Small GTP-binding proteins and regulated secretion of von Willebrand factor by endothelial cells

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CHAPTER 3

ALTERNATIVE SPLICING OF A DUPLICATED EXON GENERATES TWO ISOFORMS OF RAB6A


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ABSTRACT

The rab subfamily of small GTPases plays an essential role in the targeting of transport vesicles to their appropriate acceptor membrane. They control a variety of transport events in the biosynthetic and endocytic pathways by alternating between GDP- and GTP-bound conformation. One of the subfamily members, rab6a, is involved in retrograde transport of vesicles from the Golgi apparatus towards the ER. Here, we present the identification of an alternatively spliced variant of rab6a. Using a RT-PCR approach, we identified a novel rab6-like sequence and isolated the corresponding cDNA. The nucleotide sequence was identical to rab6a except for a stretch of approximately 100 bp, which contained several nucleotide substitutions. We show that the observed differences in nucleotide sequence originate from alternative splicing of two highly homologous exons within the rab6a gene. Incorporation of either of the two exons generates similar but distinct isoforms of rab6a, which differ in only three amino acid residues. Both rab6a and its isoform, termed rab6a', were ubiquitously expressed. Equal levels of rab6a and rab6a' mRNA were found in various human tissues and cell lines. Analysis of the biochemical properties revealed that both rab6a variants bound efficiently to GTP. Morphological analysis of HeLa cells expressing either epitope-tagged rab6a or rab6a', revealed that both proteins were mainly localized to the Golgi apparatus. Taken together, our results indicate that constitutive alternative splicing events in the rab6a gene generate two rab6a isoforms with similar functional properties.

INTRODUCTION

Vesicular transport along biosynthetic and endocytic pathways requires coordinated activity of a large number of protein families. One of these, the rab subfamily of small GTPases, is thought to play an important role in unidirectional transport of vesicles to their correct subcellular destination (Novick and Zerial, 1997). Of the many rab subfamily members identified so far in a variety of eukaryotic cells, rab6a has been found to play a major role in intra-Golgi transport (Martinez et al., 1994; Li and Warner, 1996). Rab6a is ubiquitously expressed and associates with Golgi and trans-Golgi network membranes (Goud et al., 1990; Antony et al., 1992). Using chimeric ras-rab proteins, it was found that residues in the N-terminal region of rab6a were required for targeting of rab6a to the Golgi apparatus (Beranger et al., 1994). Furthermore, anti-rab6a antibodies inhibited transport between cis and medial Golgi cisternae in an in vitro assay (Mayer et al., 1996). Extensive studies with GTP- and GDP-bound mutants of rab6a pointed towards a role for rab6a in a microtubule dependent retrograde membrane traffic within the Golgi (Martinez et al., 1997; McConlogue et al., 1996). Rab6a binds to microtubules through rabkinesin-6, which thereby may control membrane dynamics and directional of vesicular transport (Echard et al., 1998). Limited information is available about the structure of the rab6a gene. The human rab6a gene has been localized to chromosome 2q14-q21 by in situ hybridization (Rousseau-Merck et al.,
The mapping of rab subfamily members showed that their genes are distributed throughout the genome (Barbosa et al., 1995). So far, the genomic organizations of four rab genes have been reported. The protein coding regions of rab1, rab3a, rab11b and S10 are divided into 2 to 6 exons (Wichmann et al., 1989; Baumert et al., 1993; Lai et al., 1994; Zheng et al., 1997). Interestingly, conservation of the positions of exon/intron boundaries was not observed suggesting that genes encoding small GTPases of the rab subfamily diverged at an early step of evolution (Iwabe et al., 1996).

Here, we report on the alternative splicing of two homologous exons within the rab6a gene, which results in expression of two isoforms of rab6a. Functional analyses revealed that both proteins have a high affinity for GTP and are localized to the Golgi apparatus. Possible implications of our findings for the role of the two rab6a isoforms in vesicular transport are discussed.

