The yeast dynamin-related GTPase Vps1p functions in the organization of the actin cytoskeleton via interaction with Sla1p

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Summary
Recent studies have suggested that the function of the large GTPase dynamin in endocytosis in mammalian cells may comprise a modulation of actin cytoskeleton. The role of dynamin in actin cytoskeleton organization in the yeast Saccharomyces cerevisiae has remained undefined. In this report, we found that one of the yeast dynamin-related proteins, Vps1p, is required for normal actin cytoskeleton organization. At both permissive and non-permissive temperatures, the vps1 mutants exhibited various degrees of phenotypes commonly associated with actin cytoskeleton defects: depolarized and aggregated actin structures, hypersensitivity to the actin cytoskeleton toxin latrunculin-A, randomized bud site selection and chitin deposition, and impaired efficiency in the internalization of membrane receptors. Over-expression of the GTPase mutants of vps1 also led to actin abnormalities. Consistent with these actin-related defects, Vps1p was found to interact physically, and partially co-localize, with the actin-regulatory protein Sla1p. The normal cellular localization of Sla1p required Vps1p and could be altered by over-expression of a region of Vps1p that was involved in the interaction with Sla1p. The same region also promoted mis-sorting of the vacuolar protein carboxypeptidase Y upon over-expression. These findings suggest that the functions of the dynamin-related protein Vps1p in actin cytoskeleton dynamics and vacuolar protein sorting are probably related to each other.

Key words: GTPase, Dynamin, Actin, Vps1p, Sla1p

Introduction
Dynamin and dynamin-related proteins are an evolutionally conserved family of large GTPases engaged in a diversity of cellular processes, including endocytosis, intracellular protein trafficking, and organelle partitioning (Hinshaw, 2000; Danino and Hinshaw, 2001). The role of dynamin in clathrin-mediated endocytosis is first suggested in the analysis of a temperature-sensitive mutant of dynamin in Drosophila. At the non-permissive temperature, the mutant, shibire, exhibits a paralytic phenotype due to a block in endocytosis at the presynaptic membranes (Kosaka and Ikeda, 1983). The unusual accumulation of long invaginations at these membranes indicates a failure in vesicle detachment (Kosaka et al., 1983; Koenig and Ikeda, 1989). One conspicuous feature of dynamin is its ability of self-assemble into spiral-like structures around lipid tubules, which has led to the proposal that dynamin acts as a mechano-enzyme to release the clathrin-coated vesicles using its GTPase-dependent conformational changes (Sweitzer and Hinshaw, 1998; Stowell et al., 1999; Marks et al., 2001; McGavin et al., 2001; Song and Schmid, 2003).

Recent studies reveal that the function of dynamins in endocytosis might depend on their roles as actin cytoskeleton regulators. Actin cytoskeleton has been known for quite some time to be important for endocytosis in the yeast Saccharomyces cerevisiae. Yeast mutants with an abnormal or perturbed cortical actin cytoskeleton are often found to be defective in endocytosis (Munn, 2001). The evidence for actin cytoskeleton participating in endocytosis in mammalian cells has also been accumulating in recent years. Numerous studies have established the ability of dynamin to interact with various actin regulatory factors including profilin (Witke et al., 1998), and the actin-binding protein Abp1 (Kessels et al., 2001), as well as syndapin, intersectin, and cortactin, which link dynamin to the Wiskott Aldrich Syndrome protein (WASP) and the Arp2/3 complex, the major actin assembly promoters (Qualmann et al., 1999; McGavin et al., 2001; Schafer et al., 2002). Despite these findings, however, the exact function of dynamin in endocytosis remains unresolved.

In addition to conventional dynamins, there are other proteins from the dynamin family that share high homology with dynamins in their N-terminal GTPase domain but show less or no sequence conservation in other regions. These dynamin-related proteins are generally also found to have functions distinct from dynamins. For example, one of the better-studied dynamin-like proteins, DLP1, is known to be required for organelle morphology in mammalian cells (Shin et al., 1997; Yoon et al., 1998; Imoto et al., 1998; Kamimoto et al., 1998; Smirnova et al., 1998; Sever et al., 1999). So far, there is no documentation yet to suggest that these dynamin-like proteins have an actin-related function similar to the conventional dynamins.

There are three dynamin-like proteins in yeast that are structurally more related to DLP1 than to conventional
Materials and Methods

Strains, constructs, media, and general methods

The yeast strains used in this study are listed in Table 1. All gene disruptions and integrations were confirmed by PCR. The GTPase domain mutations of vps1 were generated by site-directed mutagenesis and confirmed by DNA sequencing. The plasmid constructs are listed in Table 2. The oligonucleotides used in this study are listed in Table 3. General methods, such as medium preparation, cell culture, protein extraction, immunoprecipitation and immunoblotting of the epitope-tagged proteins, were performed according to the procedures described previously (Tang et al., 1997; Tang et al., 2000). The yeast two-hybrid interaction assays followed the instructions of the manufacturer (Clontech, CA). Testing fragments were cloned into pGADT7 containing the HA tag or pGBK7 containing the Myc tag. The plasmids were then co-transformed into the strain SFY526 and expression of each fusion protein was verified by western blotting using either anti-HA or anti-Myc antibodies. CFP and YFP tags were amplified from the template plasmid pDH3 and pDH5 from Yeast Resource Center, University of Washington.

