Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis

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Small GTPases of the rab family control distinct steps of intracellular transport. The function of their GTPase activity is not completely understood. To investigate the role of the nucleotide state of rab5 in the early endocytic pathway, the effects of two mutants with opposing biochemical properties were tested. The Q79L mutant of rab5, analogous with the activating Q61L mutant of p21-ras, was found to have a strongly decreased intrinsic GTPase activity and was, unlike wild-type rab5, found mainly in the GTP-bound form in vivo. Expression of this protein in BHK and HeLa cells led to a dramatic change in cell morphology, with the appearance of unusually large early endocytic structures, considerably larger than those formed upon overexpression of wild-type rab5. An increased rate of transferrin internalization was observed in these cells, whereas recycling was inhibited. Cytosol containing rab5 Q79L stimulated homotypic early endosome fusion in vitro, even though it contained only a small amount of the isoprenylated protein. A different mutant, rab5 S34N, was found, like the inhibitory p21-ras S17N mutant, to have a preferential affinity for GDP. Overexpression of rab5 S34N induced the accumulation of very small endocytic profiles and inhibited transferrin endocytosis. This protein inhibited fusion between early endosomes in vitro. The opposite effects of the rab5 Q79L and S34N mutants suggest that rab5:GTP is required prior to membrane fusion, whereas GTP hydrolysis by rab5 occurs after membrane fusion and functions to inactivate the protein.
Key words: endosome/GTPγS/membrane traffic/rab/ras

Introduction

Several lines of evidence have implicated small GTPases of the rab/ypt1/Sec4 family (in this paper referred to as rab proteins) as key regulators of intracellular traffic (Pfeffer, 1992; Novick and Brennwald, 1993; Zerial and Stenmark, 1993). Their biological importance was first illustrated by studies on the conditional lethal mutants *ypt1* (Segev *et al.*, 1988) and *sec4* (Salminen and Novick, 1987; Goud *et al.*, 1988) in the biosynthetic pathway of yeast. In both cases, accumulation of transport vesicles was observed at the non-permissive temperature, suggesting that Ypt1p and Sec4p function in vesicle docking or fusion, but not in budding.

Later studies have identified a number of different rab proteins in various organisms, including mammals. Their localization to distinct subcellular compartments, as well as their effects on transport in several *in vitro* and *in vivo* systems, have emphasized their role in maintaining specificity of vesicular transport (Pfeffer, 1992; Zerial and Stenmark, 1993).

Like other small GTPases, rab proteins are thought to act as regulatory molecules that recognize target proteins through a nucleotide-dependent conformational change (Bourne *et al.*, 1991). One candidate for a downstream target is rabphilin-3A, a synaptotagmin-like molecule that binds specifically to the GTP-bound form of rab3a (Shirataki *et al.*, 1993). Association of rab proteins with membranes depends on a C-terminal isoprenylation and is essential for their function (Magee and Newman, 1992). The membrane association is regulated by rab GDP dissociation inhibitor (rab GDI) (Araki *et al.*, 1990; Regazzi *et al.*, 1992; Soldati *et al.*, 1993; Steele-Mortimer *et al.*, 1993; Ullrich *et al.*, 1993) and is accompanied by exchange of bound GDP with GTP (Ullrich *et al.*, 1994). Nucleotide exchange is probably catalyzed by GDP dissociation stimulators (GDSs) *in vivo* (Burstein and Macara, 1992; Burton *et al.*, 1993; Moya *et al.*, 1993). Conversely, the switch from GTP- to GDP-bound state occurs through hydrolysis of GTP, a process catalysed by GTPase activating proteins (GAPs) (Becker *et al.*, 1991; Burstein and Macara, 1992; Walworth *et al.*, 1992; Strom *et al.*, 1993).

While mutational studies have indicated that cycling between the GTP- and GDP-bound forms is critical for the function of rab proteins (Becker *et al.*, 1991; Tisdale *et al.*, 1992; Walworth *et al.*, 1989, 1992), the exact role of the GTP hydrolysis step is not clear (Goud and McCaffrey, 1991; Simons and Zerial, 1993). GTP hydrolysis might be required as a kinetic proofreading device prior to membrane fusion (Bourne, 1988), as supported by the inhibitory effect of the non-hydrolysable GTP analogue, GTPγS, in various cell-free transport assays. Thus, homotypic fusion between early endosomes, which requires rab5 (Gorvel *et al.*, 1991), is inhibited by GTPγS (Mayorga *et al.*, 1989; Bomsel *et al.*, 1990). However, intracellular membranes contain a number of different GTPases that regulate vesicular transport (Pfeffer, 1992) and, so far, it has not been possible to specifically affect rab protein function with GTPγS. Therefore, the alternative possibility still exists that rab proteins might use GTP hydrolysis as a means of switching from an 'active' to an 'inactive' conformation (Takai *et al.*, 1992; Novick and Brennwald, 1993).

To distinguish between these possibilities, we have chosen to study the role of the GTPase activity of rab5, a rate-limiting factor in the endocytic pathway. An increased level of rab5 increases the rate of endocytosis in intact cells and stimulates early endosome fusion in a cell-free assay, whereas interference with rab5 function leads to the opposite effects (Gorvel *et al.*, 1991; Bucci *et al.*, 1992). The
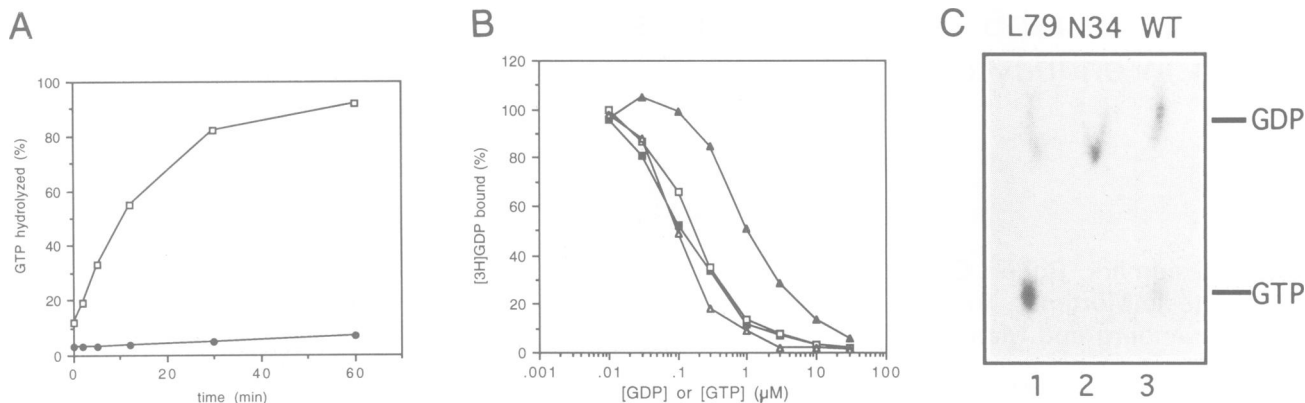
Fig. 1. Two rab5 mutants with different changes in the GTP/GDP cycle. **(A)** Measurement of the intrinsic GTPase activity of wild-type rab5 (□) and rab5 Q79L (●). **(B)** Measurement of [³H]GDP binding to wild-type rab5 (squares) and to rab5 S34N (triangles) in the presence of unlabelled GTP (filled symbols) or GDP (open symbols). **(C)** GTP:GDP ratio of rab5 and rab5 mutants in transfected cells. Cells were transfected with rab5 Q79L (lane 1), rab5 S34N (lane 2) or wild-type rab5 (lane 3), and the bound nucleotides were determined by thin-layer chromatography, as described in Materials and methods. The positions of GTP and GDP are indicated.

functional properties of rab5 and the early endocytic compartments are thus suitable to dissect the rab GTP/GDP cycle in membrane fusion. Since the GDP-bound form of rab5 is preferentially isoprenylated (Sanford *et al.*, 1993) and since rab5 appears to be correctly presented to membranes only in its GDP-bound form (Ullrich *et al.*, 1994), this problem could best be solved by studying the function of rab5 when its GTPase activity is reduced, but not completely inhibited. Using two rab5 mutants with different bound nucleotides, we here present evidence that GTP hydrolysis by rab5 occurs at a step subsequent to membrane fusion. We propose that rab5:GTP enhances the association between compatible membranes, thereby promoting their fusion, whereas rab5 GTPase activity is required to enable post-fusion inactivation and translocation of the protein to the cytosol.