**MATERIALS AND METHODS**

*Library screening and DNA sequencing*

By a RT-PCR based cloning approach with primers matching conserved domains PM3 and G2 of rab proteins, a rab6a-like cDNA fragment (Figure 1a, product 2) was isolated from human umbilical vein endothelial cells (HUVEC)(de Leeuw et al., 1998). The same procedure was carried out on human colon carcinoma cells (Caco-2), but with primers corresponding to the conserved GTP-binding regions PM1 and PM3 of the family of rab proteins (5'-GGN(A20,G80)(G20,A80)NNN(A20,G20,C60)NNNN(A20,T20,G60)(G20,C20,T60)(A20,T80)GG NAA(A/G)(A/T)C-3' and 5'-'TTC(C/T)TGNCC(A/T)GCNGT(A/G)TCCCA-3' respectively). The latter RT-PCR also resulted in a rab6a-like sequence (see results, Figure 1a, product 1). Product 2 was radiolabeled by random oligonucleotide priming (Feinberg and Vogelstein, 1983) and used as a probe to screen a human umbilical vein endothelial cell cDNA library consisting of approximately 8×10^4 independent clones. The nucleotide sequence of one isolated clone corresponding to the rab6a-like sequence was sequenced using T7 polymerase (Pharmacia-LKB, Roosendaal The Netherlands).

*Isolation and analysis of genomic DNA*

To analyze the genomic organization of rab6a, the following primers (Eurogentec, Belgium) were used: 5'→3', primer 1: ACAGTACGATTGCAATTA, primer 2: TCGTAAACTACTACAGCTG, primer 3: AATCAGGCTTCAGCTG, primer 4: CCACAGTGAGTCAGCA, primer 5: CAGGCACGAAATGGC, primer 6: ATCCCAGTTTTAGGTTC. Combinations of primers (400 pmol) were used to perform a PCR on human genomic DNA (100 ng) in a 50 µl volume in the presence of 1×PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1.5 mM MgCl₂, 200 µM of dNTPs and 5 units of Taq polymerase. The reactions were overlaid with 20 µl mineral oil, transferred to a thermal
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cycler (Perkin Elmer, Norwalk, USA) and incubated for 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 6 min with a final extension at 72 °C for 10 min. The PCR samples (10 µl) were loaded on a 2% agarose gel. For sequencing, the amplified products were extracted from the gel using a gel extraction kit (Qiagen GMBH, Hilden, Germany) and cloned into the vector pGEM-T (Promega, Madison, WI, USA).

**RNA isolation and cDNA synthesis**

Total RNA from various frozen human tissues and cultured cell lines was extracted employing standard procedures (Chirgwin et al., 1979). About 1.5 µg RNA was reverse transcribed to cDNA using random hexamers (2 µg; Pharmacia LKB, Roosendaal, The Netherlands). The coding regions of rab6a and rab6a' were amplified using the following primers: forward 5'-ATGTCCACGGGCGGA-3' and reverse 5'-CTGAAGAAGGTGGAAGATG-3'. Reaction conditions were 35 cycles of 94 °C for 1 min, 50 °C for 30 s, and 72 °C for 1 min. Relative amounts of rab6a and rab6a' were determined following digestion with PstI.

**Cells, media and cell culture**

For RNA isolation, Caco-2 TC7 cells were cultured in DMEM (Gibco/BRL, Breda, The Netherlands), supplemented with 20% fetal calf serum (FCS) and 1% non-essential acids (Gibco/BRL) and harvested one day after seeding (log phase), at 70% confluence (undifferentiated cells) or 5 days after reaching confluence (differentiated stadium). For differentiation of HT-29 cells, cells were grown without D-glucose (Darmoul et al., 1992). BeWo-b24 (choriocarcinoma; ATCC number CCL-98) and other cell lines used were grown in DMEM supplemented with 10% FCS. MEN-1 (mesothelial cells) and fibroblasts were cloned in our laboratory. HeLa cells were grown in DMEM, supplemented with 10% FCS, 1% non-essential acids, L-glutamine, sodium pyruvate (Gibco/ BRL) and 0.001% β-mercaptoethanol at 37 °C, 10% CO₂.

