Prenylation of Rab8 GTPase by type I and type II geranylgeranyl transferases

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Rab GTPases are post-translationally modified by addition of geranylgeranyl moieties to carboxyl-terminal cysteine residues. For Rab proteins ending with *xxCC*, *xCxC* and *CCxx* motifs this modification is catalysed by geranylgeranyltransferase type II (GGTaseII), and is entirely dependent on the Rab substrate being bound to Rab escort protein (REP). Several Rab proteins contain carboxyl-terminal *CaaL* prenylation motifs typical of members of the Rho family, which are modified in a REP-independent manner by geranylgeranyltransferase type I (GGTaseI). The present studies show that one such Rab protein (Rab8), which ends with a CVLL motif, is uniquely able to serve as a substrate for either REP/GGTaseII or GGTaseI in cell-free assays. The modification of Rab8 by GGTaseI did not require REP, indicating that a REP-induced conformational change is not essential for exposure of the Rab carboxyl-terminal cysteine prenylation site. To determine whether one enzyme plays a predominant role in Rab8 prenylation *in vivo*, the incorporation

of [³H]mevalonate into Rab8 was measured in human embryonal kidney 293 cells under conditions where the activity of GGTaseI, but not GGTaseII, was blocked by the peptidomimetic inhibitor GGTI-298. The GGTaseI inhibitor did not prevent prenylation of either overexpressed Myc-tagged Rab8 or endogenous Rab8, whereas prenylation of a known GGTaseI substrate with the same carboxyl-terminal motif, Cdc42Hs, was completely blocked. To rule out the possibility that the apparent prenylation of Rab8 by GGTaseII occurs only when GGTaseI activity is eliminated, metabolic labelling studies were carried out in the absence of the GGTaseI inhibitor, using a REP-binding-deficient Rab8 construct (Y78D) that cannot serve as a substrate for GGTaseII, but is indistinguishable from wild-type Rab8 as a substrate for GGTaseI. Prenylation of the Y78D mutant was reduced by 60–70% in intact cells, consistent with the conclusion that the majority of Rab8 is prenylated by the REP/GGTaseII system *in vivo*.

INTRODUCTION

Rab proteins are Ras-related GTPases that function in a variety of vesicular transport pathways in mammalian cells [1–4]. Nascent Rab proteins are modified by addition of one or two 20-carbon geranylgeranyl groups to carboxyl-terminal cysteine residues [5–7]. The post-translational modification of Rab proteins is critical for their membrane localization [8,9] and for their interaction with soluble regulatory proteins such as the Rab GDP-dissociation inhibitors (GDIs) [10]. The latter proteins form stable complexes with Rab proteins in the GDP-conformation [11] and appear to play a key role in the cycling of Rab proteins on and off intracellular membranes in conjunction with vesicle budding and fusion events [12].

The majority of Rab proteins end with *xxCC*, *xCxC* or *CCxx* carboxyl-terminal amino acid motifs and are modified by geranylgeranyl transferase type II (GGTaseII) [13]. The catalytic component of this enzyme, which consists of a heterodimer (α and β subunits), can transfer geranylgeranyl (GG) groups to the Rab substrate only if the latter is bound to a carrier protein termed Rab escort protein (REP) [7,14–17]. In cell-free systems REP remains associated with the geranylgeranylated Rab protein after the prenylation reaction is complete [14] and is capable of donating the modified Rab protein to cellular membrane [18] or phospholipid vesicles [19]. Thus in addition to being required for proper presentation of the Rab substrate to GGTaseII, REP appears to play a role in Rab membrane targeting. Several studies have established that geranylgeranylation of Rab proteins

by the REP/GGTaseII enzyme complex is highly dependent on structural features outside of the immediate Rab carboxyl-terminal prenylation site [20–24] and is sensitive to changes in the nucleotide state of the protein [25–27]. In particular, REP associates preferentially with the GDP-bound form of the Rab protein [28].

Although most Rab proteins are di-geranylgeranylated, several members of the Rab protein family (e.g. Rab8, Rab13, Rab18) contain only one target cysteine and are distinguished by a carboxyl-terminal *Caax* [amino acid sequence motif in which cysteine is followed by two aliphatic residues (*a*) and a variable terminal residue (*x*)] prenylation motif typically found in Ras-related GTPases outside of the Rab family. For instance, the carboxyl-terminal sequence of Rab 8 (CVLL) is identical to the prenylation motifs found on Cdc42Hs [29] and Rho1p [30]. In general, when small peptides or proteins contain a *Caax* motif with M, S, A or Q in the terminal position, they serve as substrates for farnesyltransferase (FTase) [31,32]. When L occupies the terminal position, proteins are modified by geranylgeranyltransferase type I (GGTaseI) [33,34]. Unlike GGTaseII, both FTase and GGTaseI can modify monomeric GTPases or small *Caax*-containing peptides in the absence of an escort protein, and neither enzyme is known to require a particular guanine nucleotide conformation of the substrate protein [35].

In the absence of direct evidence to the contrary, it has been assumed that prenylation of Rab proteins ending with a *CaaL* motif, such as Rab8, is mediated by GGTaseI [36,37]. If true, this would imply that prenylation and correct membrane targeting of

Abbreviations used: GGTaseII, geranylgeranyltransferase type II; GGTaseI, geranylgeranyltransferase type I; GDI, GDP-dissociation inhibitor; REP, Rab escort protein; HEK, human embryonal kidney; GG, geranylgeranyl group; PVDF, poly(vinylidene difluoride); MVA, mevalonate; DMEM, Dulbecco's modified Eagle's medium; *Caax*, amino acid sequence motif in which cysteine is followed by two aliphatic residues (*a*) and a variable terminal residue (*x*).