Results

Biochemical characterization of two rab5 mutants with different properties

Several studies have shown that mutations equivalent to the activating Q61L mutation in p21-ras inhibit both the intrinsic and the GAP-stimulated GTPase activity of small GTPases, but not their ability to bind nucleotides (Der *et al.*, 1986; Adari *et al.*, 1988; Tisdale *et al.*, 1992; Walworth *et al.*, 1992). In contrast, mutants corresponding to the inhibitory S17N mutant of p21-ras have a lower affinity for GTP than for GDP (Feig and Cooper, 1988; Ridley *et al.*, 1992) and appear to inhibit GDSs (Burstein *et al.*, 1992; Medema *et al.*, 1993).

We therefore introduced the corresponding mutations in rab5 (Q79L and S34N, respectively). The mutant proteins, as well as wild-type rab5, were tagged N-terminally with six histidine residues and expressed in *Escherichia coli*. We first demonstrated that His-rab5 has a similar GTPase activity as wild-type rab5 (data not shown). When purified His-tagged rab5 was immobilized on Ni^{2+} beads (Hochuli *et al.*, 1987) and then loaded with [α-³²P]GTP, it rapidly converted the nucleotide into [α-³²P]GDP (Figure 1A). We estimated the hydrolysis rate constant at 37°C to be 0.05

min^{-1}, in agreement with earlier findings (Touchot *et al.*, 1987). In contrast, the Q79L mutant hydrolysed GTP about 100-fold slower than wild-type rab5 at 37°C (Figure 1A).

The results in Figure 1B show that equal concentrations of unlabelled GDP and GTP were required to compete binding of [³H]GDP to His-tagged rab5, confirming that rab5 binds the two nucleotides with the same affinity (Touchot *et al.*, 1987). However, almost 10-fold higher concentrations of GTP than GDP were required to compete binding of [³H]GDP to rab5 S34N. This indicates that the mutant protein, like the corresponding inhibitory ras and rac mutants, has lower affinity for GTP than for GDP.

