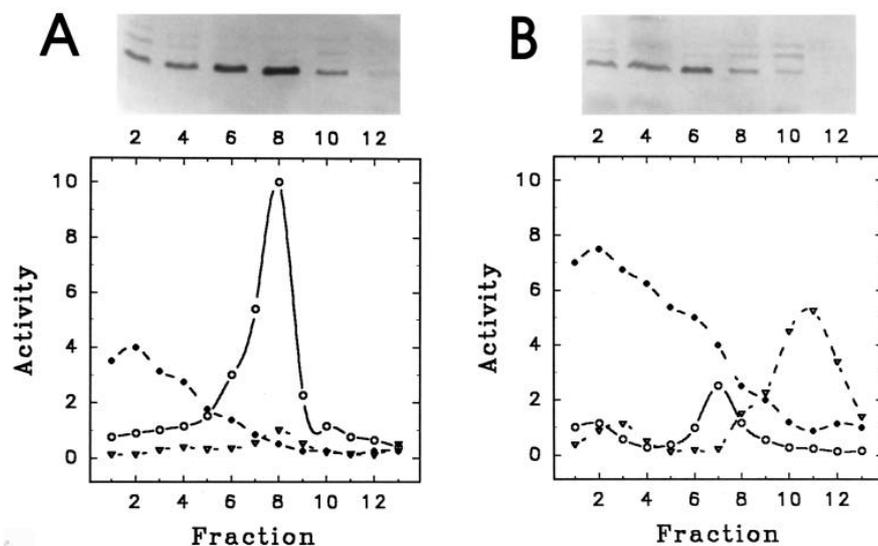
RabD, postnuclear supernatants were fractionated on Percoll gradients and the separated fractions were subjected to SDS-PAGE and western blot analysis. The RabD antigen distributed on gradients in two prominent peaks; approximately 80% of the total antigen was recovered with buoyant membranes (containing endosomes, Golgi membranes) while the remainder was in dense fractions containing the majority of lysosomal  $\alpha$ -mannosidase activity (results not shown). These results suggest that some of the cellular RabD could be associated with lysosomal/endosomal membranes.

To determine more directly if RabD was enriched in lysosomes, we purified these organelles by magnetic fractionation of cells fed colloidal iron for 15 minutes and then chased in iron-free medium for 15 minutes (Rodriguez-Paris et al., 1993). The isolates were typically enriched 25-fold in lysosomal markers. Fig. 4A represents a gel stained with Coomassie Blue to visualize proteins. Western blot analysis (B) indicated that RabD was enriched at least 5-fold in lysosomes (when corrected for differences in protein loaded) compared to crude membranes (compare lane 2 with lane 1 in Fig. 4A and B).

To determine the quantitative distribution of RabD along the endocytic pathway, fractions were purified from cells pulsed with colloidal iron and chased for various periods of time. Using this approach (Nolta et al., unpublished data), we purified early endocytic vesicles (3 minute pulse), endocytic vacuoles and lysosomes (15 minute pulse and 0-30 minute chase), and late or post-lysosomal vacuoles (15 minute pulse and 60-120 minute chase). Proteins were separated by SDS-PAGE and transferred to nitrocellulose (Fig. 4C). The relative abundance of RabD was highest in the lysosome-rich fractions (lanes 1-3 of C); RabD decreased in post-lysosomal vacuoles to <20% of the maximum (compare lanes 4 and 5 with lane 2). Also the relative levels of RabD in 3-minute vesicles (lane 7) was less than 25% of that observed for lysosomes (lane 6). These two fractions were prepared from different batches of cells because of the different pulse times used; therefore, to compare them the same amount of protein was loaded into each



**Fig. 4.** RabD associates with lysosomes and proton pump-rich membranes. Homogenate (lane 1, 100  $\mu$ g), lysosomes (lane 2, 20  $\mu$ g) and the V-H<sup>+</sup>-ATPase-rich fraction (acidosomes) (lane 3, 40  $\mu$ g) were subjected to SDS-PAGE and gels were either stained with Coomassie Blue (A) or blotted to nitrocellulose (B). (C) Magnetically purified subcompartments of the endocytic pathway were subjected to western blot analysis. Lanes 1-6 have fractions equal volumes of from one experiment in which cells that had been fed colloidal iron for 15 minutes and chased for 0 minutes (lane 1), 15 minutes (lane 2), 30 minutes (lane 3), 60 minutes (lane 4), and 120 minutes (lane 5). Lanes 6 and 7 each had 25  $\mu$ g of protein from 15-minute lysosomes and 3-minute pinosomes, respectively. Western blots were stained with affinity-purified anti-RabD antibody.