**Transient expression and Western blot detection**

Human rab6a and rab6a' proteins were expressed using the eukaryotic expression vector pcDNA3 (Invitrogen, Leek, the Netherlands). Epitope-tagged rab6a and rab6a' cDNAs were constructed by insertion of the c-myc epitope at amino terminus of both isoforms. HeLa cells were transfected using lipofectamine according to the instructions of the manufacturer. Transfected cells were cultured for 48 hours in 10-cm dishes. Cells were harvested by scraping in ice-cold PBS, pelleted by centrifugation (1000xg, 5 min), resuspended in 50 µl of PBS and directly lysed in 2x sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% (v/v) glycerol). Protein lysates were analyzed on a 12.5% (v/v) polyacrylamide gel and transferred to nitrocellulose membranes. The immuno blot was incubated with monoclonal antibody 9E10, directed against the c-myc epitope. Immunostained proteins were visualized using CPD Star chemiluminescence according to instructions provided by the manufacturer (Tropix, Bedford, MA, USA).
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Immunofluorescence
Transfected HeLa cells were cultured in 24-wells plates on glass coverslips. After 48 h, cells were washed with PBS, fixed in 1% paraformaldehyde for 1 h at room temperature and washed twice in PBS/0.05% Tween 20 (PBST). Cells were incubated with monoclonal antibody 9E10 and a polyclonal antibody directed against CTR433, a medial Golgi marker, for 1 h at room temperature and washed four times for 5 min in PBST. Coverslips were subsequently incubated with fluorescein-conjugated goat anti-mouse IgG in PBST for 1 h. Cells were mounted in Mowiol (Sigma, St Louis, MO, USA) and examined by confocal laser scanning microscopy (MRC 1000, Bio-Rad, Veenendaal, The Netherlands).

Expression of GST fusion proteins in E.coli
Constructs comprising the cDNA of GST and human rab6a or rab6a' within a single open reading frame were prepared by subcloning the rab sequences into the BamH1/Xho1 sites of the vector pGEX (Pharmacia-LKB, Roosendaal, The Netherlands). Clones expressing rab6a or rab6a' were grown to an OD$_{600}$ of 0.3-0.5 at 37 °C and induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 18 h at 30 °C. Cells were centrifuged, frozen, thawed, and lysed by sonication for 15 s on ice. After treatment with 1% Triton X-100, the lysates were spun at 10,000 rpm for 10 min, and the supernatant was collected. GST-fusion proteins were adsorbed on glutathione-Sepharose by a batch procedure, and eluted with 10 mM glutathione in 10 mM Tris/HCl (pH 7.4).

Analysis of GTP-binding properties of GST fusion proteins
Purified protein samples (1 µg/sample) were separated using 12.5% (v/v) SDS-polyacrylamide gelelectrophoresis. Gel was stained with Comassie Brilliant Blue, or proteins were transferred onto nitrocellulose membranes. The blot was incubated with 1 nM [$\alpha$-32P]GTP (1 µCi [$\alpha$-32P] GTP/ml) as described previously (Gromov and Celis, 1998). GTPγS-binding properties were determined as follows: Samples (500 ng assay) were diluted to 30 µl with 20 mM Tris/HCl buffer (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Triton X-100. To each sample 30 µl of GTPγS-binding mix (20 mM Tris/HCl buffer, pH 8.0, 1 mM EDTA, 2 mM dithiothreitol, 2 µM GTPγS, and ~1.5×10$^7$ cpm of [35S]GTPγS) was added. Nonspecific binding was assayed with samples containing 0.1 mM unlabeled GTPγS. The samples were incubated at 30 °C for 0, 5, 15, 30, 60, or 120 min and reaction was terminated by addition of 2 ml of ice-cold washing buffer (20 mM Tris/HCl, pH 8.0, 25 mM MgCl$_2$, 100 mM NaCl). The samples were filtered through nitrocellulose membranes (NC45, Schleicher & Schuell, Dassel, Germany), subsequently washed four times in ice-cold washing buffer, air dried, and counted in a water-compatible scintillation mixture (Opti-Fluor, Packard, Meriden, CT, USA). All samples were assayed in duplo.
RESULTS

