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Cloning of two genes encoding Rab7 in *Paramecium*

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Rab7 is a small GTPase that plays a crucial role in the regulation of transport from early to late endosomes and lysosomes, phagosome maturation and in lysosomal biogenesis in mammalian cells. It contains conserved and unique sequence elements that mediate its function. Two *Rab7* genes, *Rab7a* (703 bp) and *Rab7b* (707 bp) were identified in the unicellular eukaryote *Paramecium* by PCR amplification. They contain three short introns of different lengths (28–32 bp) and sequence located at identical positions in both genes. The presence of two *Rab7* genes in the *Paramecium* genome was confirmed by Southern hybridization analysis performed with six different restriction enzymes. Expression of both genes was assessed by Northern blot and RT-PCR. Two transcripts of 1.8 and 2.2 kb were identified by hybridization analysis. The cloned complementary DNAs, both of 618 nucleotides in length, encode polypeptides of 206 amino acids that are 97.6% identical and differ in their C-termini. The predicted protein sequences of Rab7a and Rab7b contain all characteristic domains essential for Rab function: the effector domain (YRATVGADF) and four GTP-binding consensus sequences (GDSGVGKT, WDTAGQ, NKLD, SAK) as well as the prenylation motif (–CC) at the C-terminus indispensable for Rab binding to the membrane. Similarity searches revealed 81.6–82.1% homology of *Paramecium* Rab7 isoforms to human Rab7 and a lack of an insert typical for the Kinetoplastida – the species that appeared earlier in evolution. *Paramecium* is the first free-living lower eukaryote in which homologues of Rab7 have been identified that exhibit features similar to those of mammalian Rab7.

Keywords: Rab7, *Paramecium*, cloning, hybridization, digoxygenin labeling, PCR/RT-PCR

Rab family of small GTPases regulate transport of endo- and exocytic vesicles and are involved in the control of their docking and fusion in mammalian cells (Chavrier *et al.*, 1991; Saraste *et al.*, 1995; Van der Sluijs & Gerez, 1999; Somsel Rodman & Wandinger-Ness, 2000). More than 60 Rab GTPases are known and they have been localized to the surface of intracellular compartments (Pereira-Leal & Seabra, 2001; Pfeffer & Aivazian, 2004).

Rab proteins contain conserved and unique sequence elements that mediate their function. The highly conserved regions include the effector domain, the guanine base- and phosphate-binding motifs and a cysteine motif which is posttranslationally modified by the geranyl-geranyl lipid group and is required for the interaction of Rab protein with GDI and membranes. Multiple factors contribute to the specificity of Rab localization to distinct intracellular membranes (Seabra & Wasmeier, 2004), including a unique sequence motif called hypervariable region (Chavrier *et al.*, 1991; Bruckert *et al.*, 2000; Moyer & Balch, 2001).

Compounds internalized by different endocytic pathways enter the late endosomal compartment in which Rab7 regulates their rate of degradation (Feng *et al.*, 1995; Bruckert *et al.*, 2000). Much attention is focused on Rab7 GTPase since a mutation in its gene evoking neuropathy of Charcot-Marie-Tooth type 2B was recently discovered (Verhoeven *et al.*, 2003). Rab7 plays an important role in the regulation of cholesterol level (Bruckert *et al.*, 2000), production of thyroid hormones (Croizet-Berger *et al.*, 2002) and accumulation of unprenylated Rab27A in choroideremia (Rak *et al.*, 2004). It is also involved in entering of viruses and bacteria (Meresse *et al.*, 1999; Sieczkarski & Whittaker, 2003). The expression level of this protein in different

***Accessions numbers**: Sequences of Rab7a and Rab7b have been deposited in the GenBank database under the following numbers: cDNA sequences: AY744503, AY644723; genomic sequences: AY050242, AY875981; amino-acid sequences: AAL08054, AAW68046.