Nucleotide exchange and GTPase activity of rab proteins are modified by cellular factors (Burstein and Macara, 1992; Walworth *et al.*, 1992; Burton *et al.*, 1993; Moya *et al.*, 1993; Strom *et al.*, 1993). We therefore determined the GDP:GTP ratios of rab5 Q79L and rab5 S34N when expressed in BHK cells. For this purpose, we transiently expressed wild-type or mutant rab5 using the T7 RNA polymerase recombinant vaccinia virus (vT7) system (Fuerst *et al.*, 1986). During the transfection period the cells were incubated with ³²PO₄³⁻ to label the endogenous pools of GTP and GDP. After extraction with detergent, the expressed proteins were immunoprecipitated with the anti-rab5 mAb 4F11H10A20 (Bucci *et al.*, 1994). Under the conditions used, rab5 is overexpressed >10-fold over the endogenous level (Bucci *et al.*, 1992), and the contribution from endogenous rab5 is therefore low. Since low but significant hydrolysis of GTP by rab5 occurs even at 4°C ($t_{1/2}$ ~60 min, data not shown), the manipulations were carried out during a time period of only 20 min. ³²P-labelled nucleotides bound to the immunoprecipitated proteins were analysed by thin-layer chromatography (Downward *et al.*, 1990; Burgering *et al.*, 1991). Immunoprecipitated wild-type rab5 contained 21% bound GTP (Figure 1C), rab5 N34S only 5%, whereas rab5 Q79L contained as much as 63% GTP. The accumulation of the GTP-bound form of rab5 Q79L is consistent with its low GTP hydrolysis rate. The mutant proteins thus appear to have similar biochemical properties in intact cells as they have *in vitro*, although a cellular GAP may partially stimulate the GTPase activity of rab5 Q79L, as shown previously with the analogous Sec4 Q79L mutant (Walworth *et al.*, 1992).
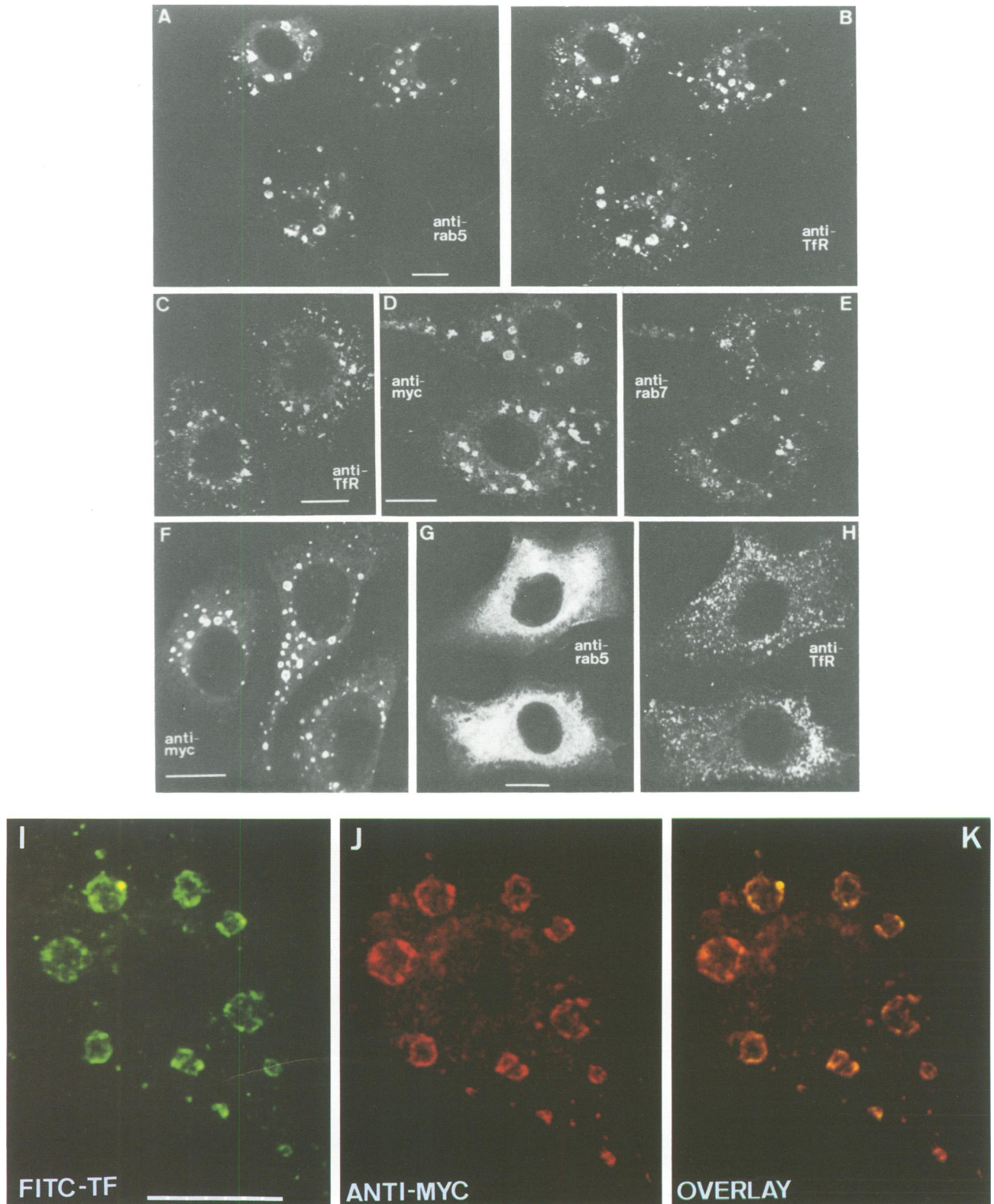
Fig. 2. Confocal immunofluorescence analysis of cells expressing wild-type rab5 and rab5 Q79L. (A−E) and (G−K), BHK cells transfected with plasmids encoding the following proteins: rab5 Q79L and the human transferrin receptor (A, B), rab5 and the human transferrin receptor (C), *myc*-rab5 Q79L (D, E), rab5 Q79L ΔCCSN and the human transferrin receptor (G, H) and *myc*-rab5 Q79L and the human transferrin receptor (I, J, K). (F) The HeLa-derived line 444, expressing *myc*-tagged rab5 Q79L at a 1:1 ratio with endogenous rab5. The cells were either permeabilized with 0.05% saponin prior to fixation (A−E), or fixed directly in 3% paraformaldehyde (Zerial *et al.*, 1992) (F−K). In the latter cases, the cells were permeabilized with 0.1% Triton X-100 prior to incubation with antibodies. The primary antibodies used are indicated in the figure. The secondary antibodies used were FITC-labelled donkey anti-rabbit IgG antibodies and rhodamine-labelled donkey anti-mouse IgG antibodies, both from DiaNova. (I−K) 20 µg/ml FITC-transferrin was bound to the cells at 4°C for 30 min. The cells were then washed three times with ice-cold PBS and then incubated at 37°C for 5 min in GMEM. Subsequently, the cells were washed three times with ice-cold PBS and fixed with 3% paraformaldehyde. (K) A superimposition of the images in (I) and (J); the yellow colour indicates areas of colocalization between the internalized FITC-transferrin and the overexpressed *myc*-rab5 Q79L. The optical sections viewed are 0.4 µm. Size bars, 10 µm.
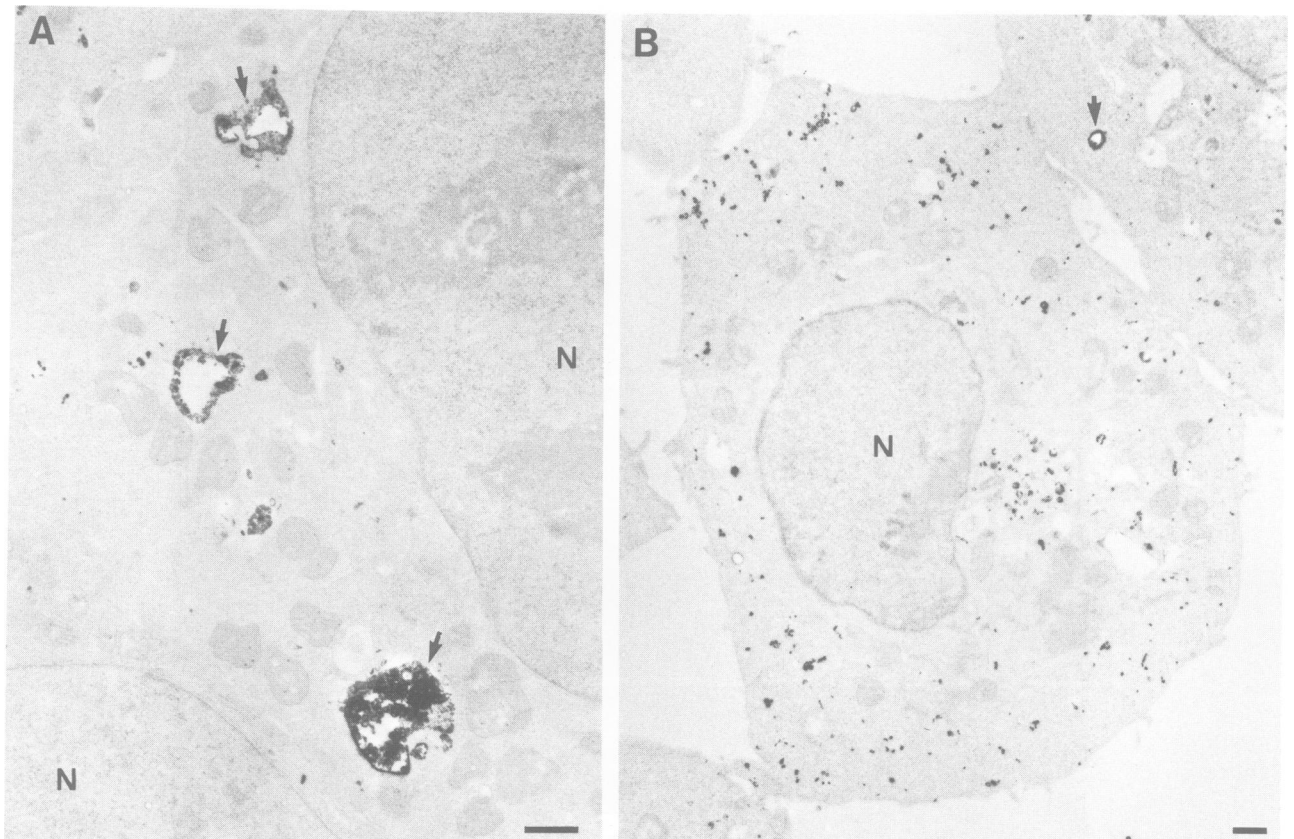
Fig. 3. Electron microscopy of cells expressing rab5 mutants. Cells overexpressing rab5 Q79L (**A**) or rab5 S34N (**B**) were incubated in the presence of 10 mg/ml HRP for 10 min, then fixed and processed for epon embedding. Semi-thick (200 nm) sections parallel to the substratum were prepared. In (A), the label is found in large, membrane-bound structures (arrows). In (B), the HRP reaction product is found in small structures, most abundant at the periphery of the cells. A few normal early endosomes (arrows) were also labelled. Bars, 1 μm.