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such Rab proteins might be accomplished without the 'escort' function of REP. However, it remains possible that certain intrinsic characteristics of the Rab structure may take precedence over the *Caax* motif and render these proteins substrates for the REP-dependent GGTaseII enzyme complex. In the present study, we address this issue by evaluating the abilities of a naturally occurring *Caal*-type Rab protein, Rab8, to serve as a substrate for GGTaseI versus GGTaseII in cell-free systems and intact human embryonal kidney 293 (HEK293) cells. The results indicate that Rab8 is uniquely able to serve as a substrate for both enzymes in cell-free systems, but is modified predominantly via the REP/GGTaseII pathway *in vivo*.

MATERIALS AND METHODS

Generation of GTPase expression vectors

The cDNA encoding Rab1B was modified as previously described to include a Myc epitope tag (EQKLISEEDL) at the amino terminus of the expressed protein [12]. The cDNA for Rab8 was obtained by PCR amplification from HeLa cell cDNA and modified to include a sequence encoding an amino-terminal Myc tag. The Y to D amino acid substitution at position 78 of the Rab8 protein sequence was engineered by PCR modification of the Rab8(wt) cDNA, using the overlap extension technique [38]. The 5' and 3' oligonucleotide primers included appropriate restriction sites so that the resulting Rab8(Y78D) PCR product could be cloned into a pCMV5 expression vector [39] containing an in-frame 5' Myc-tag sequence immediately upstream from the inserted Rab8(Y78D) sequence. The cDNA encoding Cdc42Hs [29] was provided by Richard Cerione (Cornell University, Ithaca, NY, U.S.A.) and was modified to introduce a Myc tag at the amino-terminus of the protein. The dideoxy chain termination technique (Sequenase 2.0 or Thermo Sequenase, Amersham) was used to verify the DNA sequence of all constructs. The cDNA sequences encoding Myc-tagged Rab1B, Rab8 and Cdc42Hs were subcloned into pCMV5neo [40] for expression studies in mammalian cells.

Preparation of recombinant GTPases

cDNA constructs encoding Myc-tagged Rab1B, Rab8, Rab8(Y78D) or Cdc42Hs were inserted into pET17b (Novagen) and recombinant proteins were expressed in *Escherichia coli* as described previously [12]. Bacterial cell lysates were prepared in Buffer A (50 mM Hepes, pH 7.4, 5 mM MgCl₂, 1 mM DTT, 10 μ M GDP) and stored at -80°C after addition of 20% (v/v) glycerol. Myc-tagged recombinant proteins were quantified by immunoblot analysis of *E. coli* lysates using the 9E10 mouse monoclonal anti-Myc antibody (Oncogene Sciences). ¹²⁵I-labelled goat anti-mouse IgG was applied as the secondary antibody and the bound IgG was quantified with a Molecular Dynamics PhosphorImager. Known amounts of Myc-Rab1B were included in the immunoblot analysis to provide a standard by which a particular Myc signal could be related to the actual amount of recombinant protein in the lysate.

For some studies the cDNAs encoding Rab1B and Cdc42Hs were modified by PCR to encode an amino-terminal polyhistidine (his₆) sequence and the recombinant proteins were purified. *E. coli* lysates derived from 100 ml cultures were supplemented with imidazole and NaCl to final concentrations of 10 and 200 mM respectively. Nickel-agarose spin columns (Qiagen) were equilibrated with Buffer B (50 mM NaH₂PO₄, pH 8, 300 mM NaCl, 20 mM imidazole and 10 μ M GDP). Lysates were loaded onto the columns and the resin was washed three times with 0.6 ml of Buffer B supplemented with 1 mM PMSF. Finally, his₆-tagged

proteins were eluted with Buffer B containing 250 mM imidazole. Eluted proteins were concentrated using an Amicon Centricon-10 concentrator and diluted 100-fold with Buffer A. Proteins were stored at -80°C after addition of 20% (v/v) glycerol. Rab proteins were quantified by means of the Bio-Rad protein assay and densitometric scanning of Coomassie-stained proteins after SDS/PAGE. Typically, the his₆-tagged protein represented approximately 40–50% of the total protein eluted from the nickel-agarose resin.

Geranylgeranylation of recombinant proteins *in vitro*

Varying amounts of Myc-tagged or his₆-tagged substrate proteins typically are incubated at 37°C in 50 μ l reactions containing either 20 ng recombinant REP-1 and 20 ng recombinant GGTaseII $\alpha\beta$ subunits (provided by Miguel Seabra, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) or 10 or 100 ng recombinant GGTaseI (provided by Pat Casey, Duke University, Durham, NC, U.S.A.). The GGTaseII assays were carried out in Buffer A supplemented with 0.2 mM NP-40, essentially as described by Seabra et al. [7]. GGTase I assays were performed in Buffer C (50 mM Tris, pH 7.7, 20 mM KCl, 5 mM MgCl₂, 25 μ M ZnCl₂, 1 mM DTT, 0.5 mM Zwittergent 3-14), as described by Zhang et al. [41]. Assay mixtures were preincubated at 37°C for 3 min and the reaction was started by the addition of 1 μ Ci [³H]GGPP_i (15 Ci/mmol; American Radiolabeled Chemical Corp., St. Louis, MO, U.S.A.). Reactions were terminated after 10 min, unless otherwise indicated, and proteins were separated by SDS/PAGE on a 12.5% polyacrylamide gel [42]. The dried gel was subjected to fluorography and segments of the gel containing the radiolabelled proteins were excised, solubilized in 0.75 ml of 30% H₂O₂ for 16 h at 70°C , and counted in a liquid scintillation spectrometer.

GGTaseI inhibitors

Specific inhibitors of GGTaseI (GGTI-297, GGTI-298) were generously provided by Saïd Sebt and Andrew Hamilton (H. Lee Moffitt Cancer Center, Tampa, FL, U.S.A. and University of Pittsburgh, Pittsburgh, PA, U.S.A.). The structures and properties of these inhibitors have been described previously [43]. To inhibit the activity of GGTaseI in cell-free systems, the indicated amounts of recombinant protein were assayed as described above, except that increasing concentrations of GGTI-297 (5, 25 and 50 nM) were added to the reaction mixture. The GGTI-297 (free acid) was solubilized in DMSO containing 10 mM dithiothreitol, and equivalent amounts of solvent were added to parallel control reactions without inhibitor.