Fluorescence microscopy procedures

Yeast cells were cultured in appropriate media as mentioned in figure legends (see Table 1 for yeast strains). For actin and chitin staining, cells were fixed with 3.7% formaldehyde, and stained with rhodamine phalloidin (Molecular Probes), or calcofluor (Sigma), respectively, as described elsewhere (Pringle et al., 1989). The samples were examined under a Leica DMAXA microscope equipped with a Hamamatsu C4742 digital camera. The analyses of actin organization and the budding scar patterns followed previous studies (Drubin et al., 1993; Chant and Pringle, 1995). Actin depolarization was scored only in budded cells with small- and medium-sized buds. In cases where quantification was required, at least 200 cells were counted for each sample.

Endocytosis assays and measurement of the Ste3p half-life

The assay for the uptake of uracil was carried out as described elsewhere (Zeng et al., 2001). Data were compiled from at least two independent experiments. The turnover of Ste3p was examined as follows. For strains containing the GAL1-STE3-EGFP, cells were cultured to mid-log phase at 25°C in a dropout medium supplemented with 2% raffinose. The expression of Ste3-GFP was induced by addition of 2% galactose. After 90 minutes of induction at 25°C, aliquots of cultures were shifted to 30°C or 37°C while glucose was added to the medium (to 3%) to repress the expression of Ste3p. At indicated time intervals after glucose addition, about 25 OD600 units of cells were harvested, washed, and resuspended in 400 μl of ice-cold extraction buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA) and lysed by vortexing with glass beads. The suspension was centrifuged briefly to remove cell debris and centrifuged again at a high speed (14,000 g) for 45 minutes. The pellet was resuspended in

| Table 1. Yeast strains |
|------------------------|
| Strain name | Relevant genotype |
| W303-1-A | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| W303-1-B | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| YMC450 | MATa sla1Δ::HIS3 |
| YMC451 | MATa vps1Δ::HIS3 |
| YMC452 | MATa dm1Δ::LEU2 |
| YMC453 | MATa mgm1Δ::TRP1 |
| YMC454 | MATa vps26Δ::HIS3 |
| YMC455 | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| YMC456 | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| YMC457 | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| YMC458 | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| YMC459 | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| YMC460 | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| YMC461 | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| YMC462 | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| YMC463 | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| YMC464 | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| YMC465 | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| YMC466 | MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-52 |
| YMC467 | MATa sla1Δ::HIS3[pTHY918] |
| YMC468 | MATa sla1Δ::HIS3 mgm1Δ::TRP1[pTHY918] |
| YMC469 | MATa sla1Δ::HIS3 dm1Δ::LEU2[pTHY918] |
| YMC470 | MATa sla1Δ::HIS3 vps1Δ::LEU2[pTHY918] |
| SFY526 | MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu23,112UalaG4-Gal542 gal80-538 URA3::GAL1-lacZ |
Halo assays for latrunculin-A sensitivity

The latrunculin-A (LAT-A) sensitivity was measured by the halo assay following the published procedures (Ayscough et al., 1997) with minor modifications. Cells were cultured overnight in appropriate media. The amounts of the cells from each culture were normalized related function by the recent findings that the clathrin heavy

Results

The yeast dynamin Vps1p is required for normal actin cytoskeleton organization and chitin deposition

We were attracted to the idea that Vps1p might have an actin-related function by the recent findings that the clathrin heavy
chain (Chc1p), the presumptive functional partner of Vps1p in vesicle transport, is required for normal actin organization in yeast (Henry et al., 2002), and that Scd5p, a high copy suppressor of the chc1 deletion mutant (chc1Δ), localizes to cortical actin patches and is essential for actin organization and endocytosis (Henry et al., 2002). To find out whether Vps1p is required for normal actin structures, a vps1Δ mutant was generated and analyzed with phalloidin staining. Deletion of the VPS1 gene caused a temperature-sensitive phenotype, as had been reported before (Ekena and Stevens, 1995). In the vps1Δ cells growing at 30°C, the actin cytoskeleton appeared to be largely normal in morphology but was evidently

![Fig. 1. Vps1p is required for normal actin cytoskeleton organization.](image)

(A) Actin cytoskeleton in several yeast mutants at different temperatures. Strains (YMC451) vps1Δ, (YMC452) dnm1Δ, (YMC453) mgm1Δ, (YMC454) vps26Δ and wild-type W303-1-B (W303) cultured to mid-log phase in YEPD at 24°C were shifted to 30°C or 37°C for 3 hours before being subjected to actin staining (bar, 5 μm). (B) Quantitative illustration of the populations of vps1Δ and W303 with actin abnormalities. Actin depolarization shown in the left panel was calculated only in budded cells with small- and medium-sized buds, and the actin aggregation shown in the right panel was based on the total cell population. (C) The abnormal budding pattern and chitin deposition in vps1Δ cells. Strains YMC456 (vps1Δ, diploid) and the wild type YMC455 (W303, diploid) were grown in YEPD to log phase at 24°C. Aliquots of the cultures were then shifted to either 30°C or 37°C for 3 hours before being subjected to Calcofluor staining. The budding patterns and chitin distributions of cells incubated at different temperatures were scored and shown in Table 4 (bar, 5 μm). (D) Prolonged half-life of Ste3p in vps1Δ cells. YMC451 (vps1Δ) and the wild-type W303-1-B (W303) cells that contain GAL1-STE3-EGFP were induced to express Ste3p-GFP for 90 minutes in 2% of galactose at 25°C, followed by addition of 3% glucose to repress the expression. The cultures were immediately shifted to 30°C or 37°C. The amount of Ste3p-EGFP at indicated times after glucose addition in W303 and vps1Δ cells were detected by western blotting (lower panels). The levels of Ste3-EGFP were quantified using a densitometer and plotted in upper panels.
depolarized (Fig. 1A and B). The cortical patches were more or less evenly distributed between the mother and the daughter in about 70% of budded cells with small- and medium-sized buds. A minor population (about a quarter of total cells) also contained abnormal actin aggregates, or chunks (Fig. 1B). In comparison, only about 20% of the budded cells displayed depolarized actin patches and about 7% of the total cells contained actin aggregates in the wild type at this temperature (Fig. 1A and B). At the non-permissive temperature of 37°C, the actin cytoskeleton of the mutant became grossly aberrant, with most (over 70% of the total) cells containing abnormal actin aggregates, and over 90% of the budded cells also showing depolarized localization (Fig. 1A and B). The abnormal actin morphology in the vps1 mutant was not simply a response to the high-temperature treatment, as the deletion mutants of other two dynamin-related genes, dnm1Δ and mgm1Δ, as well as the wild-type cells, generally maintained their normal cortical actin morphology and distribution patterns under the same condition (Fig. 1A and B). These results suggest that Vps1p is indeed required for normal organization of actin cytoskeleton in yeast as we speculated.

