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Isoprenylation of Rab proteins possessing a C-terminal CaaX motif

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Rab proteins are small GTPases highly related to the yeast Ypt1 and Sec4 proteins involved in secretion. The Rab proteins were found associated with membranes of different compartments along the secretory and endocytic pathways. They share distinct C-terminal cysteine motifs required for membrane association. Unlike the other Rab proteins, Rab8, Rab11 and Rab13 terminate with a C-terminal CaaX motif similar to those of Ras/Rho proteins This report demonstrates that Rab8 and Rab13 proteins are isoprenylated in vivo and geranylgeranylated in vitro. Rab11 associates in vitro geranylgeranylpyrophosphate and farnesylpyrophosphate. Our study shows that the CaaX motif is required for isoprenylation

Rab small GTPase; Isoprenylation; C-terminal CaaX motif

1. INTRODUCTION

The small Ras-related GTP-binding proteins constitute a superfamily which can be grouped into four main branches according to their sequence similarities: Ras, Rho/Rac, Rab/Sec4/Ypt and Arf [1–6]. The Rab/Sec4/Ypt family comprises more than thirty proteins which are involved in the regulation of vesicular traffic between different membrane compartments [7]. Mutants studies in *S. cerevisiae* of Sec4 and Ypt1 proteins, provided direct evidence for their involvement in vesicle transport [8–9]. The association of many Rab proteins with different subcellular compartments [10–13] and the in vivo and in vitro studies indicate a role for these proteins in the exocytic and endocytic pathways [7].

The mechanism responsible for the membrane association of these small GTPases is not clear. However, all members of the Ras superfamily (except Arf proteins) share one or two cysteines at their C-terminal end. Ras and Rho/Rac proteins families present a C-terminal CaaX sequence (C, Cys; a, mostly aliphatic, and X, any amino acid [1]. This motif allows the protein to undergo a set of post-translational modifications necessary for their anchorage into the membranes which has been shown to be a critical step for p21Ras function [14]. The cysteine is first isoprenylated by the association of an unsaturated C_{15} or C_{20} lipidic chain; then the three terminal residues are removed and, in most cases, the cysteine is carboxymethylated. The nature of the isoprenyl chain depends on the X amino acid: if it is a Met, a Ser, an Ala or a Cys, the protein appears to be farnesylated (C_{15} lipid) [15] by a farnesyl-transferase (FTase) [16]; When X is a Leu or a Phe, the protein would be geranylgeranylated (C_{20} lipid) [15] by the geranylgeranyltransferase I (GGTase I) [17].

Rab proteins present more heterogenic C-terminal motifs: CC (Rab1, Rab2, Rab9), CXC (Rab3, Rab4, Rab6) or CCXX (Rab5) [1]. So far, all prenylation studies on these Rab proteins have led to the conclusion that they are geranylgeranylated [18–19].

In contrast to the other members of the Rab/Sec4/Ypt family, three Rab proteins, more recently identified, present a CaaX motif at their C-terminus: Rab8 (CVLL), Rab13 (CSLG) and Rab11 ((C)CQNI) [4–5]. In this paper we have investigated the post-translational modifications of these three human Rab proteins. We describe the isolation of rab8 and rab11 cDNAs. The Rab8, Rab11 and Rab13 proteins were expressed in *E. coli* and showed to bind GTP. We present evidence that Rab8 and Rab13 incorporate in vivo a derivative of mevalonate and show that in vitro both proteins undergo a modification by 20 carbons geranylgeranyl groups. Rab11 seems to associate in vitro the two different isoprenoids.

2. EXPERIMENTAL

2.1 *Screening of the library*

2×10^5 plaques of a pheochromocytoma cDNA λgt10 library were screened with the oligonucleotide mixture sequence 5′-AC(A,C,G,T)AT(A,T)GG(A,C,G,T)AT(A,T)GATTTTAAG-3′ corresponding to the TIGIDFK sequence of the effector domain of Sec4. Filters were prehybridized for 3 h and hybridized overnight at 42°C in 5 × SSPE, 5 × Denhart's, 0.1% SDS, 100 μg/ml of sonicated salmon sperm DNA in presence of 0.5×10^6 cpm/filter of [γ-^{32}P]dATP-oligonucleotide. Thus filters were washed in 2 × SSC, 0.1% SDS 10 min at room temperature and 20 min at 42°C. Phage DNA was prepared, cloned in bluescript plasmid and sequenced using the T7 sequencing TM Kit (Pharmacia). We have screened the same pheochromocytoma library with an insert of 700 bp containing the whole coding region of rab6 cDNA. The probe was labeled with [α-^{32}P]dCTP using the

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random priming procedure. Hybridization was performed at 60°C and filters were washed in a solution containing 1 × SSC, 0.1% SDS for 15 min at room temperature and 30 min at 68°C.