Prenylation of Rab proteins in cultured cells

HEK 293 cells were plated at a density of 3.8×10^4 cells/cm² and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal calf serum for 24 h. The cells were then transfected with 40 μ g of pCMV expression vector encoding Myc-Rab1B, Myc-Rab8, Myc-Rab8(Y78D) or Myc-Cdc42Hs as described previously [12], except that Opti-MEM (Gibco) was used in place of DMEM during the incubation with the calcium phosphate-DNA precipitate and 10 μ M lovastatin was added to the culture medium immediately after the transfection. Six hours after the addition of lovastatin, cells were labelled by adding fresh DMEM/10% FBS containing 200 μ Ci/ml [³H]mevalonolactone (3.4 Ci/mmol) and 10 μ M lovastatin. Where indicated, the labelling medium also contained the methyl ester form of the GGTaseI inhibitor, GGTI-298 (10 μ M). Cells were harvested at various intervals after transfection and the overexpressed Myc-

tagged proteins were immunoprecipitated as described previously [12]. The relative amounts of Myc-tagged protein collected in the immunoprecipitates were compared by subjecting 10% of the immunoprecipitated protein to SDS/PAGE followed by immunoblot analysis using affinity-purified rabbit polyclonal antibodies against Rab1B (Zymed Laboratories), Rab8 (Santa Cruz Biotech), or Cdc42Hs (provided by Dr. Tony Evans). ^{125}I -labelled goat anti-rabbit IgG was used as secondary antibody and the ^{125}I -labelled blots were exposed to film and quantified by densitometric analysis of the autoradiograph. The relative incorporation of $[\text{H}]\text{MVA}$ (MVA = mevalonate) into each of the expressed Myc-tagged proteins was determined by subjecting the remaining 90% of the immunoprecipitated protein to SDS/PAGE and fluorography. Fluorographs were quantified by densitometric scanning using a Molecular Dynamics laser densitometer.

To assess the effect of GGTI-298 on the prenylation of endogenous Rab8 and Rab1B in HEK293 cells, parallel cultures were incubated with or without $10\text{ }\mu\text{M}$ GGTI-298 for 15 h in medium containing $200\text{ }\mu\text{Ci/ml}$ $[\text{H}]\text{MVA}$ and $10\text{ }\mu\text{M}$ lovastatin. Rab8 and Rab1B were immunoprecipitated sequentially from the same cell lysates using the monospecific antibodies described earlier. Immunoprecipitated proteins were resolved on an SDS gel and transferred to a poly(vinylidene difluoride) (PVDF) membrane. The blots were subjected to fluorography, and incorporation of $[\text{H}]\text{MVA}$ into the GTPases was quantified by densitometry. Immunoblot analysis was then performed, utilizing the antibodies to Rab8 or Rab1B, followed by ^{125}I -labelled goat anti-rabbit IgG, to quantify the total amount of each Rab protein on the same PVDF membrane.

RESULTS

Rab8 is a substrate for both GGTaseII and GGTaseI *in vitro*

To begin to explore the importance of the CVLL carboxyl-terminal motif in establishing the identity of Rab8 as a substrate for GGTaseI versus GGTaseII, we carried out cell-free reactions in which recombinant Myc-tagged GTPases were incubated with each enzyme. As expected, the wild-type Rab1B was efficiently geranylgeranylated only in the reaction containing REP and GGTaseII (Figures 1A and 1B). The opposite was true of Cdc42Hs, a known GGTaseI substrate with a CVLL carboxyl-terminal motif identical to that of Rab8 (Figures 1A and 1B). In contrast to the exclusive enzyme specificity exhibited by Rab1B for GGTaseII and Cdc42Hs for GGTaseI, Rab8 was able to serve as a substrate for either enzyme (Figures 1A and 1B). In the reaction catalysed by GGTaseII, Rab8 was a good substrate when compared with the Rab1B standard (Figure 1A), but in the GGTaseI reaction, Rab8 was a relatively poor substrate when measured against a comparable amount of Cdc42Hs (Figure 1B). In accord with the well-established 'escort' role of REP in the GGTaseII reaction [14,19], the incorporation of $[\text{H}]\text{GG}$ into both Rab1B and Rab8 was abolished when REP was omitted from the prenylation reaction mixture (not shown). On the other hand, addition of an equivalent amount of REP to the GGTaseI reaction neither enhanced nor inhibited the prenylation of Rab8 or Cdc42Hs by this enzyme (not shown).

It is presently unclear whether Rab carboxyl-terminal cysteine residues participate directly in the interaction with REP. If they do, this interaction could serve to mask the target cysteines until GGTaseII is recruited to the REP/Rab complex. This model predicts that the interaction of Rab8 with REP might decrease the accessibility of the carboxyl terminus to enzymes that do not interact with REP (e.g. GGTaseI). To test this model, we preincubated Myc-Rab8 in the presence or absence of a 2-fold

Figure 1 Prenylation of recombinant Myc-tagged proteins *in vitro*

The indicated amounts of Myc-Rab1B (●), Myc-Rab8 (△) or Myc-Cdc42Hs (▲) were incubated for 10 min at $37\text{ }^{\circ}\text{C}$ under assay conditions for GGTaseII (including REP) (A) or GGTase (no REP) (B). Each point is an average from duplicate assays which did not differ by more than 15%. In a separate experiment, recombinant Myc-Rab8 was prenylated by GGTaseI in the presence or absence of stoichiometric amounts of REP (C). Myc-Rab8 ($0.3\text{ }\mu\text{M}$) was preincubated for 10 min at $37\text{ }^{\circ}\text{C}$ in the presence and absence of a 2-fold molar excess of recombinant REP in Buffer B with $1\text{ }\mu\text{Ci}$ $[\text{H}]\text{GGPP}$ (see the Materials and methods section). The prenylation reaction was then initiated by the addition of 10 ng GGTaseI and allowed to proceed for 30 min and the incorporation of $[\text{H}]\text{GG}$ was visualized by fluorography and quantified by liquid scintillation spectroscopy (see the Materials and methods section). The values shown at the bottom of the figure are derived from duplicate assays which did not vary by more than 10%. The experiment was performed one additional time with similar results.

