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Biochemical properties of the YPT-related *rab*1B protein

Comparison with *rab*1A

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We recently identified a novel rat cDNA: *rab*1B, closely related to the *rab*1A cDNA and to the yeast YPT1 gene. The *rab*1B cDNA encodes a 202 amino acid protein (22.1 kDa) that was produced in *Escherichia coli* under the control of the Φ 10 promoter for the T7 RNA polymerase. The *rab*1B protein was purified in large amounts to near homogeneity in a simplified procedure. We studied the biochemical properties of *rab*1B and *rab*1A proteins. They both bind specifically GTP and GDP and possess intrinsic GTPase activities. The *rab*1B Lys21→Met mutant protein does not bind GTP, whereas the Ala65→Thr mutant has a reduced GTPase activity and is competent for autophosphorylation in the presence of GTP.

GTP binding protein; Superfamily, *ras*; Secretion

1. INTRODUCTION

Many 21-25 kDa GTP binding proteins have been described recently. Most of them belong to the *ras* superfamily [1]. Among these, the *rab* proteins [2-8] constitute a distinct branch that comprises at least 10 members in mammals. Two related 23 kDa proteins have been characterized in yeast: YPT1 and SEC4 [9-11]. Recent reports have implicated these two yeast proteins as regulatory elements in the secretory pathways of *Saccharomyces cerevisiae*. SEC4 regulates the transport of secretory vesicles between the Golgi apparatus and the plasma membrane. The functional role of the YPT1 protein (YPT1p) is not clearly demonstrated. It appears to be localized in the Golgi and thought to be involved in secretion, cell growth, sporulation and regulation of cytoplasmic Ca^{2+} concentration [12-14].

Among the mammalian *rab* proteins, two share striking similarities with YPT1p and can be considered as its mammalian counterparts: *rab*1A [2,8] and *rab*1B [4]. They present respectively 75% and 66% amino acid identity with YPT1; this score reaches 95% and 92% in the N-terminal part of the protein (residues 1-110). *rab*1A and *rab*1B share 92% amino acid identity. The expression patterns of the two corresponding genes in adult mouse tissues and in several cell lines such as CHO cells and MRC 5 fibroblasts are similar. However, *tab*1B RNAs are always found in smaller amounts than *rab*1A RNAs (B. Olofsson, personal communication).

We have previously described the purification of the bacterially produced *rab*1A protein [6]. Here we report the purification of a bacterially produced *rab*1B protein. The protein could not be solubilized from bacterial membranes when using non-ionic detergents; we, therefore, developed a method comprising an alkaline treatment step. The GTP binding and GTPase activities of the *rab*1A and *rab*1B proteins were compared. We also introduced mutations in the *rab*1B cDNA leading to mutant proteins with altered capacities for GTP binding, GTP hydrolysis and autophosphorylation.

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2. EXPERIMENTAL

2.1. *Expression of the* rab*1B protein in* E. coli *and purification*

An *Nde*I restriction site was introduced by site directed mutagenesis upstream the ATG initiator codon of the *rab*1B open reading frame. The following oligonucleotide was used: 5′ CGCTGCCGCCATATGAACCCCGAA 3′. Mutation was confirmed by DNA sequencing. The resulting 1 kb *Nde*I *Bam*HI fragment containing the whole coding sequence for *rab*1B was cloned in pET3C vector digested with *Nde*I and *Bam*HI. This construction was used to transform the *E. coli* strain BL21(DF3) pLys S [23]. Bacteria were grown in LB medium containing 100 μg/ml ampicillin and 12.5 μg/ml chloramphenicol. They were then diluted to 1:50 in LB medium containing 50 μg/ml ampicillin for 1 h followed by induction with 200 μM IPTG for 5 h. Clones expressing the *rab*1B protein were identified by Coomassie blue staining. Mutant proteins were produced and purified using the same procedure. The following oligonucleotides were used: for the Lys21 → Met mutant: 5′ GTGGGCCATGTCATGCC 3′; for the Ala65 → Thr mutant: 5′ GGGACACTACTGGTCAGG 3′. Mutations were confirmed by DNA sequencing.

