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Rab escort protein-1 is a multifunctional protein that accompanies newly prenylated rab proteins to their target membranes

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Rab proteins comprise a family of small GTPases that serve a regulatory role in vesicular membrane traffic. Geranylgeranylation of these proteins on C-terminal cysteine motifs is crucial for their membrane association and function. This post-translational modification is catalysed by rab geranylgeranyl transferase (RabGGTase), a multisubunit enzyme consisting of a catalytic heterodimer and an accessory component, named rab escort protein (REP)-1. Previous *in vitro* studies have suggested that REP-1 presents newly synthesized rab proteins to the catalytic component of the enzyme, and forms a stable complex with the prenylated proteins following the transfer reaction. According to this model, a cellular factor would be required to dissociate the rab protein from REP-1 and to allow it to recycle in the prenylation reaction. RabGDP dissociation inhibitor (RabGDI) was considered an ideal candidate for this role, given its established function in mediating membrane association of prenylated rab proteins. Here we demonstrate that dissociation from REP-1 and binding of rab proteins to the membrane do not require RabGDI or other cytosolic factors. The mechanism of REP-1-mediated membrane association of rab5 appears to be very similar to that mediated by RabGDI. Furthermore, REP-1 and RabGDI share several other functional properties, the ability to inhibit the release of GDP and to remove rab proteins from membranes; however, RabGDI cannot assist in the prenylation reaction. These data suggest that REP-1 is *per se* sufficient to chaperone newly prenylated rab proteins to their target membranes.

Key words: endosome/prenylation/rab/REP-1

Introduction

Rab proteins are a subfamily of Ras-like GTPases that play a key role in the regulation of intracellular membrane transport (Goud, 1992; Pfeffer, 1992; Takai *et al.*, 1992; Novick and Brennwald, 1993; Simons and Zerial, 1993; Zerial and Stenmark, 1993). They are found in two distinct pools: a small cytosolic pool complexed to RabGDP dissociation inhibitor (RabGDI; Regazzi *et al.*, 1992) and a large membrane-bound pool. According to the current view, RabGDI has the dual function of inhibiting GDP exchange and chaperoning rab proteins during their cycling between the cytosol and the membrane (Araki *et al.*, 1990; Sasaki *et al.*, 1990; Regazzi *et al.*, 1992; Soldati *et al.*, 1993; Ullrich *et al.*, 1993). The cycle starts with the recognition of the cytosolic rab–RabGDI complex by a specific receptor on the donor membrane or on the transport vesicle. Membrane association of the rab protein is accompanied by the release of RabGDI into the cytosol. The rab protein is then converted into the GTP-bound form by a guanine nucleotide exchange factor (GEF; Soldati *et al.*, 1994; Ullrich *et al.*, 1994). In this conformation the rab protein would be competent to interact with and activate effector molecules controlling the docking and fusion of the vesicle with its target compartment (Stenmark *et al.*, 1994b). Following GTP hydrolysis catalysed by a GTPase-activating protein (GAP), RabGDI would solubilize GDP–rab from the membrane so that the cytosolic rab–RabGDI complex could enter a new transport cycle.

Association of rab proteins with their corresponding membranes is a multistep process involving post-translational modifications and recognition of targeting sequences by organelle-specific receptors (Chavrier *et al.*, 1991; Brennwald and Novick, 1993). All rab proteins have distinctive cysteine motifs at their C-termini and, despite their structural diversity, undergo geranylgeranylation in all examined cases (for reviews see Magee and Newman, 1992; Armstrong, 1993). Two pieces of evidence show that this modification is critical for the interaction of rab proteins with membrane receptors and regulatory molecules. First, truncated rab proteins lacking the C-terminal cysteine motif are unable to associate with cellular membranes and are functionally impaired (Walworth *et al.*, 1989; Chavrier *et al.*, 1991; Gorvel *et al.*, 1991; Bucci *et al.*, 1992; Lombardi *et al.*, 1993). Secondly, replacement of the geranylgeranylation motif of rab5 (CCSN) with the farnesylation motif of Ras (CVLS) leads to a drastic reduction in binding of the mutant protein to the membrane and inefficient interaction with RabGDI (Araki *et al.*, 1991; Ullrich *et al.*, 1993; Beranger *et al.*, 1994).

The enzyme responsible for the post-translational modification of rab proteins, rab geranylgeranyl transferase (RabGGTase), also called GGTase II, was biochemically purified from rat brain and found to be a multisubunit enzyme (Seabra *et al.*, 1992a,b). It comprises a catalytic heterodimer composed of tightly associated α and β subunits of 60 and 38 kDa, respectively (Seabra *et al.*, 1992b; Armstrong *et al.*, 1993), and an accessory factor, the rab escort protein (REP). Both subunits of the catalytic component share sequence homology with the corresponding subunits of the known protein prenyl transferases, farnesyl transferase and *CAAX* geranylgeranyl transferase

(or GGTase I; reviewed in Brown and Goldstein, 1993). Molecular cloning of the accessory factor REP-1 revealed identity with the human choroideremia gene, an X-linked gene that when mutated results in one form of retinal degenerative disease, and an unexpected sequence homology with RabGDI in two regions of the molecule (Cremers *et al.*, 1990; Seabra *et al.*, 1992a, 1993; Andres *et al.*, 1993).

When the prenylation reaction was reconstituted *in vitro* using purified components, REP-1 was shown to form a complex with rab proteins, suggesting the following model: REP-1 binds newly synthesized rab proteins and presents them to the catalytic RabGGTase. Upon prenylation, REP-1 would then remove rab proteins from the catalytic site of the transferase and deliver them to an acceptor protein (Andres *et al.*, 1993). The existence of such an acceptor was deduced from the observation that *in vitro* the prenylation reaction can proceed either stoichiometrically or catalytically. If the detergent concentration in the reaction mixture is below the critical micellar concentration (cmc), the reaction proceeds until all of rab proteins complexed to REP-1 are prenylated and then ceases. In contrast, if the concentration of detergent is higher than the cmc, prenylated rab proteins dissociate from REP-1 which becomes available for new rounds of catalysis (Andres *et al.*, 1993). These findings suggested that for RabGGTase to function catalytically *in vivo*, an acceptor protein would functionally substitute the detergent *in vitro* (Andres *et al.*, 1993). One obvious candidate for such an acceptor, given its established function in mediating membrane association of prenylated rab proteins (Novick and Garrett, 1994; Soldati *et al.*, 1994; Ullrich *et al.*, 1994), is RabGDI (Andres *et al.*, 1993).

Here we have investigated the mechanism whereby newly prenylated rab proteins associate with the membrane of Streptolysin-O (SLO)-permeabilized cells. We demonstrate that REP-1 is competent to deliver *in vitro* prenylated rab proteins to their target compartments. This process is cytosol- and RabGDI-independent. Furthermore, we show that REP-1 itself acts as a GDP dissociation inhibitor for rab5, thus sharing functional as well as structural similarity with RabGDI, but that only REP-1 is competent to assist in the prenylation reaction. These results suggest that after the prenyl transfer reaction, REP-1 *per se* is sufficient to mediate binding of rab proteins to their target membranes.

Results

In vitro prenylation and purification of the rab5–REP-1 complex

To prenylate rab5 *in vitro*, histidine-tagged rab5 (his-rab5) protein expressed in *Escherichia coli* was incubated in the presence of REP-1, RabGGTase and geranylgeranyl pyrophosphate (GGPP), and in the absence of detergent, as described previously (Andres *et al.*, 1993). The reaction mixture was subjected to gel filtration chromatography on a Superose 12 column and eluate fractions were analysed by SDS–PAGE and Coomassie blue staining. Two peaks of absorbance emerged from the column at positions corresponding to apparent molecular weights of 120 and 30 kDa (Figure 1A). The first peak (fractions 16–23) contained REP-1 and his-rab5 in an ~1:1 molar ratio, as deduced from the intensity of the bands (Figure 1B). Although very faint bands corresponding to RabGGTase could be seen in these fractions, they are most probably not associated to his-rab5 and REP-1, since a complex of the four proteins would be expected to elute at ~200 kDa. When GGPP was supplemented with [^{3}H]GGPP, a peak of protein-associated radioactivity co-eluted with his-rab5 and REP-1 (Figure 1A), thus demonstrating that geranylgeranylated his-rab5 was complexed to REP-1. More than 90% of his-rab5 in complex with REP-1 was prenylated, as calculated by protein-bound radioactivity. In contrast, the second peak contained exclusively non-modified his-rab5, as revealed by the lack of protein-bound radioactivity in these fractions. Fractions 18–20 were collected and used for further studies.

