Rab22a affects the morphology and function of the endocytic pathway

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SUMMARY

Soon after endocytosis, internalized material is sorted along different pathways in a process that requires the coordinated activity of several Rab proteins. Although abundant information is available about the subcellular distribution and function of some of the endocytosis-specific Rabs (e.g. Rab5 and Rab4), very little is known about some other members of this family of proteins. To unveil some of the properties of Rab22a, one of the less studied endosome-associated small GTPases, we have expressed the protein tagged with the green fluorescent protein in CHO cells. The results indicate that Rab22a associates with early and late endosomes (labeled by a 5 minute rhodamine-transferrin uptake and the cation-independent mannose 6-phosphate receptor, respectively) but not with lysosomes (labeled by 1 hour rhodamine horseradish peroxidase uptake followed by 1 hour chase). Overexpression of the protein causes a prominent morphological enlargement of the early and late endosomes. Two mutants were generated by site-directed mutagenesis, a negative mutant (Rab22aS19N, with reduced affinity for GTP) and a constitutively active mutant (Rab22aQ64L, with reduced endogenous GTPase activity). The distribution of the negative mutant was mostly cytosolic, whereas the positive mutant associated with early and late endosomes and, interestingly also with lysosomes and autophagosomes (labeled with monodansylcadaverine). Cells expressing Rab22a wild type and Rab22aS19N displayed decreased endocytosis of a fluid phase marker. Conversely, overexpression of Rab22aQ64L, which strongly affects the morphology of endosomes, did not inhibit bulk endocytosis. Our results show that Rab22a has a unique distribution along the endocytic pathway that is not shared by any other Rab protein, and that it strongly affects the morphology and function of endosomes.

Key words: Intracellular transport, Endocytosis, GTPase, GFP, Endosomes, Rab proteins

INTRODUCTION

Intracellular transport of macromolecules between membrane-bound compartments requires highly regulated membrane fusion events. A large body of evidence indicates that membrane fusion is governed by conserved families of proteins, regardless of the trafficking pathway analyzed. The same basic mechanism is used for homotypic (e.g. fusion between early endosomes) and heterotypic (e.g. the exocytosis of synaptic vesicles) fusion. This conserved fusion mechanism takes place in cell types, as diverse as yeast and mammalian neurons (Gerst, 1999). Two main sets of proteins play crucial roles in intracellular membrane fusion: Rab and Rab-interacting proteins, and SNARE and SNARE-interacting proteins. Rabs constitute a family of small GTPases necessary for membrane fusion (Pfeffer, 1999). Their function is probably related to the recruitment of a complex of Rab-associated proteins capable of tethering the compartments that are going to fuse. Rabs also participate in vesicle budding (McLauchlan et al., 1998; Nuoffer et al., 1994) and facilitate transport of organelles along cytoskeletal filaments (McLauchlan et al., 1998; Nielsen et al., 1999). SNAREs are a group of membrane proteins associated with a wide variety of organelles. After membrane tethering, SNAREs located in the two approaching compartments form extremely stable *trans* complexes, bringing the membranes into close proximity in a process that is essential for fusion (Sutton et al., 1998; Jahn and Sudhof, 1999; Chen and Scheller, 2001).

A strict control of specificity in membrane fusion events is necessary for preserving the structural and functional organization of the cell. Both SNAREs and Rabs provide specificity for proper membrane recognition (Zerial and McBride, 2001; McNew et al., 2000). Different fusion events are mediated by distinct combinations of SNAREs and Rabs. To date, ~40 distinct Rab proteins have been identified, and each is believed to be specifically associated with a particular organelle or pathway (Novick and Zerial, 1997; Schimmoller et al., 1998; Zerial and McBride, 2001). Despite the fact that Rabs are not transmembrane proteins, their membrane association is so specific that they are used as markers for many compartments (e.g. Rab5 for early endosomes, Rab7 for late endosomes).

Rab function is regulated by cycles of GTP binding and hydrolysis that result in conformational changes in the protein that promote specific contacts with different effectors (Novick and Zerial, 1997). Transport vesicles carry Rab proteins with

bound GTP. After membrane fusion, GTP hydrolysis stimulated by specific GAPs (GTPase activating proteins) generates GDP-bound Rabs. A cytosolic protein named GDI (GDP dissociation inhibitor) extracts the Rabs from the membranes and delivers them to the compartments of origin where they are subsequently reactivated by Rab-specific, nucleotide exchange factors.

Endocytosis is a fundamental process that take place in almost all living eukaryotic cells (Mellman, 1996). Several Rabs have been described in the endocytic pathway. For some of them (e.g. Rab5) detailed studies have permitted the identification of specific effectors and regulatory factors, the postulation of molecular mechanisms for their function, and the assignment of a defined role in the endocytic pathway (Christoforidis et al., 1999; Gournier et al., 1998). Moreover, mutants of these Rabs defective in GTP hydrolysis (constitutively active mutants) and in GTP binding (dominant negative mutants) have been described and used to unveil several aspects of their function (Barbieri et al., 1996; Li and Stahl, 1993). However, comparatively little information is available for other endocytosis-related Rabs. One of the less studied members of this family is Rab22a, which was described as an endosomal associated protein in different cell lines expressing Myc epitope-tagged Rab22a (Olkkonen et al., 1993). However very little is known about its function.

We have characterized the subcellular localization of the wild-type protein, and negative and constitutively active mutants tagged with green fluorescent protein (GFP) in living cells. Our results indicate that wild-type Rab22a associates with early and late endosomes, but not with lysosomes. Overexpression of the wild-type protein and the mutants strongly affects the morphology and physiology of the endocytic pathway, suggesting a role for this Rab in the intracellular transport of endocytosed material.

MATERIALS AND METHODS

Reagents

Rhodamine-transferrin (Rh-tf), LysoTracker red, BODIPY-TR Ceramide, MitoTracker orange, rhodamine B hexyl ester and TAMRA (5-(and 6) carboxytetramethylrhodamine, succinimidyl ester) were from Molecular Probes (Eugene, OR). All other reagents were from Sigma Chemical Company (St Louis, MO). Rhodamine-labeled horseradish peroxidase (Rh-HRP) was obtained by incubating 5 mg/ml HRP with 5 mg/ml TAMRA overnight at room temperature in phosphate buffer 0.1 M, pH 8. Uncoupled rhodamine was separated by gel-filtration on a G-25 Sephadex column equilibrated with 140 mM NaCl, 10 mM phosphate buffer, pH 7 (PBS), containing 1 mg/ml bovine serum albumin (BSA). Rabbit anti-cation independent mannose 6-phosphate receptor (CI-M6PR) antibody was generously provided by S. Kornfeld (St Louis, MO). Mouse anti-membrin antibody was from StressGen Biotech. (Victoria, BC, Canada). Secondary anti-mouse and anti-rabbit antibodies labeled with Alexa Fluor 546 were purchased from Molecular Probes (Eugene, OR). The cDNA for Rab22a was kindly provided by M. Zerial (Heidelberg, Germany).

