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Distinct structural elements of rab5 define its functional specificity

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Members of the rab family of small GTPases are localized to distinct cellular compartments and function as specific regulators of vesicle transport between organelles. Overexpression of rab5, which is associated with early endosomes and the plasma membrane, increases the rate of endocytosis [Bucci *et al.* (1992) *Cell*, 70, 715-728]. From sequence alignments and molecular modelling we identified structural elements that might contribute to the definition of the functional specificity of rab5. To test the role of these elements experimentally, we transplanted them onto rab6, which is associated with the Golgi complex. The chimeric proteins were assayed for intracellular localization and stimulation of endocytosis. First, we found that the C-terminus of rab5 could target rab6 to the plasma membrane and early endosomes but it did not confer rab5-like stimulation of endocytosis. Further replacement of other regions revealed that the N-terminus, helix α2/loop 5 and helix α3/loop 7 were all required to functionally convert rab6 into rab5. Reciprocal hybrids of rab5 containing these regions replaced with those of rab6 were inactive, demonstrating that each region is essential for rab5 function. These results indicate that distinct structural elements specify the localization, membrane association and regulatory function of rab5.

Key words: endocytosis/GTPase/protein modelling/rab/ras

Introduction

A complex molecular machinery regulates vesicle-mediated transport of proteins and lipids between organelles (Pryer *et al.*, 1992; Rothman and Orci, 1992). Proteins belonging to the rab family of small GTPases are localized to distinct organelles and have been shown to function as specific regulators of intracellular transport (Pfeffer, 1992; Zerial and Stenmark, 1993). Mutant rab proteins with low affinity for GTP or with reduced GTPase activity inhibit transport both *in vitro* (Gorvel *et al.*, 1991; Plutner *et al.*, 1991; Tisdale *et al.*, 1992; Lombardi *et al.*, 1993) and *in vivo* (Goud *et al.*, 1988; Segev *et al.*, 1988; Bucci *et al.*, 1992; van der Sluijs *et al.*, 1992; Walworth *et al.*, 1992). The conformational changes occurring during the GTP/GDP cycle thus appear to be essential for the function of rab proteins. This led to the proposal that these GTPases act as molecular switches that ensure fidelity in vesicle delivery (Bourne, 1988; Bourne *et al.*, 1991).

Proteins acting downstream of rab proteins are predicted to specifically recognize only one of their two nucleotide-bound forms. For instance, rabphilin-3A, a putative target for rab3a, has been shown to interact only with the GTP-bound form of the protein (Shirataki *et al.*, 1993). Interconversion between the GTP and GDP forms occurs slowly *in vitro* but is catalysed *in vivo* by GTPase-activating proteins (GAPs) and GDP dissociation stimulators (GDSs, also called exchange factors and release proteins) (Burstein and Macara, 1992; Burton *et al.*, 1993; Moya *et al.*, 1993; Strom *et al.*, 1993). Membrane association, which requires post-translational modification with hydrophobic geranylgeranyl groups, is required for the function of rab proteins (Magee and Newman, 1992). Binding to membranes is regulated by the cytosolic protein rab GDI (Araki *et al.*, 1991; Regazzi *et al.*, 1992; Soldati *et al.*, 1993; Ullrich *et al.*, 1993), which binds only the GDP-bound form of geranylgeranylated rab proteins (Sasaki *et al.*, 1990).

So far, ~30 different members of the rab protein family have been identified. Overall, these share >30% sequence identity. The four regions constituting the nucleotide-binding pocket show the highest conservation (Valencia *et al.*, 1991). Other regions must contribute to the definition of the functional specificity of different rab proteins by interacting with specific regulators or effector molecules.

To identify candidates for such regions, we exploited information from multiple sequence alignments and constructed molecular models based on crystallographic data from other GTPases. To test experimentally the role of the structural elements identified by this analysis, we took advantage of the property of rab5, a small GTPase associated with the plasma membrane and early endosomes (Chavrier *et al.*, 1990), to increase the rate of endocytosis when overexpressed (Bucci *et al.*, 1992). In contrast, overexpression of rab6, which is associated with the Golgi apparatus and the *trans*-Golgi network (Goud *et al.*, 1990; Antony *et al.*, 1992) has no such effect. Then we replaced the defined regions of rab6 with the corresponding parts of rab5 and assayed for the gain of rab5-like function on endocytosis. In addition, we tested the reciprocal chimeras for the loss of rab5 function.

Results

Prediction of candidate regions mediating functional specificity

Multiple sequence alignments identify the N- and C-terminal regions of rab proteins as the most divergent ones (Valencia *et al.*, 1991). Based on this criterion these regions may be regarded as putative mediators of functional specificity. In addition, candidate regions can be identified by molecular modelling. This is possible because rab proteins share homology throughout most of their sequence with p21-ras,

which has been crystallized in both GTP- and GDP-bound forms (Milburn *et al.*, 1990; Pai *et al.*, 1990). Using as a framework those residues that are conserved between rab family members and p21-ras, we constructed 3-D models of the central regions of several rab proteins. A model of rab5 is shown in Figure 1A (right panel). From such modelling, four lines of evidence point to the 'effector' loop (L2), helix α2/loop 5 (α2/L5) and helix α3/loop 7 (α3/L7) as regions involved in nucleotide-dependent conformational changes and in interactions with other molecules. In this study we refer to these as 'switch' regions. First, based on multiple sequence alignments, the amino acid residues that discriminate different subfamilies and subgroups of ras-like proteins (labelled in red in Figure 1A, left panel) are localized mainly in L2, α2/L5 and the closely spaced α3/L7 (C.Sander and A.Valencia, unpublished data). Second, these regions change conformation upon GTP hydrolysis, as shown by 3-D structure analysis of p21-ras:GTP, p21-ras:GDP (Milburn *et al.*, 1990; Pai *et al.*, 1990), as well as by molecular modelling of p21-ras:GDP based on similarities with elongation factor Tu:GDP (Stouten *et al.*, 1993). Third, α2/L5 and α3/L7 in the GTPase domain of elongation factor Tu interact with another domain in a nucleotide-dependent manner (R.Hilgenfeld, personal communication). Given the structural similarities between ras-like proteins and the GTPase domain of elongation factor Tu, one might also assume that α2/L5 and α3/L7 of rab proteins are involved in protein–protein interactions. Fourth, mutational analysis of p21-ras and RAS2 have implicated L2, as well as α2/L5, in interactions with associating proteins (Adari *et al.*, 1988; Bourne *et al.*, 1991; Gideon *et al.*, 1992; Mistou *et al.*, 1992; Verrotti *et al.*, 1992).