Confocal immunofluorescence microscopy shows that rab5 Q79L drastically alters the morphology of early endosomes

To study the effects of the two rab5 mutants on the endocytic pathway, we first overexpressed rab5 Q79L in BHK cells using the vT7 system and studied its localization by confocal immunofluorescence microscopy. If GTP hydrolysis were needed for membrane fusion, then we would expect to observe a decreased size for early endosomes in the presence of this mutant. Remarkably, however, antibodies against the overexpressed protein (Figure 2A) stained large vesicular structures with a typical size of 2−5 μm, usually with a perinuclear localization. The structures stained were in general much larger than the expanded early endosomes observed upon overexpression of wild-type rab5 (Figure 2C) (Bucci *et al.*, 1992). Antibodies against the coexpressed human transferrin receptor stained the same structures strongly (Figure 2B). On the other hand, when a *myc* epitope-tagged rab5 Q79L was expressed, only a small subset of the anti-*myc* positive structures (Figure 2D) were stained by an antibody against rab7 (Figure 2E). These results indicate that most of the large vesicular structures are early endosomes and do not represent late endosomes.

To see whether the enlarged structures observed upon overexpression of the Q79L mutant are accessible to endocytic markers, we prebound FITC-conjugated transferrin to the cells at 0°C and incubated them at 37°C for 5 min to induce internalization. In control cells, the internalized FITC-transferrin was found in small vesicular

compartments, having the size of normal early endosomes (not shown). However, in cells overexpressing the Q79L mutant, the FITC-transferrin was found in the same vacuolar-like structures (Figure 2I) as those labelled with antibodies against the mutant protein (Figure 2J and K). This indicates that the structures formed upon overexpression of the Q79L mutant are accessible to an early endocytic tracer, suggesting that they represent early endosomes that are morphologically altered but functionally active.

To rule out the possibility that the morphological changes caused by rab5 Q79L are due to the titration of a common cytosolic factor (e.g. a GAP), we overexpressed a rab5 Q79L variant that lacks the C-terminal cysteine motif to prevent isoprenylation and membrane association (Magee and Newman, 1992). Although the protein was expressed at high level (Figure 2G), antibodies against the coexpressed transferrin receptor in the same cells revealed no formation of altered endosomes (Figure 2H). This indicates that post-translational modification and presumably membrane attachment are required for the morphological effects caused by the Q79L mutant. The finding that rab5 Q79L displayed an increased GTP:GDP ratio when immunoprecipitated from intact cells (Figure 1C) strongly suggests that the effect of this mutant is correlated with its low GTPase activity. This is further supported by the finding that another mutant, rab5 I53M, which is analogous to a Ypt1 mutant that is insensitive to GAP stimulation (Becker *et al.*, 1991), gives a similar phenotype as rab5 Q79L (H.Stenmark and M.Zerial, unpublished data).

We also selected stable HeLa cell lines expressing *myc*-tagged rab5 Q79L. Several independent lines were selected, each expressing the Q79L mutant at approximately the same level as wild-type rab5. Anti-rab5 and anti-*myc* antibodies stained large vacuolar compartments in the cells expressing the mutant protein (Figure 2F), but not in control HeLa cells (not shown). Although these structures were smaller than those observed in BHK and HeLa cells transfected using the vT7 system, it appears that even at a 1:1 ratio with wild-type rab5 the Q79L mutant changes the morphology of early endosomes. Cell lines expressing a 2-fold level of wild-type rab5 do not have the same effect on endosome morphology (data not shown). Therefore, in this respect the Q79L mutation causes a dominant phenotype.

Electron microscopy reveals that rab5 Q79L and rab5 S34N have opposite effects on early endosome morphology

To rule out the possibility that the large endocytic structures observed in the presence of rab5 Q79L were actually clusters of small vesicles, we analysed the cells by electron microscopy. Semi-thin epon sections of transfected cells that had internalized the fluid phase endocytic marker horseradish peroxidase (HRP) for 10 min were examined. In cells that had only been infected by vT7, the HRP was found in ring-shaped profiles and peripheral tubules, as described previously (Griffiths *et al.*, 1989; Bucci *et al.*, 1992) (data not shown). In contrast to the control cells, cells transfected with rab5 Q79L contained large HRP-positive structures (Figure 3A), several-fold larger than normal early endosomes. The large structures formed upon overexpression of rab5 Q79L were also labelled with HRP-conjugated transferrin (data not shown), in agreement with the confocal microscopy analysis. The structures appeared to contain continuous membranes and were therefore classified as being large early endosomes rather than aggregates of smaller vesicles.

In contrast, confocal microscopy with antibodies against the S34N mutant, as well as antibodies against the transferrin receptor expressed in the same cells, showed a diffuse punctate staining pattern (data not shown). Electron microscopy of cells transfected with rab5 S34N showed that HRP appeared in peripheral tubules and in small 100 nm vesicles (Figure 3B). In addition, a few characteristic early endosomes could be observed (arrow). This phenotype is reminiscent of that observed upon overexpression of rab5 N133I, a dominant negative mutant with low affinity for GTP and GDP which was proposed to delay the fusion of clathrin-coated vesicle-derived structures with early endosomes (Bucci *et al.*, 1992).

Altogether, these results indicate that overexpression of two rab5 mutants with different biochemical properties has opposite effects on the morphology of early endosomes. A mutant with decreased GTPase activity forms unusually large early endosomes, whereas a mutant with preferential affinity for GDP causes the accumulation of transport intermediates or fragmented early endosomes.

The morphological effects caused by overexpression of rab5 Q79L and rab5 S34N correlate with changes in the transferrin cycle

Having established that the two rab5 mutants change endosome morphology oppositely, we then measured their effects on the transferrin cycle (Ciechanover *et al.*, 1983b). When cells with prebound [^{125}I]transferrin were warmed to 37°C, transferrin was rapidly internalized (Figure 4A). Cells coexpressing rab5 together with the human transferrin receptor endocytosed transferrin >2-fold faster than control cells transfected with the transferrin receptor alone, as shown previously (Bucci *et al.*, 1992). Cells coexpressing rab5 S34N showed reduced uptake of transferrin. Conversely, the rate of internalization was strongly increased in cells coexpressing rab5 Q79L. These cells internalized transferrin at approximately the same rate as those transfected with wild-type rab5. Kinetic studies of four different HeLa lines that express low levels of *myc*-rab5 Q79L gave similar results, although the stimulation of endocytosis observed was less pronounced (data not shown). The biochemical measurements thus correlate well with the morphological observations. A mutant that leads to fragmentation of early endosomes causes the inhibition of endocytosis, whereas a mutant that causes the formation of large early endosome-like structures markedly increases the rate of transferrin internalization.

About half of the transferrin internalized in control cells was recycled into the medium after a 30 min incubation (Figure 4B). Cells transfected with wild-type rab5 recycled transferrin somewhat faster, as shown before, whereas the S34N mutant caused a delay in recycling that could be correlated with its inhibitory effect on endocytosis. In contrast to wild-type rab5, the Q79L mutant caused a significant inhibition of transferrin recycling. Because the rate of recycling in these cells did not balance the increased rate of uptake, we would expect the transfected cells to contain a smaller fraction of their transferrin receptors on their surface. We therefore estimated the fraction of surface receptors (van der Sluijs *et al.*, 1992). In all cases, the total number of receptors was the same (data not shown). Control cells (transfected with transferrin receptor alone) contained ~27% of their transferrin receptors on their surface (Figure 4C). This fraction was essentially the same in cells transfected with wild-type rab5 or with rab5 S34N. In contrast, cells expressing rab5 Q79L displayed only 12% of their transferrin receptors on their surface. Altogether, these results indicate that the Q79L mutant causes the accumulation of transferrin in enlarged endosomal structures. The inhibition of recycling was not detected under conditions of lower expression at which increased rate of internalization could still be observed (data not shown), suggesting that the inhibition of recycling is secondary to the increase in endocytosis rate. Under the conditions used, cells transfected with transferrin receptor plasmid contained maximally ~10^6 receptors on their surface (not shown), which is considerably less than the number shown previously to cause saturation of coated pits (3 × 10^6 cell^{-1}) (Iacopetta *et al.*, 1988; Miller *et al.*, 1991). This argues that the increased endocytosis rate in cells transfected with rabQ79L is not simply a result of their reduced number of surface-exposed transferrin receptors.

