A role for the dynamin-like protein Vps1 during endocytosis in yeast

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Summary

Dynamins are a conserved family of proteins involved in membrane fusion and fission. Although mammalian dynamins are known to be involved in several membrane-trafficking events, the role of dynamin-1 in endocytosis is the best-characterised role of this protein family. Despite many similarities between endocytosis in yeast and mammalian cells, a comparable role for dynamins in yeast has not previously been demonstrated. The reported lack of involvement of dynamins in yeast endocytosis has raised questions over the general applicability of the current yeast model of endocytosis, and has also precluded studies using well-developed methods in yeast, to further our understanding of the mechanism of dynamin function during endocytosis. Here, we investigate the yeast dynamin-like protein Vps1 and demonstrate a transient burst of localisation to sites of endocytosis. Using live-cell imaging of endocytic reporters in strains lacking vps1, and also electron microscopy and biochemical approaches, we demonstrate a role for Vps1 in facilitating endocytic invagination. Vps1 mutants were generated, and analysis in several assays reveals a role for the C-terminal self-assembly domain in endocytosis but not in other membrane fission events with which Vps1 has previously been associated.

Key words: Dynamin, Amphiphysin, Endocytosis, Rvs167, Saccharomyces cerevisiae

Introduction

Endocytosis is a highly regulated and essential process that occurs in the majority of eukaryotic cells. It is required for recycling of plasma membrane lipids and trafficking proteins, and for uptake or downregulation of cell-surface receptors. During endocytosis, the plasma membrane invaginates into the cell, resulting in the production of a vesicle that is then able to fuse with endosomes and enter the endolysosomal membrane system. This process is known to involve more than 50 proteins, which assemble transiently at sites on the plasma membrane (for a review, see Robertson et al., 2009).

Work in the model organism Saccharomyces cerevisiae has led to significant advances in our understanding of the distinct stages that take place during endocytosis in vivo. It is now widely believed that the broad stages of coat assembly (early), invagination (mid) and scission, followed by inward movement (late) are largely conserved across evolution, and that in many cases direct homologues of proteins are responsible for carrying out the same steps in the process. One notable difference between yeast and mammalian cells is that dynamins are considered to be central to the endocytic process in mammalian cells, whereas this family of proteins has been considered to be largely peripheral to endocytic function in yeast (Gammie et al., 1995; Yu and Cai, 2004).

Dynamin was originally isolated 20 years ago as a microtubule-binding protein (Shpetner and Vallee, 1989). A role in endocytosis was indicated from studies in Drosophila, when mutations in the shibire gene were seen to cause paralysis at an elevated temperature by reversibly blocking endocytosis at the nerve terminal (Koenig and Ikeda, 1989). Shibire was subsequently identified as Drosophila dynamin (Chen et al., 1991; Van Der Bliek and Meyerowitz, 1991). Although mammalian dynamins have been shown to be crucial for endocytic function, their exact mechanism of function has remained the subject of debate (Song and Schmid, 2003). Furthermore, the presence of several mammalian dynamin genes (DNM1, DNM2 and DNM3) adds additional complexity. Although dynamin-1 is thought to have a role solely in endocytosis, dynamin-2 is considered to have a broader role possibly involving the trans-Golgi network, endosomes and podosomes, as well as endocytosis (Cao et al., 2003; Cao et al., 2005; Ferguson et al., 2009; Ochoa et al., 2000), whereas the role for dynamin-3 is still largely unknown, although this protein has a more restricted tissue expression (Kruchten and McNiven, 2006). For several years, dynamin-1 was proposed to act largely in a mechanical way to encircle the neck of invaginated endocytic vesicles facilitating scission of vesicles during endocytosis (Hinshaw and Schmid, 1995). Subsequently, a regulatory role was described, in which dynamin functions earlier through its GTPase domain as a molecular switch to regulate recruitment or activity of proteins within the endocytic complex (Sever et al., 1999). Other studies have indicated that dynamin might function in both a regulatory and mechanical manner (Narayanan et al., 2005). A recent study of dynamin-1- and dynamin-2-knockout cells suggests a predominant role for dynamin in scission rather than earlier events, and has also revealed the importance of amphiphysins and actin in mammalian endocytosis (Ferguson et al., 2009).