Isolation of a cDNA sequence highly homologous to rab6a

As a preamble to study the involvement of rab proteins in the regulation of vesicular transport in human epithelial Caco-2 cells and umbilical vein endothelial cells (HUVEC), an RT-PCR was carried out with oligonucleotides corresponding to conserved domains of small GTP-binding proteins. Primer sets corresponding to conserved domains PM3 and G2 involved in GTP-binding (nomenclature according to Valencia et al., 1991) revealed the presence of a large repertoire of small GTP-binding proteins in HUVEC (de Leeuw et al., 1998). Also in Caco-2 cells a variety of partial sequences encoding rab proteins were identified, but with primers corresponding to domains PM1 and PM3 involved in nucleotide binding (manuscript in preparation). Both independent analyses revealed the presence of a partial cDNA (Figure 1a, product 1 from Caco-2 cells, product 2 from HUVEC) which showed high homology to rab6a. Using product 2 as a probe, we isolated a full-length cDNA from a human endothelial cell cDNA library. Coding DNA sequences revealed several nucleotide substitutions when compared to the rab6a sequence. Nucleotide substitutions in the rab6a-like sequence, termed

![Diagram](image)

**Figure 1.** Alignment of nucleotide sequence (A) and deduced amino acid sequence (B) of rab6a and rab6a′ in their non-identical region.
(A) Domain structure of small GTP-binding proteins. The putative consensus domains for phosphate/magnesium binding (PM1-3) and guanine nucleotide binding (G1-2) are depicted as black boxes. Product 1 and 2, generated by RT-PCR with two sets of degenerate rab specific primers amplified two distinct products flanking domain PM3, are indicated. Partial sequences of rab6a and rab6a′ cDNA were aligned. Differences in nucleotide sequences are indicated. Nucleotide sequences resulting in amino acid substitutions are depicted in bold. (B) Comparison of partial amino acid sequences of rab6a and rab6a′. Differences in amino acid sequences are depicted in bold. Region PM3, involved in nucleotide binding is underlined.
rab6a', were restricted to one region of approximately 100 bp (Figure 1a). These nucleotide substitutions resulted in 3 amino acid substitutions in the open reading frame of rab6a' when compared to rab6a. One conservative substitution (V62I) and two non-conservative changes (T60A and V65A) are positioned in the proximity of domain PM3 involved in GTP-binding.

**Figure 2.** Genomic PCR-strategy reveals alternatively spliced exons in the rab6a gene.

(A) Schematic drawing of the proposed genomic organization of rab6a, which results in two alternative spliced forms; i.e. rab6a and rab6a'. Arrows and numbers represent cDNA-based primers, constructed to reveal exon/intron positions. Primer 3 and 2 are sequence specific for rab6a'; primer 1 and 4 are specific for rab6a sequence; primers 5 and 6 are derived from regions that are identical in rab6a and rab6a' cDNA. Putative exons are depicted as black boxes. (B) PCR on human genomic DNA with different combinations of primers. Lane 1-4: Amplification with the indicated sets of primers of rab6a' cDNA. Lane 5-13: Amplification of genomic DNA using sets of primers that are indicated below the figure. (C) Partial sequence of the rab6a gene. Nucleotide sequence correspond to primers used for amplification are underlined. A unique PstI restriction present in the Rab6a' exon is depicted in bold.
Alternativ e splici ng of a dupl icated exon generates two isoforms of Rab6a

(Figure 1b).