2.2. *Expression of the Rab proteins in* E. coli

To express Rab proteins in *E coli* we performed site-directed mutagenesis [3]. The following oligonucleotides: 5′-TCGAATTCCACATATGGCGAA-3′ for Rab8, 5′-GGTGTCCGCCATATGGCCAAAG-3′ for Rab13, and 5′-GGCCGCGCATATGGGCACC-3′ for Rab11, were used to create *Nde*I sites, including the ATG initiator codon, upstream from the coding region. Mutations were confirmed by sequencing. The rab13 cDNA encoding $Rab13_{\Delta CSLG}$ protein that lacks the CSLG C-terminal motif was generated by site-directed mutagenesis using the 5′-CCAACAAGTGATCCCTGGGCT-3′ oligonucleotide. *Nde*I–*Bam*HI inserts containing the complete coding regions were cloned into the pET-3c vector and expressed in the *E coli* BL21 (De3) pLysS strain [3]. Bacterial clones expressing Rab proteins were identified by SDS-PAGE and GTP-binding overlays [20].

2.3. *Anti-sera*

New Zealand rabbits were immunized against synthetic peptides covalently coupled to ovalbumin (Neosystem, Strasbourg, France) [20]. The peptide sequences were derived from the C-terminal regions of Rab13 NKPPSTDLKTC$_{acm}$DKKNT, residues 182–197, and of Rab8 PQGSNQGVKITPDQQK, residues 182–197. Both anti-sera were specific for their respective antigene. They did not cross-react with other members of the Rab protein family [21] and Zahraoui et al., submitted).

2.4. *In vivo isoprenylation assay*

Coding regions of rab8 and rab13 cDNAs were cloned in the pcEXV-3 vector [22]. Simian Cos-7 cells, in DMEM–10% foetal calf serum, were transfected with 10 μg DNA/6 cm diameter plate using a $CaCl_2$ precipitation method [23]. 48 h after transfection, cells were cultured in the presence of 50 μM of lovastatin (a generous gift from Dr. Slater, Merk Sharp and Dohme) for 1 h. The cells were then incubated in fresh medium supplemented with 50 μM lovastatin and 150 μCi/ml of [^{3}H]mevanololactone for 20 h. Cos-7 cells were lysed at 4°C in TNE buffer (Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.15 M NaCl, 0.5% deoxycholate, 0.1% SDS, 0.5% nonidet-P40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride), and nuclei were removed by centrifugation for 10 min at 13,000 × *g*. Supernatants were precleared for 1 h using a rabbit preimmune serum and protein A sepharose CL-4B beads (Sigma). Precleared supernatants were incubated separately with anti-Rab8 or anti-Rab13 for 3.5 h at 4°C. Protein A sepharose beads were added for 2 h. Immune complexes were pelleted and washed two-fold with TNE containing 0.5 M NaCl and two-fold with TNE and resuspended in denaturation buffer, warmed and the supernatant electrophoresed. Gel was subjected to fluorographic treatment.

2.5. *In vitro isoprenylation assays*

In vitro isoprenylation assays were performed using 2 μg (100 pmol) of purified H-Ras, 100 μg (about 100 pmol) of extracts prepared from bacteria expressing Rab proteins (Rab8, Rab11, Rab13, $Rab13_{\Delta CSLG}$) by Triton X-100 treatment and sonication as described by Wagner et al. [20]. Proteins were separately incubated with 50 μg of a brain cytosolic AS_{20-60} fraction [19] in 50 μl of a solution containing 25 mM Tris-HCl, pH 7.5, 10 mM DTT, 10 mM $MgCl_2$, 50 μM leupeptin, 0.1 μM pepstatin and 10 μM $ZnCl_2$. 1 μCi of [^{3}H]GGPP or [^{3}H]FPP was added and reactions were carried out at 37°C for 1 h. Proteins were precipitated, electrophoresed and the gel were treated by fluorography as described elsewhere [19].