molar excess of REP-1 and then measured the prenylation of the protein by GGTaseI (Figure 1C). The results indicate that REP does not impair the prenylation of Rab8 by GGTaseI. In fact, high concentrations of REP enhanced the GGTaseI-catalysed incorporation of $[\text{H}]\text{GG}$ into Rab8 by about 50%. Although we did not directly confirm the binding of Rab8 to REP in these GGTaseI experiments, it should be noted that the assays were done under reaction conditions normally employed for the GGTaseII assay. Under these conditions, a 20-fold lower concentration of REP fully supported REP-dependent prenylation of both Rab8 and Rab1B by GGTaseII (Figure 1A), suggesting that the ability of Rab8 to form an initial prenylation-competent complex with REP is not markedly different from what has been observed previously for Rab1 and other Rab proteins [14,19,28].

Figure 2 Selectivity of a peptidomimetic GGTaseI inhibitor in cell-free assays and intact cells

(A) Purified his₆ Rab1B and Cdc42Hs (0.5 μ M) were incubated for 10 min at 37 °C with either GGTaseI or REP + GGTaseII, as indicated, in the presence of increasing concentrations of GGTI-297. Each point is the average of two determinations which did not vary by more than 10%. (B) Parallel cultures of 293 cells were transfected with expression vectors encoding Myc-tagged Rab1B and Cdc42Hs and the cells were incubated in medium containing 10 μ M lovastatin for 6 h. Cultures were then incubated for 15 h in medium containing [³H]MVA (200 μ Ci/ml) and 10 μ M lovastatin, with or without 10 μ M GGTI-298. The overexpressed Myc-tagged proteins were immunoprecipitated as described in the Materials and methods section. Aliquots of each immunoprecipitate were subjected to SDS/PAGE and fluorography to detect [³H]MVA incorporation into Myc-Rab1B or Myc-Cdc42Hs (upper panel). Separate aliquots of each immunoprecipitate were analysed by immunoblotting to determine the relative amount of expressed protein in each sample (lower panel). Ratios of ³H to ¹²⁵I, determined by densitometric scanning, were as follows: Rab1B: 0.94 (— inhibitor) and 0.98 (+ inhibitor); Cdc42Hs: 3.48 (— inhibitor) and 0.061 (+ inhibitor).

Effects of a specific inhibitor of GGTaseI on prenylation of Rab8

The preceding studies indicate that recombinant Rab8 can serve as a substrate for either GGTaseII or GGTaseI in cell-free systems. However, because the relative concentrations of the prenyltransferases in mammalian cells are not known, it is difficult to predict from these results whether or not both enzymes actually contribute to the modification of *Caax*-type Rab proteins *in vivo*. To address this issue, we initially employed an experimental strategy based on the selective inhibition of one of the relevant prenyltransferases. Inhibitors of GGTaseII have not yet been developed, but peptidomimetic compounds are available that selectively inhibit GGTaseI at concentrations that have little or no effect on FTase in cell-free assays (GGTI-297) or intact cells (GGTI-298) [43].

To verify the selectivity of these inhibitors for GGTaseI versus GGTaseII, we performed cell-free assays with GGTI-297. As expected, GGTI-297 proved to be a very effective inhibitor of GGTaseI-catalysed prenylation of the known GGTaseI sub-

Figure 3 Effects of a specific inhibitor of GGTaseI on prenylation of Myc-Rab8 in cell-free assays and in transfected 293 cells

(A) Recombinant Myc-Rab8 (approximately 0.2 μ M) was assayed in the presence and absence of 50 nM GGTI-297 with the indicated amounts of GGTaseI or REP/GGTaseII for 10 min at 37 °C. Proteins were subjected to SDS/PAGE and incorporation of [³H]GG was determined by liquid scintillation counting of solubilized gel slices as described in the Materials and methods section. The values shown in the graph are means of two determinations which did not vary by more than 10%. (B) Parallel cultures of 293 cells were transfected with an expression vector encoding Myc-Rab8 and then incubated in medium containing [³H]MVA and lovastatin as described in the legend for Figure 2(B). The upper panel shows the fluorograph of 90% of the immunoprecipitated [³H]MVA-labelled Myc-Rab8 samples, and the lower panel shows the immunoblot analysis performed on the remaining 10% of the same immunoprecipitated Myc-Rab8 samples. The experiment was repeated once with similar results.

strate, Cdc42Hs (IC₅₀ approximately 3–4 nM) (Figure 2A). In contrast, the same inhibitor had very little effect on the prenylation of wild-type Rab1B by GGTaseII. To further evaluate the specificity of the GGTaseI inhibitor in intact cells, we transiently overexpressed Myc-Rab1B, and Myc-Cdc42Hs in 293 cells and allowed non-prenylated substrate proteins to accumulate for a period of 6 h by including lovastatin in the culture medium. Lovastatin inhibits the activity of 3-hydroxy-3-methylglutaryl-CoA reductase [44] and blocks the synthesis of the essential isoprenoid precursor, MVA, thereby uncoupling protein synthesis from prenylation [45]. Prenylation of the accumulated substrate proteins was then initiated by adding medium containing [³H]MVA, with or without 10 μ M GGTI-298 (the methyl ester of GGTI-297), and incorporation of radioactivity into the immunoprecipitated Myc-tagged proteins was determined. The results depicted in Figure 2B clearly demonstrate that GGTI-298 is able to completely inhibit the GGTaseI-catalysed prenylation of Myc-Cdc42Hs in 293 cells

Figure 4 Effects of the GGTaseII inhibitor, GGTI-298, on prenylation of endogenous Rab8 in HEK293 cells

Parallel cultures of non-transfected HEK293 cells were incubated for 16 h in medium containing [³H]MVA (200 μ Ci/ml) and 10 μ M lovastatin, with or without 10 μ M GGTI-298, and proteins were immunoprecipitated using anti-Rab1B or anti-Rab8 antibodies as described in the Materials and methods section. Proteins were subjected to SDS/PAGE and transferred to a PVDF membrane. The amount of [³H]MVA incorporated into Rab1B or Rab8 was determined by fluorography and densitometric scanning of the blot. The results were normalized to the amount of immunodetectable protein (units of bound [¹²⁵I]-IgG) quantified by probing the same blot with antibodies to Rab1B or Rab8. Each bar depicts the mean ($\pm$ S.E.) of separate determinations from three cultures. The results of two different experiments are shown in **A** and **B**.

without impairing the GGTaseII-catalysed prenylation of Myc-Rab1B. Thus, GGTI-297 and GGTI-298 are specific inhibitors of GGTaseI.

