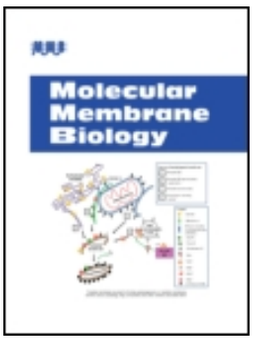

Molecular Membrane Biology

ISSN: 0968-7688 (Print) 1464-5203 (Online) Journal homepage: http://www.tandfonline.com/loi/imbc20

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To cite this article: Karin Mohrmann, & Peter van der Sluijs (1999) Regulation of membrane transport through the endocytic pathway by rabGTPases, Molecular Membrane Biology, 16:1, 81-87, DOI: 10.1080/096876899294797

To link to this article: https://doi.org/10.1080/096876899294797

Published online: 09 Jul 2009.

Molecular Membrane Biology, 1999, 16, 81–87

Regulation of membrane transport through the endocytic pathway by rabGTPases

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Summary

Small GTP binding proteins of the rab family are associated with the cytoplasmic surface of compartments of the central vacuolar system. Several of them, including rab5, rab4 and rab11, are localized to early endocytic organelles where they regulate distinct events in the transferrin receptor pathway. Whereas rab5 is controlling transport to early endosomes, rab4 and rab11 are involved in the regulation of recycling back to the plasma membrane. How GTP-hydrolysis of rab bound GTP is related to the role of these proteins in endocytosis is not yet known, but quick progress is being made towards this goal through the identification of proteins regulating the activity of these rab proteins.

Keywords: rab GTPases, effectors, endosomes, membrane recycling.

Receptor-mediated endocytosis

All eukaryotic cells internalize solutes and macromolecules from their environment in a process otherwise known as (receptor mediated) endocytosis. Ligands bind to specific receptor molecules on the cell surface and move in the lateral plane of the membrane into clathrin coated pits. Alternatively, ligand-receptor complexes are triggered to concentrate in clathrin coated pits. Clathrin coated pits pinch off the plasma membrane to form clathrin coated vesicles that rapidly loose their clathrin coat and fuse with early endosomes. The internal milieu of the early endosome is mildly acidic pH (pH = 6.2–6.5) which causes dissociation of pH-sensitive receptor ligand complexes. Receptors, such as the LDL receptor, are usually recycled back to the plasma membrane, whereas ligands enter the degradative pathway to late endosomes/lysosomes. Thus, once internalized, the low internal pH of early endosomes provides a mechanism to sort ligands from their receptors. Other ligands remain bound to their receptor in early endosomes, e.g. endocytosed lysosomal enzymes complexed to the cation independent mannose 6-phosphate receptor (CI-MPR) are transported from early endosomes to late endosomes. In the lumen of the more acidic late endosome (pH = 5.0–5.5), lysosomal enzymes dissociate from the CI-MPR. The receptor is recycled to the trans Golgi network (TGN) and the ligand is transported to lysosomes.

Although the name early endosome implies it to be a single compartment, this clearly is an oversimplification. This was initially demonstrated by Yamashiro *et al.* (1984) in CHO cells using FITC-transferrin as endocytic tracer. They observed that transferrin is first transported from the cell surface to numerous small cytoplasmic structures. After 5 min, FITC-transferrin begins to appear in crescent shaped organelles in the perinuclear area. In these so-called sorting endosomes, Fe^{3+} dissociates from FITC-transferrin and from here the tracer is transported to a paranuclear recycling endosome, a compact discrete organelle juxtaposed to the nucleus. Thus, the sorting endosome where dissociation of ligand-receptor complexes occurs, is distinct from the perinuclear recycling endosome. In this organelle, intracellular FITC-transferrin accumulates 10–15 min after internalization before it is recycled back to the cell surface. In this respect, the heterogeneity of the early endosome is analogous to that of the Golgi complex, where the different cisternae have distinct biochemical functions (Mellman and Simons 1992). The residence time of endocytosed transferrin is less than 15 min, as measured using biochemical assays in a variety of cell lines. This means that there is a short circuit pathway via which internalized transferrin is recycled with faster kinetics than via the recyling compartment to the plasma membrane.

Proteins involved in transport through early endosomes

As early endosomes are highly dynamic structures involved in multiple vesicle budding and fusion events, it is clear that these reactions have to be tightly regulated to maintain the structural and functional integrity of the compartment. Although the pathway is relatively well described at the phenomenological level, it is only in the last decade that the first proteins were identified regulating the various transport steps to and from early endosomes. Probably the best characterized regulatory proteins include members of the family of monomeric rab GTPases that were shown to be critically involved in transport between compartments of the early endocytic pathway (Novick and Zerial 1997). Although significant progress has been made in elucidating the role of rab proteins in endocytosis, it remains to be established how GTP hydrolysis of rab proteins is wired to the activity of the conserved membrane fusion machinery of SNARE proteins. Small transmembrane proteins of the SNARE family were more recently identified on early endocytic compartments (McMahon *et al.* 1993, Advani *et al.* 1998, Tang *et al.* 1998, Wong *et al.* 1998a,b), a description of their role in the endocytic pathway is, however, beyond the scope of this review.

The GTPase switch of rab proteins

After the initial discovery in Saccharomyces cerevisiae that mutations in the SEC4 (Salminen and Novick 1987) and YPT1 (Segev *et al.* 1988) genes cause secretion defects and that the products of these genes were members of the ras superfamily of small GTPases, a search for homologues was initiated in mammalian cells. Today, more than 40 of these so

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called rab GTPases are known in mammalian cells, many of which have been localized to the cytoplasmic surface of compartments of the central vacuolar system, where they appear to be involved in one or more steps in intracellular transport (Olkkonen and Stenmark 1997).