Plasmids

Canine Rab22a cDNA was amplified by PCR and subcloned as *Bam*HI fragments into the pGEX-2t plasmid (Amersham Pharmacia Biotech, Uppsala, Sweden). Rab22a mutants (Q64L and S19N) were obtained by site directed mutagenesis (QuickChange kit from Stratagene, La Jolla, USA). The mutations were verified by sequencing the inserts. Wild-type and mutant sequences were subcloned into the *Bam*HI site of the pEGFP-C1 vector (Clontech, Palo Alto, CA).

GTP binding and hydrolysis

The GTP-binding properties of Rab22a wild-type and mutants were assessed as described by Schlierf et al. (Schlierf et al., 2000). In brief, purified GST fusion proteins were immobilized on nitrocellulose membranes. The membranes were incubated in 50 mM NaH_2PO_4, pH 7.5, 10 μM $MgCl_2$, 2 mM DTT, 0.1% Triton X-100 and 4 μM ATP for 45 minutes at 20°C. [α^{32}P]GTP was added to the incubation buffer (6 μCi/ml, 5 Ci/μmol). The membranes were incubated for 2 hours at 20°C and extensively washed at 4°C with PBS containing 0.1% Tween 20 and 5 mM $MgCl_2$ (PBS-Mg). The membranes were dried and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY). The developed films were scanned and the radioactivity associated with Rabs was quantified using the TotalLab software (Newcastle, UK). To study the endogenous GTPase activity of the mutants, immobilized proteins were loaded with [γ^{32}P]GTP using the protocol described above. After washing, the membranes were either incubated in 20 mM Tris-HCl, pH 8, 1 mM DTT and 5 mM $MgCl_2$ for 1hour at 37°C (Liang et al., 2000) or maintained at 4°C in PBS-Mg. After the incubation, the membranes were washed with PBS-Mg. The dried membranes were exposed to film and the radioactivity associated with Rabs was quantified using the TotalLab software.

Cell culture, transfection and treatments

Chinese hamster ovary (CHO) cells were grown in α-minimal essential medium (αMEM) supplemented with 10% fetal bovine serum. Before transfection, the cells were plated on coverslips for 24 hours. Cells were washed three times with serum-free αMEM, and transfected with 1 μg of purified plasmidic DNA using the LipofectAmine reagent (GIBCO-BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. To generate stable cell lines expressing GFP-Rab22aWT, GFP-Rab22aS19N and GFP-Rab22aQ64L, clones resistant to 1 mg/ml G418 antibiotic (GIBCO-BRL) were selected.

Labeling of subcellular compartments

To label different compartments of the endocytic pathway, CHO cell monolayers grown in 35 mm dishes were washed three times with uptake medium (Basal Medium Eagle, 20 mM Hepes, pH 7.4, 5 mg/ml BSA). Internalization was initiated by addition of 1 ml uptake medium containing 0.5 mg/ml Rh-HRP. Early endosomes were labeled by a 5 minute uptake, late endosomes by 5 minute uptake followed by 15 minute chase, and lysosomes by a 1 hour uptake and 1 hour chase. During the chase period, the medium was changed every 15 minutes to avoid reinternalization of the recycled Rh-HRP. To label early compartments by receptor-mediated endocytosis, the cells were washed three times with PBS and incubated in uptake medium containing 20 μg/ml Rh-tf for 5 minutes at 37°C. Acidic compartments were labeled by a 10 second incubation with uptake medium containing 1 μM LysoTracker Red, a weak basic amine that accumulates in organelles with low pH. The Golgi apparatus was labeled with 2.5 μM BODIPY-TR ceramide as described (Pagano et al., 1991). The endoplasmic reticulum was stained by incubating glutaraldehyde-fixed cells with 2.5 μg/ml rhodamine B hexyl ester (Terasaki and Reese, 1992). A 30 minute incubation with 1 μM MitoTracker orange was used to label mitochondria (Burgess et al., 1994). Autophagosomes were labeled by a 30 minute incubation at 37°C with 50 μM monodansylcadaverine in uptake medium (Biederbick et al., 1995).

To immunostain compartments containing CI-M6PR (a late endosome marker) or membrin (a *cis*-Golgi marker), transfected cells were fixed in 4% paraformaldehyde dissolved in PBS containing 1 mM $MgCl_2$, 0.2 mM $CaCl_2$ (PBS-CM) for 20 minutes. The cells were

permeabilized with 0.5% Triton X-100 in PBS-CM. Primary antibodies were diluted 1/100 in PBS-CM containing 1% BSA. The secondary antibodies were diluted 1/500 in PBS-CM containing 1% BSA. All incubations were carried out for 2 hours at 20°C.

Fluorescent microscopy

Unfixed and fixed cells were observed in a Eclipse TE300 Nikon microscope equipped with a Hamamatsu Orca 100 camera operated with the MetaMorph software (Universal Imaging Corp., USA). Images were taken with three sets of filters (excitation 510-560 nm, barrier 590 nm for tetramethylrhodamine, LysoTracker Red, MitoTracker Orange, rhodamine B, Alexa Fluor 546 and BODOPY-TR ceramide; excitation 450-490 nm, barrier 520 nm for GFP; and excitation 330-380 nm, barrier 420 nm for MDC) and processed with the MetaMorph and Paint Shop Pro programs (Jasc Software, Eden Prairie, MN).

Fluid phase endocytosis assay

CHO cell monolayers in 35 mm dishes at 60-70% confluence were washed three times with serum-free α-MEM, and HRP uptake was initiated by addition of 1 ml of α-MEM containing 0.5 mg/ml HRP and 1 mg/ml BSA. After incubation at 37°C for 1 hour, the uptake was stopped by washing the cell monolayers twice with ice-cold PBS containing 1% BSA and twice with ice-cold PBS. The cells were then lysed in 500 μl of ice-cold PBS containing 0.1% Triton X-100. The cell lysates were assayed for peroxidase activity in 96-well microplates using *o*-phenylenediamine as the chromogenic substrate. Briefly, the reaction was started by adding 5 μl of the lysate to 100 μl of 0.5 M Na acetate (pH 5.0) containing 0.75 mg/ml *o*-phenylenediamine and 0.006% H_2O_2. The reaction was conducted at room temperature for 10 minutes and stopped by adding 100 μl of 0.1 N H_2SO_4. The products were quantified by measuring the $OD_{490\ nm}$ in a BioRad microplate reader. Protein content was determined by the BioRad protein assay according to the manufacturer's instructions. The HRP activity was expressed as a ratio of the protein content.