It may perhaps be argued that the abnormal actin cytoskeleton organization in vps1 cells was probably resulted from other defects of the mutant, such as protein sorting or aberrant cellular organelles, rather than from a loss of a direct role of Vps1p in actin cytoskeleton organization. One way to investigate this possibility is to examine other vps mutants that have similar phenotypes in protein sorting as vps1. The vps1 mutant belongs to the F type of vps mutants, which also include vps26. Both vps1 and vps26 have been reported to exhibit aberrant vacuolar morphologies and a severe CPY sorting defect (Raymond et al., 1992), as well as the defects in the retention of late-Golgi membrane proteins (Nothwehr et al., 1996). When vps26Δ cells were examined for actin organization, however, no observable defects in either the morphology or the distribution pattern of the actin cytoskeleton were found regardless of the growth temperature (Fig. 1A). It is therefore unlikely that the actin abnormalities in the vps1 cells could be attributed to deficiencies of the mutant in protein sorting.

It is known that defects in the actin cytoskeleton organization are often accompanied by abnormal budding patterns and altered chitin deposition. Wild-type yeast cells select bud site according to genetically determined spatial patterns: axial mainly in haploid cells and bipolar mainly in diploid cells (Casamayor and Snyder, 2002). As the yeast cell forms a bud, a ring of chitin is deposited in the cell wall at the bud site, which remains on the surface of the mother cell as a bud scar after cell division. Budding patterns can be examined and scored by staining the cell with Calcofluor, which labels chitin on the cell wall. To gather additional evidence of the actin cytoskeleton defect in the vps1 mutant, we examined the budding pattern of the vps1Δ homozygous diploid cells. As shown in Fig. 1C and Table 4, the chitin staining in the vps1Δ diploid cells was clearly mislocalized even at 24°C and 30°C (Fig. 1C, Table 4): about 30% of cells showed random budding pattern and another 20% or so contained abnormal chitin deposition, such as chitin chunks instead of rings. Conversely, over 90% of wild-type cells showed a normal budding pattern (65% bipolar and 27% axial) under the same conditions (Fig. 1C, Table 4). After a temperature shift to 37°C for 3 hours, while wild-type cells maintained the normal budding pattern, the vps1Δ cells showed a marked increase in the population with the abnormal chitin deposition (to over 40%, Fig. 1C, Table 4). Similarly, the haploid vps1Δ cells also showed mislocalized chitin deposition at 37°C (27% with random budding and 46% with abnormal chitin staining, data not shown). Therefore, the vps1Δ cells were defective in bud site selection and chitin deposition at both permissive and non-permissive temperatures.

The turn-over of the membrane receptor protein Ste3p is impaired in vps1Δ

As the integrity of actin cytoskeleton is required for endocytosis in yeast, we tested the vps1Δ mutant for its ability to carry out membrane receptor endocytosis. It is known that the a factor receptor, Ste3p, is constantly internalized in the absence of its ligand through the endocytic pathway and delivered to the vacuole for degradation without being recycled back to the cell membrane (Davis et al., 1993; Chen and Davis, 2000). The stability of the full-length Ste3p is indicative of the efficiency by which Ste3p is being endocytosed. At 37°C, most if not all of Ste3-GFP in wild-type cells was degraded within 60 minutes after the Ste3p expression was turned off (Fig. 1D). However, in vps1Δ cells, Ste3-GFP was turned over more slowly, and a substantial amount of the protein (about 70%)

### Table 3. Oligonucleotides used in this study

| Oligo  | Sequence                          |
|--------|----------------------------------|
| VPS1-Y9* | 5’TGGGCGCCGCGGATTTGATTTTTTAC 3’ |
| VPS1-Y10* | 5’TGGGCGCCGCGGATTTGATTTTTTAC 3’ |
| VPS1-Y12 | 5’TGGGCGCCGCGGATTTGATTTTTTAC 3’ |
| VPS1-Y13 | 5’TGGGCGCCGCGGATTTGATTTTTTAC 3’ |
| VPS1-Y14 | 5’TGGGCGCCGCGGATTTGATTTTTTAC 3’ |
| VPS1-Y15 | 5’TGGGCGCCGCGGATTTGATTTTTTAC 3’ |
| VPS1-Y17 | 5’TGGGCGCCGCGGATTTGATTTTTTAC 3’ |

*An AscI site is underlined. ‡A PstI site is underlined.