Abbreviations: RILP, Rab-interacting lysosomal protein; RabSF, Rab subfamily; GDI, GDP dissociation inhibitor.

cells correlates with the endocytic/phagocytic activity (Desjardins *et al.*, 1994; Bruckert *et al.*, 2000; Rupper *et al.*, 2001). Rab7 plays a crucial role in the regulation of trafficking from early to late endosomes and lysosomes, phagosome maturation and in lysosomal biogenesis (Pfeffer & Aivazian, 2004).

In the present study we describe the first members of the Rab family in the free-living ciliate *Paramecium*. Ciliates emerged early in evolution prior to yeasts, plants and animals (Sogin & Elwood, 1986). Many components of the trafficking and signaling pathways are conserved from ciliates to humans (Luporini *et al.*, 1994; Subramanian *et al.*, 1994; Wyroba *et al.*, 1995; Creutz *et al.*, 1998; Kissmehl *et al.*, 2002; Wiejak *et al.*, 2004b) but the molecular machinery underlying endocytic processes has not yet been fully elucidated in these unicellular eukaryotes. We have previously characterized components participating in the early stages of endocytosis and identified two partial genes encoding dynamin. *Paramecium* seems to be the most ancient cell in which dynamin performs its function as in the Metazoa – in clathrin-mediated endocytosis (Wiejak *et al.*, 2004b) and in agonist-induced receptor desensitization (Wiejak *et al.*, 2004a).

Here we identified a component of the late endocytic pathway by cloning two genes and complementary DNAs coding for Rab7. The predicted amino-acid sequences of *Paramecium* Rab7 exhibit very high homology to mammalian counterparts and contain the characteristic domains indispensable for Rab protein function. DNA/RNA hybridization analyses confirmed the presence of two genes and two mRNAs for Rab7 in *Paramecium*.

MATERIALS AND METHODS

Cells. *Paramecium octaurelia* strain 299s (5-day-old axenic culture) was cultivated and harvested as reported (Wyroba, 1987).

PCR/RT-PCR and cloning. PCR/RT-PCR amplifications were performed using genomic DNA (0.75 μg) isolated from *Paramecium* (Subramanian *et al.*, 1994) or cDNA, respectively, as templates in a PTC-200 DNA Engine thermocycler (MJ Research, Inc.). cDNA was obtained by reverse transcription of 1 μg of total RNA with the Enhanced Avian RT-PCR Kit with oligo-dT primers according to manufacturer's instruction (Sigma-Aldrich Chemie GmbH). Several primers were designed to the nucleotide sequence of highly conserved regions of Rab7: the effector domain, RabSF (Rab subfamily) motifs, and G3 binding domain from different species and synthesized according to the ciliate nuclear codon usage (Martindale, 1989). Subsequently, next sets of primers were constructed to the sequence of the *Rab7* gene fragments obtained during cloning. The forward primers were: 5′-TATAAAGCTACAATTGGAGCTGATTTC, 5′-CCATTCGTTGTTTTAGGA, 5′-ATGGCCAGCCAGAAGAAGCAA, and the backward ones: 5′-TCCTAAAACAACGAATGG, 5′-ACAACATCCTCCTTATTTGG, 5′-ACAACATCCTCC TTATTTGGCTTATGGTTTTTGGCTC, 5′-ACAACATCCTCCTTATTTGGTCTATTTCTTTGG GTCT. PCR setting, cloning into pGEM-T Easy vector (Promega), transformation of competent *Escherichia coli* bacteria (JM109) and selection of positive clones were performed as described previously (Wiejak *et al.*, 2004b). Plasmid DNA that contained an insert of expected molecular size was isolated by the GenElute™ HP Plasmid Midiprep Kit (Sigma-Aldrich Chemie GmbH), purified using the QIAquick Gel Extraction Kit (Qiagen) and automatically sequenced.

