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The retromer complex mediates retrograde transport of transmembrane cargo from endosomes to the trans-Golgi network (TGN). Mammalian retromer is composed of a sorting nexin (SNX) dimer that binds to phosphatidylinositol 3-phosphate-enriched endosomal membranes and a vacuolar protein sorting (Vps) 26/29/35 trimer that participates in cargo recognition. The mammalian SNX dimer is necessary but not sufficient for recruitment of the Vps26/29/35 trimer to membranes. In this study, we demonstrate that the guanosine triphosphatase Rab7 contributes to this recruitment. The Vps26/29/35 trimer specifically binds to Rab7-guanosine triphosphate (GTP) and localizes to Rab7-containing endosomal domains. Interference with Rab7 function causes dissociation of the Vps26/29/35 trimer but not the SNX dimer from membranes. This blocks retrieval of mannose 6-phosphate receptors to the TGN and impairs cathepsin D sorting. Rab5-GTP does not bind to the Vps26/29/35 trimer, but perturbation of Rab5 function causes dissociation of both the SNX and Vps26/29/35 components from membranes through inhibition of a pathway involving phosphatidylinositol 3-kinase. These findings demonstrate that Rab5 and Rab7 act in concert to regulate retromer recruitment to endosomes.

Introduction

The retromer is a phylogenetically conserved multisubunit complex that mediates retrograde transport of transmembrane cargo from endosomes to the TGN (Seaman, 2005; Bonifacino and Rojas, 2006; Bonifacino and Hurley, 2008). The best-characterized cargo for the mammalian retromer is the cation-independent mannose 6-phosphate receptor (MPR [CI-MPR]), one of two intracellular sorting receptors that participates in the delivery of acid hydrolases to lysosomes (Kornfeld, 1992). The CI-MPR binds newly synthesized acid hydrolases at the TGN and carries them within clathrin-coated vesicles to endosomes, where the hydrolases are released for eventual transport to lysosomes. The retromer functions to retrieve the unoccupied receptors to the TGN, where they engage in further cycles of acid hydrolase sorting. Depletion of retromer subunits by RNAi prevents this retrieval, leading to rerouting of the receptors to lysosomes and consequent leakage of newly synthesized acid hydrolases into the extracellular medium (Arighi et al., 2004; Carlton et al., 2004; Seaman, 2004; Rojas et al., 2007).

The mammalian retromer comprises two biogenetically distinct subcomplexes of tightly assembled subunits: a dimer composed of a still undefined combination of sorting nexin 1 (SNX1), SNX2, SNX5, and SNX6 (herein referred to as the SNX subcomplex) and a heterotrimer composed of vacuolar protein sorting 26 (Vps26), Vps29, and Vps35 (the Vps subcomplex; Haft et al., 2000; Collins et al., 2005; Hierro et al., 2007; Rojas et al., 2007). Recent studies have begun to shed light into the structure and function of the different retromer subunits. SNX1, SNX2, SNX5, and SNX6 are members of the SNX family of TGN, where they engage in further cycles of acid hydrolase sorting. Depletion of retromer subunits by RNAi prevents this retrieval, leading to rerouting of the receptors to lysosomes and consequent leakage of newly synthesized acid hydrolases into the extracellular medium (Arighi et al., 2004; Carlton et al., 2004; Seaman, 2004; Rojas et al., 2007).

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proteins (Carlton and Cullen, 2005; Cullen, 2008), which are characterized by the presence of a phox homology domain that binds phosphatidylinositol 3-phosphate (PI3P) and other phosphoinositides (Burda et al., 2002; Cozier et al., 2002; Zhong et al., 2002; Carlton et al., 2005a) and a Bin–Amphiphysin–Rvs domain that mediates dimerization and binding to highly curved membranes (Carlton et al., 2004; Carlton and Cullen, 2005). These properties endow the SNX subcomplex with the ability to bind endosomal membranes independently of the Vps subcomplex (Rojas et al., 2007).

The Vps subcomplex has recently been shown to consist of a 210-Å-long filament comprising one copy each of Vps26, Vps29, and Vps35 (Hierro et al., 2007). At one end of this filament lies Vps29, a protein with structural similarity to the arrestin family of sorting adapters (Shi et al., 2006). The other end comprises Vps29 and the C-terminal half of Vps35 (Hierro et al., 2007). Vps29 has a metallophosphoesterase fold (Collins et al., 2005; Wang et al., 2005) but little or no enzymatic activity (Collins et al., 2005; Hierro et al., 2007; but see Damen et al., 2006) caused by occlusion of the putative active site by the Vps35 C-terminal half and replacement of the key catalytic His residue by a Phe residue in the putative active site (Hierro et al., 2007). This latter domain consists of a horseshoe-shaped α-helical solenoid reminiscent of the trunk domain of adaptins (Hierro et al., 2007). Both ends of the filament are connected by the N-terminal half of Vps35 (Hierro et al., 2007). Molecular electron microscopy of this domain has shown that it consists of an extended α-helical solenoid (Hierro et al., 2007). Several lines of evidence indicate that the Vps subcomplex functions to recognize sorting signals within the cytosolic tails of retrograde cargo such as the MPRs (Nothwehr et al., 1999, 2000; Arighi et al., 2004; Seaman, 2007). The Vps35 subunit, in particular, has been proposed to interact with retrograde sorting signals (Nothwehr et al., 2000; Arighi et al., 2004), although definitive structural evidence for such interactions is still lacking.

