A WASp-binding type II phosphatidylinositol 4-kinase required for actin polymerization-driven endosome motility

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Endosomes in yeast have been hypothesized to move through the cytoplasm by the momentum gained after actin polymerization has driven endosome abscision from the plasma membrane. Alternatively, after abscission, ongoing actin polymerization on endosomes could power transport. Here, we tested these hypotheses by showing that the Arp2/3 complex activation domain (WCA) of Las17 (Wiskott-Aldrich syndrome protein [WASp] homologue) fused to an endocytic cargo protein (Ste2) rescued endosome motility in *las17*ΔWCA mutants, and that capping actin filament barbed ends inhibited endosome motility but not endocytic internalization. Motility therefore requires continual actin polymerization on endosomes. We also explored how Las17 is regulated. Endosome motility required the Las17-binding protein Lsb6, a type II phosphatidylinositol 4-kinase. Catalytically inactive Lsb6 interacted with Las17 and promoted endosome motility. Lsb6 therefore is a novel regulator of Las17 that mediates endosome motility independent of phosphatidylinositol 4-phosphate synthesis. Mammalian type II phosphatidylinositol 4-kinases may regulate WASp proteins and endosome motility.

Introduction

Endocytosis regulates signaling by cell surface receptors, including hundreds of growth factor receptors and G protein–coupled receptors (GPCRs; for review see Engqvist-Goldstein and Drubin, 2003; Qualmann et al., 2000; Sorkin and Von Zastrow, 2002; Gaborik and Hunyady, 2004). Endocytosis can attenuate signaling when internalized receptors are transported to lysosomes and degraded. Conversely, endocytosis can promote signaling, as indicated by the ability of internalized GPCRs to activate MAP kinase cascades and/or recycle to the plasma membrane for further rounds of agonist stimulation.

Internalized receptors use several mechanisms to move within minutes from the plasma membrane to lysosomes. Motor proteins such as myosin VI and myosin V can move endosomes along actin filaments near the cell cortex (Schott et al., 1999; Aschenbrenner et al., 2003; Hasson, 2003; Soldati, 2003), and kinesin and dynein can move endosomes along microtubules over longer distances (Aniento et al., 1993; Apodaca, 2001; Bananis et al., 2003). Actin polymerization can also power endosome movement (Taunton et al., 2000), similar to mechanisms that transport intracellular pathogens (Loisel et al., 1999), macropinosomes (Merrifield et al., 2001; Seastone et al., 2001), and the insulin-responsive glucose transporter Glut4 (Kanzaki et al., 2001). These actin polymerization-dependent transport mechanisms are thought to use the Arp2/3 complex to nucleate ongoing and continuous assembly of branched actin filament networks on organelle membranes (reviewed in Schafer, 2002). The WASp/SCAR/WAVE family of proteins are potent activators of the Arp2/3 complex (Pollard and Borisy, 2003), and have been implicated in promoting motility of endosomes and other organelles (Taunton et al., 2000; Southwick et al., 2003). Indeed, we have shown that Las17, the sole WASp homologue of yeast, is required for motility of endosomes containing the GPCR Ste2 (Chang et al., 2003).

Actin dynamics may power organelle motility by other mechanisms as suggested by studies of endocytosis in yeast. In one model, actin polymerization-driven internalization imparts momentum that carries endosomes through the cytoplasm (Kaksonen et al., 2003). In support of this model, the WASp homologue Las17 and the actin binding protein Abp1 are de-

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Abbreviations used in this paper: GPCR, G protein–coupled receptor; Lsb, Las17-binding protein; MSD, mean squared displacement; PI 4-kinase, phosphatidylinositol 4-kinase; PI4P, phosphatidylinositol 4-phosphate; PI4,5P2, phosphatidylinositol 4,5-bisphosphate; SCAR, suppressor of cAMP receptor; WASp, Wiskott-Aldrich syndrome protein; WAVE, WASP family Verprolin homology protein; WCA, Arp2/3 complex activating region (WASp homology 2, central region, acidic region).

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tected on cortical actin patches as they internalize but are depleted from endosomes moving away from the cortex (Kaksonen et al., 2003). However, the distance or speed with which endosomes could travel through the viscous cytoplasm by such a mechanism is unclear. In a second mechanism, a subpopulation of endosomes have been shown to bind cytoplasmic F-actin cables that treadmill, transporting endosomes to the lysosome-like vacuole (Huckaba et al., 2004).

Mechanisms regulating actin polymerization-dependent endosome and organelle motility have emerged from studies of WASp/SCAR/WAVE proteins. WASp proteins are autoinhibited and form complexes with accessory proteins including WIP and TOCA-1 (Moreau et al., 2000; Ho et al., 2004). Phosphoinositides such as phosphatidylinositol 4,5-bisphosphate (PI4,5P2; PIP2) in concert with GTP-loaded Cdc42 or Rac1 can bind and activate WASp in vitro (Kanzaki et al., 2001; Benesch et al., 2002; Sokac et al., 2003) by exposing the WASp COOH-terminal Arp2/3 complex activating region (WCA) domain (reviewed in Higgs and Pollard, 2001). Furthermore, overexpression of phosphatidylinositol 4-phosphate (PI4P)5-kinase induces N-WASP–dependent motility of endosomes and other organelles (Rozelle et al., 2000; Taunton et al., 2000). Regulation of SCAR/WAVE proteins is less well understood. SCAR/WAVE proteins are constitutively active, but can be stabilized in inactive complexes with accessory proteins (Nap1, Abi2, HSPC300; Eden et al., 2002; Innocenti et al., 2004; Steffen et al., 2004).

Here we have addressed whether endosomes in yeast move by the momentum gained upon actin polymerization-driven abscission from the plasma membrane or by continuously polymerizing actin on endosome membranes after abscission. We also address how the yeast WASp homologue Las17 is regulated to drive endosome motility. Our findings indicate that endosome motility requires continuous actin polymerization on endosomes, and that Lsb6, a type II phosphatidylinositol 4-kinase (PI 4-kinase) of previously unknown function, regulates endosome motility by interacting with Las17 rather than by catalyzing PI4P synthesis.

