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RAB GERANYLGERANYLATION OCCURS PREFERENTIALLY VIA THE PRE-FORMED REP:RGGT COMPLEX AND IS REGULATED BY GERANYLGERANYL PYROPHOSPHATE

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Running title: The alternative pathway of Rab geranylgeranylation predominates in vivo

ABSTRACT

Prenylation (or Geranylgeranylation, GG) of Rab GTPases is catalysed by Rab Geranylgeranyl Transferase (RGGT) and requires Rab Escort Protein (REP). In the classical pathway, REP associates first with unprenylated Rab which is then prenylated by RGGT. In the alternative pathway, REP associates first with RGGT; this complex then binds and prenylates Rab proteins. Here we show that REP mutants (REP1^{F282L} and REP1^{F282L/V290F}) defective in RGGT binding are unable to compete with wild-type REP in the prenylation reaction *in vitro*. When over-expressed in cells, REP wild type and mutants are unable to form stable cytosolic complexes with endogenous unprenylated Rabs. These results suggest that the alternative pathway may predominate *in vivo*. We also extend previous suggestions that GGPP acts as an allosteric regulator of the reaction. We observed that REP:RGGT complexes are formed *in vivo* and are unstable in absence of intracellular GGPP. RGGT increases the ability of REP to extract endogenous prenylated Rabs from membranes *in vitro* by stabilising a soluble REP:RGGT:Rab-GG complex. This effect is regulated by GGPP, which promotes the dissociation of RGGT and REP:Rab-GG to allow delivery of prenylated Rabs to membranes.

Key words: geranylgeranyl, prenylation, Rab GTPase, transferase, Rab Escort Protein, membrane

INTRODUCTION

RabGTPases are involved in specific stages of membrane trafficking and require correct localisation to different membrane compartments within the cell. Rab GTPases need to be geranylgeranylated on either one or two cysteines in their C-termini to localise to the correct intracellular membrane and be functional [1, 2]. This posttranslational modification is catalysed by Rab geranylgeranyl transferase (RGGT). This enzyme belongs to a family of protein prenyltransferases, which includes farnesyl transferase (FT) and geranylgeranyl transferase type I (GGT-I) [2]. All three enzymes are heterodimers consisting of one α and one β subunit and possess a single active site [3]. RGGT is unique in that it transfers two moles of geranylgeranyl pyrophosphate (GGPP) per mole of Rab substrate. Another major difference is the RGGT requirement for an associated protein, termed Rab escort protein (REP) [4-6]. In the absence of REP, the geranylgeranylation reaction does not occur [5] and early studies have proposed that newly synthesised Rab binds REP and the REP:Rab complex is the protein substrate of RGGT [4]. RGGT then transfers one or two GGPP molecules onto the Rab substrate without prior dissociation of REP:Rab complex [7, 8]. Thomä et al have shown that the lipid substrate, GGPP promotes the interaction between RGGT and REP in the absence of Rab and described an alternative pathway of Rab geranylgeranylation, whereby unprenylated Rab binds a pre-formed RGGT:REP complex [9]. Furthermore, kinetic studies suggested that GGPP reduces the affinity of RGGT for REP:Rab-digeranylgeranylated (Rab-diGG) [10], with subsequent delivery of Rab-diGG to an intracellular membrane [11].

The crystal structure of the REP1:RGGT complex showed surprisingly that the interface between REP1 and RGGT is formed by a very small area [12]. Residue F279 of REP1 protrudes deeply into a hydrophobic cavity formed by RGGT helices 8/10 and stabilises the REP1:RGGT complex as demonstrated by the inability of REP1^{F279A} mutants to associate with RGGT [1, 12]. Interestingly, this residue is not conserved in the related Rab GDP Dissociation Inhibitor (RabGDI) proteins. Furthermore, another residue in REP1 (V287) corresponds to phenylalanine in RabGDIs and was proposed to clash with RGGT. These subtle changes between REP and RabGDI explain the specificity of the REP:RGGT association [1, 12].

After dissociation from RGGT, REP keeps Rab-diGG soluble until it inserts into the membrane bilayer [1]. A related protein, RabGDI behaves similarly as it forms cytosolic complexes with prenylated Rabs. The delivery of the prenylated Rab to intracellular membranes from a REP:Rab or RabGDI:Rab complex is a process that remains ill characterised. The Rab protein is then “inserted” into the membrane bilayer where it is activated, and the REP or RabGDI protein is released into the cytosol.

To gain further insights into the events preceding the activation of Rab GTPases at membrane surfaces, we studied the role of REP, RGGT and the lipid substrate GGPP in the Rab geranylgeranylation reaction.

EXPERIMENTAL

Antibodies - Rabbit anti-GST (affinity purified) was used at 1:4000 dilution, mouse anti-Rab3 (BD Biosciences) was used at 1:1000 dilution, mouse anti-Rab5 (BD Biosciences) was used at 1:2000 dilution, mouse anti-Rab11 (BD Biosciences) was used at 1:2000 dilution, mouse anti-REP1 (hybridoma line 2F1) was used at 1:4000 dilution, chicken anti-RGGT (affinity purified)

was used at 1:4000 dilution, rabbit anti-RabGDI (affinity purified) was used at 1:1000 dilution, mouse anti-FLAG (Sigma) was used at 1:5000 dilution.

Plasmid constructs - Human REP1 sequence was cloned into pFastBacHTb using BamHI-XbaI [13]. The REP1 mutants were obtained by using the Quickchange mutagenesis kit (Stratagene). The sequences of all plasmid constructs used were confirmed by DNA sequencing.

Recombinant proteins - The recombinant proteins RGGT, REP1 and REP1 mutants were prepared by infection of Sf9 cells with recombinant baculoviruses encoding each subunit of the desired enzyme and purified by nickel Sepharose-affinity chromatography as described previously [13, 14]. All recombinant proteins were snap frozen in small aliquots and stored at -80°C until use.

Cell Culture and Transfection - Human embryonic kidney HEK 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin G, and 100 units/ml streptomycin at 37°C with 10% CO_2 . HEK 293 cells were transfected with FuGENE 6 (Roche Diagnostics) according to the manufacturer's instructions.

Membrane proteins preparation - Cell pellets were lysed in 10mM Tris pH 7.5, 1mM dithioerythrol (DTE), complete protease inhibitors (PI) (Roche) by brief sonication. The solution was clarified by centrifugation for 10 minutes at $800 \times g$ and the supernatant (S) was centrifuged for 1h at $100,000 \times g$. The membrane fraction (P) was re-suspended in 10mM Tris pH 7.5, 1mM DTE, PI, 200mM Sucrose (same volume as S) and sonicated for membrane solubilisation. The protein concentration was measured using Coomassie Plus Protein Assay reagent (Pierce).

Extraction of Rab proteins from cellular membranes - Rab proteins associated with membranes were extracted using REP1 in 50mM HEPES pH 7.5, 150mM NaCl, 5mM MgCl_2 , 1mM DTE, 1mM GDP, PI (Roche). $20\mu\text{g}$ of membrane proteins were mixed with REP1 with or without RGGT for 20 minutes at 37°C and the soluble fraction was separated from the membrane fraction by ultra centrifugation for 1h at $100,000 \times g$. Soluble and membrane proteins were resolved on 12.5% SDS-PAGE and protein concentration was quantified by densitometry using a Fuji Film Intelligent Dark Box LAS-3000 and analysed using Aida software. Each condition was analysed in duplicate.