Yeast contains three dynamin homologues (Vps1, Dnm1 and Mgm1), all of which contain the crucial N-terminal GTPase domain, a middle domain and a GTPase effector domain (GED) at the C-terminus, which has also been shown to function in oligomerisation. Similar to other dynamin-like proteins, Vps1 does not contain the PH domain, nor the C-terminal proline-rich domain, which is found in conventional dynamins (Shin et al., 1999; Vater
et al., 1992). Vps1 is involved in several membrane fusion and fission events within the Golgi, vacuole, endosome and peroxisomal systems (Hoepfner et al., 2001; Kuravi et al., 2006; Nothwehr et al., 1995; Peters et al., 2004; Vater et al., 1992), whereas Dnm1 and Mgm1 are thought to be involved in mitochondrial fusion and fission (Cerveny et al., 2007). However, a role for Dnm1 in endosomal trafficking has also been reported (Gammie et al., 1995). A role for Vps1 in early stages of endocytosis has been controversial and it is rarely listed as a protein involved in the yeast endocytic process. However, there is evidence that deletion of *VPS1* affects the morphology of cortical actin patches and that it interacts with an endocytic adaptor protein Slal1 (Yu and Cai, 2004). It is possible that the role for dynamins in yeast endocytosis has been underestimated because of their involvement in other cell processes, and also the ability of cells to undergo endocytosis in their absence.

In yeast, it is the amphiphysin proteins (Rvs167 and Rvs161) that have been considered responsible for vesicle scission. Similarly to dynamins, amphiphysins have the ability to tubulate and fragment membranes in vitro (Dawson et al., 2006). In addition to this mechanical role, the SH3 domain at the C-terminus of some amphiphysins (including Rvs167) is likely to be important in recruiting and possibly activating other endocytic proteins. Deletion of *RVS167* causes a reduction (~30%) in the number of successful vesicle scission events (Kaksonen et al., 2005), indicating that the amphiphysins are important, but might not be the only proteins involved in this event.

## Results

### Vps1 colocalises with endocytic proteins

Vps1 has a clearly demonstrated role in endosomal trafficking and in peroxisomal fission (Hoepfner et al., 2001; Nothwehr et al., 1995; Vater et al., 1992; Peters et al., 2004; Rothlisberger et al., 2009). In this study, we aimed to determine whether this fusion–fission role also functions during endocytosis. A minimum requirement for a protein to have a direct role in endocytosis is for it to show at least partial localisation to endocytic sites. In previous studies, Vps1 has been shown to localise to internal organelles, and this staining is predominant in cells expressing GFP-tagged Vps1 (Peters et al., 2004). However, partial colocalisation with an endocytic adaptor protein Slal1 has also been reported (Yu and Cai, 2004). Expression of Vps1-GFP from both high and low copy number plasmids leads to high levels of fluorescence on the internal membranes and in these cells, spots at the cell periphery are difficult to discern (our unpublished observations). In this study, we have used a strain expressing Vps1-GFP under its own promoter (Peters et al., 2004). Vps1-GFP was expressed, both as the sole source of Vps1 in a haploid cell, and also coexpressed with endogenous untagged Vps1 in a heterozygous diploid cell. In both strains, the behaviour of coexpressed RFP markers are apparently normal, and do not show the aberrant behaviours observed for the *vps1*-deletion strain (see below). To investigate colocalisation with endocytic markers, cells were generated that coexpress Vps1-GFP and either Slal1-mRFP (a marker that is present throughout endocytic invagination) or Abp1mRFP (a marker of actin at the site). These strains were analysed using both wide-field epifluorescence microscopy and TIRF microscopy. As shown in Fig. 1A (upper panels), Vps1-GFP was found in several internal organelles, which are likely to be endosomes. It also colocalised with Abp1mRFP, demonstrating that Vps1 is present at endocytic sites. By TIRF microscopy,
colocalisation with the coat protein Sla1-mRFP was also clear. However, colocalisation with both markers was only partial, suggesting that either only a subset of sites recruit Vps1 or that Vps1 is present only transiently at endocytic sites. To address this, the lifetime of Vps1 localisation was measured relative to the actin marker Abp1. Intensity profiles of multiple spots were generated (Fig. 1B). This approach revealed that Vps1 arrived about 5–6 seconds after Abp1, at a time corresponding to onset of invagination (Fig. 1B) (Liu et al., 2009). Vps1 then remained at the membrane and disassembled from the site before Abp1. Vps1 lifetime at the membrane was 8.70±4.07 seconds (mean ± s.d.; n=75 patches). This lifetime was the same whether Vps1-GFP was expressed as the sole form of Vps1, or coexpressed with untagged endogenous Vps1. The Vps1 lifetime corresponds to about half the lifetime of Abp1 at patches (Abp1 lifetime ~18 seconds) and about one third of the lifetime of Sla1 (25–30 seconds) and can explain why only partial colocalisation with these endocytic markers was observed.