Efflux assay

In order to follow HRP degradation and recycling to the media, HRP was radiolabeled with ^{125}I using chloramine T (Stahl et al., 1980). Cells transiently expressing wild-type and mutant Rab22a or GFP alone were allowed to internalize radioactive HRP for 1 hour as described above. The cells were then washed five times with PBS containing 10 mg/ml BSA to eliminate extracellular HRP. Protein degradation and recycling into the medium was measured during a 40 minute incubation at 37°C. The media were collected every 10 minutes (to avoid re-internalization) and pooled. At the end of the incubation, the cells were solubilized in PBS containing 1% Triton X-100. Trichloroacetic acid soluble and insoluble radioactivity was measured in all the samples. The amount of soluble radioactivity in both the media and the cells measured HRP digestion, and was expressed as a percentage of the total radioactivity present in the cells after the internalization period. The trichloroacetic acid precipitable radioactivity in the media measured HRP recycling, and was expressed as a percentage of the total radioactivity present in the cells after the internalization period.

RESULTS

Rab22a specifically associate with early and late endosomes but not with lysosomes

Rab22a was described several years ago as an endosome-associated GTPase (Olkkonen et al., 1993). To better characterize its subcellular localization and function in living cells, the cDNA of canine Rab22a was subcloned into the pEGFP-C1 plasmid and expressed as a fusion protein with GFP. It has been shown that the presence of GFP (or other polypeptides) at the N terminus of Rab proteins does not affect the subcellular localization and function of these proteins (Olkkonen et al., 1993; Bucci et al., 2000). Twenty-four hours after transfection of CHO cells, the fluorescent protein associated with small and large round-shaped structures, and with tubular networks (Fig. 1A,D,G,J). Rab22a clearly labeled the perimeter of several large structures, indicating that they correspond to large vesicles and not to aggregates of small compartments (arrowheads, Fig. 1D,G). Tubular projections were frequently observed (inset, Fig. 1B). Tubules growing from vesicular structures and fusion between Rab22a-labelled structures were also detected in time-lapse experiments (data not shown).

Different markers were used to identify the nature of the Rab22a-labeled structures. The GFP-tagged protein partially colocalized with early endosomes loaded by fluid phase (Rh-HRP, 5 minute uptake, Fig. 1C) and receptor-mediated (Rh-tf, 5 minute uptake, Fig. 1F) uptake. Rab22a was also found associated with late endosomes (Rh-HRP, 5 minute uptake, 15 minute chase, Fig. 1I). Localization with late endosomes was confirmed by immunocytochemistry using an antibody that recognizes the CI-M6PR (Fig. 1L). It was interesting to observe that cells overexpressing Rab22a presented enlarged structures loaded with early and late endosomal markers (compare the size of rhodamine-labeled vesicles in transfected and untransfected cells in Fig. 1C,I,L). Although the distribution of CI-M6PR, which normally localizes near the Golgi apparatus, was strongly altered, specific markers of this organelle, such as membrin (Fig. 2B) and BODIPY-TR ceramide (data not shown) were not affected. Colocalization with a lysosomal probe (Rh-HRP, 60 minute uptake, 60 minute chase, Fig. 2E) and an acidic compartment marker (LysoTracker red, Fig. 2H) was not observed. The autophagosomal marker MDC did not colocalize with Rab22a-positive structures (Fig. 2K). In addition, endoplasmic reticulum (rhodamine B hexyl ester) and mitochondria (MitoTracker orange) fluorescent probes did not colocalize with Rab22a-positive structures (data not shown).

The intracellular localization of Rab22a did not depend on the cell type or the transfection method used. Similar results were obtained in BHK21 and HeLa cells transfected by either the LipofActamine method or by electroporation (data not shown). In conclusion, wild-type Rab22a associates with early and late endosomes, causing distinct morphological alterations of these compartments. Association with other structures of the endocytic pathway was not observed.

Positive and negative mutants of Rab22a present different subcellular distribution

The function of the Rab GTPases is regulated by GTP binding and hydrolysis. Two mutants were generated by site-directed mutagenesis. A Ser to Asn mutation in the first motif of the GTP-binding domain decreases the affinity for GTP in several Rab proteins (Stenmark et al., 1994). This mutation generates dominant negative mutants by blocking the GDP-to-GTP exchange necessary for Rab activation (Feig, 1999). By contrast, a Gln to Leu mutation in the second motif inhibits the endogenous GTPase activity of several Rabs, generating constitutively active Rabs (Stenmark et al., 1994).

To assess the GTP binding and hydrolysis activity of the mutants, the proteins were expressed in *E. coli* as GST fusion