Myc-Rab3a immunoprecipitation - AtT20 cells were transfected with pCS2-Rab3 using FUGENE according to the manufacturer protocol. 24h later, the cells were scraped, washed with 1ml of PBS and lysed in buffer 10mM Tris pH 7.5, 1mM DTE, PI (see above). Myc-Rab3 was extracted from membrane using $1.6\mu\text{M}$ REP1 and $1.6\mu\text{M}$ RGGT according to the previous protocol. The isolated REP1:RGGT:Myc-Rab3 complex was mixed with or without $256\mu\text{M}$ GGPP and the solution was incubated 20 minutes at 37°C . $400\mu\text{l}$ of 50mM HEPES pH 7.5, 150mM NaCl, 5mM MgCl_2 , 1mM DTE and $1\mu\text{g}$ of rabbit anti-Myc antibody (Upstate) were added to the reaction, and the solution was incubated for 3h at 4°C . $20\mu\text{l}$ of protein A were added to precipitate the complex associated with the anti-Myc antibody (1h at 4°C). After 3 washes in the same buffer (1ml/wash), proteins were resolved on 12.5% SDS-PAGE and analysed with mouse anti-Rab3 and mouse anti-REP1 antibodies.

GST-Rab3a pull-down assay - *In vitro* prenylation of GST-Rab3a protein was performed in $50\mu\text{l}$ of 50mM HEPES, pH 7.5, 5mM MgCl_2 , 1mM DTE, 1mM Nonidet P-40 (NP-40 or IGEPAL), $1\mu\text{M}$ REP1, $1\mu\text{M}$ RGGT and $1\mu\text{M}$ GST-Rab3a at different [GGPP]. The prenylation reaction was initiated by the addition of GGPP and incubated for 20 min at 37°C . $20\mu\text{l}$ of glutathione beads were added and the solution was shaken for 20 minutes at RT. The beads were

washed 3 times in the same buffer and the proteins bound to GST-Rab3 were resolved on 12.5% SDS-PAGE.

Formation and purification of complexes on glycerol gradient - Reaction mixtures (25 μ l) contained 50mM Hepes pH7.5, 5mM MgCl₂, 1mM DTE, 1mM NP-40 and depending on the conditions, 200 μ M GGPP, 1 μ M REP1, 1 μ M RGGT and 10 μ M Rab1a. After incubation for 20 min at 37°C, reaction mixtures were diluted in 75 μ l of buffer containing 20mM Tris pH7.5, 5mM MgCl₂, 1mM DTE, 5% Glycerol and loaded onto 9ml of 5-25% glycerol gradient in 20mM Tris pH7.5, 5mM MgCl₂, 1mM DTE. Glycerol gradients were spun for 18h at 4°C in a TH641 rotor (Beckman) at 40,000 rpm. 34 fractions (250 μ l) were collected from the bottom and aliquots were subjected to 12.5% SDS-gel electrophoresis, transferred to PVDF membranes, silver stained or counted for incorporated radioactive GGPP into Rab1a proteins.

Purification of protein complexes on Gel Filtration Chromatography - Cytosolic fraction prepared as described before was loaded onto a Superdex 200 3.2/30 using a SMART system (Pharmacia Biotech Inc.). The column was equilibrated in buffer containing 50mM Tris (pH 7.5), 100mM NaCl, 8mM MgCl₂, 2mM EDTA, 1mM dithiothreitol (DTT), 10 μ M GDP at a flow rate of 40 μ l/min. The samples were injected, and the material eluting between 1.1 ml and 2.1 ml was collected in 20 μ l or 40 μ l fractions. An aliquot of fractions was subjected to SDS-gel electrophoresis, transferred to nitrocellulose filters, and the proteins identified by immunoblot analysis using the ECL system (Amersham Corp.).

In vitro prenylation assay - *In vitro* prenylation of 6xHis-Rab1a proteins in 25 μ l reaction volumes was performed as described previously [14]. 5 μ M Rab1a were incubated with 50nM RGGT and 5 μ M [³H]geranylgeranylpyrophosphate (specific activity 712 dpm/mole) in the presence of various concentrations of REP (0–4 μ M) at 37°C for 30 min. The [³H]GGPP transferred to the Rab1a was measured by scintillation counting as the precipitated radioactivity after filtration of the reaction mixtures onto 1.2 μ m glass fiber filters.

RESULTS

REP1^{F282L}, REP1^{V290F} and REP1^{F282L/V290F} are compromised in Rab prenylation activity.

The rat REP1 residues F279 and V287 were previously reported to be key amino acids for the formation of the REP1:RGGT complex [12]. We mutated the equivalent residues in the context of the human REP1 sequence (F282L, V290F and double F282L/V290F) and produced the respective recombinant mutant proteins. To test whether these REP1 mutants associate with RGGT, we used density centrifugation on a 5-25% glycerol gradient to purify the complexes formed between REP1 and RGGT (Supplemental Fig. 1). In the presence of REP1 wild-type, we observed a REP1:RGGT complex in fractions 13-16 and free REP1 in fractions 19-22. Conversely, any of the different REP1 mutants appeared in fractions 13-16, suggesting that they cannot stably associate with RGGT as previously reported [12].

Next, we tested the activities of the REP mutants using an established *in vitro* prenylation assay (Fig. 1) [6]. As expected, the enzymatic activity was severely reduced in the presence of REP1^{F282L} and REP1^{F282L/V290F} as compared to REP1 wild type (Table 1). The REP1^{V290F} mutant showed a milder effect exhibiting only a 10-fold increase in the apparent K_m of the reaction (Table 1). This result suggests that the REP1^{V290F} mutant retains some ability to act in the Rab geranylgeranylation reaction, presumably through a weak interaction with RGGT. Because the mutants display a similar K_d for REP:Rab association (data not shown), the decrease in

prenylated product could be explained by an increased K_d for the formation of the REP:RGGT complex.

Evidence for the alternative pathway of Rab geranylgeranylation. The current model for Rab prenylation suggests that newly synthesised unprenylated Rab forms a complex with REP and then the binary complex serves as substrate for RGGT. This model predicts that REP:Rab complexes will accumulate if the engagement with RGGT is disrupted. To test this prediction, we performed competition experiments between wild type and REP1^{F282L} or REP1^{F282L/V290F} in the prenylation reaction *in vitro*. We predicted that the association with RGGT should depend on the K_d of REP1:Rab and thus we should observe a decrease of prenylated products with increasing concentrations of REP mutants. Surprisingly, we did not observe a significant effect of the REP mutants on the ability of wild type REP1 to function in the prenylation reaction (Fig. 2A), despite concentrations up to 60-fold higher. The slight increase in prenylated product observed is similar to the one observed in absence of wild-type REP1 and is probably due to a residual activity of the mutants. We also performed a competition experiment at saturating concentrations of both substrates (Rab and GGPP) and REP1^{F282L/V290F} mutant (Fig. 2B). The presence of REP1^{F282L/V290F} does not appear to alter the V_{max} of REP1 wild type and has a small effect on the K_m . Those results suggest that Rab substrates in the prenylation reaction associate preferentially with the REP1:RGGT complex, previously described as the alternative pathway for Rab geranylgeranylation [9].

To address if the REP:RGGT complex is present *in vivo*, we fractionated cytosol of HEK 293 cells on a glycerol gradient. We observed two peaks for REP1 correlating with the free protein (fractions 18-21) and with the REP1:RGGT complex (fractions 14-17) (Fig. 3A). As discussed in the Introduction, a previous report established that GGPP molecules are required for REP1:RGGT complex formation [9]. We tested this by resolving the cytosolic fraction of HEK 293 cells treated with mevastatin, an inhibitor of HMG-CoA reductase, a key enzyme for the production of FPP and GGPP [15]. Depletion of cellular GGPP results in a single REP1 peak eluted as a free protein in fractions 18-21 (Fig. 3B).