Total RNA isolation. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Cell homogenization was carried out on ice in a sterile glass homogenizer using 0.25–0.5 mm glass beads (Carl Roth GmbH and Co.). Any contaminating DNA was removed by treatment with RNase-free DNase I (Promega). RNA concentration was determined spectrophotometrically.

Southern blot hybridization. DNA was isolated, digested with restriction enzymes, electrophoresed (10 μg per lane) and blotted as described before (Subramanian *et al.*, 1994). Hybridization was performed at 55°C with the cloned *Rab7a* as the probe that was labeled with digoxygenin-11-dUTP (Roche) using Nick Translation System (Promega). Chemiluminescent detection was performed as described in (Wyroba *et al.*, 1995).

Northern blot hybridization. Total RNA (20 μg) of was electrophoresed on a formaldehyde/1% agarose gel and transferred to a positively charged nylon membrane (Boehringer Mannheim). Hybridization with digoxygenin-11-dUTP-labeled *Paramecium Rab7b* cDNA as the probe was carried out at 50°C in Dig Easy Hyb (Roche) and blots were processed as in (Platek *et al.*, 1999) except for maleate buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5) used in the blocking/washing procedures.

Computer analysis. Sequence searches were performed by the BLAST algorithm (Altschul *et al.*, 1997) on NCBI databases. Multiple sequence alignment was carried out using the CLUSTAL W (1.82) program (Thompson *et al.*, 1994).

RESULTS AND DISCUSSION

Cloning of *Paramecium Rab7* genes

We identified two genes encoding Rab7 in *Paramecium* by PCR with primers designed from

the amino-acid sequence of domains conserved in Rab7 of different species. Due to deviations from the universal genetic code in ciliates (Martindale, 1989) these primers were synthesized as degenerate oligonucleotides.

Different sets of primers generated PCR products of different lengths (not shown). Subcloning and sequencing of these products revealed that PCR products of 703 bp and 706 bp encoded Rab7a (Fig. 1, lane 1) and Rab7b (Fig. 1, lane 3), respectively. Subsequently, the sets of primers that enabled identification of these genes were used for RT-PCR and generated single products of about 600 bp which upon subcloning and sequencing gave cDNA sequences encoding Rab7a (Fig. 1, lane 2) and Rab7b (Fig. 1, lane 4).

The *Rab7a* gene (accession number AY050242) of 703 bp and *Rab7b* (accession number AY875981) of 707 bp are 88.9% identical (including introns). These sequences show features typical for genes of ciliates: short introns, a high A+T content and in-frame universal stop codon (TAA and TAG) coding for glutamine (Martindale, 1989; Russell *et al.*, 1994). Both genes have three introns of different length (28–32 bp) and sequence located at identical positions (Fig. 2). The cloned cDNAs for *Rab7a* and *Rab7b* (accession numbers AY744503, AY644723) are 618 nucleotides in length and 92.6% identical.

Paramecium Rab7a and *Rab7b* contain open reading frames encoding proteins of 206 amino acids. These deduced amino-acid sequences are 97.6% identical. There are five amino acid exchanges (two of which are conserved) over the full length of the two polypeptides (Fig. 2).

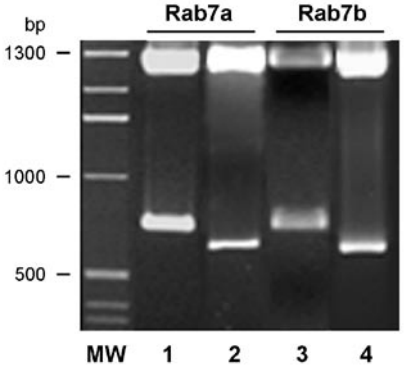

Figure 1. Agarose gel electrophoresis of isolated plasmids containing *Rab7* inserts.
Following subcloning into pGEM-T vector of PCR/RT-PCR products obtained with *Rab7*-specific primers, the plasmids were isolated from positive colonies and digested with *Eco*RI. The presence of inserts of correct sizes was detected. Lanes 1 and 3 show inserts of predicted molecular size of about 700 bp originated from PCR products which upon sequencing resulted in the cloning of *Rab7a* (lane 1) and *Rab7b* (lane 3). Lanes 2 and 4 show inserts of about 620 bp originated from RT-PCR products which upon sequencing resulted in cloning of cDNA for *Rab7a* (lane 2) and *Rab7b* (lane 4). The molecular mass standard (MW) is indicated on the left.