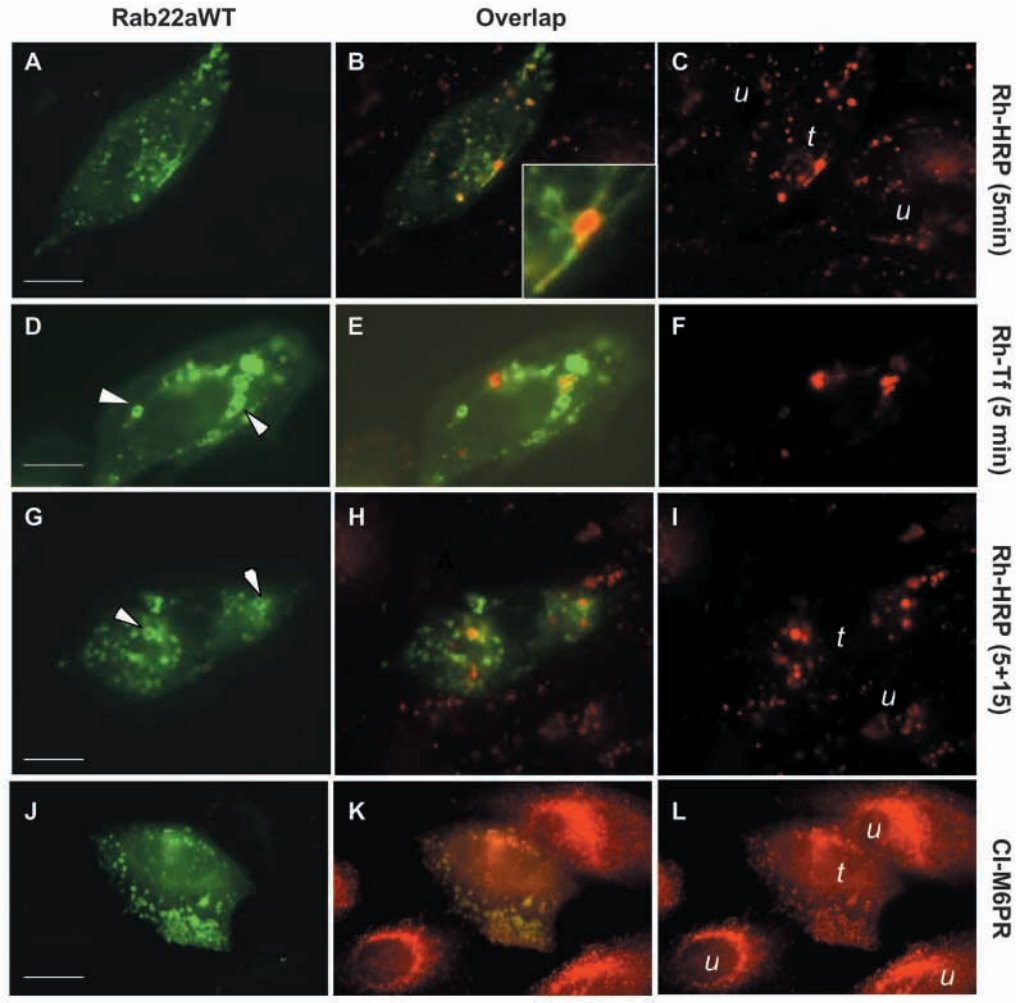

Fig. 1. GFP-Rab22a localizes to early and late endosomes. CHO cells were transfected with pEGFP-Rab22aWT and 24 hours later the distribution of the GFP fluorescence was recorded. Early endosomes were labeled by a 5 minute uptake of Rh-HRP (A-C) or Rh-Tf (D-F). Late endosomes were labeled by a 5 minute uptake of Rh-HRP followed by 15 minute chase (G-I) or by immunostaining with an anti CI-M6PR antibody (J-L). Notice the large size of some Rab22a-positive vacuoles (arrow heads) and the altered morphology of early and late compartments in cells overexpressing Rab22a (t) when compared with untransfected cells (u). The inset in B shows tubular projections attached to a large vesicle. The bars represent 7 μm.

proteins, and purified on gluthathione columns. In these recombinant proteins, the binding of radioactive GTP to Rab22aS19N was greatly reduced when compared with the wild-type protein. Conversely, the binding to Rab22aQ64L was only slightly affected by the mutation (Table 1). However, this mutant hydrolyzed only 7% of the bound GTP in 1 hour while the wild type protein hydrolyzed 80% of the nucleotide in the same assay (Table 1). These results indicate that the mutations performed have conferred the expected phenotypes to Rab22a.

When the mutants were expressed in eukaryotic cells, they showed very distinct subcellular distributions. Most of the Rab22aS19N fluorescence was distributed throughout the cytosol (80-90%), although the label showed an enriched region near the nucleus that overlapped with Golgi markers (Fig. 3B). By contrast, the positive mutant associated with strikingly large vesicles that were labeled with early and late endocytic markers (Fig. 3E,H,K,N). Some cells also showed Rab22a at the plasma membrane (arrow, Fig. 3G). Surprisingly, the positive mutant colocalized with lysosomal (Fig. 4B,E) and autophagosomal markers (Fig. 4H). The size of some Rab22aQ64L-labelled vesicles was very large. It was possible to distinguish transfected cells by the presence of enlarged structures loaded with LysoTracker and MDC (compare the size of fluorescent vesicles in transfected and untransfected cells in Fig. 4F,I).

Table 1. Binding and hydrolysis of GTP by wild-type Rab22a and mutants

	GTP binding	GTP hydrolysis
Rab22aWT	1	80%
Rab22aS19N	0.06	–
Rab22aQ64L	0.72	7%

Binding values are normalized to the binding to the wild-type protein.
Hydrolysis values are expressed as a percentage of the radioactivity remaining after incubation at 4°C.
The GTPase activity of Rab22aS19L was not measured.
Similar results were obtained in three independent experiments.

Overexpression of Rab22a alters endocytosis

The specific association of Rab22a with endocytic structures and the striking morphological alterations observed suggest that Rab22a has a role in the endocytic pathway. The intracellular accumulation of HRP internalized by fluid phase endocytosis was measured in cells expressing GFP alone (vector) and the chimeras with Rab22aWT, Rab22aS19N and Rab22aQ64L. Both wild-type Rab22a and the negative mutant significatively inhibited intracellular accumulation of HRP. Overexpression of GFP alone had no effect. Interestingly, the positive mutant did not decrease HRP accumulation (Fig. 5A).

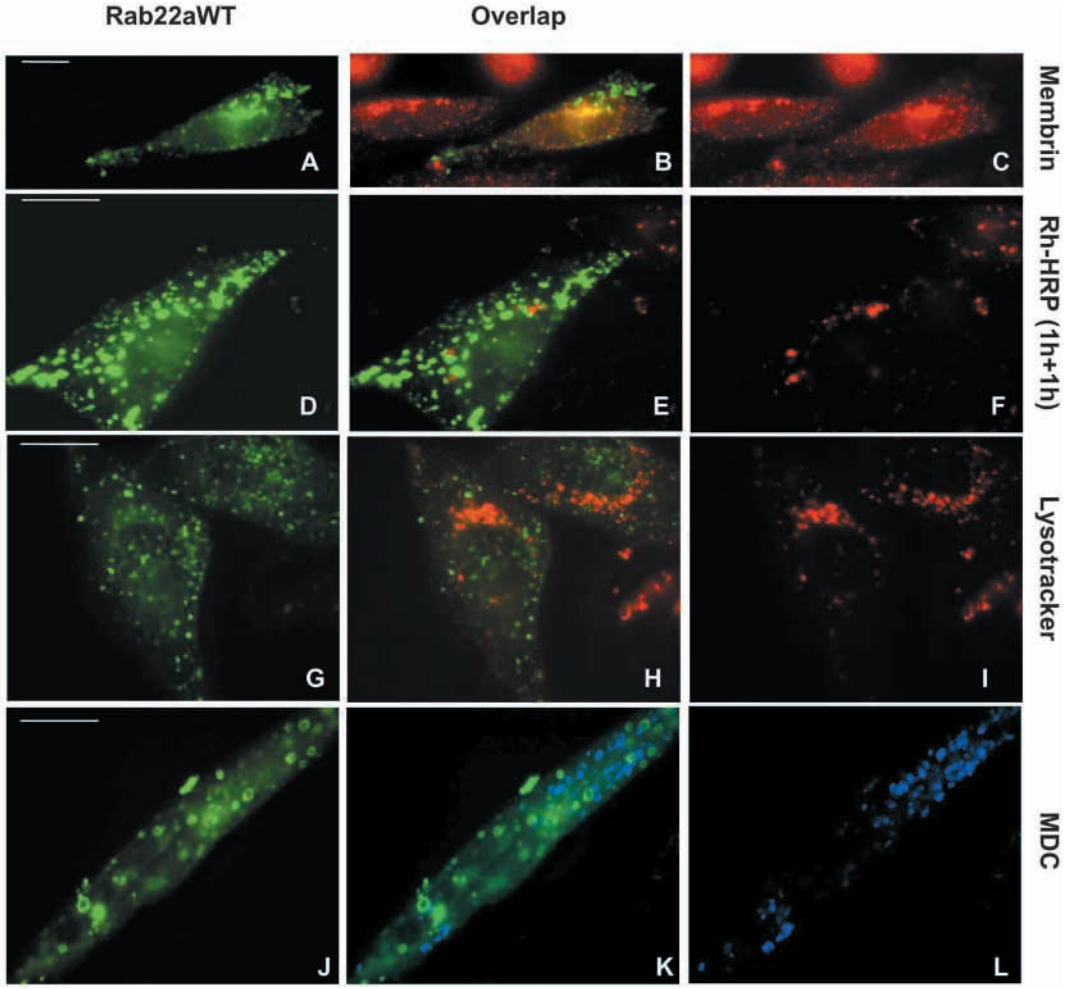

Fig. 2. GFP-Rab22a does not localize with Golgi structures or lysosomes. CHO cells were transfected with pEGFP-Rab22aWT and 24 hours later the distribution of the GFP fluorescence was analyzed. The Golgi apparatus was labeled with an antibody that recognizes membrin, a *cis* Golgi marker (A-C). Lysosomes were loaded with Rh-HRP by a 1 hour uptake followed by 1 hour chase (D-F). Acidic compartments were labeled with LysoTracker red (G-I). Autophagosomes were labeled with MDC (monodansylcadaverine) (J-L). Very scarce colocalization can be observed with the four markers used. The bars represent 7 μm.