We then analysed the elution profile of cytosolic REP, RGGT, RabGDI and Rab5 as a model Rab protein upon gel filtration (Fig. 4). Under steady-state growth conditions, cytosolic prenylated Rab5 eluted in complex with RabGDI proteins (fractions 4-8) as previously reported [16] but not with REP1 and RGGT that were eluted in fractions 2-4 (Fig. 4A). Mevastatin treatment did not modify the elution profiles of REP1, RGGT and RabGDI, while Rab5 was eluted as two peaks corresponding to prenylated/complexed (fractions 4-7) and unprenylated/free (fractions 8-11) forms of the protein (Fig. 4B). Surprisingly, unprenylated Rab5 did not form a stable complex with REP1 *in vivo*. A similar profile was observed for Rab11 (Supplemental fig. 2).

Over-expression of REP1 does not enhance REP:Rab complex formation *in vivo*. One possible explanation for the previous observations is that the concentration of REP may be far lower than that of the Rab proteins. To increase the cellular concentration of REP, we over-expressed REP1 in HEK 293 cells, and analysed the presence of REP:Rab complexes in the cytosolic fraction by gel filtration (Fig. 5). We estimate that the level of over-expressed REP1 in these experiments was at least 15-fold greater than that of endogenous REP and 4-fold over RabGDI (data not shown). In cells transfected with empty vector, Rab5 eluted in fractions (12-19) corresponding to RabGDI:Rab5 complexes, as observed above. In REP1-transfected cells, we observed that approximately 25% of Rab5 co-eluted with REP1 (fractions 2-7), with the

remainder eluting in fractions 11-19. As a control, we verified that the transfections did not affect Rab5 expression and/or its prenylation state (data not shown). In REP1-transfected cells treated with mevastatin, the elution in fractions 2-7 was lost and we observed two peaks for Rab5 (Fig.5A). A small peak corresponding to fractions 11-17, presumably prenylated Rab5 eluted in complex with RabGDI, and a larger peak corresponding to the unprenylated protein as a monomer in fractions 18-22 (Fig 5A). Those results were confirmed when purified REP1 and Rab1a proteins were incubated together in prenylation buffer and subjected to purification by gel filtration chromatography (Supplemental fig. 3). The two proteins did not co-purify even at a concentration of Rab1a 4-fold higher than that of REP1.

The elution of Rab5 in fractions 2-7 upon REP1 over-expression suggested the possibility that it represented a complex containing REP, RGGT and prenylated Rab5. To test this hypothesis, we used the REP mutants described above, REP1^{F282L}, REP1^{V290F} and REP1^{F282L/V290F}, which do not bind RGGT. Indeed, the expression of REP1 mutants at similar levels to over-expressed wild type REP1 did not induce a mobility shift in Rab5 to fractions 2-7 (Fig. 5B). This experiment suggested that the Rab5 identified in the high molecular weight fractions when wild type REP was over-expressed was due to the formation of a stable REP1:RGGT:Rab complex.

To complement these studies, we analysed the complexes formed after *in vitro* prenylation reactions. The abnormal migration of REP1 upon gel filtration chromatography prevents the use of this technique to resolve free REP from complexed REP and therefore we resorted to density ultracentrifugation using a 5-25% glycerol gradient (Fig. 6). In the absence of Rab, the presence of geranylgeranyl pyrophosphate (GGPP) results in formation of a stable REP1:RGGT complex observed in fractions 13-17 as reported before, comprising 51.2% of total REP1 (Compare Fig. 6A and 6B). In the presence of Rab1 to enable the prenylation reaction to proceed, most of the Rab1a eluted in fractions 18-21, corresponding to the complex of prenylated Rab1a (Rab1a-GG) with REP1 (49.6 % of total REP1 protein) (Fig. 6C), which was verified in experiments where [³H]GGPP was used to detect Rab1a-GG (data not shown). These results suggest that GGPP can stabilise binding between REP1 and RGGT in the absence of Rab. Once Rab is added and prenylated Rab is formed, the stability of the ternary complex is altered by GGPP, which induces RGGT dissociation.

GGPP destabilises the RGGT:REP:Rab-GG complex. These experiments suggest that GGPP acts as an allosteric regulator of RGGT:REP interaction. We thus tested the possibility that GGPP may also regulate membrane delivery and extraction of Rab proteins. Based on previous reports, we established an *in vitro* system to extract Rab proteins from membranes by REP1 [17-19]. Briefly, we first prepared cellular membranes from cultured cells by ultracentrifugation as a source of prenylated Rabs, reconstituted these membranes and incubated them with recombinant proteins. After incubation, we measured the amount of prenylated Rab3 (as a suitable model Rab), which had shifted to the soluble fraction. Firstly, we showed that REP1 is able to extract endogenous prenylated Rab3 from membranes (Supplemental Fig. 4). Secondly, the addition of RGGT in concert with REP1 enhances the extraction of Rab3 (Supplemental Fig. 4). We then performed the *in vitro* extraction experiment with varying concentrations of related isoprenoids. The maximum concentrations used in the assay were below the critical micelle concentration (CMC) [20]. The increase in GGPP concentration in the assay induced some decrease in Rab3 extraction by REP, probably due to non-specific binding of the lipid to REP1 (Fig. 7A). However, when RGGT was present in the extraction experiment, a pronounced reduction in Rab3 extraction upon GGPP addition was observed (Fig. 7A). Above 50 μ M GGPP, the profile of Rab3 extraction by REP1/RGGT was similar to REP1 alone, indicating that GGPP neutralises the

effect of RGGT in the assay (Fig. 7A). The half-maximal concentration for this effect by GGPP is $33.85 \pm 1.13 \mu\text{M}$ (Fig. 7B). When FPP was used, we observed a less dramatic effect, where the half-maximal concentration is $103.98 \pm 17.14 \mu\text{M}$. The addition of GPP or GGOH did not affect the assay, indicating the specificity of the effect (Fig. 7B). Similar results were obtained with Rab5, another model Rab (data not shown). These results are consistent with the hypothesis that GGPP could act as an allosteric regulator of REP1:RGGT:Rab3 complex stability where addition of the isoprenoid destabilises the complex and results in RGGT dissociation. Furthermore, the data suggest that pyrophosphate is an important component of the effect, possibly because it requires binding of GGPP to the RGGT binding site.

To test more directly whether GGPP destabilises the REP1:RGGT:Rab3 complex, we performed pull-down experiments. In the first experiment, we subjected GST-Rab3a to *in vitro* prenylation and pulled-down GST-Rab3a after the incubation (Fig. 8A). GST-Rab3a was incubated in presence of REP1 and RGGT for 30 minutes at 37°C at different concentrations of GGPP. The reaction was diluted in PBS and GST-Rab3a was pulled-down by addition of glutathione beads. The precipitates were identified by immunoblot and quantified by densitometry. We observed that increasing concentrations of GGPP resulted in dissociation of RGGT from GST-Rab3a but not REP1. In the second experiment, we immunoprecipitated Rab3a after extraction from cellular membranes (Figure 8B). Over-expressed myc-Rab3a was extracted from cellular membranes by REP1 and RGGT. Preliminary studies showed that the complex is rapidly formed (5 min) and stable for at least 1h at 37°C (data not shown). The isolated complex was then incubated in presence or absence of GGPP and subjected to immunoprecipitation using anti-myc antibody. In the absence of GGPP, myc-Rab3a formed a stable complex with REP1 and RGGT (Fig. 8B). However, the presence of GGPP induced loss of RGGT signal. These experiments directly demonstrate that GGPP can promote the dissociation of RGGT from the ternary complex.

