Prenylation of a Rab1B mutant with altered GTPase activity is impaired in cell-free systems but not in intact mammalian cells

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Previous studies have reached differing conclusions as to whether or not guanine-nucleotide-dependent conformational changes affect the ability of Rab proteins to undergo post-translational modification by Rab:geranylgeranyltransferase (Rab-GGTase). We now show that the ability of a Rab1B mutant [Q67L (Gln-67 → Leu)] with reduced intrinsic GTPase activity to undergo geranylgeranylation in cell-free assays depends on the guanine nucleotide composition of the system. When GTP is the predominant nucleotide in the assay, Rab1B^{Q67L} is a poor substrate. However, when GDP is present and GTP is omitted, prenylation of the Q67L mutant is comparable with that of the wild-type (WT) protein. These studies, coupled with the poor prenylation of Rab1B^{WT} in the presence of the non-hydrolysable GTP analogue guanosine 5'-[γ-thio]triphosphate, support the notion that Rab-GGTase prefers substrates in the GDP conformation. When the abilities of Rab1B^{Q67L} and Rab1B^{WT} to undergo prenylation

were compared by metabolic labelling of transiently expressed proteins in cultured human 293 cells, we did not observe a decline in prenylation of the mutant protein as predicted on the basis of the cell-free assays. Moreover, the Q67L mutant was comparable with the wild-type Rab1B in its ability to associate with co-expressed Rab GDP dissociation inhibitors in 293 cells. These findings raise the possibility that unidentified proteins present in intact cells may compensate for the reduced intrinsic GTPase activity of the Q67L mutant, allowing a significant proportion of the nascent Rab1B^{Q67L} to assume a GDP conformation. The differential prenylation of Rab1B^{Q67L} in cell-free systems versus intact cells underscores the importance of evaluating the post-translational modification of specific Rab mutants *in vivo*, where poorly characterized regulatory proteins may have a significant effect on GTPase activity or nucleotide exchange rates.

INTRODUCTION

The Rab proteins are Ras-related GTPases [1,2] that are believed to mediate the directionality and/or specificity of individual steps in the exocytic and endocytic vesicular transport pathways in mammalian cells [3–5]. Different Rab proteins are localized in discrete intracellular membrane compartments [6,7]. The association of Rab proteins with intracellular membranes requires their post-translational modification by one or two geranylgeranyl groups, which are linked to C-terminal cysteine residues [8–12]. The geranylgeranylation of Rab proteins is also essential for their interaction with cytosolic proteins termed Rab GDP-dissociation inhibitors (GDIs), which are thought to function in Rab recycling [13–15].

Rab:geranylgeranyltransferase (Rab-GGTase), also termed geranylgeranyltransferase type II, is structurally and functionally distinct from the enzymes that catalyse the prenylation of other members of the Ras superfamily, for example farnesyltransferase (FTase) and geranylgeranyltransferase type I (GGTaseI) [16–18]. While the latter enzymes can prenylate monomeric GTPase substrates, the catalytic αβ subunits of Rab-GGTase cannot transfer geranylgeranyl groups to a Rab protein unless the substrate is bound to an accessory protein termed Rab escort protein (REP) [19,20]. In cell-free assays, REP remains associated with the prenylated Rab protein unless detergent is added to the system, suggesting that, after the prenylation reaction is complete, REP may 'escort' the modified Rab protein to its appropriate

membrane site [19,21,22]. Because of the complexity of the Rab prenylation mechanism, a precise definition of the structural requirements for recognition of Rab substrates by Rab-GGTase has remained elusive. Nevertheless, it is clear that, unlike other Ras-related proteins (e.g. Ras, Rap, Rho, Rac), where the presence of a C-terminal *Caax* motif (see the abbreviations footnote) is necessary and sufficient for recognition by FTaseI or GGTaseI [23–27], Rab proteins must retain specific tertiary structural features beyond the C-terminus in order to be prenylated [9,28–32].

One of the major factors influencing the conformation of Rab and other Ras-related proteins is the bound guanine nucleotide. Thus an important question concerning the mechanism of Rab prenylation is whether or not the components of the Rab-GGTase complex interact preferentially with nascent Rab proteins in the GTP- or GDP-bound state. Studies employing non-hydrolysable analogues of GTP to address this issue have yielded conflicting answers. Sanford et al. [33] reported that GTP[S] partially impairs the geranylgeranylation of Rab5, whereas Beranger et al. [31] observed no effect of GTP[S] on the prenylation of Rab6. An alternative approach to this question has been to alter the Rab substrate by introducing mutations that reduce the intrinsic GTPase activity of the protein (e.g. substitutions at the position equivalent to Gln-61 in H-Ras). However, such studies have also arrived at differing conclusions. When modification of nascent Rab5 polypeptide was carried out in a coupled *in vitro* translation/prenylation assay, the Rab5^{Q79L}

Abbreviations used: Rab-GGTase; Rab geranylgeranyltransferase; FTase, farnesyltransferase; REP, Rab escort protein; GAP, GTPase-activating protein; GDI, GDP dissociation inhibitor; GGPP, geranylgeranyl pyrophosphate; GG, geranylgeranyl moiety; PVDF, poly(vinylidene difluoride); DMEM, Dulbecco's modified Eagle's medium; MVA, mevalonolactone; *Caax*, a C-terminal prenylation motif consisting of cysteine (C), followed by two aliphatic amino acids (a) and any amino acid (x), where x is usually leucine, methionine or serine; WT, wild-type; Q67L, Gln-67 → Leu mutant; ΔCC, lacking the target cysteine residues for geranylgeranylation; GTP[S], guanosine 5'-[γ-thio]triphosphate ('GTPγS'); DTT, dithiothreitol; FLAG, amino acid sequence DYKDDDDK.