Results

Ongoing actin polymerization powers endosome motility

To address whether endosomes move by continuously polymerizing actin after abscission from the plasma membrane, we appended the domain of the WASp homologue (Las17) that activates the Arp2/3 complex (WCA domain; COOH-terminal 90 amino acids; Winter et al., 1999; Higgs and Pollard, 2001) to the COOH terminus of the endocytic cargo Ste2 (Ste2-WCA). As a control, the WCA domain also was fused to a highly ex-

Table I. **Endosome speeds**

Strain			*n*	Average speed	Significant defect?[a]
WT	+ vector	23°	36	0.19 ± 0.02	—
lsb6Δ	+ vector	23°	50	0.09 ± 0.01	Yes
lsb6Δ	+ pLSB6	23°	48	0.20 ± 0.01	No
WT	+ Ste2-WCA	23°	58	0.14 ± 0.01	No
las17ΔWCA	+ vector	23°	72	0.09 ± 0.01	Yes
	+ Ste2-WCA	23°	67	0.18 ± 0.01	No
	+ Hxt1-WCA	23°	51	0.09 ± 0.01	Yes
lsb6Δ	+ Ste2-WCA	23°	45	0.15 ± 0.01	No
	+ Hxt1-WCA	23°	60	0.10 ± 0.01	Yes
WT	+ Gal Cap1/2	Raffinose	57	0.14 ± 0.01	—
	+ Gal Cap1/2	Galactose	57	0.08 ± 0.02	Yes
lsb6Δ	+ Lsb6 K192M	23°	28	0.16 ± 0.01	No
	+ Lsb6 D387A	23°	25	0.15 ± 0.01	No
	+ Lsb6 N392A	23°	28	0.17 ± 0.01	No
	+ Lsb6 D413A	23°	25	0.18 ± 0.02	No
	+ Lsb6 K192M (CEN)	23°	54	0.18 ± 0.01	No
	+ Lsb6 4KD	23°	35	0.19 ± 0.01	No
lsb6Δ	+ Lsb6 ΔN-terminus	23°	59	0.07 ± 0.01	Yes
	+ Lsb6 Δkinase subdomain 1	23°	47	0.11 ± 0.02	No
	+ Lsb6 Δlinker	23°	47	0.11 ± 0.01	No
	+ Lsb6 Δkinase subdomain 2	23°	47	0.14 ± 0.01	No
	+ Lsb6 ΔC-terminus	23°	59	0.13 ± 0.01	No
	+ Lsb6 N-terminus	23°	52	0.12 ± 0.01	No
	+ Lsb6 kinase subdomain 1	23°	49	0.10 ± 0.01	Yes
	+ Lsb6 N-terminus + kinase subdomain 1	23°	48	0.15 ± 0.01	No
	+ Lsb6 kinase subdomain 1 + linker	23°	52	0.06 ± 0.01	Yes
	+ Lsb6 kinase subdomain 2	23°	43	0.08 ± 0.01	Yes
	+ Lsb6 C-terminus	23°	46	0.08 ± 0.01	Yes

[a]Average endosome speed, SEM, and P values were calculated by imaging 25–72 endosomes taken from three independent transformants in each of two to four experiments.
$P < 0.005$ to paired wild-type control.

pressed glucose transporter (Hxt1-WCA) that localizes to the plasma membrane but does not undergo endocytosis when glucose is abundant (Schmelzle et al., 2004). We expressed Ste2-WCA or Hxt1-WCA in a mutant defective in endosome motility (*las17*ΔWCA, in which the chromosomal sequence encoding the Las17 WCA domain was deleted; Chang et al., 2003). Expression of Ste2-WCA or Hxt1-WCA augmented the appearance of cortical F-actin structures at the expense of cytoplasmic F-actin cables (Fig. S1 A available at http://www.jcb.org/cgi/content/full/jcb.200501086/DC1), indicating that both fusions stimulated actin polymerization at the plasma membrane.

To determine whether expression of Ste2-WCA or Hxt1-WCA could rescue the endosome motility defect of *las17*ΔWCA mutants, we introduced Ste2-GFP into these cells as an endosome marker (Stefan and Blumer, 1999) and analyzed endosome movement in these transformants by making single focal plane time-lapse movies. Endosome (n = 25–50) motility in each mutant was measured by calculating average speed, distribution of speeds, and by tracing paths of endosome motion over a 5–10-s period. Using these methods we showed previously that endosomes labeled with Ste2-GFP in wild-type cells move with an average speed of ~0.2 μm/s, travel along paths of various length with marked changes in direction, and often leave the plane of focus within a few seconds (Chang et al., 2003).

Ste2-WCA and Hxt1-WCA fusions had strikingly different effects on the motility of endosomes labeled with Ste2-GFP in a *las17*ΔWCA mutant (Table I). Because Ste2 efficiently and constitutively forms homooligomers (Overton and Blumer, 2000), Ste2-WCA and Ste2-GFP will traffic together during endocytosis. Using this approach, we found that expression of Ste2-WCA fully rescued the endosome motility defect of a *las17*ΔWCA mutant. Rescued cells displayed an average endosome speed of 0.18 ± 0.01 μm/s relative to that of 0.08 ± 0.01 μm/s in empty vector control cells. As occurs in wild-type cells, endosomes in rescued cells traveled along paths that were long and changed direction, often leaving the plane of focus after a few seconds. In contrast, although Hxt1-WCA was highly overexpressed on the plasma membrane in a pattern indistinguishable from Ste2 (Fig. S1 B available at http://www.jcb.org/cgi/content/full/jcb.200501086/DC1), it failed to rescue the endosome motility defect of *las17*ΔWCA mutants (Table I). These results are illustrated in Video S1 (endosome motility in a *las17*ΔWCA cell + vector), Video S2 (endosome motility in a *las17*ΔWCA cell + Ste2-WCA), and Video S3 (endosome motility in a *las17*ΔWCA cell + Hxt1-WCA; videos available at http://www.jcb.org/cgi/content/full/jcb.200501086/DC1).

To obtain and analyze endosome motility more quantitatively, we used an automated particle tracking program developed to study actin patch motility in yeast (Carlsson et al., 2002). The resultant data were plotted as the mean squared displacement (MSD) of endosomes (n = 50–150) over time. This analysis indicated that endosomes labeled with Ste2-GFP were poorly motile in *las17*ΔWCA cells carrying an empty vector or a plasmid overexpressing Hxt1-WCA, as indicated by relatively flat curves in MSD plots (Fig. 1). In contrast, endosomes in the *las17*ΔWCA mutant expressing Ste2-WCA exhibited wild-type motility, as indicated by significantly greater displacement over time. Because under these conditions Ste2 is recruited to endosomes whereas Hxt1 is not, these results suggested that actin polymerization on endosomes rather than the plasma membrane promotes motility.