3. RESULTS

In order to isolate a mammalian homolog of the yeast Sec4 protein, we screened a pheochromocytoma cDNA library with a degenerated oligonucleotide corresponding to the sequence TIGIDFK, present in the effector region of Sec4. We isolated a cDNA clone encoding a protein that presents 51% amino acid identity with Sec4 and 100% identity with the dog Rab8 protein [4]. A human rab8 cDNA (*MEL* gene cDNA) has been previously reported [24]. The nucleotide sequence of the two rab8 cDNAs are exactly the same – including the non-coding regions – except for three deletions in the *MEL* cDNA sequence: T_{530}, C_{545} and G_{549}, reflecting a probable sequence error; consequently, the *MEL* product and our Rab8 amino acids sequences differ by a Glu^{183} deletion and by five non-conservative amino acids substitutions at positions 177 to 181 (Fig. 1).

Using the same strategy, we have recently identified a small GTP-binding protein, named Rab13, from a human epithelial cell cDNA library (Zahraoui et al., submitted). The Rab13 protein presents 92% amino acid identity with the partial sequence of the rat Rab13 [5]. Rab13 shared 64% and 56% identity with Rab8 and Sec4 proteins, respectively. As the chromosomic localisation of *rab6* suggested the existence of a highly related gene [25], we screened the same pheochromocytoma library with a rab6 cDNA probe. We isolated a cDNA encoding a protein presenting 100% identity with the dog and human Rab11 proteins [4,26]. Rab8, Rab11 and Rab13 proteins sequences share all the structural features of the Rab family but unlike other Rab proteins, they have a carboxy-terminal CaaX motif: CVLL for Rab8, CSLG for Rab13 and (C)CQNI for Rab11.

The rab8, rab11 and rab13 cDNAs were subcloned in the pET-3c expression vector. Recombinant proteins

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cRab8        523  AAA AAA TTG GAA GGC AAC AGT CCC CAA GGG AGC AAC  558
             175   K   K   L   E   G   N   S   P   Q   G   S   N  186

hRab8        523  AAA AAA TTG GAA GGC AAC AGC CCC CAG GGG AGC AAC  558
             175   K   K   L   E   G   N   S   P   Q   G   S   N  186

hRab8 (MEL)  523  AAA AAA  TGG  AAG GCA ACA GCC  CCA  GGG AGC AAC  555
             175   K   K   W   K   A   T   A   P   G   S   N  185
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Fig. 1 Partial sequence alignment of canine Rab8 (cRab8) [4], our human Rab8 (hRab8) and hRab8/MEL [24] showing the nucleotide differences between cRab8 and our hRab8 on the one hand and hRab8/MEL on the other. The three nucleotide deletions in hRab8/MEL are underlined. The rab8 sequence is available from EMBL databases under the accessive number X56741.

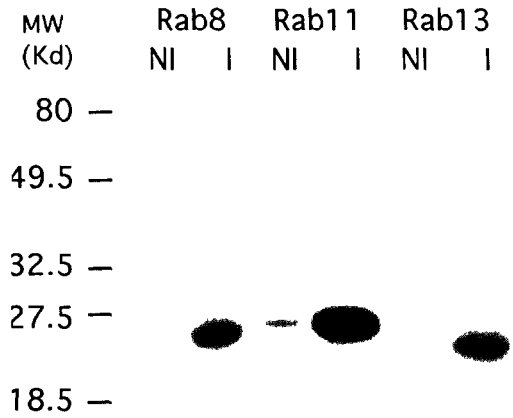

Fig. 2. GTP-binding of Rab8, Rab11 and Rab13 proteins. A fraction of non-induced (NI) or isopropyl β-D-thiogalactopyranoside (IPTG) induced (I) total bacterial lysates were separated by electrophoresis, transferred onto nitrocellulose membranes and incubated with [α-^{32}P]GTP. Films were exposed for 15 min for Rab8 and Rab11, or for 2.5 days for Rab13. A basal expression of Rab proteins could be seen in non-induced tracks, especially for Rab11.

were produced in *E. coli*, separated on a polyacrylamide-SDS gel, transferred on a nitrocellulose membrane and incubated with [α-^{32}P]GTP. Fig. 2 shows that the three proteins bind radiolabelled GTP.