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mutant was prenylated less efficiently than the wild-type protein [33]. Similarly, Scheidel et al. [34] reported that Rab6^{Q72R} was not detectably prenylated when translated in reticulocyte lysates or expressed in Sf9 cells. In contrast with these observations, studies utilizing bacterially expressed recombinant proteins as substrates in cell-free assays have not revealed a significant difference in the geranylgeranylation of Rab5^{Q79L} compared with Rab5^{WT} [35].

In the present study we provide new data to support the conclusion that Rab-GGTase favours the Rab GDP conformation, based on an assessment of the geranylgeranylation of a GTPase-deficient Rab1B mutant (Rab1B^{Q67L}) in cell-free systems of varying guanine nucleotide composition. We also show that, although the Q67L mutant is not efficiently prenylated in cell-free assays containing GTP, its prenylation is indistinguishable from that of the wild-type protein in cultured human 293 cells. The latter finding illustrates the importance of studying the post-translational modification of Rab proteins in intact mammalian cells, where the intrinsic properties of the substrate protein or the Rab-GGTase complex observed *in vitro* may be affected by the presence of unidentified regulatory proteins.

MATERIALS AND METHODS

Mutagenesis of Rab1B

Rab1B^{Q67L} was generated by PCR modification of the cDNA encoding Rab1B [36], using Deep Vent DNA polymerase (New England Biolabs) and pGEM3Zrab1B [30] as the template. The 5' and 3' anchoring oligonucleotide primers were: 5'-GTTGTA-AAACGACGGCCAGTG (matching a sequence upstream from the multiple cloning site in pGEM3Z) and 5'-ACGGGATCCT-ATCTAACAGCAGCCACCACTAGCAGA (which complements to the 3' end of *rab1B* and introduces a *Bam*H1 restriction site downstream of the stop codon). The mutator oligonucleotide was: 5'-ACAGCTGGTCTGGAAACGGTTC. The PCR product was subcloned into pGEM3Z as an *Eco*R1/*Bam*H1 insert. To obtain Myc epitope-tagged Rab1B proteins, the wild-type or mutant *rab1B* cDNAs were modified by PCR utilizing the following 5' and 3' oligonucleotide primers: 5'-GCCAGCGAATTCCATATGGAGCAGAAGCT-GATCAGCGAGGAGGACCTGAACCCCGAATATGACT-AC and 5'-ACGTCTAGAGGATCCTATCTAACAGCAGCC-ACCACTAGCAGA. The resulting PCR products were digested with *Eco*R1 and *Xba*I and subcloned into pCMV5neo [37,38] for *in vivo* expression studies or pGEM3Z (Promega) for *in vitro* transcription/translation assays. The cDNA encoding Myc-Rab1B^{ΔCC} (^{ΔCC} means lacking the target cysteine residues for geranylgeranylation) was generated by PCR from pGEMrab1B^{WT} using the 5' Myc-tagging primer described above, together with the 3' oligonucleotide: 5'-ACGGGATCCTATCTAGCCACCACTAGCAGATTTCAC. The DNA sequences of all *rab1B* constructs were verified by the dideoxy chain-termination technique using Sequenase 2.0 (United States Biochemical Co.).

Coupled *in vitro* translation/prenylation of Rab1B

RNAs were generated from wild-type and mutant *rab1B* cDNAs in pGEM3Z, utilizing the AmpliScribe T7 transcription kit (Epicentre Technologies, Madison, WI, U.S.A.). Coupled translation/prenylation assays were carried out at 30 °C for 3 h in rabbit reticulocyte lysate (Promega), as described previously [39]. Parallel reactions contained either 40 μCi of L-[³⁵S]methionine (1250 Ci/mmol; New England Nuclear), to monitor synthesis of Rab1B or 1 μCi of geranylgeranyl pyrophosphate ([³H]GGPP; 15 Ci/mmol; American Radiochemical Corp.) to monitor pre-

nylation. Translation reactions were stopped by addition of 1 ml of ethanol/HCl (9:1, v/v), and the precipitated proteins were collected by centrifugation and washed twice with 100 % ethanol. Proteins were subjected to SDS/PAGE and fluorography, and incorporation of ³H or ³⁵S into the translation products was determined by liquid-scintillation counting of solubilized gel slices [40].

Expression of recombinant Rab1B in *Escherichia coli*

Myc-tagged *rab1B* inserts were excised from pCMV5neo and subcloned into pET11a (Novagen Inc., Madison, WI, U.S.A.), using *Nde*I and *Bam*H1 restriction sites. Expression of Rab1B was induced in *E. coli* BL21(DE3)pLysS by incubation of bacterial cultures with 1 mM isopropyl β-D-thiogalactopyranoside for 1 h. Bacterial cell pellets from 500 ml cultures were usually suspended in 3 ml of Buffer A (50 mM Hepes/5 mM MgCl₂/1 mM dithiothreitol (DTT)/10 μM GDP/1 mM PMSF/25 μM leupeptin/2.8 units of aprotinin). In some experiments, bacterial lysates containing recombinant Rab1B were prepared in Buffer B, in which 10 μM GDP was replaced with 1 mM GDP, 1 mM GTP or 1 mM GTP[S], and the concentration of MgCl₂ was reduced to 10 μM. Cells were disrupted by a single freeze-thaw cycle (−80 °C), the lysate was cleared by centrifugation at 15000 g for 15 min, and the supernatant solution was stored at −80 °C after addition of glycerol (40 %, v/v). Total protein was determined by the method of Bradford [41]. The relative amount of Myc-Rab1B in each bacterial lysate was determined by immunoblot analysis, using a monoclonal antibody 9E10 against the Myc-epitope tag and ¹²⁵I-goat anti-mouse IgG, as described previously [42,43]. Bound ¹²⁵I-IgG was quantified by scanning the blots with an AMBIS 4000 radioanalytical imaging detector for 4 h. One unit of Rab1B was defined as the amount of protein giving 1000 c.p.m. of bound ¹²⁵I-IgG. Densitometry of Coomassie Blue-stained gels indicated that Myc-Rab1B^{WT} accounted for approx. 10 % of the total soluble protein in the *E. coli* lysates. By relating the Coomassie Blue staining to the results of immunoblot analysis, it was estimated that one immunological unit of Myc-Rab1B was equivalent to 0.2 μg of recombinant protein.