To test this hypothesis further, we examined whether endosome motility in wild-type cells requires polymerization from free barbed ends of actin filaments, as expected for Arp2/3 complex–driven polymerization. Accordingly, we overexpressed the barbed-end capping protein (Cap1 + Cap2) from an inducible promoter in wild-type cells (Fig. S1 C). Formation of cortical actin patches implicated in endocytic internalization is known to be preserved upon overexpression of capping protein (Amatruda et al., 1992; Kaksonen et al., 2000; Engqvist-Goldstein and Drubin, 2003). Actin patch motility was strongly inhibited by capping protein overexpression (unpublished data). Strikingly, overexpression of capping protein strongly inhibited endosome motility compared with uninduced control cells (average speed 0.08 ± 0.02 μm/s for induced versus 0.14 ± 0.02 μm/s for uninduced cells; Table I; Video S4 [endosome motility in a WT cell]; Video S5 [endosome motility in a WT cell + overexpressed capping protein]; videos available at http://www.jcb.org/cgi/content/full/jcb.200501086/DC1). Because endosomes labeled with Ste2-GFP were produced when capping protein was overexpressed, endocytic internalization was not blocked. These results coupled with those using Ste2-WCA fusions therefore provided independent lines of evidence indicating that Ste2-labeled endosomes move via continuous Arp2/3 complex–mediated polymerization of actin filaments on endosomes.

The type II PI 4-kinase Lsb6 is required for endosome motility

Little is known about the mechanisms that regulate Las17. Purified Las17 appears to be constitutively active, yet it can be inhibited in vitro by the SH3-domain proteins Bbc1 and Sla1 (Rodal et al., 2003). It remains unknown whether Las17 in vivo is constitutively active or regulated by stimulatory or inhibitory proteins or ligands.

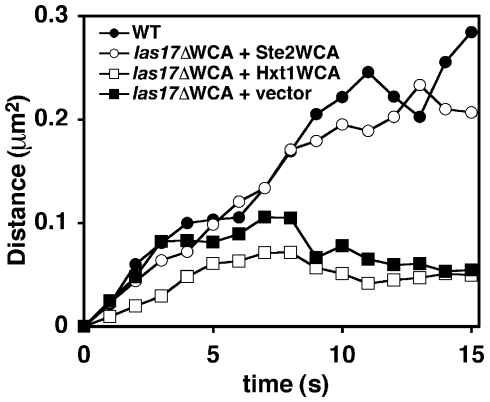

Figure 1. **Appending the WCA domain of Las17 to the endocytic cargo Ste2 rescues endosome motility in las17ΔWCA cells.** Automated particle tracking was used to analyze endosome position over time. Ste2-GFP was used as an endosome marker in this and subsequent figures. The mean square displacement of endosomes (n = 50–100) over time was calculated for the indicated wild-type and mutant cells.

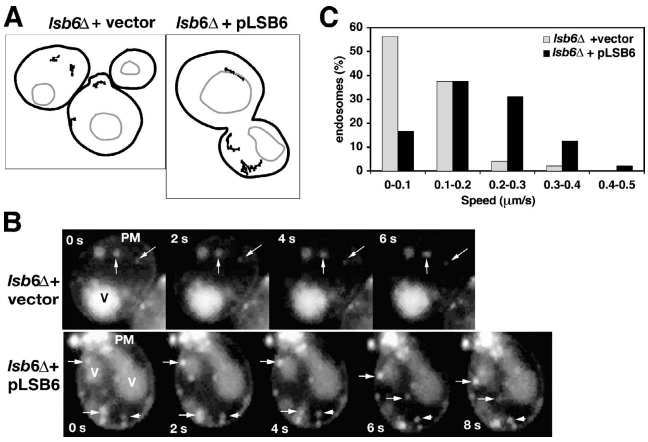

Figure 2. **Lsb6 is required for endosome motility.** (A) Tracings of endosome paths in *lsb6*Δ cells that lack or carry a plasmid (pLSB6) expressing wild-type Lsb6. (B) Gallery of fluorescence micrographs derived from Video S6 and S7 that illustrate endosome position over time in *lsb6*Δ cells lacking or carrying pLsb6. PM, plasma membrane; V, vacuole. (C) Histogram showing the distribution of endosome speeds in *lsb6*Δ cells lacking or carrying pLsb6.

To identify proteins that may activate or recruit Las17 and thereby promote motility of Ste2-containing endosomes, we analyzed mutants lacking proteins that were identified previously in a two-hybrid screen as Las17-binding proteins (Lsb; Madania et al., 1999). These proteins include Lsb1 and 2 (SH3 domain–containing proteins similar to Grb2 or Grap2), Lsb3 and Lsb4 (SH3 domain–containing proteins similar to intersectin 1), Lsb5 (VRS domain–containing protein like HRS), and Lsb6 (the single type II PI 4-kinase homologue in yeast). Endosome motility was assayed as described above in null mutants lacking each of these Las17-interacting proteins, and in double mutants lacking pairs of closely related proteins (Lsb1 and Lsb2, or Lsb3 and Lsb4).

Of the six single mutants and two double mutants analyzed, *lsb6*Δ mutants displayed significant impairment of endosome motility relative to wild-type cells. Endosomes in *lsb6*Δ and wild-type cells moved with average speeds of 0.09 ± 0.01 μm/s and 0.19 ± 0.02 μm/s, respectively (Table I). The average speed of endosome motility in *lsb6*Δ mutants was similar to that observed in cells expressing Las17 lacking its COOH-terminal WCA domain (*las17*ΔWCA, 0.09 ± 0.01 μm/s) or in wild-type cells treated with the actin-depolymerizing drug latrunculin A (0.08 ± 0.04 μm/s; Chang et al., 2003). In *lsb6*Δ mutants, the distribution of endosome speeds was shifted to lower values (Fig. 2 C), and endosomes moved along abnormally short paths that remained within the plane of focus for many seconds (Fig. 2 B). Impaired endosome motility in *lsb6*Δ mutants was rescued by expression of the wild-type *LSB6* gene on a single-copy plasmid (compare Video S6 [endosome motility in an *lsb6*Δ cell] with Video S7 [endosome motility in an *lsb6*Δ cell + pLSB6]; videos available at http://www.jcb.org/cgi/content/full/jcb.200501086/DC1). These results were confirmed by performing automated particle tracking and analyzing the data in MSD plots (Fig. S2 A). These results indicated that Lsb6 is required for motility of Ste2-containing endosomes. This is the first phenotype caused by the absence of the sole type II PI 4-kinase of yeast.

Evidence that Lsb6 functions upstream of Las17

Because Lsb6 and Las17 interact and localize to the plasma membrane (Madania et al., 1999; Eitzen et al., 2002; Han et al., 2002), they may function in a common pathway in which Lsb6 acts upstream of Las17 to promote endosome motility. We tested this hypothesis by determining whether expression of Ste2-WCA could rescue the endosome motility defect of an *lsb6*Δ mutant by bypassing the hypothesized impairment of Las17 recruitment or activation caused by the absence of Lsb6. We found that *lsb6*Δ cells expressing Ste2-WCA and Ste2-GFP exhibited nearly wild-type endosome motility (average speed was 0.15 ± 0.01 μm/s vs. 0.09 ± 0.01 μm/s for controls lacking Ste2-WCA; Table I; Video S6 [endosome motility in a *lsb6*Δ cell]). These results suggested that Las17 functions downstream of Lsb6 in a pathway leading to activation of the Arp2/3 complex, actin polymerization and endosome motility.