The Vps subcomplex does not seem capable of directly interacting with membrane lipids (Collins et al., 2005; Shi et al., 2006). Instead, it requires the SNX subcomplex for recruitment to membranes (Rojas et al., 2007). Indeed, depletion of SNX subcomplex subunits by RNAi results in dissociation of the Vps subcomplex from membranes (Rojas et al., 2007). Interactions between the two subcomplexes have been proposed to occur between the relatively unstructured N-terminal extensions of SNX1 and SNX2 (Gullapalli et al., 2004) and several sites on Vps29 and Vps35 (Haft et al., 2000; Collins et al., 2005). However, in mammalian cells, the SNX and Vps subcomplexes exist as separate entities in cytosolic and detergent extracts of cells (Rojas et al., 2007) and do not seem capable of binding to each other in vitro (Collins et al., 2005; Rojas et al., 2007). In fact, the only evidence for binding of these subcomplexes comes from weak interactions detected in the yeast two-hybrid system (Haft et al., 2000; Rojas et al., 2007) and from coprecipitation of overexpressed proteins (Gullapalli et al., 2004). This indicates that other as yet unidentified factors might contribute to the recruitment of the Vps subcomplex to membranes.

In this study, we report that the small GTPase Rab7 is a regulator of Vps subcomplex recruitment to membranes. We discovered this role in the course of affinity purification experiments aimed at identifying proteins that interact with endosomal Rabs. Immunoblot analyses revealed the presence of the retromer Vps subcomplex among the proteins eluted from a column of GST-Rab7 loaded with the nonhydrolyzable GTP analogue guanylyl-5’-imidodiphosphate (GMP-PNP). This interaction was guanine nucleotide dependent and specific to Rab7 among other endosomal Rabs. Furthermore, GST pull-down experiments with purified recombinant proteins showed that the interaction between Rab7 and the Vps subcomplex is direct. We went on to demonstrate that the Vps subcomplex localizes to endosomal domains that contain Rab7 and that depletion or dominant-negative interference of Rab7 causes dissociation of the Vps subcomplex from membranes, inhibition of CI-MPR retrograde transport, and missorting of the acid hydrolase cathepsin D. In addition, we found that Rab5 indirectly contributes to the recruitment of retromer to membranes, most likely through activation of a pathway involving the Rab5 effector phosphatidylinositol 3-kinase (PI3K) and the SNX subcomplex. Thus, both Rab5 and Rab7 act in a sequential manner to enable retromer recruitment to endosomes and its function in retrograde traffic.

Results

Guanine nucleotide-dependent interaction of Rab7 with the retromer Vps subcomplex

In the course of studies aimed at identifying Rab effectors by affinity purification followed by mass spectrometry and/or immunoblot analysis for candidate proteins, we observed that the three subunits of the mammalian retromer Vps subcomplex, Vps26, Vps29, and Vps35, bound to GST-Rab7 in the presence of the nonhydrolyzable GTP analogue, GMP-PNP (Fig. 1 A). This binding was guanine nucleotide dependent, as it was not detected in the presence of GDP (Fig. 1 A). In addition, it was specific for Rab7 because the Vps subcomplex subunits did not bind to GST-Rab4a, -Rab5a, -Rab9a, -Rab27a, or -Rab37a, all loaded with GMP-PNP (Fig. 1 A). In contrast to the Vps subcomplex, SNX1 and SNX2 did not bind to GMP-PNP–loaded GST-Rab7 (unpublished data).

To further characterize this interaction, we performed GST pull-downs of extracts from COS-7 cells expressing different combinations of epitope-tagged forms of Vps26, Vps29, and Vps35. Small amounts of HA-Vps35 were found to bind to GST-Rab7–GMP-PNP upon expression of HA-Vps35 alone or in combination with either myc-Vps26 or FLAG-Vps29 (Fig. 1 B). However, much larger amounts of HA-Vps35, together with myc-Vps26 or FLAG-Vps29, were recovered from cells coexpressing all three subunits (Fig. 1 B). None of these species bound to GST or GST-Rab4a–GMP-PNP (unpublished data), indicating that the aforementioned pull-downs were specific. These results indicated that Vps35 might be the subunit that mediates binding to Rab7. However, optimal binding requires expression of all three subunits, probably because of a requirement of Vps subcomplex assembly for proper folding of Vps35, as previously found for the recombinant proteins (Hierro et al., 2007).

To determine whether the interaction of Rab7 with the Vps26/29/35 trimer was direct, we performed GST pull-down
endogenous Vps26 to GFP-Rab5a–positive structures (Fig. 2, D–F) as previously described (Arighi et al., 2004; Seaman, 2004). Similar results were obtained for the colocalization of endogenous SNX1 with GFP-Rab7 and GFP-Rab5a (unpublished data).

