Rab11 Regulates Recycling through the Pericentriolar Recycling Endosome

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Abstract. Small GTPases of the rab family are crucial elements of the machinery that controls membrane traffic. In the present study, we examined the distribution and function of rab11. Rab11 was shown by confocal immunofluorescence microscopy and EM to colocalize with internalized transferrin in the pericentriolar recycling compartment of CHO and BHK cells. Expression of rab11 mutants that are preferentially in the GTP- or GDP-bound state caused opposite effects on the distribution of transferrin-containing elements; rab11-GTP expression caused accumulation of labeled elements in the perinuclear area of the cell, whereas rab11-GDP caused a dispersion of the transferrin labeling. Functional studies showed that the early steps of uptake and recycling for transferrin were not affected by overexpression of rab11 proteins. However, recycling from the later recycling endosome was inhibited in cells overexpressing the rab11-GDP mutant. Rab5, which regulates early endocytic trafficking, acted before rab11 in the transferrin-recycling pathway as expression of rab5-GTP prevented transport to the rab11-positive recycling endosome. These results suggest a novel role for rab11 in controlling traffic through the recycling endosome.

MEMBERS of the rab family of small GTPases play an essential role in the regulation of membrane traffic (for reviews see Goud and McCaffrey, 1991; Pfeffer, 1992; Simons and Zerial, 1993; Zerial and Stenmark, 1993). Rab proteins have been localized to many different intracellular organelles on both the endocytic and exocytic pathways of eukaryotic cells, suggesting that each step of membrane traffic might involve a different rab protein. A particularly striking result to emerge from these studies is the number of different rab proteins localized to early compartments of the endocytic pathway. Rab4, rab5, rab17, rab18, and rab20 have all been shown to associate with early endocytic compartments (Chavrier et al., 1990*b*; Lütcke et al., 1993, 1994; van der Sluijs et al., 1991), suggesting a very high level of complexity in their organization and function. In fact, it is now apparent that the early endosome comprises at least two functionally distinct and separate compartments (Ghosh et al., 1994). Internalized receptors and ligands first enter the peripheral sorting endosome. In this compartment, membrane proteins destined for degradation are sorted away from those proteins, such as the transferrin receptor, which are recycled back to the plasma membrane (Dunn et al., 1989; Ghosh and Maxfield, 1995). Recycling back to the plasma membrane can occur directly from the sorting endosome (fast cycle) or indirectly via the so-called recycling endosome (Hopkins et al., 1994). The recycling endosome has a tubular morphology and is located close to the centrioles in many cell types; this organization is maintained by microtubules (Yamashiro et al., 1984). In migrating cells, the recycling endosome may play a role in directing receptors to the leading edge of the cell (Hopkins et al., 1994). However, other studies have suggested that the transferrin receptor can return from the recycling endosome to the sorting endosome rather than to the plasma membrane (Ghosh and Maxfield, 1995), or that the entire endocytic pathway is interconnected (Hopkins et al., 1990). The exact boundaries between the recycling and sorting endosomes, their relationship, and the pathways leading from these compartments to the cell surface therefore remain unclear and analysis of these pathways is complicated by the morphological complexity of the compartments as well as their plasticity and variation between different cell types.

Given the role of rab proteins in regulating distinct steps of intracellular transport, we examined the localization of rab GTPases in relation to the recycling endosome. Rab11 has previously been shown to be associated with post-Golgi membranes, including the TGN, secretory vesicles (Urbé et al., 1993), and the apical tubulovesicles of parietal cells (Goldenring et al., 1994; for review see Urbé and Parton, 1995). In addition, the ray homologue ora3 was

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found to associate with synaptic vesicles (Volknandt et al., 1993). Here, we show that rab11 is associated with the pericentriolar recycling endosome, and rab11 mutants modify the morphology of this compartment. In addition, we identify rab11 as the first small GTPase to regulate traffic through the recycling endosome.

Materials and Methods

Cell Culture and Antibodies

Media and reagents for cell culture were purchased from GIBCO BRL (Eggenheim, Germany). CHO cells were grown in α-modified MEM containing 10% (vol/vol) heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. BHK-21 cells were cultured in Glasgow's MEM (GMEM)[1] supplemented with 10% tryptose phosphate broth, 5% (vol/vol) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

Polyclonal antiserum 121 was raised against full-length recombinant rab11 expressed in *Escherichia coli* (Urbé et al., 1993), and was affinity purified essentially as described previously (Zerial et al., 1992). Antibodies against the human transferrin receptor were purchased from Boehringer Mannheim GmbH (Mannheim, Germany).

Plasmids and Transfection

DNAs encoding rab11 (Chavrier et al., 1990*a*), rab11 mutants, rab5 (Chavrier et al., 1990*b*), rab5 Q79L (Stenmark et al., 1994), and the human transferrin receptor (Zerial et al., 1986) were cloned into pGEM1 vector (Promega, Madison, WI) under the T7 promoter. Rab11 Q70L and rab11 S25N were constructed by PCR-based mutagenesis using a modification of Landt et al. (1990). In the first amplification reaction the mutant primers 5′-GGTCTAGAGCGATACCGAGCTATAAC-3′, 5′-CTAGACCTGCTGTGTCCCATATCTG-3′ (rab11 Q70L), and 5′-GAATAA TCTCTTGTCTCGATTTAC-3′, 5′-TTATTCTTTCCAACACCAGAATCTCC-3′ (rab11 S25N) were used in combination with oligonucleotides corresponding to the SP6 and T7 promoters present in the vector. The resulting fragments were isolated by agarose gel electrophoresis and purified, and the two overlapping fragments of each mutant were linked by primer extension and amplified by a second PCR. Restriction fragments from the amplification were cloned into pGEM1-rab11, and the PCR-amplified regions were sequenced to verify the mutations and to exclude additional mutations introduced by the Taq polymerase.

For transient overexpression studies using the T7 RNA polymerase recombinant vaccinia virus (vT7) expression system, CHO and BHK-21 cells were infected with vT7 and then transfected for 4.5 h with the appropiate pGEM1 plasmids using DOTAP (Boehringer Mannheim) as described previously (Stenmark et al., 1995). Transient expression of human α-2,6-sialyltransferase (SialylT) was performed by transfecting CHO cells with a vesicular stomatitus virus (VSV)-G epitope-tagged SialylT (Rabouille et al., 1995) by using standard calcium phosphate precipitation protocols.

Confocal Immunofluorescence Microscopy

Confocal microscopy was performed essentially as described (Zerial et al., 1992). Cells grown on 10-mm round coverslips were either permeabilized with 0.05% saponin before fixation or fixed in 3% paraformaldehyde followed by permeabilization in 0.1% Triton X-100. Endogenous rab11 was detected using affinity-purified rabbit anti-rab11 antibody. Overexpressed proteins were visualized with rabbit rab11-antiserum, mouse mAb 4F11 against rab5 (Bucci et al., 1994), and P5D4 mouse mAb, which recognizes the VSV-G tag (Kreis, 1986) of SialylT. As secondary antibodies, we used FITC-labeled donkey anti-mouse antibody and rhodamine-labeled donkey anti-rabbit antibody (Dianova, Hamburg, Germany). Coverslips were mounted on Mowiol (Hoechst AG, Frankfurt, Germany) and examined in the EMBL confocal microscope, using the excitation wavelengths 529 and 476 nm. Optical sections were 0.5 μm in the *z* plane.

1. *Abbreviations used in this paper*: GMEM, Glasgow's MEM; SialylT, sialyl transferase; Tf-HRP, human transferrin conjugated to peroxidase; VSV, vesicular stomatitis virus; WT, wild type.

Internalization of FITC-Transferrin

Iron-saturated human transferrin (Sigma Immunochemicals, St. Louis, MO) was labeled with FITC as described before (Ullrich et al., 1993). For continuous FITC-transferrin internalization, transfected cells grown on 10-mm round coverslips were incubated in internalization medium (GMEM, 0.1% BSA, 10 mM Hepes, pH 7.3, 10 μg/ml cycloheximide, and 10 mM hydroxyurea) with 25 μg/ml FITC-transferrin for 30 min at 37°C. Cells were then washed once with cold PBS, extracted with cold 0.05% saponin for 5 min on ice, and fixed with 3% paraformaldehyde, first 5 min on ice and then 15 min at room temperature.