A characteristic feature of many endocytic proteins is that their lifetime at the plasma membrane is increased in the presence of the actin-monomer-sequestering drug latrunculin A (Lat-A) (Ayscough et al., 1997; Morton et al., 2000; Toshima et al., 2009). Although actin-binding proteins are unable to assemble at the site, actin-independent components localise, and remain at sites for longer. Lat-A was added to cells expressing Vps1-GFP and Abp1-mRFP (Fig. 1C, upper). As expected the Abp1-mRFP localisation was lost from sites, although Vps1 remained localised, demonstrating that its localisation was actin-independent. In the presence of Lat-A, Vps1 still colocalised with Sla1 (Fig. 1C, bottom) and its localisation was actin-independent. In the presence of Lat-A, 78% of Sla1-mRFP lifetime in patches was increased to 33.1±18.5 seconds (mean ± s.d.; n=102), supporting the idea that the majority of endocytic patches involve Vps1 function.

**Deletion of Vps1 confers defects on endocytic machinery**

Having established that Vps1 is in a position to function directly during endocytosis, we determined the effect that deletion of Vps1 has during single endocytic invagination events. The effect of *VPS1* deletion on the lifetime and behaviour of several proteins was investigated: Ent1 and Sla2, components of the endocytic coat complex; Las17, the yeast WASP homologue and activator of Arp2/3 mediated actin polymerisation; Abp1 and Sac6, components of the actin network that forms during invagination; and Rvs167, the amphiphysin proposed to be responsible for scission. As shown in Fig. 2A, lifetimes of five of the six proteins were markedly increased (see also supplementary material Movies 1 and 2).

To examine the behaviour of patches in more detail, kymographs were generated which depict movement of a patch over its lifetime. The endocytic coat proteins Sla2 and Ent1 localised longer at the plasma membrane before moving inwards (Fig. 2B). However, the movement inwards was often aberrant and patches could be seen to retract toward the plasma membrane (Fig. 2B, arrows). The WASP homologue las17 usually remains at the plasma membrane throughout invagination. In the *vps1*-deletion strain, movement in the plane of the membrane, as well as invagination and retraction could be observed. Normal and aberrant phenotypes were quantified.

**Fig. 2. The effect of vps1 deletion on known endocytic proteins.** (A) The lifetime of six endocytic proteins, Sla2, Ent1, Las17, Abp1, Sac6, and Rvs167 was analysed in the presence and absence of vps1. For wild-type cells, n=67, 22, 119, 30, 60, 35 and for vps1/Δ cells, n=19, 36, 121, 36, 69, 33 for the markers as ordered on graph. Error bars indicate s.d. **P<0.0001 for Sla2, Ent1, Las17, Abp1; ***P<0.0004 for Sac6 and 0.0002 for Rvs167.** (B) Kymographs of Las17-GFP, Sla2-GFP and Ent1-GFP. Aberrant endocytic events for Sla2 and Ent1 were defined as those showing no invagination, movement in the membrane plane, delayed scission or retraction. For Las17, aberrant events were those showing movement in the membrane plane, inappropriate invagination and retraction. (C) FM4-64 internalisation was performed as described. Total internal cell fluorescence intensity (mean ± s.d.) was measured at each time point. (D) Representative images of wild-type and vps1/Δ cells from three time points in the FM4-64-uptake assay. Arrows indicate foci of FM4-64 staining on the plasma membrane. Scale bars: 2 μm.
and the vacuoles was also delayed. Thus, previous studies, the subsequent entry of FM4-64 to endosomes which internal fluorescence was observed. As expected from persisted longer and there was a marked reduction in the rate at the time for foci to form was slower. When the foci formed they membrane staining occurred on the same time scale (1–2 minutes), of these endocytic events (Fig. 2B).

scission normally. Deletion of classified according to their ability to invaginate and undergo to determine the extent of the defect. Patches were analysed and classified according to their ability to invaginate and undergo scission normally. Deletion of vps1 resulted in a severe disruption of these endocytic events (Fig. 2B).