To assess the dynamics of association of retromer with Rab7- and Rab5-containing endosomes in live cells, we performed time-lapse fluorescence microscopy of cells transfected with plasmids encoding Vps29-YFP and either CFP-Rab7 or CFP-Rab5a (Fig. 3 and Videos 1–6, available at http://www.jcb.org/cgi/content/full/jcb.200804048/DC1). In accordance with the aforementioned immunofluorescence microscopy experiments, we visualized numerous endosomal vesicles that contained both Vps29-YFP and CFP-Rab7 (Fig. 3, A–C). Moreover, Vps29-YFP was almost always found on domains of the endosomal vesicles that also contained CFP-Rab7 (Fig. 3 B). These domains were highly dynamic and underwent aggregation and fragmentation, but Vps29-YFP remained coincident with CFP-Rab7 throughout these changes. An exception was a set of long tubules that emanated and eventually detached from the endosomes; these tubules invariably contained Vps29-YFP (Fig. 3 C, green arrows).
Requirement of Rab7 for retromer association with membranes

The interaction and colocalization of Rab7 with the retromer Vps subcomplex suggested a probable functional relationship between these proteins. Rab GTPases such as Rab7 cycle between GTP-bound, membrane-associated, and GDP-bound cytosolic states and, in doing so, function to regulate the recruitment of effector proteins to membranes. To determine whether Rab7 played a role in retromer recruitment to membranes, we used RNAi to deplete Rab7 from HeLa cells (Fig. 5 A). Immunofluorescence microscopy showed that this depletion had no effect on the distribution of the adapter protein (AP) complexes AP-3 (Fig. 5 E) and AP-1 (Fig. 5 I), which are associated with specific domains of the tubular endosomal network (Futter et al., 1998; Peden et al., 2004; Theos et al., 2005), but caused virtually complete loss of endosomal staining for Vps26 (Fig. 5, D, H, L, and P). This loss was caused by dissociation from membranes and not to degradation because the levels of Vps26 protein were unchanged by depletion of Rab7 (Fig. 5 A). The effects of Rab7 depletion on Vps26 localization were specific because combined depletion of the also endosomal Rab4a and Rab4b paralogues (Fig. 5 A) did not alter the distribution of Vps26 (Fig. 6 B). Moreover, transfection of Rab7-depleted cells with a plasmid encoding an RNAi-resistant form of GFP-Rab7 (Fig. 6, C and E, arrows) but not with a plasmid encoding GFP (Fig. 6, D and F) partially restored Vps26 association with endosomes. Interestingly, SNX1 (Fig. 5 M) and SNX2 (Fig. 5 Q) retained

but not CFP-Rab7 (Fig. 3 C, red arrows). However, the tubules seemed to arise from endosomal foci where CFP-Rab7 was most concentrated (Fig. 3 C).

Vps29-YFP was also found in association with endosomal vesicles that contained CFP-Rab5a (Fig. 3, D–F). However, these two proteins were largely segregated to different domains. These domains were also highly motile and underwent changes over time, but only rarely did Vps29-YFP coincide with CFP-Rab5a (Fig. 3, D–F). Vps29-YFP–containing tubules did form from CFP-Rab5a–positive endosomes, but the tubules themselves were devoid of CFP-Rab5a (Video 4).

Although both Vps29-YFP and CFP-Rab7 decorated the limiting membrane of endosomal vesicles, they were most often concentrated within foci that appeared to bulge from the membrane (Fig. 3, A–C). To examine the ultrastructure of these foci, we performed immunoelectron microscopy. HeLa cells were transfected with a plasmid encoding GFP-Rab7 and subsequently double-immunogold labeled for endogenous Vps26 (15-nm gold) and GFP (10-nm gold; Fig. 4). Both proteins were found on multiple buds and tubules that emanated from the vacuolar part of endosomes (Fig. 4 A and B) and that appeared as a cluster of tubules on en face sections (Fig. 4 B). These structures are characteristic of the tubular endosomal network (Bonifacino and Rojas, 2006) that mediates retromer-dependent sorting of MPRs to the TGN, likely via the long tubules observed to extend from endosomes in the live cell imaging experiments (Fig. 3 C; Zhong et al., 2002; Arighi et al., 2004; Carlton et al., 2004, 2005b; Popoff et al., 2007).

Figure 2. Immunofluorescence microscopy showing localization of Vps26 to Rab7- and Rab5-positive endosomes. HeLa cells were transfected with plasmids encoding GFP-Rab7 (A–C) or GFP-Rab5a (D–F), fixed, permeabilized, and immunostained with rabbit polyclonal antibody to Vps26 followed by Alexa Fluor 594–conjugated donkey anti–rabbit IgG. Cells were examined by confocal microscopy. (A and D) GFP fluorescence, green channel. (B and E) Alexa Fluor 594 fluorescence, red channel. (C and F) Merged images; yellow indicates colocalization. Arrows indicate examples of foci where proteins colocalize. Bar, 10 μm.
Dominant-negative overexpression reveals a role for Rab5 in retromer recruitment to membranes

In addition to RNAi, another commonly used approach to test for the dependence of cellular processes on small GTPases is to overexpress dominant-negative GDP-locked forms of the proteins.

their association with endosomes in Rab7-depleted cells, although the endosomes appeared enlarged and more disperse. These observations highlighted another important difference in the properties of the Vps and SNX subcomplexes of retromer in that only the Vps subcomplex requires Rab7 for association with endosomes.
The role of Rab5 in retromer recruitment because the existence of Rab5 activates endosomal PI3K (Christoforidis et al., 1999b), which produces PI3P, an essential cofactor for SNX1 and SNX2 association with membranes (Cozier et al., 2002; Carlton et al., 2004). In turn, SNX1 and SNX2 enable the recruitment of the retromer Vps subcomplex to membranes (Rojas et al., 2007).