Internalization of surface-bound FITC-transferrin was performed by first binding FITC-transferrin (25 μg/ml) to cells on ice for 30 min in internalization medium. Cells were washed three times with ice-cold GMEM/BSA and warmed up in internalization medium containing 5 mM nitrilotriacetic acid for different periods of time. Samples were then washed and fixed in 3% paraformaldehyde followed by permeabilization in 0.1% Triton X-100.

EM

BHK or CHO cells were cotransfected with plasmids encoding rab11 or rab11 mutants, together with pGEM-human transferrin receptor. After 5 h, the cells were washed, and then incubated continuously with 25 μg/ml human transferrin conjugated to peroxidase (Tf-HRP; Rockland Inc., Gilbertsville, PA) for 30 min at 37°C. At this concentration, only those cells expressing high levels of the transferrin receptor were strongly labeled with peroxidase reaction product. The DAB reaction and extraction procedure were adapted from the method of (Stoorvogel et al., 1996). The cells were then washed 5 × 2 min in PHEM buffer (60 mM K-Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9), and the living cells were then treated with diaminobenzidine/hydrogen peroxide in the same buffer for 10 min at room temperature to visualize the peroxidase reaction product. After further washing (5 × 4 min in PHEM), the cells were incubated with two changes of 0.005% saponin in PHEM buffer for a total time of 6 min to permeabilize the cells. The cells were then washed once with PHEM buffer, fixed with 3% paraformaldehyde in phosphate buffer, pH 7.35, and labeled with antibodies to rab11 by a modification of the usual immunofluorescence protocol. Briefly, after washing the fixed cells, they were quenched with NH_4Cl, incubated with a blocking mixture (5% FCS, 2% fish skin gelatin in PBS), and incubated for 1 h at room temperature with antibody 121 to rab11. The cells were then washed and further incubated with 10 nm protein A–gold for 1 h at room temperature before washing and fixation in 0.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.35, and embedding in Epon as previously described (Parton et al., 1991).

Measurement of Transferrin Uptake and Recycling

Iron-saturated human transferrin (Sigma Immunochemicals) was labeled with ^{125}I to a specific activity of 3×10^7 cpm/μg using Iodogen (Fraker and Speck, 1978). Cells grown in 12-mm wells were transfected for 4 h as described above and incubated in internalization medium (GMEM, 0.1% BSA, 10 mM Hepes, pH 7.3, 10 μg/ml cycloheximide, and 10 mM hydroxyurea) for 1 h at 37°C. To measure uptake and recycling of surface-bound transferrin, cells were first incubated with 15 nm ^{125}I-transferrin diluted to 100 nM with cold transferrin in internalization medium for 60 min on ice (surface binding), washed six times with cold PBS/0.1% BSA, and incubated with internalization medium containing 5 mM nitrilotriacetic acid for various times at 37°C (endocytosis/recycling). The medium was collected and surface-bound ^{125}I-transferrin was removed with 3 mg/ml pronase E (Sigma) in GMEM/10 mM Hepes, pH 7.3, for 1 h on ice (Ciechanover et al., 1983). The cells were then pelleted (2 min at 3,000 *g*), and the radioactivity associated with the cells (endocytosed), the supernatant (surface-bound), and the incubation medium (recycled) was measured by gamma counting.

To measure recycling of transferrin from the recycling endosomes, cells were first incubated with 15 nM ^{125}I-transferrin in internalization medium for 5 min at 37°C (continuous internalization), washed six times with cold PBS/0.1% BSA, and incubated with internalization medium/5 mM nitrilotriacetic acid for 30 min at 37°C (to chase out fast recycling ^{125}I-transferrin). The medium was removed, and the cells were washed and further incubated with internalization medium/5 mM nitrilotriacetic acid for 30 min at 37°C for increasing periods of time. Samples were then treated as described above.

Results

Rab11 Is Associated with the Pericentriolar Transferrin Recycling Compartment in CHO and BHK Cells

We examined the distribution of rab11 in CHO and BHK cells by immunofluorescence using a well-characterized affinity-purified antibody (Urbé et al., 1993) and confocal laser scanning microscopy. In CHO cells, the bulk of the labeling was present in a single discrete patch in the perinuclear area of the cell and a few small dispersed dots throughout the cytoplasm (Fig. 1 *a*). The staining pattern was reminiscent of the pericentriolar recycling endosome in which transferrin accumulates after internalization to steady state (Ghosh and Maxfield, 1995). We therefore incubated CHO cells with FITC-transferrin for 30 min at 37°C. The rab11 staining in the perinuclear area colocalized with the internalized FITC-transferrin as shown by confocal microscopy (Fig. 1, *a* and *b*). We then examined the distribution of rab11 in BHK cells that were used for subsequent biochemical experiments. Endogenous labeling for both rab11 and transferrin receptor was low in these cells (results not shown). We therefore transiently overexpressed wild-type (WT) rab11 and the human transferrin receptor using the vaccinia VT7 system. FITC-transferrin was again internalized continuously for 30 min at 37°C. There was good colocalization of the internalized transferrin and rab11. In the BHK cells, the perinuclear patch was less evident and the dispersed dots were more prominent (Fig. 1, *c* and *d*).

We next examined the association of transferrin with the rab11-positive compartment in a pulse chase protocol in CHO cells. FITC-transferrin was first bound at 4°C to the cell surface, washed, and then the cells were warmed to allow a pulse of labeled transferrin to enter the cell (Fig. 2). After 2 min at 37°C, the transferrin labeling was dispersed throughout the cell periphery, but after 5 min, a fraction of the transferrin already colocalized with rab11 in the perinuclear area. Note that the rab11 staining generally appeared more dispersed after low temperature incubations, possibly because of partial microtubule depolymerization (Lieuvin et al., 1994). The colocalization of internalized transferrin and rab11 increased considerably after 15 min and was maximal after 30 min at 37°C. Labeling was still present within this perinuclear compartment, although it was decreased after a 60-min incubation. These results show that rab11 is associated with the perinuclear recycling endosome.