### Table 4. The budding pattern distribution in vps1Δ and wild-type cells

| Strain (diploid) | Axial (%) | Bipolar (%) | Random (%) | Abnormal chitin staining (%) |
|------------------|-----------|-------------|------------|-----------------------------|
| 24°C             |           |             |            |                             |
| W303            | 27        | 68          | 5          | 0                           |
| vps1Δ           | 19        | 36          | 29         | 16                          |
| 30°C             |           |             |            |                             |
| W303            | 22        | 74          | 4          | 0                           |
| vps1Δ           | 18        | 32          | 31         | 19                          |
| 37°C             |           |             |            |                             |
| W303            | 27        | 65          | 8          | 0                           |
| vps1Δ           | 12        | 24          | 23         | 41                          |
remained after 90 minutes at 37°C, suggesting that the internalization of Ste3-GFP from the cell surface to the vacuole was impaired in the \textit{vps1} deletion strain. This result is in agreement with the above finding that the \textit{vps1} mutant suffered from severe actin defects at the non-permissive temperature.

The putative GTPase mutants of \textit{VPS1} exhibit similar defects as the null mutant

Among the three yeast dynamin-related proteins, Vps1p and Dnm1p are more related to mammalian dynamin than Mgm1p (Fig. 2A). The sequence similarity resides mainly in the GTPase domain, and, to a lesser degree, the GED domain (Fig. 2A). In mammalian cells, it has been shown that dynamin mutants deficient in GTP hydrolysis, such as K44E, S45N and G273D are unable to internalize transferrin (Herskovits et al., 1993; Damke et al., 1994; Damke et al., 1995; Marks et al., 2001). As these key amino acid residues are also conserved in Vps1p, mutations with same alterations were generated in \textit{VPS1} and the resulting mutants, \textit{vps1K42E}, \textit{vps1S43N} and \textit{vps1G315D}, were tagged with the Myc-epitope, expressed under its native promoter, and introduced into \textit{vps1DA} by integration. Western blot analysis confirmed that they were all expressed to a level similar to their wild-type counterpart (data not shown). All three mutants were temperature-sensitive when grown at 37°C (Fig. 2B).

We first examined the sensitivity of these mutants to the LAT-A, a drug that inhibits actin assembly by binding and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{The putative GTPase mutants of \textit{vps1} affect actin cytoskeleton. (A) Alignment of the GTPase domain and the GED domain from mammalian dynamin-1 with three yeast dynamin-related proteins. Numbers on the top indicate the amino acid positions of Vps1p. Underlined in the GTPase domain are the residues that were mutated. (B) Temperature sensitivities of the \textit{vps1DA} cells containing various GTPase mutants of \textit{vps1}. Strains YMC457 (vector/\textit{vps1DA}), YMC458 (\textit{Vps1WT}/\textit{vps1DA}), YMC459 (\textit{vps1K42E}/\textit{vps1DA}), YMC460 (\textit{vps1S43N}/\textit{vps1DA}) and YMC461 (\textit{vps1G315D}/\textit{vps1DA}) cultured at 24°C were first diluted to similar density from which further serial dilutions were made. We spotted 5 μl of each dilution onto the YEPD plates and incubated at indicated temperature for one day. (C) LAT-A sensitivity of the \textit{vps1DA} and GTPase mutants. Cells shown in (B) were cultured in YEPD at 30°C and subjected to LAT-A halo assay as described in Materials and Methods. The bar chart in the right shows the quantified result. (D) The uracil-uptake assays in the YMC458 (\textit{VPS1}) ( ), YMC457 (\textit{vps1DA}) ( ) and YMC459-461 (\textit{vps1K42E}, \textit{vps1S43N} and \textit{vps1G315D}) (▲, ▼, ◆) at both 30°C (left) and 37°C (right).}
\end{figure}
sequestering actin monomers (Coue et al., 1987). The sensitivity to LAT-A has been regarded as an indicator of the actin cytoskeleton integrity (Ayscough et al., 1997). Consistent with its actin structure abnormalities, the vps1 null mutant was about twofold more sensitive to LAT-A than the wild-type cell (the same cell containing VPS1), as shown in Fig. 2C. The three GTPase mutants of VPS1 all exhibited a similar level of sensitivity as the vps1 Δ mutant in this assay (Fig. 2C). This result suggests that the loss of VPS1 led to the instability of filamentous actin structures in vivo, and the GTPase activity of Vps1p is probably essential for its role in actin cytoskeleton organization.

These GTPase mutants were examined for their proficiency in endocytosis using the uracil uptake assay. The uracil permease, encoded by the FUR4 gene, is responsible for uptake of uracil in yeast. When protein synthesis is inhibited, Fur4p localized on the plasma membrane is rapidly internalized through the endocytic machinery, resulting in impaired uracil uptake (Volland et al., 1994). The vps1 Δ mutant, as well as the three GTPase mutants, exhibited essentially a same kinetics of uracil uptake as the wild type at 30°C (Fig. 2D, left). At 37°C, conversely, the uracil transport activity in all four mutants persisted and remained at about 40% at a time when no activity was detected in wild-type cells (Fig. 2D, right). These results showed that the GTPase mutants and the deletion mutant of VPS1 were similarly deficient in the internalization of membrane proteins at the non-permissive temperature.