For large scale preparation, bacteria from a 5 l culture were harvested by centrifugation to yield 15 g cell paste. A solubilization procedure according to the method described by Tucker et al. [17] was carried out. This method allowed to solubilize each of the other bacterially produced *rab* proteins, but in the case of *rab*1B, the protein was still present in the pellet. Insoluble materials wre resuspended in 20 ml buffer B and brought to alkaline or acid pH by adding drops of NaOH or HCl. At pH 10.5 the aspect of the suspension appeared modified. It was centrifuged for 10 min at 10000 rpm; most of the *rab*1B protein was then found in the supernatant. The supernatant was brought to 70% saturation with ammonium sulfate. The precipitate was recovered by centrifugation, dissolved in 5 ml of buffer B and centrifuged for 5 min at 10000 rpm. The resulting supernatant was dialysed against the same buffer at 4°C for 12 h. Protein concentration was determined by the method of Lowry et al. [24] using bovine serum albumin (BSA) as standard.

GTP binding and GTPase activities were performed as previously described [6].

3. RESULTS

3.1 *Expression and purification of the* rab*1B protein*

We constructed by site directed mutagenesis an *Nde*I restriction site upstream the coding region of the *rab*1B cDNA so as to have the ATG from the *Nde*I site (CATATG) serving as the translation initiation codon. The modified cDNA was inserted into an *E. coli* expression vector using the Φ 10 promoter from T7 RNA polymerase: pET3C provided by W. Studier [16]. The estimated amount of *rab*1B protein produced was ≈5% of the total *E. coli* proteins. Purifications of all the *rab* proteins produced previously in bacteria involved a first step of solubilization from bacterial structures (by sodium deoxycholate) followed by a two column-chromatography procedure [6,17]. For *rab*1B, this procedure allowed solubilization of most of the *E. coli* proteins but not of the foreign *rab*1B protein, found in the pellet (fig.1A). Insoluble materials were resuspended in buffer B and gradually brought to acid or alkaline pH. At pH 10.5, a partial dissolution of the pellet was observed. The

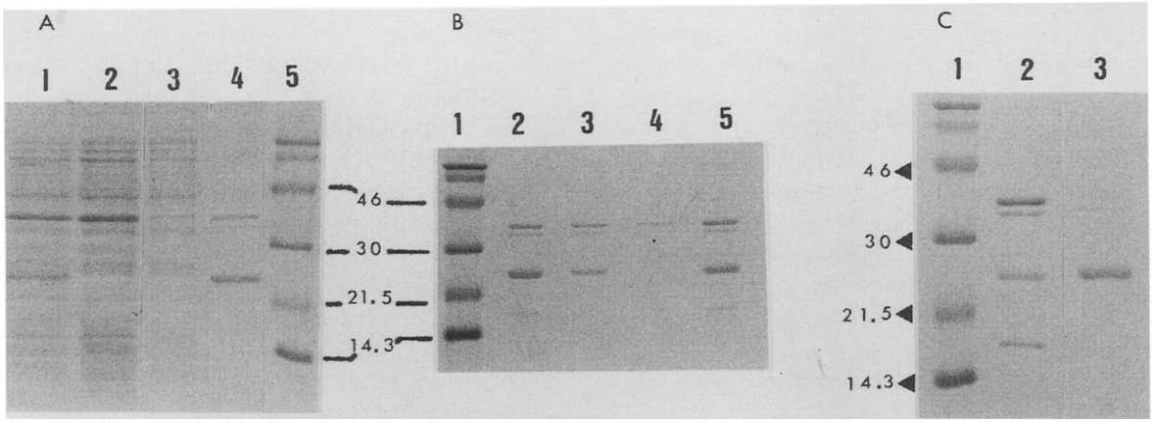

Fig.1. Purification of the bacterially produced *rab*1B protein. (A) Total proteins from induced (1) and non-induced (2) producing strains, proteins from the supernatant (3) and the pellet (4) obtained after the first step of solubilization using non-ionic detergents. (B) Proteins from the supernatant (2,4) and the pellet (3,5) obtained after alkaline (2,3) or acidic (4,5) treatments. (C) Proteins from the insoluble (2) and soluble (3) fractions following ammonium sulfate precipitation. Samples were analysed by SDS-PAGE with 15% polyacrylamide gels and stained with Coomassie blue. Molecular masses are indicated in kDa.

solution was centrifuged and the soluble phase immediately neutralized (fig.1B). It contained ≈75% of the *rab*1B protein. After precipitation by ammonium sulfate, the precipitate was resuspended, clarified by centrifugation and extensively dialysed as indicated in section 2. This procedure allowed one to obtain large amounts of *rab*1B protein with a purity higher than 95% (fig.1C) and was also used for the purification of the mutant proteins. However, the *rab*1B^{Met21} mutant was very unstable during the purification process and thus obtained in smaller amounts.

