Identification of an organelle-specific myosin V receptor


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Class V myosins are widely distributed among diverse organisms and move cargo along actin filaments. Some myosin Vs move multiple types of cargo, where the timing of movement and the destinations of selected cargoes are unique. Here, we report the discovery of an organelle-specific myosin V receptor. Vac17p, a novel protein, is a component of the vacuole-specific receptor for Myo2p, a Saccharomyces cerevisiae myosin V. Vac17p interacts with the Myo2p cargo-binding domain, but not with vacuole inheritance-defective myo2 mutants that have single amino acid changes within this region. Moreover, a region of the Myo2p tail required specifically for secretory vesicle transport is neither required for vacuole inheritance nor for Vac17p–Myo2p interactions. Vac17p is localized on the vacuole membrane, and vacuole-associated Myo2p increases in proportion with an increase in Vac17p. Furthermore, Vac17p is not required for movement of other cargo moved by Myo2p. These findings demonstrate that Vac17p is a component of a vacuole-specific receptor for Myo2p. Organelle-specific receptors such as Vac17p provide a mechanism whereby a single type of myosin V can move diverse cargoes to distinct destinations at different times.

Introduction

Myo2p moves secretory vesicles (Govindan et al., 1995; Schott et al., 1999), the Golgi (Rossanese et al., 2001), the vacuole (Hill et al., 1996; Catlett and Weisman, 1998; Catlett et al., 2000), and the mitotic spindle (Beach et al., 2000; Yin et al., 2000). The time of movement and the final destinations of these cargoes, while overlapping, are not identical. At the onset of the cell cycle, the organelles are targeted to the presumptive bud site. However, at cytokinesis, secretory vesicles are targeted to the mother-bud neck, whereas vacuoles are localized at the central regions within both the mother and bud cells. Thus, the attachment and/or activation of Myo2p on distinct organelles must be differentially regulated.

The globular tail of myosin V plays an important role in its attachment to cargo. Overexpression of the globular tail of myosin Va causes a defect in melanosome movement (Wu et al., 2002a); likewise, overexpression of the globular tail of Myo2p disrupts secretory vesicle targeting and causes cell death (Reck-Peterson et al., 1999; Schott et al., 1999).

In both cases, overexpression of this domain is thought to compete with endogenous myosin V and displace it from cargo. Additionally, cell cycle–specific phosphorylation of the myosin Va globular tail releases it from membranes (Karcher et al., 2001), suggesting that post-translational modification of the tail may be important for its attachment to cargo.

The globular tail of Myo2p contains at least two distinct cargo-binding domains, one specific for vacuole movement (Catlett and Weisman, 1998; Catlett et al., 2000), the other specific for secretory vesicles (Schott et al., 1999; Catlett et al., 2000). The vacuole-specific region was defined by seven point mutations affecting one of five amino acids between residues 1248–1307 of the Myo2p globular tail domain. The secretory vesicle binding domain was identified via sequence analysis of a set of myo2ts mutants (Schott et al., 1999), and by identification of myo2/D1459–1491, a mutant specifically defective in secretory vesicle movement (Catlett, 2000). Overexpression of the Myo2p globular tail missing the secretory vesicle–specific region disrupts vacuole inheritance, but does not affect secretory vesicle targeting. Conversely, mutations in the vacuole-binding domain cause defects in vacuole movement, but do not affect other Myo2p-related functions such as secretion. Given that specific regions of the globular tail are required for different functions of Myo2p, the existence of cargo-specific receptors for Myo2p was predicted. Here, we describe Vac17p, a novel protein that is a key component of the vacuole-specific Myo2p receptor.

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Results

To identify the vacuole-specific myosin V receptor, we designed two genetic screens. First, we sought multicopy suppressors of myo2–2 (myo2-G1248D), guessing that the impaired association of myo2–2p with vacuole (Catlett and Weisman, 1998) might be overcome by increasing levels of the receptor or of other proteins required for Myo2p-mediated vacuole movement. The second screen sought mutations in genes other than MYO2 that would restore vacuole inheritance in a vacuole-specific myo2 tail mutant, myo2-N1304S; and was based on the hypothesis that a weakened protein–protein interaction caused by myo2-N1304S might be restored by a compensatory mutation in the binding partner. Furthermore, the location of the point mutation in the binding partner might reveal additional information indicating which region of the receptor contacts the globular tail of Myo2p. We obtained VAC17 as a multicopy suppressor of myo2–2 and the point mutant VAC17-S57F as a genomic mutation suppressing myo2-N1304S. VAC17 had also been identified as the corresponding wild-type gene of the vacuole inheritance mutant vac17 (Tang et al., 2003) and as encoding a protein interacting with the vacuolar membrane protein Vac8p (Tang et al., 2003).