PI 4-kinase activity of Lsb6 is dispensable for endosome motility

Lsb6 is the only type II PI 4-kinase in yeast (Han et al., 2002; Shelton et al., 2003). However, yeast also possesses two type III PI 4-kinases, Stt4 and Pik1, each of which is essential for cell growth (Audhya et al., 2000). Whereas Lsb6 synthesizes a small fraction (~5%) of the total PI4P pool, the remainder of the PI4P pool is produced by Stt4 on the plasma membrane and by Pik1 on Golgi membranes (Han et al., 2002). These plasma membrane and Golgi PI4P pools have distinct functions because overexpression of Stt4 does not rescue the phenotype of *pik1* mutants and vice versa (Han et al., 2002).

To determine whether Lsb6 synthesizes PI4P to promote endosome motility, we generated and analyzed several point mutants defective in PI 4-kinase activity. Like other type II PI 4-kinases, the kinase domain of Lsb6 is interrupted by a linker such that motifs 1, 2, 3, and 4 of the catalytic domain are located in the NH_2-terminal portion of the molecule and motifs 6 and 7 in the COOH-terminal region (Fig. 3 A and Fig. S3). Flanking the two kinase subdomains are noncatalytic NH_2- and COOH-terminal extensions. Studies of the rat type II PI 4-kinase have identified residues required specifically for catalysis; substitution of any of these residues eliminates enzyme activity (Barylko et al., 2002). Accordingly, we targeted the equivalent amino acids either singly or together in NH_2-terminally HA-tagged Lsb6 to yield the following mutants: K192M, D387A, N392A, D413A, and a quadruple mutant (4KD) bearing all four of these substitutions (Fig. 3 A). Each mutant form of HA-Lsb6 of the expected molecular mass was well expressed (Fig. 3 B). We used assay conditions optimized to detect the PI 4-kinase activity of Lsb6 and to minimize activity of the other PI 4-kinases Stt4 and Pik1 (Han et al., 2002). Under these conditions, we found that extracts from cells expressing wild-type HA-Lsb6 showed robust PI 4-kinase activity relative to the low level of activity detected in extracts from *lsb6*Δ cells expressing vector alone (Fig. 3 C). In contrast, extracts derived from cells expressing any of the kinase domain point mutants lacked PI 4-kinase activity above the background (Fig. 3 C).

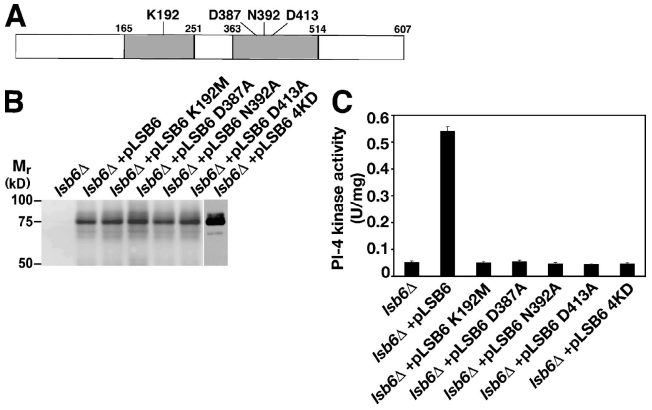

Figure 3. **Catalytically inactive Lsb6 point mutants.** (A) Schematic of Lsb6 indicating the two halves of the kinase domain (shaded). Residues changed singly or together to alanine to generate kinase-inactive forms of Lsb6 are indicated. (B) Expression of HA-tagged wild-type or mutant Lsb6 in *lsb6*Δ cells detected with an anti-HA antibody. (C) PI 4-kinase activity detected in extracts of *lsb6*Δ cells carrying wild-type or mutant Lsb6; data shown are the average of two experiments.

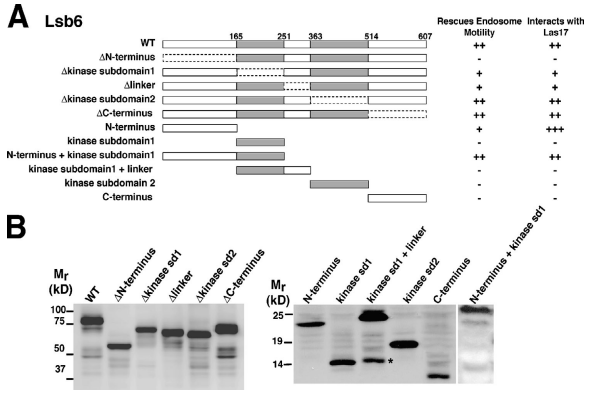

Figure 4. **Lsb6 deletion mutants.** (A) Schematic of Lsb6 deletion mutants. The two halves of the kinase domains are indicated in gray. The ability of each construct to rescue endosome motility (−, no rescue, +, partial rescue, ++, full rescue; Table I) or interact with Las17 (−, no interaction, +, weak interaction, ++, wild-type interaction, +++, stronger than wild-type interaction; see Fig. 5) is also indicated. (B) Expression of HA-tagged Lsb6 mutant constructs in *lsb6*Δ cells. The expected sizes of wild-type and mutant forms of HA-tagged Lsb6 in the left panel are: WT, 75 kD; ΔN-terminus, 50 kD; Δkinase subdomain 1, 55 kD; Δlinker, 55 kD; Δkinase subdomain 2, 52 kD; and ΔC-terminus, 60 kD. The expected sizes of the HA-Lsb6 constructs shown in the second panel are: NH_2 terminus, 22 kD; kinase subdomain 1, 13 kD; kinase subdomain 1+ linker, 25.7 kD; kinase subdomain 2, 18 kD; COOH terminus, 11.5 kD, and NH_2 terminus + kinase subdomain 1, 34 kD. The asterisk indicates a degradation product.

Remarkably, expression of any of these kinase-inactive forms of HA-Lsb6 from high copy or low copy plasmids rescued the endosome motility defect of *lsb6*Δ cells (Table I; compare Video S6 [endosome motility in an *lsb6*Δ cell] to Video S8 [endosome motility in an *lsb6*Δ cell + pHA-Lsb6 4KD]). These results indicated that the PI 4-kinase activity of Lsb6 is dispensable for endosome motility.

PI4P generated by Stt4 or Pik1 is dispensable for endosome motility

Although the PI 4-kinase activity of Lsb6 is dispensable for endosome motility, the preceding experiments could not exclude a role for PI4P production by Stt4 or Pik1. Accordingly, we examined endosome motility in a *pik1* or *stt4* temperature-sensitive mutant or in an *lsb6*Δ *stt4*-ts double mutant. The *pik1* and *stt4* temperature-sensitive mutant phenotypes were confirmed according to published assays that scored aberrant vacuolar morphology or actin patch depolarization, respectively (Audhya et al., 2000). Inactivation of these PI 4-kinases by temperature shift, however, did not result in defective endosome motility (unpublished data). These results suggested that Pik1 and Stt4 are dispensable for endosome motility.