To perform the biochemical assay in a more homogeneous population of cells, stably transfected cells were selected by resistance to G418. The clones that overexpressed wild-type and the dominant negative mutant Rabs accumulated significantly less HRP than untransfected cells. The constitutively active mutant did not inhibit HRP accumulation, confirming what was observed in transiently transfected cells (Fig. 5B).

HRP accumulation is the balance between the fluid phase internalization of the enzyme and the amount that is lost from the cell either by recycling back to the medium or by digestion in lysosomes. To assess whether the mutants were affecting these two processes, HRP was radiolabeled and the digestion of the enzyme was followed by the appearance of acid soluble radioactivity in the system, and the recycling by the release of acid precipitable radioactivity into the medium. The results indicate that there was not a significant difference between control cells and cells transfected with the vector or the different Rab22a constructs (Fig. 5C). This observation indicates that the effects of Rab22a on HRP accumulation can be mostly attributed to the process of internalization.

In conclusion, the results indicate that overexpression of Rab22a affects not only the morphology of the endocytic pathway but also the efficiency of fluid phase endocytosis.

DISCUSSION

Membrane associated and soluble macromolecules internalized by endocytosis are initially transported to early endosomes. This dynamic organelle is constantly receiving material from the plasma membrane and sorting macromolecules to late compartments for degradation or recycling the material back to the cell surface either directly or through a specialized perinuclear recycling compartment (Mellman, 1996). The precise mechanism by which sorting to the different organelles is accomplished and, at the same time, the functional identity of the compartments is maintained is still not well understood. Whatever model is proposed, it will require vesicle formation, cytoskeletal-mediated transport, and membrane recognition and fusion. Rabs are essential elements in the molecular mechanisms underlying all these events (Novick and Zerial, 1997; Somsel and Wandinger-Ness, 2000). Therefore, it is not surprising that a plethora of Rab proteins have been localized in the endocytic pathway (Somsel and Wandinger-Ness, 2000; Mohrmann and van der Sluijs, 1999). Rab5 is a central factor for the homeostasis of the early endocytic compartment. Dominant negative mutants of this protein inhibit endocytosis and constitutively active mutants generate large early endosomes (Bucci et al., 1992). In vitro, Rab5 promotes homotypic fusion among early endosomes

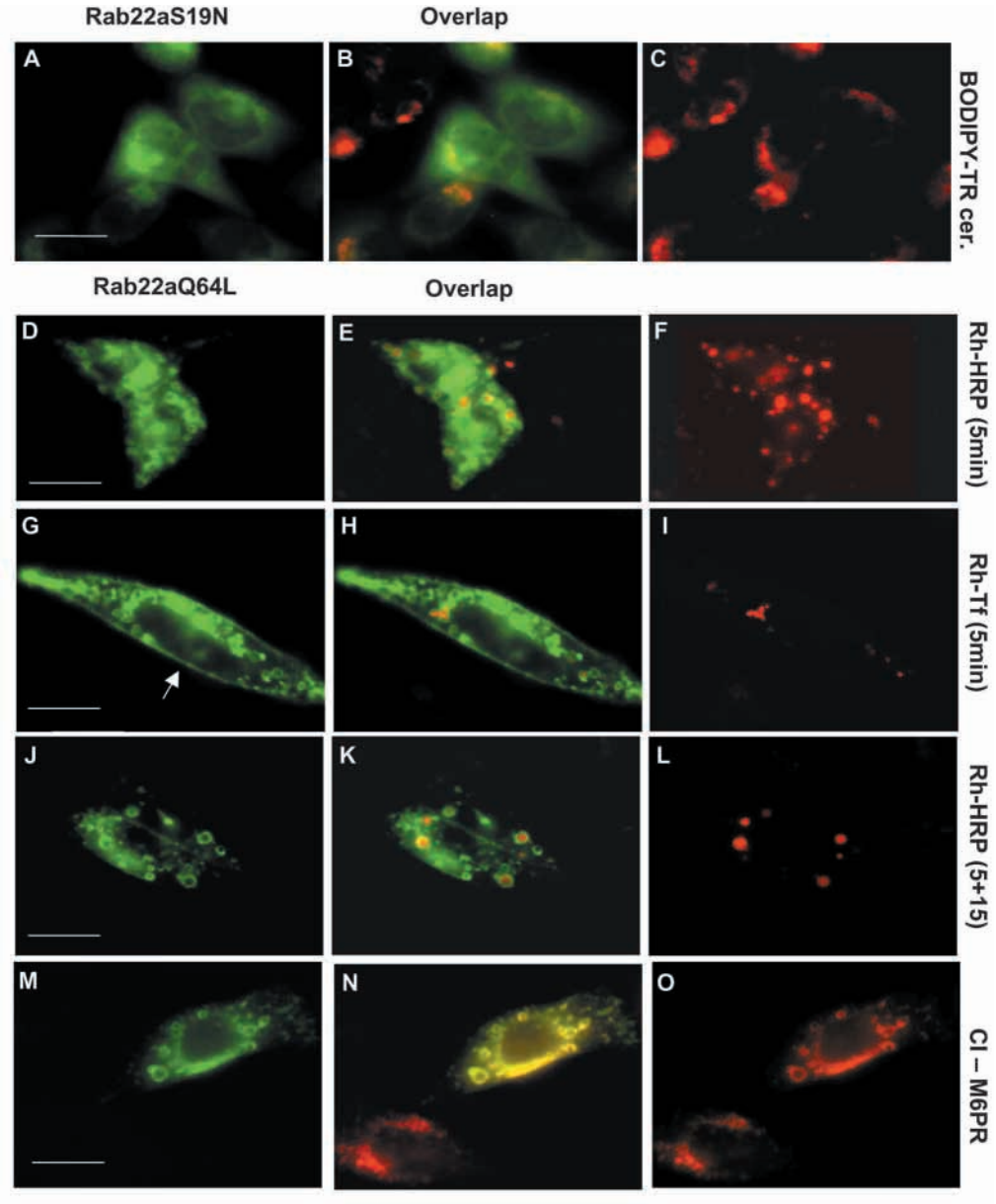

Fig. 3. Rab22aS19N, a mutant with low affinity for GTP, is mostly cytosolic, whereas Rab22aQ64L, a mutant with low GTPase activity, localizes in large vesicles that are labeled by early and late endosomal markers. Cells expressing GFP-Rab22aS19N presented a diffuse cytosolic fluorescence. The protein was enriched in a perinuclear region that colocalized with a Golgi marker (BODIPY-TR ceramide) (A-C). The distribution of the Rab22aQ64L mutant was completely different. This GTPase-deficient mutant localized to large ring-shaped vacuoles that were labeled by a 5 minute uptake of Rh-HRP (D-F) or Rh-Tf (G-I). The protein also localized to late endosomes labeled by a 5 minute uptake of Rh-HRP followed by a 15 minute chase (J-L) or by immunocytochemistry using an anti CI-M6PR antibody (M-O). In some cells, Rab22aQ64L was also present at the plasma membrane (arrow). The bars represent 7 μm.