Surprisingly, VAC17-S57F suppressed the vacuole inheritance defect of all the vacuole-specific myo2 tail mutants (Fig. 1 and Table I). These findings prompted us to perform a directed screen for other mutations in VAC17 that could suppress the vacuole inheritance defect of myo2-N1304S. We identified a second amino acid substitution (VAC17-I140V) that showed allele specificity, as it only suppressed the vacuole inheritance defects of myo2–2, myo2-N1304S, and myo2-N1307D (Fig. 1 and Table I).

The above genetic interactions and the requirement for Vac17p in vacuole inheritance (Fig. 2; Tang et al., 2003) suggest that Vac17p plays a key role in Myo2p-mediated vacuole movement. Moreover, the vac17Δ strain displays no growth defects, suggesting that Vac17p is not required for secretory vesicle transport (Tang et al., 2003). Next, we tested whether Vac17p is required for movement of other cargo carried by Myo2p. Inheritance of the late Golgi and peroxisomes requires Myo2p, as defects in the inheritance of both organelles are observed in the myo2–26 mutant (Fig. 2; Hoepfner et al., 2001; Rossanese et al., 2001). In contrast, inheritance of these organelles is normal in the vac17Δ mutant (Fig. 2). Myo2p also functions in nuclear spindle orientation (Beach et al., 2000; Yin et al., 2000). Consistent with these observations, myo2–2 cells are defective in nuclear migration, as demonstrated by a significant increase in binucleate cells (Fig. 2 C, column 7). However, nuclear partitioning is normal in vac17Δ. We also investigated mitochondrial inheritance in...

Table I. VAC17-S57F, VAC17-I140V, and high copy VAC17 suppress the vacuole inheritance defects of selected vacuole-specific myo2 tail mutants

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Vacuole inheritance was measured by counting the percentage of cells with an inherited vacuole in the bud. Control strains with vector alone, low copy (pRS416), or high copy (pRS426) gave similar values, but only pRS416 is shown. Each strain was counted at least three times, with a minimum total of 200 cells scored. ND, not determined.
**Vac17p is required for inheritance of vacuoles, but not for inheritance of Golgi, peroxisomes, mitochondria, or nuclei.**

(A) Fluorescence micrographs of the indicated organelles and strains. Examples of buds with (arrow) and without (arrowhead) the relevant organelles are indicated. Vacuoles were labeled for 1 h with FM4–64 and chased in fresh media for 2 h. Golgi were visualized with Sec7p-GFP. Peroxisomes were visualized with GFP fused to the peroxisome-targeting signal PTS1 (GFP-PTS1). Nuclei were labeled using DAPI. Images of Sec7p-GFP– and GFP-PTS1–labeled cells were obtained by merging five Z-axis planes spaced by 0.75 μM. **myo2–66** is defective in vacuole, Golgi, and peroxisome inheritance. Vacuole inheritance is reported as percentage of cells with an inherited vacuole in the bud. Golgi inheritance was assessed in cells with buds less than one third the size of the mother. Peroxisome inheritance was assessed in cells with buds less than half the size of the mother. For each strain, cells were scored from at least two independent experiments. (C) Quantification of nuclear segregation. Each strain was counted in four independent experiments. Cell types are reported as percentage of total cells.

**Vac17Δ** cells and observed no defect (unpublished data). Thus, Vac17p appears to be solely required for vacuole movement.

As predicted for a receptor for Myo2p, Vac17p interacts with the Myo2p globular tail (Fig. 3 A). Notably, although the interaction between full-length Vac17p (1–425) and the Myo2p globular tail (1113–1574) was weak, removal of the secretory vesicle–binding domain from the Myo2p tail (Δ1459–1491) consistently increased Vac17p–Myo2p interactions (Fig. 3 A). Note that these fusion proteins were expressed to similar levels (Fig. 3 B). This suggests that Myo2p attachment to Vac17p may be regulated to ensure that only one type of cargo binds at any given moment. Likewise, compared with
full-length Vac17p, specific truncations of Vac17p increased Vac17p–Myo2p interactions. This behavior suggests that regions outside of the Myo2p binding domain of Vac17p function in the negative regulation of Vac17p–Myo2p interactions.