**Fig. 5.** Distribution of RabD antigen on sucrose density gradients. A homogenate of  $10^9$  stationary-phase cells was subjected to low-force centrifugation ( $1 \times 10^4 g \times$  minutes) and the resulting pellet (A) and supernatant (B) fractions were centrifuged on parallel 25% to 45% (w/w) sucrose density gradients as described (Nolta et al., 1991). Equal volumes of alternative fractions were electrophoresed on 12% Laemmli slab gels, transferred to nitrocellulose, and incubated with a 1:100 dilution of the affinity-purified antibody to RabD using an HRP-linked anti-rabbit antibody for detection. V-H<sup>+</sup>-ATPase (open circles), acid phosphatase (filled circles) and alkaline phosphatase (triangles) activities are plotted as percentage of total homogenate (Nolta et al., 1991). The gradient in A contained 82% of cellular V-H<sup>+</sup>-ATPase activity, 14% of alkaline phosphatase, 36% of acid phosphatase and 64% of RabD antigen.

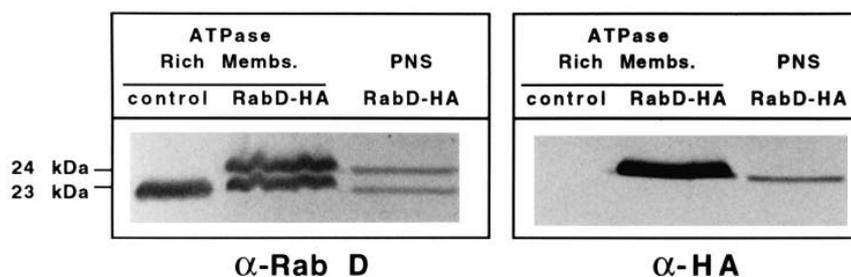
well. Since the mass of total protein in 3-minute fractions is 10-fold less than the amount of protein in 15 minute lysosomes, the abundance of RabD in the early fraction is probably <10% of that in lysosomes. We conclude that RabD was more abundant in lysosomes than in early or late endocytic compartments.

The specific activity of V-H<sup>+</sup>-ATPases is 5-fold higher in lysosomes than in the total organelle pellet (Rodriguez-Paris et al., 1993); this enrichment is similar to that observed for RabD in lysosomes. To determine if RabD and proton pumps were colocalized, buoyant V-H<sup>+</sup>-ATPase-rich membranes (acidosomes) were prepared by sucrose density gradient centrifugation (Nolta et al., 1991) and subjected to western blot analysis (Fig. 4). This fraction apparently contains the tubular membrane network (spongiomes) derived from the contractile vacuole complex (Nolta and Steck, 1994). The specific activity of the V-H<sup>+</sup>-ATPase in these membranes is enriched 30- to 40-fold and the most prominent proteins stained with Coomassie Blue are subunits of the proton pump (lane 3, Fig. 4A). Western blots indicated that RabD was also enriched at least 20-fold in these membranes compared to crude membranes (compare lane 3 with lane 1 in Fig. 4A and B).