Newly synthesized rab proteins are posttranslationally modified on carboxy terminal cysteine residues. Following synthesis, rab proteins associate with the cytosolic rab escort protein (REP) (Andres *et al.* 1993). REP-bound rab is next delivered to the heterodimeric enzyme geranylgeranyltransferase type II and subjected to isoprenylation of both cysteines in the CXC, CC and CCXY motifs. Geranylgeranyl groups render a rab protein hydrophobic and are required for reversible membrane association (Alexandrov *et al.* 1994, Wilson *et al.* 1996). A subgroup of rab proteins with the CXC motif is further modified on the last cysteine by carboxymethyl transferase. However, this modification is not required for membrane association. The doubly geranylgeranylated rab protein is thought to remain associated with REP, that delivers the GTPase to a specific organelle or transport vesicle. Upon binding to unknown receptors (Ullrich *et al.* 1994, Ayad *et al.* 1997, Dirac-Svejstrup *et al.* 1997), REP is dissociated and the rab in its GDP-bound form is converted through the action of a guanine nucleotide exchange protein to the GTP-bound state. Membrane-bound rabGTP is stabilized by the recruitment of cytosolic effector proteins (cf below) that, via an incompletely understood mechanism, regulate the activity of a downstream v/t SNARE complex. Possibly, the rabGTP-dependent assembly of docking factors might confer vectoriality to vesicular transport between two compartments. At some point in time, intrinsic GTP hydrolysis of the rab protein, assisted by a GTPase activating protein converts the active rab back to the GDP-bound form. Membrane bound rabGDP is next released from the membrane by the GDP dissociation inhibitor (GDI) and translocated to the cytosol as a rab-GDI complex (Ullrich *et al.* 1993). GDI now assists in the initiation of a new cycle by targeting bound rab to a specific organelle analogous to the role of REP in the delivery of newly synthesized rab to the organelle (figure 1).

Several rab proteins are localized to organelles of the early endocytic pathway. Rab4 and rab5 are both associated with early endosomes and differentially regulate membrane transport through this compartment (Chavrier *et al.* 1990, van der Sluijs *et al.* 1991). Rab11 is on the pericentriolar recycling endosome (Ullrich *et al.* 1996) and the TGN (Urbe *et al.* 1993) and regulates recycling of the transferrin receptor to the plasma membrane.

Rab5

Several isoforms of rab5 have been identified (Bucci *et al.* 1995) and named rab5a, rab5b, and rab5c, of which rab5a is the best characterized isoform. Most likely, the three isoforms have the same intracellular localization. Although initially identified on early endosomes, the presence of rab5a was also demonstrated on the plasma membrane and clathrin coated vesicles. In Saccharomyces cerevisiae, the rab5 homologues Ypt51p, Ypt52p and Ypt53p are associated

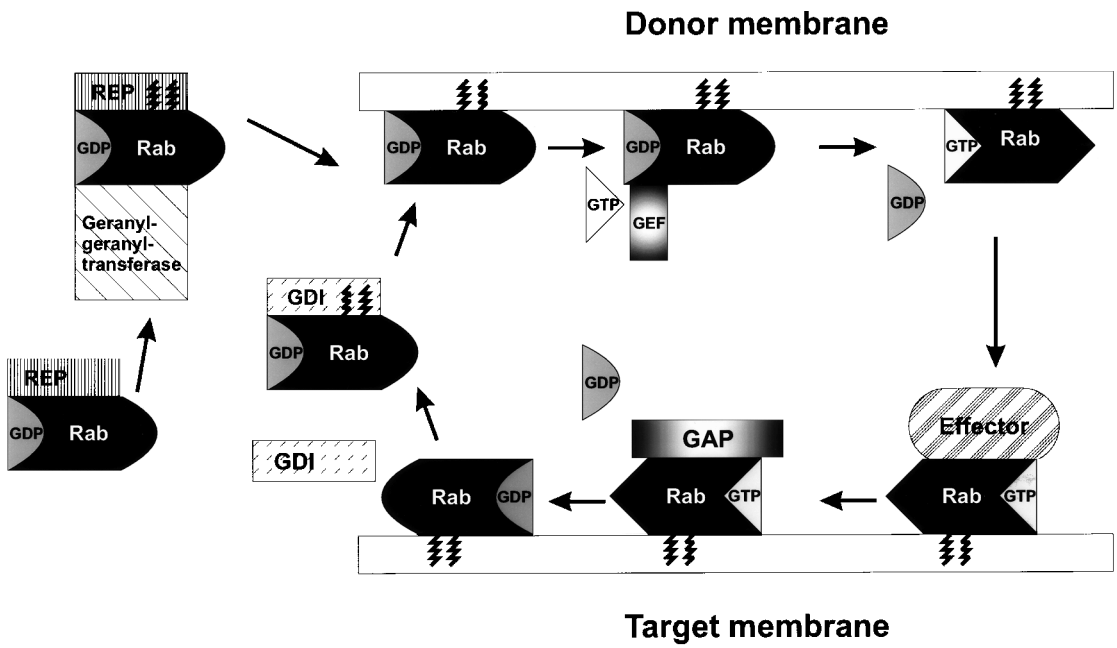

Figure 1. Functional cycle of rabGTPases. Shortly after synthesis, newly synthesized rab protein associates with the Rab Escort Protein (REP) which serves to deliver rabGDP to geranylgeranyltransferase type II. Upon prenylation rabGDP-REP complex is targeted to a membrane receptor on the donor organelle or transport vesicle and REP is displaced by a membrane-bound displacement protein. Subsequently, rabGDP is activated to rabGTP by a guanine nucleotide exchange protein which prevents association with REP and GDP Dissociation Inhibitor (GDI). The transport vesicle buds off the donor compartment and is transported to the target organelle. Docking of the transport vesicle at this site requires rabGTP and recruited effector proteins. Catalyzed GTP hydrolysis by a GTPase activating protein converts rabGTP to inactive rabGDP that is subsequently extracted from the membrane by GDI. GDI serves to maintain rabGDP soluble in the cytoplasm and deliver it for the next cycle to the donor organelle.

with early endosomes and are required for transport to early endosomes (Kruger *et al.* 1994) and protein sorting to the vacuole (Horazdovski *et al.* 1994). The YPT51, YPT52 and YPT53 genes are not essential, since single, double and even triple deletion strains appear to be viable albeit at reduced growth rates.