As a further means of exploring the role of phosphoinositide synthesis in endosome motility, we examined temperature-sensitive *mss4* mutants, which are defective in the sole PI4P 5-kinase in yeast. At nonpermissive temperature, the *mss4* mutant lost PI4P 5-kinase activity, as indicated by cytosolic localization of a PI4,5P2 sensor (PLC-PH-GFP; Wild et al., 2004). Under these conditions, defects in endosome motility were not observed (unpublished data). Therefore, PI4,5P2 produced by Mss4 is dispensable for endosome motility.

The NH_2-terminal half of Lsb6 mediates endosome motility and Las17 interaction

Because the kinase activities of Lsb6 and other PI 4-kinases are dispensable for endosome motility, we hypothesized that Lsb6 promotes endosome motility by interacting with Las17. To test this hypothesis, we determined which domains of Lsb6 are necessary and sufficient for endosome motility and Las17 interaction.

Accordingly, we generated a series of deletion mutants of HA-Lsb6 expressed from plasmids in *lsb6*Δ cells (Fig. 4). All Lsb6 deletion constructs exhibited undetectable PI 4-kinase activity (unpublished data). Analysis of endosome motility in *lsb6*Δ mutants expressing these constructs indicated that the NH_2-terminal region flanking the catalytic domain was necessary for endosome motility (Table I). This result is illustrated by comparing Video S9 (endosome motility in an *lsb6*Δ cell + pHA-Lsb6ΔN-terminus) and Video S7 (endosome motility in an *lsb6*Δ cell + pLsb6). In contrast, deletion of other regions of Lsb6 did not affect endosome motility (Table I). This result is illustrated by comparing Video S10 (endosome motility in an *lsb6*Δ cell + pHA-Lsb6ΔC-terminus) and Video S7 (endosome motility in an *lsb6*Δ cell + pLsb6). Both kinds of results were confirmed quantitatively by using automated particle tracking and MSD plots (Fig. S2). Expression of the NH_2-terminal domain of Lsb6 rescued endsome motility less well than a construct containing both the NH_2-terminal domain and the first half of the kinase domain (Table I). Taken together, these results indicated that the region of Lsb6 containing the NH_2-terminal domain and first half of the kinase domain is necessary and sufficient for full activity.

To explore the mechanism by which Lsb6 promotes endosome motility, we determined whether the domains of the protein required for endosome motility also mediate interaction with Las17. For analysis of protein–protein interactions, we used yeast two-hybrid assays rather than coimmunoprecipitation experiments because conditions that solubilized Las17 failed to solublize Lsb6 and vice versa. The NH_2-terminal domain of

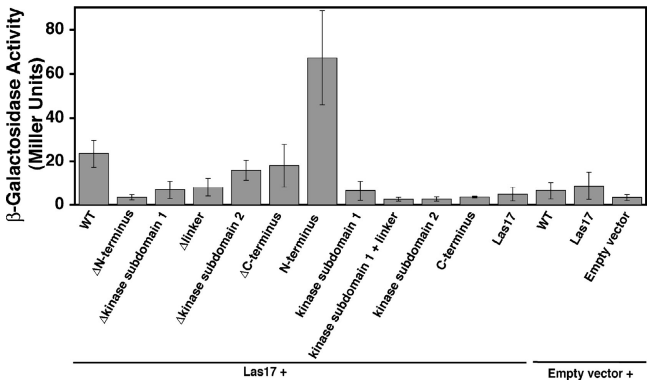

Figure 5. **The NH_2 terminus of Lsb6 interacts with Las17.** Interaction of the indicated Lsb6 and Las17 constructs was assayed by performing yeast two-hybrid experiments. Results of β-galactosidase assays are average ± SD for at least three independent experiments.

Lsb6 was required for interaction with Las17 (Fig. 5). Loss of the NH_2-terminal half of Lsb6 eliminated Las17 interaction, whereas deletions removing the COOH-terminal region of Lsb6 preserved Las17 interaction. Furthermore, the NH_2-terminal region preceding the catalytic domain of Lsb6 was sufficient to interact strongly with Las17 (Fig. 5). Accordingly, the results indicated that the NH_2-terminal half of Lsb6 powers endosome motility by interacting directly or indirectly with Las17.

Discussion

Here we provide new insight into mechanisms that regulate endocytic transport of signaling receptors. Our findings suggest that endosomes bearing the GPCR Ste2 in yeast are transported by actin polymerization on endosome membranes. We show that this mechanism requires a type II PI 4-kinase and a WASp homologue that activates the Arp2/3 complex. Whereas phosphoinositides have been shown previously to activate mammalian N-WASp and stimulate organelle motility (Rohatgi et al., 2000; Rozelle et al., 2000; Taunton et al., 2000; Benesch et al., 2002), our findings show that the catalytic activity of the yeast type II PI 4-kinase, Lsb6, is dispensable for endosome motility. Lsb6 instead functions by interacting directly or indirectly with the WASp homologue Las17, leading to activation of the Arp2/3 complex, actin filament assembly and endosome motility. This is the first direct evidence indicating that a type II PI 4-kinase regulates endosome transport, and that this class of enzyme has an important function apart from the ability to synthesize phosphoinositides.

Actin polymerization-driven endosome motility

Extensive evidence has demonstrated that actin assembly in yeast is critical for endocytic internalization and transport, and that endosomes do not use myosin motors for transport (for review see Engqvist-Goldstein and Drubin, 2003; Munn, 2000). Endosomes that have abscised from the plasma membrane have been proposed to move independently of actin polymerization because Las17 and certain actin binding proteins are depleted from endocytic F-actin patches as they internalize from the cell cortex (Kaksonen et al., 2003). In this model, the force of internalization is hypothesized to impart momentum to endosomes, moving them through the cytoplasm. However, whether this mechanism could account for the distance and speed with which endosomes travel through the viscous cytoplasm is unclear.

In contrast, results presented here and elsewhere indicate that Ste2-containing endosomes are transported by ongoing and continual polymerization of actin filaments from their membrane surface. First, the actin polymerization toxins latrunculin A and jasplakinolide rapidly (<5 min) inihibit endosome motility (Chang et al., 2003; unpublished results). Second, endocytic internalization is insufficient to support endosome motility because the WCA domain of Las17 is dispensable for Ste2 internalization whereas it is required for motility of Ste2-containing endosomes (Chang et al., 2003). This conclusion is also supported by results of the present study showing that endosome motility but not endocytic internalization is inhibited when actin filament barbed ends are capped by overexpressing capping protein. Third, attaching the WCA domain of Las17 to the endocytic cargo Ste2 rescues the motility defect of *las17*ΔWCA cells. Fourth, Ste2-containing endosomes can stop and start, make sharp turns or reverse direction (Chang et al., 2003), indicating that movement is not inertial.