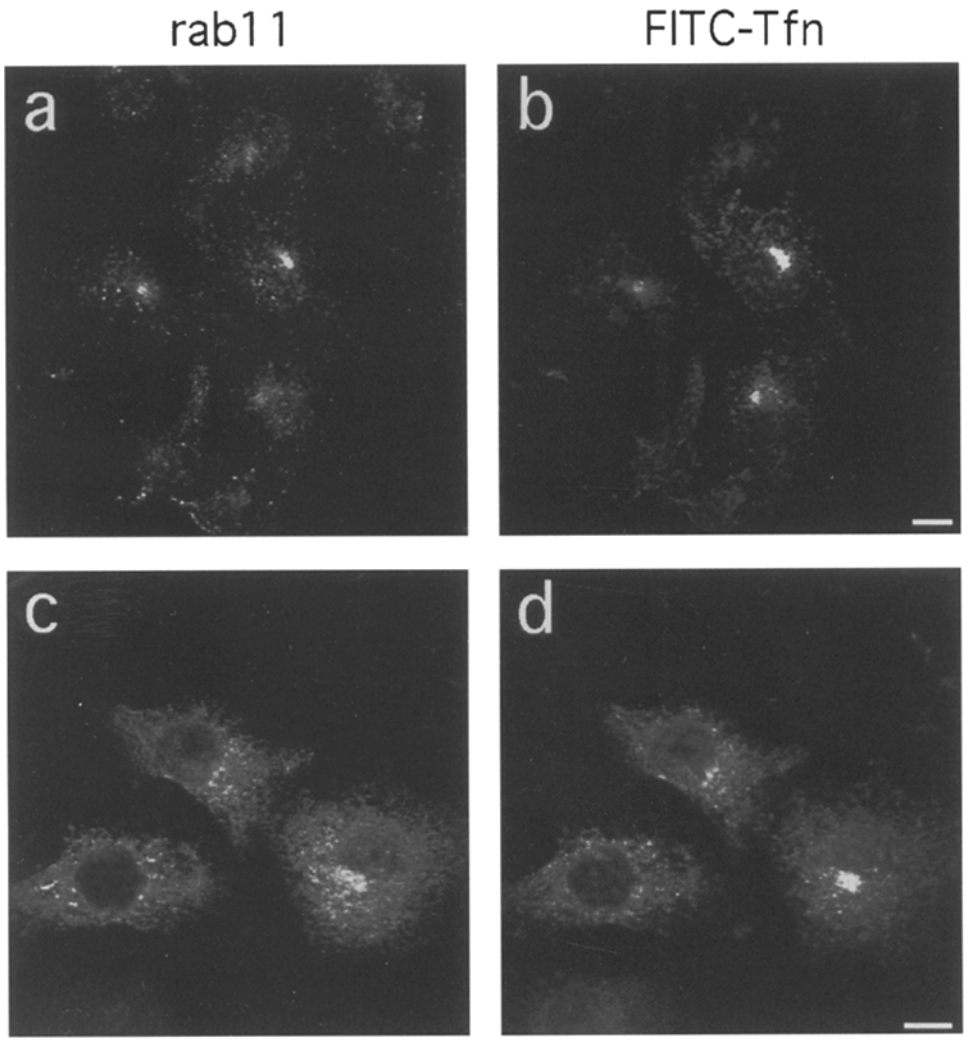


Figure 1. Localization of rab11 and internalized FITC-transferrin in CHO and BHK cells. Confocal microscopy analysis shows that both endogenous and overexpressed rab11 colocalize with internalized FITC-transferrin in CHO and BHK cells, respectively. CHO (*a* and *b*) and BHK (*c* and *d*) cells were infected with vT7 and transfected with plasmids encoding human transferrin receptor alone (*a* and *b*) or transferrin receptor plasmid in combination with rab11 plasmid (*c* and *d*). Cells were then incubated with 25 μg/ml FITC-transferrin for 30 min at 37°C, washed once with cold PBS, permeabilized with 0.05% saponin, and fixed with 3% paraformaldehyde before incubation with antibodies. We used rabbit anti-rab11 as a primary antibody and rhodamine-labeled donkey anti-rabbit as a secondary antibody. Bars, 10 μm.

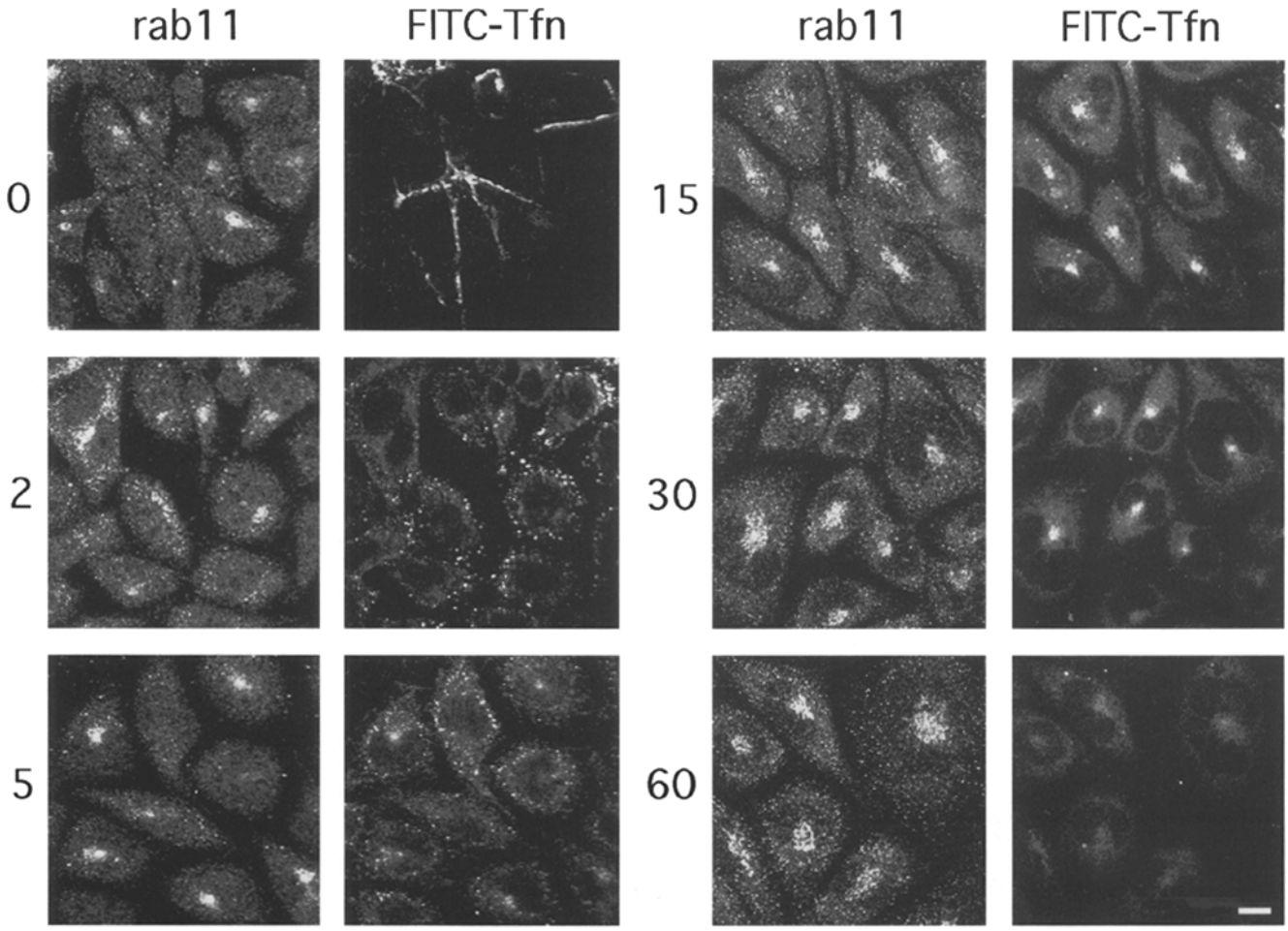


Figure 2. Time course of FITC-transferrin internalization into the rab11-positive recycling endosome. Confocal microscopy analysis of CHO cells that internalized surface-bound FITC-transferrin for increasing periods of time. Cells were first incubated with FITC-transferrin (25 μg/ml) for 30 min on ice, washed, and warmed up for the indicated periods of time (0–60 min) at 37°C. Samples were then washed, fixed, and stained for rab11. Bar, 10 μm.

We have previously shown that rab11 is associated with the TGN, secretory granules, and constitutive exocytic vesicles in PC12 cells by subcellular fractionation and EM analysis (Urbé et al., 1993). Since earlier studies (Hopkins et al., 1983; Yamashiro et al., 1984; Connolly et al., 1994) have shown that the recycling endosome is in the proximity of, but distinct from, the Golgi apparatus, we examined the localization of rab11 with respect to the two organelles. We therefore compared the localization of rab11 with that of internalized FITC-transferrin and α-2,6-SialylT, a marker for the trans-Golgi cisterna and the TGN (Roth et al., 1985). VSV-G epitope-tagged SialylT (Rabouille et al., 1995) was transiently overexpressed in CHO cells, and FITC-transferrin was continuously internalized for 30 min at 37°C. Consistent with previous observations, whereas the perinuclear compartment labeled with FITC-transferrin was positioned closely to that stained for SialylT, the two markers showed only very limited overlap (Fig. 3, *a–c*). The rab11 staining (Fig. 3, *d* and *f*, *arrows*) only partially colocalized with that of SialylT (Fig. 3, *e* and *f*), and some structures in the perinuclear area, presumably corresponding to the recycling endosome, were negative for the Golgi marker. To increase segregation of the two pools of rab11, we preincubated cells with nocodazole to disrupt the microtuble network and disperse the Golgi apparatus (Rogalski and Singer, 1984; Turner and Tartakoff, 1989) and the recycling endosome (Sakai et al., 1991; McGraw et al., 1993). Both rab11 and SialylT were found to redistribute from the perinuclear area towards the periphery of the cell and again exhibited only partial colocalization (Fig. 3, *g–i*). Identical results were obtained with BHK cells (not shown). These results suggest that rab11 is present on both the Golgi complex and the recycling endosome.