The predicted protein sequences of Rab7a and Rab7b contain all the characteristic and highly conserved domains essential for Rab function: the effector (YRATVGADF) and nucleotide binding domains by phosphate (GDSGVGKT, WDTAGQ) and guanine (NKLD, SAK) as well as the prenylation motif (–CC) at the C-terminus (Fig. 2). This posttranslational modification is present in all Rab proteins and is essential for membrane binding (Bruckert *et al.*, 2000).

These are the first genes encoding Rab GTPases to be identified in *Paramecium*. Only short gene fragments homologous to Rab1, Rab2 and Rab5 were previously amplified by others (Fraga & Hinrichsen, 1994), whereas we cloned the effector and nucleotide binding domain of Rab1 and Rab11 (accession numbers AY228708 and AY228707, respectively). In the ongoing project of *Paramecium* Genome Sequencing (Sperling *et al.*, 2002) of another strain — *P. tetraurelia* d4-2 (http://www.genoscope.cns.fr) — some sequences exhibiting homology to *Rab7* genes cloned by us may be found.

Many Rab GTPases seem to be products of gene duplication (Stenmark & Olkkonen, 2001). However, up to now, Rab7 isoforms have been identified only in human and plants. Rab7b — a new human isoform of Rab7 GTPase recently just reported — was shown to be involved in monocytic differentiation of acute promyelocytic leukemia cells (Yang *et al.*, 2004). Two flowering plants *Nicotiana tabacum* and *Lotus japonicum* express more than one isoform of Rab7 (four and three, respectively) (Haizel *et al.*, 1995; Borg *et al.*, 1997). Recent gene duplication has previously been reported in *Paramecium*: two isoforms of proton-translocating pyrophosphatase that display 94% homology (Perez-Castineira *et al.*, 2002) were identified. We described two genes encoding dynamin — another protein essential in the endocytic processes (Wiejak *et al.*, 2004b).

Southern blot analysis

Southern blot analysis of *Paramecium* DNA digested with *Eco*RI, *Xba*I, *Eco*RV, *Bgl*II, *Hin*dIII and *Pst*I using the cloned *Rab7a* as the probe was performed (Fig. 3). The hybridization pattern revealed an excessive number of hybridizing DNA fragments (Fig. 3) in comparison to the number of restriction sites in this gene (Table 1). Although there are no restriction sites for *Eco*RI and *Xba*I in *Rab7a*, two bands were observed in each case. Only one restriction site was mapped for *Bgl*II and *Eco*RV in *Rab7a* gene, but three DNA hybridizing species were detected. Finally, there are two restriction sites for *Hin*dIII and *Pst*I each, whereas five and four DNA fragments, respectively, were observed.