In vitro, rab5a and the rab5aQ79L GTP hydrolysis deficient mutant stimulate homotypic early endosome fusion (Stenmark *et al.* 1994). In contrast, the dominant negative rab5aN133I mutant with a low affinity for guanine nucleotides, and rab5aS34N that preferentially binds GDP, both inhibit early endosome fusion (Stenmark *et al.* 1994, Gorvel *et al.* 1991). The fact that the XTP binding mutant rab5aD136N stimulates early endosome fusion and even more when bound to the non-hydrolyzable analogue XTPγS shows that endosome fusion does not require XTP or GTP-hydrolysis (Barbieri *et al.* 1994, Rybin *et al.* 1996). It has, therefore, been proposed that rab5 may act as a timer in controlling the fusion of early endosomes (Aridor and Balch 1996, Rybin *et al.* 1996). Although seemingly attractive, it remains to be seen whether the timer model for rab action in membrane fusion is valid *in vivo* or for other rab proteins regulating heterotypic membrane fusion.

One of the most striking phenotypes obtained after expression of rab5a and rab5aQ79L in mammalian cells is the expansion of early endocytic organelles, whereas expression of rab5aS34N and rab5aN133I causes fragmentation of early endosomes. Wild type rab5a and rab5aQ79L selectively increase the rate of fluid-phase endocytosis and transferrin uptake, while rab5aS34N and rab5aN133I inhibit transferrin endocytosis (Bucci *et al.* 1992, Stenmark *et al.* 1994), suggesting that rab5 is involved in the fusion of coated vesicles with early endosomes. In polarized epithelial cells transfected rab5 localized to apical and basolateral endosomes and enhanced fluid phase endocytosis from both plasma membrane domains (Bucci *et al.* 1994). This indicates that rab5 is involved in both apical and basolateral endocytosis. Consistent with the previously observed parallels in membrane transport between polarized MDCK cells and cultured hippocampal neurons (Dotti and Simons 1990), rab5a colocalizes with synaptophysin-labelled structures and somatodendritic and axonal early endosomes (de Hoop *et al.* 1994). Apart from a role in vesicle fusion, rab5 also appears to regulate the formation of transport vesicles. In the search for novel cytosolic proteins required for coated pit invagination at the plasma membrane, McLaughlan *et al.* (1998) purified a stimulating activity that upon protein sequencing turned out be the rab5-GDI complex. Possibly, the coordination of the activity of rab proteins at both vesicle budding and fusion sites would provide a feedback loop to ensure that the amount of vesicles consumed through fusion with a target organelle is balanced by the number of vesicles formed from the donor organelle.

Rab4

Two isoforms of rab4 have been cloned and named rab4a and rab4b. Using subcellular fractionation and immunofluorescence microscopy it was previously shown that rab4a is localized to early endosomes (van der Sluijs *et al.* 1991, Bottger *et al.* 1996). Overexpression of wild-type rab4 in mammalian cells reduces the intracellular accumulation of the fluid-phase tracer horse radish peroxidase and transferrin receptors are redistributed from endosomes to the plasma membrane (van der Sluijs *et al.* 1992a). Furthermore, the discharge of iron from transferrin in early endosomes is inhibited, suggesting that internalized transferrin is mistargeted to a population of non-acidic vesicles and tubules. Rab4 thus appears to control the function or formation of endosomes involved in recycling of internalized transferrin back to the plasma membrane. In pulse chase experiments using FITC-labelled transferrin, it was shown at early time points that the fluorescent label first appeared in rab4 containing endocytic organelles before it was transported to a tubulovesicular organelle in the area of the microtubule organizing centre (Daro *et al.* 1996). This compartment was found to be enriched in the v-SNARE cellubrevin, which is involved in regulating transferrin recycling (Galli *et al.* 1994). Thus, the rab4 containing endosomal compartment in the transferrin-recycling route is biochemically distinct from the pericentriolar recycling endosome (Daro *et al.* 1996).

As rab4 is relatively depleted from recycling endosomes it was difficult to assess whether it controls transport from sorting endosomes to the recycling endosome or direct recycling to the plasma membrane. Several lines of evidence, however, suggest that the indirect recycling pathway from early endosomes to the recycling endosome is regulated by rab4. First, *in vivo*, the Q67Lrab4 GTP hydrolysis deficient mutant which is thought to be stabilized in its association with the target organelle, is associated with a perinuclear compartment (Mohrmann and van der Sluijs, in preparation). Secondly, cotransfection of rab4 and its effector rabaptin4 causes the expansion of a perinuclear compartment that is enriched in cellubrevin, and contains the majority of internalized Cy3 labelled transferrin at steady state. These are the hallmarks of the perinuclear recycling endosome and suggest that rab4 acts in the indirect recycling pathway (Nagelkerken and van der Sluijs, submitted).

Although it is thought that isoforms of ubiquitously expressed rab proteins have similar functions, it is unknown whether these isoforms have similar or distinct spatiotemporal expression patterns in tissues of multicellular organisms. Interestingly, the levels of rab4 appear to be regulated during development in the rat pancreas (Valentijn *et al.* 1997). Unfortunately, it is not clear whether one of the rab4 isoforms, or both, are subject to this regulation and whether regulation occurs at the transcriptional or at the protein level. At least one difference is obvious from comparing the rab4a and rab4b amino acid sequences. The carboxy terminal hypervariable region of rab4a, but not of rab4b, contains a concensus sequence for phosphorylation by $p34^{cdc2}$ kinase (Bailly *et al.* 1991). Indeed, rab4a is reversibly phosphorylated on Ser196 during mitosis by $p34^{cdc2}$ kinase or another cell cycle dependent kinase (van der Sluijs *et al.* 1992b). Phosphorylation occurs on both the cytoplasmic and membrane-bound pools of rab4a (Gerez and van der Sluijs, submitted) and results in the accumulation of rab4a in the cytoplasm where it presumably can not participate in endosomal functions. Membrane transport through the endocytic and biosynthetic pathways in higher eukaryotes is inhibited during mitosis (Warren 1993). Several proteins of the vesicular transport

machinery regulating transport through the Golgi complex are phosphorylated and do not exhibit their normal interphase interactions (Lowe *et al.* 1998). It is thought that phosphorylation of key components is responsible for the inhibition of membrane transport in the endocytic pathway. Mitotic phosphorylation of rab4 might, therefore, be part of the mechanism to downregulate endocytosis during mitosis.