Rab11 Shows Partial Colocalization with Overexpressed WT rab5, but Not with Mutant GTP-bound rab5

Rab5 has been localized to the early sorting endosome of mammalian cells, where it colocalizes both with internalized fluid-phase markers (Chavrier et al., 1990*b*) and with transferrin receptor (Bucci et al., 1992). We examined the distribution of rab5 in relation to rab11. Rab5 and rab11 were overexpressed in CHO cells, and their distributions were compared by double-label confocal microscopy. Under these conditions, rab5 is overexpressed 5–15-fold over the endogenous level (Bucci et al., 1992). Overexpressed

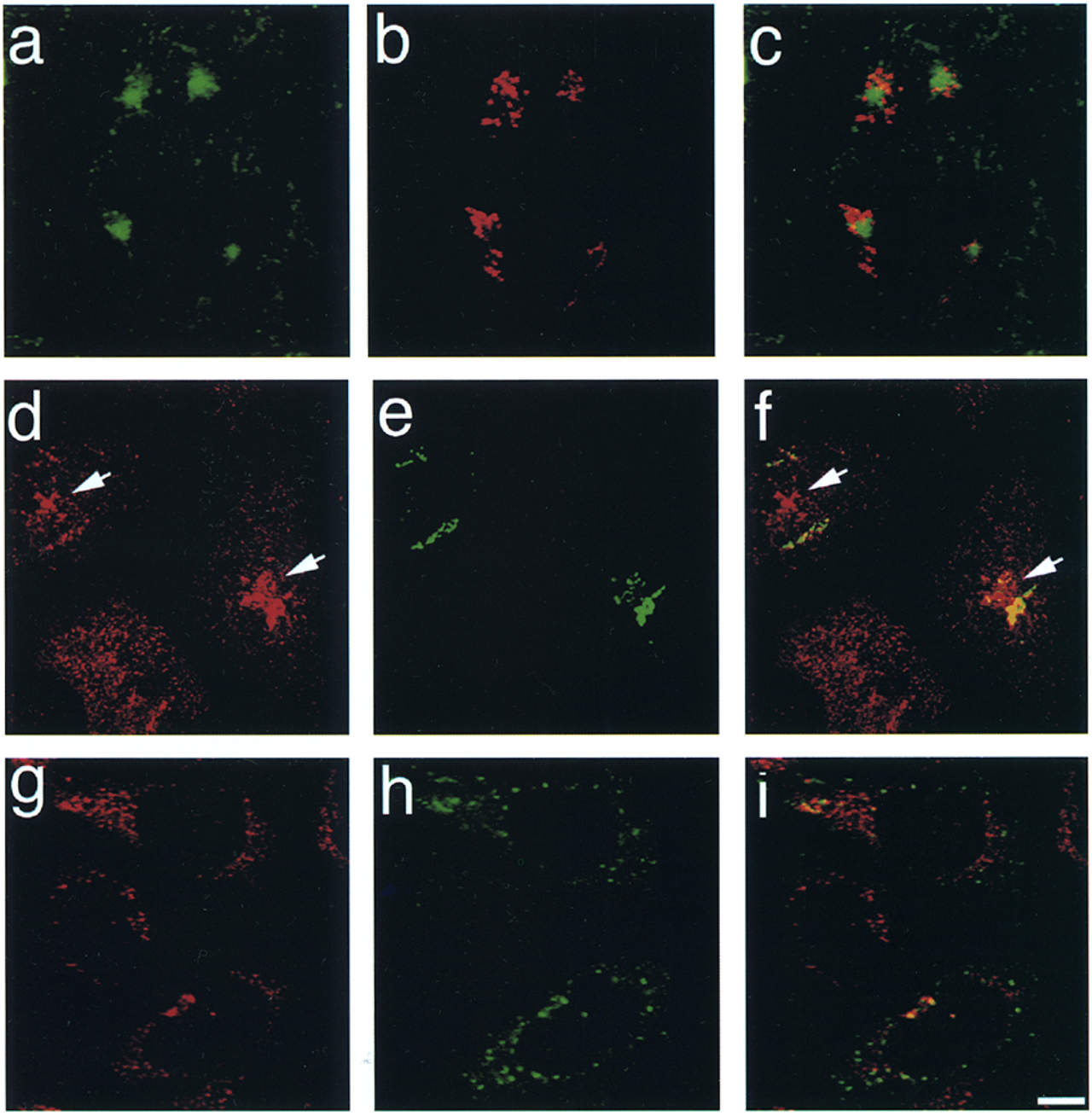


Figure 3. Localization of rab11 and the Golgi marker SialylT in CHO cells. CHO cells were transfected with plasmid encoding VSV-G epitope tagged SialylT. (*a–c*) Cells were then incubated for 30 min with FITC-transferrin at 37°C and stained with P5D4 mouse mAb, which recognizes the VSV-G tag. (*a*) FITC-transferrin, (*b*) SialylT, (*c*) overlay. (*d–f*) Transfected cells were stained for rab11 (*d*) and SialylT (*e*). *f* shows the overlay. (*d* and *f*) Arrows indicate the localization of the rab11-positive recycling endosome. (*g–i*) Transfected cells were incubated for 3 h with 20 μM nocodazole and stained for rab11 (*g*) and SialylT (*h*). (*i*) Overlay. Bar, 10 μm.

WT rab5 and WT rab11 showed a high level of colocalization in the pericentriolar region of the cell (Fig. 4, *a* and *b*). To further explore the overlap between the two proteins, we took advantage of the effects induced by the rab5Q79L mutant on the early endosome compartment. Expression of rab5Q79L, which is preferentially in the GTP-bound state, induces the formation of characteristic enlarged endosomes (Stenmark et al., 1994). Moreover, this mutant inhibits the kinetics of transferrin recycling and, consequently, reduces the pool of transferrin receptor on the cell surface. We therefore investigated whether the morphological alterations on the early endosomes induced by rab5Q79L also affect the rab11-positive recycling compartment. In contrast to WT rab5, rab5Q79L showed very limited colocalization with overexpressed rab11; the characteristically enlarged rab5Q79L-positive, ring-shaped endosomes were clearly negative for rab11 (Fig. 4, *c* and *d*). We then examined the routing of transferrin in these cells by expression of the human transferrin receptor together with rab5Q79L. Internalized FITC-labeled transferrin

strongly labeled the enlarged rab5Q79L-positive endosomes, as shown previously (Stenmark et al., 1994), but poorly labeled the rab11-positive recycling compartment (Fig. 4, *e–h*). In addition, the transferrin-labeled structures appeared more dispersed in the rab5Q79L-expressing cells (Fig. 4 *f*; nontransfected cells, i.e., lacking the expanded endosomes, have a perinuclear accumulation of transferrin indicated by large arrows, whereas the small arrow points to the enlarged early endosomes of transfected cells). This result suggests that transferrin enters the rab5-positive sorting endosome before the rab11-positive recycling endosome. In addition, the expression of the GTP-bound form of rab5 inhibits the transport of transferrin to the rab11-positive compartment, suggesting that the disruption of the normal GTP/GDP rab5 cycle indirectly affects traffic out of the sorting endosome to the recycling compartment.