Over-expression of the GTPase mutants of VPS1 leads to actin defects and cell death at 37°C

The GTPase mutants of dynamin have been reported to exert a dominant-negative effect on endocytosis and actin dynamics when over-expressed in mammalian cells. For example, the dynamin 2 mutant K44A, defective in GTP binding and hydrolysis, inhibits actin dynamics upon over-expression (Schafer et al., 2002; Orth et al., 2002). To test whether over-expression of the putative GTPase mutants of VPS1 could similarly exert a negative effect on actin cytoskeleton in yeast, the Myc-tagged VPS1, vps1 K42E, vps1 S43N and vps1 G315D were each placed under the control of the GAL1 promoter and integrated into wild-type cells. Western blotting confirmed that all four proteins were expressed to similar levels upon induction with galactose (data not shown). These cells were first examined for their actin structures. After 4 hours of induction in galactose at 30°C, all three GTPase mutants, vps1 K42E, vps1 S43N and vps1 G315D, incited obvious actin depolarization and aggregation (Fig. 3A). Over-expression of the wild-type VPS1, conversely, had little or no effect (Fig. 3A). Quantitative analysis showed that only about 20% of cells

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![Fig. 3.](image-url) The actin defects caused by over-expression of the GTPase domain mutants of vps1. (A) Over-expression of the GTPase domain mutants caused abnormal actin structures. Strains integrated with the following GAL1-driven vps1 constructs, YMC463 (Gal-Vps1WT), YMC464 (Gal-vps1 K42E), YMC465 (Gal-vps1 S43N) and YMC466 (Gal-vps1 G315D), as well as YMC462 (vector), were cultured to mid-log phase in the presence of 2% raffinose (strain name in brackets, see Table 1). Galactose was added to 2% to induce the expression of the vps1 mutants for 4 hours at 30°C. Cells were then collected for actin staining (Bar, 5 μm). (B) Over-expression of the GTPase mutants caused lethality in wild-type cells. The strains as described in (A) were grown on glucose-containing plates and replica-plated onto galactose plates and incubated at 37°C for 1 day before being photographed. (C) Cells over-expressing the vps1 GTPase mutants are hypersensitive to LAT-A. The strains as described in (A) were plated on the galactose-containing medium at 30°C and the LAT-A with the indicated concentration was applied (left panel). A summary of the halo assay is shown in the graph at the right.
were able to maintain a normal actin-patch morphology and distribution in each of the three strains over-producing the GTPase mutants. Over-expression of the three GTPase mutants also caused lethality to the host cells at 37°C, as shown in Fig. 3B. Consistent with the abnormal actin phenotypes, over-expression of these GTPase mutants also resulted in hypersensitivity to LAT-A (Fig. 3C). These findings suggest that, similar to the dynamin mutants of mammalian cells, the GTPase mutants of vps1 also affect stability of filamentous actin structures upon over-expression in yeast cells.

Vps1p interacts with the actin regulatory protein Sla1p. A number of Src-homology-3 (SH3) domain-containing proteins, such as amphiphysin, endophilin, syndapin and intersectin, are known to interact with dynamin in mammalian cells (David et al., 1996). Intersectin is particularly interesting as it contains five SH3 domains and two Eps15 homology (EH) domains, structurally reminiscent of the yeast Pan1p-Sla1p complex identified in our laboratory to be required for actin cytoskeleton organization and endocytosis (Tang et al., 1997). It was thus speculated that the Pan1p-Sla1p complex could be a linkage between Vps1p and the actin regulatory machinery and the possible interaction between Vps1p and Sla1p, an SH3 domain-containing protein, was investigated. We first tested if disruption was lethal, whereas sla1ΔΔ and vps1ΔΔ double disruption was lethal, whereas sla1ΔΔ dnm1ΔΔ and sla1ΔΔ mgm1ΔΔ were not. Further analysis revealed that the third SH3 domain and the C-terminal repeats of Sla1p, two regions in Sla1p that are known to be required for its function in actin cytoskeleton organization (Ayscough et al., 1999), were essential for supporting the viability of the vps1Δ mutant (Fig. 4B and C). We next tested whether the Vps1p and Sla1p could physically interact with each other using the co-immunoprecipitation assay. Protein extracts from wild-type cells containing pGal1-myc-VPS1 and/or single copy of HA-tagged SLA1 on a separate vector were subjected to immunoprecipitation by the anti-HA antibody. HA-Sla1p migrated on the SDS-PAGE as a band with a molecular mass of 150 kDa. When the anti-HA immunoprecipitates were probed with the anti-Myc antibody, the 80 kDa Myc-Vps1p was readily detected (Fig. 5A, lane 5). The Myc-Vps1p was not found in the control sample made from the cell lysate containing no HA-SLA1 (Fig. 5A, lane 6). This showed that Vps1p and Sla1p could indeed associate with each other in vivo.

The Vps1p-Sla1p complex, however, was only weakly detectable if Vps1p was not over-produced from the GAL1 promoter (Fig. 5B, lane 10). We wondered whether this was partly because the interaction between Vps1p and Sla1p was a highly transient event in vivo. If so, it might be possible to increase the probability of the complex formation using the putative GTPase mutants described above, as the rate of dynamic exchange between different forms of Vps1p had to be decreased by these mutations. To test this hypothesis, the co-immunoprecipitation experiments were performed again using the HA-tagged Sla1p and different Myc-tagged GTPase mutants of Vps1p, each expressed from its native promoter. The expression levels of these proteins were similar to their wild-type counterpart (Fig. 5B). Indeed, the various Vps1p mutant proteins now became more readily detectable in the HA-Sla1p immunoprecipitates without having to be over-expressed. Notably, the Vps1pG315D mutant, which corresponded to the original mutation in shibire (Baba et al., 1999), interacted with Sla1p more strongly than the other two mutants (Fig. 5B, lane 16).

