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Molecular basis for the control of motor-based transport of MHC class II compartments

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Chapter 5

Rab7-RILP-ORP1L receptors couple late endosomal transport and fusion
In preparation

Rab7-RILP-ORP1L receptors couple late endosomal transport and fusion

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Fusion between late endosomal compartments is crucial for degradation of endocytosed material. Motor-based vesicular transport brings late endosomal membranes into close proximity prior to their tethering/docking and fusion. We propose that the control of these two consecutive processes is combined in one single tripartite complex on late endosomal membranes, the Rab7-RILP-ORP1L complex. RILP establishes the molecular link between Rab7 and the dynein-dynactin microtubule motor for centripetal vesicular transport. ORP1L, a second Rab7 effector, controls the interaction of RILP with p150^{Glued}, a subunit of dynactin. In addition, Rab7 has also been implicated in tethering/docking and fusion of late endosomal compartments, but how this is regulated is unclear. We show that Vam6p, a specific tethering/docking factor that promotes fusion of late endosomal compartments, is also recruited to Rab7 on late endosomal membranes in a RILP-dependent manner. Furthermore, we show that ORP1L governs this, suggesting a common regulatory mechanism for late endosomal transport and tethering/docking and fusion. This would provide coordinated spatio-temporal control and continuity for these two consecutive steps within the late endocytic route.

INTRODUCTION

Rab GTPases are targeted to specific subcellular compartments and orchestrate the spatio-temporal regulation of intracellular endomembrane trafficking by regulating vesicular transport, docking/tethering of organelle bilayers, and ultimately their fusion (Jordens et al., 2005; Somsel Rodman and Wandinger-Ness, 2000).

Examples of Rab GTPases implicated in recruiting (directly or through effector molecules) motor proteins to specific membranes for vesicular transport include (Jordens et al., 2005): Rab4, Rab5, and Rab6, which recruit members of the kinesin superfamily of motor proteins; Rab8, Rab11, and Rab27a, recruit myosin motor proteins; and Rab6 and Rab7, support the recruitment of the minus end-directed motor dynein. Rab7 associates to membranes of late endosomal structures (Jordens et al., 2001; Meresse et al., 1995), including late endosomes, lysosomes, major histocompatibility class II-containing

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compartments (MIICs) (Jordens et al., 2001), phagosomes (Harrison et al., 2004; Marsman et al., 2004), early melanosomes (Jordens et al., 2006), and cytolytic granules (Stinchcombe et al., 2006). The Rab7 effector Rab7-interacting lysosomal protein (RILP) interacts with $p150^{Glued}$, a subunit of dynactin, to recruit the dynein-dynactin motor to late endosomal membranes. The Rab7-RILP complex thus acts as a specific receptor for the minus-end dynein motor. After dynein recruitment to late endosomal membranes, active centripetal transport of late endosomal compartments still requires the concerted activities of two other proteins: an additional Rab7 effector, oxysterol-binding protein (OSBP)-related protein 1L (ORP1L), which is part of the tripartite complex Rab7-RILP-ORP1L; and βIII spectrin, which acts as a second receptor for dynein-dynactin on late endosomal membranes. ORP1L is thought to target the Rab7-RILP-$p150^{Glued}$-dynein complex to βIII spectrin on late endosomal membranes leading to full activation of minus-end directed transport (Johansson et al., 2007). In addition, ORP1L responds to changes in late endosomal cholesterol content with variations in its conformational state, thereby controlling access of dynein-dynactin to its receptor Rab7-RILP (Chapter 3 of this thesis).

Rab GTPases and their effectors are also implicated in orchestrating the assembly of complexes that regulate tethering/docking and fusion of vesicular membranes (Cai et al., 2007; Grosshans et al., 2006). For example, the early endosomal Rab GTPase-effector complex, Rab5-EEA1, interacts with syntaxin-13, a t-SNARE required for early endosomal homotypic fusion. Rab7/Ypt7p is involved in tethering/docking and fusion of late endosomal compartments. The homotypic fusion and vacuole protein sorting (HOPS) complex is recruited to Rab7/Ypt7p-bearing membranes to assemble soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes (Luzio et al., 2007), an event that leads to bilayer fusion (Sollner et al., 1993). However, it is not clear how this is regulated. In addition, the HOPS complex appears to act as an effector of Rab5 and a guanine nucleotide exchange factor (GEF) for Rab7 in the process of Rab5-Rab7 conversion for early-to-late endosome maturation (Rink et al., 2005).