Morphological Alterations of the Recycling Endosome in CHO Cells That Overexpress Mutants of rab11

Previously, we showed that mutant forms of rab5 that are predominantly in the GTP-bound (rab5Q79L) or GDP-bound state (rab5S34N) dramatically change the morphology of the early endosome and affect trafficking from the cell surface to the endosome (Stenmark et al., 1994). We therefore examined whether comparable mutants of rab11 would affect the morphology of the recycling endosome in CHO cells. The recycling endosome was labeled to steady state by a continuous 30-min incubation with FITC-transferrin in cells expressing rab11 WT, rab11Q70L (preferentially in the GTP-bound state), or rab11S25N (preferentially GDP-bound; Fig. 5). In WT rab11–expressing cells (Fig. 5, *a* and *b*), the transferrin-labeled compartment comprised dispersed peripheral elements and a highly concentrated small patch of transferrin-containing elements in the perinuclear area. In cells expressing rab11Q70L (Fig. 5, *c* and *d*), the distribution of labeled elements was greatly shifted towards the perinuclear area and few labeled peripheral elements were evident. In contrast, cells expressing rab11S25N (Fig. 5, *e* and *f*) showed little perinuclear concentration of labeled elements and mostly peripheral dispersed staining. Similar results were obtained with BHK cells (data not shown). Since the extent of colocalization between the rab11 S25N-mutant and FITC-transferrin decreased considerably (Fig. 5, *e* and *f*; areas of colocalization are indicated by arrows), we tested whether the exit of FITC-transferrin from the sorting endosomes is retarded in these cells. CHO cells overexpressing rab11S25N and WT rab5 were allowed to internalize FITC-transferrin for 3 or 30 min and were stained for rab5. FITC-transferrin internalized for 3 min showed a high degree of colocalization with rab5 in early endosomes (Fig. 5, *g* and *h*). Even after 30 min of labeling, there was a high degree of overlap between the two markers (Fig. 5, *i* and *j*), suggesting that exit of FITC-transferrin from the sorting endosome is slowed down by the rab11 S25N mutant. These results indicate that the rab11 mutants have opposite effects on the recycling endosomes, comparable to the effects of rab5 mutants on the sorting endosomes.

We then used an EM approach modified from that of Stoorvogel et al. (1996) to examine the distribution of

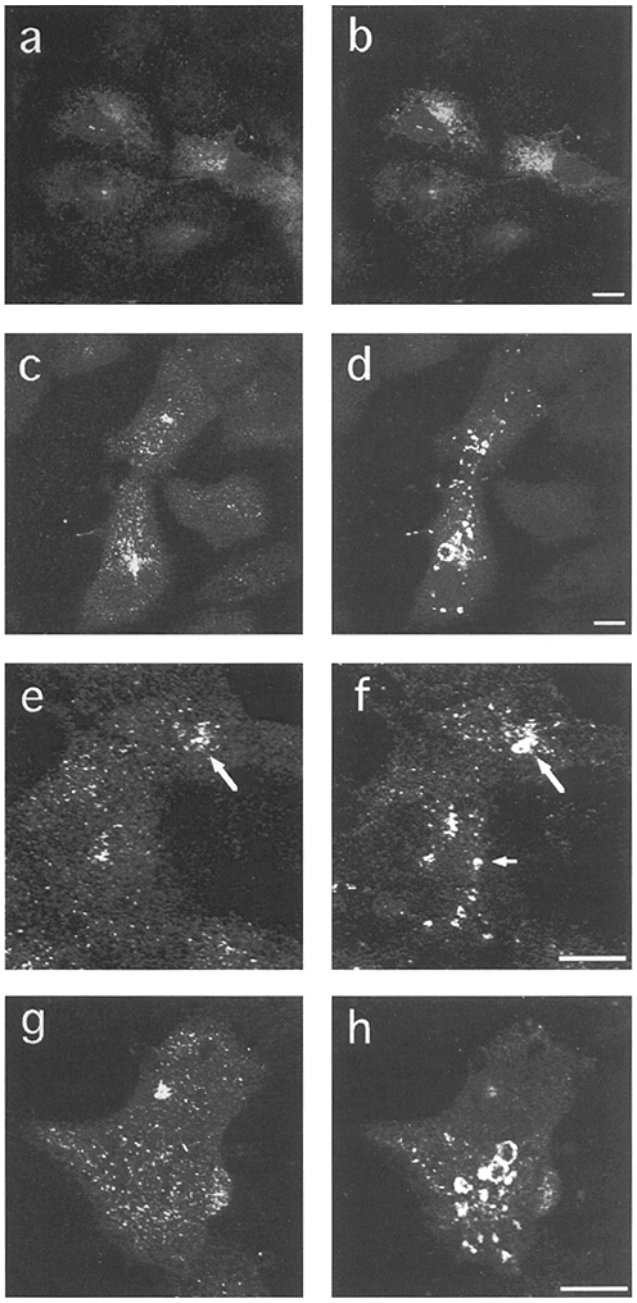


Figure 4. Localization of rab11 and internalized FITC-transferrin in cells expressing rab5 and rab5 Q79L. CHO cells were cotransfected with plasmids encoding rab11 and rab5 (*a* and *b*), rab11 and rab5 Q79L (*c* and *d*), and rab5 Q79L and human transferrin receptor (*e–h*). In *e–h*, cells were additionally incubated with FITC-transferrin for 30 min at 37°C as described in Fig. 1. After extraction and permeabilization, cells were stained for confocal microscopy analysis with rabbit rab11 antiserum (*a* and *c*, *e* and *g*) and mouse anti-rab5 mAb (*b* and *d*), using rhodamine-labeled donkey anti–rabbit antibody and FITC-labeled donkey anti–mouse antibody as secondary antibodies. (*f* and *h*) The localization of FITC-transferrin. (*e* and *f*) Large arrows indicate the localization of the recycling endosome in nontransfected cells, and the short arrow points at the enlarged endosomes in transfected cells. Transfected cells are positively recognized by the presence of the characteristic enlarged early endosomes induced by rab5Q79L. Bars, 10 μm.