These observations are consistent with two hypotheses. First, Las17 activates the Arp2/3 complex on the plasma membrane, producing endosomes with uncapped actin filaments that continue to polymerize and depolymerize, thereby powering movement. Alternatively, Las17 activates the Arp2/3 complex directly on endosome membranes, thereby stimulating ongoing actin filament assembly that drives motility. Although the Arp2/3 complex, Las17 and F-actin have yet to be localized on Ste2-labeled endosomes, these proteins may be present below the detection limit because endosomes are small and actin filaments are short.

Does yeast have more than one endocytic process?

To integrate our findings with those of previous investigations, we suggest that yeast cells possess at least two endocytic processes distinguished by their internalization sites and mechanisms of endosome transport via the actin cytoskeleon, analogous to the diverse roles of the mammalian actin cytoskeleton in endocytosis (reviewed in Engqvist-Goldstein and Drubin, 2003). One process mediates endocytosis of the GPCR Ste2; whether other cargo use this mechanism remains to be determined. In this mechanism, Ste2 internalizes at small invaginations associated with relatively little actin, as shown by immunoelectron microscopy (Mulholland et al., 1999). As Ste2-containing endosomes form, they continue to nucleate actin filaments by a mechanism requiring Las17 and the Arp2/3 complex (Chang et al., 2003; results of the present investigation), thereby transporting endosomes along irregularly shaped paths to the lysosome-like vacuole. Such nonlinear motility may also allow endocytic cargo to recycle to the plasma membrane, as occurs with the GPCR Ste3 (Davis et al., 1993; Luo and Chang, 2000).

In a second process, endocytic internalization occurs at cortical F-actin patches, as observed using FM 4–64 or GFP-

labeled actin-binding proteins. Here, Pan1, Las17, type I myosins (Myo3 and Myo5), and other proteins activate the Arp2/3 complex (Evangelista et al., 2000; Geli et al., 2000; Lechler et al., 2000; Duncan et al., 2001; Young et al., 2004), thereby nucleating actin filament assembly and endosome scission from the plasma membrane. A subpopulation of the endosomes produced by this mechanism are transported to the vacuole along actin cables that treadmill (Huckaba et al., 2004). This transport process may efficiently couple secretion and endocytosis during polarized cell growth because cortical actin patches colocalize with the ends of actin cables and because actin cables deliver secretory vesicles to cortical sites of cell growth (Pruyne et al., 1998, 2002; Evangelista et al., 2002).

Type II PI 4-kinases in endocytosis and vesicular trafficking

Type II PI 4-kinases are a newly appreciated family of enzymes characterized by catalytic domains dissimilar to those of other PI kinases (Minogue et al., 2001; Wei et al., 2002). Mammalian cells express α and β isoforms of type II PI 4-kinase that are products of distinct genes. Type II PI 4-kinases are peripheral membrane proteins that associate with the plasma membrane, ER, Golgi apparatus, endosomes, synaptic vesicles, and F-actin (Balla et al., 2002; de Graaf et al., 2002; Guo et al., 2003; Wang et al., 2003; Waugh et al., 2003; Carloni et al., 2004). The type IIα isoform produces a pool of PI4P that recruits protein scaffolds implicated in cytoskeletal organization and synaptic vesicle budding (Guo et al., 2003), or that regulates the clathrin adaptor AP-1 on Golgi membranes (Wang et al., 2003). Otherwise, the functions of type II PI 4-kinases are poorly understood.

Before our study, Lsb6, the sole type II PI 4-kinase of yeast, had been characterized biochemically (Han et al., 2002; Shelton et al., 2003). However, its function was unknown because *lsb6*Δ mutants did not exhibit growth defects (Han et al., 2002; Shelton et al., 2003), in contrast to the lethal growth deficits of mutants defective in the type III PI 4-kinases Stt4 or Pik1. However, we show herein that Lsb6 is required for the motility of endosomes containing Ste2, a novel function for type II PI 4-kinases. Strikingly, we find that the PI 4-kinase activity of Lsb6 is completely dispensable for endosome motility. Instead, Lsb6 promotes endosome motility by interacting directly or indirectly with the WASp homologue Las17, which in turn activates the Arp2/3 complex, resulting in actin filament assembly on endosome membranes. This conclusion is supported by results showing that the NH_2-terminal half of Lsb6 is necessary and sufficient for endosome motility and interaction with Las17. The NH_2 terminus of Lsb6 is highly conserved among fungi, with two clusters of conserved hydrophobic residues that may play a role in this process (Fig. S4).

Several findings suggest that Lsb6 may promote endosome motility by activating Las17. First, Lsb6 and Las17 localize to plasma and vacuole membranes (Madania et al., 1999; Eitzen et al., 2002). Second, Las17 functions downstream of Lsb6 because appending the WCA domain of Las17 to Ste2 rescues the endosome motility defect of *lsb6*Δ mutants. Third, Lsb6 may activate rather than recruit Las17 because Las17 is localized normally in *lsb6*Δ mutants (unpublished data). Biochemical studies are underway to determine whether Lsb6 stimulates the ability of Las17 to activate the Arp2/3 complex.

In conclusion, our findings support the hypothesis that endosomes bearing the GPCR Ste2 move by force generated from ongoing polymerization of actin filaments on endosome membranes via the action of the WASP homologue Las17 and the Arp2/3 complex. This mechanism requires a type II PI 4-kinase, Lsb6, functioning independently of its enzymatic activity as an upstream regulator of Las17. Because Lsb6, Las17, and the Arp2/3 complex are highly conserved in eukaryotes, their counterparts in other organisms may have critical roles regulating endocytic transport of many signaling receptors, transporters, or intracellular pathogens.