3.2. *GTP binding abilities of the* rab*1A and* rab*1B proteins*

Amino acid sequences of the *rab*1A and *rab*1B proteins in the 4 domains presumed to be involved in the binding of GTP/GDP are very conserved (fig.2). We investigated their respective GTP binding abilities by a filter assay procedure. Conditions for binding were determined by selecting optimal conditions for factors known to affect the binding properties of other well characterized G proteins [18] (fig.3). For *rab*1A, optimal binding was observed in the presence of 1 mM EDTA and low

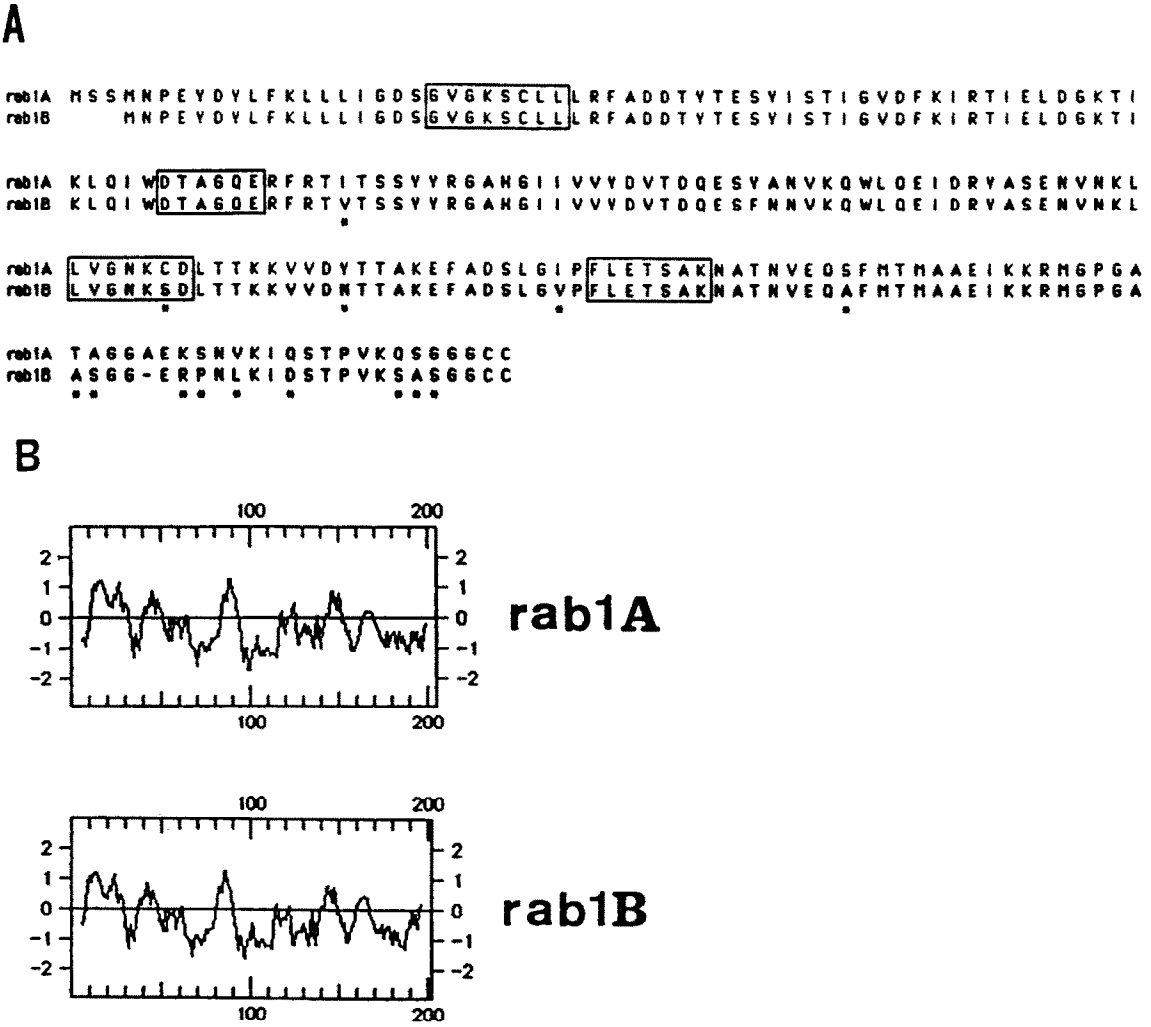

Fig.2. (A) Alignment of the *rab*1A and *rab*1B protein sequences. The *rab*1B sequence is deduced from the cloned rat cDNA. The *rab*1A amino acid sequences deduced from the human and rat cDNA sequences were identical. *, Differences between the two sequences. The 4 regions involved in the binding of GTP/GDP are boxed. (B) Hydrophobicity patterns of the *rab*1A and *rab*1B proteins using the Kyte and Doolittle dot matrix.

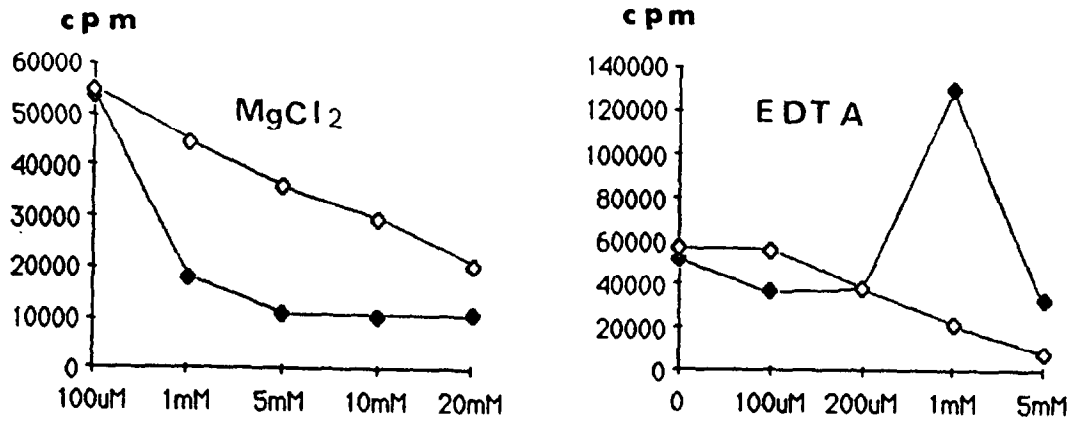