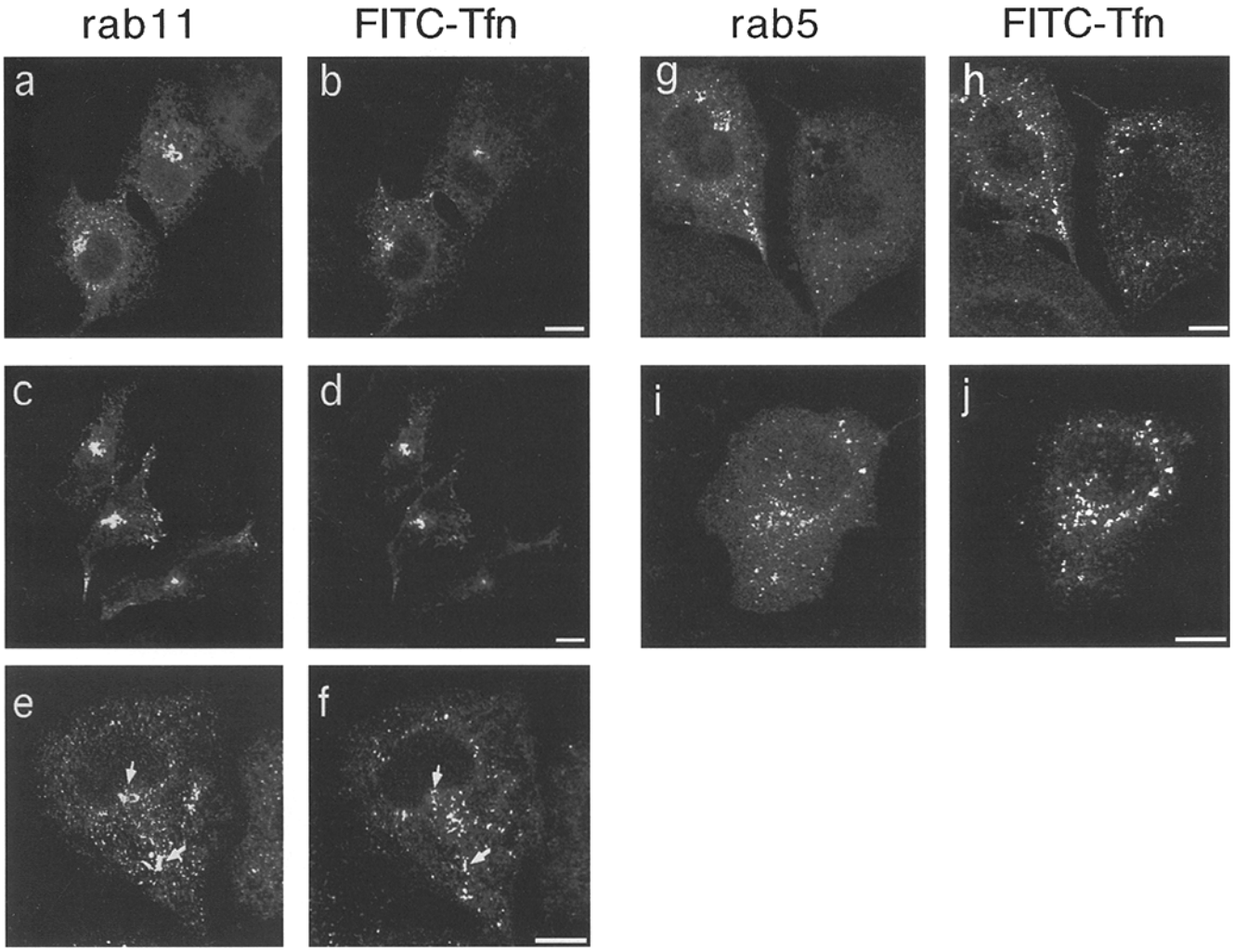
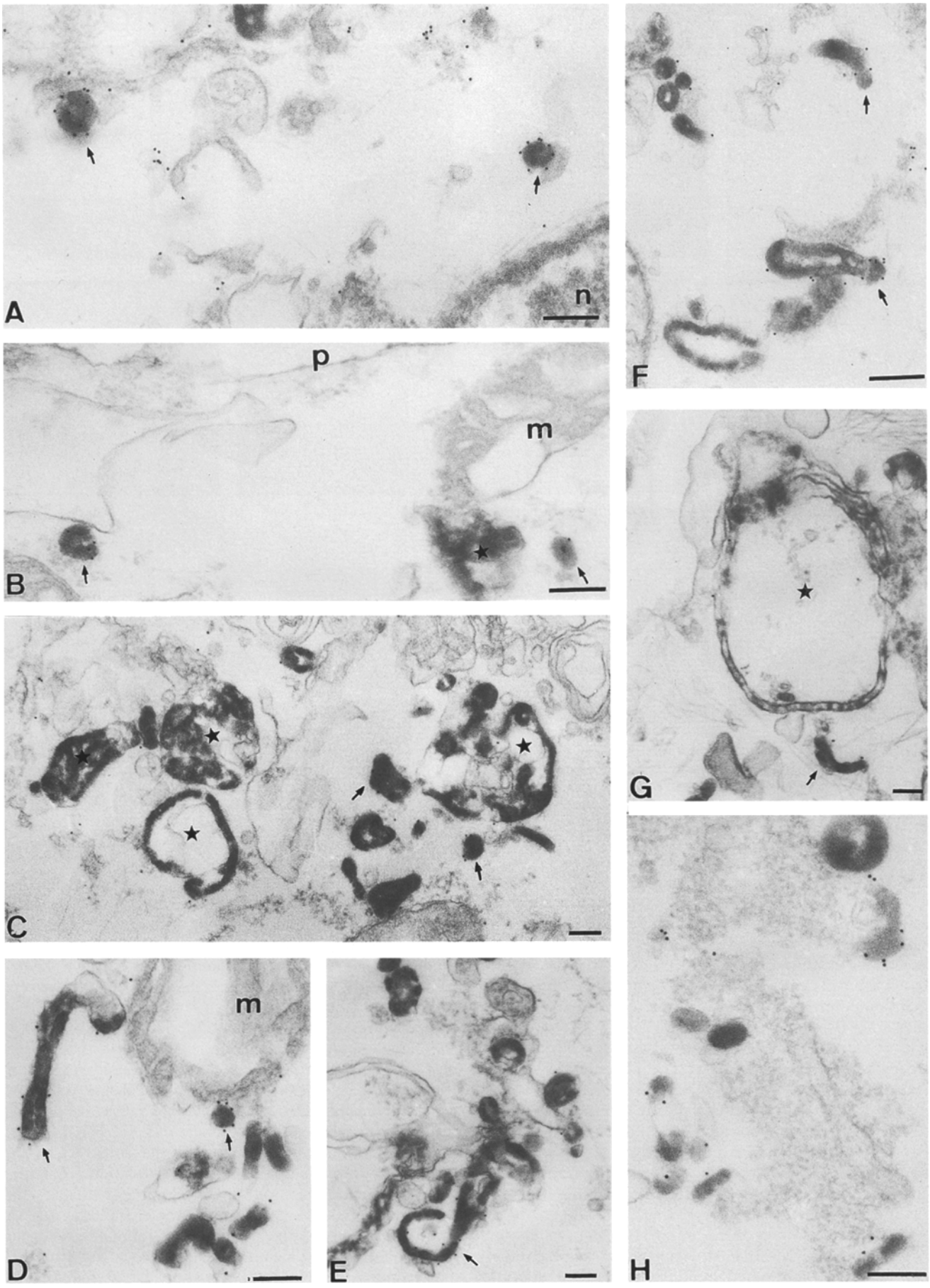


Figure 5. Morphological alterations of the transferrin-positive recycling endosome in cells expressing WT rab11 or rab11 mutants. CHO cells were cotransfected with plasmids encoding human transferrin receptor together with rab11 (*a* and *b*), rab11 Q70L (*c* and *d*), rab11 S25N (*e* and *f*), or rab11 S25N together with rab5 wt (*g–j*). FITC-transferrin was then internalized for 5 min (*g* and *h*) or 30 min (*a–f*, *i* and *j*) at 37°C, and cells were stained for rab11 (*a*, *c*, and *e*) and mouse anti-rab5 mAb (*g* and *i*), as described in Fig. 1. Bars, 10 μm.

rab11 mutant proteins with respect to internalized Tf-HRP at the ultrastructural level. WT rab11 (results not shown), rab11Q70L, or rab11S25N, were coexpressed with the human transferrin receptor in BHK cells. Tf-HRP was then internalized continuously for 30 min at 37°C. We then treated the cells with DAB before fixation to fill the Tf-HRP–containing endosomes with the electron-dense peroxidase-DAB reaction product. This stabilized the endosomal membrane allowing us to extract the cells with saponin before fixation. This procedure provided a number of advantages over conventional localization methods. First, the use of Tf-HRP allowed us to easily locate the double-transfected cells, since fluorescence studies indicated that essentially all cells that express the transferrin receptor also express the rab11 proteins (endogenous transferrin-binding activity is very low in BHK cells, allowing the easy detection of transfected cells). Second, we could compare the distribution of the different rab11 proteins and the morphology of the Tf-HRP–labeled structures in the same samples. Third, this method greatly facilitated the identification and labeling of small vesicles and tubules, such as those containing transferrin, which would have been difficult with an "on section" labeling technique. Finally and most importantly, we could express rab11 or rab11 mutants to high levels, but avoid the problem of filling the cytosol with non–membrane-associated rab11 by the use of a saponin extraction step.

Overexpressed rab11Q70L, rab11S25N, and WT rab11 (not shown) were specifically associated with the surface of Tf-HRP–containing endosomal tubules and vesicles in transfected cells (Fig. 6). No labeling was observed in non-transfected cells where the endogenous rab11 was not detectable with this antibody concentration. The labeling was not distributed uniformly over the entire Tf-HRP–positive endosomal population, but was highly concentrated on a subpopulation of 80–100-nm diameter vesicular elements as well as 50–80-nm diameter tubular profiles. The larger more complex endosomes containing internal membranes showed significant but much lower labeling. These endosomes consisted of a ring-shaped, membrane-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enclosed cisternae surrounding an electron-lucent space (for example, see Fig. 6 *G*) or had a multivesicular appearance (see Fig. 6 *C*). A quantitative analysis showed that the average density of labeling on rab11-positive small vesicles and tubules was more than 10-fold higher than on the limiting membrane of the large multivesicular regions after overexpression of either mutant. The plasma membrane and most other organelles showed much lower labeling, even in cells expressing high levels of rab11. Some Tf-HRP–negative vesicles and tubular profiles in the peri-Golgi area, however, were consistently labeled for rab11 (results not shown). The labeling pattern and morphology of the labeled elements were similar after overexpression of WT rab11t (results not shown), rab11Q70L, or rab11S25N. Note that the differences observed at the light microscopic level were less evident by EM. This suggests that the rab11 mutants might cause redistribution of transferrin-labeled elements rather than a gross change in morphology analagous to that seen with rab5 mutants (Bucci et al., 1992). The results show that rab11 associates with a subpopulation of transferrin-labeled endosomal membranes. The morphology of the rab11-positive structures and their labeling with internalized transferrin is consistent with the association of rab11 with the recycling endosome.