Having shown that vps1 deletion caused aberrant behaviour at the level of a single endocytic patch, we investigated the effect of vps1 deletion in the whole cell. Although some studies have indicated no internalisation defects in cells lacking vps1 expression (Geli and Riezman, 1998), others have demonstrated effects of vps1 deletion on uptake of uracil permease (Fur4) activity (Yu and Cai, 2004). Deletion of many genes encoding proteins with accepted roles in endocytosis, such as Cap1, Srp1, Abp1, are reported to have no defect in several assays (Gheorghe et al., 2008; Huang et al., 1999; Kaksonen et al., 2005; Maldonado-Baez et al., 2008). However, the assays are often performed on fixed cells or over long time courses that do not detect more subtle, kinetic changes in uptake. To circumvent these issues, uptake of the lipophilic dye FM4-64 was performed under continuous observation following addition of dye to cells. Images were recorded over 15 minutes and total internal fluorescence intensity was measured (Fig. 2C). The plasma membrane was the first structure to be labelled following addition of FM4-64 (Fig. 2D). Foci of dye could then be seen in the plane of the plasma membrane before internal staining was observed (arrows). In wild-type cells, FM4-64 was seen next in endosomes and vacuoles. In vps1Δ cells, although plasma membrane staining occurred on the same time scale (1–2 minutes), the time for foci to form was slower. When the foci formed they persisted longer and there was a marked reduction in the rate at which internal fluorescence was observed. As expected from previous studies, the subsequent entry of FM4-64 to endosomes and the vacuoles was also delayed. Thus, vps1 deletion affects internalisation of a bulk lipid marker, and the behaviour of single endocytic patches.

Overlapping roles of Vps1 and amphiphysin

The exception to the increased patch lifetime in vps1Δ cells (Fig. 2A) was the lifetime for the amphiphysin Rvs167. Furthermore, the retraction behaviour observed in kymographs for the various endocytic proteins (Fig. 2B) was reminiscent of the behaviour noted for rvs167Δ cells (Kaksonen et al., 2005). These data suggest that the function of Vps1 could be linked to, or overlap with, that of amphiphysins, as has been suggested from studies in mammalian cells (Ferguson et al., 2009; Itoh et al., 2005; Yamada et al., 2009). To investigate possible interplay between Vps1 and Rvs167, single deletion strains were crossed to determine any genetic interaction. Each deletion caused a slight temperature-sensitive growth phenotype (Fig. 3A). However, when combined, the mutations had a synergistic effect, and cells did not grow at 37°C. Such a phenotype is often suggested to indicate an overlapping role within a process (Tong et al., 2001).

Imaging of cells expressing Rvs167-GFP revealed an effect of vps1 deletion on Rvs167-GFP localisation. In wild-type cells, Rvs167-GFP showed robust localisation to endocytic sites, whereas in vps1Δ cells, Rvs167 localised to endocytic sites, but its intensity was reduced (Fig. 3B). This effect was quantified by measuring fluorescence intensities of patches and cytoplasmic background in wild-type and vps1Δ cells. The intensity of Rvs167-GFP patches and cytoplasmic background in wild-type and vps1Δ cells were recorded and plotted. Mean patch intensity: wild type, 885±508 units, n=128; vps1Δ, 429±227 units, n=53; P<0.0001. (D) Vps1-GFP was coexpressed with Rvs167-mRFP and intensity curves generated. Weighted averages of spot intensity were calculated and plotted. (E) Kymographs of Sac6-RFP in wild-type, vps1Δ, rvs167Δ cells and in vps1Δ/Δ/Δ cells. Percentage of spots that retracted toward the membrane was counted. n=210, wild type; 46, vps1Δ; 52, rvs167Δ; 48, vps1Δ/Δ/Δ/Δ cells.
proteins are reduced when coexpressed, further suggests that the presence of two bulky fluorophores compromises their close interactions within the patch.