Analysis of the Vac17p truncations showed that the Myo2p binding domain on Vac17p lies between residues 97–170. In further support of the above two-hybrid analysis, deletion of this region (vac17Δ109–190) abrogates vacuole inheritance (Tang et al., 2003).

Deletion analysis of Myo2p showed that removal of residues 1297–1307 abolished Vac17p–Myo2p interactions (Fig. 3 C). However, this short sequence is not sufficient for Vac17p binding, as the shortest segment of the tail that interacts with Vac17p (residues 1113–1568) encompasses nearly the entire globular tail (Fig. 3 C).

To determine if the vacuole inheritance defects seen in the myo2 tail mutants result from poor interaction of the mutant Myo2p with Vac17p, we examined interaction of Vac17p

Figure 3. Vac17p interacts with Myo2p.
(A) Vac17p and the globular tail of Myo2p interact in a yeast two-hybrid test. The Myo2p globular tail (WT) or the Myo2p globular tail missing the secretory vesicle binding domain (Δ1459–1491), fused to the GAL4 DNA binding domain (GBD) were tested for their ability to interact with the indicated coding regions of VAC17 fused with the GAL4 DNA activation domain (GAD). Plasmids were cotransformed into the yeast strain PJ69–4A. Transformants were plated onto SC-LEU-TRP, replica-plated onto SC-LEU-TRP-ADR-HIS-3AT, and grown for 2 d at 24°C. The regions tested are represented by boxes, with the corresponding amino acid positions indicated. CC, coiled-coil regions; PEST, PEST sequence (see Fig. 2 A of Tang et al., 2003). Protein expression of all noninteracting constructs was confirmed by Western analysis (not depicted). (B) The globular tail missing the secretory vesicle binding domain interacts more strongly with Vac17p. Western blot of the GBD-Myo2 globular tail and the GBD-Myo2 globular tail (Δ1459–1491) fusion proteins (bottom) and endogenous Myo2p (top) indicates that both fusion proteins are expressed at similar levels. The Western blot shown is representative of seven independent experiments. (C) Plasmids encoding the GAL4 DNA binding domain fused with the indicated coding regions of the MYO2 globular tail were tested for their ability to interact with the GAL4 DNA activation domain fused with the portion of VAC17 encoding residues 1–170. WB, vacuole binding site in the Myo2p globular tail (1297–1307). Protein expression of all noninteracting constructs was confirmed by Western analysis (not depicted). (D) The vacuole-specific Myo2p point mutations disrupt the ability of Myo2p to interact with Vac17p. The yeast two-hybrid test was performed as in C, using the GAL4 DNA binding domain fused with the Myo2p globular tail containing the indicated point mutations and the GAL4 DNA activation domain fused with the portion of VAC17 encoding residues 1–355. (E) Western blot analysis of the expression of the wild-type and mutant GAL4 binding domain-Myo2p globular tail fusion proteins (bottom) and endogenous Myo2p (top). The Western blot shown is representative of the results obtained in three independent experiments.
(1–355) with myo2p tail fusions of five representative mutants (myo2–2G1248D, D1297N, L1301P, N1304D, and N1304S). None of these constructs interacts significantly with Vac17p (Fig. 3D). Western blot analysis indicates that all of these mutant Myo2p fusion proteins were expressed to a similar degree as wild type (Fig. 3E). Together with the requirement of Vac17p in vacuole inheritance, these observations suggest that the interaction of Vac17p and Myo2p is normally required for vacuole movement.

We further analyzed the relationship between Vac17p–Myo2p interactions and vacuole inheritance by testing the interaction of additional myo2 mutant vacuoles with Vac17p. As part of a separate paper (Catlett, N.L., unpublished data), we obtained a collection of intragenic suppressors of the myo2–2 (G1248D) vacuole inheritance defect via random PCR mutagenesis. Eight of these suppressors restore vacuole inheritance to >80% when present as the sole copy of MYO2. Each suppressor, except the pseudorevertant G1248N, contains both G1248D and a second point mutation. We constructed myo2 tail DNA binding-domain fusions of these suppressors and found that all eight simultaneously restored vacuole inheritance and Vac17p–Myo2p interactions. This finding demonstrates that the vacuole inheritance defect in myo2–2 is directly related to a loss of Vac17p–Myo2p interactions.