To analyze the interplay between PI3K and Rab7 in the regulation of retromer recruitment to membranes, we overexpressed a constitutively active GTP-locked form of Rab7 (GFP-Rab7–Q67L) and examined the effects of adding the PI3K inhibitor wortmannin. In control cells, wortmannin addition caused dissociation of both Vps26 (to 28% of levels in untreated cells) and SNX1 from membranes (Fig. 7, J–L; and Fig. S1) as previously reported (Arighi et al., 2004; Rojas et al., 2007). However, in cells overexpressing GFP-Rab7–Q67L, Vps26 (Fig. 7 K, arrows; and Fig. S1) but not SNX1 (Fig. 7 L, arrows; and Fig. S1) remained partially associated with membranes in the presence of wortmannin (76% of levels in untreated cells; Fig. S1 E). These results indicated that the nonphysiologically high level of activity of GFP-Rab7–Q67L can override the absence of PI3P for recruitment of the retromer Vps subcomplex but not the SNX subcomplex to endosomes. Thus, interaction with Rab7 and regulation by Rab5 through a PI3K–PI3P–SNX1/2 pathway cooperate to recruit the retromer Vps subcomplex to membranes.

**Involvement of Rab7 in the trafficking of MPRs and their acid hydrolase cargo**

Because retromer functions in the retrograde transport of the CI-MPR from endosomes to the TGN, we expected that interference with Rab7 would alter the trafficking of the receptors. Indeed, we found that the steady-state distribution of the CI-MPR changed from a juxtanuclear cluster of small vesicles (Fig. 8, A and C) to a more disperse collection of large vesicles (Fig. 8, B and D) upon depletion of Rab7 by RNAi. Incubation of control live cells with antibody to the CI-MPR at 37°C showed that the cell surface receptors were internalized and underwent retrograde transport to achieve a distribution similar to that at steady state (i.e., a mix of endosomes and TGN; Fig. 8, E and G; Seaman, 2004; Rojas et al., 2007). However, in Rab7-depleted cells, the internalized CI-MPR remained trapped in enlarged peripheral endosomes (Fig. 8, F and H). These endosomes contained the transferrin receptor (TfR; Fig. 8, I–K), identifying them as early endosomes. In contrast to retromer depletion (Arighi et al., 2004; Rojas et al., 2007), however, Rab7 depletion did not affect the levels of the CI-MPR (Fig. 8 O), indicating that the receptor was not delivered to lysosomes for degradation but accumulated in early endosomes.

The trapping of the CI-MPR in early endosomes should result in its depletion from the TGN, leading to mis-sorting of newly synthesized acid hydrolases. Immunoblot analysis of one such hydrolase, cathepsin D, showed that this was indeed the case. In control cells, most of the cathepsin D occurred as the 31-kD mature form with only small amounts of the 53-kD precursor and 47-kD intermediate forms (Fig. 9). Only trace amounts of the precursor form were secreted into the medium in control cells (Fig. 9 ). This efficient processing and intracellular retention of cathepsin D reflects the integrity of the normal mechanism for trafficking of the enzyme from the TGN all the way to lysosomes. Depletion of Rab7, like depletion of Vps26 (Seaman, 2004),
caused substantial increases in the amount of precursor and intermediate forms present within the cells and of precursor form secreted into the medium (Fig. 9), which were indicative of impaired transport of the enzyme to lysosomes. These results are consistent with the role of Rab7 in regulating retromer function and CI-MPR sorting in endosomes.

Discussion

The results of our study indicate that Rab5 and Rab7 function in a sequential manner to regulate the recruitment of the retromer Vps subcomplex to endosomes. Rab5 does not bind the Vps subcomplex (Fig. 1), although overexpression of dominant-negative Rab5 causes dissociation of the subcomplex from endosomes (Fig. 7). In contrast, Rab7 binds retromer in a guanine nucleotide–dependent manner (Fig. 1). In addition, depletion of Rab7 by RNAi (Figs. 5 and 6) or overexpression of dominant-negative Rab7 (Fig. 7) results in Vps subcomplex dissociation from membranes. These latter perturbations also interfere with trafficking of the CI-MPR (Fig. 8) and its cargo, cathepsin D (Fig. 9), in line with previous findings (Press et al., 1998). Thus, Rab7 constitutes the missing link in the mechanism that controls retromer recruitment to endosomes and its function in cargo sorting. These findings expand the range of processes that are known to be regulated by Rab5 and Rab7 by implicating these Rabs in retrograde transport from endosomes to the TGN.

A model for the regulation of retromer by Rab5 and Rab7

Fig. 10 depicts a model for the mechanism of recruitment of the mammalian retromer complex to endosomes based on the data presented here and in previous studies. Exchange of GTP for GDP causes Rab5 to associate with early endosomes (Ullrich et al., 1994) and to recruit a large number of effector proteins...
Bar, 10 μm. donkey anti-rabbit IgG. Images were captured by epifluorescence microscopy. rabbit polyclonal antibody to Vps26 followed by Alexa Fluor 594-conjugated Vps26 (A–D) was assessed by indirect immunofluorescent staining using or canine GFP-Rab7 are indicated by arrows. The cellular distribution of GFP-Rab7 (C and E) or GFP (D and F). Examples of cells expressing GFP cells were transfected with plasmids encoding RNAi-resistant (i.e., canine) GFP-Rab7 (C–F). At 36 h after the second treatment with siRNA to Rab7, some cells were transfected with plasmids encoding RNAi-resistant [i.e., canine] GFP-Rab7 [C and E] or GFP [D and F]. Examples of cells expressing GFP or canine GFP-Rab7 are indicated by arrows. The cellular distribution of GFP-Rab7 (A–D) was assessed by indirect immunofluorescent staining using rabbit polyclonal antibody to Vps26 followed by Alexa Fluor 594-conjugated donkey anti-rabbit IgG. Images were captured by epifluorescence microscopy. Bar, 10 μm.