DISCUSSION

Rab geranylgeranylation is a complex reaction that precedes their membrane association. The reaction is catalysed by RGGT with the assistance of REP since RGGT does not recognise the substrate directly. The classical model for Rab geranylgeranylation predicts the formation of the REP:Rab complex prior to association with RGGT [1, 4]. An alternative model suggests that a REP:RGGT complex forms before binding to unprenylated Rab proteins [9]. Our present results *in vitro* and in cells suggest that the alternative pathway may be the predominant pathway for Rab geranylgeranylation.

We generated mutant REPs that are unable to associate with RGGT to test the role of the REP:RGGT engagement in Rab prenylation. This led to a number of unexpected observations. One was that the mutant REPs failed to compete with wild type REP in the prenylation reaction even at 60-fold higher concentrations, suggesting that the preferential pathway of the reaction *in vitro* is through a preformed REP:RGGT complex (Fig. 2). Another unexpected result was the absence of a complex between unprenylated Rab proteins and REP. The over-expression of REP mutants defective in RGGT binding should have resulted in soluble REP:Rab complexes but no such complexes could be observed (Fig. 5). Furthermore, in cells where the production of GGPP was blocked by an HMGC_oA Reductase inhibitor, unprenylated Rab5 and Rab11 could not

stably associate with REP (Fig. 5). However, upon prenylation, the REP:Rab-GG complex was elicited (Figs. 5 and 6).

Thomä et al first reported based on kinetic analysis that GGPP can act as an allosteric regulator of RGGT activity, thus preventing product inhibition of the reaction [10]. The present studies confirm and extend those findings. Firstly, we confirmed *in vitro* that GGPP addition can stabilise the RGGT:REP interaction in the absence of Rab and that prenylation of Rab in the presence of excess GGPP leads to release of REP:Rab-di-GG complex (Figs. 3 and 6). Secondly, we show that addition of RGGT to REP can greatly improve the efficiency of Rab extraction by stabilising a cytosolic RGGT:REP:Rab-di-GG complex (Figs. 7, 8 and Supplemental Fig. 4). Thirdly, we show that the addition of GGPP to this extraction assay counteracts the RGGT effect, which results from GGPP-induced dissociation of REP and RGGT (Fig. 7).

The results presented here also demonstrate that mammalian REP1 is able to extract endogenous prenylated Rabs from membranes as effectively as RabGDI (Fig. 7 and data not shown). Early studies using semi-permeabilised cells suggested that both REP and RabGDI could extract Rabs from membranes [17]. Later studies using yeast REP (Mrs6p) in an extraction assay *in vitro* suggested that Mrs6p could not extract Rabs from membranes [21] or less effectively than RabGDI [22]. The discrepancies with the previous studies may relate to functional differences between species. Nevertheless, it remains to be clarified whether mammalian REPs play any role in Rab recycling *in vivo* given their activity *in vitro*.

Altogether, these results suggest a complex series of events prior to membrane delivery of Rab GTPases, as follows. GGPP binds to RGGT and promotes the formation of a REP:RGGT complex, which is recognised by newly synthesised unprenylated GDP-bound Rab. One or two rounds of GG modification are catalysed by RGGT, after which the binding of a new molecule of GGPP promotes the dissociation of the REP:Rab-GG. Rab-GG is delivered to the membrane and free REP is recycled for interaction with RGGT loaded with GGPP.

FOOTNOTES

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ABBREVIATIONS

RGGT, Rab geranylgeranyl transferase, REP1, Rab escort protein 1, RabGDI, Rab GDP dissociation inhibitor, Rab-GG, geranylgeranylated Rab, GGPP, geranylgeranyl pyrophosphate, FPP, farnesyl pyrophosphate, GPP, geranyl pyrophosphate, GGOH, geranylgeraniol.

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17 Alexandrov, K., Horiuchi, H., Steele-Mortimer, O., Seabra, M. C. and Zerial, M. (1994)
Rab escort protein-1 is a multifunctional protein

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FIGURE LEGENDS

Fig. 1. *In vitro* prenylation of recombinant Rab1a protein in presence of REP1 mutants. Each reaction contained RGGT (50nM), Rab1a (5 μ M), GGPP (5 μ M) (specific activity 710 dpm/pmol) and increasing concentrations of REP1 (●), REP1^{F282L} (○), REP1^{V290F} (▼), REP1^{F282L/V290F} (Δ). The values represent the means \pm the standard error of the mean determined from duplicate determinations of two independent experiments.

Fig. 2. A. Competition for Rab1a prenylation by REP1 mutants. Reaction mix contains REP1 (50nM), RGGT (50nM), GGPP (5 μ M), Rab1a (100nM) and increasing concentrations of (●) REP1^{F282L} and (○) REP1^{F282L/V290F}. After 20 min incubation at 37°C, the proteins were precipitated and analysed by filter binding assay. As controls, the prenylation reactions were run with (▼) REP1^{F282L} and (Δ) REP1^{F282L/V290F} alone. The values represent the mean \pm the standard error of the mean determined from duplicate determinations of two independent experiments and is representative of two other independent experiments. The yield for prenylation of Rab1a substrate was calculated to be 30%. **B. Competition for Rab1a prenylation by REP1^{F282L/V290F}.** Reaction mix contains REP1 (50nM), RGGT (50nM), GGPP (5 μ M) and increasing concentrations of Rab1a with (○) or without (●) REP1^{F282L/V290F} (4 μ M). After 20 min incubation at 37°C, the proteins were precipitated and analysed by filter binding assay. The values for Rab1a prenylation obtained for the mix of REP1 proteins (wild-type + mutant) were corrected by the values obtained for Rab1a prenylation for REP1^{F282L/V290F} alone. The values represent the means \pm the standard error of the mean determined from duplicate determinations of two independent experiments. The yield for prenylation of Rab1a substrate was calculated to be 30%.

Fig. 3. Analysis of *in vivo* REP1:RGGT complex formation. Cytosolic extracts (1.25 mg) obtained from HEK 293 cells after cytosol/membrane partitioning (*see experimental procedures*) were resolved on Glycerol gradient (5-25%) for 18h, 40,000 rpm, 4°C. The protein complexes were analysed using mouse anti-REP1 and chicken anti-RGGT antibodies. **A.** Control cells. **B.** Cells treated with 20 μ M mevastatin for 24h.

Fig. 4. Analysis of *in vivo* proteins associated with prenylated and unprenylated Rab5. HEK 293 cytosolic extracts (100 μ g) were resolved on S200 gel exclusion column using a SMART SYSTEM (Pharmacia). The eluted fractions (40 μ l) were analysed by Western blot with mouse anti-Rab5, mouse anti-REP1, chicken anti-RGGT and rabbit anti-RabGDI α/β antibodies. Loaded fractions: 1.40 to 1.96 ml. **A.** Control cells. **B.** Cells treated with 20 μ M mevastatin for 24h.

Fig. 5. A. Analysis of *in vivo* complexes for overexpressed REP1 protein. HEK 293 cells were transfected with REP1 (N-term FLAG tagged) as described under material and methods and a soluble (S) HEK 293 extract (100 μ g) was resolved on S200 gel exclusion column using a SMART SYSTEM (Pharmacia). The eluted fractions (20 μ l) were analysed by Western blot with mouse anti-Rab5, anti-REP and anti-FLAG antibodies. Loaded fractions: 1.40 to 1.82 ml. **B. Analysis of *in vivo* complexes for REP1 mutants.** HEK 293 cells were transfected with the different REP1 mutants as described under Material and Methods and analysed as previously described. Loaded fractions: 1.40 to 1.76 ml.