Cytosol from rab5 Q79L-overexpressing cells stimulates homotypic early endosome fusion in vitro, in spite of its low content of isoprenylated protein

Both kinetic and morphological data suggest that rab5 Q79L may cause an increased rate of fusion events in the endocytic pathway. To study the effects of the mutant protein on
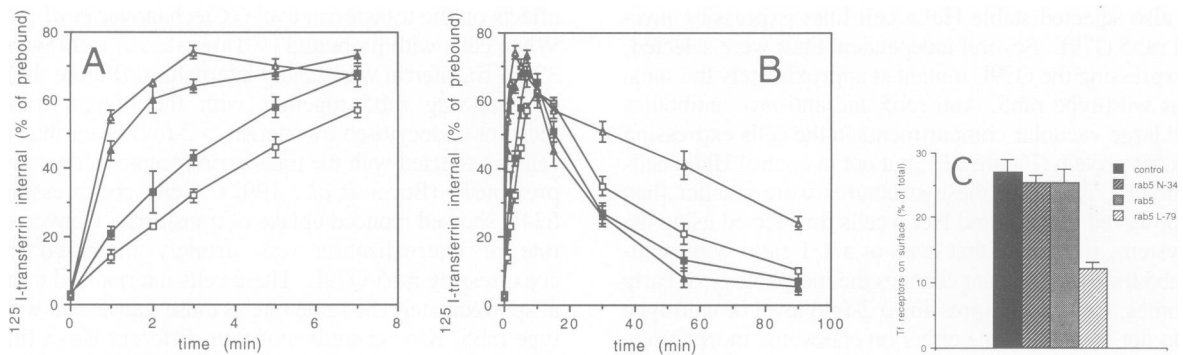
Fig. 4. Effect of rab5 mutants on the transferrin cycle. (**A**) Internalization of prebound transferrin at early time points in cells transfected with the human transferrin receptor alone (■) or together with rab5 (▲), rab5 S34N (□) or rab5 Q79L (△). (**B**) Internalization and recycling of transferrin in transfected cells. Same experiment as in (A), but the internal [^{125}I]transferrin was determined after longer periods of time. (**C**) Effect of rab5 and rab5 mutants on the cellular distribution of the transferrin receptor. BHK cells were transfected with transferrin receptor alone, or together with rab5, rab5 S34N or rab5 Q79L, as indicated. [^{125}I]transferrin (300 nM) was then bound at 0 or 37°C for 2 h, the cells were washed three times with cold PBS and the cell-associated radioactivity was measured. The proportion of transferrin receptors on the surface was calculated as the ratio between the [^{125}I]transferrin bound at 0 versus 37°C. Error bars represent the range in duplicate (A, B) or triplicate (C) experiments.

membrane fusion more directly, we tested its activity in an established cell-free assay (Davey *et al.*, 1985; Gruenberg and Howell, 1986; Gorvel *et al.*, 1991). Earlier studies have shown that fusion between two populations of early endosomes *in vitro* can be stimulated by cytosol from rab5-overexpressing cells and inhibited by cytosol containing the dominant negative N133I mutant and by anti-rab5 antibodies (Gorvel *et al.*, 1991).

We therefore prepared cytosols from vT7 infected and transfected cells and tested their effect on the fusion between early endosomes. One population of early endosomes was isolated from BHK cells that had internalized avidin for 5 min, whereas the other endosome fraction was isolated from cells that had internalized biotinylated HRP for 5 min. The fusion assay was carried out in the presence of cytosol from transfected cells, supplemented with rat liver cytosol to obtain highly reproducible experimental conditions (Steele-Mortimer *et al.*, 1994). The extent of vesicle fusion in the presence of cytosol from transfected cells is reflected by the number of complexes formed between the avidin molecules in the first endosome population and the HRP biotin in the second. These complexes were extracted with detergent containing biotinylated insulin to quench unreacted avidin, and then immunoprecipitated with anti-avidin antibodies. In this way, fusion activity could be detected by measuring HRP activity in the immunoprecipitates (Gorvel *et al.*, 1991).

Cytosol from cells overexpressing wild-type rab5 stimulated fusion strongly compared with control cytosol from vT7 infected cells (Figure 5). As an inhibitory control we used cytosol containing overexpressed rab5 N133I. This cytosol inhibited the fusion, as shown earlier (Gorvel *et al.*, 1991). The S34N mutant also inhibited fusion consistent with its effect on cell morphology, similar to that of rab5 N133I. In contrast, the Q79L mutant stimulated fusion strongly, supporting the view that the large early endocytic profiles observed with this mutant reflect an increased level of fusion. However, the extent of stimulation was lower than with wild-type rab5. An explanation for this came by inspecting the level of post-translationally modified rab5 Q79L in the cytosol.

The cytosolic fraction of rab proteins is found in complex with rab GDI (Araki *et al.*, 1990; Regazzi *et al.*, 1992; Soldati *et al.*, 1993; Steele-Mortimer *et al.*, 1993; Ullrich *et al.*, 1993). Since rab GDI interacts specifically with the
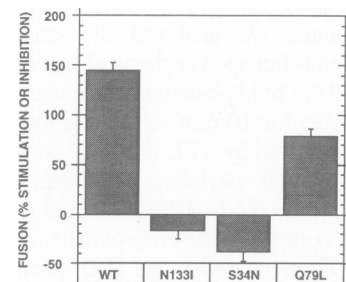

Fig. 5. Effects of rab5 mutants on the fusion between early endosomes *in vitro*. The ability of cytosol from cells overexpressing the proteins indicated to stimulate homotypic fusion between early endosomes is indicated as stimulation in per cent of that obtained with control cytosol (cytosol from cells infected with vT7, and then mock-transfected). Error bars represent the range between triplicate determinations.
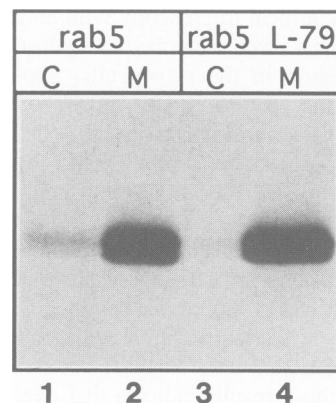

Fig. 6. Intracellular distribution of isoprenylated rab5 and rab5 Q79L. Cells were infected with vT7 and transfected with rab5 (lanes 1 and 2) or rab5 Q79L (lanes 3 and 4) in the presence of [^{14}C]mevalonic acid, as described in Materials and methods. Postnuclear supernatants from the cells were separated into cytosol (lanes 1 and 3) and membrane (lanes 2 and 4) fractions. The fractions were analysed by SDS–PAGE followed by fluorography.