Figure 6. Specificity and rescue of Vps26 dissociation from membranes in RNAi-treated cells. HeLa cells were treated twice at 24-h intervals with an inactive siRNA [control; A] or siRNAs to both Rab4a and Rab4b [B] or to Rab7 [C–F]. At 36 h after the second treatment with siRNA to Rab7, some cells were transfected with plasmids encoding RNAi-resistant [i.e., canine] GFP-Rab7 [C and E] or GFP [D and F]. Examples of cells expressing GFP or canine GFP-Rab7 are indicated by arrows. The cellular distribution of GFP-Rab7 (A–D) was assessed by indirect immunofluorescent staining using rabbit polyclonal antibody to Vps26 followed by Alexa Fluor 594-conjugated donkey anti-rabbit IgG. Images were captured by epifluorescence microscopy. Bar, 10 μm.

(Christoforidis et al., 1999a). A key Rab5 effector is the class III PI3K (Christoforidis et al., 1999b), which in mammalian cells is composed of a catalytic Vps34 subunit and a regulatory p150 subunit (Panaretou et al., 1997). PI3K phosphorylates phosphatidylinositol to PI3P, a phosphoinositide that is highly enriched in endosomal membranes (Gillooly et al., 2000). PI3P, in turn, functions to recruit many other downstream effectors, among which are the components of the retromer SNX subcomplex (Burd et al., 2002; Cozier et al., 2002; Zhong et al., 2002, 2005; Arighi et al., 2004; Carlton et al., 2004, 2005b). These proteins bind to PI3P via their phox homology domains and to curved membranes via their Bin–Amphiphysin–Rvs domains in a process that is referred to as coincidence detection (Carlton et al., 2004; Carlton and Cullen, 2005). The SNX subcomplex contributes to the recruitment of the Vps subcomplex probably by virtue of interactions between the largely unstructured N-terminal extensions (Gullapalli et al., 2004) and several binding sites on the Vps29 and Vps35 subunits (Collins et al., 2005). However, these interactions are too weak to fully account for the association of the Vps subcomplex with endosomes (Rojas et al., 2007). That is where Rab7 comes into play. Another Rab5 effector is the homotypic fusion and vacuole protein sorting–Vps-C complex, which functions as a Rab7 guanine nucleotide exchange factor (Rink et al., 2005). Activation of Rab7 by exchange of GTP for GDP results in binding of Rab7-GTP to membranes, thus providing an additional attachment point for the Vps subcomplex to associate with endosomes. This cooperation of the SNX subcomplex and Rab7 represents another layer of coincidence detection that further specifies the recruitment of the Vps subcomplex to particular endosomal domains. Rab7 also interacts with and activates PI3K (Stein et al., 2003), ensuring sustained production of PI3P for SNX subcomplex recruitment after dissociation of Rab5 from endosomes. The relative contributions of the SNX proteins and Rab7 to Vps subcomplex recruitment vary in other organisms. In the yeast Saccharomyces cerevisiae, the two SNX1/2 orthologues, Vps5p and Vps17p, are much more tightly associated with the corresponding Vps subcomplex subunits, to the point that all five subunits can be coprecipitated from cell extracts (Seaman et al., 1998; Gokool et al., 2007). At the other end of the range is Entamoeba histolytica, which has a Vps subcomplex that interacts with Rab7 but apparently has no SNX-like proteins encoded in its genome (Nakada-Tsukui et al., 2005). The interaction of E. histolytica Rab7 with the Vps subcomplex has another feature that appears unique to this organism in that Rab7 binding requires the long C-terminal extension of Vps26 that is not present in the mammalian and yeast orthologues (Nakada-Tsukui et al., 2005). This is in contrast to the interaction of the mammalian proteins, which involves binding of Rab7 to the fully assembled Vps subcomplex probably through direct contact with Vps35 (Fig. 1 B).