In summary, sequence comparison and protein modelling, combined with information from functional studies on other GTPases, identify five candidate regions for conferring functional specificity to rab proteins: the N- and C-termini, and the regions L2, α2/L5 and α3/L7.

The C-terminus of rab5 confers targeting but not functional specificity

To test the functional importance of the candidate regions of the rab5 molecule, our strategy was to transplant them onto another rab protein (Figure 1B and Table I), express the hybrid proteins in BHK cells and test for their ability to stimulate endocytosis. We chose rab6 as an acceptor molecule for two reasons: (i) rab5 and rab6 are localized to different compartments (Chavrier *et al.*, 1990; Goud *et al.*, 1990; Antony *et al.*, 1992); and (ii) having constructed 3-D models of rab6 as well as ra6/rab5 chimeras (not shown), our structural analysis suggested that rab6 would be able to accommodate the L2, α2/L5 and α3/L7

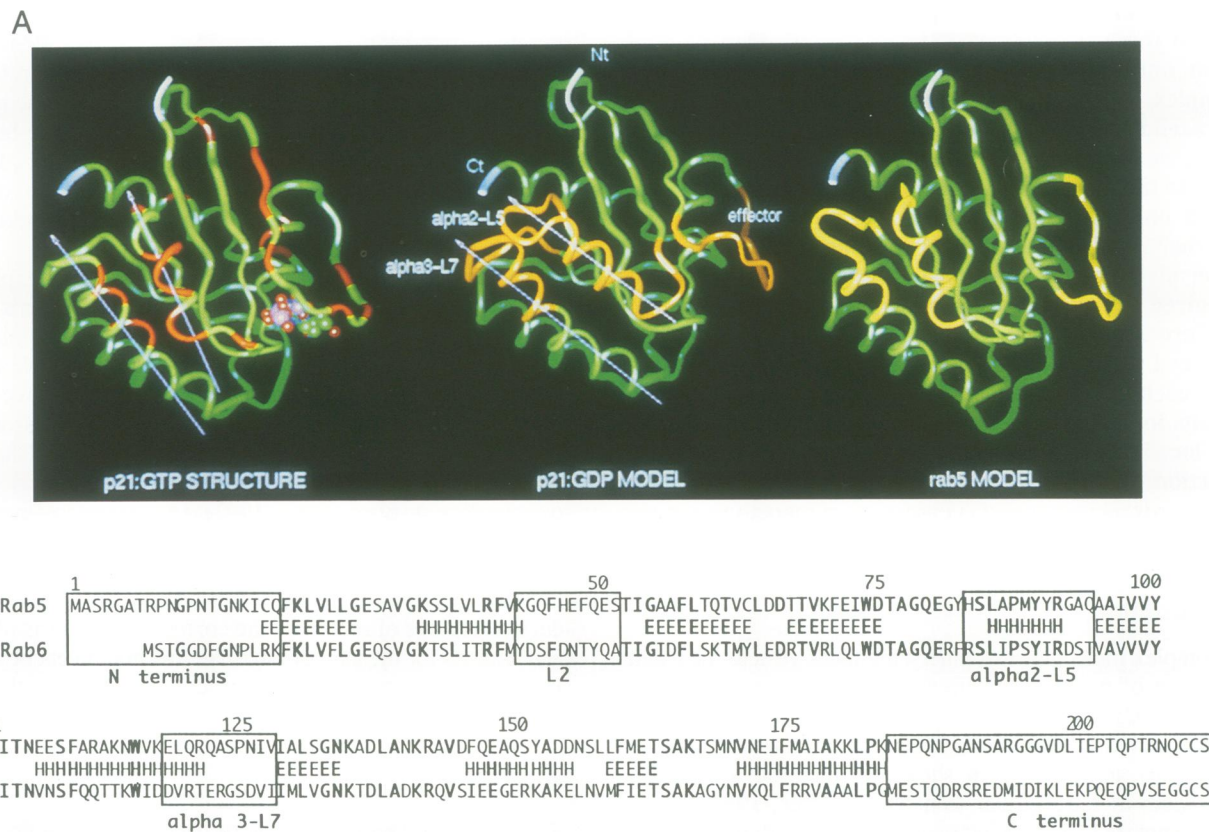

Fig.1. (**A**) Colour ribbon plots of the structures of ras:GTP, ras:GDP (model) and rab5 (model). Left: 3-D structure of ras-p21 (Pai *et al.*, 1990) showing the position of the bound GTP (in space filling model), the axis of α2 and the axis of α3. The regions coloured red correspond to the 12 residues that discriminate best between the different subgroups of ras-like proteins. Centre: 3-D model of the proposed structure of p21-ras bound to GDP (nucleotide not shown). The main differences compared with the ras:GTP structure are shown in orange. The C-terminus, the N-terminus, helix α2/loop 5, helix α3/loop 7 and the effector loop (L2) are indicated. Right: model of rab5. The fragments transplated to rab6 are shown in yellow. Some loops, for instance L7, are structurally different from those of p21-ras, due to the presence of insertions in the sequence of rab5 with respect to ras. (**B**) Sequence comparison between MDCK rab5 (upper sequence) and human rab6 (lower sequence), and the secondary structure of the rab5 model, as predicted by Kabsch and Sander (1983). H, α-helix; E, β-strand. The regions that have been swapped between the two proteins are boxed and named. Identical residues are shown in bold.

of rab5 with minimal distortion of its overall structure (see Materials and methods).