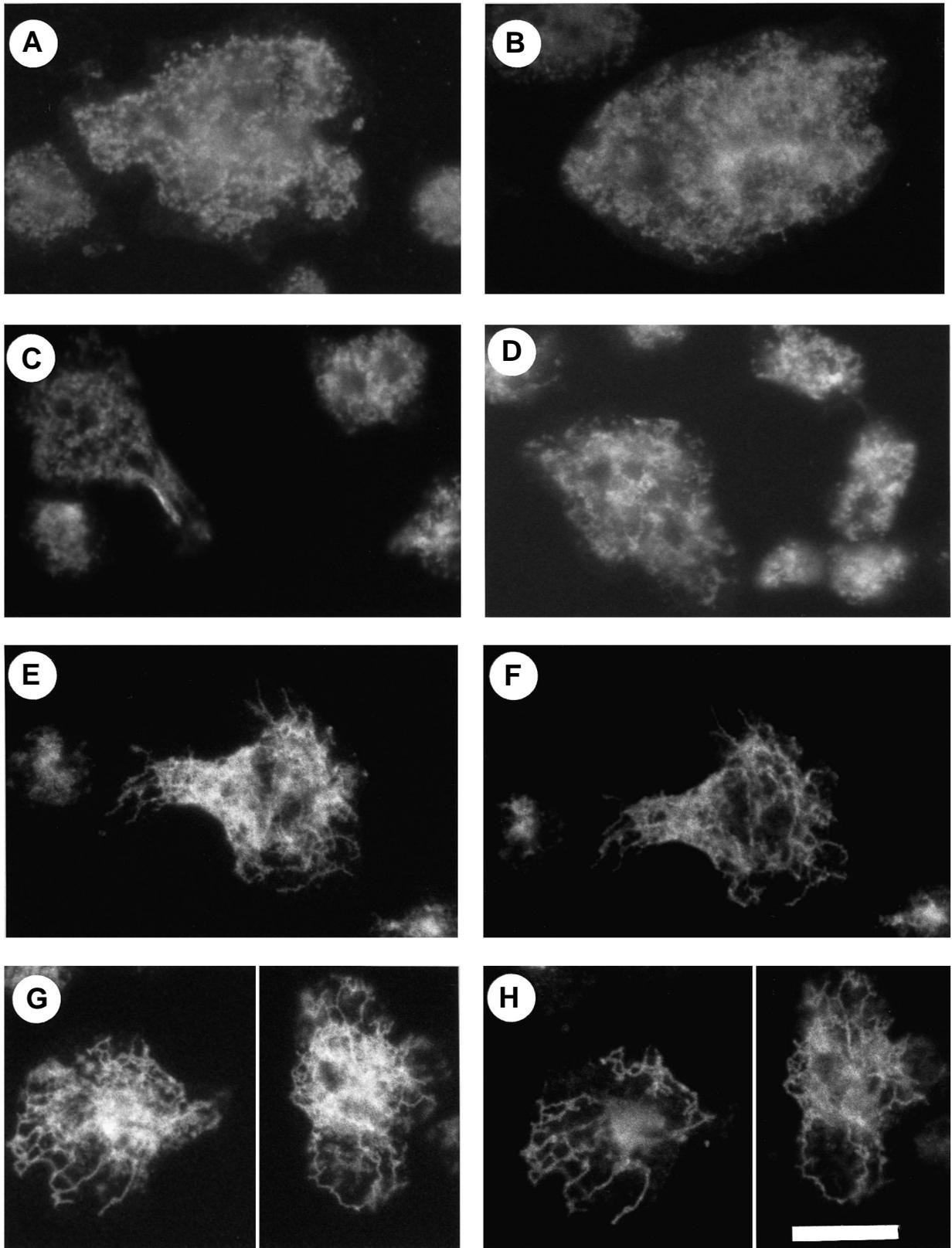
Sucrose density gradient fractionation (Fig. 5) of low-speed

pellet membranes (A) and membranes remaining in the supernatant (B) also indicated that the distribution of RabD paralleled the distribution of the ATPase activity, although the relative amount of RabD was higher than that of ATPase in the lysosomal fractions. The distribution of the V-H<sup>+</sup>-ATPase activity differed from lysosomal acid phosphatase and contractile vacuole bladder alkaline phosphatase. Together, these data support the hypothesis that RabD and the V-H<sup>+</sup>-ATPase colocalize in intracellular membranes.

To eliminate the possibility that the RabD antibody was non-specifically recognizing another Rab4-like protein in the contractile vacuole system, we inserted into the rabD cDNA a fragment encoding an 11 amino acid peptide representing a Flu virus HA epitope recognized by the monoclonal antibody 12CA5. The epitope was positioned at the N terminus of the RabD protein. The plasmid containing this insert was used to transform wild-type cells and a single G418-resistant colony was isolated. Western blot analysis (Fig. 6) indicated that, in addition to the 23 kDa RabD protein, this cell line produced a 24 kDa protein recognized by both the RabD antibodies and the HA antibody. The 23 kDa and 24 kDa (epitope tagged) proteins were both greatly enriched in ATPase-rich membranes (Fig. 6), supporting the hypothesis that RabD is enriched in the contractile vacuole system.



**Fig. 6.** An epitope-tagged RabD protein is also enriched in ATPase-rich membranes. We engineered a cDNA to express the RabD protein containing the amino acid Flu virus HA epitope (recognized by the monoclonal antibody 12CA5) at the extreme N terminus. The plasmid containing this chimeric construct was used to transform cells and a G418-resistant colony was isolated. ATPase-rich membranes were purified from control and transformed cell lines (RabD-HA) and, along with postnuclear supernatants (PNS), subjected to SDS-PAGE (100  $\mu$ g) and western blot analysis. Western blots were decorated with antibodies to RabD and to the HA epitope.