Endosomal domains involved in retromer function

The aforementioned steps take place as the endosomes undergo the process of Rab conversion (Rink et al., 2005), in which Rab5 is replaced by Rab7 during endosomal maturation. Rab5 is associated with the plasma membrane (Sato et al., 2005) and early endosomes (Chavrier et al., 1990). It is at this early endosomal stage that PI3K starts producing PI3P and that the SNX subcomplex can be first recruited to endosomes. Only during the brief interval when Rab5 is being replaced by Rab7 do both Rabs coexist on the same endosome (Rink et al., 2005; Vonderheit and Helenius, 2005). The arrival of Rab7 completes the set of regulators needed for recruitment of the Vps subcomplex to membranes. Upon dissociation of Rab5, Rab7 assumes complete control of Vps subcomplex recruitment via continued activation of the PI3K–PI3P–SNX pathway and direct interaction with the Vps subcomplex (Fig. 10). This coincidence of events regulated by Rab7 accounts for the predominant localization of the Vps subcomplex to Rab7 domains (Fig. 3 and Videos 1–6). All of these events occur while the proteins are associated with the vacuolar aspect of the endosomes. However, both Rab7 and retromer are
also found on a convoluted set of buds and tubules adjacent to the vacuolar aspect of endosomes (Fig. 4). At some point, both the SNX and Vps subcomplexes of retromer begin to leave the endosomes on long tubular extensions that eventually detach (Fig. 3 C and Videos 3 and 4). A population of endosome to TGN carriers has also been shown to form from such sites (Mari et al., 2008). This process probably starts at the stage of the transitional Rab5–Rab7 endosomes, as some retromer-containing tubules can be seen to emanate from Rab5-positive endosomes (Video 4). However, conversion to Rab7 status brings about an intensification of retromer–tubule formation. Remarkably, Rab7 does not accompany retromer into the long tubules (Fig. 3 C and Video 3), suggesting that the stabilizing role of Rab7 must be taken over by some other factor. This role is likely fulfilled by cargo proteins such as the CI-MPR, as is the case for other cargo–adapter interactions (Fig. 10; Hirst et al., 2007).

Rab7 as a master regulator of multiple endosomal processes

Rab7 has several other effectors in addition to retromer and homotypic fusion and vacuole protein sorting–Vps-C. Among these are Rab-interacting lysosomal protein (Cantalupo et al., 2001) and ORP1L (Johansson et al., 2005), which form a complex that interacts with dynein–dynactin motors to effect minus end–directed tracking of late endosomes on microtubules (Johansson et al., 2007). Another Rab7 effector is Rabring7, an ubiquitin ligase that promotes targeting of internalized EGF receptors for lysosomal degradation (Sakane et al., 2007). Notably, all of these effectors other than retromer are known to mediate processes that take place in the vacuolar part of late endosomes and that lead to lysosomes. Thus, our findings uncover a new role for Rab7 in controlling an essentially distinct process, the diversion of cargo away from lysosomes and into recycling tubules. Not only does Rab7 control this retrograde transport pathway, but it can actually be found on a tubular network adjacent to endosomes (Fig. 4). Thus, Rab7 acts as a master control switch that regulates multiple endosomal processes. Along these lines, Rab7 has been recently shown to modulate retrograde axonal transport of vesicles containing neurotrophins and their receptors in motor neurons (Deinhardt et al., 2006). It is perhaps because of the involvement of Rab7 in protein transport
The retrieval of unoccupied CI-MPR to the TGN was long thought to occur from late endosomes. Indeed, an ensemble of the late endosomal Rab9 and its effector TIP47 were shown to play a role in this process (Carroll et al., 2001). However, there was also evidence that at least some retrieval took place from early pathways that lead both toward and away from lysosomes that interference with Rab7 function does not result in targeting of the CI-MPR to lysosomes, as is the case for retromer depletion (Arighi et al., 2004; Carlton et al., 2004; Seaman, 2004; Rojas et al., 2007), but in its accumulation in endosomes (Fig. 8).

**Endosomal sites of retrieval to the TGN**

The retrieval of unoccupied CI-MPR to the TGN was long thought to occur from late endosomes. Indeed, an ensemble of the late endosomal Rab9 and its effector TIP47 were shown to play a role in this process (Carroll et al., 2001). However, there was also evidence that at least some retrieval took place from early
endosomes (Press et al., 1998). This latter view was bolstered by the demonstration that the mammalian retromer, which mediates CI-MPR retrieval, is associated with a population of endosomes that have the morphological features of intermediates in the maturation from early to late endosomes (Arighi et al., 2004; Carlton et al., 2004; Seamann, 2004; Bujny et al., 2007; Popoff et al., 2007). This is consistent with the finding that some retromer localizes to Rab5-positive endosomes (Figs. 2 and 3 and Videos 4–6). GTP analog treatment from early to late endosomes (Arighi et al., 2004; Carlton et al., 2004; Seaman, 2004; Bujny et al., 2007; Popoff et al., 2007).

Figure 9. Rab7 depletion impairs processing of cathepsin D. HeLa cells were treated twice at 24-h intervals with inactive siRNA (control; lane 1) or siRNAs to the proteins indicated on top. At 24 h after the second round of siRNA treatment, cells were rinsed with PBS and incubated in serum-free culture medium for 24 h. The medium was collected and precipitated with trichloroacetic acid, and the resulting pellets were dissolved in Laemmli sample buffer. Cell extracts and media samples were analyzed by 4–20% SDS-PAGE and immunoblotting with rabbit polyclonal sample buffer. Samples were analyzed by SDS-PAGE and immunoblotting.