To confirm the differential organelle localization of rab5 and rab6, we coexpressed rab6 and N-terminally myc epitope-tagged rab5 in BHK cells using the T7 RNA polymerase recombinant vaccinia virus (vT7) system (Fuerst *et al.*, 1986). The localization of the two molecules was visualized by confocal immunofluorescence microscopy. Anti-rab6 antibodies diluted to recognize overexpressed, but not endogenous, protein stained vesicular structures close to the nucleus (Figure 2A), consistent with the earlier reported localization of rab6 to the Golgi complex and *trans*-Golgi network (Goud *et al.*, 1990; Antony *et al.*, 1992). In contrast, the anti-myc epitope antibody (Figure 2B) gave a typical rab5 staining of the plasma membrane and early endosomes (Chavrier *et al.*, 1990; Bucci *et al.*, 1992). Overexpression of rab5 increased by more than 2-fold the rate of fluid-phase endocytosis, measured as uptake of horseradish peroxidase (HRP), whereas overexpression of a rab5 mutant (rab5 I-133) strongly inhibited HRP uptake (Bucci *et al.*, 1992). In contrast, overexpression of rab6 did not affect the rate of fluid-phase endocytosis (Figure 5, see below). Therefore, rab6 shares neither the localization nor the function of rab5.

Next we investigated whether the targeting of rab6 to the plasma membrane and early endosomes can alone confer rab5 function. Since earlier studies had demonstrated an important role of the C-terminus of rab proteins for their localization (Chavrier *et al.*, 1991; Brennwald and Novick, 1993), we replaced the C-terminal 32 residues of rab6 with the corresponding region of rab5 (hybrid 6/5-1, Table I). When hybrid 6/5-1 was coexpressed with myc-tagged rab6, the two staining patterns showed little overlap (compare Figure 2C and D), although we cannot exclude the possibility that a minor fraction of 6/5-1 was localized to the Golgi region. In contrast, when 6/5-1 was coexpressed with tagged rab5, the two proteins colocalized to a large extent (Figure 2E and F). The results indicate that the C-terminus of rab5 can target rab6 to the plasma membrane and early endosomes. To determine whether it would also confer rab5 function to rab6, we measured the effect of 6/5-1 on endocytosis of HRP. As shown in Figure 5, overexpression of 6/5-1 did not affect HRP uptake. This indicates that the C-terminus of rab5, although sufficient to target a rab6 hybrid to early endosomes and the plasma membrane, is not sufficient to transmit rab5 function.

Table I. Rab6/rab5 chimeras

Hybrid protein	Region of rab6 replaced with that of rab5				
	N	L2	α2/L5	α3/L7	C
6/5-1	−	−	−	−	+
6/5-2	−	−	+	−	+
6/5-3	−	−	+	+	+
6/5-4	−	+	−	−	−
6/5-5	−	+	+	−	+
6/5-6	−	−	+	+	−
6/5-7	−	+	−	−	+
6/5-8	−	+	+	+	+
6/5-9	−	−	−	+	+
6/5-10	−	+	−	+	+
6/5-11	+	−	+	+	+
6/5-12	+	+	−	+	+
6/5-13	+	+	+	+	+
6/5-14	+	−	−	−	−
6/5-15	+	−	−	−	+
6/5-16	+	−	+	−	+
6/5-17	+	+	−	−	+
6/5-18	+	−	−	+	+
6/5-19	+	+	−	+	+
	Region of rab5 replaced with that of rab6				
	N	L2	α2/L5	-aα3/L7	C
5/6-1	−	−	−	−	+
5/6-2	+	−	−	−	−
5/6-3	−	−	+	−	−
5/6-4	−	−	−	+	−
5/6-5	−	−	+	+	−

The table lists the various chimeras made and shows the regions that were swapped between the two proteins (indicated with +).
N, N-terminus; C, C-terminus.

Role of the switch regions α2/L5 and α3/L7 in membrane association

Since the C-terminus of rab5 cannot alone confer rab5 function onto rab6, we decided to replace in addition the regions predicted to undergo a conformational change upon GTP hydrolysis (Figure 1A and Table I). We started by replacing α2/L5. Immunofluorescence analysis of the overexpressed hybrid, 6/5-2 (Table I), gave a surprising result. When cells were permeabilized with saponin to wash out cytosolic protein, 6/5-2 was not detected (Figure 3A). In contrast, when the cells were permeabilized after fixation a strong cytoplasmic staining was observed (Figure 3C), indicating that 6/5-2 was found in the cytosol. This interpretation was confirmed by subcellular fractionation studies (see Materials and methods) which showed that the 6/5-2 protein was detected in the cytosolic, but not in the membrane, fraction (data not shown).

Geranylgeranylation of one or two C-terminal cysteines is required for membrane association of rab proteins (Magee and Newman, 1992). Inefficient membrane localization of 6/5-2 could thus, in principle, be due to a lack of this post-translational modification. This was tested by measuring isoprenylation *in vitro* in reticulocyte lysate in the presence of [^{3}H]geranylgeranyl pyrophosphate. As a control for the translation efficiency, parallel samples were translated in the presence of [^{35}S]methionine. The results displayed in Figure 4 (compare upper and lower panels) show that rab5 (lane 1) and the hybrid 6/5-7 (lane 6) which contains the C-terminus and L2 of rab5 (see later), were efficiently modified. 6/5-2 (lane 4) was geranylgeranylated less efficiently than rab5, but to the same extent as rab6 (lane 2) and 6/5-1 (lane 3). Assuming that the conditions *in vitro* resemble those *in vivo* (Hancock *et al.*, 1991; Khosravi-Far *et al.*, 1991; Kinsella *et al.*, 1991; Kinsella and Maltese, 1992; Peter *et al.*, 1992), these data suggest that inefficient membrane association of 6/5-2 is not caused by lack of geranylgeranylation.