The HOPS complex is a conserved large multisubunit complex that comprises four class C VPS proteins Vam1p/Vps11p, Vam9p/Vps16p, Vam6p/Vps39p, and the Sec1/Munc-like protein Vam5p/Vps33p, as well as two class B VPS proteins, Vam6p/Vps39p and Vam2p/Vps41p, and likely plays a range of roles in intracellular trafficking, including tethering/docking and fusion of late endosomal bilayers (Caplan et al., 2001; Poupon et al., 2003; Ungermann et al., 2000; Wurmser et al., 2000). In yeast, the class C VPS/HOPS complex functions as an effector of the Rab GTPase Ypt7p/Rab7 and interacts with unpaired SNAREs required for vacuole fusion (Collins et al., 2005).

In mammalian cells, overexpression of Vam6p, a subunit of the HOPS complex, resulted in tethering/docking and fusion of late endosomal compartments (Caplan et al., 2001). It was proposed that Vam6p exerts its function either downstream of, or in parallel to, Rab7, but the precise mechanism remains unclear (Caplan et al., 2001). Moreover, Vam6p was also found associated with microtubules and fusion of EEA-1-positive early endosomes, suggesting a connection between SNARE-dependent fusion and the microtubule cytoskeletal network in the endocytic route (Richardson et al., 2004).

Here, we show that recruitment of Vam6p to late endosomal membranes is RILP-dependent, possibly through a direct RILP-Vam6p interaction. Furthermore, we show that ORP1L governs this interaction, suggesting a common regulatory mechanism for late endosomal transport, docking/tethering, and fusion. This points to a model where the tripartite Rab7-RILP-ORP1L complex orchestrates the control of both late endosomal transport and

fusion, thereby providing spatio-temporal coordination for these two consecutive processes.

RESULTS

RILP-dependent dynamic recruitment of Vam6p to late endosomal membranes

Overexpression of Vam6p has been reported to induce tethering/docking and fusion of late endosomal membranes (Caplan et al., 2001). However, the mechanism by which Vam6p, and possibly other subunits of the HOPS complex, are recruited to late endosomal membranes remains unresolved. To test whether RILP, which establishes the molecular bridge between Rab7 and the dynein-dynactin motor for microtubular transport, could also be involved in the recruitment of Vam6p, we co-transfected MelJuSo cells with Vam6p and RILP or ΔN-RILP, a truncated mutant of RILP that binds to GTP-Rab7 but fails to recruit the dynein motor (Jordens et al., 2001; Wu et al., 2005) and analyzed the intracellular distribution of Vam6p by confocal laser-scanning microscopy. Ectopically expressed Vam6p is predominantly cytosolic and does not significantly localize to CD63-positive compartments. However, upon RILP co-expression, but not ΔN-RILP or ORP1L, Vam6p is found associated to lysosomal membranes and extensively co-localizing with RILP on compact perinuclear organelle clusters (Figure 1). This suggests that RILP is crucial for the recruitment of Vam6p to late endosomal membranes.

To determine whether Vam6p and RILP may be part of a common physical complex, MelJuSo cells were transfected with mRFP-tagged Vam6p and GFP-tagged RILP or ΔN-RILP and subjected to immunoprecipitation with the anti-GFP antibody or an irrelevant rabbit IgG. Immunoblotting of the isolates with the anti-mRFP antibody revealed co-immunoprecipitation of mRFP-Vam6p

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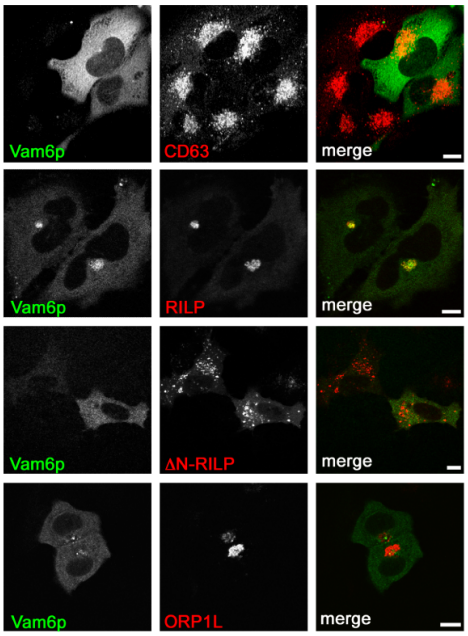
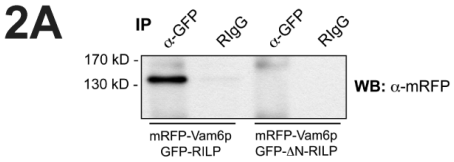