Table II. **Yeast strains used in this study**

Strain	Genotype	Source
BY4741	MAT**a** his3Δ1 leu2Δ met15Δ ura3Δ	M. Linder
BY4741 *lsb1*Δ	MAT**a** his3Δ1 leu2Δ met15Δ ura3Δ lsb1Δ::kanMXR	M. Linder
BY4741 *lsb2*Δ	MAT**a** his3Δ1 leu2Δ met15Δ ura3Δ lsb2Δ::kanMXR	M. Linder
BY4741 *lsb3*Δ	MAT**a** his3Δ1 leu2Δ met15Δ ura3Δ lsb3Δ::kanMXR	M. Linder
BY4741 *lsb4*Δ	MAT**a** his3Δ1 leu2Δ met15Δ ura3Δ lsb4Δ::kanMXR	M. Linder
BY4741 *lsb5*Δ	MAT**a** his3Δ1 leu2Δ met15Δ ura3Δ lsb5Δ::kanMXR	M. Linder
BY4741 *lsb6*Δ	MAT**a** his3Δ1 leu2Δ met15Δ ura3Δ lsb6Δ::kanMXR	M. Linder
BY4741 *lsb1*Δ, *lsb2*Δ	MAT**a** his3Δ1 leu2Δ met15Δ ura3Δ lsb1Δ::kanMXR lsb2Δ::kanMXR	This lab
BY4741 *lsb3*Δ, *lsb4*Δ	MAT**a** his3Δ1 leu2Δ met15Δ ura3Δ lsb3Δ::kanMXR lsb4Δ::kanMXR	This lab
BY4741 *lsb3*Δ, *lsb5*Δ	MAT**a** his3Δ1 leu2Δ met15Δ ura3Δ lsb3Δ::kanMXR lsb5Δ::kanMXR	This lab
pik1Δ	MAT**a** pik1Δ::TRP1 ade2-101och his3-d200 leu2-1 lys2-801a trp1-d ura3-52	J. Thorner
pik1-83	MAT**a** pik1-83:TRP1 ade2-101och his3-Δ200 leu2-1 lys2-801a trp1-d ura3-52	J. Thorner
SEY6210	MAT**a** ura3-5 trp1-903 his3-Δ200 leu2-3 112, lys 2-801	S. Emr
AAY102	MAT**a** stt4Δ::HIS3 ura3-5 trp1-903 his3-Δ200 leu2-3 112, lys 2-801 suc2Δ9 [pLEU2 CEN stt4-4]	S. Emr
AAY201	MAT**a** mss4Δ::HIS3MX6 ura3-5 trp1-903 his3-Δ200 leu2-3 112, lys 2-801 suc2Δ9 [pRS416 URA3 CEN MSS4]	S. Emr
AAY202	MAT**a** mss4Δ::HIS3MX6 ura3-5 trp1-903 his3-Δ200 leu2-3 112, lys 2-801 suc2Δ9 [pRS416 URA3 CEN mss4-2]	S. Emr
KBY58	MAT**a** ste2::LEU2 sst1-Δ5 leu2-3,112 ura3-52 his3-Δ1 trp1	This lab
KBY66&ret;	MAT**a** ste2::leu2 sst1-Δ5 leu2-3,112 ura3-52 his3-Δ1 trp1 las17ΔWCA::12myc kanR	This lab
PJ69-4A	MAT**a** trp1-901 leu2-3, 112 ura3-52 his3Δ-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-LacZ	M. Johnston
FM178	MAT**a** trp1-289 leu2-3, 112 ura3-52 his3Δ-1 met2	M. Johnston

Materials and methods

Yeast strains, plasmids, and growth conditions

Yeast strains are listed in Table II. Yeast cultures were grown at 30°C, except for temperature-sensitive mutants that were grown at 25°C and then shifted to nonpermissive temperature (37°C). An *lsb6*Δ strain was generated in various genetic backgrounds by replacing the open reading frame with a kanamycin resistance gene. Knockouts were verified by growth on G418-containing plates; genomic PCR confirmed that the locus of interest was replaced.

Plasmids are listed in Table III. For construction of pRS316-Lsb6, the promoter, terminator, and open reading frame of *LSB6* was PCR-amplified from genomic DNA (chromosome X from coordinates 237180 to 239003) with NotI and BamHI restriction sites and cloned into pRS316. For construction of pRS423PGK-HA-Lsb6, the open reading frame from pRS316-Lsb6 was PCR amplified with two HA tags designed into the 5′ primer with BamHI sites. This fragment was cloned into pRS423PGK (Overton and Blumer, 2000). For construction of kinase-dead Lsb6 point mutants, site-directed mutagenesis was done according to the manufacturer's instructions (Quikchange Site Directed Kit; Strategene). For construction of Lsb6 deletion mutants, PCR-based deletion mutagenesis was done according to the manufacturer's instruction (ExSite; Strategene). To generate two hybrid vectors, wild-type Lsb6, kinase-dead mutants, or deletion constructs were subcloned from the appropriate pRS423PGK constructs into pGBDU-C1 as BamHI fragments downstream and in-frame of the DNA binding domain (James et al., 1996). To generate the pGAD-Las17 construct, Las17 was PCR amplified from pRS313Las17 (Chang et al., 2003) with a single amino-terminal myc tag and cloned into pGAD-C1 (James et al., 1996) as a BamHI fragment. To generate WCA fusion proteins, the 90–amino acid COOH terminus of Las17 was PCR amplified from genomic DNA with NheI restriction sites and cloned into NheI sites in pRS313Ste2 or pRS426Hxt1 (Stefan et al., 1998; Overton and Blumer, 2000). All constructs were verified by DNA sequencing and by immunoblotting.

Table III. **Plasmids used in this study**

Plasmid	Source
pRS426 Ste2-GFP	This lab
pRS426 Ste2-CFP	This lab
pRS313 Lsb6	This lab
pRS313 Ste2-WCA	This lab
pRS313 Ste2-WCA-YFP	This lab
pRS426 Hxt1-WCA	This lab
pRS426 Hxt1-WCA-YFP	This lab
pUG34 PLCPH2GFP	A. Wild
pRS423 PGK HA-Lsb6	This lab
pRS423 PGK HA-Lsb6 K192M	This lab
pRS423 PGK HA-Lsb6 D387A	This lab
pRS423 PGK HA-Lsb6 N392A	This lab
pRS423 PGK HA-Lsb6 D413A	This lab
pRS313 PGK HA-Lsb6 K192M	This lab
pRS423 PGK HA-Lsb6 4KD (K192M, D387A, N392A, K192M)	This lab
pRS423 PGK HA-Lsb6 ΔN-terminus	This lab
pRS423 PGK HA-Lsb6 Δkinase subdomain 1	This lab
pRS423 PGK HA-Lsb6 Δlinker	This lab
pRS423 PGK HA-Lsb6 Δkinase subdomain 2	This lab
pRS423 PGK HA-Lsb6 ΔC-terminus	This lab
pRS423 PGK HA-Lsb6 N-terminus	This lab
pRS423 PGK HA-Lsb6 kinase subdomain 1	This lab
pRS423 PGK HA-Lsb6 kinase subdomain 1 + linker	This lab
pRS423 PGK HA-Lsb6 kinase subdomain 2	This lab
pRS423 PGK HA-Lsb6 C-terminus	This lab
pGAD MYC-Las17	This lab
pGBDU HA-Lsb6	This lab
pGBDU HA-Lsb6 K192M	This lab
pGBDU HA-Lsb6 D387A	This lab
pGBDU HA-Lsb6 N392A	This lab
pGBDU HA-Lsb6 D413A	This lab
pGBDU HA-Lsb6 ΔN-terminus	This lab
pGBDU HA-Lsb6 Δkinase subdomain 1	This lab
pGBDU HA-Lsb6 Δlinker	This lab
pGBDU HA-Lsb6 Δkinase subdomain 2	This lab
pGBDU HA-Lsb6 ΔC-terminus	This lab
pGBDU HA-Lsb6 N-terminus	This lab
pGBDU HA-Lsb6 kinase subdomain 1	This lab
pGBDU HA-Lsb6 kinase subdomain 1 + linker	This lab
pGBDU HA-Lsb6 kinase subdomain 2	This lab
pGBDU HA-Lsb6 C-terminus	This lab
PBJ115 GAL1/10 Cap1/2	J. Cooper