**Fig. 7.** Immunofluorescence microscopy to determine the intracellular location of the 100 kDa ATPase antigen and the RabD antigen. Cells were fixed as described in Materials and Methods (method two, A and B; method three, C-H) and stained with antibodies to RabD (A,B,F and H) or the 100 kDa antigen (C,D,E and G). Goat anti-rabbit antibodies coupled with rhodamine or goat anti-mouse antibodies coupled with fluorescein were added to detect RabD and the ATPase subunit, respectively. Bar, 5  $\mu$ m. (A-D) Different fields; (E-F and G-H) the same fields.

### Cellular localization of RabD determined by immunofluorescence microscopy

Immunofluorescence microscopy was also used to determine the intracellular location of RabD and the 100 kDa V-H<sup>+</sup>-ATPase subunit. Permeabilized cells were incubated with rabbit anti-RabD antibodies and mouse anti-100 kDa antigen antibodies, followed by the addition of secondary antibodies coupled with rhodamine (goat anti-rabbit antibodies) and fluorescein (goat anti-mouse antibodies). The RabD antigen was localized to a tubular-vesicular network distributed throughout the entire cell; the plasma membrane was not labeled (Fig. 7A and B). The 100 kDa antigen had a similar distribution (C and D). Finally, double-label immunofluorescence microscopy indicated that RabD (F and H) and the 100 kDa protein (E and G) colocalized in this extensive reticular network. Although all of the RabD antigen appeared to distribute with the 100 kDa antigen, some of the tubular elements appeared to be relatively enriched in the ATPase subunit. We suggest that both of these antigens were associated with spongiome membranes of the contractile vacuole system.

The distribution of RabD appeared more tubular-vesicular and extended more throughout the cell in Fig. 7A and B compared to the more singularly tubular pattern seen in F and H. Fixation method 2 (optimal for RabD) was used for the experiments shown in A and B while fixation method 3 (optimal for the ATPase but suboptimal for RabD) was used for the experiments in C-G, which accounts for this difference.

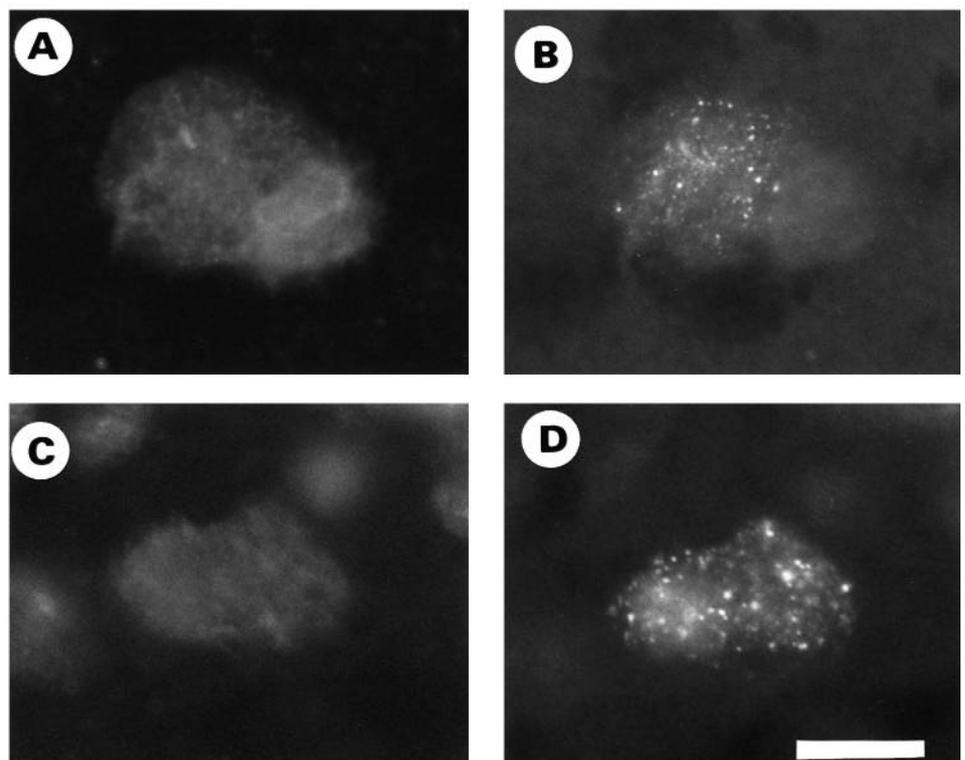
### Localization of RabD by immunoelectron microscopy

Although RabD was detected in lysosomes by subcellular fractionation techniques, double-label immunofluorescence microscopy indicated that little RabD colocalized with

lysosomal  $\alpha$ -mannosidase; that is, RabD associated with reticular membranes while  $\alpha$ -mannosidase was found in punctate vacuoles (Fig. 8). This issue was therefore pursued by immunoelectron microscopy. Fig. 9A indicates that the majority of the magnetically isolated lysosomes were decorated with gold particles recognizing anti-RabD antibodies. The antigen was detected both on the limiting membrane and on the membrane vesicles in the lumen of these vacuoles.