(Gorvel et al., 1991; Li and Stahl, 1993). Rab4, however, is important for the recycling of macromolecules to the cell surface either through the direct pathway or through the recycling compartment (van der Sluijs et al., 1992). Rab11 associates with the recycling compartment of many cell types. Disruption of Rab11 function alters the recycling of internalized material to the cell surface and transport to the recycling compartment (Ren et al., 1998; Ullrich et al., 1996). It is also important for trancytosis in epithelial cells (Wang et al., 2000). The dynamics of association of these three Rabs with transferrin-containing vesicles has been studied in living cells (Sonnichsen et al., 2000). Comparatively fewer Rab proteins are known to participate in the trafficking to lysosomes. Rab7 is involved in the transport to late compartments (Vitelli et al., 1997; Mukhopadhyay et al., 1997) and probably in the maintenance of lysosomal morphology and function (Bucci et al., 2000), whereas Rab9 is necessary for transport between late endosomes and the trans-Golgi network (Lombardi et al., 1993). Other Rab proteins (i.e. Rab17, Rab18, Rab20 and Rab25) are specific to polarized cells and participate in the transport of macromolecules across epithelia (Somsel and Wandinger-Ness, 2000; Mohrmann and van der Sluijs, 1999; Novick and Zerial, 1997). Very little is known about Rab22a, a protein originally described as associated with early and late endosomes (Olkkonen et al., 1993).

In CHO cells expressing GFP-tagged Rab22a, markers internalized by fluid phase (Rh-HRP) and receptor mediated (Rh-tf) endocytosis reach Rab22a positive structures very rapidly, indicating that Rab22a localizes to early endosomes. Chasing the fluid phase marker for 15 minutes, a procedure that forces the label out of Rab5-positive structures, did not eliminate the colocalization with Rab22a, suggesting that this Rab also associates with late endosomes. This observation was confirmed by immunocytochemistry using an antibody against the CI-M6PR, a bona fide late endosome marker. Interestingly, Rab22a did not localize to lysosomes, as inferred from the

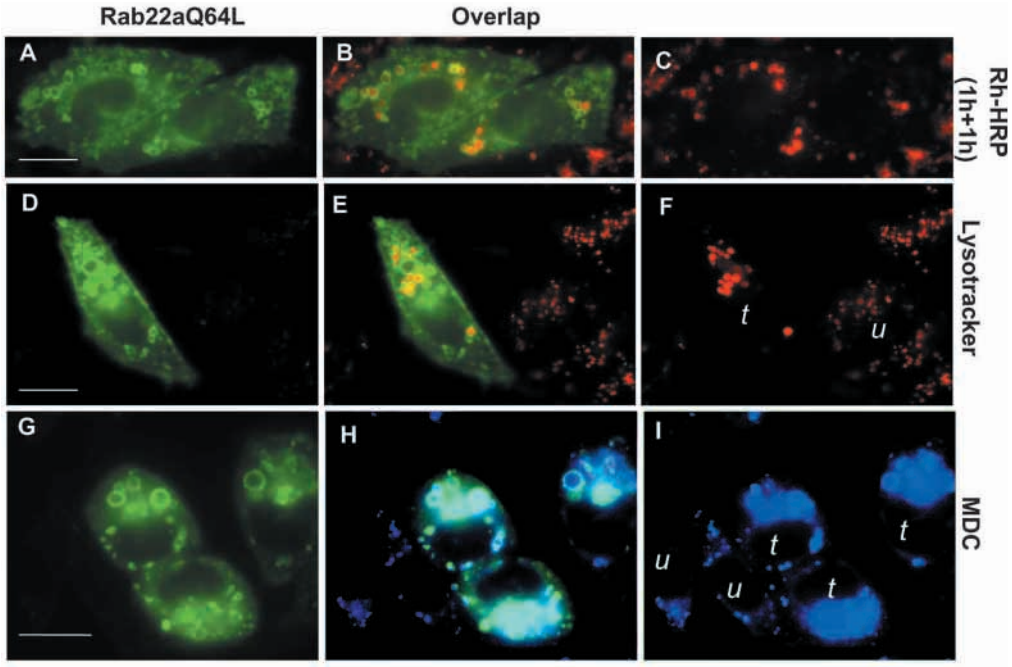

Fig. 4. Rab22aQ64L, the GTPase-deficient mutant, colocalizes not only with early and late endosomes, but also with lysosomes and autophagosomes. Lysosomes were loaded with Rh-HRP by a 1 hour uptake followed by a 1 hour chase (A-C). Acidic compartments were labeled with LysoTracker red (D-F). Autophagosomes were labeled with MDC (monodansylcadaverine) (G-I). Enlarged lysosomal and autophagosomal compartments were evident in cells overexpressing Rab22aQ64L (t) when compared with untransfected cells (u). The bars represent 7 μm.

lack of colocalization with Rh-HRP chased for 1 hour, or with acidic compartments labeled with LysoTracker. Morphologically, Rab22a positive structures ranged from tubular networks in the periphery of the cell to large ring-shaped vesicles distributed all over the cytoplasm.

The first and only report about Rab22a subcellular localization describes the association of the protein with early and late endosomes, although a precise definition for the late compartments labeled with Rab22a was not provided (Olkkonen et al., 1993). According to the original observation, Rab22a presented only slight colocalization with two late endocytic markers (Rab7 and the CI-M6PR) by immunofluorescence. However, Rab22a was localized by immunoelectron microscopy to vesicles containing a fluid phase marker chased to lysosomes by an overnight incubation. Our results indicate instead that the wild-type protein binds to early and late endosomes but not to lysosomes. In conclusion, despite some discrepancy between the original report and our results, possibly owing to the differences in the markers used, we confirmed that Rab22a is located in early and late endosomes. This localization within the endocytic pathway is not shared by any other Rab protein. Most Rabs that participate in endocytosis are engaged in the regulation of early endosome fusion and recycling. Rab7, which associates with late compartments, is not present in early endosomes, and colocalizes with LysoTracker and lysosomal markers in specific cell types (Bucci et al., 2000).