We next examined the effects of these compounds on prenylation of Rab8. In cell-free assays, GGTI-297 (50 nM) completely inhibited the GGTaseI-catalysed incorporation of [³H]GG into recombinant Myc-Rab8, while having no substantial effect on the GGTaseII-catalysed prenylation of the same substrate (Figure 3A). Having confirmed that GGTI-297 selectively inhibits GGTaseI-mediated prenylation of Rab8 *in vitro*, we transfected 293 cells with a construct encoding Myc-Rab8 and measured the effects of GGTI-298 on prenylation of the over-expressed protein in intact cells (Figure 3B). The results show that, as was previously demonstrated for Rab1B, the incorporation of [³H]MVA into Myc-Rab8 was not reduced by the GGTaseII inhibitor.

To determine whether the apparent modification of Myc-Rab8 by GGTaseII observed in the preceding experiment might occur as a consequence of saturation of the GGTaseI pathway when Rab8 is overexpressed, we tested the effects of GGTI-298 on the prenylation of endogenous Rab8 immunoprecipitated from non-transfected 293 cells. As shown in Figure 4, the inhibitor had no significant effect on the incorporation of [³H]MVA into endogenous Rab8 at a concentration (10 μ M) that completely

Figure 5 Effects of a mutation in the switch-2 region of Rab8 on prenylation by GGTaseI and REP/GGTaseII in a cell-free assay

The indicated amounts of recombinant Myc-Rab8(wt) and Myc-Rab8(Y78D) were incubated for 10 min at 37 $^{\circ}$ C under the following conditions: **(A)** GGTaseII reaction conditions (20 ng REP, 20 ng GGTaseII α/β) or **(B)** GGTaseI reaction conditions (100 ng GGTaseI). Each point is an average of duplicate determinations, which did not vary by more than 10%.

blocked prenylation of Cdc42Hs (Figure 2B). As expected, incorporation of [³H]MVA into endogenous Rab1B in the non-transfected cells was not reduced by GGTI-298 (Figure 4).

Effects of a mutation that prevents Rab interaction with REP on prenylation of Rab8

The preceding studies suggest that Rab8 is prenylated predominantly through the action of the REP/GGTaseII complex, since prenylation of the protein shows no decline under conditions where the activity of GGTaseI is suppressed in intact cells. However, the inhibitor studies do not completely eliminate the possibility that Rab8 is normally prenylated by GGTaseI, and that when the latter enzyme is inhibited there is an efficient compensatory prenylation of the Rab8 substrate by GGTaseII. Recent studies have shown that prenylation by alternative enzymes can indeed occur in the case of a FTase substrate, K-RasB, which is modified by GGTaseI when FTase is inhibited [46,47]. In light of this possibility, we employed an entirely different approach to examine the enzyme specificity of Rab8 prenylation in intact cells. The strategy was based on our recent finding that amino acid substitutions in the predicted Rab1B 'switch-2' domain (e.g. Y78D in the region corresponding to the α 2 helix in H-Ras [48]) prevent REP association and thereby

Figure 6 Effects of a mutation in the switch-2 region of Myc-Rab8 on prenylation of the protein in intact cells

Cultures of 293 cells were transfected with expression vectors encoding Myc-Rab8(wt) or Myc-Rab8(Y78D) and incubated in medium containing 10 μM lovastatin for 6 h. Cultures were then incubated for an additional 15 h in medium containing $[^3\text{H}]\text{MVA}$ (200 $\mu\text{Ci}/\text{ml}$) and 10 μM lovastatin. (A) Some 10% of the total cell lysate used for immunoprecipitation was subjected to immunoblot analysis with monoclonal anti-Myc antibody to assess the expression of Myc-tagged Rab8. (B) Aliquots (10%) of each immunoprecipitate were subjected to immunoblot analysis with a rabbit antibody against Rab8 to compare the relative amounts of Myc-tagged Rab8 recovered in each sample. Average recovery ratios (units of Rab8 in the immunoprecipitate/units of Myc in the cell lysate) were 1.31 for Rab8(wt) and 1.15 for Rab8(Y78D). (C) The remaining 90% of each immunoprecipitate was subjected to SDS/PAGE and fluorography to detect $[^3\text{H}]\text{MVA}$ incorporation into the Myc-Rab8(wt) or Myc-Rab8(Y78D). (D) The values for $[^3\text{H}]\text{MVA}$ incorporation were normalized to the amount of immunodetectable Myc-Rab8 (bound ^{125}I -IgG in panel B) in each sample. Each bar depicts the average of two separate determinations derived from parallel cultures.

abolish the ability of the Rab protein to serve as a substrate for GGTaseII in cell-free assays or intact cells [24]. Since substrate recognition by GGTaseI does not require a REP complex and depends mainly on the carboxyl-terminal CaaL tetrapeptide sequence, we reasoned that introduction of a similar switch-2 mutation into Rab8 should have no effect on prenylation by GGTaseI, while preventing prenylation by GGTaseII. To test these predictions, we introduced the Y78D mutation into Rab8 (the amino acid numbering in Rab8 is the same as in Rab1B) and compared the abilities of recombinant Myc-Rab8(Y78D) and Myc-Rab8(wt) to be modified by REP+GGTaseII or GGTaseI in the standard cell-free assays. As expected, the Y78D mutation prevented Rab8 from serving as a substrate for the REP-dependent GGTaseII (Figure 5A). In contrast, the mutation did

not impair the ability of Rab8 to serve as a substrate for GGTaseI (Figure 5B).