From the model (Figure 1A, right panel), α2/L5 was predicted to interact with the end of α3 as well as with L7, and it is possible that α2/L5 of rab5 is not compatible with α3/L7 of rab6. Therefore we tested if the replacement of α3/L7 in addition to α2/L5 (hybrid 6/5-3, Table I) would restore membrane association of the hybrid protein. When

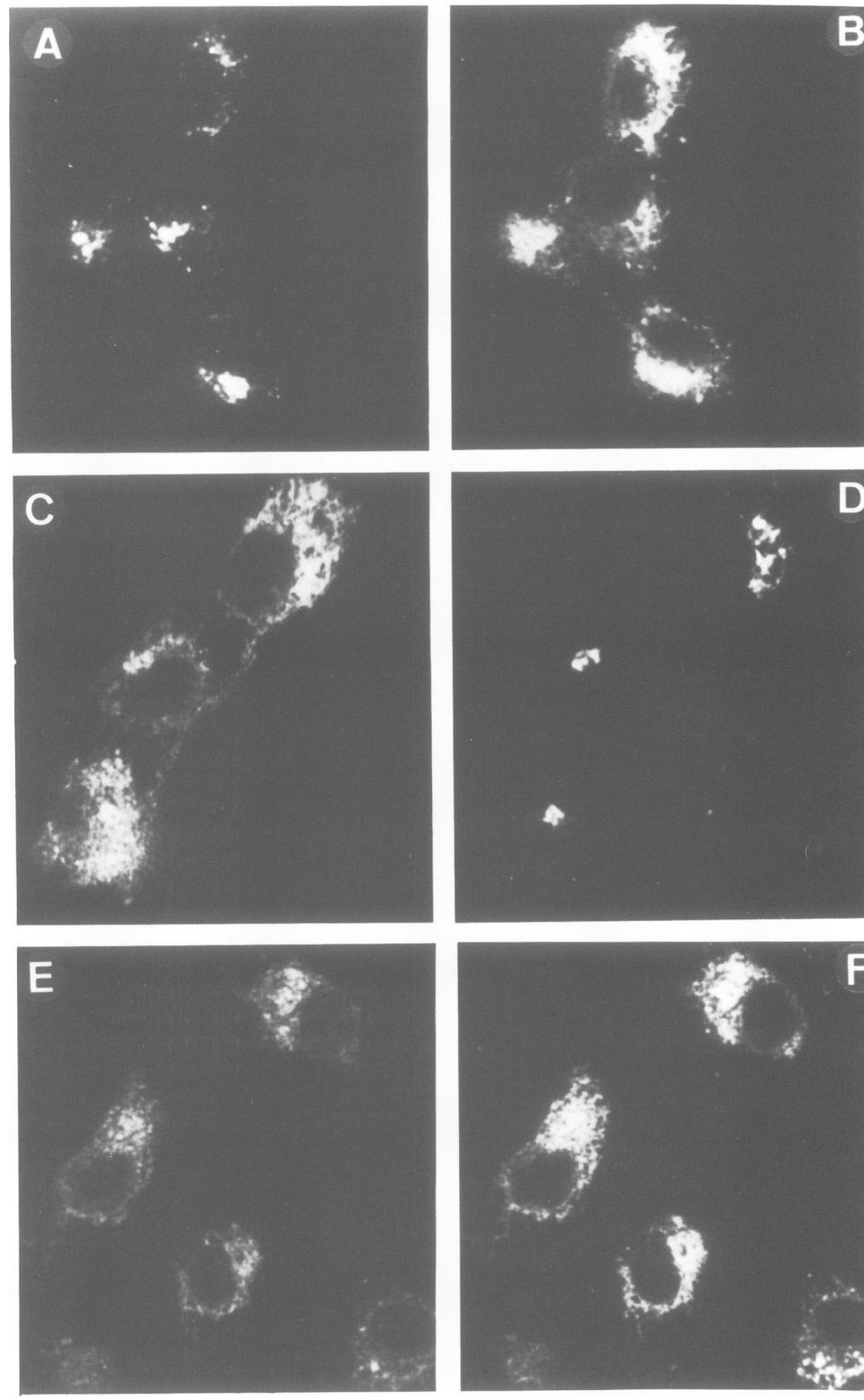

Fig. 2. Confocal double immunofluorescence localization of rab5, rab6 and hybrid 6/5-1. BHK cells on coverslips were infected with vT7 and then cotransfected with rab6 and myc-rab5 (**A** and **B**), 6/5-1 and myc-rab6 (**C** and **D**), or 6/5-1 and myc-rab5 (**E** and **F**). After 4 h of transfection, cells were permeabilized with saponin and then fixed with paraformaldehyde (see Materials and methods). Overexpressed proteins were visualized by incubation with antibodies against rab6 (A and E), myc epitope (B, D and F) or rab5 (C), followed by incubation with FITC-labelled (A, C and E) or rhodamine-labelled (B, D and F) secondary antibodies. The field size is 60 μm.

analysed by immunofluorescence, 6/5-3 was found associated with membranes of saponin-treated BHK cells (Figure 3D) and colocalized with the transferrin receptor to the plasma membrane and early endosomes (data not shown). Thus, α3/L7 of rab5 restores both membrane binding and specific localization of 6/5-2. This region might therefore be required for proper interaction with α2/L5. In addition, both α2/L5 and α3/L7 of rab5 might be required together to interact