Binding of rab–REP-1 complex to permeabilized MDCK cells

Having purified the his-rab5–REP-1 complex, we then tested its possible interaction with cellular membranes using an *in vitro* assay. MDCK cells were permeabilized with SLO and extensively depleted from cytosol as described (Ullrich *et al.*, 1994). The permeabilized cells were then incubated with different concentrations of *in vitro* prenylated his-rab5–REP-1 complex for 30 min at 37°C, washed and the cell-associated proteins detected by immunoblot analysis. Exogenous and endogenous rab5 could be distinguished, since exogenous his-rab5 displayed a slower electrophoretic mobility (30 kDa band) than that of the endogenous protein (27 kDa band; Figure 2A). Increasing amounts of his-rab5 associated with the membranes of permeabilized cells in a concentration-dependent manner, reaching saturation at ~50 nM (Figure 2A and B). Under these conditions ~25% of exogenously added his-rab5 bound to membranes, resulting in a >10-fold increase over endogenous protein. Very low binding was detected with intact cells. No decrease in the amount of membrane-associated endogenous rab5 was detected, even at the highest concentration of added complex. By quantitative immunoblot (Figure 2B) we estimated that <1% of REP-1 bound to the membrane of either permeabilized or unpermeabilized cells (compare the relative intensities of the REP-1 and his-rab5 bands in lane 1 of Figure 2A). Thus, REP-1 was released into the medium upon binding of his-rab5 to the membrane. Most importantly, neither RabGDI nor ATP nor other cytosolic factors were required for membrane association of his-rab5, and RabGGTase appeared to have no effect on this process (see also below). Similarly, no requirement for cytosolic factors was observed when an enriched early endosome fraction (see Materials and methods) was used as the source of acceptor membranes (data not shown).

To investigate the general role of REP-1 in mediating both isoprenylation and membrane targeting of different rab proteins, we performed similar studies using rab7. As for rab5, rab7 was isoprenylated *in vitro*, consistent with previous results showing that RabGGTase can isoprenylate rab proteins with structurally distinct cysteine motifs (Cremers *et al.*, 1994). Incubation of permeabilized MDCK cells in the presence of rab7–REP-1 complex led to membrane binding of exogenous rab7 in a dose-dependent manner (data not shown). Saturation of binding was obtained at a concentration of ~60–70 nM, in good

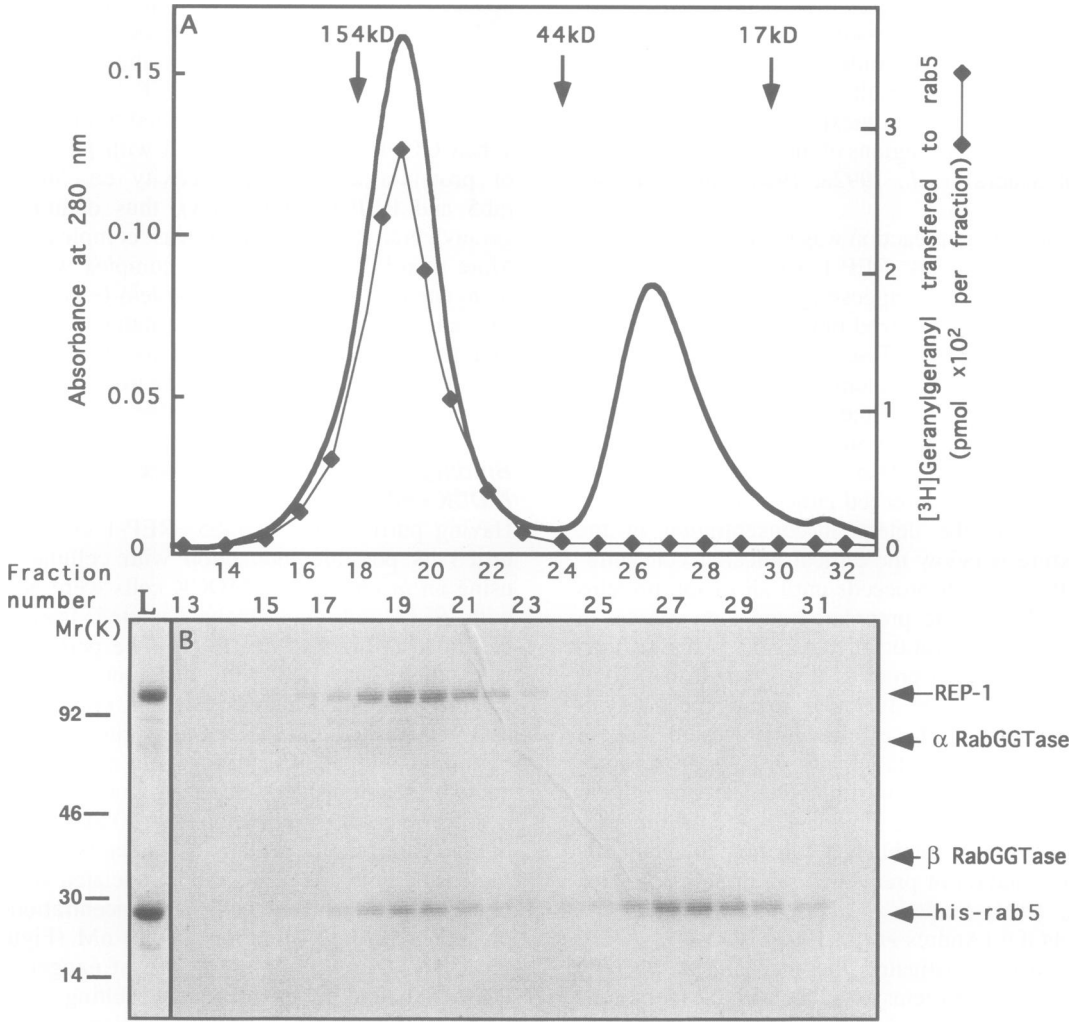

Fig. 1. Purification of [^{3}H]geranylgeranylated his-rab5−REP-1 complex by gel filtration column chromatography. Recombinant his-rab5 purified from *E.coli* was incubated in the presence of REP-1, RabGGTase and [^{3}H]GGPP, and loaded onto a Superose 12 gel filtration column as described in Materials and methods. (**A**) Elution profile showing absorbance at 280 nm (thick line) and protein-bound radioactivity (◆). Relative molecular masses are indicated by arrows. (**B**) Coomassie blue-stained SDS−polyacrylamide gel showing in lane L 10 μl of the sample loaded on the column and 30 μl of fractions 13–32 eluted from the column. Protein-bound radioactivity appeared in the fractions containing both rab5 and REP-1 (fractions 17–21), whereas fractions 25–30 containing only the rab5 protein were essentially devoid of counts. The gel was calibrated with the indicated molecular size markers. Arrowheads show the positions of the α and β subunits of RabGGTase on the gel.

agreement with the concentration determined for rab5. These data demonstrate that *in vitro* prenylated rab5 and rab7 proteins complexed to REP-1 are competent for membrane association.

Specificity of REP-1-mediated rab5 and rab7 membrane targeting

To rule out the possibility that rab5 was retained in the permeabilized cells non-specifically, we tested the binding properties of non-prenylated his-rab5−REP-1 complex. His-rab5 was incubated with REP-1 in the absence of RabGGTase and GGPP and subjected to gel filtration chromatography as described above. The chromatographic profile of the two proteins was essentially identical to that of his-rab5−REP-1 (data not shown). When permeabilized MDCK cells were incubated with unmodified his-rab5−REP-1 complex, no membrane association of exogenous his-rab5 was observed (Figure 2A, lane 10). Thus, while REP-1 can complex with both modified and unmodified rab5, it can only mediate membrane association of the geranylgeranylated protein.

Similar to the RabGDI-mediated process (Ullrich *et al.*, 1993, 1994), REP-1-mediated binding of rab5 to the membrane was temperature- and time-dependent, but cytosol-independent. Binding was not detectable at 4°C, largely suppressed at 10–20°C and essentially restored at 25°C (Figure 3A). A time course experiment conducted at 37°C showed that saturation was reached within 10–15 min (Figure 3B).