The Effect of rab11 Mutants on Transferrin Recycling

The localization of rab11 to the recycling endosome suggests a possible role in controlling traffic into or out of this compartment. We therefore examined the role of rab11 on the transferrin cycle by transient expression of WT rab11 and mutants together with the transferrin receptor. For these experiments, we used BHK cells, since these cells are more efficiently transfected compared to CHO cells and rab11 has an identical distribution in both cell types (see Fig. 1). The endogenous level of transferrin-binding activity in these cells is also low, allowing the effects of rab11 mutants to be examined in double-transfected cells (WT rab11 or mutants plus transferrin receptor) with a relatively low background from nontransfected cells. Iodinated transferrin was bound to the cell surface of the doubly transfected BHK cells, and the kinetics of internalization and externalization (recycling) was measured in cells expressing each of the rab11 proteins. As shown in Fig. 7 *a*, the initial rate of internalization was unaffected by the expression of WT rab11 and rab11 mutants. There was a small but consistent effect on transferrin recycling to the cell surface, however, especially evident with rab11 S25N, which was only detectable after longer incubation times.

In view of these results and the localization of rab11 to the recycling compartment, we performed a similar experiment but used conditions designed to preferentially study traffic through the recycling endosome. BHK cells were incubated with a 5-min pulse of labeled transferrin, washed to remove unbound ligand, and incubated a further 30 min at 37°C to allow transferrin to recycle out of the cell or chase to a later compartment. We then measured the recycling from this compartment. As shown in Fig. 7 *b*, after labeling the cells in this manner, expression of the rab11-S25N mutant caused a marked inhibition of transferrin recycling. Moreover, in contrast to the results when transferrin is prebound to the surface, the effects of rab11S25N overexpression are evident from the earliest time points examined. Overexpression of WT rab11 and rab11Q70L also significantly inhibited transferrin recycling, but their effect was more moderate than that of the rab11S25N mutant. Taken together, these results suggest that rab11 controls the traffic through the recycling endosome.

Discussion

Increasing evidence has shown that the recycling endosome is a morphologically and functionally distinct early endosomal subcompartment (for review see Gruenberg and Maxfield, 1995). The recycling endosome differs in several respects from the sorting endosome: (*a*) it shows a more pericentriolar distribution; (*b*) it is organized by microtubules; and (*c*) it exhibits a more tubular morphology. Until now, however, the compartment could only be defined functionally by the presence of recycling membrane proteins and the lack of receptors destined for degradation (Dunn et al., 1989; Ghosh and Maxfield, 1995). The further study of this compartment has been severely hampered by the lack of molecular markers, and no specific components of the molecular machinery involved in the traffic through this compartment have been identified. In the present study, we show that rab11 is a good candidate for such a component. Rab11 is associated with the pericentriolar recycling endosome and regulates the "slow cycle" of transferrin recycling through this compartment. In addition, we have shown that rab11 acts distal to rab5 in the endocytic pathway, but that rab5 dysfunction can affect traffic to the recycling endosome.

Several rab proteins that operate in the early stages of endocytic traffic have now been characterized functionally. Rab4 appears to be a regulator of the fast cycle of transferrin recycling (Daro et al., 1996; van der Sluijs et al., 1992). Rab4 is present on early sorting endosomes but absent from the pericentriolar recycling endosomes, and appears to regulate the early recycling step from the sorting endosome to the plasma membrane. In contrast to rab4, rab5 operates on the inward leg of endocytic traffic be-

Figure 6. Immunoelectron microscopic localization of Rab11-GTP and Rab11-GDP in BHK cells. BHK cells were cotransfected with the human transferrin receptor and rab11 Q70L (*A* and *B*) or rab11 S25N (*C–H*). They were then incubated with Tf-HRP to label the endocytic compartments of the doubly transfected cells. The cells were then further processed to detect the HRP reaction product and the membrane-associated overexpressed rab11 protein (10 nm gold), as described in the Materials and Methods section. The HRP reaction product was crosslinked to the inside of the endosomal membrane before permeabilization, giving a peripheral staining in some endosomal elements (e.g., see *D* and *F*). Labeling for rab11 Q70L or rab11 S25N is mainly associated with Tf-HRP–labeled vesicular and tubular profiles (*arrows*). Negligible labeling is associated with the ER surrounding the nucleus (*n*), with the mitochondria (*m*), and with the plasma membrane (*p*). The gold labeling associated with the Tf-HRP–labeled profiles is very heterogeneous and is mainly associated with small vesicles and tubules, and not with the larger multivesicular or cisternal domains (*stars*). Bars, 200 nm.