Blot overlay assay for GTP binding and hydrolysis

The ability of recombinant Myc-Rab1B proteins to bind and hydrolyse GTP was analysed by ligand blotting using a modification of an established [α -³²P]GTP overlay assay [44]. Aliquots of *E. coli* lysate containing equal amounts of Myc-Rab1B^{Q67L} or Myc-Rab1B^{WT} (determined by immunoblot analysis) were run on an SDS/12.5 %-polyacrylamide minigel. Immediately following electrophoresis, the gel was soaked in 50 mM Tris/HCl (pH 7.5)/20 % (v/v) glycerol for 30 min, with one buffer change after 15 min. The proteins were then transferred electrophoretically to a nitrocellulose membrane at 100 mA for 16 h in 10 mM NaHCO₃/3 mM Na₂CO₃, pH 9.4. The membrane was blocked for 30 min in 50 mM NaH₂PO₄ (pH 7.5)/10 μM MgCl₂/2 mM dithiothreitol/0.2 % Tween-20/4 μM ATP (GTP-binding buffer). Fresh GTP-binding buffer containing 1 μCi/ml [α -³²P]GTP (3000 Ci/mmol; New England Nuclear) was then added and the incubation was continued for 2 h. The membrane was washed with GTP-binding buffer for 1 h with four buffer changes, and the dried membrane was subjected to autoradiography for 16 h. In order to determine the extent of hydrolysis of bound [α -³²P]GTP, the zones containing Rab proteins were excised from the blot and the bound nucleotides were eluted by incubation at 67 °C for 15 min in 5 mM EDTA/2 mM DTT/2 % SDS/0.5 mM GDP/0.5 mM GTP.

Equal aliquots of the eluates were subjected to TLC on PEI-F (polyethyleneimine fluorescent)-cellulose plates (J. T. Baker) developed in 0.75 M KH_2PO_4 , pH 3.4. After autoradiography, the [^{32}P]GDP and [^{32}P]GTP were quantified by scanning the plates with an AMBIS detector.

Partial purification of Rab-GGTase

A protein fraction enriched in Rab-GGTase activity was prepared by subjecting rat brain cytosol (100000 g supernatant solution) to ammonium sulphate fractionation and ion-exchange chromatography on a Mono Q 5/5 column (Pharmacia) [16]. Column fractions were dialysed against 20 mM Hepes (pH 7.2)/10 mM NaCl/0.1 mM Nonidet P40/1 mM DTT, and aliquots (10 μl) of each fraction were assayed for Rab-GGTase, GGTase I and FTase activities [16], using the following recombinant fusion proteins (approx. 1 μg) as substrates: MBP-Rab1B (for Rab-GGTase), MBP-G25K (for GGTaseI), and MBP-H-Ras (for FTase) [9]. Fractions enriched in Rab-GGTase activity were pooled and stored at -80°C .

Geranylgeranylation of recombinant Rab1B with rat brain cytosol.

Initial attempts to assess the ability of recombinant Rab1B to undergo geranylgeranylation *in vitro* were carried out with rat brain cytosol as a source of enzyme. Aliquots of *E. coli* lysate in Buffer A (6 μl) containing no Rab protein (blank) or 0.15 μg (0.75 units as defined by immunoblot assay) of Myc-Rab1B^{WT} or Myc-Rab1B^{Q67L} were added to 43 μl of rat brain cytosol (prepared in 50 mM Hepes, pH 7.4, 10 μM MgCl_2 , 1 mM PMSF) and 1 μCi of [^3H]GGPP. The reaction was carried out for 3 h at 30°C , and was terminated by addition of SDS sample buffer [45]. All samples were subjected to SDS/PAGE and fluorography. The gel segments between 26 and 30 kDa, which contained the radiolabelled Rab proteins, were excised and solubilized by heating at 65°C for 16 h in 0.75 ml of 30 % H_2O_2 . Radioactivity incorporated into the Rab proteins was determined by liquid-scintillation counting. The results were expressed as d.p.m. of [^3H]geranylgeranyl moiety ([^3H]GG) transferred per unit of Myc-Rab1B.

Geranylgeranylation of recombinant Rab1B with partially purified Rab-GGTase

Two different assay systems were employed for evaluation of recombinant Rab1B proteins as substrates for partially purified Rab-GGTase. In the first system, 12 μl of *E. coli* lysate (in Buffer A) containing 0.3 μg (1.5 immunological units) of Myc-Rab1B^{WT} or Myc-Rab1B^{Q67L} were combined with 20 μl of partially purified Rab-GGTase, 5 μl of 10 $\times$ reaction buffer [0.5 M Hepes (pH 7.4)/50 mM MgCl_2 /100 μM GDP/10 mM DTT] and the final volume was adjusted to 48 μl with water. Reactions were started by addition of 2 μl of [^3H]GGPP (1 μCi) and terminated immediately after addition of [^3H]GGPP (zero time), or after incubation for 10 min or 20 min with radiolabelled substrate at 37°C . Blank reactions were carried out for the same time intervals with *E. coli* lysate lacking expressed Rab protein. The remainder of the assay was carried out as described above.