Fig.3. Mg^{2+} ion and EDTA effects on binding of GTP to the *rab*1A (♦) and rab1B (◇) proteins. Purified proteins were incubated for 20 min at 37°C in [α^{32}P]GTP containing buffer in the presence of the indicated concentrations of $MgCl_2$ or EDTA. Fixed radioactivity measured by a filter assay is indicated in counts per min. Results are the mean of 3 independent experiments.

Mg^{2+} concentrations whereas, in the case of *rab*1B, the optimal binding was observed in the absence of EDTA and with low Mg^{2+} concentration. These results are clearly different from those observed for the *ras* and the Aplysia *rho* proteins [19]. Various EDTA concentrations had no effects on both *rab*1A and *rab*1B GTP binding capacities (data not shown). N-ethylmaleimide (NEM) has been shown to inhibit the GTP binding ability of some members of the *ras* superfamily [20,21]. Low (1 mM) or high (10 mM) concentrations of NEM do not change the GTP binding properties of the *rab*1A and *rab*1B proteins. The GTP binding of either *rab*1A or *rab*1B proteins was not efficiently competed by ATP, UTP, CTP or TTP (less than 10% inhibition was observed) whereas GTP, GDP or GTPγS were very efficient competitors (fig.4). These results suggest that the *rab*1 proteins specifically bind GTP and GDP. The Lys21 → Met mutant protein has no detectable capacity to bind GTP. The Ala65 → Thr mutant is not affected in its GTP binding capacity.