**A duplicated exon is present within the rab6a gene**

Since nucleotide substitutions were confined to a region of 100 basepairs, we hypothesized that expression of the rab6a' sequence results from alternative splicing within the rab6a gene. Thus, the restricted differences in nucleotide sequences found between rab6a and rab6a' might represent two homologous but distinct exons present in the rab6a gene. Mutually exclusive incorporation of one of these exons would then generate either rab6a or rab6a'. According to this model, introns should flank both exons and one intron should be present in between the two homologous exons (Figure 2a). To investigate the organization of the rab6a gene, we used a PCR-based strategy. Specific oligonucleotide primers were designed based on the cDNA sequences of rab6a (primer 1 and 4) and rab6a' (primer 2 and 3; Figure 2a). In addition, two oligonucleotide primers, corresponding to regions flanking the stretch of 100 bp that differs between the two rab6a isoforms were used (Figure 2a, 5 and 6). Different combinations of primers were used to amplify part of the rab6a gene using human genomic DNA as template. Primer sets 1/4 and 2/3 both amplified a product of approximately 100 bp. These results indicate that the stretch of 100 bp that differs between the two rab6a isoforms is contained within a single exon for each of two variants (Figure 2b, lane 5 and 6). Primer combinations 1/2 and 3/4 should determine the order in which these two exons are positioned in the rab6a gene. As can be seen in Figure 2b, combination 3/4 amplified a band of about 250 bp (lane 8), whereas primers 1 and 2 did not yield detectable amounts of product (lane 7). This suggests that the exon for rab6a' precedes the rab6a exon and that both exons are separated by a short intron. Primer 5 or 6 in combination with the specific rab6a or rab6a' primers resulted all in an amplified product of 2 kb or more (lanes 10, 11, 12 and 13). This indicates the presence of long introns of about 2 kb flanking the duplicated exons. Combination 5 and 6 did not yield a product, probably due to the inability of Taq polymerase to amplify a product calculated to be approximately 4.5 kb. To confirm the proposed organization of the rab6A gene we determined the sequence of the obtained PCR products. Sequence analysis revealed the presence of two homologous but distinct exons, separated by an intron of 66 bp (Figure 2c). The rab6a' exon is positioned upstream of the rab6a exon and both are flanked by an intron of about 2 kb. The exon/intron boundary sequences conform to the GT-AG rule (Krawczak et al., 1992).

**The rab6a and rab6a' exon are subjected to constitutive alternative splicing**

In the previous paragraph, we have shown that alternative splicing may generate two rab6a isoforms. We investigated whether both rab6a isoforms are expressed in a tissue and/or cell type specific manner. Primers, corresponding to the 5' and 3' UTR regions were used to amplify rab6a and rab6a' from cDNA obtained from a variety of adult human tissues and cell lines. We made use of an unique PstI restriction site, specifically present in the rab6a' cDNA (Figure 2c). to discriminate between both rab6a isoforms. The undigested amplified product, containing one or both isoforms was present in all tissues and cell lines examined (Figure 3, PstI -). Digestion of amplified products resulted in three bands (Figure 3, PstI +); the upper
band corresponds to the undigested rab6a cDNA (675 bp) and the two lower bands (405 and 270 bp) represent rab6a’ cDNA. This result shows that rab6a and rab6a’ are co-expressed in all tissues and cell lines examined. Interestingly, no differences in expression levels between both isoforms were observed. To test for possible differentiation-dependent expression of rab6a isoforms, their presence was examined in non-differentiated and differentiated epithelial Caco-2 and HT29 cells (Figure 3, lanes 21 and 23, and lanes 17, 19 and 25 respectively). Also in these cells, no differences in the amount of rab6a and rab6a’ mRNA was observed.