Alteration of cellular localization of Sla1p by vps1 mutations. Sla1p interacts with several actin regulatory proteins and co-localizes with cortical actin patches in wild-type cells (Tang et al., 1997; Tang et al., 2000; Zeng et al., 2001; Danino et al., 2001; Howard et al., 2002; Warren et al., 2002; Gourlay et al., 2003). The localization at the cortical actin patches is important for the function of Sla1p in actin organization and

Fig. 4. Synthetic lethality between vps1Δ and alleles of sla1 with actin defects. (A) The viabilities of various mutants after their SLA1-containing plasmid pTHY918 was lost on a 5-FOA plate: strains YM467 (sla1Δ), YM468 (sla1Δ mgm1Δ), YM469 (sla1Δ dnm1Δ) and YM470 (sla1Δ vps1Δ) grown on SC-Ura plate (left) at 24°C were replica-plated onto a 5-FOA plate (right) and incubated at 24°C for 2 days. (B) The schematic structures of Sla1p and its deletion constructs to be used in the following experiments. (C) The test of the strain YM470 (sla1Δ vps1Δ) to lose the SLA1-containing plasmid pTHY918 in the presence of various sla1 deletion constructs: pKS514 (vector, lane 1), pYGS203 (sla1ΔΔSH3, lane 2), pTHY1114 (sla1ΔΔSH3, lane 3), pTHY1115 (sla1ΔΔSH3, lane 4) and pTHY1089 (sla1ΔΔ, lane 5).
endocytosis (Warren et al., 2002; Gourlay et al., 2003). In light of our new finding that Sla1p and Vps1p could form a complex in vivo, we investigated whether Vps1p could affect the normal subcellular localization of Sla1p. The SLA1 gene in vps1Δ and the three GTPase mutants described above were tagged with GFP by integration. The exponentially growing cells of these strains at 30°C were examined for actin structures and Sla1p-GFP localization. In wild-type cells, most of the Sla1p signals were localized to cortical actin patches that were well polarized in small- and medium-size-budded cells (Fig. 6A, Vps1WT). In all four vps1Δ mutants, however, actin and Sla1p signals were both disturbed. The cell-cortex-associated Sla1p was evidently diminished and most of the signals were not concentrated at the site of polarized growth (Fig. 6A). While the actin structures and the distribution patterns became abnormal, as described earlier, the co-localization between actin and Sla1p was no longer obvious in these mutants (Fig. 6A). Based on these findings, it is concluded that the normal cellular location of Sla1p depends on the proper function of Vps1p.

Overexpression of the C-terminal region of Vps1p leads to detrimental effects

The N-terminal region of Vps1p is the GTPase domain and the C-terminal region is the GED domain based on its sequence homology with the mammalian dynamin (Fig. 7A). Both domains are required for the function of Vps1p in vacuolar protein sorting (Vater et al., 1992). Using the two-hybrid assay system, the regions within Vps1p that are involved in the interaction with Sla1p were mapped. As shown in Table 5, Vps1p interacted with Sla1p through a C-terminal region comprising the GED domain (amino acids 357-704 and amino acids 566-704). The N-terminal GTPase domain of Vps1p (amino acids 1-355) also showed a positive, albeit weaker, interaction (Table 5).

To analyze the importance of the C-terminal region of Vps1p, we first examined whether the ectopic expression of the C-
terminal region could cause any actin defects. Various regions of Vps1p (Fig. 7A) were tagged with CFP and placed under the inducible promoter pGAL1. All constructs gave rise to similar expression levels in vivo when induced by galactose (data not shown). As shown in Fig. 7B, expression of a region containing the central and the C-terminal parts of Vps1p (amino acids 276-704) resulted in temperature sensitivity at 37°C. Deletion of the most-C-terminal GED domain from this construct mitigated this growth inhibitory effect (Fig. 7B, amino acids 276-616). Thus, the GED domain was at least part of the cause for the cell death upon over-expression at 37°C. Conversely, the GED domain alone was not sufficient (Fig. 7B, amino acids 614-704), and it had to be combined with some 200 amino acids of the upstream sequence to result in a cell growth defect (Fig. 7B, amino acids 357-704). In comparison, over-expression of either the GTPase domain or the full-length protein did not cause a temperature sensitive phenotype (Fig. 7B).

The cellular localization of various regions of Vps1p was also examined in parallel with that of Sla1p. Consistent with the finding that Vps1p and Sla1p could be immunoprecipitated as a complex, the full-length Vps1p (amino acids 1-704) was found to be partially co-localized with Sla1p after 4 hours of induction by galactose at 30°C (Fig. 7C, row 6). Under the same condition, the two GED-domain-containing constructs that caused temperature sensitivity (amino acids 276-704 and amino acids 357-704) both gave rise to more irregular or aggregated Vps1p signals that remained largely overlapped with the abnormally accumulated Sla1p (Fig. 7C, rows 1 and 4). Cells over-expressing the central region without the GED domain (amino acids 276-616) only showed one or two dots of the Vps1p signal in the cytosol, which did not alter the normal localization of Sla1p (Fig. 7C, row 2). Furthermore, over-expression of the N-terminal-most GTPase domain (amino acids 1-355) or the very C-terminal GED domain (amino acids 614-704), both had no cell growth inhibitory effect, yielded no discrete Vps1p signals at all (Fig. 7C, rows 3 and 5). These patterns of domain localization indicated that the GED domain along with some upstream sequence in the central region of Vps1p is indispensable for the protein’s co-localization with Sla1p. This is consistent with results from the two-hybrid interaction demonstrating this region as being involved in the interaction with Sla1p.

As the proper localization of Sla1p is important for actin cytoskeleton organization, it was anticipated that the over-expression of the two GED domain containing constructs (amino acids 276-704 and amino acids 357-704) that caused cell lethality at 37°C and mislocalization of Sla1p will also compromise the function of the actin cytoskeleton. This was ascertained by testing the LAT-A sensitivity of these cells. As shown in Fig. 7D, over-expression of these two constructs indeed led to a hypersensitivity to LAT-A (Fig. 7D, 4 and 7).