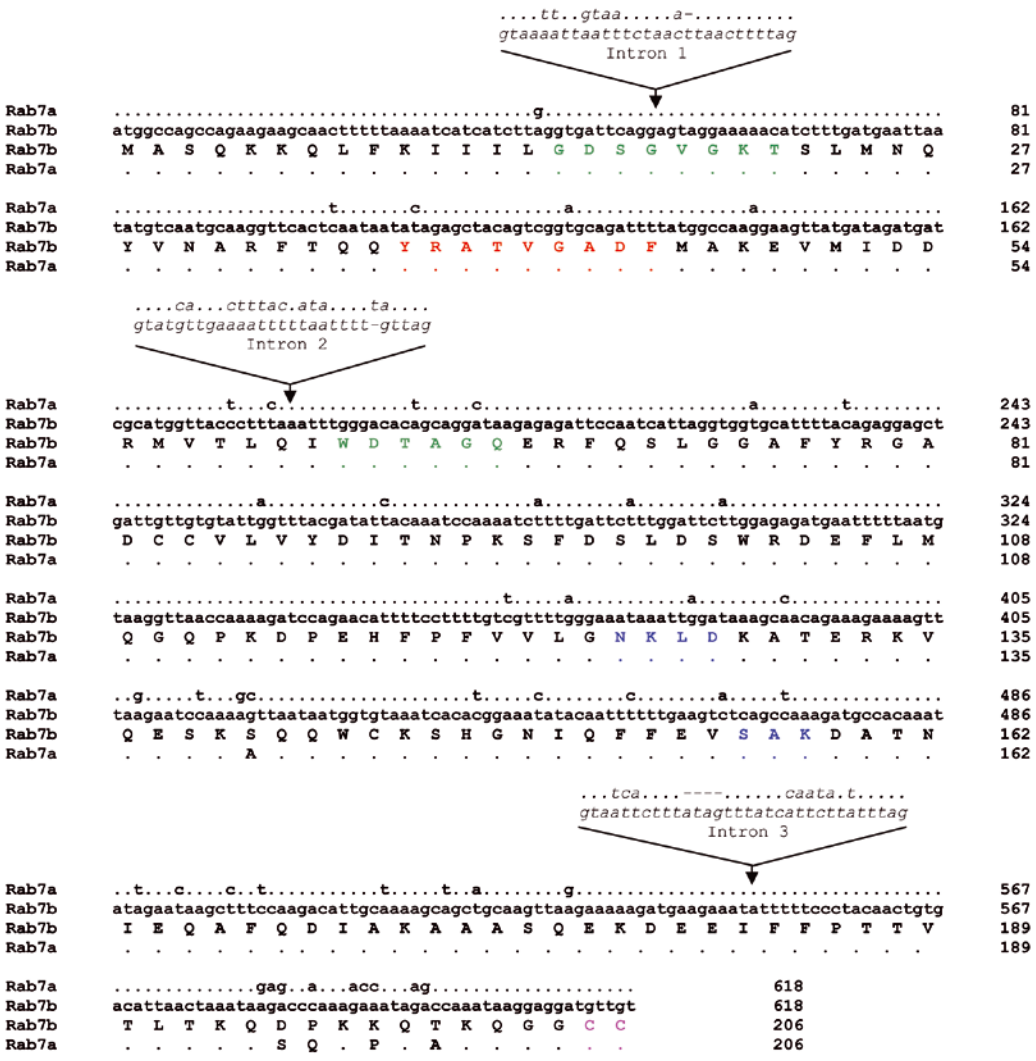

Figure 2. Nucleotide and deduced amino-acid sequences of *Paramecium Rab7a* and *Rab7b*.
The top two lines in each group show nucleotide sequences derived from cDNAs (accession numbers AY744503, AY644723) and the bottom two lines show the respective deduced amino-acid sequences (single-letter code). Dots indicate identical nucleotides or amino acids. Positions of three introns (italics) deriving from the genomic sequences (accession numbers AY050242, AY875981) are indicated by arrows: the top line in *Rab7a* gene and the bottom in *Rab7b* gene. Evolutionarily conserved domains are shown: effector domain (red), guanine base- (blue) and phosphate-binding motifs (green) and cysteine motif (pink).

However, the *Rab7b* gene contains only one restriction site for *Hin*dIII (Table 1). Thus, a comparison of the number of the hybridizing DNA fragments with the number of restriction sites for six different restriction enzymes in both cloned *Rab7* genes confirms that two genes encoding this protein exist in *Paramecium* genome.