Fig. 6. Silver staining for REP1:RGGT complex formation. Each reaction mix contains REP1 (1 μ M), RGGT (1 μ M) and as indicated on the figure, GGPP (200 μ M) and Rab1a (10 μ M). The solutions were incubated for 30 min at 37°C and resolved on Glycerol gradient (5-25%) for 18h, 40,000 rpm, 4°C. The protein complexes were analysed by silver staining.

Fig. 7. A. Inhibition of Rab3 extraction by GGPP. AtT20 total membranes (20 μ g) were incubated with REP1 (1.6 μ M) with (●) or without (○) RGGT (1.6 μ M) for 10 min at 37°C. Then, increasing concentrations of GGPP were added to the solution and the soluble Rab3 proteins were separated from membrane bound Rab3 proteins by ultracentrifugation at 100,000 x g. The values represent the means \pm the standard error of the mean determined from duplicate determinations of two independent experiments and is representative of two other independent experiments. **B. Isoprenoid functions in Rab3 extraction *in vitro*.** AtT20 total membranes (20 μ g) were incubated with REP1 (1.6 μ M) and RGGT (1.6 μ M) for 10 min at 37°C. Then, increasing concentrations of GGOH (x), GPP (■), FPP (●), or GGPP (▼) were added to the solution. After 20 min soluble Rab3 proteins were separated from membrane bound Rab3 proteins by ultracentrifugation at 100,000 x g. The experimental data obtained were fitted to exponential decay equation. The values represent the mean \pm the standard error of the mean determined from duplicate determinations of two independent experiments and is representative of four independent experiments.

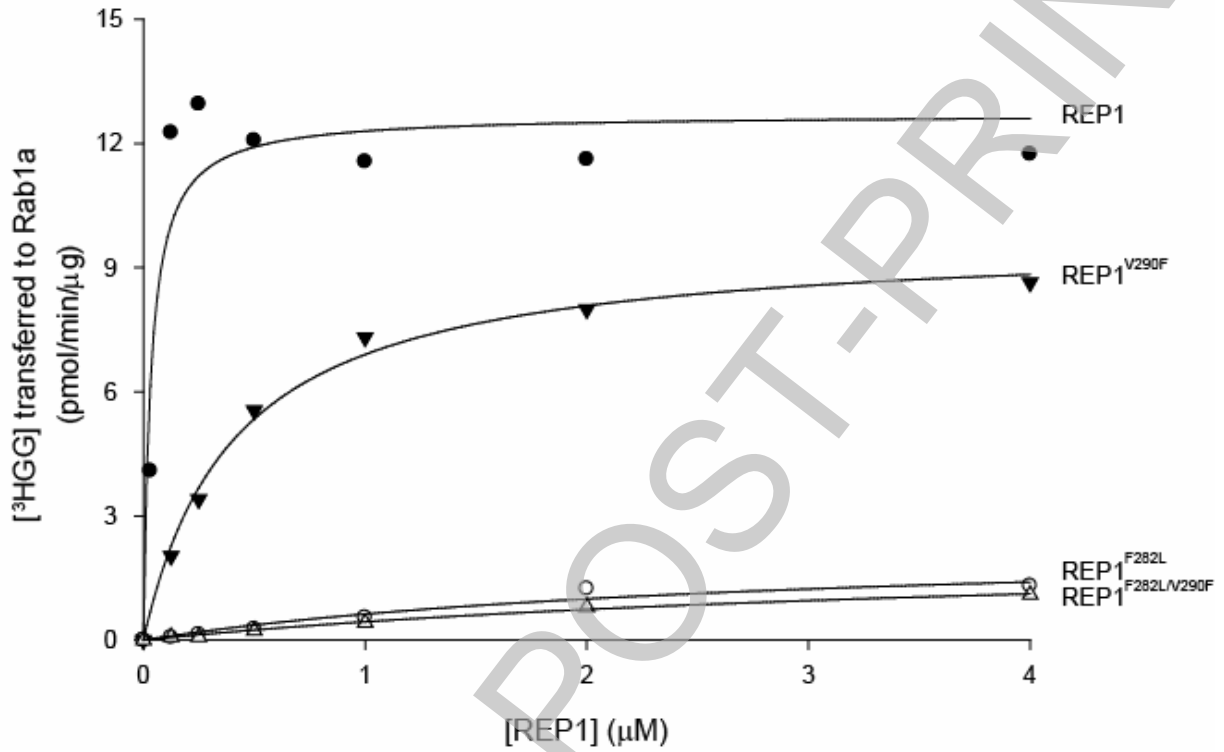
Fig. 8. A. GST-Rab3 pulled-down for increasing concentrations of GGPP. REP1 (1 μ M), RGGT (1 μ M) and GST-Rab3 (1 μ M) were mixed with increased concentrations of GGPP for 30 min at 37°C. The protein complexes were pulled down by addition of glutathione beads and analysed by Western blotting using anti-GST, anti-RGGT, anti-Rab3 and anti-REP1 antibodies. The amount of RGGT pulled-down was quantified by densitometry, normalised to the amount of GST-Rab3 and plotted against the GGPP concentrations. **B. Myc-Rab3 immunoprecipitation.** AtT20 membranes (100 μ g) were incubated with REP1 (1.6 μ M) and RGGT (1.6 μ M) for 20 min at 37°C and the soluble complexes were separated by ultracentrifugation at 100,000 x g. The soluble complexes were mixed with or without GGPP (256 μ M) and immunoprecipitated with rabbit anti-Myc antibody. The proteins were analysed by Western blot using anti-Rab3 and anti-REP1 mouse antibodies. AtT20 total extract (20 μ g), recombinant REP1 (5ng) and RGGT (1 μ g) (Control proteins) were used as controls for Western blot analysis.

Table 1: Determination of Michaelis constants (K_m and V_{max})

	K_m for Rab1a (μM)	V_{max} for Rab1a ($\mu\text{M}/\text{h}$)
REP1	0.032 ± 0.025	33.2 ± 3
REP1 ^{F282L}	2.8 ± 1.45	6.4 ± 1.8
REP1 ^{V290F}	0.41 ± 0.04	26 ± 0.82
REP1 ^{F282L/V290F}	4.16 ± 0.96	6.6 ± 0.84

The constant values represent the means \pm the standard error of the mean determined from duplicate determinations of two independent experiments.