GDP-bound and isoprenylated form of rab proteins (Sasaki *et al.*, 1990), we would expect it to interact inefficiently with rab5 Q79L:GTP, resulting in a decreased amount of isoprenylated rab5 Q79L in the cytosol. We therefore investigated whether isoprenylated rab5 Q79L had a different

distribution between membranes and cytosol than wild-type rab5. For this purpose, transfected cells were incubated in the presence of [^{14}C]mevalonate, and the postnuclear supernatants were separated into membrane and cytosol fractions. Both isoprenylated rab5 and rab5 Q79L were found mainly in the membrane fraction and in similar amounts (Figure 6, lanes 2 and 4). In contrast, significantly more isoprenylated wild-type rab5 than rab5 Q79L was found in the cytosol fractions (compare lanes 1 and 3). Collectively, these results indicate that wild-type rab5 and rab5 Q79L are isoprenylated to the same extent *in vivo*, but less of the isoprenylated Q79L mutant is found in the cytosol.

The lower cytosolic concentration of isoprenylated rab5 Q79L might explain why cytosol from rab5 Q79L-transfected cells stimulates fusion somewhat less than rab5 cytosol (Figure 5). Correcting for the lower amount of post-translationally modified mutant protein in the cytosol used, we find that rab5 Q79L is at least as efficient as wild-type rab5 in stimulating early endosome fusion *in vitro*.

Discussion

Here, we have addressed a crucial question concerning the function of rab5: what is the role of the protein's GTPase activity? According to one view, rab GTPase activity could be required prior to membrane fusion. By analogy with elongation factor-Tu in protein synthesis, it might serve as a proofreading mechanism ensuring that fusion takes place only if membrane—membrane interactions are stable enough to outlast GTP hydrolysis (Bourne, 1988). Alternatively, GTP hydrolysis may be required merely to convert the 'active' rab:GTP into the 'inactive' rab:GDP (Takai *et al.*, 1992; Novick and Brennwald, 1993). To distinguish between these two possibilities, we have studied the effects of two rab5 mutants with different perturbations of their GTP/GDP cycle. Our experimental systems measure membrane fusion events in the early endocytic pathway that are modulated by rab5, and that can be followed biochemically and morphologically. The fragmentation of endosomes, inhibition of endocytosis and inhibition of endosome fusion caused by rab5 S34N, which has preferential affinity for GDP and is thought to inhibit a GDS, suggest that rab5:GDP is unable to promote membrane fusion. Conversely, the ability of rab5 Q79L, which has reduced GTPase activity, to induce the formation of highly enlarged early endocytic structures and to stimulate endocytosis *in vivo* and endosome fusion *in vitro*, suggests that rab5:GTP stimulates membrane fusion. We therefore conclude that rab5:GTP, rather than rab5 GTPase activity by itself, is required for fusion.

Rab5 has been found on clathrin-coated vesicles (Bucci *et al.*, 1992), and our data are consistent with the possibility that rab5:GTP is involved in docking between endocytic vesicles and early endosomes. The accumulation of putative transport intermediates observed with various inhibitory rab mutants suggests that these proteins interfere with vesicle docking or fusion, though the exact step affected has not been defined. In the cases of rab5 N133I (Bucci *et al.*, 1992) and rab5 S34N (this study), inhibition of endocytosis and the accumulation of small vesicles containing an endocytic marker are observed. There is evidence that the S17N mutation in p21-ras inhibits a GDS (Medema *et al.*, 1993), and it is conceivable that rab5 S34N acts in a similar way, thereby leading to a decreased level of wild-type rab5:GTP

in the membrane. The inhibitory effect of rab5 S34N thus argues that the GTP-bound form of rab5 is required prior to fusion.

Rab3a Q81L was found to contain only slightly more GTP bound than wild-type rab3a *in vivo* (Brondyk *et al.*, 1993). The finding that rab5 Q79L stimulated HRP uptake to the same extent as wild-type rab5 was thus thought to reflect a similar GTP:GDP ratio *in vivo* (Li and Stahl, 1993). However, here we demonstrate that rab5 Q79L has as much as 3-fold more GTP bound than wild-type rab5 *in vivo*. Furthermore, we show that the mutant (i) expands the size of early endosomes more strongly than the wild-type protein, (ii) is a potent stimulator of homotypic fusion between early endosomes and (iii) has an altered distribution between membranes and cytosol. All results are consistent with the view that rab5 GTPase activity is not required for membrane fusion. The fact that the GTPase activity of rab5 Q79L is not completely inhibited *in vivo* probably explains why the protein is efficiently isoprenylated and is targeted to the correct membranes. After GTP hydrolysis, rab5:GDP would be removed from the early endosome membrane by rab GDI (Ullrich *et al.*, 1993), which could recycle rab5 back to the plasma membrane via the cytosol. Rab5:GTP that remains on the early endosome membrane might promote association, and thereby fusion, with neighbouring early endosomes. This would account for the increased size of early endosomes upon expression of a mutant with reduced GTPase activity. Such a protein would reside longer in the early endosome membrane in the GTP-bound form and be less efficiently removed by rab GDI, in agreement with the finding that only a low concentration of isoprenylated rab5 Q79L is found in the cytosol (Figure 6).

When endocytic vesicles fuse with early endosomes, soluble components may be released that are rate limiting for the formation of new coated vesicles. This might explain why overexpression of rab5, which stimulates fusion (Gorvel *et al.*, 1991), also increases the initial rate of internalization (Bucci *et al.*, 1992). The ability of rab5 Q79L, like rab5, to stimulate endocytosis thus agrees with the idea that it is able to promote membrane fusion. Assuming that rab5 functions in fusion events in the endocytic pathway (Bucci *et al.*, 1992), how would we explain the inhibitory effect of rab5 Q79L on recycling of transferrin? We favour the possibility that inhibition of rab5 GTPase activity may prevent disassembly of the vesicle docking machinery and thereby block assembly of the machinery required for budding. Another explanation could be that an increased homotypic fusion between early endosomes interferes with the spatial organization of a compartment that regulates recycling (Yamashiro *et al.*, 1984). A less likely possibility is that vesicles destined for recycling might fuse back with the early endosome in the presence of the Q79L mutant. In contrast to the observed changes in endosome morphology, the inhibitory effect on recycling was dependent on a high expression of the mutant protein. This suggests that the increased size of early endosomes induced by this mutant is primarily due to stimulation of membrane fusion rather than to inhibition of budding of recycling vesicles.

A yeast mutant containing Sec4 Q79L as its only copy of Sec4 accumulated small vesicles reminiscent of transport intermediates and displayed slowed TGN to plasma membrane transport (Walworth *et al.*, 1992). The apparent discrepancy between the effects obtained with Sec4 Q79L and rab5 Q79L could reflect a principal difference in how

Sec4 and rab5 regulate membrane traffic. Alternatively, it could result from differences in the experimental strategies employed (gene disruption versus overexpression), although the latter possibility is unlikely in view of the dominant effect of rab5 Q79L. However, we favour an explanation that would be consistent with a role for rab:GTP in vesicle docking: with rab5 Q79L, an accumulation of the protein on the acceptor (early endosome) membrane would lead to an increased fusion between early endosomes. In contrast, for Sec4 Q79L, an accumulation of the protein on the acceptor (plasma) membrane would not give rise to enlarged vesicles. Rather, it would result in a partial depletion of Sec4 Q79L from TGN-derived vesicles. This would lead to incomplete docking between the vesicles and the plasma membrane. Alternatively, the mutant might occupy a limited number of vesicle docking sites. Similar scenarios may explain why Ypt1 D44N, which is activated poorly by Ypt1-GAP, accumulates ER and ER-derived vesicles (Becker *et al.*, 1991), and why rab2 Q65L inhibits ER−Golgi transport (Tisdale *et al.*, 1992). Our results agree with these findings on the importance of an intact GTP/GDP cycle for rab function.