To investigate whether the three Rab proteins were isoprenylated in vivo, we tested the incorporation of [^{3}H]mevalonate derivatives (mevalonate is an isoprenoid precursor). For this purpose, simian Cos-7 cells were transfected with the pcEXV-3 vectors containing rab8 and rab13 cDNAs. The corresponding Rab proteins were transiently expressed in these cells. Transfected Cos cells were pretreated for 1 h with lovastatin. This drug, an inhibitor of isoprenoid metabolism, considerably inhibits mevalonic acid synthesis and consequently reduces the incorporation of endogenous isoprenyl groups. Cells were then incubated in fresh medium containing [^{3}H]mevanololactone (MVA) and Rab proteins were isolated by immunoprecipitation with specific anti-Rab antibodies and SDS-PAGE. Incorporation of the ^{3}H-lipid moieties into Rab8 and Rab13 was detected by fluorography. In both cases, the ^{3}H- labeled bands correspond to proteins exhibiting the expected molecular mass of Rab8 and Rab13 (Fig. 3). The low ^{3}H-band corresponding to Rab13 probably reflects the feeble surexpression level of Rab13. Indeed, when Cos-7 cells transiently expressing Rab13 were metabolically labeled with [^{35}S]Met/Cys mixture (Translabel, Amersham) and the protein was immunoprecipitated, we observed that Rab13 surexpression level is about 2-fold above control (data not shown).

Having established that Rab8 and Rab13 proteins were isoprenylated in vivo, it was important to determine the nature of the incorporated isoprenoid group. We performed in vitro farnesylation and geranylgeranylation assays using [^{3}H]farnesyl pyrophosphate (FPP) and [^{3}H]geranylgeranyl pyrophosphate (GGPP). Rab8 and Rab13 proteins were produced in *E. coli* and the extracts were incubated with a $A_{20\text{-}60}$ cytosolic fraction derived from a bovine brain, in presence of either [^{3}H]FPP or [^{3}H]GGPP. The $A_{20\text{-}60}$ fraction contains prenyl-transferases necessary for the reactions [19]. We found that Rab8 and Rab13 incorporated [^{3}H]GGPP (Fig. 4). We did not observe a significant incorporation when the proteins were incubated with [^{3}H]FPP. No association of any labeled isoprenoid was seen in control reactions performed either with only $A_{20\text{-}60}$ fraction (Fig. 4) or with a lysate prepared from bacteria transformed with pET-3c vector alone (data not shown). As a positive control for farnesylation, Fig. 4 shows that purified H-Ras, as expected and in agreement with the published results [15], is only farnesylated.

We next determined whether the CaaX motif is absolutely required for isoprenylation. We constructed a Rab13 mutant that lacks the CaaX C-terminal box, $Rab13_{\Delta CSLG}$. This truncated protein was produced in *E. coli* (Fig. 5A) and the bacterial extract was used in the in vitro isoprenylation assay in presence of [^{3}H]GGPP. In contrast to the wild type Rab13, no incorporation of [^{3}H]GGPP was detected by fluorography into the $Rab13_{\Delta CSLG}$ (Fig. 5B). This result indicates that the CSLG motif is an essential component of the prenylation signal.

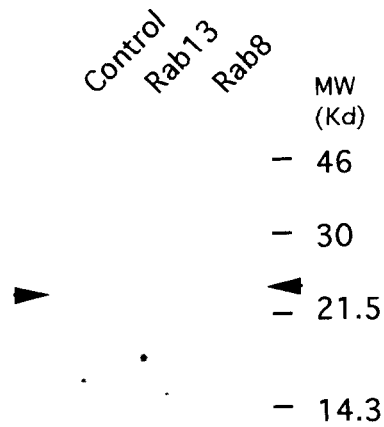

Fig. 3. In vivo isoprenylation of Rab8 and Rab13. Cos-7 cells were transfected with pcEXV/rab8 (Rab8), pcEXV/rab13 (Rab13) or pcEXV alone (Control) and were labeled with [^{3}H]mevanololactone in presence of lovastatin. Cellular lysates were immunoprecipitated using anti-Rab8 (Rab8) or anti-Rab13 (Rab13, Control) antibodies. Immunocomplexes were then electrophoresed on a SDS-polyacrylamide gel and subjected to fluorography. Arrowheads indicate immunoprecipitated Rab8 and Rab13 proteins. Film was exposed 50 days.