Immunoelectron microscopy also demonstrated the presence of RabD in V-H<sup>+</sup>-ATPase-rich membranes of the contractile vacuole system (Fig. 9B and C). Consistent with the immunofluorescence results, the tubular membranes (marked with asterisks) of a low-speed pellet containing most of the ATPase activity were positive for RabD (Fig. 9C). These tubular elements tended to vesiculate during purification on sucrose gradients (generating the fraction previously termed acidosomes; Nolte et al., 1991); however, as expected (Fig. 9B) RabD was also detected in these membranes.

Large vesiculated membranes presumably contractile vacuole 'bladders' were essentially devoid of RabD antigen; however, remnant spongiome membranes still associated with the bladders were labeled by immunogold (Fig. 9D). We have found that the pellets of unfractionated homogenates contained abundant contractile vacuole complexes (Nolte and Steck, 1994; Fig. 10A). Immunoelectron microscopy of these membranes (spongiomes and bladders) demonstrated that both RabD (10 nm gold, arrowheads) and the 100 kDa V-H<sup>+</sup>-ATPase antigen (20 nm gold, short arrows) were present on the same tubular membranes (Fig. 10B) and absent from the bladder membranes, consistent with the results obtained using purified fractions. Fig. 10C represents the contractile vacuole complex decorated with RabD antibodies only.



**Fig. 8.** Immunofluorescence microscopy of RabD and lysosomal  $\alpha$ -mannosidase. Cells were fixed as described in Materials and Methods (method one) and stained with antibodies to RabD (A and C) or  $\alpha$ -mannosidase (B and D). The secondary antibodies described in the legend to Fig. 7 were used to detect the primary antibodies. This method of fixation is optimal for visualization of lysosomal  $\alpha$ -mannosidase but the RabD antigen is not as readily stained (as shown in Fig. 7). Bar, 5  $\mu$ m.

Immunogold also labeled RabD in tubular membranes in whole cells (Fig. 10D and E). D presents a cross-section through a bundle of these membranes (they appear vesicular) while E shows transverse sections that appear tubular. These results are consistent with the intracellular pattern observed for RabD by immunofluorescence microscopy (Fig. 7).

## DISCUSSION

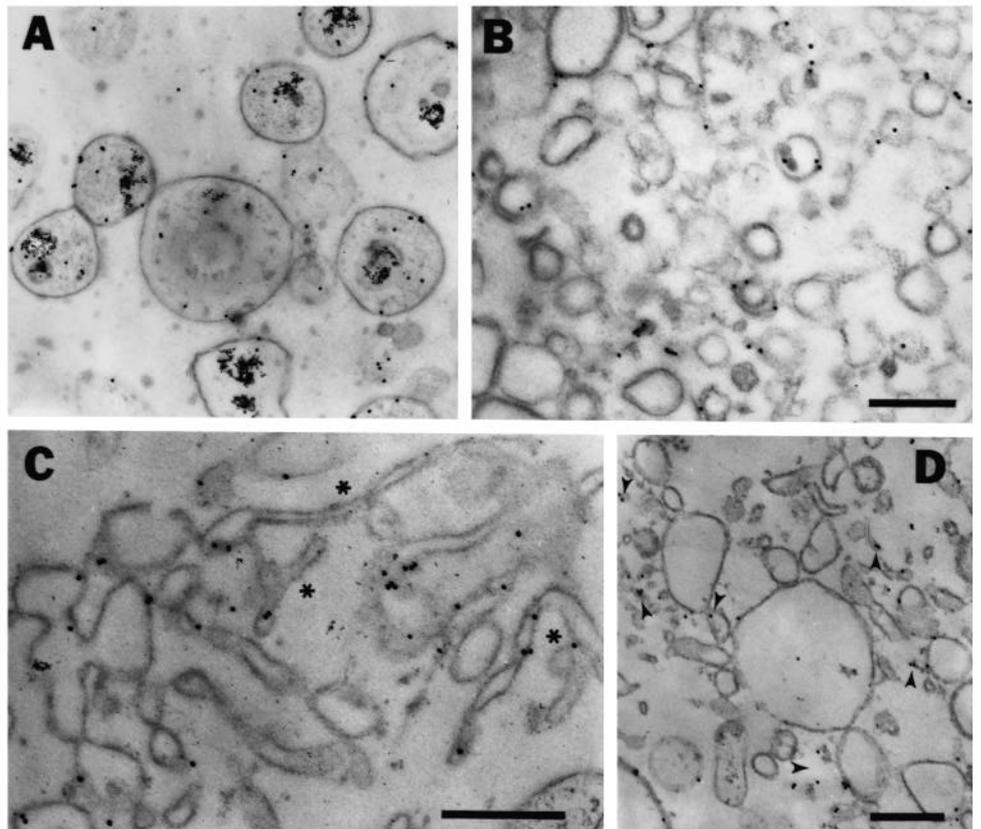
In this report, we show that *D. discoideum* contains a single gene (*rabD*) encoding a Rab4-like GTPase expressed at a relatively constant level during growth and development. Subcellular fractionation and immunoelectron microscopy indicated that RabD was enriched in lysosomes; much less of the protein was found in early and late endocytic vacuoles. Unexpectedly, most of the RabD protein colocalized with the V-H<sup>+</sup>-ATPase in tubular membranes apparently associated with contractile vacuole complexes. These studies suggest that RabD may regulate membrane flow along both the endocytic pathway and the unexplored pathway that generates and maintains the contractile vacuole system of *D. discoideum*.