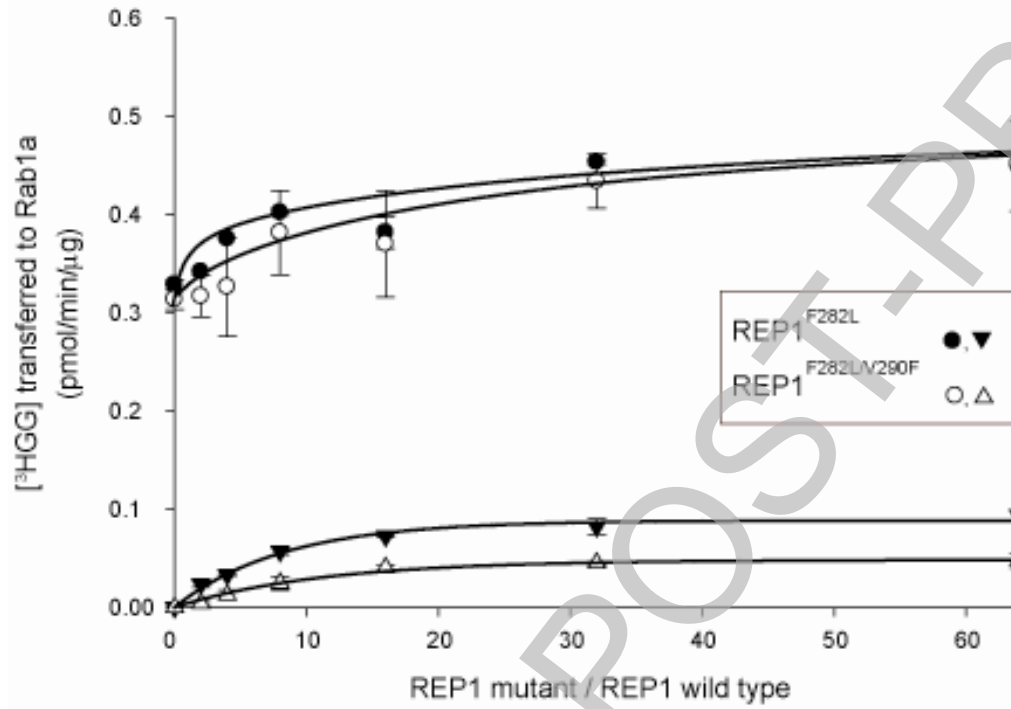
Figure 1



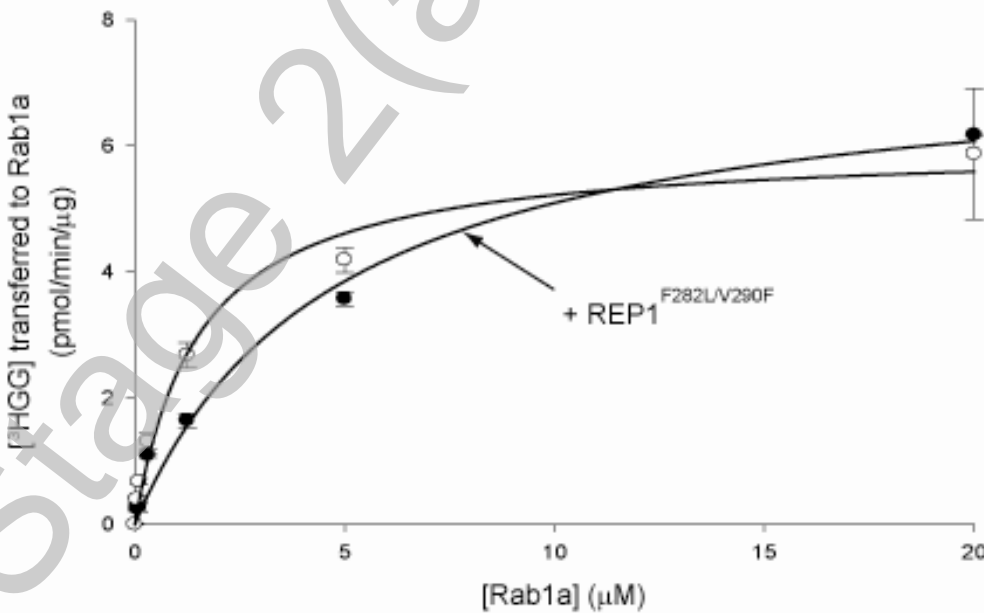
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Figure 2

A

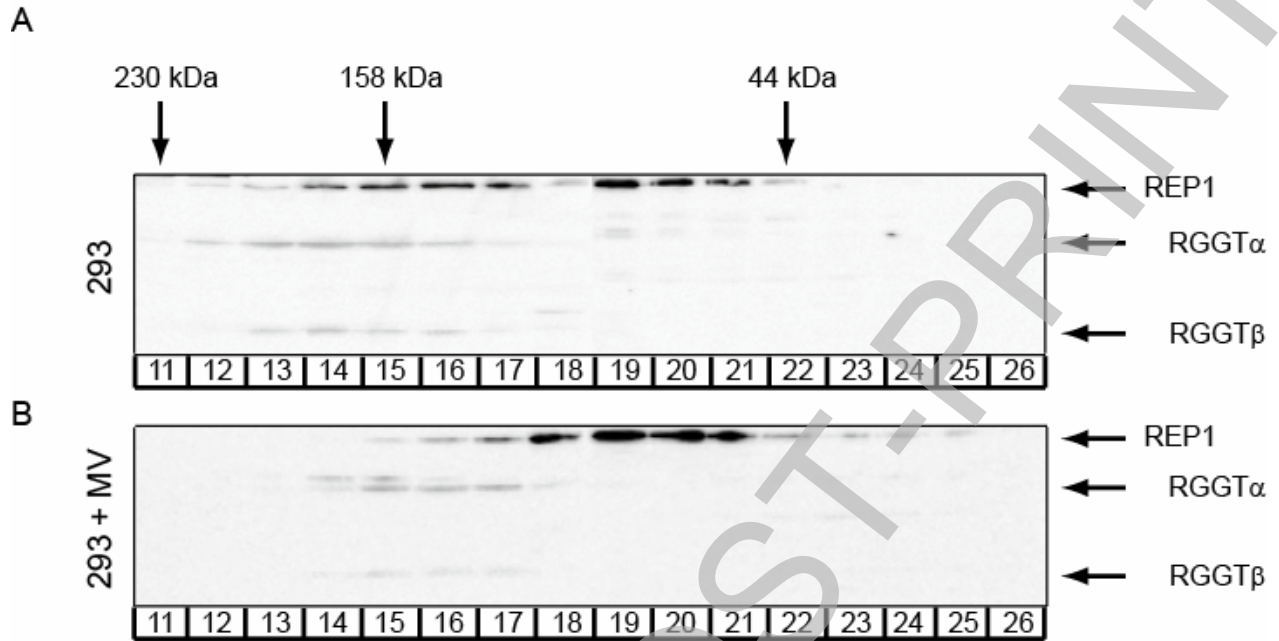


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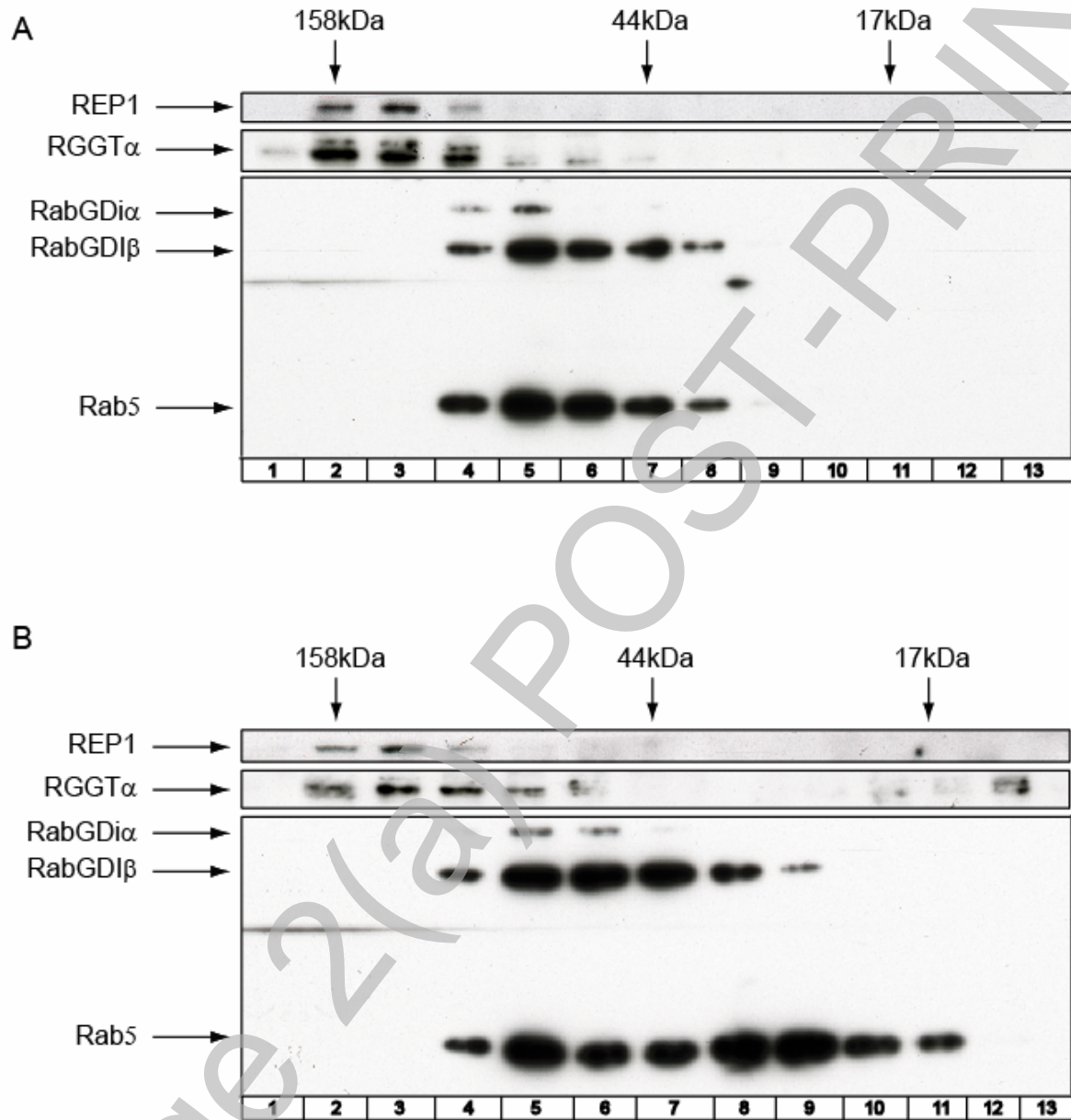
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Figure 3