In the second assay system, which was designed to assess the impact of guanine nucleotides on the prenylation reaction, the *E. coli* lysates containing the substrate proteins were prepared in Buffer B (low MgCl_2) with 1 mM GDP, GTP or GTP[S]. Aliquots (5 μl) of each lysate, containing 0.3 μg of substrate protein, were combined with 20 μl of partially purified Rab-GGTase and 5 μl of 10 $\times$ reaction buffer [0.5 M Hepes (pH 7.4)/10 mM DTT] and

the volume was adjusted to 43 μl with water. The reaction mixture was preincubated for 15 min at 37°C and then 5 μl of 50 mM MgCl_2 was added. The prenylation reaction was then initiated by addition of 2 μl of [^3H]GGPP (1 μCi) and the reaction was terminated after 10 min at 37°C .

Prenylation of Rab1B in cultured 293 cells

Transformed human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and grown in 6 cm dishes in Dulbecco's modified Eagle's medium (DMEM) with 10 % (v/v) fetal-calf serum. Cells were transfected with 15 μg of pCMVrab1B^{WT}, pCMVrab1B^{Q67L}, or pCMVrab1B^{ACC}, using the calcium phosphate precipitation technique described by Gorman et al. [46]. At 3 h after addition of the DNA, the cultures were shocked by exposure to 15 % (v/v) glycerol in PBS for 30 s and were allowed to recover for 1 h in DMEM containing 10 % fetal-calf serum. Parallel cultures transfected with each Myc-Rab1B construct were incubated for either 12 h or 24 h in 3 ml of medium containing 200 $\mu\text{Ci}/\text{ml}$ [^3H]mevalonolactone (MVA) (3.4 Ci/mmol), beginning immediately after recovery from the glycerol shock. During the labelling period, 10 μM lovastatin was added to the culture medium to block endogenous isoprenoid synthesis. Cells were harvested, washed three times with HBSS and lysed in 100 μl of RIPA [100 mM Tris/HCl (pH 7.4)/2 mM EDTA/0.1 % (w/v) SDS/0.5 % (w/v) deoxycholate/0.5 % (v/v) Nonidet P40/1 mM PMSF/5 μM leupeptin/1 μM pepstatin/0.3 μM aprotinin]. Each lysate was diluted 1:5 by the addition of 100 mM Tris/HCl (pH 7.4)/2 mM EDTA, with the same proteinase inhibitors as in RIPA. Insoluble material was removed by centrifugation at 12000 g for 30 min and the cleared cell lysates were incubated for 2 h at 4°C with the 9E10 anti-Myc monoclonal antibody (Oncogene Sciences) at a concentration of 5 μg of lysate/ml. The immune complexes were collected by incubation with Protein-A-Sepharose coated with goat anti-mouse IgG (Cappel) for 1 h. The Sepharose beads were pelleted by centrifugation and washed once with RIPA, twice with 5-fold-diluted RIPA, and once with 100 mM Tris/HCl (pH 7.4)/2 mM EDTA/100 mM NaCl. The Myc-tagged Rab1B proteins were then eluted in SDS sample buffer and resolved by SDS/PAGE. Proteins were transferred to poly(vinylidene difluoride) (PVDF) membrane, and tritium incorporation into the Rab proteins was quantified with a Molecular Dynamics PhosphorImager system, using the tritium screen. Each tritium value (peak volume) was normalized to the amount of immunoprecipitated Myc-tagged Rab1B present on the same blot. This was determined by immunoblotting the same membrane with an affinity-purified antibody against Rab1B (Zymed Laboratories, South San Francisco, CA, U.S.A.) and quantifying the amount of bound secondary ^{125}I -IgG with the PhosphorImager. Before forming a ratio between the tritium and bound ^{125}I -IgG values, the tritium values for Myc-Rab1B^{WT} and Myc-Rab1B^{Q67L} were corrected for background radioactivity in the immunoprecipitates by subtracting values obtained for Myc-Rab1B^{ACC}.

Assay for Interaction of Rab1B with GDI

The ability of expressed Rab proteins to form stable complexes with epitope-tagged bovine brain Rab-GDI (GDI α) in transfected 293 cells was assessed by means of a co-immunoprecipitation assay described previously [47]. Briefly, 293 cells growing in 10 cm dishes were transfected with DNA mixtures containing 15 μg of pCMVFLAG-GDI α combined with either pCMVrab1B^{WT} (15 μg), pCMVrab1B^{ACC} (40 μg) or pCMVrab1B^{Q67L} (15 μg) (FLAG is the N-terminal amino acid

sequence DYKDDDDK). At 48 h after transfection, cultures were harvested and cytosolic fractions were prepared. About 10 % of the cytosol was used for quantification of the total expressed GDI and Myc-Rab1B proteins by immunoblot analysis. The remaining cytosol was incubated with 100 μ l of a 50 % suspension of Anti-FLAG M2 affinity gel (IBI/Eastman Kodak) and the bound FLAG-GDI protein complexes were eluted with 100 μ l of 0.1 M glycine. The eluted proteins were resolved by SDS/PAGE in 11 % polyacrylamide gels and transferred to PVDF membrane. The upper half of the membrane was immunoblotted with an affinity-purified rabbit polyclonal antiserum generated against a keyhole-limpet haemocyanin-coupled synthetic peptide corresponding to amino acids 428–447 of the bovine Rab-GDI sequence (Zymed Laboratories). The lower half of the membrane was immunoblotted with the affinity-purified rabbit antibody against Rab1B. Quantification of bound primary IgG was accomplished with a PhosphorImager after incubation of the blots with 125 I-labelled goat anti-rabbit IgG. In the studies described in the present paper, recoveries of FLAG-GDI in the glycine eluates from the anti-FLAG affinity resin were approx. 25–30 % of the total present in the cytosol.