Analysis of VAC17-I140V also strongly supports the hypothesis that Vac17p–Myo2p interactions are required for vacuole inheritance. The I140V mutation is located in the Myo2p binding site. VAC17-I140V restored vacuole inheritance in myo2-N1304S from ~10 to 50% (Table I). Moreover, it showed allelic specificity, restoring vacuole inheritance in myo2–2 (G1248D), N1304S, and N1307D, but not in other vacuole-specific myo2 point mutants (Table I). Next, we tested whether VAC17-I140V, in addition to restoring vacuole inheritance, restored interaction with myo2–2 and myo2-N1304S (Fig. 4D). A VAC17-I140V two-hybrid construct (97–260) restored interaction with both myo2–2 and myo2-N1304S. A longer fusion, VAC17-I140V (1–260) also acted with the globular tail domain of myo2–2. The suppression of myo2–2 by I140V is not due to overexpression of Vac17p because its levels are similar to the wild-type protein (unpublished data). Also, it is unlikely that the S57F mutation now allows Vac17p to interact with Myo2p at a site distinct from the globular tail. Using the yeast two-hybrid test, we found no interaction between Vac17p-S57F and the Myo2p tail containing both the globular domain and the coiled-coil region or the coiled-coil region alone (unpublished data).

By definition, the vacuole-specific receptor for Myo2p would recruit the motor to the vacuole. Thus, using double-labeled immunofluorescence microscopy, we tested whether Vac17p is required for the association of Myo2p with vacuoles. In addition, we used Western analysis to measure the level of Myo2p on isolated vacuoles. For these experiments, WT, vac17Δ, and VAC17-ΔPEST cells were compared. The removal of the PEST sequence stabilizes Vac17p, increasing the levels of Vac17p on the vacuole (Tang et al., 2003). This mutant, expressed from a low copy plasmid, was used instead of high copy VAC17 because the number of multicopy plasmids present varies widely from cell to cell. Like overexpression of Vac17p from 11 to 37% (n = 187), but does not affect vacuole inheritance in myo2-N1304S (n = 232).

In wild-type cells, Myo2p concentrates at sites of polarized growth and is also present as small cytoplasmic spots, with a subset of spots colocalizing with the vacuolar membrane (Hill et al., 1996 and Fig. 5A). Thus, the low levels of vacuolar Myo2p seen by immunofluorescence microscopy are insufficient to determine whether less Myo2p is present on vacuoles in vac17Δ cells. However, increasing Vac17p levels with the Vac17p-stabilizing mutant VAC17-ΔPEST (Tang et al., 2003) dramatically increased the levels of Myo2p on the vacuole (Fig. 5C). Consistent with the immunofluorescence microscopy images, when compared with wild type, significantly higher levels of Myo2p copurified with VAC17-ΔPEST mutant vacuoles. Moreover, levels of Myo2p on vac17Δ vacuoles were significantly lower (Fig. 5D).
interact with the globular tail of myo2-N1304S shares similarities to what is currently known about myosin which in turn binds directly to Vac8p (Fig. 5 F).

PATAES with the vacuole via direct interaction with Vac17p, Vac8p (Tang et al., 2003). We propose that Myo2p associ-

myristylated and palmitoylated vacuolar membrane protein through interaction of its COOH terminus with the vacuole. Moreover, Vac17p resides on the vacuole membrane (Tang et al., 2003), and it is required for Myo2p association with the vacuole (Catlett and Weisman, 1998). However, in this work, the defect in myo2–2p association with the vacuole appears less severe. The vacuole isolation conditions for the current work included additional protease inhibitors that were not used in the previous work. Vacuoles isolated from vac17–1, or vac8Δ cells without the addition of these extra protease inhibitors showed no significant difference in Myo2p levels as compared with wild-type vacuoles (Catlett, N.L., unpublished data).

Discussion
Several lines of evidence suggest that Vac17p is a key component of the vacuole-specific Myo2p receptor. Vac17p is re-

quired for vacuole inheritance, and residues 96–170 of Vac17p bind directly to the globular tail of Myo2p. Furthermore, Vac17p is localized on the vacuole membrane (Tang et al., 2003), and it is required for Myo2p association with the vacuole. Moreover, Vac17p resides on the vacuole membrane through interaction of its COOH terminus with the myristylated and palmitoylated vacuolar membrane protein Vac8p (Tang et al., 2003). We propose that Myo2p associates with the vacuole via direct interaction with Vac17p, which in turn binds directly to Vac8p (Fig. 5 F).