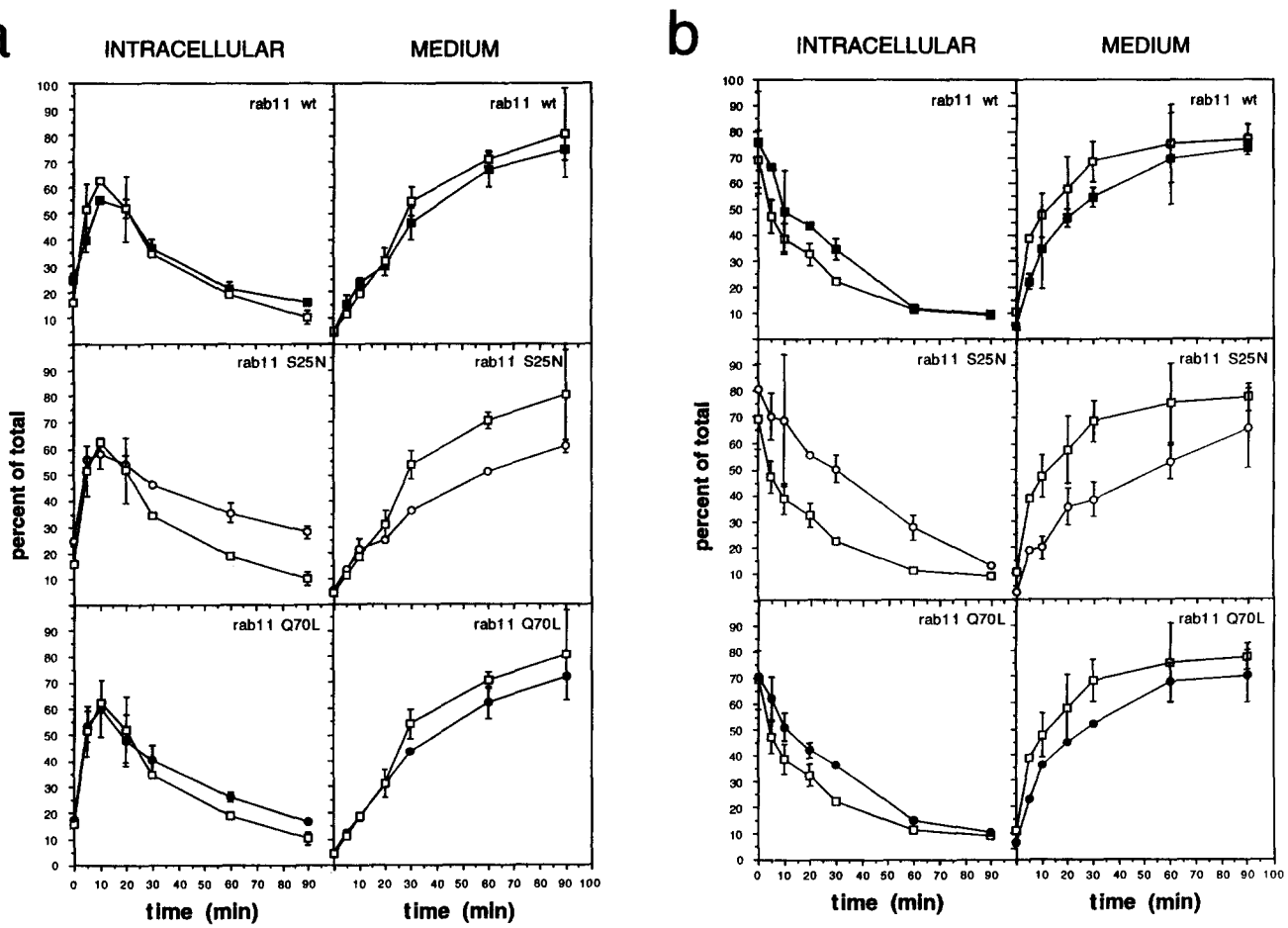

Figure 7. Kinetic analysis of the transferrin cycle in cells overexpressing WT rab11 or rab11 mutants. BHK cells transfected with human transferrin receptor alone (control, □) or together with WT rab11 (■), rab11 S25N (○), or rab11 Q70L (●). (*a*) Cycling of surface-bound transferrin: cells were allowed to bind ^{125}I-transferrin for 1 h on ice. Unbound ligand was removed, and cells were incubated at 37°C for the indicated periods of time to allow internalization of surface-bound ^{125}I-transferrin. (*b*) Recycling of transferrin from the recycling endosome: cells were first allowed to internalize ^{125}I-transferrin continuously for 5 min at 37°C. Cells were washed and then further incubated for 30 min at 37°C, to chase ^{125}I-transferrin into the recycling endosome. Finally, cells were washed again and incubated at 37°C for the indicated periods of time. (*a* and *b*) Intracellular ^{125}I-transferrin was calculated from the residual cell-associated radioactivity after pronase treatment. Measurements are plotted as percentage of total ^{125}I-transferrin (i.e., the sum of intracellular; surface-bound = pronase sensitive; recovered in medium). In each panel, the WT rab11 wt–, or mutant–expressing rab11 cells are shown together with the control cells (transferrin receptor alone). Here, a typical experiment is shown in which each time point represents the mean of duplicate samples, and the bar is the SEM. The experiments were repeated three times with similar results.

tween the plasma membrane and the sorting endosome (Bucci et al., 1992). The present study shows that a functional rab5 protein is required for subsequent transport to the rab11-positive recycling compartment. First, expression of the GTPase-deficient Q79L-mutant rab5 causes accumulation of transferrin internalized under steady-state conditions in the enlarged rab5-positive compartment rather than in the rab11-positive recycling endosome. Second, rab11 is highly segregated from overexpressed rab5Q79L, but colocalizes partially with overexpressed WT rab5. Taken together, the data suggest that hydrolysis of rab5-bound GTP is not required for the fusion of coated vesicles with the early endosome, but that the GTP–GDP cycle is required for the normal functioning of the early endosome, including traffic to the recycling endosome. The rab5 mutant may not only impair transport to the recycling endosome, but may also cause disruption of this compartment because of impaired membrane flow. This is consistent with previous studies showing a decreased recycling after expression of the rab5Q79L mutant (Stenmark et al., 1994).

Which is the transport step regulated by rab11? One possibility, suggested by the effects induced by the rab11 mutants, is that this GTPase regulates traffic coming from the sorting endosome to the recycling endosome. According to this model, expression of the rab11S25N mutant would inhibit the kinetics of transferrin transport to the recycling endosome, cause a dispersion of the transferrin-containing endosomal elements, and accumulate FITC-transferrin in the rab5-positive sorting endosomes. The fact that rab11S25N did not consequently increase the rate of recycling suggests that this pool of transferrin cannot be diverted into the fast recycling pathway.

On the other hand, overexpression of WT rab11 and the rab11Q70L mutant would instead be expected to increase the efficiency of this transport reaction. Why then do these

two proteins moderately inhibit transferrin recycling? It is possible that in WT rab11 and rab11Q70L overexpressing cells, less transferrin may recycle from the sorting endosome directly to the cell surface (short cycle), and a higher fraction may transit through the recycling endosome (long cycle), thereby resulting in an apparent inhibition of recycling. The morphological analysis is also consistent with this hypothesis, since the FITC-transferrin–labeled elements were more compact after expression of the GTP-bound mutant. The effects of rab11 on the recycling endosome therefore appear similar to those of rab5 on the sorting endosome (Bucci et al., 1992). Consistent with this interpretation, our morphological studies showed that rab11 is highly enriched on endosomal tubules and vesicles, but is also present at low levels on the multivesicular endosomal vacuolar domain. In addition, rab5 and rab11 show partial colocalization when both are overexpressed in CHO cells. Plasma membrane labeling with WT or mutant rab11 was also very low compared to the labeling associated with endosomal tubules. Rab4, which is thought to control sorting endosome to plasma membrane traffic, however, is also undetectable on the plasma membrane (van der Sluijs et al., 1992). Recent studies showing that transferrin receptors are not only transported (anterogradely) from the sorting endosome to the recycling endosome, but also in the opposite (retrograde) direction (Ghosh and Maxfield, 1995), emphasize the complexity of these traffic pathways and the need for more molecular markers.

The above model, which assumes an exclusive role for rab11 between the sorting and recycling endosome, is complicated by the observation that rab11 has been localized to a number of different compartments. We have shown here that in CHO cells, rab11 is present on FITC-transferrin–labeled recycling endosomes as well as on the Golgi apparatus, consistent with our previous studies in PC12 cells documenting the association of rab11 with the TGN, secretory granules, and constitutive exocytic vesicles (Urbé et al., 1993). In addition, a rab11 marine ray homologue is present on synaptic vesicles (Volknandt et al., 1993), and in gastric parietal cells, rab11 is associated with the H^+-K^+-ATPase–rich tubulovesicular compartment that underlies the plasma membrane (Goldenring et al., 1994). Upon stimulation, the H^+-K^+-ATPase is delivered into the secretory canalicular membrane, apparently through fusion of the tubules with the canalicular plasma membrane (Forte and Yao, 1996; but see Pettitt et al., 1996 for an alternative view). Rab11 shows a concomitant redistribution into the H^+-K^+-ATPase fraction upon stimulation (Goldenring et al., 1994). These observations raise the intriguing possibility that rab11 might not only be involved in constitutive recycling in fibroblasts, but also in the regulated reycling of membrane in secretory cells. Since increasing evidence suggests that synaptic vesicles are derived from early endosomes (Kelly, 1991), this would be consistent with the present study showing the involvement of rab11 in recycling from the endosome to the plasma membrane.

The association of rab11 with the TGN in CHO cells (this study) and with the TGN and post-Golgi compartments in PC12 cells (Urbé et al., 1993) raises the additional possibility that rab11 plays a role in traffic to or from the Golgi complex. Increasing evidence suggests the involvement of endosomal compartments in trafficking routes from the TGN (Leitinger et al., 1995; Futter et al., 1995; Sariola et al., 1995), and it cannot be ruled out that the effects of rab11 mutants shown in the present study could be the result of an indirect effect on traffic between the recycling endosome and the Golgi complex. This possibility is supported by recent studies showing a role for the rab11 homologues ypt31 and ypt32 in traffic through the Golgi complex in yeast (Benli et al., 1996). It should also be noted that rab11 may not be active on all compartments with which it is associated. Rab6 has also been localized to different subcellular compartments, including the Golgi complex (Goud et al., 1990), synaptic vesicles, and the presynaptic membrane (Jasmin et al., 1992), but recent functional evidence has suggested a role in retrograde Golgi transport and not in post-Golgi transport (Martinez et al., 1994).

In conclusion, the sequential role of rab5, rab4, and rab11 along the endocytic and recycling pathway lends further support to the idea that the sorting and recycling endosomes are distinct compartments. In particular, the identification of rab11 as regulator of transport through the recycling endosome should facilitate the molecular and functional characterization of this compartment and its connections with the biosynthetic trafficking route.

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