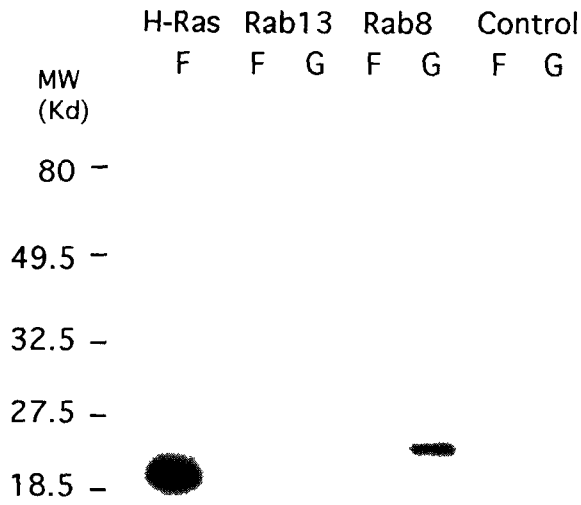

Fig. 4 In vitro prenylation of Rab8 and Rab13. Purified H-Ras protein, Rab13 or Rab8 bacterial extracts and no Rab protein (Control) were used for the assay in the presence of bovine brain cytosol fraction and 1 μCi of [^{3}H]FPP (F) or of [^{3}H]GGPP (G). Proteins were then precipitated and subjected to SDS-PAGE. Gel was treated by fluorography and films were exposed for 3 (H-Ras) and 15 days (Rab8, Rab13, Control) H-Ras does not incorporate [^{3}H]GGPP (data not shown).

We extended our analysis to Rab11 protein which possesses, like RhoB (CCKVL) protein, a (C)CaaX motif at its C-terminal end (CCQNI) [1]. Previous results have shown that Rab11 was isoprenylated in vivo but the nature of the incorporated isoprenoid was not determined [27]. We found that Rab11 protein produced in *E. coli*, incubated with [^{3}H]FPP or [^{3}H]GGPP as described in Section 2, incorporated in vitro both isoprenoid lipids, indicating that Rab11 may be geranylgeranylated and farnesylated (Fig. 6).

4. DISCUSSION

Rab proteins have striking heterogenous C-terminal cystein motifs. In this study, we demonstrated that Rab proteins displaying, like p21Ras, C-terminal CaaX boxes (Rab8, Rab11 and Rab13) are prenylated in vivo and in vitro. We also showed that the C-terminal CaaX motif is an essential structural component for signal prenylation since the truncated protein Rab13$_{\Delta CSLG}$ do not undergo geranylgeranylation. Similarly to Ras, the sequence of the post-translational modifications: isoprenylation, proteolytic cleavage and probably carboxymethylation could occur sequentially on the Rab CaaX boxes.

It appears that small GTPases presenting a Caa(M/S/A/C) C-terminal motif are modified by FTase whereas proteins terminating with a Caa(L/F) box are substrate

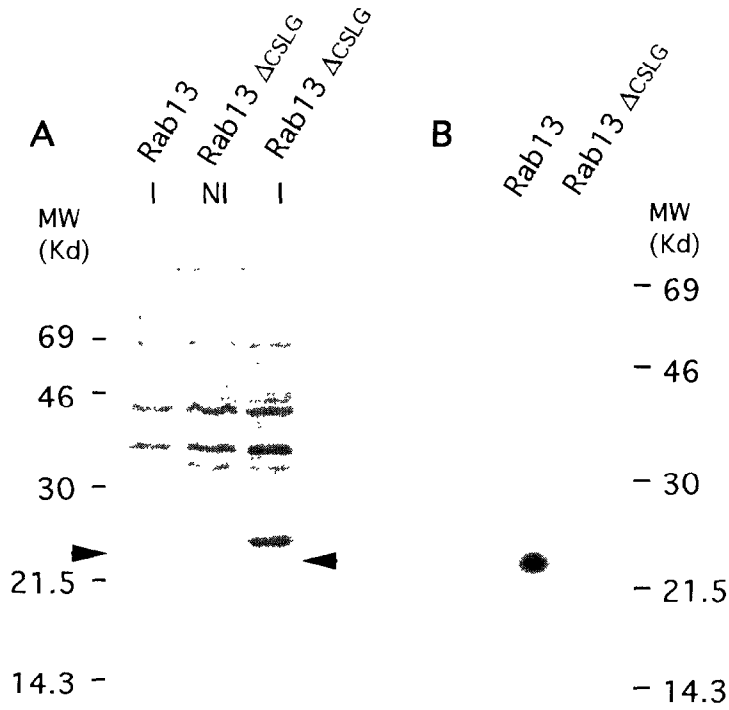

Fig. 5. (A) Coomassie blue staining of bacterial extracts expressing Rab13 and Rab13$_{\Delta CSLG}$. IPTG induced (I) or non-induced (NI) bacteria were centrifuged and resuspended in 100 μl of water 12 μl were denaturated and electrophoresed on a SDS-polyacrylamide gel. Arrowheads indicate bands corresponding to Rab13 and Rab13$_{\Delta CSLG}$. Both proteins were recognized by the Rab13 antipeptide (data not shown). (B) Rab13 or Rab13$_{\Delta CSLG}$ bacterial extracts were used for in vitro geranylgeranylation assay as indicated in Fig 4. Proteins were precipitated, electrophoresed and gel was subjected to fluorographic treatment Film was exposed for 7 days