Materials and methods

Recombinant DNA procedures

The construction of pEGFP-Rab7, pEGFP-Rab9a, and pEGFP-Rab27a was described previously (Neeft et al., 2005). A GST-Rab9a construct was provided by S. Pfeffer (Stanford University, Stanford, CA). A Rab37a cDNA was obtained from E. Masuda (Rigel Inc., San Francisco, CA) and ligated in frame into the BamH I and EcoR I sites of pGEX1X1T. A GFP-Rab5a construct was generated by in-frame cloning of canine Rab5a cDNA (provided by M. Zerial, Max Planck Institute, Dresden, Germany) into the Kpn I and BamH I sites of the pEGFP-C1 vector (BD Biosciences). Complementary DNA encoding Rab7 and Rab4a were amplified by PCR from a canine cDNA library and cloned in frame into the Kpn I–Apal and Bgl II–Kpn I sites, respectively, of the pEGFP-C1 vector. The QuikChange Site-Directed Mutagenesis kit (Stratagene) was used to produce the following mutants: GFP-Rab4a–S22N, and GFP-Rab5a–S34N. GFP-Rab7–T22N, and GFP-Rab7–Q67L. The CFP-Rab5a construct was generated as previously described (Sonnichsen et al., 2000). The Vps29–YFP construct was generated by PCR amplification of human Vps29 (residues 1–182) and cloning it into the Not I (at both 5′ and 3′ ends) site of the pEYFP-N1 vector (BD Biosciences). The pEYFP-N1 and pEYFP-Vps29 construct was obtained by PCR amplification of full-length human Vps29 cDNA from a previously described pcDNA3.1-Vps29-myc construct and cloned into the Not I (at both 5′ and 3′ ends) site of the pEYFP-N1 vector. Full-length human Vps26 cDNA was cloned in frame into the BamH I and EcoR I sites of the pRK5-myc vector. (HA)3-Vps35-pEF6/V5 has been described previously (Hierro et al., 2007).

GST affinity purification and pull-down experiments

A membrane extract was prepared by homogenizing 100 mg of pig kidney in 1 vol of 20 mM Heps, pH 7.5, 100 mM NaCl, 5 mM MgCl2, and 1 mM DTT (buffer A) supplemented with 10 μg/ml DNase, 10 μg/ml RNase, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 5 μg/ml pepstatin. Soluble cell extracts and supernatants were collected and later incubated with GST-Rab7–GMP-PNP (or GST or GST-Rab4a–GMP-PNP) in buffer A supplemented with the appropriate nucleotide and 250 mM NaCl, and finally diluted fivefold with buffer A and dialyzed overnight against 5 mM MgCl2, and 1 mM DTT. Bound proteins were analyzed by SDS-PAGE and immunoblotting.

For pull-down experiments, we transfected individually or together pRK5-myc-Vps26, pEF-BOS-FLAG-Vps29, and pEF-HA-Vps35 in COS-7 cells using Lipofectamine-2000 (Invitrogen). After 24 h, the cells were washed with ice-cold PBS and lysed in 20 mM Heps, pH 7.5, 100 mM NaCl, 5 mM MgCl2, and 0.2% (wt/vol) NP-40 for 10 min on ice. Lysates were spun down at 10,000 g in a benchtop centrifuge (Eppendorf). Supernatants were collected and later incubated with GST-Rab7–GMP-PNP (or GST or GST-Rab4a–GMP-PNP as negative controls) bound to glutathione–agarose beads for 2 h in an end over end device. Samples were subsequently washed six times with lysis buffer, the excess buffer was drained with a 30.5-gauge needle, and bound proteins were eluted by boiling in Laemmli sample buffer. Samples were analyzed by SDS-PAGE and immunoblotting with rabbit polyclonal antibodies to the myc, FLAG, and HA tags.

For in vitro binding experiments with purified recombinant proteins, GST-Rab7 and GST-Rab9a were produced in E. coli BL21 (DE3) (see Zymo 5052 autoinduction medium at 25°C [the Cterminal 23 amino acid residues from Rab9a were removed for better yields]). GST-Rab proteins were immobilized on glutathione–Sepharose 4B beads, eluted according to the manufacturer’s instructions, concentrated with a centrifugal concentrator (Amicon-15; Millipore), and purified on a Superdex 200 16/60 gel filtration column. For pull-down assays, 60 nmol of each GST-Rab was immobilized by incubation with 40 μl glutathione–Sepharose 4B beads in PBS, 5 mM MgCl2, 1 mM DTT, 0.1% (wt/vol) NP-40, and 20 μM GDP. 300 nmol recombinant Vps35/26/29 trimer, which was produced and purified as described previously (Hierro et al., 2007), was incubated in buffer A containing 50% (wt/vol) BSA and 100 μM of the respective nucleotide (GTP or GDP). Bound proteins were analyzed by SDS-PAGE and immunoblotting.
Hepes, pH 7.5, 1.5 M NaCl, 20 mM EDTA, 1 mM DTT, and 5 mM of each nucleotide (GDP for proteins bound to the GTP/S-loaded GST-Rab and vice versa). Eluates were mixed with an equal volume of NuPAGE sample buffer and processed for SDS-PAGE and immunoblotting.

Antibodies
We used mouse monoclonal antibodies to the following proteins: Rab4, SNX1, SNX2, AP-1-γ1, and actin (BD Biosciences), CHMP4 (luminal domain; AbD Serotec, and AP-3–γ1 [SA4; Developmental Studies Hybridoma Bank]. We also used rabbit polyclonal antibodies to the following proteins: Rab7 (provided by G. Schiavo, Cancer Research UK, London, UK; Deinhardt et al., 2006), CHMP4 cytosolic tail [Kametaka et al., 2005], Vps26, Vps29, and Vps35 (provided by C.R. Haft, National Institutes of Health, Bethesda, MD; Haft et al., 2000), cathepsin D (EMD), GFP (Invitrogen), giantin (Covance), Vps29 (provided by C.R. Haft, National Institutes of Health, Bethesda, MD; Deinhardt et al., 2006), CI-MPR cytosolic tail (Kametaka et al., 2005), Vps26, Vps29, and Vps35 (provided by G. Schiavo, Cancer Research UK, London, UK; Deinhardt et al., 2006), CI-MPR cytosolic tail (Kametaka et al., 2005), Vps26, Vps29, and Vps35 (provided by G. Schiavo, Cancer Research UK, London, UK; Deinhardt et al., 2006), CI-MPR cytosolic tail (Kametaka et al., 2005), Vps26, Vps29, and Vps35 (provided by G. Schiavo, Cancer Research UK, London, UK; Deinhardt et al., 2006).