The ability of a GTPase-deficient rab5 mutant to support membrane fusion, together with the fact that arf proteins are the target of GTPγS inhibition in an intra-Golgi transport assay (Taylor *et al.*, 1992), indicates that the inhibitory activity of GTPγS in early endosome fusion assays (Mayorga *et al.*, 1989; Bomsel *et al.*, 1990) is mediated by GTPases distinct from rab5 (Pfeffer, 1992). The proposed effect of rab5:GTP on fusion between early endosomes is consistent with the view of the early endosome as a highly dynamic compartment (Hopkins *et al.*, 1990). While we here have studied mutants of rab5, the picture is complicated by the fact that other early endosome-associated rab proteins exist (Simons and Zerial, 1993) which could be involved in defining a subcompartmentalization of the early endosome through regulation of specific association/fusion events. The use of rab5 Q79L and similar rab mutants may give us further insights into this issue in the future.

Materials and methods

Plasmids and transfection

DNAs encoding rab5 (Chavrier *et al.*, 1990), rab5 mutants and the human transferrin receptor (Zerial *et al.*, 1986) were positioned behind the T7 promoter in pGEM-1. Rab5 Q79L and rab5 S34N were made by directed mutagenesis of rab5 DNA, using a PCR-based protocol (Landt *et al.*, 1990) and the mutant primers 5'-GCTATGGTATCGTTCTAGACCAGCTGT-ATCCC-3' and 5'-GCTGTTGGCAAAA*A*TAGCCTAGTGCTTCG-3', respectively. SP6 and T7 promoter primers were used as the outer primers in the amplifications. Rab5 was tagged with a c-myc epitope (Evan *et al.*, 1985) using PCR with the mutant primer 5'-CAGTGAATTCGCCATG*G*-*AACAAAAACTCATCTCAGAAGAGGA*TCT*GC*AGGCTAATCGAGGA-GCAACAAG-3' and an SP6 promoter primer (Stenmark *et al.*, 1994). Rab5 Q79L ΔCCSN was prepared by PCR amplifying pGEM-rab5 using the primer 5'-CTGAGGATCCTTACTGACTCCTGGTTGGCTGCG-3' and a T7 promoter primer.

In all cases, suitable restriction fragments from the PCR-amplified DNAs were cloned into pGEM-rab5 or pGEM-rab5 Q79L, and the PCR-amplified regions were sequenced to verify the mutations and to exclude the presence of PCR errors.

For expression using the vT7 system, BHK-21 and HeLa cells were infected with vT7 for 30 min at 25°C, and then transfected with plasmid DNA containing the gene of interest behind a T7 promoter using DOTAP (Boehringer Mannheim), as described previously (Bucci *et al.*, 1992).

Preparation of cell lines expressing myc-tagged rab5 Q79L

A 2 kb EcoRI fragment was cloned from pGEM-1/myc-rab5 Q79L into the EcoRI site in the retroviral expression plasmid pLXSN, under control of the 5' long terminal repeat (Miller and Rosman, 1989). The resulting plasmid was transfected into the ecotropic packaging cell line GP+E-86 (Markowitz *et al.*, 1988) using DOTAP. Clones resistant to 0.8 mg/ml of the antibiotic G418 (Gibco) were selected. Recombinant ecotropic virus was obtained from these clones by harvesting medium from confluent dishes and was stored at −80°C. Virus from selected clones was used to infect another packaging cell line, PA317 (Miller and Buttimore, 1986), for production of amphotropic virus. Amphotropic virus was obtained by harvesting medium from pools of infected PA317 cells. HeLa cells at low density were infected with the amphotropic recombinant virus and incubated in the presence of 0.8 mg/ml G418 for 14 days with one medium change. All infections were performed in the presence of 4 μg/ml polybrene (Sigma). Clones resistant to the antibiotic were picked and tested for expression of the rab5 mutant by immunofluorescence analysis with the anti-myc epitope antibody 9E10 (Evan *et al.*, 1985). Four clones expressing rab5 Q79L in an ∼1:1 ratio with endogenous rab5, as indicated by Western blot analysis, were selected. The cells exhibited normal growth properties and were maintained in normal HeLa medium without G418.

Expression of rab5 and rab5 mutants in E.coli

DNAs encoding Rab5 S34N, rab5 Q79L and wild-type rab5 were cloned into the NdeI−BamHI sites of 6HisT-pET11 (Hoffman and Roeder, 1991) and expressed in BL-21 (DE3) cells. The His-tagged proteins were purified on Ni^{2+}−agarose (Quiagen) according to the manufacturer's instructions.

Confocal and electron microscopy

For confocal microscopy, cells grown on 10 mm round coverslips were either permeabilized with 0.05% saponin prior to fixation or fixed directly in 3% paraformaldehyde, as described (Zerial *et al.*, 1992). Overexpressed proteins were visualized with affinity-purified antibodies against a rab5 C-terminal peptide (Chavrier *et al.*, 1990), purified mouse anti-myc mAb 9E10 (Evan *et al.*, 1985) or the mouse anti-human transferrin receptor mAb B3−25 (Boehringer Mannheim). Endogenous rab7 was detected with an affinity-purified antibody raised against a C-terminal peptide of rab7 (Chavrier *et al.*, 1990). The secondary antibodies used were a rhodamine-labelled donkey anti-mouse antibody and a FITC-labelled donkey anti-rabbit antibody (both from DiaNova). Cells were mounted on Mowiol and viewed in the EMBL confocal microscope, using the excitation wavelengths 529 and 476 nm.

Electron microscopic analysis of cells transiently expressing rab5 constructs was performed exactly as described previously (Bucci *et al.*, 1992).

Measurement of transferrin uptake and recycling

Iron-saturated human transferrin (Sigma) was labelled with ^{125}I to a specific activity of 5000−20 000 c.p.m./ng using the iodogen method (Fraker and Speck, 1978). The labelled transferrin was bound to cells in 12 mm wells on ice for 30−90 min in GMEM containing 10 mM HEPES, pH 7.3, and 1 mg/ml bovine serum albumin. The cells were then warmed in 500 μl GMEM containing 10 mM HEPES, pH 7.3, 5 mM nitrilotriacetic acid, 10 μg/ml cycloheximide and 10 mM hydroxyurea. The medium was collected, and surface-bound ^{125}I-transferrin was removed with 3 mg/ml pronase E (Sigma), as described (Ciechanover *et al.*, 1983a), in GMEM containing 10 mM HEPES, pH 7.3, for 1 h at 0°C. The cells were recovered by centrifuging at 3000 r.p.m. for 2 min in an Eppendorf centrifuge. The radioactivity associated with the cells (endocytosed [^{125}I]transferrin) in the pronase-medium (surface-bound [^{125}I]transferrin) and in the incubation medium (recycled transferrin) was detected with an NE 1612 γ-counter (Nuclear Enterprises).