Point mutations in wild-type Rab22a caused substantial alterations in the interaction of the protein with GTP. The change of a serine in position 19 to asparagine decreased the affinity of the protein for GTP, as has been observed for several other Rab

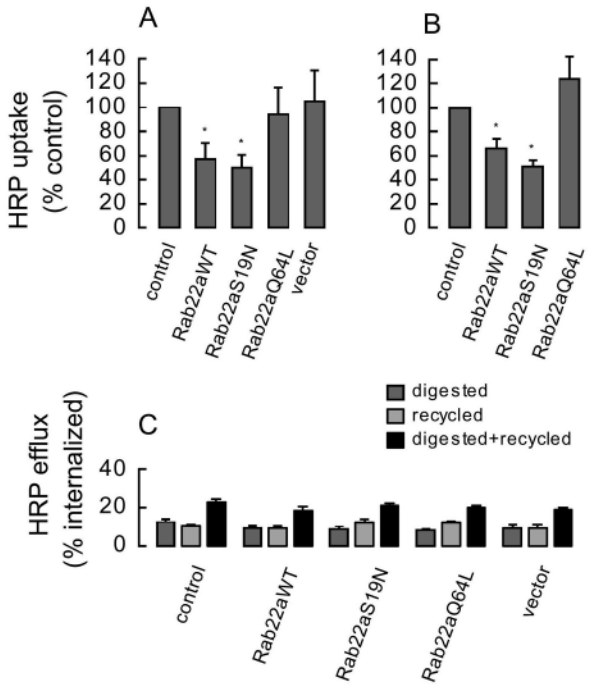

Fig. 5. Overexpression of Rab22a affects endocytosis. (A) CHO cells transiently expressing GFP alone (vector) or GFP fused to Rab22aWT, Rab22aS19N and Rab22aQ64L were allowed to endocytose HRP for 1 hour. The cells were washed and the amount of peroxidase associated with the cells was measured. The uptake was expressed as a percentage of the HRP endocytosed by untransfected cells (control). (B) The same experiment was performed with CHO clones stably expressing Rab22a wild-type and the mutants. (C) CHO cells that had internalized ^{125}I-HRP as in A were washed and incubated for 40 minutes. The amount of HRP digested (dark gray bars) and recycled into the medium (light gray bars) was estimated from trichloroacetic acid soluble and precipitable radioactivity as explained under Material and Methods. The data are expressed as a percentage of HRP present in the cells after the internalization period. Black bars show the percentage of HRP that is lost from the cells by digestion and recycling. The data represent the mean±s.e.m. of five experiments. Asterisks indicate significant differences from the control (P<0.05).

proteins (Stenmark et al., 1994). These mutants are locked in the GDP bound form that binds to GDI, a cytosolic protein that extracts Rab proteins from membranes. The mainly cytosolic distribution of GFP-Rab22aS19N is therefore consistent with the GDP-bound conformation of the protein. The significance of the perinuclear localization of this mutant is at present unknown. It would be interesting if it could be related to the localization of Rab22a exchange factors, proteins for which the dominant negative mutants show an enhanced affinity (Feig, 1999). The substitution of the glutamine at position 64 by a leucine decreases the endogenous GTPase activity of Rab22a, as has been reported for other members of the Ras superfamily (Stenmark et al., 1994). These mutants are maintained in the active form and, consequently, membrane fusion is stimulated causing the formation of large vesicles. Overexpression of GFP-Rab22aQ64L caused the formation of numerous large vesicles that in some cells filled most of the cytoplasm. Interestingly, these vesicles contained not only early and late endosomal markers, but also lysosomal and autophagosomal markers.

Overexpression of Rab22aWT and Rab22aQ64L produced a remarkable enlargement of several endocytic compartments. It has been suggested that the geometry of membrane-bound compartments plays an important role in intracellular transport (Mellman, 1996). Receptors and other membrane proteins accumulate in tubular elements, owing to their large surface/volume ratio, whereas soluble contents accumulate in vesicular structures. However, in a recent report it has been shown that trafficking can occur despite the presence of large vesicles induced by overexpression of constitutively active Rab5 (Ceresa et al., 2001). In fact, the formation of enlarged compartments may be a normal process upon strong stimulation of intracellular transport. EGF, which promotes endocytosis in NR6 cells by activating Rab5a, leads to the formation of large Rab5-positive vesicles, similar to those observed by overexpression of the active mutant of this GTPase (Barbieri et al., 2000).

Our results are consistent with a role for Rab22a in the trafficking between early and late endosomes. The presence of the protein in these two compartments, but not in lysosomes suggests that its function is to facilitate the exit from sorting endosomes of endocytosed material destined for degradation in lysosomes. The positive mutant is probably retained on the membranes after fusion has occurred, and is transported to lysosomes where it may alter the morphology of this compartment by promoting fusion as has been shown for Rab7 (Bucci et al., 2000).

Endocytosis requires a balanced set of Rab proteins and the correct regulation of the GDP-GTP cycle of these proteins. Overexpression of GFP-Rab22aWT diminished HRP accumulation in cells. The dominant negative mutant was also inhibitory, but the positive mutant did not affected endocytosis. At present these observations can be interpreted assuming a role for Rab22a in early-to-late endosome transport. Overexpression of the wild-type protein may partially deplete the cell of early endosomes and the negative mutant may block transport to late endosomes. Both effects can decrease the efficiency of the endocytic pathway, the former because transport through functional early endosomes is a limiting step in endocytosis (Bucci et al., 1992), and the latter because the endocytosed material cannot proceed to lysosomes. The lack of inhibition by the positive mutant may reflect a combination of inhibitory and stimulatory effects on endocytosis. As the wild-type protein, active Rab22a may accelerate transport to late compartments, but as it cannot dissociate from membranes, it may promote mixing between early and late compartments, preventing the disappearance of early compartments necessary to sustain endocytosis. A more careful examination of the dynamics of endocytosis in cells overexpressing different Rab22a constructs will be necessary to understand fully the role of this GTPase on cellular trafficking.

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REFERENCES

Barbieri, M. A., Li, G., Mayorga, L. S. and Stahl, P. D. (1996). Characterization of Rab5:Q79L-stimulated endosome fusion. *Arch. Biochem. Biophys.* **326**, 64-72.

Barbieri, M. A., Roberts, R. L., Gumusboga, A., Highfield, H., Alvarez-Dominguez, C., Wells, A. and Stahl, P. D. (2000). Epidermal growth factor and membrane trafficking. EGF receptor activation of endocytosis requires rab5a. *J. Cell Biol.* **151**, 539-550.

Biederbick, A., Kern, H. F. and Elsasser, H. P. (1995). Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. *Eur. J. Cell Biol.* **66**, 3-14.

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

Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J. and van Deurs, B. (2000). Rab7: a key to lysosome biogenesis. *Mol. Biol. Cell* **11**, 467-480.