Figure 1. RILP-mediated recruitment of Vam6p to late endosomal membranes. MelJuSo cells were co-transfected with mRFP-Vam6p and GFP-RILP, GFP-ΔN-RILP, or GFP-ORP1L, as indicated, prior to confocal microscopy. Immunofluorescence confocal microscopy for the first row of the panel was done using anti-CD63 antibodies. n>100 for each condition. Scale bars, 10μm.

with GFP-RILP (Figure 2A). The Vam6p-RILP interaction was further confirmed by incubating immobilized recombinant His-RILP on a metal affinity resin with lysates from MelJuSo cells overexpressing GFP-Vam6p. Immunoblotting analysis with the anti-GFP antibody revealed that GFP-Vam6p interacted *in vitro* with purified His-RILP (Figure 2B). These data suggest that RILP and Vam6p not only co-localize on late endosomal membranes but are also part of a common physical complex.

RILP interacts directly with the C-terminal region of $p150^{Glued}$ of dynactin to recruit dynein-dynactin for centripetal microtubular transport. An analogous scenario could be envisaged



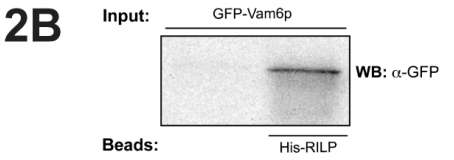
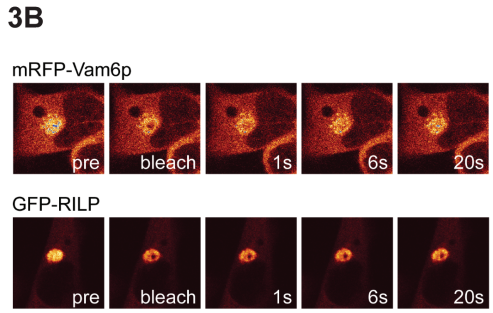


Figure 2. Vam6p co-isolates with RILP. (A) Immunoprecipitated proteins were isolated from lysates of MelJuSo cells transfected with mRFP-Vam6p and GFP-RILP or GFP-ΔN-RILP using anti-GFP antibodies (a-GFP) or irrelevant rabbit IgG (RIgG), and immunobloted using anti-mRFP antibodies (a-mRFP), as indicated. (B) Metal affinity beads or purified His-RILP immobilized on metal affinity beads (indicated as Beads:) were used to pull-down GFP-Vam6p from MelJuSo cell lysates (indicated as Input:). Anti-GFP antibodies (α-GFP) were used for immunoblotting.



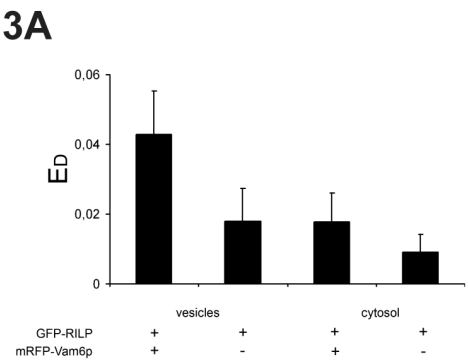


Figure 3. Dynamic interaction of Vam6p with RILP on late endosomal membranes. (A) FRET between GFP-RILP and mRFP-Vam6p. MelJuSo cells were transfected with GFP-RILP or co-transfected GFP-RILP and mRFP-Vam6p, as indicated. These cells were co-cultured with MelJuSo cells stably expressing H2B-GFP as a reference marker with a lifetime of 2.6 ns. The fluorescence lifetime of GFP was determined on GFP-RILP-bearing vesicles that were immotile during data acquisition or on cytosolic GFP-RILP, as indicated. The donor FRET efficiencies (E_D) between GFP- and mRFP-tagged proteins were plotted in the bar chart. Means + SD are shown. (B) Dynamics of Vam6p and RILP on late endosomal membranes analyzed by FRAP. pre: before photobleaching; bleach: immediately after photobleaching; 1s, 6s, and 20s, indicate time elapsed (in seconds) since photobleaching. Quantification of recovery time ($T_{1/2}$, in seconds) and immobile fraction calculated from the recovery curves.

for the recruitment of the HOPS complex for docking/tethering of late endosomal membranes, with Vam6p establishing a direct interaction with RILP. Given that Vam6p was precipitated on immobilized purified His-RILP from cell lysates, we cannot exclude the possibility that other factors present in the reaction mixture were present at the RILP-Vam6p interface.