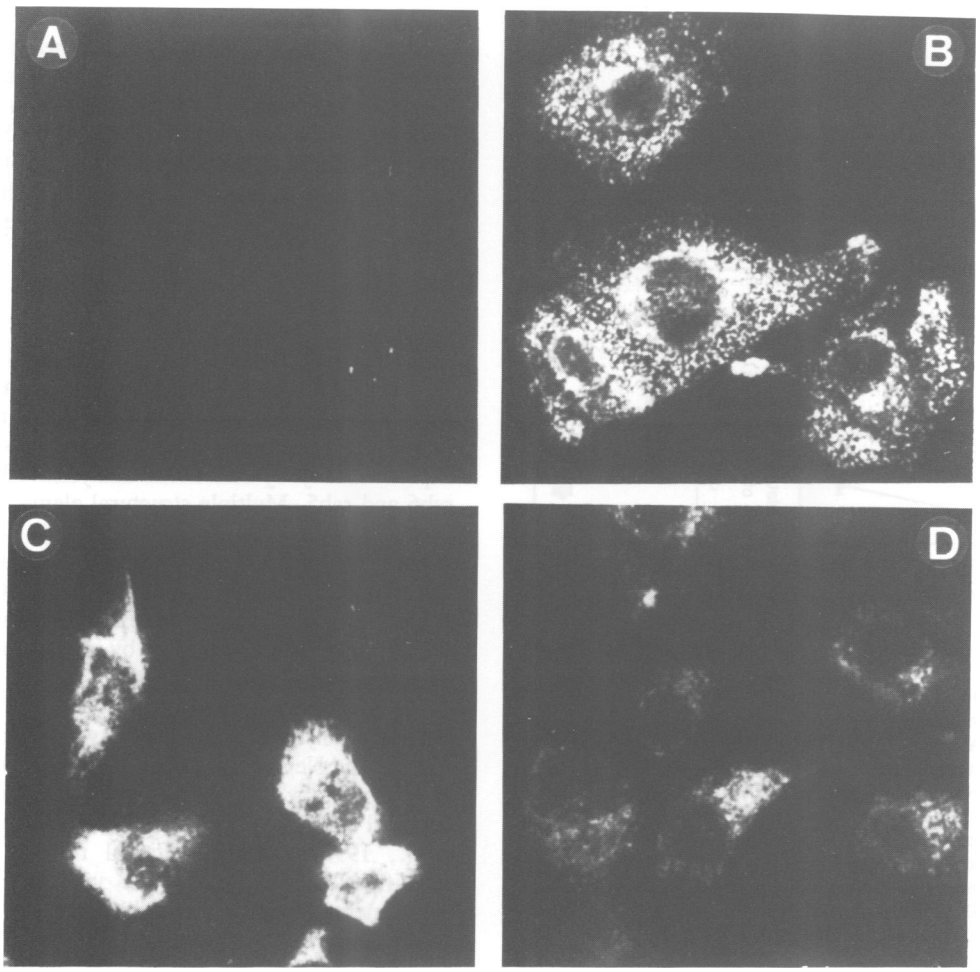

Fig. 3. Localization of hybrids 6/5-2 and 6/5-3. Cells were infected with vT7 and then cotransfected with 6/5-2 and the human transferrin receptor (**A**–**C**) or 6/5-3 and the human transferrin receptor (**D**). After transfection, cells were either permeabilized with saponin prior to paraformaldehyde fixation (A, B and D) or fixed directly (C). Overexpressed hybrid proteins were visualized with antibodies against the C-terminus of rab5 (A, C and D). In (B), the same cells as in (A) were incubated with antibodies to the overexpressed human transferrin receptor. This served as an additional control that the cells were transfected, because >95% of double-transfected cells overexpress both proteins (Chavrier *et al.*, 1991). The field size is 60 μm.

with rab5 accessory molecules. The latter possibility is supported by the finding that 6/5-2 binds [^{32}P]GTP on blots (data not shown), suggesting that the structure of 6/5-2 is not severely impaired. Moreover, no misfit could be detected by molecular modelling. Altogether, these results suggest that α2/L5 and α3/L7 are both required for correct membrane association of rab5.

Structural elements of rab5 that are important for regulation of endocytosis

Next we investigated whether the presence of both α2/L5 and α3/L7 of rab5 could confer rab5 function. For this purpose we measured the effect of overexpressed 6/5-3 on endocytosis. Surprisingly, 6/5-3 strongly inhibited HRP uptake (Figure 5), almost to the same extent as the dominant negative rab5 I-133 mutant (Bucci *et al.*, 1992). This result indicates that while the presence of α2/L5 and α3/L7 of rab5 allows 6/5-3 to interact with rab5 accessory molecules, these regions are not sufficient to confer rab5 function. This suggests that other domains of rab5 are required as well. Since L2 changes conformation upon GTP hydrolysis, we constructed several hybrid molecules containing L2 of rab5 in combination with other regions (Table I). As with 6/5-3,

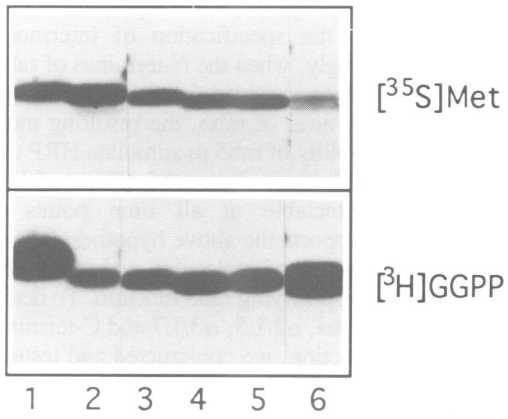

Fig. 4. *In vitro* translation and geranylgeranylation of rab5, rab6 and rab6/rab5 hybrids. Rab5 (lane1), rab6 (lane2), 6/5-1 (lane 3), 6/5-2 (lane 4), 6/5-3 (lane 5) or 6/5-7 (lane 6) were translated in rabbit reticulocyte lysate containing either [^{35}S]methionine (upper panel) or [^{3}H]geranylgeranyl pyrophosphate (lower panel). The translation products were analysed by SDS–PAGE and fluorography. The exposure times were 16 h (upper panel) and 30 days (lower panel) respectively.

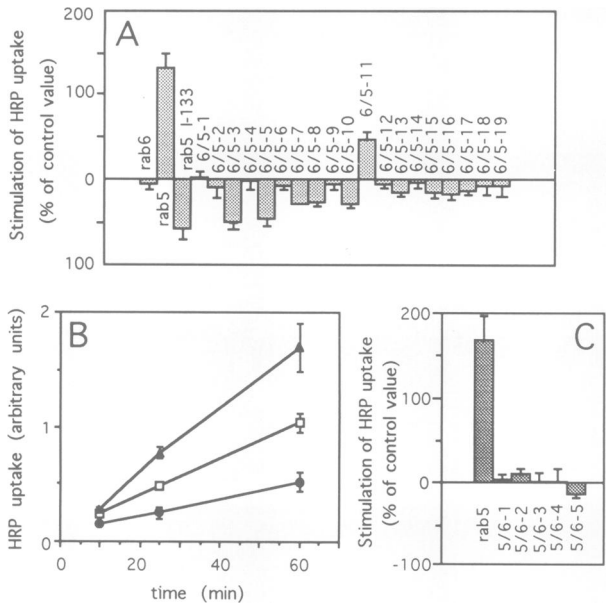

Fig. 5. Effect of overexpressed proteins on HRP endocytosis. BHK cells were infected with vT7, then transfected with the plasmids encoding the proteins indicated. The intracellular accumulation of HRP after 1 h incubation at 37°C was measured as described in Materials and methods. In (**A**) and (**C**) the values of HRP uptake are expressed as per cent stimulation of HRP uptake with respect to mock-transfected cells (transfection reagent alone without DNA). In (**B**) the HRP uptake is calculated as HRP activity per μg of cellular protein (arbitrary units). The error bars indicate the standard error of the mean of three to four independent samples. (A) Stimulation of HRP uptake by rab5, rab6, rab5 I-133 and the 6/5 series of chimeras. (B) Time course of HRP uptake in BHK cells transfected with rab5 (▲), 6/5-11 (□) or no DNA (●). (C) Stimulation of HRP uptake by the 5/6 series of chimeras.

expression of most of these proteins, in particular 6/5-5, 6/5-7, 6/5-8 and 6/5-10, inhibited HRP uptake (Figure 5A).