RESULTS

Geranylgeranylation of Rab1B^{Q67L} versus Rab1B^{WT} in reticulocyte lysates

Sequence alignments indicate that many structural elements in Ras are conserved in other low-molecular-mass GTP-binding proteins [48,49]. Therefore, the known three-dimensional structure of H-Ras [50,51] has been used extensively as a guide for creating functional alterations in Rab proteins by site-directed mutagenesis. Amino acid substitutions at position Q61 (e.g.

Q61L) markedly reduce the intrinsic GTPase activity of H-Ras [52]. Since equivalent substitutions have the same effect on Rab proteins [35,53], we decided to use the Rab1B^{Q67L} mutant to study the guanine-nucleotide-dependent conformational specificity of Rab GGTase. To facilitate immunoprecipitation analysis of the Rab1B proteins in intact cells, they were modified by addition of an N-terminal Myc tag. In preliminary studies we determined that addition of the Myc-tag has no effect on the ability of Rab1B^{WT} to serve as a substrate for Rab-GGTase (results not shown). We began by comparing the geranylgeranylation of wild-type and Q67L Rab1B translation products in rabbit reticulocyte lysate (Figure 1). As in the case of intact mammalian cells, reticulocyte lysates contain GTP in excess of GDP, with the estimated concentration of GTP being around 0.1 mM [54]. Translation was monitored by incorporation of [35 S]methionine into the Myc-Rab1B polypeptides, while prenylation of the nascent polypeptides by endogenous Rab-GGTase in the reticulocyte lysate was monitored in parallel assays supplemented with [3 H]GGPP. The results clearly demonstrate that the Myc-Rab1B^{WT} and Myc-Rab1B^{Q67L} mRNA constructs were translated with comparable efficiency, yielding [35 S]methionine-labelled polypeptides of approx. 27 kDa. However, after a 3 h incubation with [3 H]GGPP, the incorporation of [3 H]GG into the Q67L mutant was only 15 % of that observed for the wild-type protein (Figure 1). In agreement with previous studies [32,33], prenylation of the wild-type translation product was reasonably efficient in this system, with an estimated stoichiometry of 0.7 mol of GG/mol protein. If one assumes that the endogenous Rab-GGTase in the reticulocyte lysate catalyses a dual geranylgeranyl modification of Rab1B, as has been established for Rab proteins ending with the xxCC motif [55], this would mean that approx. 35 % of the substrate protein was modified during the course of the translation/prenylation reaction.

Figure 1 *In vitro* translation and prenylation of Rab1B^{Q67L} versus Rab1B^{WT}

mRNAs encoding the indicated Myc-Rab1B proteins were translated in rabbit reticulocyte lysates containing [35 S]methionine (upper panel, '[35 S]Met') or [3 H]GGPP (lower panel, '[3 H]GG'). Reactions were terminated after 3 h at 30 °C and aliquots of the translation products were subjected to SDS/PAGE and fluorography. The amount of each [35 S]methionine-labelled translation product loaded on the gel was 10 % of the amount of the [3 H]GG-labelled product from a parallel reaction. The 3 H/ 35 S ratio for Rab1B^{Q67L} was 15 % of the value calculated for Rab1B^{WT}. Based on the known specific activities of the [3 H]GGPP and [35 S]methionine, and an estimated 5 μ M concentration of endogenous methionine in the reticulocyte lysate, the calculated stoichiometry of prenylation for wild-type Myc-Rab1B in the experiment shown was 0.7 mol of GG incorporated/mol of protein. Similar results were obtained in two additional translation experiments.

Geranylgeranylation of Recombinant Rab1B^{Q67L} versus Rab1B^{WT}

Since previous prenylation studies using bacterially expressed recombinant Rab5^{Q79L} [35] have failed to confirm the defective prenylation of nascent Rab5^{Q79L} synthesized in reticulocyte lysates, we extended our studies to include a comparison of the geranylgeranylation of recombinant Myc-Rab1B^{Q67L} versus Myc-Rab1B^{WT} in different cell-free assays. To avoid any changes in the properties of the Q67L mutant that might occur during purification, the recombinant Rab1B substrates were immediately quantified by immunoblot analysis of *Escherichia coli* lysates and then added directly to the prenyltransferase assays. Although this approach precluded a detailed kinetic analysis of the GTPase activities of the wild-type and mutant proteins, we were able to confirm the predicted defect in the ability of the Q67L mutant to hydrolyse GTP by performing ligand-binding studies of the recombinant proteins renatured on nitrocellulose membranes (Figure 2). Specifically, these results showed that, while both Myc-Rab1B^{WT} and Myc-Rab1B^{Q67L} were able to bind [α - 32 P]GTP (Figure 2A), only the wild-type protein was able to convert the bound nucleotide into [32 P]GDP (Figure 2B). Incidentally, it might be noted that the overall amount of [α - 32 P]GTP bound to the Q67L mutant was reduced by approx. 50 % compared with the wild-type protein (Figure 2A). Since the recombinant Rab proteins probably lose most of their endogenous bound nucleotide during SDS/PAGE, this could indicate that the renatured Q67L mutant has a modestly reduced binding affinity for GTP. However, it remains to be established whether this behaviour of the renatured protein reflects that of the native Rab1B^{Q67L} in solution.

Figure 2 Analysis of the guanine-nucleotide-binding and hydrolysis properties of Rab1B^{Q67L}

(A) Aliquots of *E. coli* lysate containing Myc-Rab1B^{WT} or Myc-Rab1B^{Q67L} were subjected to SDS/PAGE on duplicate SDS/12.5%-polyacrylamide gels and the proteins were transferred to nitrocellulose membranes. One membrane was immunoblotted with a monoclonal antibody against the Myc epitope and the bound IgG was revealed by enhanced chemiluminescence. The duplicate membrane was incubated with α -[³²P]GTP. (B) The bound [³²P]-labelled guanine nucleotides were eluted from the Rab proteins on the blot depicted in (A) and one-third of each eluate was subjected to TLC to separate [³²P]GTP from [³²P]GDP. After autoradiography, regions of the thin-layer plate containing GDP and GTP were quantified. The ratios [³²P]GTP/[³²P]GDP were as follows: Rab1B^{WT}, 0.99; Rab1B^{Q67L}, 7.5. The experiment was repeated once with essentially identical results.