![Figure 3](image_url)

Figure 3. Levels of rab6a and rab6a’ mRNA in various human tissues and cell lines. A RT-PCR with 5' and 3' UTR encoding primers was performed on total RNA isolated from various adult human tissues and cell lines. The amplified products of ~675 bp (Pst1 -) were digested with Pst1 (Pst1 +), a restriction site present in rab6a’ but not in rab6a cDNA (see Figure 2, bold). Both digested fragments (405 bp and 270 bp) represent rab6a’ and the undigested product corresponds to rab6a. Control PCRs and digestions were performed on rab6a and rab6a’ cDNA (Lane 2-5).

GTP-binding properties of rab6a and rab6a’

The three amino acid changes that were found between the rab6a and rab6a’ proteins flank domain PM3, which is highly conserved in the ras superfamily of small GTPases (Figure 1b). This region has been indicated to change dramatically in conformation upon binding to GTP and GDP (Schweins and Wittinghofer, 1994). Therefore, we examined the GTP-binding properties of rab6a and rab6a’. GST-fusion proteins of rab6a and rab6a’ were expressed in E.coli. The recombinant fusion proteins were partially purified by glutathione-Sepharose. On SDS-PAGE, the fusion proteins appeared as a single band migrating at an apparent molecular weight of approximately 49 kDa, which corresponds to the calculated molecular weight for GST-rab6a and GST-rab6a’ (Figure 4a). GTP-overlay indicated that rab6a and rab6a’, and not GST alone, bound [γ-32P]GTP in a specific manner (Figure 4b). To elucidate the binding affinity to GTP, we used the fusion proteins in a GTP-binding assay with the use of nonhydrolyzable radiolabeled GTPγS (Figure 4c). The experiments illustrated that recombinant rab6a and rab6a’ bound [35S]GTPγS in a saturable and specific manner. The time
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Figure 4. GTP-binding assays show similar affinity of rab6a and rab6a' for GTP.
GST fusion proteins were prepared for rab6a and rab6a'. One μg of purified GST-rab6a and GST-rab6a' were subjected to SDS-PAGE. (A) Gel was stained with Comassie brilliant blue or (B) blotted and incubated with 1 nM [α-32P]GTP. (C) Time-course of [35S]GTPγS-binding to rab6a and rab6a'. About 500 ng of GST-rab6a (△) and GST-rab6a' (+) were incubated with [35S]GTPγS at 30 °C. At indicated times, the binding activities were measured as described in Materials and Methods. As a control, GTPγS-binding was measured to GST (×) alone or GST-rab6a in the presence of an excess of unlabeled GTPγS (▼). The results show the means of two independently performed assays. (D) Myc-tagged rab6a and rab6a' were expressed in HeLa cells. Protein lysates were analyzed by immunoblotting with anti-myc 9E10 antibody.

The course of binding reached its maximum already after 5 minutes (Figure 4c). Binding of [35S]GTPγS to rab6a and rab6a' could be competed by an excess of unlabeled GTP (Figure 4c). These results suggest that GST-rab6a and GST-rab6a' have similar GTP-binding properties.
Intracellular localization of rab6a and rab6a´

To examine the intracellular localization of both proteins, we expressed N-terminal myc-tagged rab6a and rab6a´ in HeLa cells. Western blot analysis of cell lysates revealed that both rab6a and rab6a´ occurred as a doublet with an apparent molecular weight of approximately 25 kDa (Figure 4d). The heterogeneity observed for both rab6a variants may be explained by the presence of unprenylated protein in transfected HeLa cells that overexpress rab6a. Immunofluorescence studies were performed to determine the subcellular localization of rab6a and rab6a´ (Figure 5, see page 103). As can be seen in figure 5, no significant differences in subcellular localization of rab6a and rab6a´ were observed. Both rab6a isoforms are mainly localized to the Golgi apparatus and colocalize CTR433, a marker for the medial-Golgi.