Discussion

Modulation of actin cytoskeleton is now appreciated to be a critical aspect of dynamin’s function in endocytosis. In yeast, the issue of whether a dynamin-like factor acts to link actin cytoskeleton to vesicle formation has been left unresolved. The recent evidence for clathrin to be required for actin cytoskeleton to vesicle formation has been left unresolved. The recent evidence for clathrin to be required for actin cytoskeleton to vesicle formation has been left unresolved. The recent evidence for clathrin to be required for actin cytoskeleton to vesicle formation has been left unresolved. The recent evidence for clathrin to be required for actin cytoskeleton to vesicle formation has been left unresolved.

Vps1p is required for normal actin organization in yeast

So far, studies of Vps1p have been mainly focused on its role in protein trafficking at TGN. Here we demonstrate for the first time that Vps1p also affects the organization of cortical actin patches. Even though the defects of actin cytoskeleton organization in the vps1 mutants were more severe at the non-permissive temperature, the majority of the mutant cells already exhibited a phenotype of actin patch depolarization at a lower temperature of 30°C. It has been shown previously that the actin depolarization is a specific phenotype and cannot be
Fig. 7. The effects of over-expression of the vps1 C-terminal region. (A) The structures of Vps1p and its deletion constructs used in the experiments below. Numbers on the top indicate the amino acid positions. (B) Over-expressions of the C-terminal region of Vps1p caused cell death at 37°C. Wild-type cells containing various deletion constructs of VPS1 (pGAL1-VPS1-C1-C4, pGAL1-VPS1-N1), as well as the full length VPS1 (pGAL1-VPS1) were grown on glucose containing medium and replicated to galactose plates and incubated at 37°C for one day. (C) The localization of Sla1p was affected by the over-expression of the C-terminal regions of Vps1p. The endogenous copy of SLA1 in wild-type cells was tagged with YFP tag at its C-terminus and the resulting integrant was transformed with various deletion constructs of VPS1 indicated in A. The resulting transformants were cultured in raffinose-containing media to the mid-log phase before galactose was added and incubated for another 3 hours at 30°C. The localization of the various domains of Vps1p along with that of Sla1p was examined under Leica microscopy. Note the partial co-localization of Sla1p with Vps1p and its domains in 1, 4 and 6 (bar, 5 μm). (D) The strains as in (B) were analyzed for their sensitivities to LAT-A. The cells were plated on the galactose-containing medium at 30°C and the LAT-A with the indicated concentrations was applied (left panel). The results were summarized graphically in the right panel.
simply attributed to poor cell growth (Karpova et al., 1998). Some populations of the vps1 cells also accumulated abnormal actin aggregates in the cytosol at 30°C, and this phenotype became a dominant one among the cells incubated at 37°C, with over 70% of the total cells displaying aggregations of actin structures. These aberrant actin structures were somewhat similar to the actin abnormalities previously observed in other actin defective mutants, such as sla1 and pan1. In comparison, disruption of the other two yeast dynamin encoding genes, DNM1 and MGM1, did not result in any obvious actin defects under all the conditions they were examined, suggesting that the actin-related function is unique to Vps1p among the three yeast dynamin-like proteins. The importance of Vps1p for the actin cytoskeleton was also illustrated by the fact that the vps1D mutants were hypersensitive to the actin cytoskeleton toxin LAT-A. The sensitivity to LAT-A has been regarded as an indicator of the actin cytoskeleton integrity, and has been applied to studies of the actin cytoskeleton in a variety of mutants (Ayscough et al., 1997; Belmont and Drubin, 1998; Singer et al., 2000). Therefore, there is little doubt that Vps1p is required for normal actin cytoskeleton organization in yeast.

The similar actin defects, such as aggregated actin structures and hypersensitivity to LAT-A, were manifested by cells of the putative GTPase mutants of vps1, as well as the cells over-expressing these mutants. The three GTPase mutations used in our studies were created according to mutations characterized previously, which abolished the GTPase activity in mammalian dynamins, although whether they indeed resulted in a loss of the GTPase activity of Vps1p has not been determined. Nevertheless, it could be at least tentatively concluded that the GTPase activity of Vps1p is important for its role in actin organization.

The vps1D cells were also found to be defective in bud site selection and chitin deposition at all temperatures tested, a phenotype that is often associated with the perturbation of actin cytoskeleton (Novick and Botstein, 1985; Yang et al., 1997). However, as mutations in genes involved in vesicular transport, such as vesicle formation at TGN, transport between ER and Golgi, or later steps of secretion, also affected budding patterns (Ni and Snyder, 2001), this phenotype of the vps1 cells is probably due to a combination of defects in actin cytoskeleton and vesicular transport.

Vps1p is required for the efficient internalization of some membrane proteins

It has been well known that the integrity of actin...
cytoskeleton is important for membrane protein endocytosis. In order to gather additional evidence for the role of Vps1p in actin cytoskeleton, we also analyzed the half-life of Ste3p-GFP and the internalization of Fur4p in the vps1 mutant. At 30°C, the turnover of Ste3p in vps1Δ cells was somewhat slower than in the wild-type. This phenomenon, however, can be explained by the known vacuolar sorting defect of the mutant (Raymond et al., 1992), rather than attributed to an endocytosis defect, because the internalization of Fur4p was found to proceed with virtually the same kinetics as that of wild-type cells at this temperature. Conversely, it is likely that the significant retardation of the uracil uptake and the marked increase of the half-life of the Ste3p at 37°C were principally due to the severe actin abnormalities at this temperature, as the Fur4p internalization assay is independent of the vacuolar status. It is worth noticing that the degree of the endocytosis defect in the vps1 mutants was comparable with that of the che1 mutant (Tan et al., 1993). The putative GTPase mutants of vps1 exhibited similar endocytosis defects as the deletion mutant, underlining again the importance of the GTPase activity of Vps1p in this process.