Figure 3 Geranylgeranylation of recombinant Rab1B in cell-free assays

(A) Equal amounts of *E. coli* lysate containing the indicated recombinant Myc-Rab1B proteins were incubated for 3 h in an assay system containing rat brain cytosol as a source of Rab-GGTase activity. For each protein, two assays were performed, with the results of each assay indicated by a separate bar. (B) Lysates containing recombinant Rab1B were mixed with partially purified Rab-GGTase and reactions were terminated after 10 min or 20 min. The amount of [³H]GG transferred to the Rab protein from [³H]GGPP was determined and the results were normalized to the amount of immunodetectable Myc-tagged Rab1B protein added to the reaction (see the Materials and methods section). Each point represents a mean ($\pm$ S.E.M.) derived from four parallel reactions.

We initially compared the geranylgeranylation of the recombinant Myc-Rab1B proteins by incubating them with rat brain cytosol and [³H]GGPP for 3 h (Figure 3A). Under these conditions the stoichiometry of GG incorporation into the wild-type protein was approx. 0.48 mol/mol protein, suggesting that approx. 24 % of the substrate was converted into the digeranylgeranylated form. In contrast, the Q67L mutant was a poor substrate for geranylgeranylation, in good agreement with the results of the reticulocyte-lysate assay. Further interpretation of these findings is complicated by the complexity of the assay system and the difficulty of predicting the nucleotide state of the Rab protein during the prenylation reaction. Recombinant Rab proteins are thought to reside predominantly in the GDP state when isolated from *E. coli* [56]. However, since brain cytosol contains a high concentration of GTP [57], and may also harbour

Table 1 Effects of guanine nucleotides on geranylgeranylation of recombinant Rab1B^{WT} and Rab1B^{Q67L}

E. coli lysates containing Myc-Rab1B^{WT} or Myc-Rab1B^{Q67L} were prepared in the presence of 1 mM GTP, GDP or GTP[S]. Aliquots of lysate containing comparable amounts of Myc-Rab1B (approx. 0.3 μ g) were then preincubated under low-magnesium conditions and assayed in reaction mixtures containing partially purified Rab-GGTase and 100 μ M (final concns.) of the indicated nucleotides. Reactions were allowed to proceed for 10 min at 37 °C. Results are the means ($\pm$ S.E.M.) for three separate determinations.

Rab1B	Nucleotide added	[³ H]GGPP incorporated (d.p.m./unit of Rab1B)	Inhibition (%) (GTP versus GDP)
Q67L	GDP	23296 $\pm$ 621	—
	GTP	5498 $\pm$ 117	76.4
WT	GDP	16021 $\pm$ 179	—
	GTP[S]	2878 $\pm$ 252	82.0

nucleotide-exchange factors capable of operating on Rab1B [58], it is possible that significant GTP loading of the substrate protein could occur during the incubation. Thus, to obtain a better estimate of the rate of Rab1B prenylation under more controlled conditions, the recombinant proteins were also assayed in a system containing partially purified Rab-GGTase. In this assay the rate of incorporation of [³H]GG into Myc-Rab1B^{WT} was linear for 20 min, with a stoichiometry of 0.2 mol of GG/mol of substrate protein at the end point. Under these conditions the recombinant Myc-Rab1B^{Q67L} showed a striking improvement in its ability to serve as a prenyl acceptor relative to the wild-type protein (Figure 3B). In considering the possible reasons for this change, we noted that the use of purified Rab-GGTase markedly reduced the concentration of GTP in the assay system compared with the previous assays containing large amounts of brain cytosol or reticulocyte lysate.

To explore further the influence of the nucleotide composition of the lysis and assay solutions on the ability of Rab1B^{Q67L} to undergo prenylation by Rab-GGTase, we prepared recombinant Myc-Rab1B^{Q67L} in lysis buffers containing either 1 mM GDP or 1 mM GTP, and then preincubated the protein for 15 min in low-magnesium buffer to promote nucleotide exchange before initiating the prenylation reaction. Because guanine nucleotide concentrations in the millimolar range have been reported to interfere with Rab-GGTase activity [31], the final concentrations of GDP or GTP were reduced to 100 μ M in the prenylation reaction. The results, which are summarized in Table 1, indicate that addition of GTP markedly reduced the ability of the Q67L mutant to serve as a substrate for Rab-GGTase, while the protein remained a good substrate when incubated in the presence of identical concentrations of GDP. We attributed the reduced geranylgeranylation of Myc-Rab1B^{Q67L} in this experiment to the displacement of bound GDP by the added GTP and the inability of the mutant protein to hydrolyse GTP. If this assumption is correct, then addition of a non-hydrolysable GTP analogue to the wild-type protein under identical conditions should have a significant impact on its geranylgeranylation. The results of such a study are shown in Table 1. Addition of GTP[S] impaired the geranylgeranylation of Myc-Rab1B^{WT} to approximately the same extent as seen when GTP was added to the Q67L mutant. In summary, the data presented in Table 1 appear to confirm the hypothesis that the guanine nucleotide state of Rab1B influences its interactions with the Rab-GGTase enzyme complex, with the GDP-bound form of the protein being the favoured substrate.