Analysis of Rab7 expression and multiple sequence alignment

Expression of *Paramecium* Rab7 genes was assessed by RT-PCR – as described above (Fig. 2) — and by Northern blotting.

Northern blot analysis of total RNA extracted from *Paramecium* cells probed with the *Rab7b* cDNA revealed the presence of two transcripts of about 1.8 and 2.2 kb (Fig. 4) that most probably corresponded to the two Rab7 isoforms since the cloned cDNAs for Rab7a and Rab7b were 92.6% identical (Fig. 2). Two messenger RNAs for Rab7 were previously found in human, mouse and rat (Vitelli *et al.*, 1996).

Multiple alignment based on NCBI GenBank data indicates that the *Paramecium* Rab7a and Rab7b exhibit 62.3–63.3% identity to human, rat and mouse counterparts, 61.2–61.7% to Rab7 from *Dictyostelium discoideum*, 64.4–64.6% to *Trypanosoma cruzi* Rab7 and 60.1–60.2% to that from *Leishmania major* (Fig. 5). *Paramecium* Rab7 isoforms display 48.1–52.4% identity to Ypt7 of *Saccharomyces cerevisiae*, the yeast homologue of Rab7 involved in vacuolar transport (Wichmann *et al.*, 1992). In the GenBank there is a sequence of a *Rab7* gene of another ciliate *Tetrahymena thermophila*, but there is no data about its expression and func-

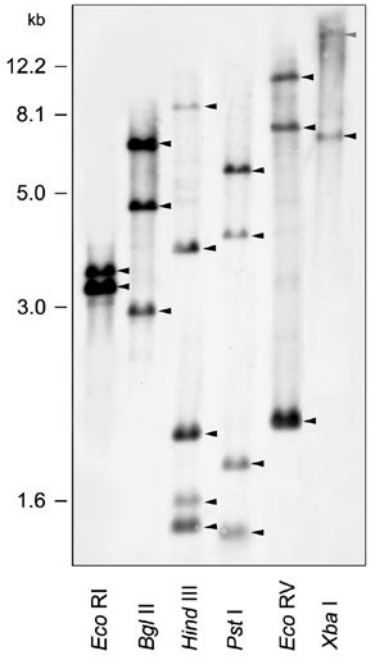

Figure 3. Southern hybridization analysis of *Paramecium* DNA restriction digests with *Rab7*-specific probe.
Paramecium DNA at 10 μg per lane was digested with *Eco*RI, *Xba*I, *Eco*RV, *Bgl*II, *Hin*dIII and *Pst*I, respectively, run in 0.8% agarose gel, transferred onto nylon filters and hybridized with the cloned *Paramecium Rab7a* gene as the probe labeled with digoxygenin-11-dUTP.

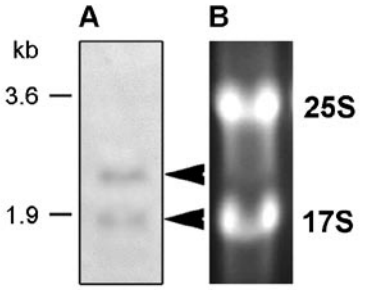

Figure 4. Northern blot analysis of *Paramecium Rab7* mRNA.
A. Total RNA (20 μg) was electrophoresed in a denaturing 1% agarose gel and transferred to a nylon membrane. Hybridization was carried out with digoxygenin-labeled *Paramecium Rab7b* cDNA as the probe. Two identified transcripts are indicated by arrows. **B**. Migration of ribosomal RNAs (25S and 17S) visualized by ethidium bromide staining performed prior to blotting and hybridization shown in A.

tion in this cell. The deduced amino-acid sequence displays 71.8% identity to *Paramecium* Rab7.