The RILP-Vam6p interaction was further analyzed using FRET (fluorescence resonance energy transfer) in living MelJuSo cells expressing GFP-RILP and mRFP-Vam6p. FRET can occur when fluorophores with spectral overlap are in close proximity (<8 nm) (Förster, 1948). We measured FRET by FLIM (fluorescence lifetime imaging microscopy. FLIM allows the detection of the time between

photon absorbance by the donor fluorophore and emission. This decreases when energy transfer to the acceptor fluorophore occurs. Consistent with our previous conclusions, the calculated donor FRET efficiencies (E_D) between GFP-RILP and mRFP-Vam6p indicate that the two molecules are in close proximity on vesicular membranes (Figure 3A).

We next analyzed the relative dynamics of Vam6p and RILP on late endosomal membranes using FRAP (fluorescence recovery after photobleaching). We bleached a portion of membrane-associated mRFP-Vam6p in living MelJuSo cells expressing also HA-tagged RILP. Next, we measured in time the recovery of fluorescence in the bleached spot. The recovery relates to the dynamic equilibrium responsible for the replacement of non-fluorescent/bleached mRP-Vam6p in the spot for fluorescent mRFP-Vam6p from the cytosolic pool and, possibly, membrane-associated pool from the surroundings of the spot. This provides information on the dynamics of Vam6p presence on RILP-harboring membranes. The same method was applied to study to the dynamics of RILP presence on membranes of cells expressing GFP-RILP. We observed that the immobile fraction (calculated from the recovery curves) was ~20% and ~65% for mRFP-Vam6p and GFP-RILP, respectively, and that mRFP-Vam6p cycled faster than mRFP-Vam6p, as reflected by the determined $T_{1/2}$ (Figure 3B).

Taken together these data indicate that Vam6p is recruited dynamically to late endosomal membranes in a RILP-dependent manner.

RILP is required for Vam6p-induced clustering and tethering/docking of late endosomal bilayers

To examine the effect of Vam6p recruitment to RILP-bearing late endosomal membranes in more detail, we performed ultrastructural analyses on MelJuSo cells co-expressing HA-tagged RILP or ΔN-RILP and GFP-tagged Vam6p. Immunoelectron microscopy on cryosections revealed that Vam6p is found predominantly associated to late endosomal membranes in cells co-expressing RILP (Figure 4a). In contrast, in cells co-expressing the truncated mutant ΔN-RILP, Vam6p association to membranes is dramatically reduced (Figure 4b). Furthermore, co-expression of RILP, but not ΔN-RILP, and Vam6p induces massive clustering of late endosomal compartments with their bilayers appearing tethered/docked (Figures 4a, 4c, and 4d).

ORP1L controls the recruitment of Vam6p to RILP-bearing late endosomal membranes

We have recently shown that ORP1L controls $p150^{Glued}$ binding to the Rab-RILP receptor and thus late endosomal transport and positioning (Chapter 3 of this thesis). Co-expression of RILP with a truncated mutant of ORP1L lacking the C-terminal OSBP-related domain (ΔORD) prevented recruitment of $p150^{Glued}$ by RILP and resulted in centrifugal relocation of RILP-positive compartments within the cell. In contrast, we have previously observed that this phenotype could be rescued if the OSBP-related domain (ORD) in ORP1L was exchanged for a tandem of ORP1L pleckstrin-homology (PH) domains. This chimera possibly mimics the ORD of ORP1L in a membrane-associated state (Chapter 3 of this thesis). To test whether ORP1L is also involved in the control of Vam6p recruitment to RILP-positive membranes, we transfected MelJuSo cells with GFP-Vam6p and mRFP-ΔORD, or mRFP-ORP1L and HA-RILP, or mRFP-ΔORD and HA-RILP, or mRFP-ΔORDPHDPHD and HA-RILP, prior to fixation and analyses by immunofluorescence confocal microscopy with anti-HA antibodies (Figure 5). Vam6p, RILP, and ORP1L, extensively co-localized