For quantification of Vps26 colocalization with GFP-Rab7 or GFP-Rab5a, each set of images was corrected for cross talk. We captured 512 x 512-pixel (x0.5 x 0.5 pixel) confocal sections (8 bit) through the whole volume of 24 cells. After image thresholding, the extent of colocalization was obtained by calculating the Manders’ coefficients (M1) and the corresponding standard deviation (Bolte and Cordelieres, 2006) with the intensity correlation analysis plug-in developed for the Image J 1.36b software (National Institutes of Health). The percentage of colocalization was obtained by multiplying M1 by 100.

To quantify cytosolic and membrane-bound levels of Vps26, we measured the mean total fluorescent intensity from DMSO-treated cells, GFP-Rab5aS34N–transfected cells, GFP-Rab7–T22N–transfected cells, and untransfected or GFP-Rab7–Q67L–transfected cells treated with wortmannin. We took advantage of the fact that endogenous cytosolic proteins but not membrane-bound proteins are partially extracted on immunofluorescent staining of fixed permeabilized cells (Reinacher-Schick and Gumbiner, 2001). Therefore, we captured 512 x 512-pixel confocal (8 bit) sections through the whole volume of the cells. Using Image J 1.36b software, we obtained 32-bit stacks from the individual confocal sections. We measured the mean total fluorescence intensity per cell surface area and the corresponding standard deviation of the cellular Vps26 fluorescence signal of at least 25 individual cells for each condition.

Figure 10. Schematic representation of the regulation of retromer by Rab5 and Rab7. The scheme depicts a section of an endosome with its vacuolar and tubular aspects. The sequence of reactions is represented in the context of the progression from Rab5- to Rab7-positive endosomes and from the vacuolar to the tubular aspect. Details of this model are described in the Discussion section. BAR, Bin–Amphiphysin–Rvs; HOPS, homotypic fusion and vacuole protein sorting; PX, phox homology.

Cell transfection, immunofluorescence microscopy, and quantitative analyses
For expression of GFP-tagged proteins, Hela cells (American Type Culture Collection) grown on coverslips to 50% confluency were transfected in 24-well plates with 0.8 μg of plasmids encoding GFP, GFP-Rab7, GFP-Rab5a, GFP-Rab7–Q67L, GFP-Rab4a–S22N, GFP-Rab5a–S34N, or GFP-Rab7–T22N using Lipofectamine-2000. In some experiments, control cells or cells expressing GFP-Rab7–Q67L were incubated with 0.1% DMSO or 200 nM wortmannin in DMSO for 30 min. Cells were fixed, permeabilized, and immunostained with rabbit polyclonal antibody to Vps26 followed by Alexa Fluor 594–conjugated donkey anti-rabbit IgG. For CHMP4 antibody uptake assays, cells were rinsed twice with MEM/BSA and incubated with mouse monoclonal antibody to the luminal domain of 10 μg/ml CHMP4 in MEM/BSA for 2 h at 37°C (Rojas et al., 2007). Procedures for immunofluorescent staining of fixed and permeabilized cells were performed as previously described (Rojas et al., 2007).

Colocalization images shown in Fig. 2 and Fig. 8 (I–N) were obtained using a confocal microscope (TCS SP2; Leica) equipped with 488-nm Ar–Kr, 543/594-nm He–Ne, and 633-nm He–Ne lasers. Images were acquired in a sequential mode using a 63× Plan Apochromat NA 1.4 oil objective and the appropriate filter combination. Settings used were as follows: photomultipliers at 400–700 V, Airy = 1, zoom = 3–4, and Kalman filter (n = 4). Images shown in Figs. 5–7 and 8 (A–G) were obtained with an epifluorescence microscope (AX10; Carl Zeiss, Inc.) with a Plan Apochromat 63× NA 1.3 oil objective equipped with a charge-coupled device camera (AxioCamMRC; Carl Zeiss, Inc.). All images were saved as TIFF files, contrast was adjusted with Photoshop (version 7.0; Adobe), and images were imported into Illustrator (CS; Adobe).
Sections were analyzed under an electron microscope (Philips CM120; FEI), and digital acquisitions were made with a numeric camera (Keen View; Soft Imaging System).

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HeLa cells grown to 30% confluency were transfected twice at 24-h intervals with 80 pmol siRNA oligonucleotides (SMARTpool reagents to Vps26, Rab7, Rab4a, Rab4d, and glycerolaldehyde 3-phosphate dehydrogenase [control]; Thermo Fisher Scientific) using Oligofectamine (Invitrogen). For most experiments, cells were analyzed 48 h after the second round of transfection. In rescue experiments, cDNA encoding canine GFP–Rab7 or either CFP–Rab5a (Videos 1–3) or GFP–Rab7 (Videos 4–6), as described in Materials and methods. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200804048/DC1.

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