The inhibitory effect of these hybrid molecules suggests that some additional structural element is required for rab5-like function. Therefore we also replaced the N-terminus of the hybrid proteins with that of rab5, because the N-termini of the two proteins are highly divergent and might contribute to the specification of intermolecular interactions. Interestingly, when the N-terminus of rab5 was grafted onto the inhibitory hybrid 6/5-3 containing α2/L5, α3/L7 and the C-terminus of rab5, the resulting molecule (6/5-11) shared the ability of rab5 to stimulate HRP uptake, albeit with lower efficiency than rab5 (Figure 5A). The stimulation was detectable at all time points tested (Figure 5B). This supports the above hypothesis that 6/5-3 interacts with rab5-associated molecules, and suggests a role for the N-terminus in specifying rab5 function. To determine whether the N-terminus, α2/L5, α3/L7 and C-terminus are required for rab5 function, we constructed and tested a set of reciprocal rab5 molecules where these regions were replaced with those of rab6. Figure 5C shows that all expressed hybrids failed to stimulate endocytosis. The finding that the rab5 mutant containing the C-terminus of rab6 (hybrid 5/6-1) was also inactive indicates that the hypervariable C-terminus is required for both correct localization (Chavrier *et al.*, 1991; this study) and function of rab5.

Unexpectedly, 6/5-13, which has L2 of 6/5-11 replaced with L2 of rab5, showed no stimulation of endocytosis. This could either reflect an unpredicted structural problem in this chimera, or the requirement for another rab5 region acting in concert with L2 (see Discussion). Since a number of other constructs containing the rab5 N-terminus did not increase HRP uptake (Figure 5A and C), it appears that a combination of the N-terminus, α2/L5, α3/L7 and C-terminus of rab5 is required for its functional specificity. These regions may play distinct roles in targeting, membrane association and regulatory activity.

Discussion

To identify structural elements that define the functional specificity of rab5, we constructed a 3-D model to predict candidate regions. The predictions were tested experimentally by functional analysis of hybrids between rab6 and rab5. Multiple structural elements were found to be necessary to specify rab5 function. These include the hypervariable N- and C-termini, as well as regions undergoing conformational changes upon GTP hydrolysis. A likely interpretation of these results is that the functional specificity of rab5 is conferred by interactions with a number of accessory proteins.

Membrane association of rab proteins requires geranylgeranylation of one or two C-terminal cysteines (Magee and Newman, 1992). Unlike p21-ras, isoprenylation of rab proteins depends on sequences upstream to the C-terminal cysteine motif (Kinsella and Maltese, 1992; Peter *et al.*, 1992). These additional regions have not been identified so far, but our *in vitro* geranylgeranylation experiments suggest a possible role for L2. Rab5 was geranylgeranylated much more efficiently than rab6, and the modification efficiency of rab6/rab5 chimeras correlated with the presence of L2 of rab5. Therefore, it is possible that L2 interacts with the geranylgeranyl transferase.

Different rab proteins are associated with distinct organelles (Pfeffer, 1992; Zerial and Stenmark, 1993), and earlier work has implicated their hypervariable C-terminus as a localization signal (Chavrier *et al.*, 1991; Brennwald and Novick, 1993). Consistent with this, we found that the C-terminus of rab5 can retarget the bulk of rab6 from the Golgi complex to the plasma membrane and early endosomes. Furthermore, replacement of the C-terminus of rab5 with that of rab6 leads to functional inactivation, thus indicating that this region is required for rab5 function. Specific molecules in the membrane are likely to interact with this sequence.

However, membrane association of rab proteins may also depend on their nucleotide state (Araki *et al.*, 1990; Pfeffer, 1992). In support of this, replacement of the switch region α2/L5 of rab6 with α2/L5 from rab5 yielded a protein that was cytosolic, despite containing the C-terminus of rab5, binding GTP and being geranylgeranylated *in vitro*. The inefficient membrane association of this protein could be due to an altered interaction with a factor controlling its nucleotide state. Candidate factors are GDI, GAP and GDS, which discriminate between the two nucleotide-bound forms of the molecule (Sasaki *et al.*, 1990; Burstein and Macara, 1992; Gideon *et al.*, 1992; Mistou *et al.*, 1992; Verrotti *et al.*, 1992). In addition, structural analysis indicates that α2/L5 interacts with α3/L7, and the inefficient membrane association was in fact corrected by the additional replacement of α3/L7. It thus appears that α2/L5 and α3/L7

cooperate to maintain the necessary conformation for recognition by regulatory factors. In addition, structural criteria (Stouten *et al.*, 1993) suggest that both regions are likely to interact with such molecules.

According to the view that rab proteins act as molecular switches (Bourne, 1988; Bourne *et al.*, 1991), their effector proteins would recognize regions that alter conformation upon GTP hydrolysis. In our model (Figure 1A), three structural elements, L2, α2/L5 and α3/L7, change conformation when GTP is hydrolysed. In the endocytosis assay, both α2/L5 and α3/L7 of rab5 were necessary but not sufficient for rab5-like function. This appeared to require the N-terminus as well, because (i) the addition of the N-terminus of rab5 converted an inhibitory chimera into one that stimulated HRP uptake, and (ii) the replacement of the N-terminus of rab5 with that of rab6 leads to loss of rab5 function. The present finding agrees with a recent report where removal of the rab5 N-terminus by trypsinization appeared to abolish early endosome fusion *in vitro*. Further, early endosome fusion was inhibited by a peptide corresponding to the N-terminus of rab5 (Steele-Mortimer *et al.*, 1994). These results suggest that the N-terminus, together with α2/L5 and α3/L7, interact with regulators or target molecules. Surprisingly, neither the N- nor the C-termini of rab proteins are highly conserved through evolution (Haubruck *et al.*, 1989; Wichmann *et al.*, 1992; Armstrong *et al.*, 1993). Yet, mammalian rab1a and rab5 proteins can functionally replace yeast Ypt1p and Ypt5p, respectively (Haubruck *et al.*, 1989; Armstrong *et al.*, 1993). The reason could be that N- and C-termini have autonomous function, conferred by a few conserved amino acids or conserved structural features. Furthermore, our data suggest that the N- and C-termini are necessary in conjunction with other parts of the molecule. Therefore specific interactions between all these regions can be envisaged.