Rab4 and rab5 associate with distinct but overlapping early endosomal structures

Although rab4 and rab5 are both associated with early endocytic organelles, their comparative distribution within the same cells has not been assessed before. Because the available antibodies against rab4 and rab5 do not allow the detection of the endogenous proteins in morphological assays, VSV-G tagged rab5 and NH-tagged rab4 (Nagelkerken *et al.* 1997) were stably transfected in CHO cells. The cells were processed for immunofluorescence microscopy and labelled with the specific epitope tag antibodies. The cells were then examined by confocal scanning laser microscopy. As shown in figure 2, rab4 (green) is mainly localized around the nucleus and scattered structures throughout the cytoplasm. Rab5 (red), in contrast, is localized to more peripheral locations in the cytoplasm. It is clear from figure 2 that rab4 and rab5 have significantly overlapping distributions in the perinuclear area, as evidenced by the yellow colour of the merged images. In addition, a class of singly labelled rab4 structures, not containing rab5, and vice versa, a class of rab5 labelled structures not containing rab4, co-exist in the peripheral cytoplasm. Thus, rab5 and rab4 appear to localize to distinct but overlapping early endocytic organelles. As overexpression of rab5 and rab4 produced opposing effects on transport through the endocytic pathway the role of the two GTPases in an *in vitro* assay reconstituting homotypic early endosome fusion and fission were also assessed. As expected, recombinant rab5 stimulates early endosome fusion (Gorvel *et al.* 1991). Although rab4 does not affect this fusion event, it specifically enhances fission of the intermediate that arose from the rab5-dependent fusion of early endosomes (Chavrier *et al.* 1997). These results are consistent with the notion that rab5 regulates the influx of molecules into early endosomes, whereas rab4 is important for the exit of molecules from this compartment.

Rab11

In addition to rab5 and rab4, a third rab protein is associated with early endocytic organelles. Rab11 is localized to the TGN and the pericentriolar recycling endosome. Rab11S25N inhibits transport of internalized transferrin from the recycling endosome compartment to the plasma membrane and causes fragmentation of this compartment into the cytoplasm. Transfection of rab11Q70L leads to compaction of the recycling endosome, but also inhibits recycling of transferrin (Ullrich *et al.* 1996). Through the use of a low temperature block to inhibit transport between sorting endosomes and the recycling endosome, Ren *et al.* (1998) recently showed that rab11S25N inhibits transferrin recycling, whereas rab11Q70L and wild type rab11 do not show this inhibitory effect. This suggests that activation of rab11 by GTP is required for the exit of the transferrin receptor, regardless of whether this occurs towards the recycling endosome, from the recycling endosome to the TGN, or directly from the early sorting endosomes to the plasma membrane. In conclusion, it is not entirely clear which transport steps during membrane recycling is (are) regulated by rab11. Nevertheless, the picture emerges that at least two rab proteins, rab4 and rab11 are required for recycling of transferrin from sorting endosomes to the plasma membrane.

Rab7 and rab9 regulate transport to and from late endocytic compartments

Late endosomes are a crossroads for transport of CI-MPR to the TGN and of lysosomal enzymes and material to be degraded to the lysosomes (Cornfeld and Mellman 1989). Not surprisingly, several rab proteins including rab7 (Chavrier *et al.* 1990) and rab9 (Lombardi *et al.* 1993), but not rab4 and rab5 (Bottger *et al.* 1996) have been localized to this compartment. In cells transfected with the inhibitory mutants

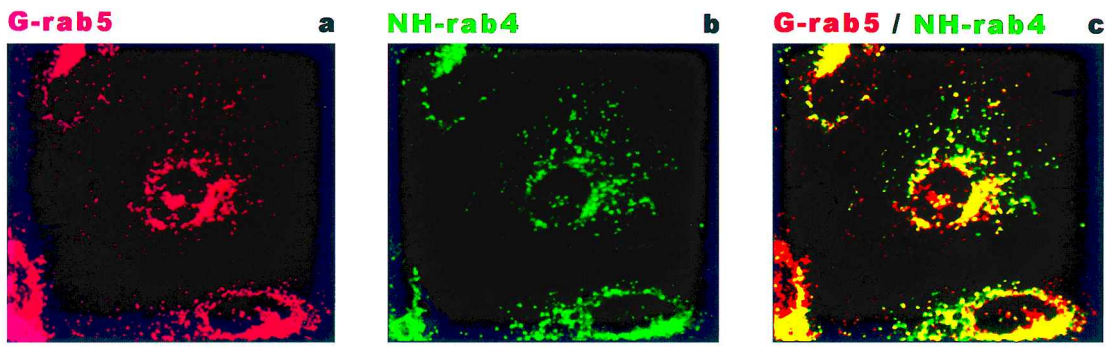

Figure 2. Rab4 and rab5 have distinct but overlapping distributions. CHO cells were stably transfected with NH tagged rab4 and VSV-G tagged rab5. Transfectants were grown on coverslips and double-labelled with rabbit antibodies against the NH tag and the mouse monoclonal P5D4 against the cytoplasmic tail of VSV-G protein. After washing, the cells were counterstained with DTAF-labelled goat anti-rabbit IgG (green, rab4) and TRITC-labelled goat anti-mouse IgG (red, rab5) and examined with a confocal scanning laser microscope. Note that in the merged image (C), in addition to organelles only containing rab4 or rab5, there are also membranes that appear to contain both small GTPases.

rab7N125I and rab7S22N, internalized VSV-G protein becomes trapped in early endosomes (Feng *et al.* 1995). These mutants also partially inhibit the cleavage of paramyxovirus SV5 haemagglutinin neuraminidase, the degradation of low-density protein and cause the accumulation of cathepsin D and CI-MPR in an early endocytic compartment (Press *et al.* 1998). Thus, mammalian rab7 is involved in transport from early to late endosomes. In contrast, Ypt7p, the Saccharomyces cerevisiae homologue of rab7, primarily regulates transport from late endosomes to the vacuole (Wichmann *et al.* 1992), suggesting that transport pathways to late endocytic compartments are not completely conserved between mammals and yeast. Alternatively, as rab7 and Ypt7p are not identical, differences in the amino acid composition might confer distinct properties to the two proteins. Rab9 is involved in transport from late endosomes to the TGN. *in vitro* transport of CI-MPR from late endosomes to the TGN is enhanced by addition of recombinant rab9 and selectively inhibited by antibodies against rab9 (Lombardi *et al.* 1993) or its effector protein p40 (Diaz *et al.* 1997). In cells transfected with the rab9S21N mutant, recycling of the CI-MPR from late endosomes to the TGN as well as delivery of newly synthesized lysosomal enzymes to late endosomes are inhibited (Riederer *et al.* 1994).