Evidence showing that the rab proteins were correctly targeted was provided by examining the intracellular localization of exogenous rab5 and rab7 in permeabilized cells by confocal immunofluorescence microscopy (Figure 4). A431 cells were chosen for these studies due to the availability of organelle markers and because binding of *in vitro* prenylated his-rab5 mediated by REP-1 to these cells exhibited the same properties as to MDCK II cells (data not shown). A431 cells were permeabilized with

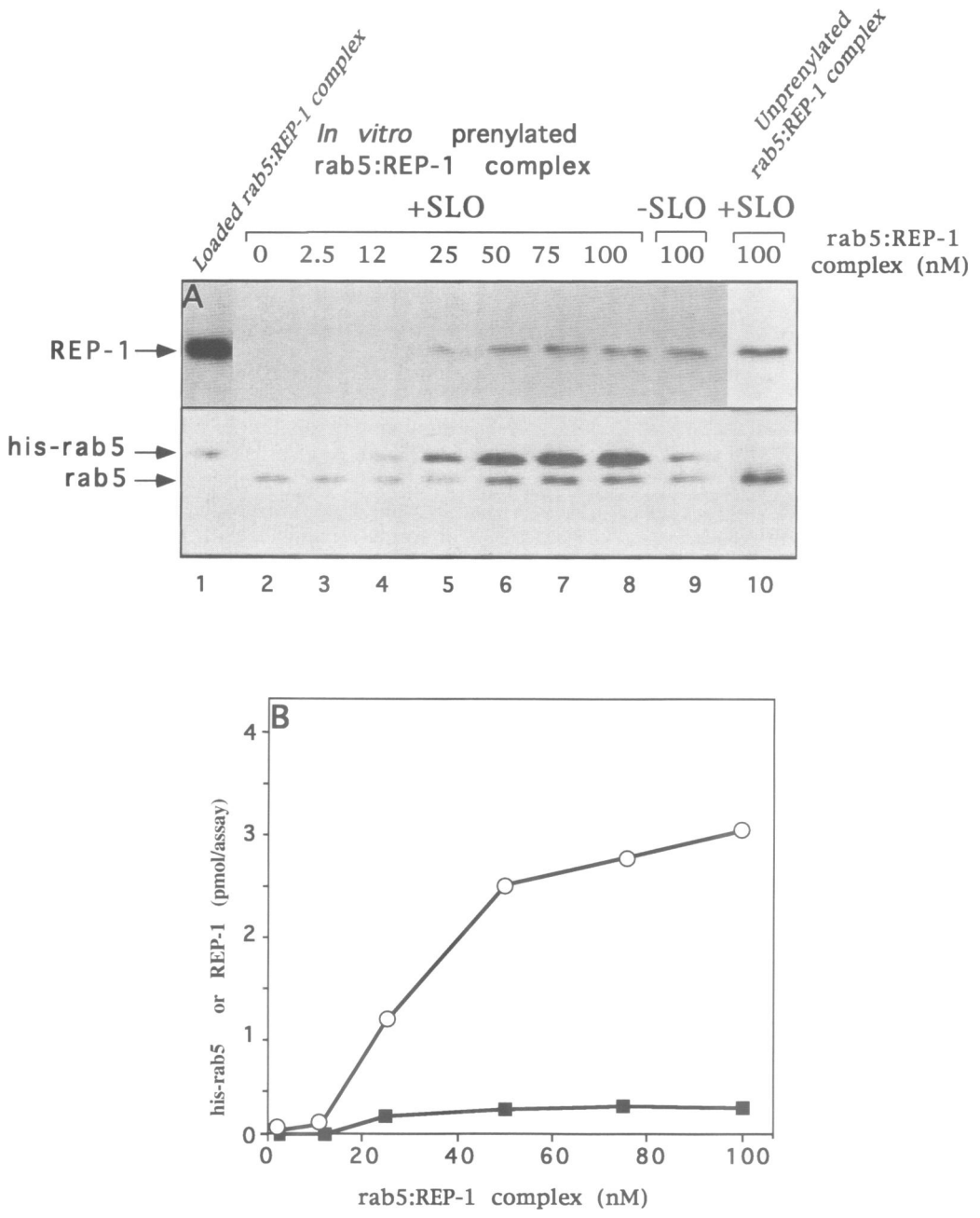

Fig. 2. Dose-dependent saturation of rab5 binding to the membrane of SLO-permeabilized MDCK II cells. Permeabilized (lanes 2–8 and 10) and unpermeabilized (lane 9) cells were incubated with various concentrations (0–100 nM) of purified *in vitro* prenylated (lanes 2–9) or unprenylated (lane 10) his-rab5−REP-1 complex at 37°C for 30 min. (**A**) Cell-associated rab5 and REP-1 were detected by immunoblot analysis using specific affinity-purified anti-rab5 and anti-REP-1 antibodies. Lane 1 contains 0.5 pmol of loaded complex. (**B**) Quantification of his-rab5 (○) and REP-1 (■) binding to permeabilized cells. Rab5 and REP-1 were quantified using purified recombinant proteins as standards. Bound antibodies were visualized by the ECL and quantified by densitometry (Molecular Dynamics).

SLO under mild conditions (see Materials and methods) and incubated in the presence or absence of his-rab5−REP-1 complex, rab7−REP-1 complex, or both. After extensive washing, the cells were fixed; rab5 and rab7 proteins were visualized using specific affinity-purified antibodies at concentrations sufficient to detect only the exogenous proteins (Chavrier *et al.*, 1991; Bucci *et al.*, 1992; Ullrich *et al.*, 1994). Consistent with previous findings, exogenous rab5 co-localized with transferrin receptor on the plasma membrane and on enlarged early endosomes (Figure 4A and B; Chavrier *et al.*, 1991; Bucci *et al.*, 1992; Ullrich *et al.*, 1994). Conversely, little co-localization of exogenous rab5 with lamp-1 was observed (Figure 4C and D). Exogenous rab7 bound to vesicular structures in the perinuclear region of the cell, as anticipated given its localization to late endosomes, but did not co-localize with either transferrin receptor (Figure 4E and F) or with exogenous rab5 (data not shown). These results

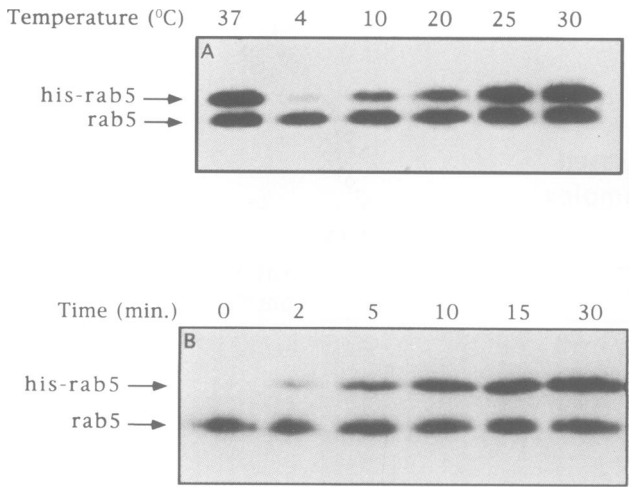

Fig. 3. Temperature- (**A**) and time-dependent (**B**) binding of exogenous rab5 to the membrane of SLO-permeabilized MDCK II cells. Permeabilized cells were incubated with purified *in vitro* prenylated his-rab5−REP-1 complex at a concentration of 40 nM at the indicated temperatures (A) or for different periods of time at 37°C (B). Cell-associated rab5 was detected by immunoblot analysis as described in Materials and methods.

indicate that rab5 and rab7 were delivered by REP-1 to their correct endocytic compartments in permeabilized A431 cells.

Prenylated rab5−REP-1 complex stimulates early endosome fusion in vitro

Having shown that rab5 was correctly targeted to the membrane by the rab5−REP-1 complex, we next investigated whether the protein was functionally active. The enlarged early endosomes observed in permeabilized cells incubated with the complex (Figure 4) suggested that this was the case. We wanted to confirm this point using a quantitative biochemical assay. Previous studies have shown that rab5 is a rate-limiting factor in the machinery which regulates fusion of early endosomes *in vitro* (Gorvel *et al.*, 1991). Here we compared the activity of his-rab5−REP-1 complex with that of rab1a−REP-1 complex in the same *in vitro* fusion assay performed under limiting cytosol concentrations (Stenmark *et al.*, 1994b). Rab1a has been shown to function in the endoplasmic reticulum to Golgi transport (Nuoffer *et al.*, 1994) and is expected to have no effect on the lateral fusion between early endosomes. We first verified that REP-1 could support the *in vitro* prenylation of rab1a, consistent with previous data (Andres *et al.*, 1993), as well as its association with the membrane of permeabilized cells (data not shown). As expected, addition of the rab1a−REP-1 complex did not have detectable activity in the endosome fusion assay (Figure 5). In contrast, the his-rab5−REP-1 complex stimulated early endosome fusion by 120%, whereas non-prenylated his-rab5, or REP-1 alone, had no effect. Thus, the rab5−REP-1 complex is functionally active.