With evidence in hand that the Y78D mutation selectively prevents prenylation of Rab8 by GGTaseII, but not GGTaseI, we extended these studies to transfected 293 cells. Prenylation of Myc-Rab8(wt) versus Myc-Rab8(Y78D) was compared by labelling the expressed proteins with $[^3\text{H}]\text{MVA}$. We hypothesized that if Rab8 is normally substrate for GGTaseI in intact cells, the Y78D mutation should have little or no effect on incorporation of the prenyl precursor into the altered protein compared with wild-type Rab8. As shown in Figure 6, incorporation of $[^3\text{H}]\text{MVA}$ into Myc-Rab8(Y78D), normalized to the amount of Rab8 recovered in the immunoprecipitate, was reduced by 60–70% compared with the Myc-Rab8(wt). This indicated that most of the prenylation of Rab8 in 293 cells is accomplished by means of the REP-dependent GGTaseII pathway. In a separate experiment the incorporation of $[^3\text{H}]\text{MVA}$ into the Y78D mutant was found to be further reduced from 30 to 10% of the wild-type Rab8 by inclusion of GGTI-298 in the culture medium (data not shown). Thus the residual prenylation of Rab8(Y78D) appears to be due to GGTaseI-catalysed modification.

The Y78D mutant exhibited a slower mobility on SDS gels compared with the wild-type Rab8 (Figures 6A and 6B). We do not know the basis for this observation, but it does not appear to be related to differences in the prenylation of the Y78D mutant, since the $[^3\text{H}]\text{MVA}$ -labelled protein showed the same mobility shift (Figure 6C). The possibility that the reduced prenylation of Rab8(Y78D) was due to general instability of the Y78D mutant rather than a specific inability to serve as a substrate for REP/GGTaseII seems unlikely. The overall expression of Myc-Rab8(Y78D) was only slightly reduced compared with Myc-Rab8(wt) (Figure 6A) and the proportion of expressed protein recovered in the immunoprecipitate was the same for both constructs. Moreover, separate studies have shown that the Y78D mutation does not markedly impair guanine nucleotide binding to Rab1B(Y78D) [24] or Rab8(Y78D) (J. H. Overmeyer and W. A. Maltese, unpublished work).

DISCUSSION

The results of the present study demonstrate that Rab8 can be modified by two distinct geranylgeranyl transferases. In this respect, Rab8 differs from previously studied members of the Rab family, which can be modified only by GGTaseII. Rab8 was modified efficiently by GGTaseII in cell-free systems and intact cells, despite the presence of the classic GGTaseI prenylation motif, CVLL. Moreover, the mono-prenylation of Rab8 by GGTaseII was completely dependent on the presence of REP in the reaction, in accord with earlier work on di-geranylgeranylated Rab proteins ending with xxCC, xCxC and CCxx motifs [13]. Thus the carboxyl-terminal motif Cxxx, which is present in several other Rab proteins besides Rab8, can be added to the list of different cysteine configurations that are recognized by GGTaseII. The diversity of prenylation motifs among the Rab proteins highlights an interesting difference between GGTaseII and the other known mammalian protein prenyltransferases, FTaseI and GGTaseI. The latter enzymes exhibit considerable positional specificity with respect to the acceptor cysteine in the carboxyl-terminal prenylation site. For example, peptides ending with a Cxxx pattern are recognized while those with a xCxx motif are not [32,34,49]. In contrast, the expanding number of different carboxyl-terminal cysteine motifs that can be prenylated by GGTaseII suggests that once a Rab protein is bound to REP, substrate recognition by the catalytic component, GGTaseII, is

not restricted to a specific cysteine arrangement within the terminal tetrapeptide sequence.

It remains unclear why Rab proteins must associate with REP to be prenylated by GGTaseII, whereas various other GTPases in the Ras superfamily can be prenylated as monomers. One obvious possibility is that unique structural characteristics of nascent Rab proteins render the carboxyl-terminal target cysteines inaccessible, so that a REP-induced conformational change is required to expose the prenylation site to the catalytic subunits of GGTaseII. The present finding that Rab8 can be geranylgeranylated by GGTaseI in a REP-independent manner suggests that, at least in the case of Rab8, the carboxyl-terminal cysteine is not masked in the monomeric protein. The observation that preincubation of Rab8 with a 2-fold molar excess of REP did not impede prenylation of this protein by GGTaseI further suggests that when REP binds to the Rab8 substrate, the CVLL motif remains accessible for interaction with either GGTaseII or GGTaseI. Finally, since a molar excess of REP did not interfere with the prenylation of Rab8 by GGTaseI, it is reasonable to infer that the Rab structural domains occupied by REP probably are distinct from those required for interaction with the $\alpha\beta$ heterodimer of GGTaseI.

Although our studies demonstrate that Rab8 can be modified by GGTaseI under certain conditions, they also indicate that, compared with Cdc42Hs, it is a relatively poor substrate for this enzyme. One possible explanation for this finding is that conformational features unique to members of the Rab family hinder the interaction with GGTaseI. This is consistent with emerging evidence that GGTaseI may recognize structural elements in addition to the carboxyl-terminal *CaaL* motif. Specifically, Zhang et al. [50] recently demonstrated that while full-length N-Ras can serve as a substrate for GGTaseI, a carboxyl-terminal N-Ras peptide containing the *Caax* motif cannot.