The molecular basis of Myo2p attachment to the vacuole shares similarities to what is currently known about myosin Va attachment to melanosomes. Melanophilin, a recently discovered rab effector, is a component of the melanosome-specific myosin Va receptor (Fukuda and Kuroda, 2002; Hume et al., 2002; Nagashima et al., 2002; Provance et al., 2002; Wu et al., 2002b). Melanophilin binds directly to myosin V, and interacts with melanosomes via interaction with Rab27a. Thus, both Vac17p and melanophilin bind directly to myosin V and attach to their respective membranes via interaction with an acylated protein.

Despite these similarities, there are several differences between attachment of myosin Va to melanosomes and Myo2p attachment to vacuoles. Melanophilin and Vac17p do not share any sequence similarity. Moreover, no obvious melanophilin homologues were found in the yeast genome database. Similarly, no Vac17p homologues were found in higher eukaryotes. In addition, Rab27a and Vac8p are not related proteins. Vac8p is not a small GTPase; rather, it likely plays its role via interaction with binding partners. It appears that Vac8p brings specific protein complexes to distinct regions of the vacuole (Wang et al., 2001). Moreover, although both Vac8p and Rab27a are acylated, Rab27a is geranylgeranylated at its COOH terminus, and Vac8p is myristylated and multiply-palmitoylated at is NH2 terminus. These diverse types of modifications are likely to serve distinct functions (Melkonian et al., 1999; Zacharias et al., 2002).

It is possible that the differences in these receptors arise because their respective membranes are found in distant organisms or because the melanosome is a specialized lysosomal-like organelle, whereas the yeast vacuole/lysosome serves more generalized functions.
Alternatively, these myosin V receptors may each contain additional proteins. A portion of the vacuole-specific region of Myo2p (residues 1291–1313) is weakly conserved with all vertebrate myosins and is highly conserved among the three classes of vertebrate myosin Vs (Catlett et al., 2000; unpublished data). This suggests that this region in vertebrate myosin Vs may bind a protein receptor. Melanophilin, which binds to the melanocyte-specific exon F that is outside of the globular tail domain, may also bind to this conserved region; alternatively there may be yet another protein that binds this region and functions together with melanophilin and Rab27a. Notably, both the globular tail domain plus exon F are needed to observe the dominant-negative effects caused by overproduction of the myosin Va tail (Wu et al., 2002b).

Similarly, the vacuole-specific receptor reported here may also include a specific rab protein. Rab27a has been shown to be required for myosin Va binding to melanosomes, and Rab11a is required for myosin Vb binding to recycling endosomes (Lapierre et al., 2001). Moreover, the rabs, Sec4p (Schott et al., 1999; Ortiz et al., 2002), and Ypt31/Ypt32 (Ortiz et al., 2002) may play a role in Myo2p movement of secretory vesicles.

The discovery of organelle-specific myosin V receptors demonstrates that myosin V attaches to membranes via protein–protein interactions. Moreover, that Vac17p is not required for movement of other Myo2p cargo shows that within a single cell type, there are specific receptors for distinct membrane cargo. Perhaps these organelle-specific receptors compete with each other for access to the myosin V tail. Further study of Vac17p–Myo2p interactions will help elucidate how organelle-specific receptors regulate myosin V attachment to its diverse cargoes.

Materials and methods
The strains used in this paper are listed in Table II.
Plate assay for vacuole inheritance

The plate assay for vacuole inheritance (Gomes de Mesquita et al., 1996; Catlett et al., 2000) measures the maintenance of active carboxypeptidase Y (CPY), a vacuolar protease, after the inactivation of PEP4. PEP4 encodes the first enzyme in the vacuolar protease cascade required for CPY activation. Strains were cotransformed with pGAL-PEP4 and the indicated libraries, plasmids, or vector controls. Transformants were replica-plated to selective media containing 3% galactose, transferred to media containing 2% glucose, and then assayed for CPY activity. Wild-type colonies (VAC+/H11001) have active CPY and are red, whereas vacuole inheritance defective colonies (vac−/H11002) have inactive CPY and are white.