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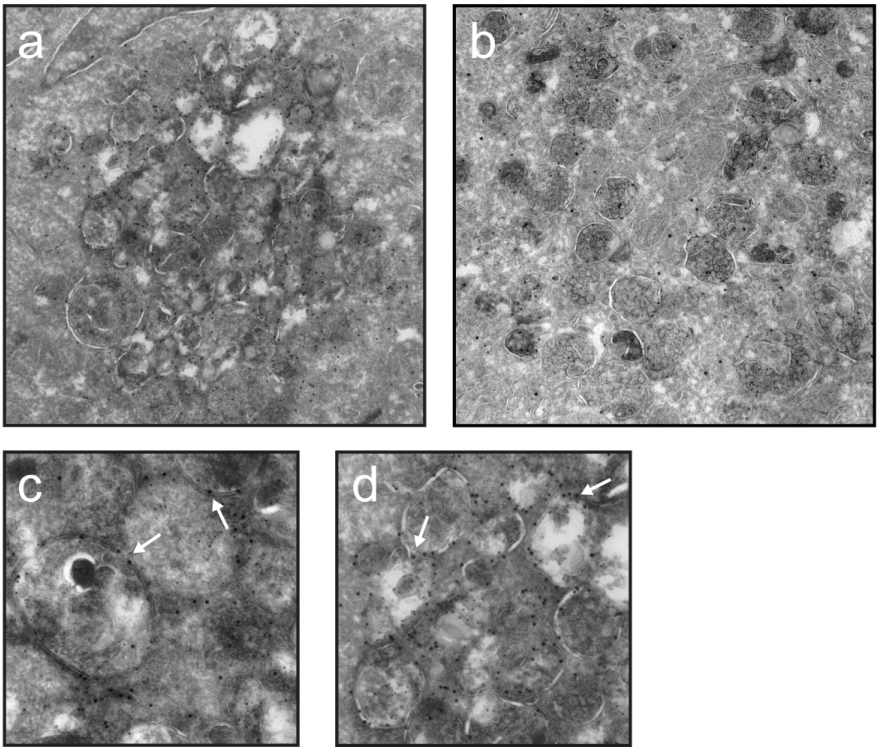

Figure 4. Vam6p is recruited to late endosomal RILP-positive membranes and induces their docking/tethering. Immunoelectron microscopy analyses on cryosections from MelJuSo cells expressing GFP-Vam6p and (a) HA-RILP or (b) ΔN-RILP. Gold particles label anti-GFP antibodies. Successful expression of RILP or ΔN-RILP was judged based on the characteristic distinct phenotypes. (c) and (d) show magnifications of representative micrographs from cells expressing GFP-Vam6p and HA-RILP showing tethering/docking of Vam6p-positive bilayers (indicated by arrows).

on juxtanuclear clusters of late endosomal organelles. Conversely, co-expression of Vam6p, RILP, and ΔORD, caused the relocation of late endosomal compartments to the periphery of the cell, as expected. Moreover, despite RILP overexpression, ΔORD excluded Vam6p from late endosomal membranes rendering it predominantly cytosolic. Vam6p recruitment to late endosomal membranes, as well as their clustering at the minus end of microtubules, could be rescued by expression of the ΔORDPHDPHD chimera (Figure 5). Taken together these data suggest that ORP1L controls Vam6p recruitment to RILP-positive membranes.

RILP couples late endosomal transport to tethering/docking of late endosomal bilayers

RILP overexpression induces minus end-directed transport by recruiting the dynein-dynactin motor and results in dense clustering of late endosomal compartments in the juxtanuclear area (Jordens et al., 2001). Concomitantly, RILP induces recruitment of

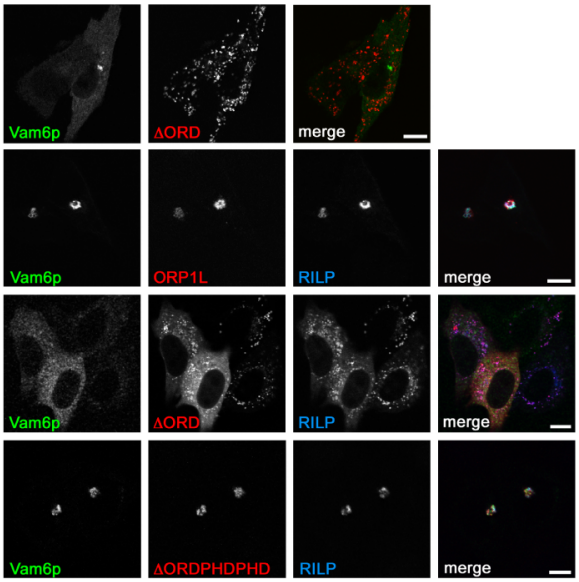

Figure 5. ORP1L controls recruitment of Vam6p to the Rab7-RILP receptor on late endosomal membranes. MelJuSo cells were co-transfected with GFP-Vam6p and mRFP-ΔORD, GFP-Vam6p and HA-RILP and mRFP-ORP1L, GFP-Vam6p and HA-RILP and mRFP-ΔORD, or GFP-Vam6p and GFP-RILP and mRFP-ΔORDPHDPHD, as indicated, prior to immunofluorescence confocal microscopy using anti-HA antibodies. n>100 for each condition. Scale bars, 10μm.