REP-1 forms a stable complex with newly synthesized rab5 protein in vivo

Having shown that REP-1 can mediate membrane association of rab proteins *in vitro*, we next investigated whether a complex between newly synthesized rab proteins and REP-1 can also be detected *in vivo*. To address this question we transiently expressed rab5 at a high level in NRK cells using the T7 RNA polymerase recombinant vaccinia virus expression system, as described previously (Bucci *et al.*, 1992). Under the experimental conditions used, the bulk of cytosolic rab5 corresponds to the non-prenylated form of the protein (Gorvel *et al.*, 1991; Peter *et al.*, 1992). Rab5 was immunoprecipitated from the cytosol of transfected and control cells with a mouse anti-rab5 mAb (Bucci *et al.*, 1994); immunoprecipitates were separated on SDS−PAGE and analysed by immunoblot staining using an anti-REP-1 mAb. Co-immunoprecipitation of REP-1 and rab5 was observed from the cytosol of rab5 overexpressing, but not from control cells where the amount of endogenous cytosolic rab5 complexed to REP-1 is presumably too low to be detected (Figure 6). No co-immunoprecipitation of rab5 (data not shown) or of REP-1 (Figure 6, lane 5) was obtained in the presence of the peptide against which the anti-rab5 antibody was raised, or using a control serum. This result indicates that REP-1 forms a complex with newly synthesized rab proteins *in vivo*.

REP-1 shares functional properties with RabGDI

The data so far described indicate that REP-1 can chaperone various rab proteins to the membrane as has been shown previously for RabGDI (Soldati *et al.*, 1994; Ullrich *et al.*, 1994). Based on these findings, the sequence homology shared by REP-1 and RabGDI may reflect some common functional features. To address this question, we investigated whether REP-1 can perform the two functions ascribed to RabGDI, namely inhibition of GDP dissociation and the ability to extract rab proteins from the membrane.

We first assayed the ability of REP-1 to inhibit the dissociation of [^{3}H]GDP and [^{35}S]GTPγS from prenylated and unprenylated rab5. Figure 7 shows that REP-1 indeed inhibited GDP dissociation from rab5 in a time- and dose-dependent fashion. However, while RabGDI was active only on prenylated rab5, as expected (Araki *et al.*, 1991), REP-1 displayed GDI activity on both prenylated and unprenylated rab5 (Figure 7B and D). The specificity of this activity was demonstrated by the lack of effect obtained with bovine serum albumin (BSA) at similar concentrations. Inhibition was dose-dependent and reached a plateau at 1 μM for REP-1. REP-1 was three times more potent than RabGDI at a concentration of 0.25 μM. In contrast, neither REP-1 nor RabGDI affected the dissociation of [^{35}S]GTPγS from either modified or unmodified rab5 (data not shown). We conclude that REP-1 has GDI activity on both the non-prenylated and prenylated forms of rab5, consistent with its ability to bind both forms of the molecule.

Secondly, we show that treatment of permeabilized MDCK II cells in the presence of different concentrations of REP-1 or RabGDI led to dose-dependent removal of rab5 from membranes with approximately the same efficiency (Figure 8). This effect was already visible at a concentration of 0.25 μM and was maximal at 1 μM. As controls we used RabGGTase and GST-tagged RhoGDI, which interact with small GTPases of the rho but not of the rab subfamily (Takai *et al.*, 1992). Neither had any effect on the membrane association of rab5. REP-1 was

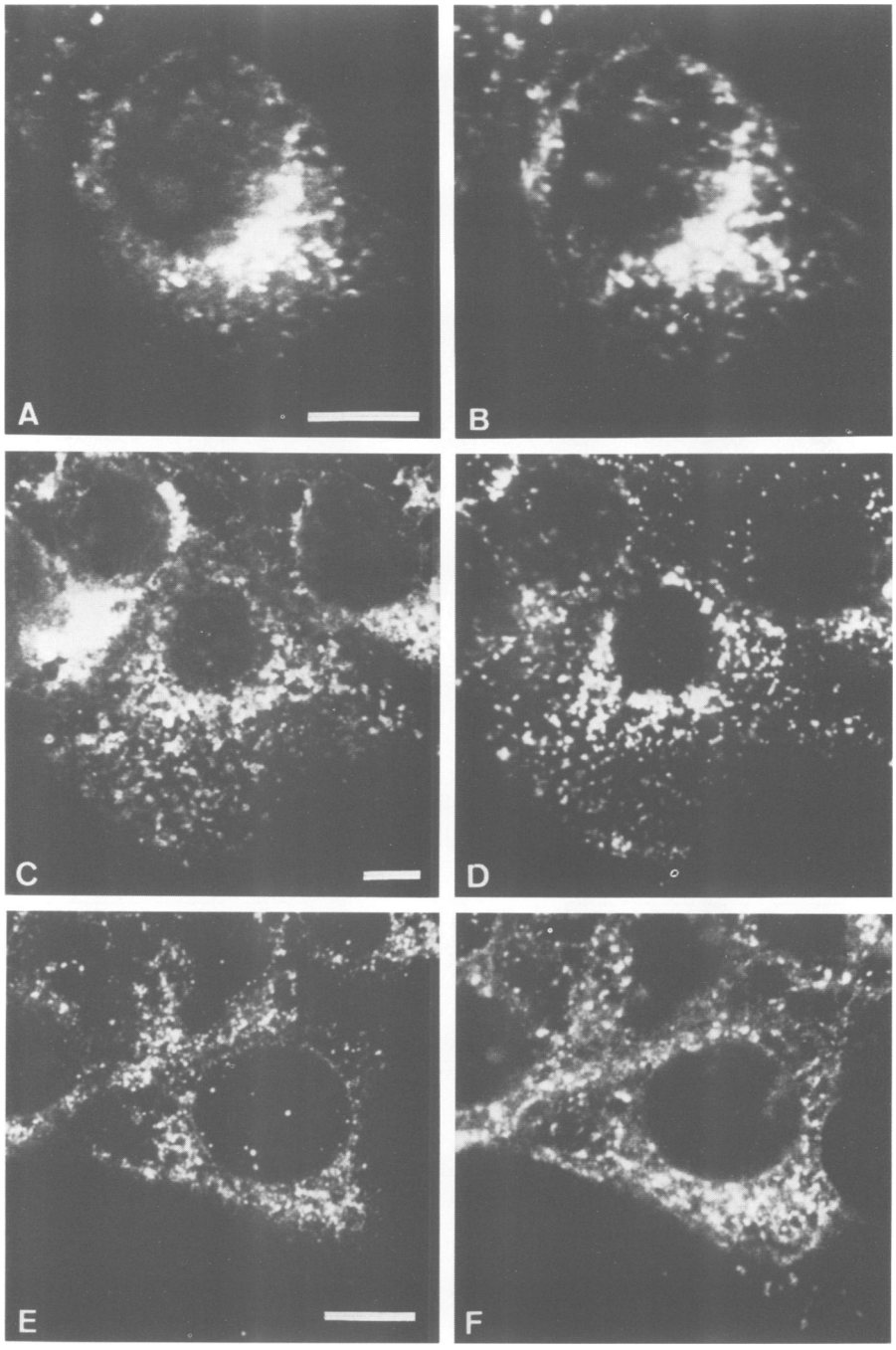

Fig. 4. Localization of exogenous his-rab5 and rab7 in permeabilized A431 cells. Cells were permeabilized with SLO and incubated with 100 nM of purified *in vitro* prenylated his-rab5−REP-1 (**A**−**D**) or rab7−REP-1 (**E** and **F**) complexes for 10 min at 37°C. Membrane-bound rab proteins and endosomal markers were detected by indirect double immunofluorescence using affinity-purified antibodies: (A) rabbit polyclonal anti-rab5, (C) mouse monoclonal anti-rab5, (B and F) mouse monoclonal anti-human transferrin receptor, (D) rabbit polyclonal anti-human lamp-1 and (E) rabbit polyclonal anti-rab7 antibodies. Secondary antibodies were FITC-labelled donkey anti-rabbit and rhodamine-labelled donkey anti-mouse antibodies. Coverslips were viewed with the confocal microscope developed at EMBL, as described previously (Ullrich *et al.*, 1994). Scale = 10 μm.

also capable of removing rab2 and rab7 from the membranes of permeabilized MDCK II cells (data not shown), thus indicating a general role for REP-1 in interacting with different rab proteins.