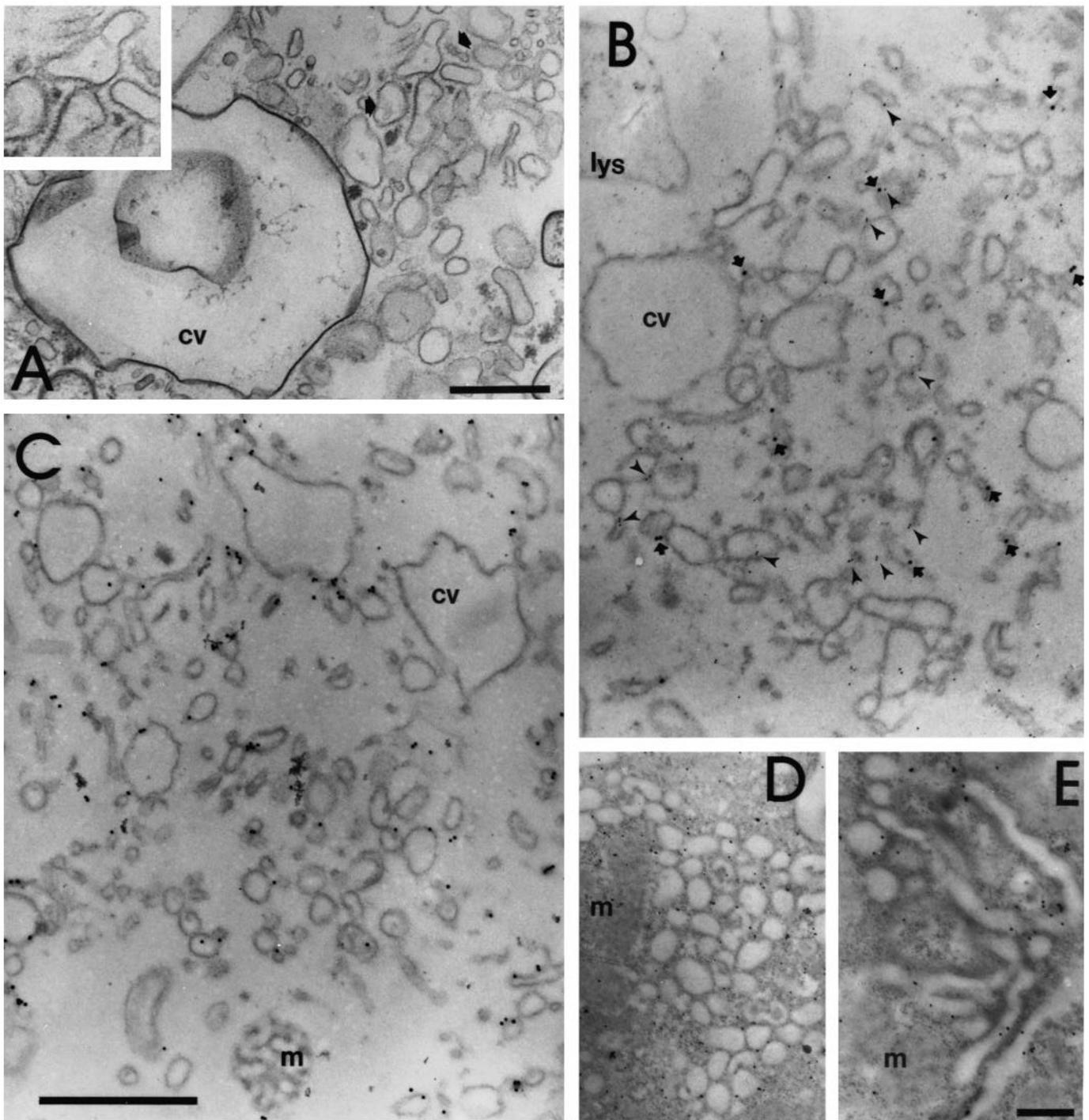
Rab4 (associated with early endosomes) and Rab2 (associated with the *cis*-Golgi network) are more closely related to each other in amino acid sequence than to other known Rab proteins. RabD from *D. discoideum* displayed 70% amino acid identity to both Rab2 and Rab4 and is more closely related to Rab4 and Rab2 than they are to each other. However, RabD may function more like Rab4 because: (1) it is found in lysosomes; and (2) the effector domain of RabD is almost identical to the effector domain of Rab4, differing at only one