Screen for multicopy plasmids suppressing the myo2–2 vacuole inheritance defect

Multicopy library pools RB378 and RB380, derived from YEp24 (URA3, 2µ), and the pRS202-based library (URA3, 2µ) were gifts from Drs. David Botstein (Stanford University, Stanford, CA) and Philip Hieter (University of British Columbia, Vancouver, BC), respectively. pNLC16 (pGAL-PEP4-HIS3) was generated by subcloning the EcoRI/SalI fragment from a URA-based pGAL-PEP4 plasmid (Vida et al., 1990) into pRS413 (HIS3, CEN). LWY5518 yeast were cotransformed with pNLC16 and library DNA. Approximately 13,000, 45,000, and 19,000 transformants were screened from the pRS202 library and the RB378 and RB380 pools, respectively.

Figure 5. Elevation of Vac17p levels on the vacuole membrane causes a corresponding increase of Myo2p on the vacuole membrane. (A–D) Indirect immunofluorescence of Myo2p (green) and the 60-kD subunit of the yeast vacuolar ATPase (red). (A) Wild-type; LWY6726, pVAC17 (low copy plasmid). (B) vac17Δ; LWY6726, pRS416 (vector control). (C) VAC17-ΔPEST; LWY6726, pVAC17-ΔPEST (low copy plasmid). (D) myo2–2, LWY5518. Bar, 5 μm. Arrowheads indicate the low levels of Myo2p on the vacuole seen in wild-type cells (small yellow spots), whereas arrows indicate the increased levels of Myo2p on the vacuole seen in the VAC17-ΔPEST mutant cells. Vacuole inheritance is delayed in DBY1398 and the related strain LWY6726. Thus, the vacuole is not always juxtaposed with the site of bud emergence or the tips of small-budded cells and the sites of Myo2p accumulation on the vacuole membrane are not always coincident with these sites. These strains facilitate distinguishing between enrichment of Myo2p on the vacuole membrane versus enrichment at sites of polarized growth. (E) Western blot analysis of the levels of Myo2p (top) and yeast vacuolar ATPase 100-kD subunit (bottom) found on isolated vacuoles. A vector control (pRS416) was present in the first three strains. WT, LWY7235; myo2–2, LWY5516; vac17Δ, LWY5798; VAC17-ΔPEST, LWY5798, pVAC17-ΔPEST (low copy plasmid). The Western blot is representative of the results obtained in three independent experiments. (F) Model for Myo2p association with the vacuole. Vac17p binds directly to a vacuole-specific region of the globular tail domain of Myo2p. Vac17p interacts with the vacuole membrane via interaction with Vac8p. See Tang et al. (2003) for a detailed analysis of Vac17p association with Vac8p.
Vacuole inheritance was assessed with the CPY plate assay. As expected, PEP4 was obtained; we also obtained MYO2. In addition, three isolates were obtained that restored vacuole inheritance to ~30% when assessed by fluorescence microscopy. Each contained a plasmid with multiple ORFs including full-length VAC17 (YCL063W). VAC17 was identified as the corresponding wild-type gene for the vac mutant vac17-1, and VAC17 is required for vacuole inheritance (Tang et al., 2003). Thus, a multicopy plasmid containing VAC17 alone was tested, and suppressed the vacuolar inheritance of myo2-2 to a similar degree as the three candidate plasmids.

Screen for extragenic suppressors of the myo2-N1304S vacuole inheritance defect

myo2Δ::TRP1 yeast carrying plasmids pmyo2-N1304S and pGal-PAL-PEP4-URA3 were mutagenized with ethyl methanesulfonate as described previously (Winston, 1990). Vacuole inheritance was assessed with the CPY plate assay. Vac+ suppressor candidates were isolated, and the original pmyo2-N1304S plasmid was replaced with unmutagenized pmyo2-N1304S to distinguish between suppression due to a new mutation in the original myo2-N1304S plasmid or a mutation in another gene. One extragenic suppressor was identified out of 110,000 colonies. A heterozygous diploid of the suppressor candidate strain, with the corresponding wild-type gene for the vac gene, was constructed (see following paragraph) and transformed into the vacuole inheritance segregated 2:2, indicating that the suppression arose from a single point mutation. Therefore, a genomic library of this suppressor was constructed (see following paragraph) and transformed into the myo2- N1304S starting strain. Complementing plasmids were recovered and sequenced. The suppressing plasmid encoded VAC17, which is required for vacuole inheritance (Tang et al., 2003). Thus, a multicopy plasmid containing VAC17 alone was tested, and suppressed the vacuolar inheritance of myo2-2 to a similar degree as the three candidate plasmids.