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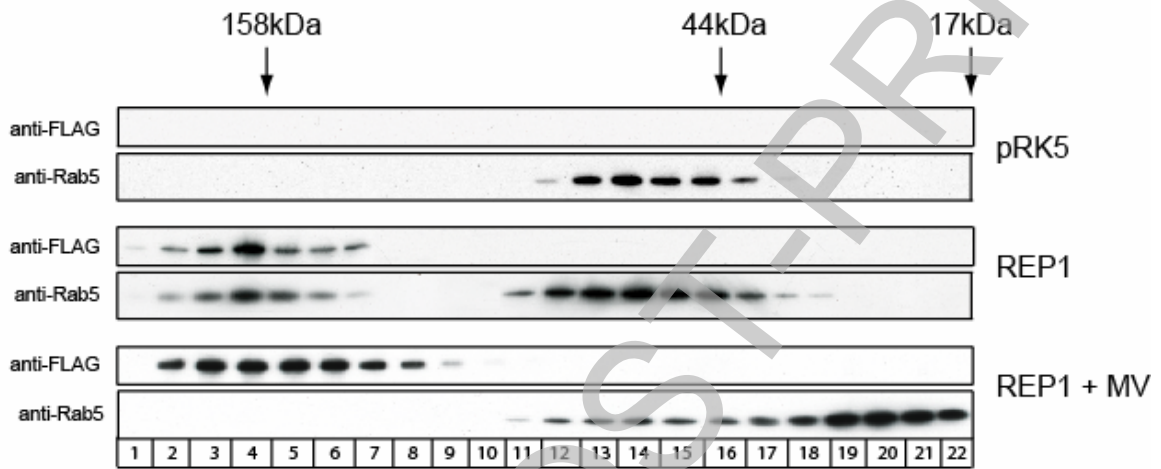
Figure 4



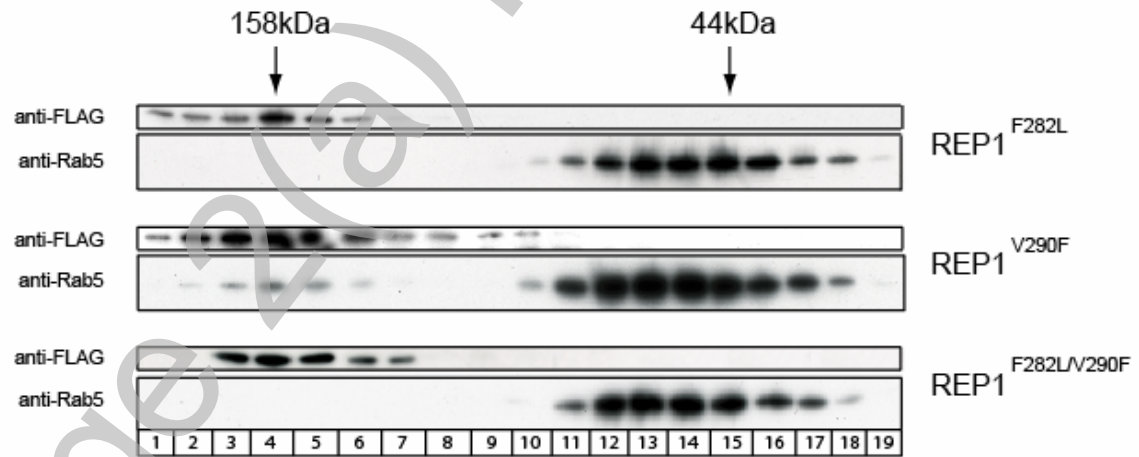
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Figure 5