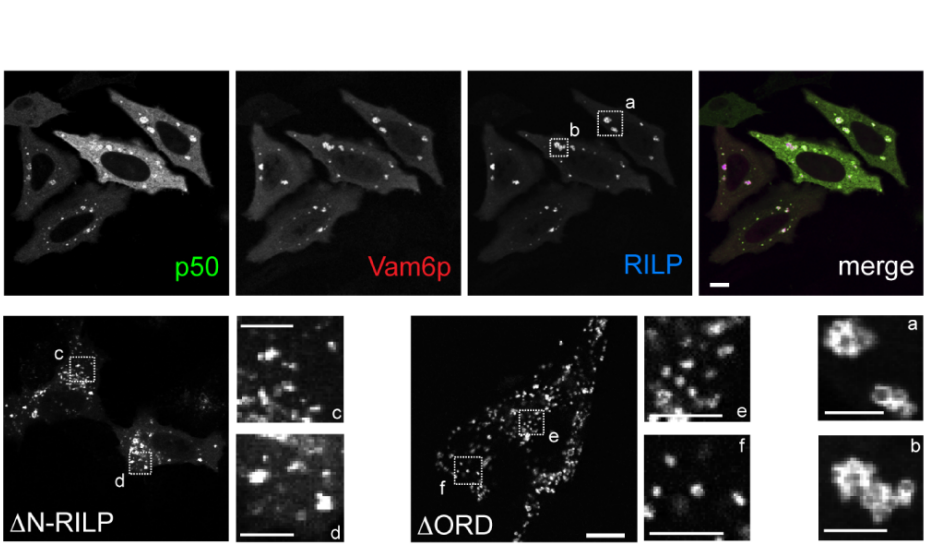

Figure 6. Uncoupling of late endosomal transport and fusion by p50^{dynamitin} overexpression. MelJuSo cells were co-transfected with GFP-p50^{dynamitin}, mRFP-Vam6p and HA-RILP, as indicated. (a) and (b): Magnification of clusters of RILP-positive vesicles. (c) and (d): Magnification of ΔN-RILP-positive vesicles from MelJuSo cells expressing mRFP-Vam6p and GFP-ΔN-RILP. (e) and (f): Magnification of ΔORD-positive vesicles from MelJuSo cells expressing mRFP-Vam6p and GFP-ΔORD. n>100 for each condition. Scale bars, 10μm.

Vam6p to late endosomal membranes and induces their tethering/docking (Figures 1 and 4). It is thought that late endosomal transport brings bilayers into close proximity to affect the efficiency of trafficking steps such as tethering/docking and fusion (Caviston and Holzbaur, 2006; Jordens et al., 2005). We tested whether selective abrogation of centripetal dynein-based transport of late endosomal organelles was accompanied by an absence of tethering/docking of bilayers. We made use of $p50^{dynamitin}$ overexpression, which inactivates dynein transport by disassembling dynein's adaptor dynactin (Burkhardt et al., 1997; Johansson et al., 2007). We then tested whether Vam6p is still able to induce tethering/docking. GFP-$p50^{dynamitin}$ and mRFP-Vam6p were overexpressed in MelJuSo cells with HA-RILP, to induce recruitment of Vam6p. Cells were fixed and analyzed by immunofluorescence confocal microscopy with anti-HA antibodies. Vam6p and RILP extensively co-localized on vesicle clusters. However, given that the dynein-dynactin motor was inhibited by GFP-$p50^{dynamitin}$ overexpression, these clusters were no longer present in the juxtanuclear area but dispersed throughout the cell (Figure 6, top panel). A closer examination of the RILP/Vam6p-positive clusters (Figure 6a and 6b) reveals that these appear to comprise a higher number of vesicles than those of MelJuSo cells expressing ΔN-RILP or ΔORD, truncation mutants that prevent the recruitment of Vam6p to late endosomal membranes (Figures 6c–f). Furthermore, the diameter of each individual vesicle appears to be larger in cells where Vam6p was efficiently recruited to late endosomal membranes (Figures 6a–f), suggesting that Vam6p could have promoted vesicular fusion and thus an increase in vesicle size.