DISCUSSION

In this study, we provide evidence for the existence of two homologous but distinct exons within the rab6a gene, which results in the expression of two rab6a isoforms by alternative splicing. So far, alternative splicing of homologous but distinct exons has not been described for other GTP-binding proteins. Alternative mRNA splicing has been previously suggested for the rab28 gene, based on an insertion of 95 bp found in a homologous rab28 cDNA sequence (Brauers et al., 1996). However, this insertion is assumed to be generated by an additional exon rather than by mutual exclusive incorporation of a duplicated exon. It would be interesting to investigate, whether exon duplication in the rab6a gene has arisen early in evolution or by recent DNA duplication. Early conserved divergence of homologous exons was found for the chicken and human SNAP-25 gene (Bark, 1993; Bark and Wilson, 1994). Interestingly, this specific neuronal protein, like rab proteins, also plays an important role in the docking/fusion machinery of transport vesicles to acceptor membranes. It was suggested that this exon switch may modify membrane binding properties of SNAP-25. Splicing isoforms, which are expressed from duplicated exons within one gene have been reported for a variety of genes and/or families, like AMPA-selective receptors (Sommer et al., 1990; Monyer et al., 1991), the human T-cell receptor gamma locus (Buresi et al., 1989), and the alpha subunit of prolyl 4-hydroxylase (Helaakoski et al., 1994).

The cDNA sequences of rab6a and rab6a´ differ in a stretch of about 100 bp containing the conserved PM3 region involved in GTP-binding. However, the open reading frames only showed three amino acid substitutions in the corresponding part of rab6a´ when compared to rab6a. This would suggest a selective evolutionary pressure on the preservation of the triplets responsible for maintaining an appropriate conformation of the GTP-binding domain. On the other hand, the non-preserved three amino acids might induce functional differences between rab6a and rab6a´. The conservative change of valine into an isoleucine at position 62 in rab6a´ might be of less relevance for distinct properties of the protein. A database search revealed
that each member of the rab family contains one of the above mentioned amino acids on the corresponding position. However, the non-conservative substitutions T₅S₆A and V₈₈A of rab₆₆' might influence the structural composition and/or functional behaviour. At the altered positions, only the rab₆₆ protein might be subject to phosphorylation as it contains a putative serine/threonine phosphorylation motif \[\text{RxxT(V)}\] (Hardie, 1993). It has been reported that cells can regulate their intracellular membrane transport by phosphorylation of rab proteins. Mitotic interruption of vesicular transport seemed to have an effect on rab₄, which was shown to be C-terminally phosphorylated leading to relocalization of the protein from endosomes to the cytosol (van der Sluijs et al., 1992; Ayad et al., 1997), whereas rab₆₆ remained unphosphorylated under the same conditions (Bailly et al., 1991). However, rab₆₆ was found to be phosphorylated through activation of platelets by thrombin, an inducer of secretion (Karniguiuan et al., 1993).

So far, our analysis did not indicate functional diversity between rab₆₆ and rab₆₆'. Both proteins were expressed ubiquitously. Splicing of the duplicated exon appears to be random generating equal levels of rab₆₆ and rab₆₆' in all tissues and cells presently investigated. The amino acid substitutions did not effect the affinity for GTP, and both rab₆₆ isoforms were found to be mainly localized to the Golgi membranes. Recently, an effector molecule for rab₆₆, rabkinesin-6, which binds predominantly to rab₆₆ in its GTP-bound form, has been identified (Echard et al., 1998). Whether both rab₆₆ isoforms interact in a similar manner with rabkinesin-6 or other effector molecules has to be established. Future studies should reveal whether expression via alternative splicing of two rab₆₆ isoforms, reflects a mechanism for cells to regulate intra-Golgi vesicular transport.

REFERENCES


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