Quantitative immunoblots showed that the intracellular concentration of REP-1 in A431 cells is ~0.2 μM, a value approximately five times lower than that estimated for RabGDI in this and other cell lines (Ullrich *et al.*, 1993). Nevertheless, the intracellular concentration of REP-1 is close to that required for the interaction with rab proteins *in vitro*.

While REP-1 and RabGDI share some functional properties, RabGDI would be expected not to substitute REP-1 in the prenylation reaction since, unlike REP-1, it does not interact efficiently with non-prenylated rab proteins (Araki *et al.*, 1991). To control this, we measured the amount of [^{3}H]GG transferred to rab5 by RabGGTase in the presence of different concentrations of RabGDI or

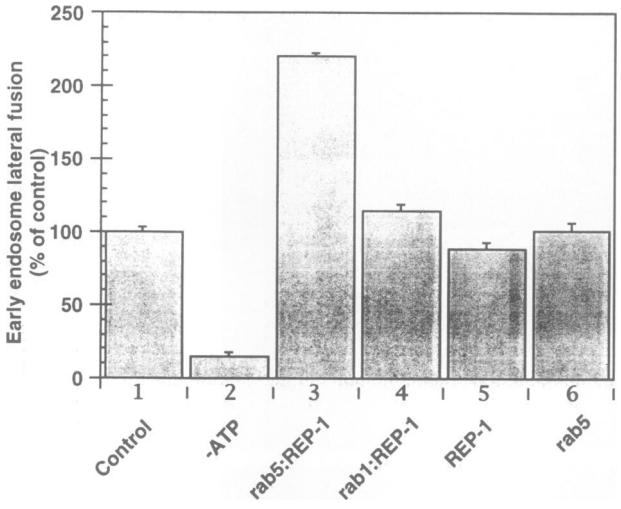

Fig. 5. Effect of his-rab5−REP-1 complex in a cell-free assay of endosome fusion. The *in vitro* fusion assay was carried out as described in Materials and methods. Rab5−REP-1 complex, rab1−REP-1 complex, REP-1 and non-prenylated rab5 were added at a concentration of 260 nM immediately prior to incubation at 37°C. The stimulation of fusion is expressed as a percentage of the fusion obtained in the absence of added complex or protein (lane 1).

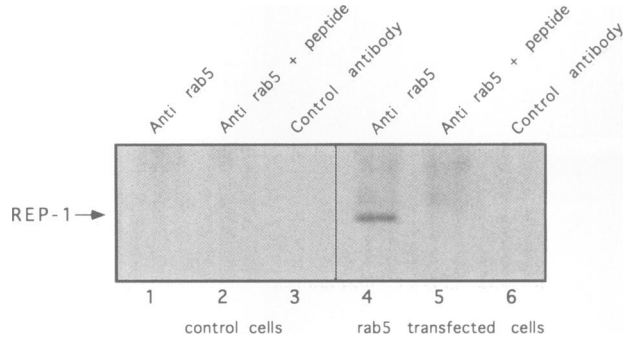

Fig. 6. Co-immunoprecipitation of REP-1 with newly synthesized rab5. Rab5 was transiently overexpressed in NRK cells using the T7 RNA polymerase recombinant vaccinia virus expression system. Rab5 was immunoprecipitated from cytosol of either control (lanes 1–3) or transfected cells (lanes 4–6) using mouse anti-rab5 mAb. Co-immunoprecipitated REP-1 was visualized by immunoblot analysis as described in Materials and methods. As controls, the immunoprecipitation of rab5 (data not shown) and REP-1 was performed in either the presence of the peptide used for raising the anti-rab5 antibody (lanes 2 and 5), or using an irrelevant antibody (lanes 3 and 6).

REP-1. As shown in Figure 9, the addition of REP-1 resulted in a dose-dependent increase in the amount of [^{3}H]GG bound to rab5, whereas the addition of RabGDI had no effect. Hence, RabGDI cannot functionally substitute REP-1 in the prenylation reaction.

Guanine nucleotide exchange accompanies REP-1-mediated membrane association of rab5

We have recently reconstituted the RabGDI-mediated binding of rab5 to the membrane *in vitro* (Ullrich *et al.*, 1994). The association of rab5 with the acceptor membrane was found to be accompanied by (i) the release of RabGDI, (ii) the formation of a transient membrane-bound rab5−GDP intermediate and (iii) its subsequent

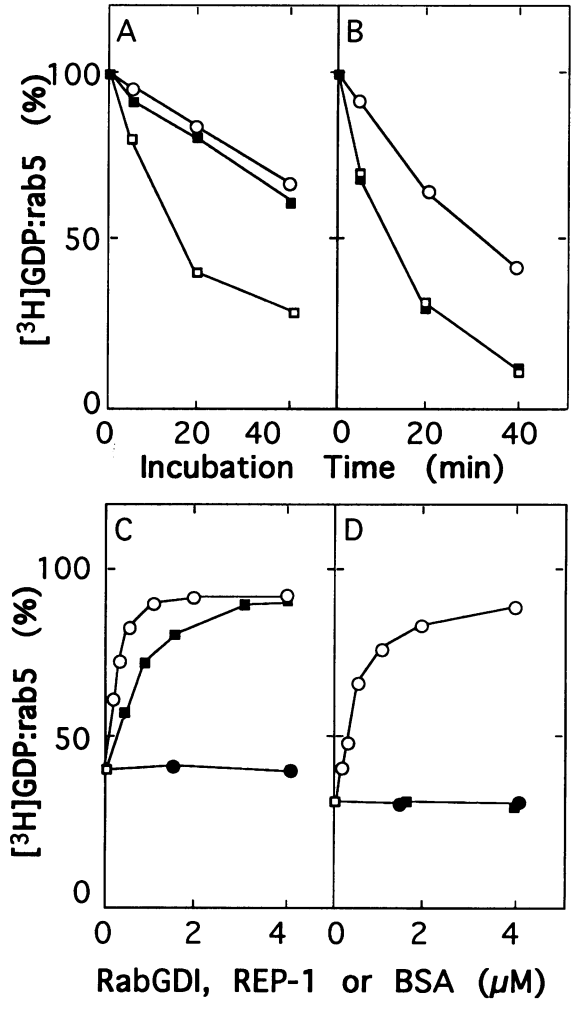

Fig. 7. Effect of REP-1 on the dissociation of [^{3}H]GDP from prenylated (**A** and **C**) and non-prenylated rab5 (**B** and **D**). [^{3}H]GDP-prebound rab5 (50 nM, 20 000 c.p.m./pmol) was incubated for various periods of time in buffer alone and with 0.5 μM REP-1 or 1.5 μM RabGDI (A and B) or in the presence of various concentrations of REP-1, RabGDI and BSA (C and D) for 20 min. After the incubation, [^{3}H]GDP bound to rab5 was measured using the filter method, as described (Sasaki *et al.*, 1990). The data are expressed as the percentage of rab5-bound [^{3}H]GDP prior to incubation. REP-1 (○); RabGDI (■); buffer only (□); BSA (●).

conversion into the GTP-bound form. We therefore determined whether REP-1 delivers rab5 to the membrane through a similar (i.e. multistep) mechanism. To this end, geranylgeranylated rab5 was purified from the membrane of overexpressing Sf9 cells, pre-loaded with [^{3}H]GDP (Ullrich *et al.*, 1994) and incubated with an equimolar amount of REP-1, followed by gel filtration chromatography. Two peaks were eluted from the column: a minor one corresponding to an apparent molecular weight of 600 kDa (void volume) and a major one of 120 kDa. SDS−PAGE analysis indicated that the fractions corresponding to the first peak contained rab5, REP-1 and minor impurities (data not shown). Due to their elution volume these fractions are likely to contain protein aggregates not removed by centrifugation. In contrast, the second peak contained REP-1 and rab5 in an apparent 1:1 molar ratio and most of the protein-bound radioactivity.