Previous studies have established that the peptidomimetic compounds GGTI-297 and GGTI-298 selectively inhibit GGTaseI versus FTase in cell-free assays and intact cells respectively [43]. In the present study we extend these observations by demonstrating that these compounds do not inhibit GGTaseII at concentrations that block GGTaseI. These findings confirm earlier work showing that GGTaseII cannot recognize short peptides that mimic the carboxyl-terminal sequences of Rab proteins [8,49]. One may envision several possible models that could explain the inability of the *CaaL* peptidomimetic inhibitors to block GGTaseII activity against *CaaL*-type Rab substrates. One possibility, suggested by the broad range of different cysteine configurations that can be prenylated by GGTaseII, is that substrate recognition by the $\alpha\beta$ subunits of this enzyme depends not on a specific carboxyl-terminal cysteine motif, but on a unique 'prenylation signal' structure formed by the specific orientation of terminal cysteine residue(s) combined with one or more upstream elements of the Rab protein. Based on the strict requirement for REP in the GGTaseII reaction, one might further speculate that the formation of such a 'prenylation signal' structure depends on a conformational change that is induced when REP binds to the Rab protein. An alternative possibility is that REP induces a conformational change in the $\alpha\beta$ subunits of GGTaseII, so that the enzyme becomes catalytically active only after the full REP/GGTaseII/Rab complex is assembled and the substrate binding site is already occupied by the Rab carboxyl-terminal cysteine domain. At present there is insufficient information to discriminate between these possibilities.

Technical limitations make it difficult to determine with absolute certainty which geranylgeranyl transferase normally

modifies the majority of Rab8 and other *Caax*-type Rab proteins in intact cells. However, the results obtained using two different experimental approaches strongly suggest that REP/GGTaseII is the predominant pathway. Under conditions where GGTI-298 was used to block the activity of GGTaseI in cultured HEK293, there was no substantial impairment of geranylgeranylation of either overexpressed Myc-Rab8 or endogenous Rab8. It remains possible that the apparent prenylation of Rab8 by GGTaseII is a technical artifact that occurs only when GGTaseI activity is eliminated by the inhibitor. However, this scenario seems highly unlikely in view of the marked reduction in prenylation observed when the REP-binding-deficient Rab8(Y78D) mutant was studied in 293 cells. In particular, since the mutant was similar to wild-type Rab8 as a substrate for GGTaseI, we would have expected to see little or no decrease in its prenylation if most of the Rab8 expressed in 293 cells was modified by GGTaseI.

Although the present studies indicate that Rab proteins ending with a *CaaL* motif are modified predominantly by GGTaseII *in vivo*, there did appear to be a minimal level of GGTaseI-mediated geranylgeranylation of Rab8(Y78D) in 293 cells. This observation may have some practical application in studies aimed at defining the mechanisms underlying the localization of nascent Rab proteins to specific intracellular membranes. Although it is clear from earlier work that the signals which direct Rab proteins to different compartments reside predominantly in their carboxyl-terminal hypervariable domains [51,52], it is well established that geranylgeranylation is a prerequisite for membrane association [8,9]. Thus it has been difficult to examine the role of REP in the targeting mechanism, because point mutations that abolish the interaction between REP and Rab also prevent prenylation by GGTaseII [24]. However, it may now be possible to use naturally-occurring or genetically engineered Rab-*CaaL* constructs to determine whether the escort function of REP is indispensable. Given the similarities between REP and GDI in putative Rab binding domains [53], it is also likely that mutations in the switch-2 region (e.g. Y78D) will prevent the association of GGTaseI-modified Rab-*CaaL* constructs with endogenous cellular GDIs. Therefore, correct localization of these altered Rab proteins would suggest that specific subcellular targeting depends solely on information contained in the carboxyl-terminal hypervariable regions of the Rab monomers. Studies aimed at testing these possibilities are currently in progress.

We thank Patrick Casey for the recombinant GGTaseI, Miguel Seabra for REP and GGTaseII, and Saïd Sebt and Andrew Hamilton for GGTI-297 and GGTI-298. This study was supported by National Institutes of Health Grant CA34569 from the United States Public Health Service.

REFERENCES

- 1 Zerial, M., Parton, R., Chavrier, P. and Frank, R. (1992) *Methods Enzymol.* **219**, 398–408
- 2 Novick, P. and Brennwald, P. (1993) *Cell* **75**, 597–601
- 3 Pfeiffer, S. R. (1994) *Curr. Opin. Cell. Biol.* **6**, 522–526
- 4 Nuoffer, C. and Balch, W. E. (1994) *Annu. Rev. Biochem.* **63**, 949–990
- 5 Kinsella, B. T. and Maltese, W. A. (1991) *J. Biol. Chem.* **266**, 8540–8544
- 6 Khosravi-Far, R., Lutz, R. J., Cox, A. D., Conroy, L., Bourne, J. R., Sinensky, M., Balch, W. E., Buss, J. E. and Der, C. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6264–6268
- 7 Seabra, M. C., Goldstein, J. L., Sudhof, T. C. and Brown, M. S. (1992) *J. Biol. Chem.* **267**, 14497–14503
- 8 Kinsella, B. T. and Maltese, W. A. (1992) *J. Biol. Chem.* **267**, 3940–3945
- 9 Peter, M., Chavrier, P., Nigg, E. A. and Zerial, M. (1992) *J. Cell. Sci.* **102**, 857–865
- 10 Musha, T., Kawata, M. and Takai, Y. (1992) *J. Biol. Chem.* **267**, 9821–9825
- 11 Pfeiffer, S. R., Dirac-Svestrup, A. B. and Soldati, T. (1995) *J. Biol. Chem.* **270**, 17057–17059