amino acid position. The cloning of a separate cDNA encoding a protein very similar to Rab2 lends additional support to this hypothesis. The *D. discoideum rab2* cDNA was isolated using an oligonucleotide probe coding for the amino acid sequence upstream of and overlapping the effector region of human Rab2 (Zahraoui et al., 1989). This method was also successful in cloning a *D. discoideum* cDNA encoding a potential homolog of canine Rab7 (Bush and Cardelli, 1994). This suggests the general applicability of this approach to cloning *rab* genes from nonmammalian systems that encode proteins homologous to human and canine Rab proteins.

In animal cells, Rab4 appears to be concentrated in early endosomes as opposed to lysosomes and other organelles. The significance of RabD in the lysosomes of *D. discoideum* is therefore of interest. One explanation might be that most of the lysosomal RabD is actually contained within the lumens of lysosomes (i.e. is autophagic); in particular, on the internal membranes of multivesicular bodies. Consistent with this hypothesis is the observation that the lumens of lysosomes in *Dictyostelium* appear to acquire fragments of acidosomes/spongiomes by autophagy, as judged by the high density of V-H<sup>+</sup>-ATPase antigens on the luminal membrane of multi-vesicular bodies (Nolta et al., 1993). Perhaps, *D. discoideum* contains another Rab4-like protein that functions to regulate vesicle traffic along the endocytic pathway. However, immunoelectron microscopy also indicated that a significant percentage of the RabD antigen was localized to the outer membrane limiting lysosomes. RabD, like Rab4, may therefore function to regulate membrane traffic along the endocytic pathway. Neither pre-lysosomal sorting nor a compartment corresponding to the early endosomes of animal cells has been

**Fig. 9.** Immunocytochemical analysis of RabD in isolated organelles. Membrane fractions were washed, pelleted, fixed and embedded in Lowicryl. Sections were reacted with affinity-purified RabD antibodies (1:50) dilution) and decorated with anti-rabbit antibodies conjugated to 20 nm gold colloidal gold. (A) Lysosomes were purified magnetically from cells fed colloidal iron for 15 minutes and chased for 15 minutes (Rodriguez-Paris et al., 1993). (B) Acidosomes were the peak of V-H<sup>+</sup>-ATPase activity in Fig. 5A (see Nolta et al., 1991). (C) Crude acidosomes/spongiomes were the low-speed membrane pellet used as the input to the experiment shown in Fig. 5A (see Nolta et al., 1991; and Nolta and Steck, 1994). (D) Contractile vacuole bladders were the peak of alkaline phosphatase activity in Fig. 5B. The membranes with RabD antigen (arrowheads in D) are presumed to be spongiome remnants (see Nolta and Steck, 1994). Bars, 0.5  $\mu$ m.