Other rab proteins associated with endocytic organelles

Several other, less well-studied rab proteins are associated with endocytic structures. Rab17 is localized to the plasma membrane of kidney proximal tubule epithelial cells and the expression of rab17 is induced during cell polarization in the developing kidney (Lütcke *et al.* 1993). Recently, it was shown that rab17 co-localized with internalized transferrin in the perinuclear recycling endosome of BHK-21 cells and with the apical recycling endosome in polarized Eph4 cells (Zacchi *et al.* 1998). Basolateral to apical transport of the transferrin receptor and of a chimeric Fc receptor was increased in cells transfected with rab17N131I, or rab17Q77L, suggesting a role for rab17 in regulating traffic through the apical recycling endosome (Zacchi *et al.* 1998). Others have shown an inhibition of transcytosis of the polymeric IgA receptor in MDCK cells transfected with rab17 (Hunziker and Peters 1998). The results of these two studies, however, can not be directly compared, since different rab17 constructs were expressed in the cells. Rab18 and Rab20 were detected on the apical dense tubules, underlying the apical plasma membrane of kidney tubule epithelial cells and rab22 is mainly localized to large perinuclear vesicle-like structures (Olkkonen *et al.* 1993). The role of these rab proteins in endocytosis has not been investigated and awaits functional expression studies.

Proteins regulating the activity of early endosomal rabGTPases

Understanding the mechanism how rab4 and rab5 regulate transport through early endocytic organelles, requires the identification of accessory proteins regulating the activity of the two GTPases and the identification of their downstream target effector molecules. Using the yeast two-hybrid system, Zerial and colleagues identified two effector proteins (Stenmark *et al.* 1995, Gournier *et al.* 1998). Rabaptin5 is a cytosolic 100 kDa protein with several putative coiled-coil domains that specifically interacts with the active GTP-bound form of rab5. The extensive α-helical coils cause homodimerization of rabaptin5. A fraction of rabaptin5 colocalizes with rab5 on peripheral early endosomes, and transfection of rab5Q79L causes recruitment of rabaptin5 to swollen early endosomes. The rab5 binding site is situated in the carboxy terminal 73 amino acids of rabaptin5 and is sufficient for rab5-dependent recruitment to early endosomes. Overexpression of rabaptin5 alone already promotes the expansion of the endosomes, as seen for rab5 and rab5Q79L. Rabaptin5 is essential for early endosome fusion, since cytosol depleted of rabaptin5 inhibits endosome fusion (Stenmark *et al.* 1995). Rabaptin5 contains a distinct binding domain for rab4 in the the amino-terminus (Vitale *et al.* 1998). This suggests that rabaptin5 may connect the activities of rab4 and rab5, the two GTPases associated with early endosomes. Rabaptin5 binds to the TSC2 gene product tuberin, which accelerates GTP hydrolysis on rab5 and inhibits endocytosis (Xiao *et al.* 1997). Mutations in the TSC2 and TSC1 genes are associated with tuberosclerosis. Tuberin is in a complex with hamartin (van Slegtenhorst *et al.* 1998), the product of TSC1, the second gene associated with the disease (van Slegtenhorst *et al.* 1997). Currently, the putative role of the hamartin-tuberin protein complex in the regulation of the endocytic pathway is being investigated. A novel rabaptin was recently identified in a two hybrid screen using rab4Q67L as a bait (Nagelkerken and van der Sluijs, submitted). Rabaptin4 is highly homologous to rabaptin5 and specifically interacts with rab4GTP. Rabaptin4 contains a rab4 binding domain in the amino terminus, and rab5 binding sites in the amino terminus as well as the carboxy terminus. Rabaptin4 and rabaptin5 heterodimerize (van der Sluijs, unpublished observation) suggesting that rabaptins may provide a scaffold to coordinate the activity of rab proteins in the early endocytic pathway.

Recently, Zerial and colleagues identified rabaptin5β. This 62 kDa cytosolic protein shares 42% sequence identity with rabaptin5 (Gournier *et al.* 1998). Rabaptin5β is also recruited to endosomes in a GTP-dependent manner. The relative expression levels of rabaptin5 and rabaptin5β vary among different cell types. Rabaptin5β does not heterodimerize with rabaptin5 and forms a distinct complex with the rab5-specific guanine nucleotide exchange factor rabex5 (Horiuchi *et al.* 1997). Cytosol immunodepleted of rabaptin5β only partially inhibits early endosome fusion *in vitro*. Stronger inhibition is seen when rabaptin5 in addition to rabaptin5β is depleted, suggesting that maximal fusion efficiency at least requires both proteins. These results indicate that both effectors cooperate in the regulation of the first step in endocytosis (Gournier *et al.* 1998). During apoptosis, rabaptin5 is proteolytically cleaved by members of the family of apoptotic caspases. The selective cleavage of rabaptin5 is responsible for the loss of early endosome fusion activity (Cosulich *et al.* 1997) *in vitro* and is possibly related to, or part of the mechanism giving rise to, general membrane fragmentation in apoptotic cells. It is not known yet whether rabaptin4 and rabaptin5β are to caspase-mediated cleavage during apoptosis.