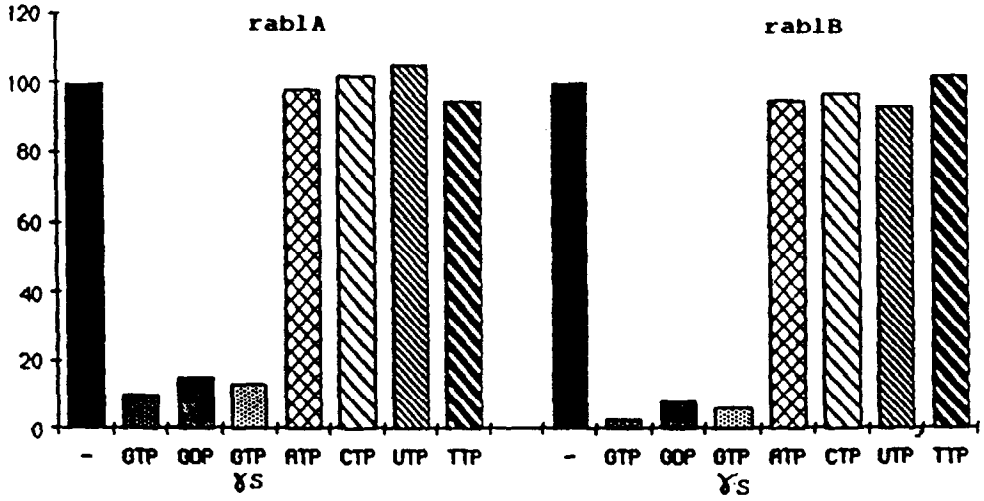

Fig.4. Competition of GTP binding to *rab*1A or *rab*1B proteins. Purified *rab*1A or *rab*1B proteins (1 μM final concentration) were incubated for 20 min at 37°C with [α^{32}P]GTP in the absence or presence of the indicated competing nucleotides (100 μM final concentration). Results are the mean of 2 independent experiments.

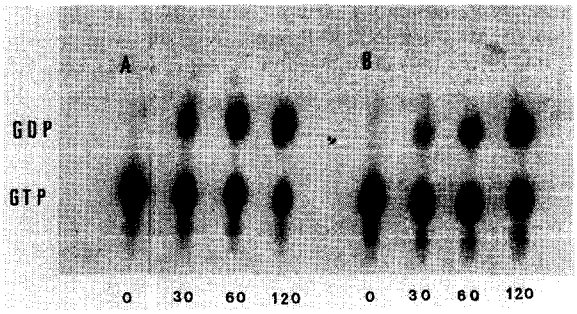

Fig.5. GTP hydrolysis by the *rab*1A (A) and *rab*1B (B) proteins. Purified proteins (1 μM) were incubated for 10 min with [α^{32}P]-GTP in the presence of 1 mM EDTA. The GTPase activity was started by addition of 10 mM $MgCl_2$. Aliquots were removed from the incubation mixture at the time points indicated. Samples were loaded on PEI plates and GTP and GDP separated by chromatography.

3.3. *GTPase activities of the* rab*1A and* rab*1B proteins*

Equal amounts of 'active proteins', as determined by their GTP binding abilities, were incubated with [α^{32}P]GTP. GTP hydrolysis was followed by the generation of GDP for up to 2 h (fig.5). GDP spots were excised and radioactivity measured (fig.5C). The rate of GTP hydrolysis (expressed in mol GTP hydrolysed per mol protein in 1 min = min^{-1}) was estimated for *rab*1A and *rab*1B to respectively 0.009 min^{-1} and 0.006 min^{-1}. This rate is comparable to that observed for the wild type yeast YPT protein [22]. The GTPase activity of the *rab*1B^{Thr65} mutant protein was reduced six-fold (fig.6) as compared to that of the wild type.

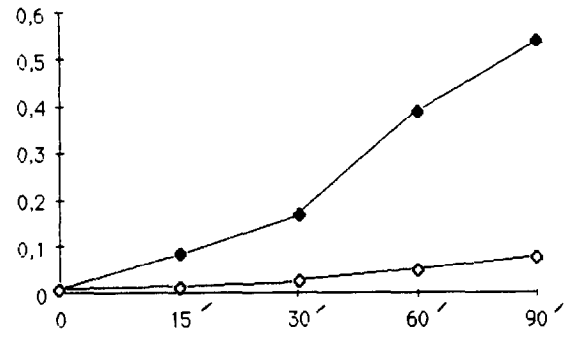

Fig.6. Kinetics of GTP hydrolysis. GTP hydrolysis catalysed by the normal (♦) and mutant^{Thr65} (◇) *rab*1B proteins was followed as described previously. Spots corresponding to GDP were excised from the PEI plates and counted.