DISCUSSION

Rab GTPases are key elements of the vesicular transport and endomembrane trafficking machineries (Caviston and Holzbaur, 2006; Jordens et al., 2005; Pfeffer, 2001; Zerial and McBride, 2001). They are of strategic importance in determining organelle identity by associating to specific organelle membranes. This is a dynamic association dependent on the ability of Rab GTPases to cycle between an active GTP-bound state and an inactive GDP-bound state. Thus, Rab GTPases, either directly or by organizing the recruitment of soluble effector molecules, bring spatial and temporal resolution to intracellular membrane trafficking reactions.

Motor-dependent vesicular transport is thought to facilitate membrane tethering/docking upstream of SNARE-dependent fusion by bringing vesicular membranes into close contact (Jordens et al., 2005; Somsel Rodman and Wandinger-Ness, 2000). In principle, a single RabGTPase could orchestrate the recruitment of both the transport and tethering/docking machineries to a specific organelle. This would provide concerted control for two consecutive steps in the course of intracellular vesicle trafficking.

We show here how the recruitment of subunits of macromolecular complexes involved in late endosomal transport and tethering/docking is regulated by single tripartite receptor—Rab7-RILP-ORP1L—whose assembly on late endosomal membranes is dictated by the small GTPase Rab7. The dynein-dynactin motor is recruited to Rab7 membranes in a RILP-dependent manner (Johansson et al., 2007). This is further controlled by ORP1L which regulates access of the motor complex to RILP (Chapter 3 of this thesis). Our data point to an analogous regulatory mechanism operating in the recruitment of the tethering/docking machinery upstream of bilayer fusion. We show that Vam6p, a subunit of the HOPS complex required for SNARE assembly, also depends on RILP for its recruitment to Rab7 late endosomal membranes. It is likely that RILP and Vam6p

establish a direct interaction, although binding studies using purified recombinant proteins have to be performed to define the binding interface between RILP and Vam6p. In addition, we show that ORP1L is able to control the access of Vam6p to RILP. Furthermore, given that $p50^{dynamitin}$ overexpression inhibited centripetal transport but not tethering/docking of vesicles, the RILP-dependent recruitment of Vam6p appears to be independent of dynein's function. This obviously does not exclude that dynein-based transport influences the efficiency of membrane tethering/docking events.

The cholesterol sensor ORP1L responds with conformational changes to variations in late endosomal content thereby acting as a switch that controls access of dynein to the Rab7-RILP receptor (Chapter 3 of this thesis). Whether ORP1L is also responding to cholesterol content variations to control the access of Vam6p/HOPS complex to its receptor Rab7-RILP remains to be tested. However, this appears to be likely since the ΔORDPHDPHD chimera, which mimics the high-cholesterol content conformational state of ORP1L, rescues the recruitment of Vam6p to the Rab7-RILP which is disrupted by ΔORD expression.

The existence of a common regulatory mechanism for sequential reactions, such as transport and tethering/docking, is consistent with the recently proposed models for Rab conversion/cascades and organelle maturation (Caviston and Holzbaur, 2006; Grosshans et al., 2006; Markgraf et al., 2007) where RabGTPases and their effectors coordinate consecutive reactions along the endocytic route and may provide an integrated system for the spatial and temporal specificity in intracellular trafficking.

EXPERIMENTAL PROCEDURES

REAGENTS

Rabbit anti-GFP and rabbit anti-mRFP antibodies were generated using purified His-mRFP or His-GFP recombinant proteins as immunogens, respectively. Cross-reactivity was excluded by Western blot analyses with various mRFP- or GFP-labelled fusion proteins. Rabbit polyclonal anti-CD63 was described elsewhere (Vennegoor et al., 1985). Rabbit polyclonal anti-HA antibody (Y11:sc-805) was from Santa Cruz Biotechnology.

cDNA CONSTRUCTS AND VECTORS

Human Vam6p was a kind gift of J. Bonifacino (Caplan et al., 2001) and was subcloned into a pEGFP-C1 (BD Biosciences Clonetech, USA) and a custom made pmRFP-C1 (Johansson et al., 2007) vectors, for N-terminal GFP and mRFP tagging, respectively. Rab7, RILP, ORP1L, and $p50^{dynamitin}$ cDNA constructs have been described previously (Johansson et al., 2005; Johansson et al., 2007; Jordens et al., 2001; Marsman et al., 2004).