Several studies on p21-ras have implicated the 'effector domain', which includes L2, in interactions with regulators and effector molecules (Bourne *et al.*, 1991). Likewise, peptides corresponding to the effector domain of rab3a affect transport in several *in vitro* assays (Plutner *et al.*, 1990; Oberhauser *et al.*, 1992; Padfield *et al.*, 1992). In this study, addition of L2 from rab5 to 6/5-11, the hybrid protein that stimulated HRP uptake, impaired its ability to function in a rab5-like manner. This suggests that L2 of rab5 requires the presence of an additional part of rab5 (not included in this study) to interact correctly with accessory molecules. Such joint interactions between L2 and other regions with GAP and GDS molecules have been suggested for other small GTPases (Burstein and Macara, 1992; Gideon *et al.*, 1992; Mistou *et al.*, 1992). This might also explain why 6/5-11 could not stimulate endocytosis to the same extent as rab5, and constructing a hybrid that has complete rab5 function would probably involve replacement of most of the 135 residues that distinguish rab5 from rab6. Consistent with the above, the hybrid protein containing L2 and the C-terminus of rab5 significantly inhibited HRP uptake, suggesting an altered interaction between L2 and accessory molecules.

Recent studies have identified several regions that functionally distinguish two yeast counterparts of rab proteins, Ypt1 and Sec4. In one study, L2, L7 and the C-terminus of Ypt1 were found to confer Ypt1 function to Sec4 (Brennwald and Novick, 1993). However, another study obtained a similar result by swapping L2, α3 and L7 (Dunn *et al.*, 1993). This is in agreement with our results. However, unlike our study, neither of these reports identified any important role for the N-terminus and α2/L5. The different experimental strategies used could explain some of the discrepancies. The results with Sec4 and Ypt1 were based on the ability of hybrid molecules to restore the growth of deletion mutants at various temperatures, thus identifying hybrid proteins capable of performing a minimal Ypt1 or Sec4 function. In contrast, we measured the function of rab6/rab5 hybrids using a quantitative biochemical assay. Moreover, the sequence similarity between rab6 and rab5 (47%) is lower than that between Ypt1 and Sec4 (66%), and one may assume that it is more difficult to confer rab5 function to rab6 than Ypt1 function to Sec4. Regarding our data, we also believe that the different results obtained reflect the complexities in the interactions between rab proteins and their accessory proteins: if several factors participate in overlapping interactions with distinct structural elements of rab proteins, then one may expect to find the requirements for specific regions to vary somewhat, depending on the experimental design.

In conclusion, our results suggest that the functional specificity of rab5 is maintained by multiple structural elements. The fact that the replacement of 65 out of a total of 208 residues in rab6 resulted in a protein with partial rab5 function indicates that we have identified some of the most important regions. These appear to play distinct roles in interactions with the geranylgeranyl transferase, specific receptors, regulatory factors and effector molecules. In the 3-D model (Figure 1A, right), the structural elements we found necessary for rab5-like function (the N-terminus, the C-terminus, α2/L5 and α3/L7) are all present on the same side of the molecule. It is thus possible that this part, although distant from the nucleotide-binding site, forms an entity that is involved in interactions with regulators or target molecules. A similar model was recently proposed in the case of α subunits of heterotrimeric G proteins (Conklin and Bourne, 1993). The identification of molecules interacting with rab proteins is now underway (Matsui *et al.*, 1990; Burstein and Macara, 1992; Burton *et al.*, 1993; Moya *et al.*, 1993; Shirataki *et al.*, 1993; Strom *et al.*, 1993), and the hybrid proteins described here should be useful in future studies on specific interactions between rab5 and its accessory proteins.

Materials and methods

Protein modelling

Molecular models of rab5, rab6 and rab6/rab5 chimeras were based on the known 3-D structure of ras-p21:GTP (Pai *et al.*, 1990), the model of ras:GDP (Stouten *et al.*, 1993) and the sequences of the different ras-like proteins (Valencia *et al.*, 1991). We derived molecular models of rab6 and rab5 using the structure of p21-ras as a framework (Pai *et al.*, 1990). The modelling was guided by the multiple sequence alignment of all ras-like proteins in the central 166 residues (N- and C-terminus excluded) (Valencia *et al.*, 1991). The alignment has no major ambiguities because the four nucleotide-binding loops (corresponding to the consensus sequences GxxxxGKS, DTAG, NKCD and SAK) always maintain the correct frame. There are also other conserved residues in all ras-like proteins that allow a good sequence alignment throughout the protein. The explicit 3-D models were built with WHATIF (Vriend, 1990). During the substitution of each sidechain we tried to maintain as many atoms as possible in position. L2 and L7, which contain insertions compared with p21-ras, were modelled according to the most similar loops in the database. The structure of the final model of rab5 is shown in Figure 1A (right). The full model was subject to a short run of molecular dynamics and normalization by energy

minimization using GROMOS (van Gunsteren and Berendsen, 1987) to avoid bad angles and clashes. All models of rab6, rab5 and rab6/rab5 chimeras passed a quality test of protein structures (Vriend, 1990). The coordinates are available via ftp from the EMBL file server (ftp.embl-heidelberg.de, directory: /pub/databases/protein_extras/models).