When the fractions containing the [^{3}H]GDP−rab5−

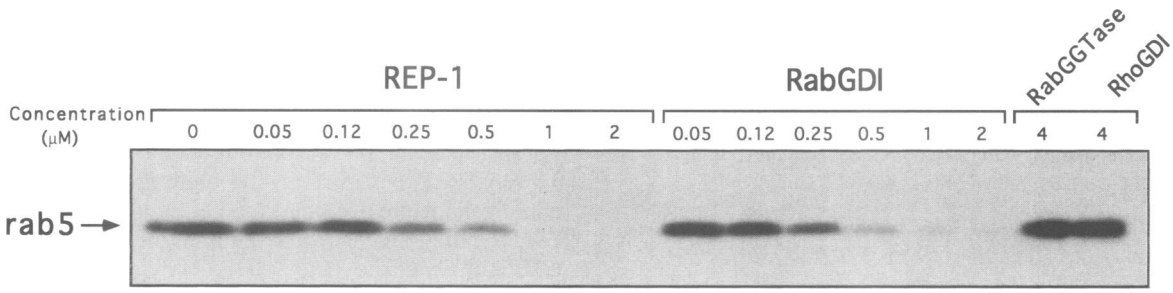

Fig. 8. Removal of endogenous rab5 protein by REP-1 and RabGDI from the membranes of SLO-permeabilized MDCK cells. Cells were permeabilized with SLO and incubated for 30 min at 37°C in the presence of either various concentrations of REP-1 and RabGDI or with 4 μM GST−RhoGDI and RabGGTase. The fraction of cell-associated rab5 protein remaining after incubation was visualized by immunoblot analysis using affinity-purified anti-rab5 antibodies and ECL.

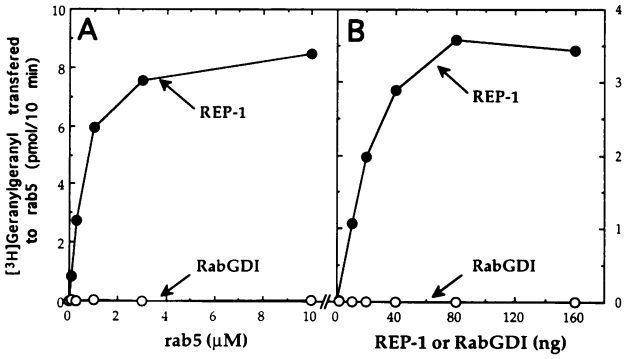

Fig. 9. Geranylgeranylation of rab5 by RabGGTase in the presence of REP-1 or RabGDI. (**A**) Various concentrations of rab5 were incubated in 50 μl of a mixture containing 100 ng RabGGTase and either 100 ng REP-1 (●) or 100 ng RabGDI (○). (**B**) Rab5 at a concentration of 5 μM was supplemented with 20 ng RabGGTase, and the indicated amounts of either REP-1 (●) or RabGDI (○). After incubation at 37°C for 10 min, RabGGTase activity was measured as described in Materials and methods.

REP-1 complex were tested in the *in vitro* assay, binding of rab5 to the membrane of permeabilized MDCK cells occurred in a concentration- and time-dependent manner identical to that observed for *in vitro* prenylated rab5−REP-1 complex (data not shown). RabGGTase was not present in the fraction and, therefore, was not required for this process. As shown for RabGDI, membrane association of rab5 was accompanied by release of REP-1. The radioactive GDP bound to rab5 in the soluble and membrane fraction was measured. Increasing levels of rab5 in the GDP-bound form were found to associate with the membrane of permeabilized cells, reaching maximal levels within 15 min and decreasing to background levels after 60 min (Figure 10A). Considering that at this time point membrane association of rab5 was already saturated, these data suggest that [^{3}H]GDP was released from rab5 and replaced with GTP. To demonstrate this directly, cells were incubated with GDP−rab5−REP-1 complex in the presence of [^{35}S]GTPγS for different time periods. Membrane-associated rab5 was then quantitatively immunoprecipitated and the radioactivity bound to rab5 was determined. In permeabilized but not in unpermeabilized cells, a time-dependent increase in rab5-bound [^{35}S]GTPγS was observed (Figure 10B). The nucleotide exchange profile was virtually identical to the one established for the rab5−RabGDI complex (Soldati *et al.*, 1994; Ullrich

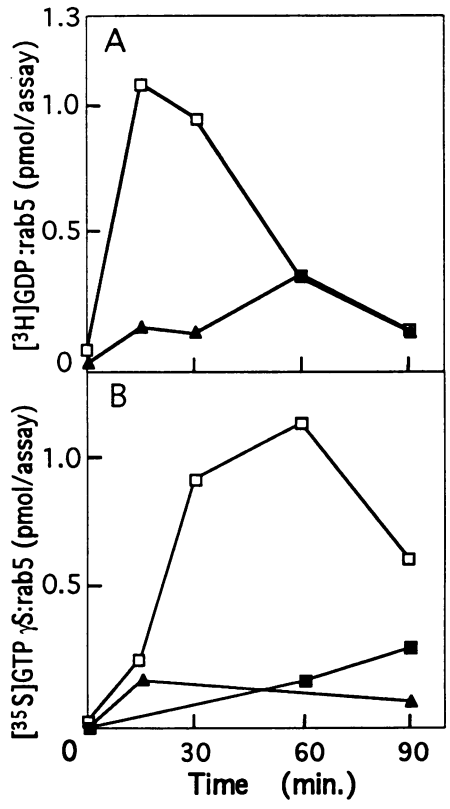

Fig. 10. REP-1-mediated binding of rab5 to the membranes of permeabilized MDCK cells is accompanied by guanine nucleotide exchange. (**A**) Time course of [^{3}H]GDP−rab5 binding to the membrane of SLO-permeabilized cells. SLO-permeabilized (□) and unpermeabilized (▲) cells were incubated in the presence of *in vitro* assembled [^{3}H]GDP−rab5−REP-1 complex (58 nM) for various periods of time. After washing, the amount of [^{3}H]GDP−rab5 associated with the cells was measured as described in Materials and methods. (**B**) Time course of [^{35}S]GTPγS binding to rab5 on the membrane of permeabilized cells. SLO-permeabilized (□) and unpermeabilized (▲) cells were incubated in the absence or presence of 58 nM unlabelled rab5−REP-1 complex and 50 μM [^{35}S]GTPγS for the indicated periods of time. The fraction of [^{35}S]GTPγS bound to membrane-associated rab5 was measured after immunoprecipitation using mouse anti-rab5 mAb. Permeabilized cells with rab5−REP-1 complex (□); unpermeabilized cells with rab5−REP-1 complex (▲); permeabilized cells without rab5−REP-1 complex (■).

et al., 1994). These findings strongly suggest that REP-1 and RabGDI mediate membrane association of rab proteins through the same mechanism.

Discussion

Previous *in vitro* studies have suggested that the accessory component of rab geranylgeranyl transferase, REP-1, functions cyclically in the isoprenylation of rab proteins. According to the proposed model, REP-1 forms a complex with newly synthesized rab proteins, presents them to the catalytic component of the enzyme and removes the prenylated proteins from this component following the transfer reaction (Seabra *et al.*, 1992a; Andres *et al.*, 1993). The observation that detergents promote multiple rounds of catalysis *in vitro*, by dissociating prenylated rab–REP-1 complexes and inducing the recycling of REP-1, has suggested that a cellular protein acceptor recognizes isoprenylated rab proteins and dissociates them from REP-1 *in vivo*. Given its function in complexing isoprenylated rab proteins and mediating their membrane association, RabGDI seemed an ideal candidate for such a protein acceptor (Andres *et al.*, 1993). In this report we provide evidence that REP-1 is complexed to newly synthesized rab5 *in vivo*, and that it is competent to mediate the association of *in vitro* prenylated rab1a, rab5 and rab7 with the membrane of permeabilized cells. This process is concentration-, time- and temperature-dependent, but does not require ATP. *In vitro* prenylated rab proteins are correctly targeted and functionally active, as revealed by the stimulatory effect of rab5 on the lateral fusion of early endosomes in a cell-free assay. Surprisingly, we demonstrate that dissociation from REP-1 and binding of rab proteins to the membrane do not require RabGDI, RabGGTase or other cytosolic factors, but that REP-1 itself shares several functional properties of RabGDI. In addition, the mechanism of REP-1-mediated membrane association of rab5 appears to be very similar to that mediated by RabGDI (Ullrich *et al.*, 1994).