Vps1p may function in actin cytoskeleton through interaction with Sla1p

Among the three yeast dynamin knockout mutants, vps1Δ was not only the only mutant that showed actin cytoskeleton defects, but also the only mutant that conferred a synthetic lethality with sla1Δ. Synthetic lethality is a common phenomenon among mutants with actin cytoskeleton defects and has been observed in many cases such as abp1 sla1 and abp1 sla2 (Holtzman et al., 1993), pan1 end3 (Tang et al., 1997), pan1 sla1 (Tang and Cai, 1996), to name a few. This phenotype is thought to reflect, at least in part, a functional relationship of the cytoskeleton machinery components. The relationship between Vps1p and Sla1p was further revealed by the experiments that established the physical interaction of the two proteins.

The interaction between Vps1p and Sla1p is probably the molecular basis for the role of Vps1p in actin cytoskeleton. Sla1p is known to play an important role in regulation of actin cytoskeleton, by interacting with several proteins capable of promoting actin assembly, such as Pan1p, Las17p and Abp1p (Tang et al., 1997; Tang et al., 2000; Howard et al., 2002; Warren et al., 2002; Gourlay et al., 2003). Vps1p might affect actin cytoskeleton dynamics through the interaction with Sla1p. We found that the proper localization of Sla1p was disturbed in vps1Δ and each of the three vps1 GTpase mutants at the semi-permissive temperature of 30°C (Fig. 6). Sla1p became more defused and depolarized, and no longer unmistakably co-localized with actin patches, which themselves were changed into abnormal structures in these mutants. The Vps1p-Sla1p interaction, nevertheless, might take place in a transient and dynamic manner, as the stable complex becomes more easily detectable in the cells either overproducing Vps1p, or containing the GTpase mutants of Vps1p, than in the wild-type cells. Sla1p interacts more favorably with the Vps1G315D mutant than with the other two GTpase mutants probably because this mutation affects the conformational flexibility of the GTpase more significantly than the other two mutations, as inferred from studies of the equivalent mutants of dynamin A (Niemann et al., 2001).

The two-hybrid interaction analysis revealed that Vps1p interacts with Sla1p through a C-terminal region comprising the GED domain. This region of Vps1p is probably crucial for the function of this protein in actin cytoskeleton, because the over-expressed truncation constructs (which disturbed the normal localization of Sla1p and caused temperature sensitive growth and a hypersensitivity to LAT-A) must contain the GED domain along with some upstream sequence to produce these effects. The correlation between the ability to alter the normal localization pattern of Sla1p, and the ability to cause LAT-A hypersensitivity and temperature sensitivity among various regions of Vps1p, again, supports the notion that the interaction between the two proteins is important for the integrity of actin cytoskeleton. The GED domain alone (amino acids 614-704) was unable to interact with Sla1p in the two-hybrid assays (Table 5). Some additional upstream sequence has to be included for the interaction to occur (amino acids 566-704). This is consistent with the temperature-sensitive growth test (Fig. 7B) and LAT-A sensitivities assay (Fig. 7D). These findings further support the importance of the interaction with Sla1p in the function of Vps1p.

Is the actin-related function of Vps1p required for protein transport?

Although Vps1p has been known to be required for a number of cellular processes, its most well-known function is in the protein trafficking at the Golgi complex. It is postulated that Vps1p promotes vesicle formation at the Golgi in an analogous way to dynamin functioning in endocytosis at the plasma membrane (Nothwehr et al., 1995; Conibear and Stevens, 1995). Similar to endocytosis, protein transport from Golgi might also involve actin cytoskeleton. In mammalian cells, it is known that the actin cytoskeleton network is required for the morphology and cellular positioning of the Golgi apparatus (Valderrama et al., 1998), and disruption of the actin cytoskeleton network causes a concomitant collapse of the Golgi complex (Valderrama et al., 2000). The actin regulators Cdc42 and Arp2/3 are also known to be important for Golgi trafficking processes (Kroschewski et al., 1999; Musch et al., 2001; Stannes, 2002). In yeast, the Golgi complex has been linked to actin cytoskeleton by the identification of proteins that are required both for the protein trafficking at the TGN and for the integrity of actin cytoskeleton. These include Chc1p, Vps54p, Pik1p and Grd20p. Like the vps1 mutant, mutants of che1, grd20 and vps54 all exhibited depolarization and aggregation of cortical actin patches at 37°C and, in the case of grd20, a significant delay in the turnover of the membrane receptor Ste3p (Spelbrink and Nothwehr, 1999; Walch-Solimena and Novick, 1999; Henry et al., 2002; Fiedler et al., 2002). The identification of Vps1p as a new factor from TGN to be required for normal actin organization further supports the connection between protein transport and actin cytoskeleton. The mechanism by which the actin assembly is integrated into the process of protein transport at TGN remains unknown. Thus far, we have not been able to separate the actin-related function of Vps1p from the function required for vacuolar transport by mutations. The same region of Vps1p was responsible, upon over-expression, for inciting actin
structural abnormalities and for promoting CPY secretion, suggesting that both processes required an unimpaired Vps1p-Sla1p interaction. However, it remains possible that the actin cytoskeleton and protein transport processes involve two different populations of Vps1p molecules, and Sla1p might only interact with one of them.

Whether or not Sla1p participates in the protein sorting or cargo transport at the Golgi apparatus has not been formally addressed in this report. The limited evidence available so far suggests that it does. A recent report demonstrates that Sla1p interacts with a cis-Golgi membrane protein Kre6p and is required for the correct localization of this protein (Li et al., 2002). We also found that Sla1p was required for the correct localization of another Golgi resident protein Kex2p (data not shown). Nevertheless, this is an important question and certainly worth of further studies.

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