Figure 4. Prenylation of Myc-Rab1B proteins expressed in mammalian cells

Parallel cultures of HEK 293 cells were transfected with pCMV*rab1B*^{WT}, pCMV*rab1B*^{Q67L}, or pCMV*rab1B*^{ΔCC} and the cells were incubated with [³H]MVA for 12 h or 24 h. Myc-Rab proteins were immunoprecipitated from the cell lysates, subjected to SDS/PAGE and transferred to PVDF membrane. (A) The results of PhosphorImager analysis for one set of blots (24 h samples) are shown. The lower panel depicts the [³H]MVA-labelled Myc-Rab1B proteins and the upper panel depicts the results after probing the same blot with a rabbit antibody to Rab1B, followed by ¹²⁵I-labelled goat anti-rabbit IgG. (B–D) The quantitative results from two separate experiments are shown, with each point derived from an immunoprecipitation from a single culture. In (B) the [³H]MVA incorporated into the immunoprecipitated Myc-Rab1B^{WT} or Myc-Rab1B^{Q67L} was expressed as a ratio to the immunodetectable Rab1B (bound ¹²⁵I-IgG) in each sample. In (C) and (D) the values for total immunodetectable Myc-Rab1B and total [³H]MVA incorporation are displayed separately.

Prenylation of Rab1B^{Q67L} in cultured cells

To compare the abilities of Myc-Rab1B^{WT} and Myc-Rab1B^{Q67L} to undergo geranylgeranylation in intact mammalian cells, 293 cells were transfected with plasmids encoding Myc-tagged Rab1B^{WT}, Rab1B^{Q67L} or Rab1B^{ΔCC} and the medium was supplemented with lovastatin and the isoprenoid precursor [³H]MVA. Since lovastatin blocks mevalonate synthesis, the radiolabelled precursor taken up from the medium was the principal carbon source for synthesis of GGPP during the post-transfection period. Upon harvesting the cells, the expressed Myc-tagged Rab1B proteins were isolated from other endogenous prenylated proteins by immunoprecipitation with an antibody against the Myc epitope. To control for variations in recovery of the wild-type and mutant Rab1B, the amount of [³H]MVA incorporated into each immunoprecipitated protein was normalized to the total Myc-Rab1B protein recovered in the sample. As shown in Figure 4(A), the immunoprecipitation with anti-Myc monoclonal anti-

Figure 5. Association of Myc-Rab1B^{Q67L} and Myc-Rab1B^{WT} with FLAG-GDIα in 293 cells

Parallel cultures were co-transfected with pCMV plasmids encoding FLAG-GDIα and either Myc-Rab1B^{WT}, Myc-Rab1B^{Q67L} or Myc-Rab1B^{ΔCC}. (A) About 10% of the cytosol from the transfected cells was subjected to immunoblot analysis. The upper region of the blot was incubated with affinity-purified antibody to GDIα and the lower region of the blot was incubated with affinity-purified antibody against Rab1B. (B) FLAG-GDIα/Rab1B complexes were eluted from the anti-FLAG resin as described in the Materials and methods section and the entire eluate was subjected to SDS/PAGE and immunoblot analysis. The upper part of the blot was incubated with anti-GDIα antibody and the lower part of the blot with anti-Rab1B antibody. Goat anti-rabbit ¹²⁵I-IgG was used as secondary antibody to allow quantification and revelation of immunodetectable proteins. Immunoblots were analysed using a PhosphorImager. Similar results were obtained in two additional experiments. The percentage of cytosolic Myc-Rab1B that was captured as a complex with FLAG-GDIα eluted from the anti-FLAG beads was as follows: WT, 15 ± 3% (S.E.M.) and Q67L, 12 ± 2% (S.E.M.). The ratio of Myc-Rab1B to FLAG-GDI in the eluted complex was: wild-type, 0.85 ± 0.15 (S.E.M.) and Q67L, 1.28 ± 0.3 (S.E.M.). There was no immunodetectable Rab1B^{ΔCC} eluted with FLAG-GDI.

body effectively isolated the expressed Myc-Rab1B proteins from endogenous Rab1B and other prenylated Ras-related proteins that normally migrate at 20–30 kDa in SDS gels. Specifically, the precipitates from cells expressing Myc-Rab1B^{ΔCC}, which lacks the target cysteine residues for geranylgeranylation, showed virtually no ³H in the region of the blot containing Rab1B, whereas parallel cultures expressing Myc-Rab1B^{WT} showed readily detectable [³H]MVA incorporation. Of particular note was the finding that prenylation of the expressed Myc-Rab1B^{Q67L} was comparable with that observed for the wild-type protein. This was confirmed when we calculated the specific radioactivity of the expressed Myc-Rab1B^{WT} and Myc-Rab1B^{Q67L} pools (i.e. the ratio of incorporated [³H]MVA to immunodetectable Myc-Rab1B) in two separate experiments (Figure 4B). Although a precise determination of the stoichiometry of prenylation in these studies is precluded by uncertainties regarding the intracellular MVA and GGPP pools and the molar amount of Rab1B in the immunoprecipitates, the data depicted in Figure 4 suggest that prenylation of Myc-Rab1B in transfected 293 cells was occurring under conditions of excess substrate protein. Thus the relative amount of expressed Myc-Rab1B in the immunoprecipitates (Figure 4C) and the whole-cell lysates (not shown) was maximal by 12 h after transfection, whereas the incorporation of [³H]MVA into the expressed proteins continued to increase in linear fashion up to 24 h (Figure 4D). Consistent with this conclusion, we recently observed that transfected 293 cells still contain a large cytosolic pool of non-prenylated Myc-Rab1B protein when examined at 24 h after transfection [47].