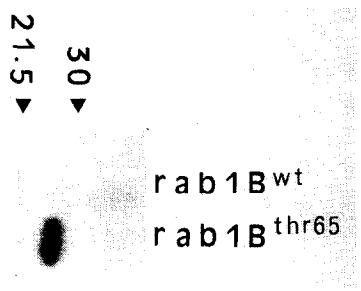

Fig.7. Autophosphorylation of the *rab*1B^{Thr65} mutant proteins. 1 μg of purified wild type and mutant proteins were incubated at 37°C with [α^{32}P]GTP (3 μM final concentration) and subjected to 15% SDS-PAGE. The gel was dried and autoradiographed.

As is the case for the *ras* proteins, the Ala65 → Thr substitution confers an autophosphorylating activity to the *rab*1B protein (fig.7).

4. DISCUSSION

We recently isolated two cDNAs: *rab*1A and *rab*1B coding for related products homologous to the yeast YPT1 protein. The *rab*1A and *rab*1B amino acid sequences contain the 4 conserved regions that have been shown, in the case of p21 *ras*, to interact with the guanine nucleotides. The *rab*1A protein was produced in *E. coli*, its GTP binding capacity and its intrinsic GTPase activity were demonstrated [6]. Here we report the production and purification of the *rab*1B protein and compare its biochemical properties to those of *rab*1A. The two proteins were produced by using cDNAs from different species: human for *rab*1A and rat for *rab*1B. However the rat and human *rab*1A cDNAs code for identical amino acid sequences and thus the *rab*1A and *rab*1B proteins may be compared as products from the same species. *rab*1B is also a GTP binding protein and possesses an intrinsic GTPase activity. We investigated the effects of several factors, known to modify the properties of some GTP binding proteins, on *rab*1A and *rab*1B. Both are sensitive to high Mg^{2+} concentrations. In contrast, it was shown that the GTP binding capacities of bacterially produced *ras* and *rho* proteins are enhanced by Mg^{2+} (maximum binding at 0.6 mM)

[19]. More surprising are the effects of EDTA on the GTP binding capacity of *rab*1A: maximum binding is observed at 1 mM concentration. This result was reproducible with proteins obtained from 3 distinct purification procedures. Neither EGTA nor NEM affected the GTP binding properties of *rab*1A and *rab*1B proteins. NEM is a thiol specific reagent that was shown to inhibit the binding of *rab*1A and K – *ras* but not of *rab*3A proteins [20,21]. Lys21 → Met and Ala65 → Thr mutations in the *rab*1B proteins confer properties similar to those obtained with the corresponding mutations in the YPT1 protein.

Rates of GTP hydrolysis were slightly different for *rab*1A and *rab*1B: 0.009 min^{-1} and 0.006 min^{-1}, respectively. For *rab*B^{Thr65} it was measured to 0.0009 min^{-1}. Estimated rates were described for P21 *ras*: 0.02 min^{-1}; *rho*: 0.1 min^{-1}; yeast YPT1: 0.006 min^{-1}; Gly^{17} mutant YPT1: 0.05 min^{-1}; Thr^{65} mutant YPT1: 0.0015 min^{-1} and *rab*3A (smg25A): 0.04 min^{-1} [19,21,22]. The difference between *rab*1A and *rab*1B can be considered as minimal when compared to the variations observed for the other members of the *ras* superfamily.

One might argue that *rab*1B could be denaturated during the specific purification procedure and greatly affected in its biochemical properties. However, when the GTP binding assays for *rab*1A and *rab*1B were performed at pH 11, similar decreases in binding ($\approx$ 15%) were observed (data not shown). We may thus conclude that there is no striking difference in the GTP binding and GTPase activities of the bacterially produced *rab*1A and *rab*1B proteins. In vivo, there could exist different post-translational events modulating the properties of the proteins. This raises the question of the specific functional role of two highly homologous proteins expressed in the same cell. For many proteins of the *ras* superfamily, the C-terminal end is thought to specifically interact with membrane structures. The sequences and hydrophobicity patterns (fig.2b) of the *rab*1A and *rab*1B proteins are almost identical except in the C-terminal end. A putative explanation could be that these two proteins are identical in most of their biochemical properties but may have different subcellular localizations while playing related functions.

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