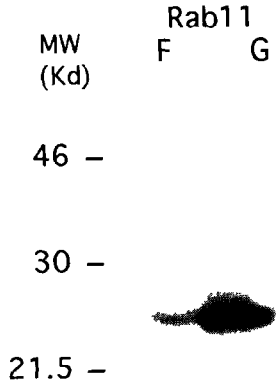

Fig. 6. In vitro prenylation of Rab11. Rab11 bacterial extract was incubated in the presence of bovine brain cytosol fraction and either 1 μCi of [^{3}H]FPP (F) or of [^{3}H]GGPP (G). Proteins were recipitated and separated by SDS-PAGE. Gels were treated by fluorography and films were exposed for 12 days.

of GGTase I [15]. In both cases, in vivo and in vitro prenylation studies have suggested that the CaaX tetrapeptide was sufficient to ensure the association of the isoprenoid [28]. The rab proteins ending with CC or CXC motifs seem to be prenylated, probably like Rab3A on both cysteines [18], by another enzyme, the Rab GGTase also noted GGTase II [29].

The geranylgeranylation of Rab8, probably by GGTase I, is in concordance with previous data on other small GTPases that end with a CaaL box [15]. Previous results have shown that a Ras protein terminating with a CAIG tetrapeptide sequence instead of the CVLS box, was farnesylated [28]. Accordingly, and since the nature of the isoprenoid group is partly determined by the last amino acid of the CaaX box [15], Rab13 (CSLG) would be farnesylated. Our results showed only a geranylgeranylation of Rab13. One possible explanation is that amino acids situated within and/or upstream from the C-terminal motif could modulate the prenylation signal. Rab13 could also be modified by an enzyme different from GGTase I which would recognize, like GGTase II, long parts, or even the entire conformation of the substrate protein [30]. Indeed, in the case of some Rab proteins it has been shown that the C-terminal motif is essential but not sufficient for signal prenylation [27,30–31]. RhoB (CCKVL) is the unique protein known to be geranylgeranylated and farnesylated [22]. Rab11, which presents a similar C-terminal (CCQNI) sequence motif, is also modified by both prenyl-transferases. However, compared to farnesylation, geranylgeranylation is clearly predominant. Further studies would be required to determine whether Rab11 farnesylation occurs also in vivo. Rab11, like RhoB, could be prenylated by enzymes different from FTase and GGTase I. The finding that the RhoB mutant protein bearing the SCKVL motif at its carboxy-terminus was only farnesylated [22] is in contradiction with the expected isoprenylation of proteins presenting a CaaL box [15]. The CCaaX motif of Rab11 and RhoB, probably with amino acids sequences upstream from the C-terminal motif, might allow proteins to be modified by both types of prenyl-transferases, FTase and GGTase.

Although the biological significance of these post-translational modifications are not fully understood, it has been demonstrated that the farnesylation of rhodopsin kinase and geranylgeranylation of β-adrenergic receptor kinase are required for their membrane translocation and activity [32]. For Ras proteins, in addition to farnesylation, palmitolation of cysteines or the presence of a polybasic region upstream from the CaaX box is required for their plasma membrane localization [33]. In the case of Rab proteins, it has been shown that the hypervariable C-terminal region specifies their localization in appropriate secretory and endocytic compartments [34]. The presence of the prenyl group is required for both membrane attachment and function. The isoprenoid group might serve to anchor the Rab proteins in the lipid bilayer. It might also mediate the interaction with regulatory proteins necessary for the association/dissociation of Rab proteins from specific compartments. Proteins termed GDIs (for GDP dissocation inhibitor) that inhibit the release of GDP from Rab3A and Rab11 have been identified. These GDIs have the capacity to dissociate the GDP-Rabprenylated form from membranes [35–36]. It has been proposed that the complex Rab-GDI formed is then recognized by another membrane protein (Receptor, Guanine exchange factor) which would catalyze the specific insertion of Rab proteins to the membrane. It appears that Rab localization involves common post-translational modifications, specific sequence recognition and probably interaction with yet unknown target proteins.

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