The regions to be replaced in L2, α2/L5 and α3/L7 were those that (i) were sufficiently different between rab5 and rab6 and (ii) would not produce clashes or bad contacts with the rest of the protein. Thus, we did not replace the beginning of α3, even if some possibly important residues are there, so as to avoid problems in the interaction of α3 with the rest of the protein. Clashes and bad contacts were checked in the model of the rab6/rab5 chimeras and also in the models of p21-ras:GDP. For example, α2 contacts more strongly with α3 in the model of ras:GDP than in the GTP structure and we decided not to replace most of α3 so as to avoid possible bad contacts. There are a few important residues in the central part that are not contained within the regions tested in this study. These include residues 37, 39 and 42 in β2, a region close in space to α2/L5 that can form part of the same site of recognition, and residues 149 and 157 in α5 which forms part of an unexplored region of the proteins close to the C-terminus (see Figure 1A, left). Here, we restricted ourselves to change only 29% of the 102 residues that are different between rab5 and rab6 in the central region (Figure 1B). The N- and C-termini were also replaced, given that there is no available information about the 3-D structure of these regions, and that they are very different in length and sequence. No modelling was attempted, but the proximity of the N- and C-termini to α2/L5 and α3/L7 makes it possible that they have functional interactions with these regions.

Plasmids

The human rab6 cDNA (Touchot *et al.*, 1987) in pGEM-1 was mutated with DNA encoding the N-terminus, L2, α2/L5, α3/L7 or C-terminus of MDCK rab5 (Chavrier *et al.*, 1990) using the PCR and synthetic mutant oligonucleotides (Saiki *et al.*, 1988). PCR-amplified fragments were cloned into suitable restriction sites in the rab6 plasmid. A similar strategy was used to construct chimeras of rab5 containing the N-terminus, α2/L5, α3/L7 or C-terminus of rab6. Rab5, rab6 and chimeras were likewise tagged with the c-myc epitope (Evan *et al.*, 1985) using the PCR. In all cases, the PCR-amplified regions were sequenced to verify the mutations and to exclude PCR errors.

Antibodies

Anti-myc epitope. A mouse hybridoma line producing the antibody 9E10 (Evan *et al.*, 1985) was a gift from Dr Stephen Fuller. The antibody was produced as mouse ascites fluid and purified on protein A–Sepharose CL-4B (Pharmacia) as described (Harlow and Lane, 1988).

Anti-rab5. Serum obtained from rabbits immunized with a synthetic C-terminal rab5 peptide was affinity-purified as described (Chavrier *et al.*, 1990; Zerial *et al.*, 1992).

Anti-rab6. Rabbits were immunized with rab6 purified from Sf9 cells infected with rab6-recombinant baculovirus, and serum was affinity purified on columns containing immobilized rab6 (Goud *et al.*, 1990; Antony *et al.*, 1992). Control experiments showed that anti-rab5 did not recognize rab6, and anti-rab6 did not crossreact with rab5. Purified mouse mAbs against the human transferrin receptor (clone B3/25) were purchased from Boehringer Mannheim, Germany. FITC-labelled donkey anti-rabbit and rhodamine-labelled donkey anti-mouse affinity-purified and affinity-adsorbed antibodies were obtained from DiaNova, Germany.

Transfection

BHK-21 cells were seeded into 24-well Falcon plates 18–22 h prior to the experiments. Cells were infected for 30 min with vT7 and transfected with plasmid DNA for 3.5–4.0 h using DOTAP (Boehringer) as described earlier (Bucci *et al.*, 1992).

Confocal scanning immunofluorescence microscopy

Cells on 10 mm round coverslips overexpressing rab constructs were either permeabilized with saponin before paraformaldehyde fixation or fixed directly with paraformaldehyde, as indicated in the legend to Figure 3. Free aldehyde groups were then blocked with ammonium chloride and the coverslips were incubated with anti-rab6, anti-myc, anti-human transferrin receptor or anti-rab5 antibodies (Zerial *et al.*, 1992). FITC-labelled donkey anti-rabbit or rhodamine-labelled donkey anti-mouse antibodies were used as secondary antibodies. The cells were viewed using the EMBL compact confocal microscope with the excitation wavelengths 476 and 529 nm. The thickness of the sections viewed was 0.4 μm.

Analysis of membrane association

BHK cells in 3 cm dishes were infected with vT7 and transfected for 3.5 h with plasmids encoding myc epitope-tagged wild-type rab5, 6/5-2 or 6/5-3. The cells were scraped into PBS and homogenized in 100 μl homogenization buffer (0.25 mM sucrose, 3 mM imidazole, pH 7.1) by 20 strokes through a 25G canule. The cytosol and membranes were separated by centrifugation at 60 000 r.p.m. for 30 min at 4°C in a TLA-100 rotor (Beckmann), followed by extraction with 1% Triton X-100 in PBS and precipitation of proteins with 5% trichloroacetic acid. The precipitates were analysed by immunoblot analysis using the mAb 9E10.

In vitro geranylgeranylation

For *in vitro* transcription, 1 μg plasmid DNA, linearized with *Hin*dIII, was transcribed with 25 U T7 RNA polymerase (NEB) for 1 h at 40°C in 10 μl reaction mixture containing 0.5 mM of each of the four nucleoside triphosphates, 40 mM Tris–HCl pH 7.6, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT and 1 U RNasin (Promega). Aliquots (1 μl) of the transcription mixture were translated in 50 μl nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of 50 μCi [^{35}S]methionine (Amersham) according to the manufacturer's instructions. Alternatively, translation was carried out in 100 μl reactions using unlabelled methionine, but in the presence of 2 μCi [^{3}H]geranylgeranyl pyrophosphate (ARC). In these cases, after 1 h at 30°C, the translation mixture was adjusted to 5 mM $MgCl_2$/1 mM DTT, followed by an additional incubation at 37°C for 1 h. The [^{3}H]geranylgeranylated translation products were immunoprecipitated using rab5 or rab6 antibodies (control experiments showed that the immunoprecipitation was quantitative), and the immunoprecipitates were analysed by 12% SDS–PAGE together with 1 μl aliquots of the [^{35}S]methionine-labelled reactions (Ullrich *et al.*, 1993). The gels were treated with Entensify (Dupont), and Kodak XAR-5 films were exposed to dried gels at −80°C.

Measurement of HRP endocytosis

BHK cells were transfected for 4 h and then incubated in the presence of 1 mg/ml HRP (1000 U/mg, Sigma) for 1 h at 37°C, and the endocytosed HRP was assayed as described (Bucci *et al.*, 1992). HRP uptake values were calculated as per cent of that endocytosed in mock-transfected cells (which were infected with vT7 and treated with DOTAP, but without added DNA) and were corrected for differences in transfection efficiency (which ranged between 50 and 90%), as determined by immunofluorescence microscopy of parallel samples.

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