The prenylation motif (–CC) present in *Paramecium* Rab7 isoforms is the same as in *D. discoideum*, whereas other protozoa, *T. thermophila* and *Entamoeba histolytica* (Welter *et al.*, 2002), as well as yeast and mammals have a CXC motif (Pereira-Leal & Seabra, 2001). Isoforms of Rab7 (EhRab7) have recently been identified in *E. histolytica*, some of them with atypical C-terminus and no cysteine (Saito-Nakano *et al.*, 2005).

The overall predicted protein sequences of both *Paramecium* Rab7 isoforms are more similar to the mammalian counterparts than to Rab7 deriving from some parasitic protozoa. A survey of NCBI GenBank data revealed that two Rab7 sequences from the Kinetoplastida *T. cruzi* and *L. major* contain an insertion of 20 residues starting at position 147 that precedes the G4 domain (Denny *et al.*, 2002). Such an insertion is not present either in *Paramecium* or in evolutionary younger *D. discoideum* and mammalian Rab7 proteins (Fig. 5). Interestingly, this region of the gene is close to the hot spot for mutations in human Rab7 at Asp161/Thr and Val162/Met causing ulcero-mutilating neuropathy of Charcot-Marie-Tooth type 2B (Verhoven *et al.*, 2003; Houlden *et al.*, 2004). There is also a short deletion in a region preceding the G3 motif in Kinetoplastida Rab7 (Fig. 5) which is not present in Rab7 of *Paramecium* and those of higher eukaryotes (Denny *et al.*, 2002). This region may contribute to different location/function of Rab7 proteins (Dunn *et al.*, 1993). In fact, Araripe and coworkers (2004) localized Rab7 in *T. cruzi* at the Golgi apparatus using a specific anti-peptide antibody.

Two conserved motifs characteristic for Rab GTPases, RabF and RabSF (Pereira-Leal & Seabra, 2001), may be found in *Paramecium* Rab7 proteins (Fig. 5). Effectors and regulators of Rab proteins bind both to the RabF motifs in the switch I and II

Table 1. Comparison of the number of restriction sites in *Paramecium Rab7* genes with the number of hybridizing DNA fragments in Southern blot analysis shown in Fig. 3, performed with cloned *Rab7a* gene as the probe.

Restriction enzymes		*Eco*RI	*Bgl*II	*Hin*dIII	*Pst*I	*Eco*RV	*Xba*I
Number of	restriction sites in *Rab7a*	–	1	2	2	1	–
	restriction sites in *Rab7b*	–	–	1	–	–	–
	hybridizing DNA fragments	2	3	5	4	3	2