Our findings, documenting the sequential participation of REP-1 in isoprenylation and delivery to the membrane of rab1, rab5 and rab7, allow us to formulate a model for the membrane targeting of newly synthesized rab proteins. Given the ability of REP-1 to act as a GDI also on non-modified rab proteins, it is likely that REP-1 preferentially forms a complex with the GDP-bound form of rab proteins and, after prenylation by the catalytic subunit of RabGGTase, it delivers them in the GDP-bound form to the membrane. Here the GDP–rab–REP-1 complex is recognized by a specific receptor molecule which causes dissociation of the complex and release of REP-1 into the cytosol. The membrane-bound GDP–rab protein is then converted into the active GTP-bound conformation by a guanine nucleotide exchange factor.

Inhibition of GDP dissociation, solubilization of rab proteins from the membrane and delivery of rab proteins to the membrane were considered functional properties specific for RabGDI. Here we have shown that REP-1 can inhibit the release of GDP from both unmodified and prenylated rab5 and remove rab proteins from the membrane. The demonstration that REP-1 and RabGDI share biochemical features provides strong support for the idea that REP-1 substitutes for RabGDI in the delivery of newly prenylated rab proteins to the membrane. The REP-1-mediated process displays at least three similar characteristics to the one mediated by RabGDI. First, newly synthesized rab proteins also appear to be complexed to REP-1 (Figure 6) and presented to the membrane in the GDP-bound conformation, similar to cytosolic rab proteins complexed to RabGDI. Secondly, the acceptor protein postulated to dissociate prenylated rab proteins from REP-1 appears to be localized to the membrane, as for the acceptor of rab proteins complexed to RabGDI. Thirdly, REP-1-mediated rab5 binding to the membrane exhibited identical properties, i.e. concentration dependence, binding kinetics, generation of the membrane-bound GDP intermediate and GDP/GTP exchange, to the RabGDI-mediated process. This striking similarity strongly suggests that these two pathways may converge on the same membrane target molecules. This hypothesis is supported by preliminary observations indicating that saturating concentrations of REP-1–rab5 complex inhibit RabGDI-mediated membrane association of rab5 (K.Alexandrov and M.Zerial, unpublished results). The apparent requirement for cytosolic rab proteins to be stabilized in the GDP-bound conformation, either complexed to RabGDI or to REP-1, may conceivably reflect a pressure to maintain them in the inactive form, i.e. unable to interact with effector proteins, until they can interact with the acceptor protein present in the target compartment and eventually become activated (Soldati *et al.*, 1994; Ullrich *et al.*, 1994).

Based on these observations, it is likely that REP-1 delivers nascent rab proteins to the membrane, whereas RabGDI acts in the recycling of rab proteins following this event. This possibility is supported by the observation that RabGDI cannot substitute REP-1 in the isoprenylation reaction *in vitro*. We cannot rule out, however, that REP-1 may also participate in the constitutive shuttling of rab proteins between the membrane and the cytosol. While the concentration of cytosolic REP-1 appears to be ~5-fold lower than that of RabGDI in the cells we have studied, its value is in the range of the measured affinity for rab proteins *in vitro*. Recent work has demonstrated that most of cytosolic RabGDI is complexed to multiple rab proteins (Regazzi *et al.*, 1992); the ratio of free REP-1 to rab–REP-1 has yet to be determined.

Recent results suggest that the membrane dynamics of rab proteins are regulated by multiple factors. The choroideremia-like gene (Cremers *et al.*, 1992) has been shown to code for a second rab escort protein, REP-2 (Cremers *et al.*, 1994), and novel RabGDI isoforms have been identified from mouse skeletal muscle and rat brain (Nishimura *et al.*, 1994; Shisheva *et al.*, 1994). Thus, in light of our findings, at least four different proteins, i.e. RabGDI, RabGDI-2/β, REP-1 and REP-2, could modulate the membrane association of rab proteins. The available data suggest that these molecules can interact with specific rab proteins (Cremers *et al.*, 1994; Nishimura *et al.*, 1994; Shisheva *et al.*, 1994). However, why are distinct types of rab binding proteins, such as REP and GDI, required? One possibility is that several factors perform a fine control of rab protein cycling. Different REP and GDI proteins may interact with different regulatory molecules. For instance, such regulation might involve selective phosphorylation, as has been suggested for RabGDI (Steele-Mortimer *et al.*, 1993). In addition, this molecular repertoire may be required to maintain a balance between membrane association and extraction. RabGDI and REP-1 can both solubilize and deliver rab proteins to the mem-

brane. Elevated levels of these factors may shift the equilibrium, resulting in the removal of rab proteins from the membrane and the inhibition of transport. For instance, concentrations of RabGDI causing extraction of rab proteins from the membrane led to the inhibition of intra-Golgi transport (Elazar *et al.*, 1994). It is also possible that the concentration required for a single molecule, such as REP-1, to interact with several factors, isoprenylate rab proteins and escort them to the membrane during their recycling would have to be so high (>1 μM in the rab-free form) that it would lead to the removal of rab proteins from the membrane. The distribution of these activities among distinct factors has the advantage of lowering the relative concentration of each component, thus keeping membrane insertion and release of rab proteins into the cytosol correctly balanced. Further experiments will be required to test this hypothesis and to identify the acceptor components mediating membrane association of rab proteins.

Materials and methods

Expression and purification of recombinant proteins

His-tagged canine rab1a protein and human his-rab5a were constructed, expressed in *E.coli* and purified as described (Andres *et al.*, 1993; Cremers *et al.*, 1994). Canine rab7 expressed in *E.coli* was a gift from Dr Metcalf (V.Brachvogel *et al.*, manuscript in preparation). Canine rab5 was expressed in Sf9 cells using the pVL1393 baculovirus transfer vector. The purification of post-translationally modified and unmodified rab5 from membranes and cytosol, respectively, of Sf9 cells will be described elsewhere (Horiuchi *et al.*, 1994). REP-1 and RabGGTase were expressed and purified as described previously (Andres *et al.*, 1993; Cremers *et al.*, 1994). His–RabGDI and GST–RhoGDI fusion proteins were expressed in *E.coli* and purified as described previously (Hancock and Hall, 1993; Ullrich *et al.*, 1994).

Purification of in vitro prenylated and non-prenylated rab–REP-1 complexes

Ten μM of rab5a, rab7 or rab1a expressed in *E.coli* were incubated with 1 μM RabGGTase, 3 μM REP-1 and 60 μM GGPP (American Radiolabeled Co.) in the presence of buffer containing 78 mM KCl, 50 mM HEPES–KOH pH 7.2, 7 mM $MgCl_2$, 10 mM EGTA, 8.37 mM $CaCl_2$, 1 mM DTT, 1 μM GDP and 0.005% Triton X-100 in a final volume of 550 μl. After incubation for 30 min at 30°C, the reaction mixture was loaded onto a Superose 12 10/30 column (Pharmacia) equilibrated in buffer A (78 mM KCl, 50 mM HEPES–KOH pH 7.2, 4 mM $MgCl_2$, 10 mM EGTA, 8.37 mM $CaCl_2$, 1 mM DTT, 1 μM GDP) supplemented with 0.005% Triton X-100. The material eluting between 4 and 18 ml was collected in 0.4 ml fractions. Fractions 18–20 containing most of the complex were combined and stored in aliquots at −80°C until use. In some experiments, GGPP was supplemented with [^{3}H]GGPP (American Radiolabeled Co.; 33 000 d.p.m./pmol). To measure protein-bound radioactivity, column fractions were processed on glass fibre filters as described below. To purify the non-prenylated rab5–REP-1 complex, 10 μM of purified rab5 from *E.coli* were incubated with 3 μM REP-1 in the presence of buffer A and 0.005% Triton X-100. After incubation for 30 min at 30°C, the reaction mixture was subjected to gel filtration and fractions were treated as described for *in vitro* prenylated rab5–REP-1 complex.

Purification of in vitro assembled [^{3}H]GDP–rab5–REP-1 complex

To prepare [^{3}H]GDP-bound rab5–REP-1 complex, 1.5 μM [^{3}H]GDP-bound post-translationally modified rab5 was incubated with 1.5 μM REP-1; [^{3}H]GDP–rab5–REP-1 complex was purified by gel filtration chromatography as described for the rab5–RabGDI complex (Ullrich *et al.*, 1994).