A

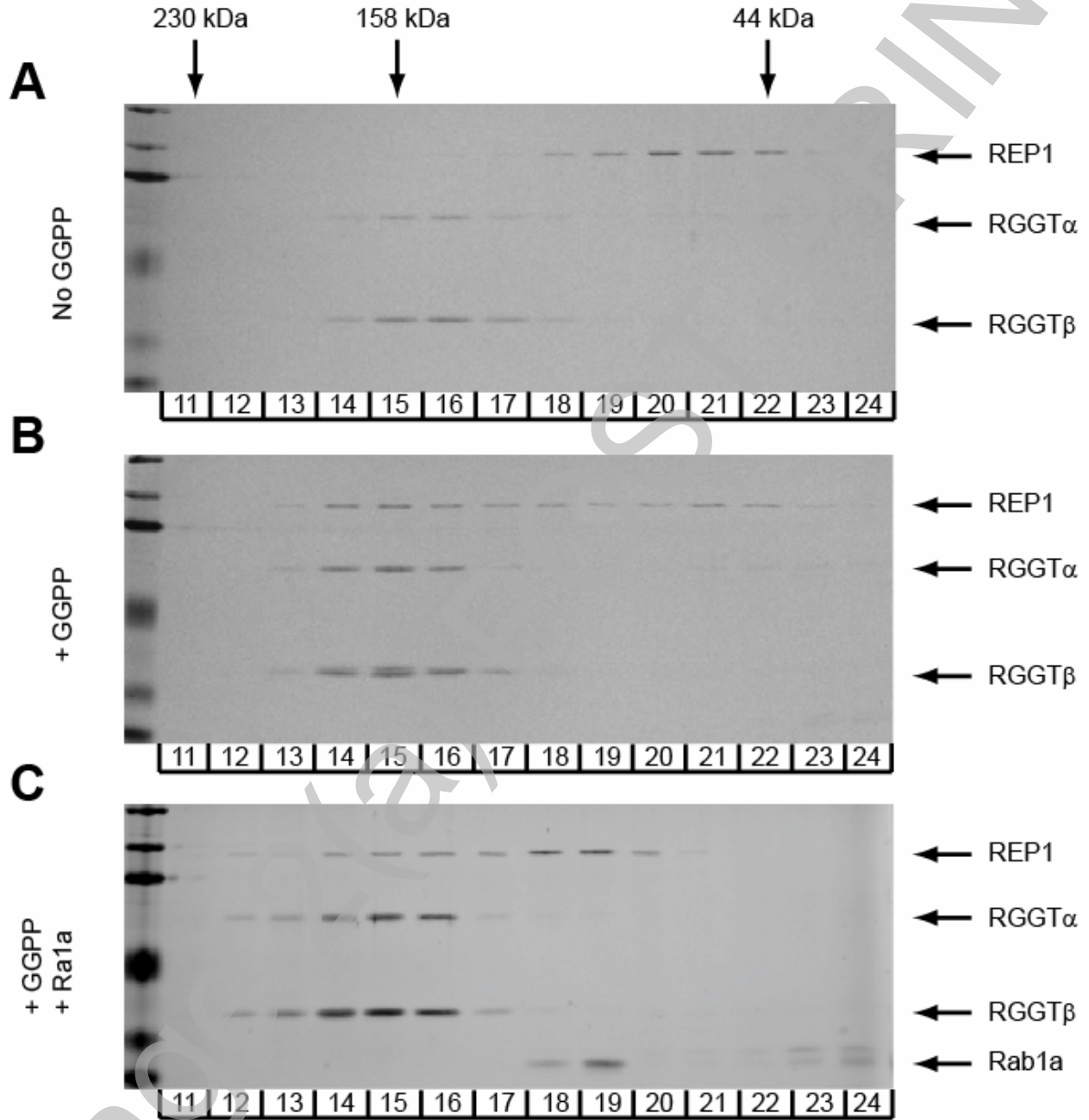


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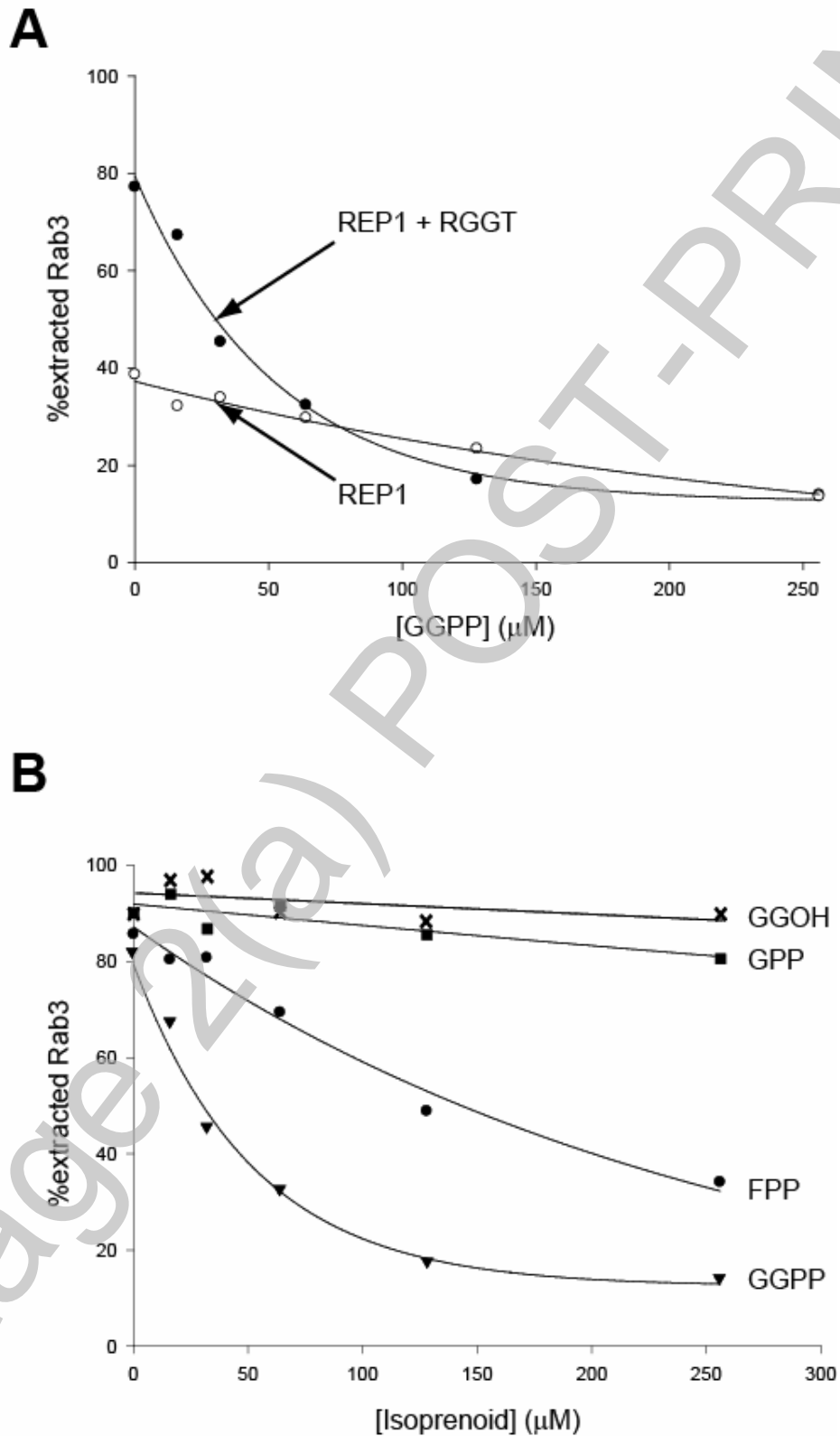
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Figure 6



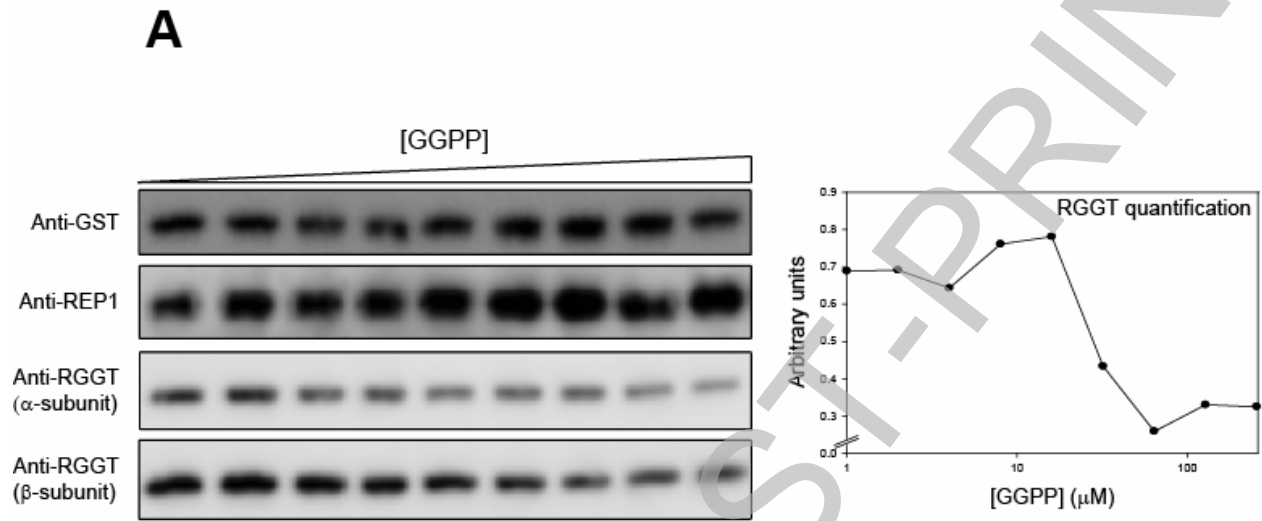
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Figure 7



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Figure 8



B