To investigate the effect of deletion of rvs167 and vps1 on endocytosis, kymographs were generated in strains expressing the actin marker Sac6-RFP (Fig. 3E). This marker usually has a short lifetime with a sharp inward movement 1–2 seconds after arrival at the plasma membrane, followed by disassembly after scission. Defects were observed in all mutant strains, but those in the double mutant were most prevalent. Because retraction events have been quantified previously for cells lacking rvs167, we sought to compare this specific phenotype. Retraction events were defined as an endocytic event in which inward movement has taken place but in which scission does not occur and movement back towards the membrane is observed. As shown in Fig. 3C, retraction was seen in 31% of rvs167Δ endocytic events, which is similar to previously reported results (Kaksonen et al., 2005); in 39% of events in vps1Δ cells and in 59% of events in rvs167Δvps1Δ cells.

**Invaginations do not form correctly in the absence of vps1**

To investigate the function of Vps1 during invagination in more detail, an ultrastructural analysis using electron microscopy was performed. In this analysis, three main types of invagination at the plasma membrane were identified. These were defined as shallow (depth <40 nm) with the neck of the opening wide compared with the tubule diameter; pronounced (40–60 nm) when the neck of the tubule was the same or only slightly wider than the tubule diameter; deep (>60 nm), with the tubule neck about the same as the tubule diameter. In wild-type cells, 38% of invaginations fell in the pronounced category (Fig. 4A). Interestingly, in vps1Δ cells, the number of pronounced invaginations was significantly reduced to 24% (n=151 invaginations for wt and 91 for vps1Δ). During the analysis, it was noted that in wild-type cells, the majority of invaginations occurred perpendicular to the membrane whereas in vps1Δ cells, invaginations were often bent in appearance and the angle of invagination deviated from the perpendicular (Fig. 4B). This observation was quantified by measuring the angle of deviation of invaginations from a line drawn perpendicular to the membrane. As shown in Fig. 4C, there was a significant increase in the angle of invagination in the absence of vps1. The mean angle score for the wild type (8.93±9.24, mean ± s.d., n=54) was significantly lower than that in vps1Δ cells (18.55±12.66, n=54; P=0.0000169).

These data suggest that Vps1 functions to facilitate directed invagination leading to effective scission. This is supported by a model in which Vps1 forms a collar structure at the neck of an invaginating tubule in an analogous manner to dynamin in mammalian cells or in shibire mutant cells (Koenig and Ikeda, 1989). In this regard, a repeat structure can be detected on some of the deep invaginations seen in wild-type cells (Fig. 4D), but not in any deep or pronounced pits in vps1Δ cells.

**Vps1 mutations reveal defects in endocytosis and endosomal trafficking**

Previous work has shown a function for the N-terminal GTPase domain of Vps1 in intracellular membrane-trafficking events, but the role of its C-terminal domain has not been addressed. The mutants generated are depicted in Fig. 5A and were based on mutations in dynamin-1. Sequence alignment of dynamin-1 and Vps1 is shown in supplementary material Fig S1. The mutations have been reported to influence the GTPase activity, self assembly or other aspects of dynamin-1 function (Damke et al., 1994; Herskovits et al., 1993; Sever et al., 1999; Song et al., 2004a; Song et al., 2004b; Vater et al., 1992; Yu and Cai, 2004). Mutations, corresponding positions in human dynamin-1 and temperature sensitivity of the vps1Δ cells transformed with plasmids carrying the mutations are listed in Table 1.

The first analysis of the mutants used a recently published assay for endocytic recycling, in which a reporter construct, containing the SNARE protein Snc1 fused to both an invertase activity at its N-terminus and GFP at its C-terminus, is transformed into vps1Δ cells (Burston et al., 2009). This assay allows differentiation between endosomal trafficking and endocytic internalisation defects. If Vps1 function is predominantly intracellular, for example