Finally the early endosome associated antigen EEA1 (Stenmark *et al.* 1996), was recently shown to be a third rab5 effector, and required for homotypic early endosome fusion (Jones *et al.* 1998). In addition to an amino-terminal rab5 binding domain, EEA1 contains a carboxy-terminal FYVE finger that is required for early endosome association as well as PtdIns(3) binding (Gaullier *et al.* 1998, Simonsen *et al.* 1998). The presence of two spatially separate binding domains for rab5 and PtdIns(3) might promote specific recruitment of EEA1 to early endosomes and confer directionality to transport through the endocytic pathway. As rab5 is localized to the plasma membrane, coated vesicles and early endosomes, it now will become interesting to establish where the distinct effectors act *in vivo* and how their activities are coordinated.

Conclusion

In conclusion, it is anticipated that the robust approaches used to identify the first proteins regulating membrane transport in the early endocytic pathway, in the near future will assist in unravelling the molecular mechanism underlying the biogenesis of early endosomes.

Acknowledgements

We thank our colleagues at the Department of Cell Biology for helpful suggestions. This work was supported by grants from the Netherlands Organization for Medical Research (to PvdS). PvdS is an investigator of the Royal Netherlands Academy of Arts and Sciences.

References

Advani, R. J., Bae, H. R., Bock, J. B., Chao, D. S., Doung, Y. C., Prekeris, R., Yoo, J. S., and Scheller, R. H., 1998, Seven novel mammalian SNARE proteins localize to distinct membrane compartments. *Journal of Biological Chemistry*, **273**, 10317–10324.

Alexandrov, K., Horiuchi, H., Steele-Mortimer, O., Seabra, M., and Zerial, M., 1994, Rab escort protein-1 is a multifunctional protein that accompanies newly prenylated rab proteins to their target membranes. *EMBO Journal*, **13**, 5262–5273.

Andres, D. A., Seabra, M., Brown, M., Armstrong, S. A., Smeland, T. E., Cremers, F. P. M., and Goldstein, J. L., 1993, cDNA cloning of component A of rab geranylgeranyl transferase and demonstration of its role as a rab escort protein. *Cell*, **73**, 1091–1100.

Aridor, M., and Balch, W. E., 1996, Membrane fusion: timing is everything. *Nature*, **383**, 220–221.

Ayad, N., Hull, M., and Mellman, I., 1997, Mitotic phosphorylation of rab4 prevents binding to a specific receptor on endosomes. *EMBO Journal*, **16**, 4497–4507.

Bailly, E., Touchot, N., Zahraoui, A., Goud, B., and Bornens, M., 1991, $p34^{cdc2}$ protein kinase phosphorylates two small GTP-binding proteins of the rab family. *Nature*, **350**, 715–718.

Barbieri, M. A., Li, G., Colombo, M. I., and Stahl, P. D., 1994, Rab5, an early acting endosomal GTPase supports *in vitro* endosome fusion without GTP hydrolysis. *Journal of Biological Chemistry*, **269**, 18720–18722.

Bottger, G., Nagelkerken, B., and van der Sluijs, P., 1996, Rab4 and rab7 define distinct endocytic compartments. *Journal of Biological Chemistry*, **271**, 29191–29197.

Bucci, C., Lütcke, A., Mortimer, O. S., Olkkonen, V. M., Dupree, P., Chiariello, M., Bruni, C. B., Simons, K., and Zerial, M., 1995, Co-operative regulation of endocytosis by three rab5 isoforms. *FEBS Letters*, **366**, 65–71.

Bucci, C., Parton, R., Mather, I., Stunnenberg, H., Simons, K., and Zerial, M., 1992, The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell*, **70**, 715–728.

Bucci, C., Wandinger-Ness, A., Lütcke, A., Chiarello, M., Bruni, C. B., and Zerial, M., 1994, Rab5a is a common component of the apical and basolateral endocytic machinery in polarized epithelial cells. *Proceedings of the National Academy of Sciences (USA)*, **91**, 5061–5065.

Chavrier, P., Parton, R. G., Hauri, H. P., Simons, K., and Zerial, M., 1990, Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell*, **62**, 317–329.

Chavrier, P., van der Sluijs, P., Mishal, Z., Nagelkerken, B., and Gorvel, J. P., 1997, Early endosome dynamics characterized by flow cytometry. *Cytometry*, **29**, 41–49.

Cosulich, S. C., Horiuchi, H., Zerial, M., Clarke, P. R., and Woodman, P. G., 1997, Cleavage of rabaptin-5 blocks endosome fusion during apoptosis. *EMBO Journal*, **16**, 6182–6191.

Daro, E., van der Sluijs, P., Galli, T., and Mellman, I., 1996, Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling. *Proceedings of the National Academy of Sciences (USA)*, **93**, 9559–9564.

de Hoop, M., Huber, L. A., Stenmark, H., Williamson, E., Zerial, M., Parton, R. G., and Dotti, C. G., 1994, The involvement of the small GTP-binding protein rab5a in neuronal endocytosis. *Neuron*, **13**, 11–22.

Diaz, E., Schimmoller, F., and Pfeffer, S. R., A novel rab9 effector required for endosome to TGN transport. *Journal of Cell Biology*, **138**, 283–290.

Dirac-Svejstrup, A. B., Sumizawa, T., and Pfeffer, S. R., 1997, Identification of a GDI displacement factor that releases endosomal Rab GTPases from Rab-GDI. *EMBO Journal*, **16**, 465–472.

Dotti, C. G., and Simons, K., 1990, Polarized sorting of viral glycoproteins to the axonal and dendrites of hipocampal neurons in culture. *Cell*, **62**, 63–72.

Feng, Y., Press, B., and Wandinger Ness, A., 1995, Rab7: an important regulator of late endocytic membrane traffic. *Journal of Cell Biology*, **131**, 1435–1452.

Galli, T., Chilcote, T., Mundigl, O., Binz, T., Niemann, H., and DeCamilli, P., 1994, TTX mediated cleavage of cellubrevin impairs exocytosis of transferrin receptor containing vesicles in CHO cells. *Journal of Cell Biology*, **125**, 1015–1024.

Gaullier, J. M., Simonsen, A., D'Arrigo, A., Bremnes, B., Stenmark, H., and Aasland, R., FYVE fingers bind PtdIns(3)P. *Nature*, **394**, 432–433.