- 12 Wilson, A. L., Erdman, R. A. and Maltese, W. A. (1996) *J. Biol. Chem.* **271**, 10932–10940
- 13 Farnsworth, C. C., Seabra, M., Ericsson, L. H., Gelb, M. H. and Glomset, J. A. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11963–11967
- 14 Andres, D. A., Seabra, M. C., Brown, M. S., Armstrong, S. A., Smeland, T. E., Cremers, F. P. M. and Goldstein, J. L. (1993) *Cell* **73**, 1091–1099
- 15 Seabra, M., Reiss, Y., Casey, P. J., Brown, M. S. and Goldstein, J. L. (1991) *Cell* **65**, 429–434
- 16 Seabra, M. C., Brown, M. S., Slaughter, C., Sudhof, T. C. and Goldstein, J. L. (1992) *Cell* **70**, 1049–1057
- 17 Armstrong, S. A., Seabra, M. C., Sudhof, T. C., Goldstein, J. L. and Brown, M. S. (1993) *J. Biol. Chem.* **268**, 12221–12229
- 18 Alexandrov, K., Horiuchi, H., Steele-Mortimer, O., Seabra, M. C. and Zerial, M. (1994) *EMBO J.* **13**, 5262–5273
- 19 Shen, F. and Seabra, M. C. (1996) *J. Biol. Chem.* **271**, 3692–3698
- 20 Khosravi-Far, R., Clark, G. J., Abe, K., Cox, A. D., McLain, T., Lutz, R. J., Sinensky, M. and Der, C. J. (1992) *J. Biol. Chem.* **267**, 24363–24368
- 21 Wilson, A. L. and Maltese, W. A. (1993) *J. Biol. Chem.* **268**, 14561–14564
- 22 Beranger, F., Cadwallader, K., Profiri, E., Powers, S., Evans, T., de Gunzburg, J. and Hancock, J. F. (1994) *J. Biol. Chem.* **269**, 13637–13643
- 23 Sanford, J. C., Pan, Y. and Wessling-Resnick, M. (1995) *Mol. Biol. Cell.* **6**, 71–85
- 24 Overmeyer, J. H., Wilson, A. L., Erdman, R. A. and Maltese, W. A. (1998) *Mol. Biol. Cell* **9**, 223–235
- 25 Sanford, J. C., Pan, Y. and Wessling-Resnick, M. (1993) *J. Biol. Chem.* **268**, 23773–23776
- 26 Wilson, A. L., Sheridan, K. M., Erdman, R. A. and Maltese, W. A. (1996) *Biochem. J.* **318**, 1007–1014
- 27 Schiedel, A. C., Barnekow, A. and Mayer, T. (1995) *FEBS Lett.* **376**, 113–119
- 28 Seabra, M. C. (1996) *J. Biol. Chem.* **271**, 14398–14404
- 29 Shinjo, K., Koland, J. G., Hart, M. J., Narasimhan, V., Johnson, D. I., Evans, T. and Cerione, R. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9853–9857
- 30 Madaule, P., Axel, R. and Myers, A. M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 779–783
- 31 Reiss, Y., Stradley, S. J., Gierasch, L. M., Brown, M. S. and Goldstein, J. L. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 732–736
- 32 Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J. and Brown, M. S. (1990) *Cell* **62**, 81–88
- 33 Casey, P. J., Thissen, J. A. and Moomaw, J. F. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8631–8635
- 34 Yokoyama, K., Goodwin, G. W., Ghomaschi, F., Glomset, J. A. and Gelb, M. H. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5302–5306
- 35 Casey, P. J. and Seabra, M. C. (1996) *J. Biol. Chem.* **271**, 5289–5292
- 36 Soldati, T., Riederer, M. A. and Pfeffer, S. R. (1993) *Mol. Biol. Cell.* **4**, 425–434
- 37 Joberty, G., Tavittian, A. and Zahraoui, A. (1993) *FEBS Lett.* **330**, 323–328
- 38 Higuchi, R. (1989) in *PCR Technology* (Ehrlich, H. A., ed.), pp. 61–70, Stockton Press, New York
- 39 Andersson, S., Davis, D. L., Dahlback, H., Jornvall, H. and Russell, D. W. (1989) *J. Biol. Chem.* **264**, 8222–8229
- 40 Krupinski, J., Lehman, T. C., Frankenfield, C. D., Zwaagstra, J. C. and Watson, P. A. (1992) *J. Biol. Chem.* **267**, 24858–24862
- 41 Zhang, F. L., Moomaw, J. F. and Casey, P. J. (1994) *J. Biol. Chem.* **269**, 23465–23470
- 42 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 43 Vogt, A., Qian, Y., McGuire, T., Hamilton, A. D. and Sefti, S. M. (1996) *Oncogene* **13**, 1991–1999
- 44 Alberts, A. W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J. and Springer, J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3957–3961
- 45 Repko, E. M. and Maltese, W. A. (1989) *J. Biol. Chem.* **264**, 9945–9952
- 46 Rowell, C. A., Kowalczyk, J. J., Lewis, M. D. and Garcia, A. M. (1997) *J. Biol. Chem.* **272**, 14093–14097
- 47 Whyte, D. B., Kirschmeier, P., Hockenberry, T. N., Nunez-Oliva, I., James, L., Catino, J. J., Bishop, W. R. and Pai, J.-K. (1997) *J. Biol. Chem.* **272**, 14459–14464
- 48 Valencia, A., Chardin, P., Wittinghofer, A. and Sander, C. (1991) *Biochemistry* **30**, 4637–4648
- 49 Moores, S. L., Schaber, M. D., Mosser, S. D., Rands, E., O'Hara, M. B., Garsky, V. M., Marshall, M. S., Pomplano, D. L. and Gibbs, J. B. (1991) *J. Biol. Chem.* **266**, 14603–14610
- 50 Zhang, F., Kirschmeier, P., Carr, D., James, L., Bond, R. W., Wang, L., Patton, R., Windsor, W. T., Syto, R., Zhang, R. and Bishop, R. W. (1997) *J. Biol. Chem.* **272**, 10232–10239
- 51 Chavrier, P., Gorvel, J.-P., Stelzer, E., Simons, K., Gruenberg, J. and Zerial, M. (1991) *Nature (London)* **353**, 769–772
- 52 Stenmark, H., Valencia, A., Martinez, O., Ullrich, O., Goud, B. and Zerial, M. (1994) *EMBO J.* **13**, 575–583
- 53 Schalk, I., Zeng, K., Wu, S.-K., Stura, E., Matteson, J., Huang, M., Tandon, A., Wilson, I. A. and Balch, W. E. (1996) *Nature (London)* **381**, 42–48