**Table 1. Mutations generated in Vps1 and corresponding positions in dynamin-1**

<table>
<thead>
<tr>
<th>Human dynamin-1</th>
<th>Yeast Vps1</th>
<th>Temperature sensitive in yeast?</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>G4SN</td>
<td>S43N</td>
<td>+</td>
<td>(Marks et al., 2001)</td>
</tr>
<tr>
<td>T65A</td>
<td>T63A</td>
<td>+</td>
<td>(Song et al., 2004a)</td>
</tr>
<tr>
<td>R66C</td>
<td>R64C</td>
<td>+</td>
<td>(Marks et al., 2001)</td>
</tr>
<tr>
<td>T141Q</td>
<td>T183Q</td>
<td>+</td>
<td>(Song et al., 2004a)</td>
</tr>
<tr>
<td>G146S shibire ts2</td>
<td>G188S</td>
<td>+</td>
<td>(Narayanan et al., 2005)</td>
</tr>
<tr>
<td>G273D shibire ts1</td>
<td>G315D</td>
<td>+</td>
<td>(Damke et al., 1995)</td>
</tr>
<tr>
<td>I699K</td>
<td>I649K</td>
<td>+</td>
<td>(Song et al., 2004b)</td>
</tr>
<tr>
<td>K694A</td>
<td>K653A</td>
<td>−</td>
<td>(Song et al., 2004b)</td>
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<tr>
<td>R725A</td>
<td>R684A</td>
<td>±</td>
<td>(Sever et al., 1999)</td>
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<tr>
<td>K730A</td>
<td>K689A</td>
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<td>(Sever et al., 1999)</td>
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in endosomal fusion–fission events, then its loss will result in the accumulation of fused Sncl and invertase inside cells, so that when cells are incubated with appropriate substrate, colonies will be white. If the primary function of the protein is in endocytic internalisation, then uptake of the SNARE will be delayed, more invertase will be exposed at the surface and in the assay colonies will appear dark brown. In wild-type cells, there was continuous cycling of Sncl, and the cells appeared light brown when assayed. Cells in which vps1 was deleted were transformed with an empty plasmid or with plasmids carrying wild-type or mutant vps1. In the assay, there was a clear outcome, with vps1Δ colonies remaining largely white (Fig. 5B). Those cells expressing wild-type VPS1 restored trafficking, such that the expected light-brown colouring was observed. The mutants fell into three classes. Those that lie in the N-terminal half caused a phenotype that was similar to vps1Δ, indicating the importance of the GTPase function in endosomal trafficking or recycling. Most of the mutants in the C-terminal domain were similar to the wild type, suggesting that C-terminal functions are less important for endosomal recycling. However, the mutant I649K was darker than the wild type, indicating a more predominant endocytic phenotype.

Two mutants were analysed further. These were the mutants T63A (representative of GTPase function) and I649K (from the GTPase effector or self-assembly domain). Three assays were used to report on the functional contribution of the N- and C-terminal domains in intracellular trafficking events. First, the localisation of the GFP fusion in the invertase–Sncl reporter was examined in wild-type and mutant strains (Fig. 5C; n=100 cells for plasma membrane staining counts). In wild-type cells, or cells in which VPS1 was re-expressed in the null strain, GFP localised to the plasma membrane in 93% of cells and to large internal organelles (probably endosomes) but there was relatively little background signal from small vesicles. In vps1Δ cells, the majority of GFP appeared to localise to small vesicles with less in larger organelles and only 13% of cells had plasma membrane staining, indicating an endosomal trafficking defect. Expression of the T63A GTPase mutant was similar to the null phenotype, with predominantly small vesicles and a few endosomes (8% cells had plasma membrane staining). The mutant in the C-terminal region (I649K) had a predominantly plasma membrane and endosomal localisation (87% cells had plasma membrane staining). Therefore, the GTPase domain is crucial for endosomal recycling.
Defects in trafficking to the vacuole were also shown by FM4-64 staining (Fig. 5D). vps1Δ cells have been classed as class F vacuolar mutants and they have a rather heterogeneous phenotype, with both fragmented vacuoles and vacuoles with aberrant morphologies (Raymond et al., 1992). A common feature of these cells is a weakly stained vacuole with clusters of vacuoles around it. In cells expressing VPS1, uptake of FM4-64 showed clear localisation to vacuolar membranes (Fig. 5D). In vps1Δ, there was a lower level of vacuolar localisation and a high level of staining, especially of clustered endosomes around the vacuole (Fig. 5D, arrows). The GTPase-domain mutant T63A was phenotypically similar to the vps1Δ strain. The C-terminal mutation I649K showed clear vacuolar membrane staining that was similar to the wild type, indicating relatively normal trafficking.

Finally, we addressed whether the Vps1 mutants would rescue the characteristic phenotype of peroxisomes in vps1ΔΔmm1Δ cells in which only one large peroxisome is observed when using a peroxisomal reporter GFP (Motley and Hettema, 2007). In support of the idea that the GTPase activity functions to drive membrane fusion–fission reactions, the mutant affecting this part of the protein was also defective in peroxisomal fission. Again, the I649K mutant in the C-terminal region of the protein was able to rescue the peroxisome defect and restore the normal number of peroxisomes in cells (Fig. 5E). To verify that the effects observed in the assays were not due to absence of protein as a result of instability and degradation, protein extracts were made from wild-type cells and from vps1Δ cells carrying VPS1, T63A, I649K and empty plasmids. The level of Vps1 protein expressed was comparable in all strains, apart from the null (Fig. 5F).