Cell permeabilization and complex binding

MDCK II cells were grown to confluence in 24-well plastic dishes (3×10^6 cells/well) and permeabilized with SLO as described (Ullrich *et al.*, 1993, 1994). After permeabilization, cells were placed on ice and incubated with buffer A for 15 min to be depleted of cytosol. Unless otherwise indicated, cells were incubated with rab5–REP-1 complex at 37°C for 30 min. Cells were then washed with buffer A for 15 min with three buffer changes and the amounts of cell-associated rab5 and REP-1 were measured by immunoblot using the ECL method (Amersham) as described (Ullrich *et al.*, 1994).

[^{3}H]GDP release and [^{35}S]GTPγS binding assays

Permeabilized cells were incubated with 7 pmol of *in vitro* assembled [^{3}H]GDP–rab5–REP-1 complex (58 nM, 1.3×10^4 c.p.m./pmol) in the presence of 50 μM GTP; cell-associated [^{3}H]GDP–rab5 was measured as described (Ullrich *et al.*, 1994). For GTPγS binding assays, SLO-permeabilized or unpermeabilized cells were incubated in the presence of 7 pmol of unlabeled rab5–REP-1 complex and 50 μM [^{35}S]GTPγS (1.0×10^4 c.p.m./pmol). As controls, permeabilized cells were also incubated with [^{35}S]GTPγS in the absence of rab5–REP-1 complex. The amount of [^{35}S]GTPγS–rab5 was measured after immunoprecipitation as described previously (Ullrich *et al.*, 1994).

Assay for RabGDI activity

The assay was performed according to the method of Sasaki *et al.* (1990) with slight modifications. Briefly, [^{3}H]GDP- or [^{35}S]GTPγS-bound post-translationally modified or unmodified rab5 (20 000 c.p.m./pmol, 50 nM) was incubated with 0–4 μM RabGDI-1 or REP-1 and the protein-bound radioactivity was measured by the filtration method (Sasaki *et al.*, 1990). The extraction of membrane-bound rab proteins from permeabilized cells was performed as described by Ullrich *et al.* (1993).

Assay for RabGGTase activity

RabGGTase activity was measured by determining the amount of [^{3}H]geranylgeranyl transferred from [^{3}H]GGPP (3000 d.p.m./pmol) to rab5, as described in Seabra *et al.* (1993). The standard reaction mixture contained the following concentrations of components in a final volume of 50 μl: 50 mM HEPES–NaOH (pH 7.2), 5 mM $MgCl_2$, 1 mM Nonidet P-40, 1 mM dithiothreitol (DTT), 5.5 μM [^{3}H]GGPP (3000 d.p.m./pmol), and the indicated amounts of rab5, RabGGTase, REP-1 and RabGDI. After incubation for 10 min at 37°C, the amount of ethanol/HCl-precipitable radioactivity was measured by filtration on a glass fibre filter, as described previously (Seabra *et al.*, 1993).

Immunofluorescence analysis

A431 cells were grown on glass coverslips, permeabilized with SLO and treated with either rab5–REP-1 or rab7–REP-1 complex for 10 min, essentially as described for rab5–RabGDI complex previously (Ullrich *et al.*, 1994). Double immunofluorescence staining was carried out as described previously (Chavrier *et al.*, 1991). As primary antibodies we used affinity-purified rabbit anti-rab5 polyclonal antibodies (Stenmark *et al.*, 1994a,b), mouse anti-rab5 mAbs (Bucci *et al.*, 1994), rabbit anti-rab7 polyclonal antibodies (Chavrier *et al.*, 1991), mouse anti-human transferrin receptor mAbs (Boehringer) and rabbit anti-human lamp-1 polyclonal antibodies (Carlsson *et al.*, 1988). The secondary antibodies used were FITC-labelled donkey anti-rabbit antibodies and rhodamine-labelled donkey anti-mouse antibodies (Dianova). Coverslips were viewed with the confocal microscope developed at EMBL. The excitation wavelengths were 476 and 529 nm.

In vitro early endosome fusion assay

In vitro prenylated rab5–REP-1 and rab1a–REP-1 complexes were purified as described above. The buffer in all samples used here was changed to buffer B (12.5 mM HEPES pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$ and 1 mM DTT) using a Sephadex G-25 fast desalting column (Pharmacia) according to the instructions of the manufacturer. The eluate was then concentrated ~12 times with an Amicon microconcentrator to a final concentration of 10 μM. Aliquots were stored frozen at −80°C. An established cell-free assay (Gruenberg and Howell, 1988; Gorvel *et al.*, 1991; Steele-Mortimer *et al.*, 1994) was used to measure the fusion between early endosomes. Briefly, avidin and biotinylated horseradish peroxidase (bHRP) were internalized separately into two cell populations. The cells were then homogenized and early endosomal fractions were prepared using a flotation gradient (Gorvel *et al.*, 1991). In the assay, the avidin- and bHRP-labelled endosome fractions were combined at 4°C in the presence of rat liver cytosol (4 mg/ml final concentration) and ATP. Rab5–REP-1 complex and rab1a–REP-1 complex were added to the assay at a final concentration of 260 nM. Fusion was then allowed to proceed for 45 min at 37°C. At the end of the reaction, the avidin–bHRP complex formed upon fusion was

immunoprecipitated with an anti-avidin antibody in the presence of detergents. An excess of biotin was present during immunoprecipitation to quench any free avidin. The extent of fusion was quantified by measuring the enzymatic activity of the immunoprecipitated bHRP.

Estimation of the cytosolic concentration of RabGDI and REP-1

A431 cells were grown on 100 mm dishes to confluency. They were then scraped from the dish into PBS and homogenized by passing through a 22 gauge syringe needle. A post-nuclear supernatant was prepared by centrifugation (10 min, 800 *g*) and then ultracentrifuged (1 h, 100 000 *g*) to generate cytosol and membrane fractions. Cytosol fractions were resolved on SDS–polyacrylamide gel and analysed by quantitative immunoblot, using purified RabGDI and REP-1 as standards. Protein concentrations were determined by densitometric scanning of Coomassie blue-stained SDS–polyacrylamide gels or the Bradford method (Bio-Rad) using BSA as a standard. For detection of the protein we used anti-GDI polyclonal antiserum (Ullrich *et al.*, 1994) and 2F1 monoclonal anti-REP-1 antibodies (see below) with the enhanced chemiluminescence (ECL) detection system or [I^{125}]protein A conjugate (Amersham). Essentially the same results were obtained with both methods. The relative intensities of the visualized protein bands were estimated by densitometry and phosphorimaging for radiolabelled samples (Molecular Dynamics). mAb 2F1 was produced by immunizing mice with a recombinant fusion protein containing glutathione-S-transferase fused to the C-terminal 415 amino acids of human REP-1 produced as described previously (Andres *et al.*, 1993). Hybridoma selection and IgG purification were performed as described (Herz *et al.*, 1990).

Immunoprecipitation

NRK cells grown on plastic dishes were infected with T7 RNA polymerase recombinant vaccinia virus and transfected with pGEM-rab5 plasmid as described (Bucci *et al.*, 1992; Stenmark *et al.*, 1994b). After 2.5 h of infection, cycloheximide was added to culture medium to a final concentration of 10 μg/ml for 1.5 h. Cells were then washed three times with medium and incubated for an additional 1 h. Transfected and control cells were scraped into buffer C (50 mM HEPES–KOH pH 7.2, 10% glycerol, 2 mM $MgCl_2$, 100 mM NaCl, 2 mM 2-mercaptoethanol, 1 μM GDP, 1 mM phenylmethylsulfonyl fluoride) and cytosol was prepared as described (Gorvel *et al.*, 1991; Stenmark *et al.*, 1994b). Monoclonal anti-rab5 antibody 4F11, as well as a control antibody P10 (raised against the kinesin domain of a MAP-like protein from *Xenopus* eggs kindly provided by Dr Karsenti, EMBL), were cross-linked to protein A–Sepharose (Pharmacia) as described by Harlow and Lane (1988). Equal amounts (87 μg) of transfected or control cytosolic proteins were incubated with cross-linked antibody for 1.5 h at 4°C. In control samples, the peptide used for raising this antibody (Bucci *et al.*, 1994) was added at a final concentration of 100 μM. The beads were extensively washed with buffer B and bound proteins were eluted by the addition of Laemmmli's SDS buffer. Samples were resolved on a 12% SDS–polyacrylamide gel. Samples were analysed by immunoblot using anti-rab5 and anti-REP-1 mAbs. Bound antibodies were visualized by the ECL method (Amersham).

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