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Construction of the genomic suppressor library

myo2-N1304S was integrated into the genome of the suppressor strain (myo2Δ::TRP1, pMYO2-URA3) via transformation and homologous recombination of a linear 5-kb fragment containing full-length myo2-N1304S (obtained from pmyo2-N1304S cut with CiaI, DraII, and Scal). The integran was selected by growth on 5-ﬂuoro-orotic acid plates and inability to grow on SC-TRP-URA plates. Size-fractionated (8–12 kb) genomic DNA from a parental strain was digested with NcoI and ligated into the corresponding fragment from pRS416::TRP1::pMYO2. This work; parental strain DBY1398 (5-AAAAGGATCC

 Yeast two-hybrid analysis

Fusion of the GAL4 activation domain with VAC17 (pGAD-VAC17) is described in Tang et al. (2003). For pRS416-VAC17(1–170), pRS416-VAC17 was cut with BstBI and religated, creating K171N, T172L, and N173-AAAAGGATCC. For pGBD-VAC17(97–170), the ~300-bp EcoRI fragment from pRS416-VAC17(1–170) was cloned into the EcoRI site of pGAD-C1 (James et al., 1996). For pRS416-VAC17(1–170), the corresponding fragment from pRS416-VAC17(1–170) was cloned into the EcoRI site of pGAD-Cl (James et al., 1996). For pRS416-VAC17(1–170), the corresponding fragment from pRS416-VAC17(1–170) was cloned into the EcoRI site of pGAD-Cl (James et al., 1996). For pRS416-VAC17(1–170), the corresponding fragment from pRS416-VAC17(1–170) was cloned into the EcoRI site of pGAD-Cl (James et al., 1996). For pRS416-VAC17(1–170), the corresponding fragment from pRS416-VAC17(1–170) was cloned into the EcoRI site of pGAD-Cl (James et al., 1996).
gated into pGBD-MYO2 1113–1574 missing the corresponding region. For pGBD-myo2 1113–1568, the primers SDP 85 (5'-GACCTGTGCG-CCAATAATGCGTACAGGG-3') and SDP 8R (5'-CGCTTGTGAC-GCTTTGCGGACAAAGTC-3') were used to generate a stop codon at amino acid 1568. The ~1.5-kb EcoRI and ClaI fragment was subcloned into pGBD-C1-myo2 1113–1574 missing the corresponding region.

Random mutagenesis of VAC17
To isolate VAC17 suppressors of myo2-N1304S, PCR mutagenesis was performed using Taq DNA polymerase (Boehringer). Full-length VAC17 was PCR amplified from pRS415-VAC17 using the primer set TF6v (see above) and TF2v (5'-AAACTGCAAGATGGCAGCCGACTTAG-3'). LWY6631 (pCAL-PEP4–URA) was cotransformed with the mutated VAC17 PCR products and a VAC17 plasmid cut with Musel and Spel to remove most of VAC17.

In vivo labeling of organelles
Yeast vacuoles were labeled with N-[3-(triethylammoniumpropyl)]-4(6)(4-diethylaminophenyl)hexatrienyll] pyridinium dibromide (FM4-64; Molecular Probes, Inc.) as described previously (Catlett et al., 2000). Low and high copy VAC17 were expressed from the pRS415 and pRS426 plasmids, respectively (Tang et al., 2003). Nuclei were observed with 4'-DAPI dihydrochloride hydrate (Sigma-Aldrich) as described previously (Sherman et al., 1986).

Immunofluorescence labeling and vacuole purification
Indirect immunofluorescence was performed essentially as described previously (Hill et al., 1996; Catlett et al., 2000). Goat anti-Myo2p tail antisem (Catlett, 2000) was affinity purified as previously described (Reck-Peterson et al., 1999). Fixed cells were incubated with affinity-purified goat anti-Myo2p tail antibody (1:200), followed by Alexa 488-donkey anti-goat IgG (1:200). Vacuole membranes were labeled with mouse anti-yeast v-ATPase 60-kD subunit (1:200), followed by Rhodamine red donkey anti-mouse IgG (1:200). Secondary antibodies and anti-yeast v-ATPase were purchased from Molecular Probes, Inc.

Vacuoles were isolated on a Ficoll flotation gradient as described previously (Govindan et al., 1995). The role of Myo2, a yeast class V myosin, in vesicular transport. J. Cell Biol. 128:1055–1068.


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