Overall, these data demonstrate the importance of the GTPase domain in known Vps1 functions in endosomal and vacuolar trafficking and peroxisome morphology. By contrast, the I649K C-terminal mutation conferred an endocytic phenotype in the invertase terminal mutation conferred an endocytic phenotype in the invertase

Wild-type Vps1 tubulates membranes in vitro

The electron microscopy data suggested that Vps1 acts to facilitate the shallow-to-pronounced invagination structure in vivo and/or to ensure directed inward growth of the invaginating tubule. We then aimed to determine whether purified Vps1 has the capacity to bind to and possibly tubulate membranes in vitro. This would add support to the idea of Vps1 performing this key role directly in vivo, rather than through recruitment of other activities. Vps1 was purified as described, and incubated with liposomes (+GTP). Binding to liposomes was assessed by centrifugation of membranes. As shown in Fig. 7A, Vps1 pelleted in the presence of liposomes (upper panel), but not in their absence (lower panel),
demonstrating the ability of Vps1 to bind liposomes. Next, the effect on liposome structure was investigated. Vps1 was incubated with liposomes and GTPγS and applied to EM grids. As shown in Fig. 7B, the presence of Vps1 had a marked effect on liposome structure, with tubules decorated with Vps1 clearly visible after incubation. These tubules were also formed at a ten-times lower concentration of Vps1 (supplementary material Fig. S2). Finally, we addressed whether self-assembly of Vps1 has a role in its ability to tubulate membranes. The mutation I649K corresponds to the I690K mutation in human dynamin and this mutation has been shown to affect oligomerisation of dynamin (Song et al., 2004b). I649K Vps1 was purified, and tested for its ability to oligomerise in the presence of GTPγS in a centrifugation-based assay. In contrast to the lipid-binding assay, which was performed in the presence of GTP, use of GTPγS stabilises self assembly of Vps1. Fig. 7C shows that although Vps1 could self assemble, allowing it to pellet under the conditions described, I649K Vps1 did not pellet, confirming the oligomerisation defect in this mutant. Mutant Vps1 was, however, able to bind liposomes (Fig. 7A). Analysis by EM reveals that Vps1 I649K did not tubulate membranes (Fig. 7B), demonstrating the importance of self-assembly in the tubulation process. EM analysis of wild-type Vps1 in the presence of GTPγS but without liposomes showed no tubules (supplementary material Fig. S2), confirming that tubules are liposome dependent.

Discussion

Dynamin is considered to be a central protein in endocytosis in metazoan cells, and the absence of this role in yeast has raised many questions (see Ferguson et al., 2009; Geli and Riezman, 1998; Robertson et al., 2009). Here, we present data that we believe strongly support a role for the dynamin-like protein Vps1 in the endocytic process in Saccharomyces cerevisiae. Additional evidence for Vps1 function in endocytosis has also been recently reported by Nannapaneni and colleagues (Nannapaneni et al., 2010).

Given our findings, it seems somewhat surprising that such an endocytic defect was not previously reported. We think that there are several reasons for this, including the promiscuous nature of Vps1 function in several other membrane-trafficking steps and also because deletion of vps1 does not show obvious defects in uptake of commonly used reporters such as Lucifer Yellow (Geli and Riezman, 1998). Lack of clear defects in these classical endocytic assays is not, however, uncommon, with deletion of many endocytic genes causing no apparent defects. In terms of a role for Vps1 in endocytosis, critically, we show the Vps1 colocalises with other endocytic proteins (Fig. 1A), and second that the vps1 deletion strain has clear defects in behaviour of endocytic reporter proteins and a kinetic defect in uptake of the dye FM4-64 (Fig. 2). Three questions were then addressed: (1) When does Vps1 function during endocytosis? (2) How does it function? (3) What are the roles of its N- and C-terminal domains in this function?