CELL CULTURE

Monolayers of MelJuSo cells were maintained in Iscoves Modified Dulbecco's Medium (IMDM) medium (Gibco) supplemented with 5% FCS, in 5% CO_2 at 37°C.

CONFOCAL MICROSCOPY ON FIXED SAMPLES

Transfected cells were fixed at 48 hr post-transfection with 4% formaldehyde in PBS for 30 min and permeabilized for 5 min with 0.05% Triton X-100 in PBS, at room temperature. Nonspecific binding of antibodies was blocked by 0.5% BSA/PBS for 40 min, after which cells were incubated with a primary antibody in 0.5% BSA/PBS for 1 hr at room temperature. Bound primary antibodies were visualized with Alexa Fluor secondary antibody conjugates (Invitrogen). Cells were mounted in Vectashield mounting medium (Vector Laboratories, USA). The specimens were analyzed with confocal laser scanning microscopes (TCS-SP1, -SP2 or AOBS) equipped with HCX PL APO 63x/NA 1.32 and HCX PL APO lbd.bl 63×/NA 1.4

oil-corrected objective lenses (all from Leica, Mannheim, Germany). The acquisition software used was Leica LCS.

FRAP and FLIM

FRAP experiments were performed as described previously (Jordens et al., 2001; Reits et al., 2000).

FLIM experiments were performed with MelJuSo cells stably expressing GFP-ORP1L-mRFP cultured on Delta T dishes (Bioptechs). Prior to measurements, cells were mounted in bicarbonate-buffered saline medium (140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 23 mM $NaHCO_3$, 10 mM [D-]glucose, and 10 mM Hepes, pH 7.3) and analyzed at 37°C in a 5% CO_2 culture hood surrounding the objective stage of the microscope. Images were taken on a Leica inverted DM-IRE2 microscope with a HCX PL APO 63x/NA 1.35 glycerol corrected objective lens equipped with a Lambert Instruments (LI) frequency domain lifetime attachment (Leutingewolde, The Netherlands), controlled by the manufacturer's LI FLIM software. GFP was excited with ~4 mW of 488 nm light from a LED modulated at 40 MHz, and emission was collected at 490–550 nm using an intensified charge-coupled device camera (CoolSNAP HQ; Roper Scientific). To calculate the GFP lifetime, the intensities from 12 phase-shifted images (modulation depth ~70%) were fitted with a sinus function, and lifetimes were derived from the phase shift between excitation and emission. For internal controls, cells were co-cultured with MelJuSo cells expressing H2B–GFP only. Lifetimes were referenced to a 1 μM solution of rhodamine-G6 in saline that was set at a 4.11 ns lifetime. The donor FRET efficiency E_D was calculated as $E_D = 1 -$ (measured lifetime/GFP lifetime in control cells) (Zwart et al., 2005).

ELECTRONMICROSCOPY

MelJuSo cells were fixed in a mixture of 4% formaldehyde (w/v) and 0.1% (v/v) gluteraldehyde in 0.1 M phosphate buffer, pH 7.2. Ultra-thin cryosections were incubated with the anti-GFP antisera. Secondary antibodies were coupled to gold. Images were made using a Philips CM 10 electron microscope.

PROTEIN IMMUNOPRECIPITATION AND PULL-DOWN

MelJuSo cells transfected with mRFP-Vam6p were washed with ice-cold PBS and scraped into 400 μL of cell lysis buffer (20 mM Hepes, 150 mM NaCl, 2 mM $MgCl_2$, 0.5% Triton X-100, pH 7.5) supplemented with Complete EDTA-free Protease Inhibitor Cocktail. Cell lysates were obtained by incubation on ice for 10 min followed by centrifugation for clearing. The supernatants were then incubated for 25 min on protein G-Sepharose 4 FF resin (Amersham Biosciences) followed by incubation with anti-GFP antisera or an irrelevant rabbit IgG as control for 2 hr. The antibody complexes were then precipitated with protein G-Sepharose 4 FF resin and washed extensively with the cell lysis buffer. The immunoprecipitates were subjected to immunoblotting with anti-mRFP antibodies.

For the pull-down of GFP-Vam6p on immobilized purified His-RILP, MelJuSo cells were transfected with GFP-Vam6p and lysates were obtained as described above. Cleared lysates were then incubated either with empty Talon Co^{2+}-affinity resin or with His-RILP immobilized on the same type of resin. After recovery of the resins by centrifugation and extensive washing with lysis buffer, the precipitated proteins were analyzed by immunoblotting with anti-GFP antisera. His-RILP production and purification was described before (Johansson et al., 2007).

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