GTPase assay

Aliquots of 10 μl Ni^{2+} agarose were incubated with 1 pmol His-rab5 or His-rab5 Q79L in buffer A (20 mM Tris−HCl, pH 7.8, 100 mM NaCl, 5 mM MgCl$_2$, 1 mM Na-phosphate and 10 mM 2-mercaptoethanol) for 20 min at 25°C. Bound nucleotide was eluted by washing once with 1 M guanidine−HCl and then twice with ice-cold buffer A. 1 pmol [α-^{32}P]GTP (400 Ci/mmol, Amersham) in 20 μl buffer A was then bound to the immobilized protein for 10 min at 0°C. To each aliquot were then added 50 μl buffer A, and the samples were incubated at 37°C for various time periods. After incubation, the samples were washed rapidly three times with ice-cold buffer A, and 8 μl buffer B (0.2% SDS, 2 mM EDTA, 10 mM GDP, 10 mM GTP, pH 7.5) were added. The samples were heated for 2 min at 70°C, and 2 μl aliquots were spotted onto PEI−cellulose sheets (Merck) for thin-layer chromatography in 0.6 M Na-phosphate buffer, pH 3.5. The radioactive spots corresponding to [α-^{32}P]GDP and [α-^{32}P]GTP were quantified using a Phosphorimager (Applied Biosystems), and GTP hydrolysis was calculated as the signal in the GDP spot relative to the total signal.

[³H]GDP binding assay

Aliquots of 10 μl Ni^{2+} agarose were incubated with 2 pmol His-rab5 or His-rab5 S34N, and bound nucleotide was eluted as described above. [³H]GDP (100 nM) was added in 100 μl buffer A containing various concentrations of unlabelled GDP or GTP. The samples were kept at 4°C under rotation for 1 h, then washed three times with cold buffer A. The radioactivity associated with the beads was determined by scintillation counting.

Determination of the GTP:GDP ratio

The analysis was performed essentially as described earlier for p21-ras (Downward et al., 1990; Burgering et al., 1991). BHK cells in 3 cm tissue culture dishes were infected with vT7 and transfected with rab5, rab5 S34N or rab5 Q79L, as described above, except that the transfection was carried out in phosphate-free Dulbecco's modified Eagles medium containing 0.5 mCi/ml [³²P]orthophosphate (Amersham). 4 h post-transfection, the cells were washed three times with cold PBS and then lysed in lysis buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, 100 mM NaCl, 5 mM MgCl$_2$, 1 mM PMSF, 0.1 mM GTP, 1 mM ATP and 10 mM Na-phosphate). Nuclei and debris were removed by centrifugation at 15 000 g for 2 min, and the supernatant was subjected to immunoprecipitation with 4 μl 4F11 H10A20 ascites fluid on 10 μl protein A–Sepharose CL-4B (Pharmacia) for 6 min at 4°C. The beads were then washed three times with wash buffer (50 mM HEPES, pH 7.4, 500 mM NaCl, 5 mM MgCl$_2$) containing 1% Triton X-100 and then three times with wash buffer containing 0.1% Triton X-100 and 0.005% SDS. The bound nucleotides were then eluted in 8 μl sample buffer (2 mM EDTA, 2 mM DTT, 0.2% SDS, 5 mM GDP and 5 mM GTP) for 2 min at 70°C. All manipulations, from lysis of the cells to elution in sample buffer, were carried out at 4°C, over a time period of 20 min. 3 μl of the samples were spotted onto 0.1 mm PEI–cellulose TLC plates (Merck) which were developed for 40 min in 0.6 M Na-phosphate, pH 3.4. The plates were dried and placed in autoradiography cassettes containing intensifying screens. For visualization of the ³²P-labelled GTP and GDP, Kodak XAR-5 films were exposed at −80°C for 4 days. A Phosphorimager (Applied Biosystems) was used to determine the GTP:GDP ratios, taking into account that the specific activity of [³²P]GDP is 2/3 that of [³²P]GTP.

Preparation of BHK cytosol containing overexpressed rab5 proteins

For cytosol preparation, four 10 cm dishes of BHK cells were infected with vT7 and transfected with rab5 or mutant rab5 as described above. 6.5 h after transfection, postnuclear supernatants were prepared as described (Gorvel et al., 1991), except that no protease inhibitors were added. Cytosol was obtained by centrifuging the postnuclear supernatant at 22 000 r.p.m. for 15 min at 4°C, and then centrifuging this supernatant at 65 000 r.p.m. for 15 min at 4°C. Both centrifugations were carried out in a TL-100 table top centrifuge (Beckman), using a TLA 100.2 rotor. The protein concentration of the cytosol was then measured using the Bio-Rad protein assay, and 50 μl aliquots were stored in liquid nitrogen.

Cell-free assay of early endosome fusion

Early endosomes labelled with the fluid phase markers avidin (Molecular Probes) and biotinylated HRP (Gruenberg et al., 1989) were prepared by incubating BHK cells for 5 min at 37°C in the presence of either 3.3 mg/ml avidin or 1.7 mg/ml biotin-HRP, and then homogenizing the cells and separating the early endosomes on floatation gradients (Gruenberg et al., 1989; Gorvel et al., 1991). The avidin- and biotin-HRP-labelled endosomes, 50 μl (20−25 μg) of each, were mixed with 25 μl (625 μg) rat liver cytosol (Aniento et al., 1993), 25 μl (100 μg) BHK cytosol (prepared from cells overexpressing rab5 proteins, as described above) and 8 μl biotin-insulin, and adjusted to 12.5 mM HEPES (pH 7.5), 1.5 mM MgOAc, 3 mM imidazole, 1 mM dithiothreitol and 50 mM KOAc. Then 4 μl of an ATP-regenerating system (1:1:1 mixture of 100 mM ATP, pH 7.0, 800 mM creatine phosphate and 4 mg/ml creatine phosphokinase) was added, except where ATP was depleted, in which case 15 μl hexokinase in 0.25 M glucose was added. The reaction mixture was incubated for 5 min on ice, followed by 45 min at 37°C. The samples were then brought to 0.2% Triton X-100 and kept for 30 min on ice. Immunoprecipitation of the avidin-biotin−HRP complex, which is formed when fusion occurs, and the enzymatic activity of the immunoprecipitated HRP was quantified (Bomsel et al., 1990).

Measurement of isoprenylation

For the analysis of isoprenylation, cells in 35 mm tissue culture dishes were infected with vT7 and transfected with rab5 or rab5 Q79L DNA as described above. 90 min after the addition of DNA, 5% fetal calf serum and 5 μCi [¹⁴C]mevalonate (Amersham) were added, and the cells were incubated further at 37°C for 4 h. The cells were then washed twice with PBS, scraped into PBS and homogenized in 100 μl intracellular transport (ICT) buffer

(78 mM KCl, 4 mM MgCl$_2$, 8.37 mM CaCl$_2$, 10 mM EGTA, 1 mM DTT, 50 mM HEPES−KOH, pH 7.2) by 20 strokes through a 25G canule. Nuclei were removed by centrifugation at 3000 g for 2 min, and the postnuclear supernatant was separated into a membrane and a cytosol fraction by centrifugation at 100 000 g for 30 min at 4°C. Both fractions were extracted with 1% Triton X-100, and rab5 was immunoprecipitated from both fractions using 2 μl ascites fluid of the mouse hybridoma clone 4F11 H10A20 which had been prebound to 10 μl aliquots of protein A−Sepharose CL4B (Pharmacia). The immunoprecipitates were analysed by SDS−PAGE under reducing conditions, followed by fluorography.

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