Interaction of Rab1B^{Q67L} with Rab-GDI in cultured cells

The foregoing studies indicated that Myc-Rab1B^{Q67L} was able to assume the GDP-bound conformation and undergo geranyl-

geranylation in 293 cells, thus meeting two of the major requirements for interaction with cytosolic Rab-GDI. To test the possibility that the Q67L mutant could form a complex with GDI *in vivo*, we employed an assay in which Myc-tagged Rab1B proteins were transiently co-expressed with FLAG-tagged GDI in transfected cells and the FLAG-GDI was subsequently collected from the cytosol on anti-FLAG affinity beads. As shown in Figure 5, the expressed wild-type, Q67L and Δ CC proteins were all readily detected in the cytosol preparations and the levels of expression of FLAG-GDI were comparable in all of the cultures (Figure 5A). Immunoblot analysis of the protein complexes eluted from the anti-FLAG affinity beads revealed that Myc-Rab1B^{WT} and Myc-Rab1B^{Q67L} were co-precipitated with FLAG-GDI, whereas the non-prenylated Myc-Rab1B^{ACC} was not detected (Figure 5B). Moreover, both the percentage of cytosolic Myc-Rab1B collected in the FLAG-GDI complex and the ratio of Myc-Rab1B to FLAG-GDI in the complex were comparable for the Q67L and wild-type proteins.

DISCUSSION

We show here that geranylgeranylation of a Rab1B mutant with reduced intrinsic GTPase activity varies significantly depending on the guanine nucleotide composition of the assay system. The mutant is a poor substrate for geranylgeranylation under cell-free conditions where GTP is the predominant nucleotide: e.g. in reticulocyte lysates, brain cytosol or buffers containing GTP. In contrast, the Q67L mutant is a relatively good substrate when extracted from *E. coli* and assayed under conditions where GDP is the only nucleotide present. Addition of the non-hydrolysable GTP analogue GTP[S] to reactions containing Rab1B^{WT} inhibits prenylation to the same extent as that observed when GTP is added to reactions containing Rab1B^{Q67L}. Taken together, these results support the conclusion that Rab1B in the GDP conformation is the preferred substrate for Rab-GGTase. Our results are consistent with previous comparative studies of Rab5^{Q79L} versus Rab5^{WT} in reticulocyte lysates, where prenylation of the nascent Q79L translation product was diminished [33], while prenylation of the corresponding recombinant protein (presumably isolated in the GDP state) was not detectably affected [35].

The molecular basis for the apparent preference of the Rab-GGTase for the GDP-bound substrate remains to be determined. Two major structural domains of Ras proteins are known to undergo conformational changes when the guanine-nucleotide-binding site is occupied by GTP versus GDP. These have been designated as the Switch-1 region (loop-2 and part of β 2) and Switch-2 region (loop-4 and α 2) [59,60]. One possibility is that the analogous nucleotide-sensitive domains in Rab proteins interact directly with the catalytic $\alpha\beta$ subunits of Rab-GGTase [18]. Alternatively, since REP plays an essential role in presenting Rab proteins to the catalytic component of Rab-GGTase [19], it is possible that the GTP-bound form of Rab has a reduced affinity for REP. Discrimination between these possibilities will require detailed studies of the physical interaction of Rab proteins with individual proteins of the Rab-GGTase complex.

The inability of our metabolic labelling studies to confirm a reduction in the prenylation of Rab1B^{Q67L} versus Rab1B^{WT} in cultured 293 cells suggests that the interaction of nascent Rab proteins with Rab-GGTase *in vivo* may differ in some fundamental way from what occurs in cell-free systems. In this regard, it is important to recognize that Rab proteins can interact with several accessory proteins that regulate GTP hydrolysis and guanine nucleotide exchange [3], and that such interactions may alter the properties of Rab proteins in intact cells. Of particular

note is our finding that Rab1B^{Q67L} and Rab1B^{WT} were essentially indistinguishable with respect to their ability to associate with Rab-GDI (Figure 5). Since interaction with GDI is absolutely dependent on geranylgeranylation of the Rab protein [13,15], this finding provides strong support, independent of metabolic labelling, for the existence of a substantial pool of prenylated Rab1B^{Q67L} in 293 cells. Furthermore, since GDI binds preferentially to Rab proteins in the GDP conformation [61], the results of the GDI-binding assay indicate that the Q67L mutant manifests significant GTPase activity *in vivo*. A similar conclusion was reached in a study of Rab3A [53], where the percentage of total Rab3A^{Q81L} in the GDP-bound state in cultured cells was similar to that determined for Rab3A^{WT}, despite the markedly reduced GTPase activity exhibited by the mutant in cell-free assays. This discrepancy was attributed to the fact that Rab3A^{Q81L} retained the capacity to interact with Rab3A-GAP (where GAP is GTPase-activating protein), which can stimulate the GTPase activity of the mutant protein as much as 16-fold over basal levels [53]. The Rab3A-GAP activity operates effectively on non-prenylated Rab3A protein [62]. Thus it is reasonable to speculate that a Rab1B-specific GAP in intact 293 cells might act on the nascent Q67L protein, allowing a substantial proportion of the mutant protein to attain the GDP-conformation preferred by Rab-GGTase and Rab-GDIs [4,15]. The possible absence of an appropriate mammalian Rab-GAP activity in insect cells might explain why others have been unable to detect prenylation of a GTPase-deficient human Rab6 mutant (Q72R) in Sf9 cells [34]. Further insight into the possible involvement of Rab-GAPs in regulating the nucleotide-dependent conformation of newly synthesized Rab polypeptides will come with the isolation and characterization of these important regulatory proteins. However, until more is known about the influence of such proteins on the prenylation process, conclusions regarding the ability of specific Rab mutants to undergo post-translational modification should be drawn only after an analysis of their behaviour in intact mammalian cells.

Note added in proof (received 26 July 1996)

While this paper was in press, Seabra [63] reported that REP interacts preferentially with the GDP-bound forms of several Rab proteins.

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