Gorvel, J. P., Chavrier, P., Zerial, M., and Gruenberg, J., 1991, rab5 controls early endosome fusion *in vitro*. *Cell*, **64**, 915–925.

Gournier, H., Stenmark, H., Rybin, V., Lippe, R., and Zerial, M., 1998, Two distinct effectors of the small GTPase rab5 cooperate in endocytic membrane fusion. *EMBO Journal*, **17**, 1930–1940.

Horazdovski, B. F., Busch, G. R., and Emr, S., 1994, VPS21 encodes a rab5-like GTP binding protein that is required for the sorting of yeast vacuolar proteins. *EMBO Journal*, **13**, 1297–1309.

Horiuchi, H., Lippe, R., McBride, H. M., Rubino, M., Woodman, P., Stenmark, H., Rybin, V., Wilm, M., Ashman, K., Mann, M., and Zerial, M., 1997, A novel rab5 GDP/GTP exchange factor complexed to rabaptin-5 links nucleotide exchange to effector recruitment and function. *Cell*, **90**, 1149–1159.

Hunziker, W., and Peters, P. J., 1998, Rab17 localizes to recycling endosomes and regulates receptor mediated transcytosis in epithelial cells. *Journal of Biological Chemistry*, **273**, 15734–15741.

Jones, A. T., Mills, I. G., Scheidig, A. J., Alexandrov, K., and Clague, M. J., 1998, Inhibition of endosome fusion by wortmannin persists in the presence of activated rab5. *Molecular Biology of the Cell*, **9**, 323–332.

Kornfeld, S., and Mellman, I., 1989, The biogenesis of lysosomes. *Annual Review of Cell Biology*, **5**, 483–525.

Kruger, S. B., Stenmark, H., Dusterhoft, A., Philippsen, P., Yoo, J. S., Gallwitz, D., and Zerial, M., 1994, Role of three rab5-like GTPases, Ypt51p, Ypt52p, and Ypt53p, in the endocytic and vacuolar protein sorting pathways of yeast. *Journal of Cell Biology*, **125**, 283–298.

Lombardi, D., Soldati, T., Riederer, M., Zerial, M., and Pfeffer, S., 1993, Rab 9 functions in transport between late endosomes and the trans Golgi network. *EMBO Journal*, **12**, 677–682.

Lowe, M., Nakamura, N., and Warren, G., 1998, Golgi division and membrane traffic. *Trends in Cell Biology*, **8**, 40–44.

Lütcke, A., Jansson, S., Parton, R., Chavrier, P., Valencia, A., Huber, L., Lehtonen, E., and Zerial, M., 1993, Rab17, a novel small GTPase, is specific for epithelial cells and is induced during cell polarization. *Journal of Cell Biology*, **121**, 553–564.

McLaughlan, H., Newell, J., Morrice, N., Osborne, A., West, M., and Smythe, E., 1998, A novel role for rab-GDI in ligand sequestration into clathrin-coated pits. *Current Biology*, **8**, 34–45.

McMahon, H. T., Ushkayov, Y. A., Edelmann, L., Link, E., Binz, T., Niemann, H., Jahn, R., and Südhof, T. C., 1993, Cellubrevin is a ubiquitous ttx substrate homologous to a putative synaptic vesicle fusion protein. *Nature*, **364**, 346–349.

Mellman, I. and Simons, K., 1992, The Golgi complex: *in vitro* veritas? *Cell*, **68**, 829–840.

Nagelkerken, B., Mohrmann, K., Gerez, L., van Raak, M., Leijendekker, R. L., and van der Sluijs, P., 1997, A novel epitope tag for the detection of rab GTPases. *Electrophoresis*, **18**, 2694–2698.

Novick, P. and Zerial, M., 1997, The diversity of rab proteins in vesicle transport. *Current Opinion in Cell Biology*, **9**, 496–504.

Olkkonen, V., and Stenmark, H., 1997, Role of rabGTPases in membrane traffic. *International Reviews of Cytology*, **126**, 1–85.

Olkkonen, V. M., Dupree, P., Killisch, I., Lütcke, A., Zerial, M., and Simons, K., 1993, Molecular cloning and subcellular localization of three GTP binding proteins of the rab subfamily. *Journal of Cell Science*, **106**, 1249–1261.

Press, B., Feng, Y., Hoflack, B., and Wandinger-Ness, A., 1998, Mutant rab7 causes the accumulation of cathepsin D and cation independent mannose 6-phosphate receptor in an early endocytic compartment. *Journal of Cell Biology*, **140**, 1075–1089.

Ren, M., Xu, G., Zeng, J., Chiarandini, C. L., Adesnik, M., and Sabatini, D. D., 1998, Hydrolysis of GTP on rab11 is required for the direct delivery of transferrin from the pericentriolar recycling compartment to the cell surface but not from recycling endosomes. *Proceedings of the National Academy of Sciences (USA)*, **95**, 6187–6192.

Riederer, M., Soldati, T., Shapiro, A. D., Lin, J., and Pfeffer, S. R., 1994, Lysosome biogenesis requires rab9 function and receptor recycling from endosomes to the trans Golgi network. *Journal of Cell Biology*, **125**, 573–582.

Rybin, V., Ullrich, O., Rubino, M., Alexandrov, K., Simon, I., Seabra, M., Goody, R., and Zerial, M., 1996, GTPase activity of rab5 acts as a timer for endocytic membrane fusion. *Nature*, **383**, 266–269.

Salminen, A. and Novick, P. J., 1987, A ras-like protein is required for a post Golgi event in yeast secretion. *Cell*, **49**, 527–538.

Segev, N., Mullholland, J. and Botstein, D., 1988, The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. *Cell*, **52**, 915–924.

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J. M., Brech, A., Callaghan, J., Toh, B. H., Murphy, C., Zerial, M., and Stenmark, H., 1998, EEA1 links PI(3)K function to rab5 regulation of endosome fusion. *Nature*, **394**, 494–498.

Stenmark, H., Aasland, R., Toh, B. H., and D'Arrigo, A., 1996, Endosomal localization of the autoantigen EEA1 is mediated by a Zinc-binding FYVE finger. *Journal of Biological Chemistry*, **271**, 24048–24054.