**Fig. 10.** Immunocytochemical analysis of spongiomes with RabD and V-H<sup>+</sup>-ATPase antibodies. (A-C) Duplicate samples of an unfractionated homogenate were centrifuged at 18,000 rpm for 30 minutes in a Sorvall SS-34 rotor; the pellets were fixed and embedded either in Epon or in Lowicryl as described (Nolta et al., 1993; Nolta and Steck, 1994). (A) Epon section of an intact contractile vacuole complex, identified by its large main vacuole (cv), surrounded by a spongiome network covered with proton pump pegs 12 nm in diameter (broad arrows). Inset: spongiome at high magnification, highlighting the V-H<sup>+</sup>-ATPase projections. (B) Lowicryl section of a double-labeled contractile vacuole complex. Decoration with a monoclonal antibody against band 1 of the proton pump complex (Fok et al., 1994) was revealed by secondary labeling with 20 nm immunogold (short arrows); the affinity-purified antibody against RabD was secondarily labeled with 10 nm immunogold (arrowheads). (C) A Lowicryl section of a contractile vacuole complex reacted with the affinity-purified antibody against RabD as in (B). (D and E) Intact cells were processed as above and reacted with RabD antibodies followed by immunogold decoration. m, mitochondria; lys, lysosomes; cv, contractile vacuole bladder. Bars: (A) 0.5  $\mu$ m; (B and C) 1  $\mu$ m; (D and E) 1  $\mu$ m.

documented in *Dictyostelium*; it is thus not clear whether Rab4 functions in this manner in this amoeba. However, RabD may regulate the formation of lysosomes from early endocytic vesicles or it may be important in the biogenesis of post-lysosomal vacuoles.

Approximately 10% of the total cellular V-H<sup>+</sup>-ATPase is associated with the endocytic pathway in *Dictyostelium* (Rodriguez-Paris et al., 1993); most of the remainder of the proton pumps are associated with spongiomes/acidosomes (Heuser et al., 1993; Nolta and Steck, 1994). Although these pumps have been thought to populate both spongiomes (tubular membranes) and large contractile vacuole bladder membranes (Heuser et al., 1993; Fok et al., 1993), more recent studies have indicated that intact V-H<sup>+</sup>-ATPase is mainly confined to spongiomes and not bladders (Nolta et al., 1993; Nolta and Steck, 1994; see also Fig. 10A). The immunofluorescence and immunoelectron microscopic studies described here support that conclusion. In fact, immunofluorescence indicated that the 100 kDa ATPase subunit associated with an extensive reticular network distributed throughout the cell. Although earlier studies showed the V-H<sup>+</sup>-ATPase antigen on short tubular structures (Heuser et al., 1993; Fok et al., 1993), they underestimated the extensive nature of this reticular network. The reason for this discrepancy is unclear but may be related to the different fixation methods used. We also found that the 100 kDa antigen did not appear to be localized in large contractile vacuole bladder membranes; although previous authors inferred the presence of this antigen and another ATPase subunit in these membranes (Fok et al., 1993; Heuser et al., 1993). Spongiome membranes that have collapsed around the vacuole, generating a ring like structure (Nolta and Steck, 1994), might explain the earlier observations made at the light microscopic level.

Subcellular fractionation, immunofluorescence microscopy and immunoelectron microscopy all indicated that RabD and the 100 kDa ATPase subunit colocalized in tubular spongiomes. The specificity of the RabD antibody was confirmed by demonstrating that an exogenously expressed epitope-tagged RabD protein was also greatly enriched in tubular spongiomes. Furthermore, the RabD antigen like the 100 kDa antigen was enriched at least 20-fold in purified spongiomes/acidosomes compared to a 5-fold enrichment in endo-lysosomes. This finding might explain why double-label immunofluorescence showed little colocalization of RabD with known lysosomal markers. Perhaps the large excess of RabD and the 100 kDa antigens in spongiomes obscured the immunofluorescence signal generated by these antigens in lysosomes and endosomes. Alternatively, RabD in endo-lysosomal compartments may be associated with but morphologically distinct from the vacuolar structures containing  $\alpha$ -mannosidase.

Given the confinement of Rab4 in mammalian cells to early endosomes (van der Sluijs et al., 1991), we must consider the significance of the concentration of the closely related RabD on spongiomes in *Dictyostelium*. It can be speculated that despite their sequence similarity, RabD and Rab4 may differ in their intracellular functions. The main function of RabD may be to regulate membrane flow to and from the contractile vacuole system, and other Rab proteins may regulate endocytosis and lysosome biogenesis. In addition, RabD might regulate vesicle traffic between the contractile vacuole complex and the endocytic pathway (see, for example, Padh et

al., 1991a,b). Presumably the codistribution of RabD and V-H<sup>+</sup>-ATPases is more than coincidental; hence RabD may regulate the distribution and/or function of the proton pumps directly or indirectly. Current molecular genetic approaches designed to express mutant forms of the RabD proteins should help clarify these issues.

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