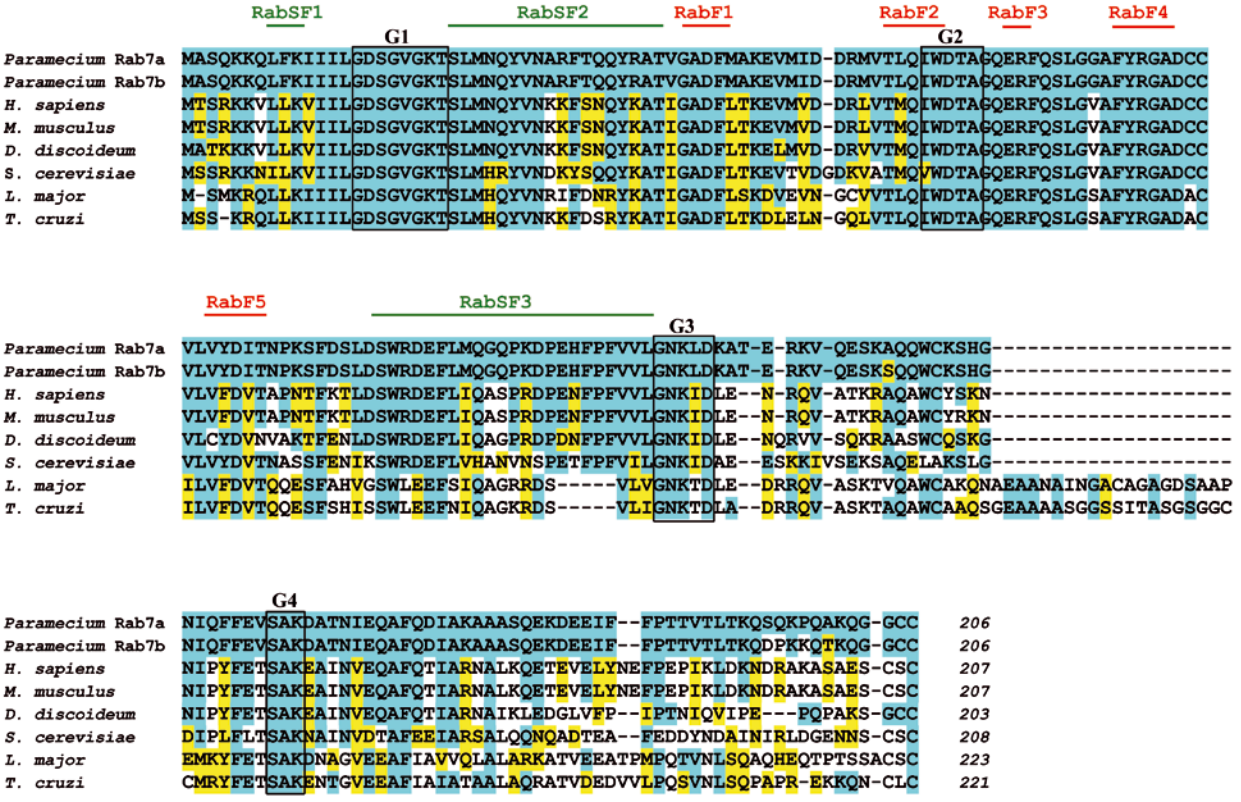

Figure 5. Multiple alignment of the deduced amino-acid sequences of *Paramecium* Rab7a (accession number AAL08054) and Rab7b (accession number AAW68046) with Rab7 proteins from different species using CLUSTAL W (1.82) program.
Homo sapiens (NP_004628), *Mus musculus* (NP_033031), *Dictyostelium discoideum* (AAA80152), *Saccharomyces cerevisiae* (P32939), *Leishmania major* (CAB75350) and *Trypanosoma cruzi* (AAD32707). Amino acids are shown in single-letter code. Identical residues are marked blue and similar ones — yellow. Four GTP binding sites (G1–G4) are boxed. Five Rab-specific motifs (RabF) and three Rab subfamily regions (RabSF) are indicated.

regions, to discriminate between active and inactive conformations, and to RabSF regions for specificity (Pereira-Leal & Seabra, 2001; Stenmark & Olkkonen, 2001).

Previously, when we cloned the effector domain of the first gene encoding *Paramecium* Rab7 (which later we defined as Rab7a), an antibody against human Rab7 was used for localization studies that revealed labeling of the phagosomal membrane in *Paramecium* (Surmacz *et al.*, 2003) and Rab7 colocalization with its effector protein, RILP (Rab-interacting lysosomal protein)*.

RILP induces recruitment of the dynein-dynactin motor to late endosomes/lysosomes and enables the movement of these organelles along microtubules (Jordens *et al.*, 2001). Thus, our result is consistent with the concept of Rab7 participation in the transport of internalized compounds from the early to the late endocytic/lysosomal compartment as observed in mammalian cells (Pfeffer & Aivazian, 2004). Now, when two genes encoding Rab7 in *Paramecium* have been identified, elucidation of the exact biological role of both isoforms will require an extensive proteomic analysis, bearing in mind that there are only five amino acid exchanges over the full length of the two polypeptides. It is worth noticing that four of those substitutions are located in the C-terminal hypervariable region that mainly specifies the location/function of Rab7 proteins (Chavrier *et al.*, 1991; Bruckert *et al.*, 2000; Moyer & Balch, 2001).

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