Stenmark, H., Parton, R., Steele-Mortimer, O., Lütcke, A., Gruenberg, J., and Zerial, M., 1994, Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. *EMBO Journal*, **13**, 1287–1296.

Stenmark, H., Vitale, G., Ullrich, O., and Zerial, M., 1995, Rabaptin-5 is a direct effector of the small GTPase rab5 in endocytic membrane fusion. *Cell*, **83**, 423–432.

Tang, B. L., Tan, A. E. H., Lim, L. K., Lee, S. S., Low, D. Y. H. and Hong, W., 1998, Syntaxin 12, a member of the sdyntaxin family localized to the endosome. *Journal of Biological Chemistry*, **273**, 6944–6950.

Ullrich, O., Horiuchi, H., Bucci, C., and Zerial, M., 1994, Membrane association of rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. *Nature*, **368**, 157–160.

Ullrich, O., Reinsch, O., Urbe, S., Zerial, M., and Parton, R., 1996, Rab11 regulates recycling through the pericentriolar recycling endosome. *Journal of Cell Biology*, **135**, 913–924.

Ullrich, O., Stenmark, H., Alexandrov, K., Huber, L., Kaibuchi, K., Sasaki, T., Takai, Y., and Zerial, M., 1993, Rab GDI as a general regulator for the membrane association of rab proteins. *Journal of Biological Chemistry*, **268**, 18143–18150.

Urbe, S., Huber, L. A., Zerial, M., Tooze, S. A., and Parton, R. G., 1993, Rab11, a small GTPase associated with both constitutive and regulated secretory pathways in PC12 cells. *FEBS Letters*, **334**, 175–182.

Valentijn, J. A., Civita, D. Q., Gumkowski, F. D., and Jamieson, J., 1997, Rab4 associates with the actin terminal web in developing rat pancreatic acinar cells. *European Journal of Cell Biology*, **72**, 1–8.

van der Sluijs, P., Hull, M., Huber, L. A., Male, P., Goud, B., and Mellman, I., 1992b, Reversible phosphorylation-dephosphorylation determines the localization of rab4 during the cell cycle. *EMBO Journal*, **11**, 4379–4389.

van der Sluijs, P., Hull, M., Webster, P., Goud, B., and Mellman, I., 1992a, The small GTP binding protein rab4 controls an early sorting event on the endocytic pathway. *Cell*, **70**, 729–740.

van der Sluijs, P., Hull, M., Zahraoui, A., Tavitian, A., Goud, B., and Mellman, I., 1991, The small GTP binding protein rab4 is associated with early endosomes. *Proceedings of the National Acadamy of Sciences (USA)*, **88**, 6313–6317.

van Slegtenhorst, M., de Hoogt, R., Hermans, C., Nellist, M., Janssen, B., Verhoef, S., Lindhout, D., van den Ouweland, A., Halley, D., Young, J., Burley, M., Jeremiah, S., Woodward, K., Nahmias, J., Fox, M., Ekong, R., Osborne, J., Wolfe, J., Povey, S., Snell, R. G., Cheadle, J. P., Jones, A. C., Tachataki, M., Ravine, D., Sampson, J. R., Reeve, M. P., Richardson, P., Wilmer, F., Munro, C., Hawkins, T. L., Sepp, T., Ali, J. B. M., Ward, S., Green, A. J., Yates, J. R. W., Kwiatkowska, J., Henske, E. P., Short, M. P., Haines, J. H., Jozwiak, S., and Kwiatkowski, D. J., 1997, Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. *Science*, **277**, 805–808.

van Slegtenhorst, M., Nellist, M., Nagelkerken, B., Cheadle, J., Snell, R., van den Ouweland, A., Reuser, A., Sampson, J., Halley, D., and van der Sluijs, P., 1998, Interactions between hamartin and tuberin, the TSC1 and TSC2 gene products. *Human Molecular Genetics*, **7**, 1053–1057.

Vitale, G., Rybin, V., Christoforidis, S., Thornqvist, P. O., McCaffrey, M., Stenmark, H., and Zerial, M., 1998, Distinct rab-binding domains mediate the interaction of rabaptin-5 with GTP-bound rab4 and rab5. *EMBO Journal*, **17**, 1941–1951.

Warren, G., 1993, Membrane partitioning during cell division. *Annual Review of Biochemistry*, **62**, 323–48.

Wichmann, H., Hengst, L., and Gallwitz, D., 1992, Endocytosis in yeast: evidence for the involvement of a small GTP binding protein (Ypt7p). *Cell*, **71**, 1131–1142.

Wilson, A. L., Erdman, R. A., and Maltese, W. A., 1996, Association of rab1b with GDI is required for recycling but not initial membrane targeting of the rab protein. *Journal of Biological Chemistry*, **271**, 10932–10940.

Wong, S. H., Xu, Y., Zhang, T. and Hong, W., 1998a, Syntaxin 7, a novel syntaxin member associated with the early endosomal compartment. *Journal of Biological Chemistry*, **273**, 375–380.

Wong, S. H., Zhang, T., Xu, Y., Subramaniam, V. N., Griffiths, G. and Hong, W., 1998b, Endobrevin, a novel synaptobrevin/VAMP-like protein preferentially associated with early endosome. *Molecular Biology of the Cell*, **9**, 1549–1563.

Xiao, G. H., Shoarinejad, F., Jin, F., Golemis, E., and Yeung, R. S., 1997, The tuberous sclerosis 2 gene product, tuberin, functions as a rab5 GTPase activating protein (GAP) in modulating endocytosis. *Journal of Biological Chemistry*, **272**, 6097–6100.

Yamashiro, D. J., Tycko, B., Fluss, S. R. and Maxfield, F. R., 1984, Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell*, **37**, 789–800.

Zacchi, P., Stenmark, H., Parton, R. G., Orioli, D., Lim, F., Giner, A., Zerial, M., and Murphy, C., 1998, Rab17 regulates membrane trafficking through apical recycling endosomes in polarized epithelial cells. *Journal of Cell Biology*, **140**, 1039–1053.