Burgess, S. M., Delannoy, M. and Jensen, R. E. (1994). MMM1 encodes a mitochondrial outer membrane protein essential for establishing and maintaining the structure of yeast mitochondria. *J. Cell Biol.* **126**, 1375-1391.

Ceresa, B. P., Lotscher, M. and Schmid, S. L. (2001). Receptor and membrane recycling can occur with unaltered efficiency despite dramatic rab5(Q79L)-induced changes in endosome geometry. *J. Biol. Chem.* **276**, 9649-9654.

Chen, Y. A. and Scheller, R. H. (2001). SNARE-mediated membrane fusion. *Nat. Rev. Mol. Cell Biol.* **2**, 98-106.

Christoforidis, S., McBride, H. M., Burgoyne, R. D. and Zerial, M. (1999). The Rab5 effector EEA1 is a core component of endosome docking. *Nature* **397**, 621-625.

Feig, L. A. (1999). Tools of the trade: use of dominant-inhibitory mutants of Ras-family GTPases. *Nat. Cell Biol.* **1**, E25-E27.

Gerst, J. E. (1999). SNAREs and SNARE regulators in membrane fusion and exocytosis. *Cell Mol. Life Sci.* **55**, 707-734.

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 J.* **17**, 1930-1940.

Jahn, R. and Sudhof, T. C. (1999). Membrane fusion and exocytosis. *Annu. Rev. Biochem* **68**, 863-911.

Li, G. P. and Stahl, P. D. (1993). Structure-function relationship of the small GTPase rab5. *J. Biol. Chem.* **268**, 24475-24480.

Liang, Z., Mather, T. and Li, G. (2000). GTPase mechanism and function: new insights from systematic mutational analysis of the phosphate-binding loop residue Ala30 of Rab5. *Biochem. J.* **346**, 501-508.

Lombardi, D., Soldati, T., Riederer, M. A., Goda, Y., Zerial, M. and Pfeffer, S. R. (1993). Rab9 functions in transport between late endosomes and the trans Golgi network. *EMBO J.* **12**, 677-682.

McLauchlan, H., Newell, J., Morrice, N., Osborne, A., West, M. and Smythe, E. (1998). A novel role for Rab5-GDI in ligand sequestration into clathrin-coated pits. *Curr. Biol.* **8**, 34-45.

McNew, J. A., Parlati, F., Fukuda, R., Johnston, R. J., Paz, K., Paumet, F., Sollner, T. H. and Rothman, J. E. (2000). Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature* **407**, 153-159.

Mellman, I. (1996). Endocytosis and molecular sorting. *Annu. Rev. Cell Dev. Biol.* **12**, 575-625.

Mohrmann, K. and van der Sluijs, P. (1999). Regulation of membrane transport through the endocytic pathway by rabGTPases. *Mol. Membr. Biol.* **16**, 81-87.

Mukhopadhyay, A., Barbieri, A. M., Funato, K., Roberts, R. and Stahl, P. D. (1997). Sequential actions of Rab5 and Rab7 regulate endocytosis in the Xenopus oocyte. *J. Cell Biol.* **136**, 1227-1237.

Nielsen, E., Severin, F., Backer, J. M., Hyman, A. A. and Zerial, M. (1999). Rab5 regulates motility of early endosomes on microtubules. *Nat. Cell Biol.* **1**, 376-382.

Novick, P. and Zerial, M. (1997). The diversity of Rab proteins in vesicle transport. *Curr. Opin. Cell Biol.* **9**, 496-504.

Nuoffer, C., Davidson, H. W., Matteson, J., Meinkoth, J. and Balch, W. E. (1994). A GDP-bound of rab1 inhibits protein export from the endoplasmic reticulum and transport between Golgi compartments. *J. Cell Biol.* **125**, 225-237.

Olkkonen, V. M., Dupree, P., Killisch, I., Lutcke, A., Zerial, M. and Simons, K. (1993). Molecular cloning and subcellular localization of three GTP-binding proteins of the rab subfamily. *J. Cell Sci.* **106**, 1249-1261.

Pagano, R. E., Martin, O. C., Kang, H. C. and Haugland, R. P. (1991). A novel fluorescent ceramide analogue for studying membrane traffic in animal cells: accumulation at the Golgi apparatus results in altered spectral properties of the sphingolipid precursor. *J. Cell Biol.* **113**, 1267-1279.

Pfeffer, S. R. (1999). Transport-vesicle targeting: tethers before SNAREs. *Nat. Cell Biol.* **1**, E17-E22.

Ren, M., Xu, G., Zeng, J., De Lemos-Chiarandini, C., 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 sorting endosomes. *Proc. Natl. Acad. Sci. USA* **95**, 6187-6192.

Schimmoller, F., Simon, I. and Pfeffer, S. R. (1998). Rab GTPases, directors of vesicle docking. *J. Biol. Chem.* **273**, 22161-22164.

Schlierf, B., Fey, G. H., Hauber, J., Hocke, G. M. and Rosorius, O. (2000). Rab11b is essential for recycling of transferrin to the plasma membrane. *Exp. Cell Res.* **259**, 257-265.

Somsel, R. J. and Wandinger-Ness, A. (2000). Rab GTPases coordinate endocytosis. *J. Cell Sci.* **113**, 183-192.

Sonnichsen, B., De Renzis, S., Nielsen, E., Rietdorf, J. and Zerial, M. (2000). Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J. Cell Biol.* **149**, 901-914.

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

Stahl, P. D., Schlesinger, P. H., Sigardson, E., Rodman, J. S. and Lee, Y. C. (1980). Receptor-mediated pinocytosis of mannose-glycoconjugates by macrophages: characterization and evidence for receptor recycling. *Cell* **19**, 207-215.

Sutton, R. B., Fasshauer, D., Jahn, R. and Brunger, A. T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature* **395**, 347-353.

Terasaki, M. and Reese, T. S. (1992). Characterization of endoplasmic reticulum by co-localization of BiP and dicarbocyanine dyes. *J. Cell Sci.* **101**, 315-322.

Ullrich, O., Reinsch, S., Urbe, S., Zerial, M. and Parton, R. G. (1996). Rab11 regulates recycling through the pericentriolar recycling endosome. *J. Cell Biol.* **135**, 913-924.

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

Vitelli, R., Santillo, M., Lattero, D., Chiariello, M., Bifulco, M., Bruni, C. B. and Bucci, C. (1997). Role of the small GTPase Rab7 in the late endocytic pathway. *J. Biol. Chem.* **272**, 4391-4397.

Wang, X., Kumar, R., Navarre, J., Casanova, J. E. and Goldenring, J. R. (2000). Regulation of vesicle trafficking in madin-darby canine kidney cells by Rab11a and Rab25. *J. Biol. Chem.* **275**, 29138-29146.

Zerial, M. and McBride, H. (2001). Rab proteins as membrane organizers. *Nat. Rev. Mol. Cell Biol.* **2**, 107-117.