To ascertain when Vps1 functions during endocytosis, the cortical lifetime of Vps1 was measured and found to be 8.7 seconds. This is short compared with many other endocytic proteins such as Slal (~25–30 seconds and Abp1 ~15–18 seconds), but more similar to timing of Myo3 and Myo5 (~10–12 seconds), the actin-binding protein Sac6 (10–12 seconds) and the amphiphysins (~9–10 seconds) (Gheorghe et al., 2008; Jonsdottir and Li, 2004; Kaksonen et al., 2003; Kaksonen et al., 2005). Timing of its arrival at the membrane, about 5 seconds after Abp1, indicate that it is not functioning in recruitment of endocytic coat or early acting F-actin polymerisation factors and most likely has a role at the onset or during the invagination step of endocytosis. This appears to be, at least qualitatively, similar to the situation in Swiss-3T3 cells in which dynamin fluorescence increased sharply and transiently at clathrin-labelled spots just before inward movement (Merrifield et al., 2002).

The question of the mechanism of function then arises. The behaviour of several endocytic proteins was studied in vps1Δ cells. Of these, one had a reduced level of localisation and a shorter lifetime in patches (Figs 2, 3). This was the amphiphysin protein Rvs167. However, in colocalisation studies, we were not able to distinguish arrival times to patches, indicating that Vps1 is more likely to maintain Rvs167 at the membrane rather than
recruit it (Fig. 3D). There is currently no evidence that Vps1 interacts directly with Rvs167. However, there are clear links between the proteins. One candidate for linking Vps1 and Rvs167 function is SlA1, which has reported interactions with both proteins (Stamenova et al., 2004; Yu and Cai, 2004). Alternatively, Vps1 might induce changes to the membrane itself that favour stable binding of Rvs167. From the ultrastructural analysis (Fig. 4), one potential role for Vps1 is in the switch from a shallow wide-necked pit to a parallel-sided invaginating tubule. The in vitro data (Fig. 7) also demonstrated that Vps1 has the capacity to bind to liposomes and to tubulate them. Taken together, we suggest a model in which Vps1 is recruited to membranes in an actin-independent manner, but after F-actin polymerisation has been initiated. As rapid actin polymerisation drives inward membrane growth, Vps1 potentially in concert with Rvs167, could bind to the membrane and form an oligomserised structure to bring the sides of the tubule essentially parallel. This action would ensure that the force from actin polymerisation is driving directly into the cell around the tubule. If a parallel-sided tubule is not formed, directed inward movement would be less likely, explaining why angles of invagination are more varied in the vps1Δ strain. Such disorganised forces might also be expected to cause the more erratic patch behaviour that is observed. The idea of a sheath of protein along the invaginated tubule region had been suggested in endocytotic modelling studies (Liu et al., 2009). This protein sheath is proposed to act as a filter to effect a separation of lipid species and thus generate lipid phase boundary forces, which, in concert with the force from actin polymerisation, could be responsible for membrane scission. Liu and colleagues propose that this sheath is entirely composed of amphiphysin (Liu et al., 2009). Our data suggest that it could be initiated or maintained by the function of Vps1.

Finally, the role of distinct domains within Vps1 was investigated. Ten mutant forms of Vps1 were generated and expressed. The sites and amino acid changes made were based on mutations previously generated in mammalian dynamin or from the original Drosophila shibire mutations. The effects of two of these mutations T63A and I649K, which correspond to dominant-negative mutations in mammalian cells, were investigated. Taken together, the data indicate that the N-terminal GTPase domain of Vps1 is crucial for its function in endosomal recycling, vacuolar uptake of FM4-64 and in peroxisomal fission. Interestingly, mutations in the C-terminal domain did not compromise the function of Vps1 in these assays. The effect of Vps1 mutations in endocytosis was, however, markedly different. Not only does the GTPase domain have an important role in successful invagination, but the C-terminal region is also involved. For all endocytic markers studied, the I649K mutation in the C-terminal region caused effects as severe as those in the GTPase region. Biochemical studies (Fig. 7) reveal that I649K mutation in the C-terminal domain of Vps1 prevents self-assembly. Thus, self-assembly is important for endocytic function of Vps1, but does not appear to be required for the other trafficking roles investigated. This is a key result that defines the role of the yeast dynamin during endocytosis compared with its other roles in cell-membrane trafficking.

**Materials and Methods**

**Materials**

Unless stated otherwise, chemicals were obtained from Sigma. Media were from Melford Laboratories (Ipswich, Suffolk, UK) (yeast extract, peptone, agar) or Sigma (minimal synthetic medium and amino acids). Lat-A and FM4-64 were from Molecular Probes.
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