Antibodies and immunoblotting methods

Anti-HA (HA.11) and anti-myc (9E10) antibodies were purchased from Covance and used according to the manufacturer's instructions. Capping protein antibody was a gift of J. Cooper (Washington University School of Medicine, St. Louis, MO) and used according to published directions (Amatruda et al., 1992). HRP-conjugated secondary antibodies were purchased from GE Healthcare and used according to manufacturer's instructions. Fresh transformants were grown overnight to saturation and then harvested and washed in cold water. Cells were lysed at 4°C with glass beads in cold lysis buffer (10% glycerol, 0.1 M NaCl, 50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1 mM EGTA) with fresh protease inhibitors (0.1 mg/ml PMSF, 0.1 mg/ml benzamidine, 0.75 mg/ml TAME, 1 ng/ml pepstatin A, 0.1 mg/ml leupeptin, and 50 ng/ml aprotinin) until 90% lysis was achieved as indicated by phase contrast microscopy. Lysates were spun at 5,000 *g* for 5 min at 4°C and clarified lysates were removed. Protein concentration was determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories). SDS-PAGE electrophoresis and immunoblotting was performed according to the manufacturer's instructions (Bio-Rad Laboratories).

PI 4-kinase assays

Cells were grown to exponential phase (OD600 ≈ 0.5). We grew *lsb6*Δ cells in synthetic complete (SC) medium and *lsb6*Δ cells containing plasmids in synthetic complete medium lacking histidine and uracil. Cell extracts were prepared as described previously (Han et al., 2002). For immunoblot analysis, cell extracts were resolved by SDS-PAGE using a 10% slab gel, transferred to a PVDF membrane, and subjected to reaction with anti-HA antibodies at a concentration of 1 μg/ml. PI 4-kinase activity was measured for 10 min at 30°C in the presence of 50 mM Tris-maleate (pH 7.0), 2.5 mM [γ-^{32}P]ATP (10,000 cpm/nmol), 10 mM $MgCl_2$, 0.2 mM PI, 3.2 mM Triton X-100, and 20 μg of cell extract protein in a total volume of 0.1 ml (these are optimal conditions for Lsb6 kinase activity relative to other PI 4-kinases as described in Han et al. (2002). The ^{32}P-labeled chloroform-soluble products of the reaction were dried and used for scintillation counting. All assays were conducted in triplicate and were linear with time and protein concentration. A unit of PI 4-kinase activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min. Specific activity was defined as units per mg of protein.

Microscopy

Rhodamine-phalloidin (Invitrogen) and FM 4–64 (Invitrogen) were used according to the manufacturers' directions. The plasmid pUG34-PLCPH2GFP was used according to published methods (Wild et al., 2004). Yeast cells were grown in synthetic dextrose media overnight, subcultured, and allowed to grow overnight to an OD600 of 0.25–0.4. Cells were then washed twice and concentrated in nonfluorescent media. For temperature-sensitive mutants, all mutants were grown in synthetic media and switched to nonpermissive temperature for the indicated times, washed twice with nonfluorescent media, and visualized immediately on a heated microscope stage. For capping protein overexpression, cells were grown in synthetic media containing raffinose overnight. Induction was done by adding galactose to a final concentration of 2%, and cells were allowed to induce to 2 h until maximal expression was achieved as indicated by immunoblotting. Cells were mounted on glass slides, covered with a glass coverslip, and viewed with an Olympus IX70 inverted epifluorescence microscope and a cooled CCD camera (RC300; Dage-MTI). Single focal plane images were obtained using U-MNG (rhodamine-phalloidin) and U-MWIBA (GFP) filter sets captured using NIH Image (http://rsb.info.nih.gov/nih-image). Each frame was taken by integrating for 300–500 ms; frames were separated by 500–700 ms until 50 frames had been acquired. For experiments using temperature-sensitive mutants, a plastic tent was set up such that the microscope system was heated for 2–3 h before the experiment and maintained at the indicated nonpermissive temperature for the duration of the experiment.

Quantification of endosome motility

Time-lapse images of cells expressing Ste2-GFP were analyzed manually and where indicated by using automated procedures. For manual analysis, four sequential frames from movies were chosen and three endosomes were tracked in the XY plane. Very bright late endosomes near the vacuole or GFP aggregates on the plasma membrane were not scored. As endosomes in wild-type cells are highly motile, four frames in any given movie were sufficient to analyze motility of several endosomes. The number of endosomes per cell was variable as a function of growth conditions (unpublished data). The distance traveled by a given endosome between each time point was calculated based on pixel coordinates (10.2 pixels/micron), which allowed the average speed of each endosome to be calculated. For a given cell type or experimental condition, the average endosome speed, standard error and P value were calculated from data obtained by imaging 35–72 endosomes (Table I) using the unpaired Student's *t* test. A P value < 0.005 when compared with matched wild-type controls was considered significant. For each experiment, three independent transformants were isolated, visualized, and the data quantified. Each experiment was repeated two to four times.

When using an automated particle tracking program to analyze endosome motility (TrackerX; Carlsson et al., 2002), we transferred time-lapse images into text files using ImageJ 1.32g (http://rsb.info.nih.gov/objectimage). Endosomes were tracked for a minimum of 5 s as described previously (Carlsson et al., 2002); data obtained from 60 to 140 endosomes were averaged per experiment. Because ~70% of endosomes in wild-type cells moved rapidly and left the plane of focus over a period of 25 s, shorter time courses (10–15 s) were used. Conversely, poorly motile endosomes in mutants remained in the plane of focus much longer.

Yeast two hybrid assays

Two hybrid plasmids were introduced into PJ69-4A cells as described by the manufacturer (CLONTECH Laboratories, Inc.). At least three transformants were isolated and assayed for β-galactosidase activity in extracts using *o*-nitrophenyl-BD-galactopyranoside (ONPG; Sigma-Aldrich) as substrate. Results shown are the average of at least three independent experiments.

Online supplemental material

Online supplemental materials are available at http://www.jcb.org/cgi/content/full/jcb.200501086/DC1.

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