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V-ATPase-dependent luminal acidification is required for endocytic recycling of a yeast cell wall stress sensor, Wsc1p

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ABSTRACT

Wsc1p is a major cell wall sensor protein localized at the polarized cell surface. The localization of Wsc1p is maintained by endocytosis and recycling from endosomes back to the cell surface, but changes to the vacuole when cells are subjected to heat stress. Exploiting this unique property of Wsc1p, we screened for yeast single-gene deletion mutants exhibiting defects in Wsc1p trafficking. By expressing 3GFP-tagged Wsc1p in mutants with deleted genes whose function is related to intracellular trafficking, we identified 5 gene groups affecting Wsc1p trafficking, impaired respectively in endocytic internalization, multivesicular body sorting, the GARP complex, endosomal maturation/vacuolar fusion, and V-ATPase. Interestingly, deletion of the *VPH1* gene, encoding the V_o subunit of vacuolar-type H^+-ATPase (V-ATPase), led to mis-localization of Wsc1p from the plasma membrane to the vacuole. In addition, disruption of other V-ATPase subunits (*vma* mutants) also caused defects of Wsc1p trafficking and vacuolar acidification similar to those seen in the *vph1Δ* mutant. Moreover, we found that deletion of the *VPS26* gene, encoding a subunit of the retromer complex, also caused a defect in Wsc1p recycling and mis-localization of Wsc1p to the vacuole. These findings clarified the previously unidentified Wsc1p recycling pathway and requirement of V-ATPase-dependent luminal acidification for Wsc1p recycling.

1. Introduction

Endocytosis and recycling of membrane proteins are important processes regulating various intracellular signaling pathways, such as those responsible for receptor-mediated proliferation, differentiation, and cell wall integrity. Upon internalization from the plasma membrane, cargo proteins are usually delivered to the early endosome, known as the sorting endosome, and sorted to recycling endosomes that bring the cargo back to the plasma membrane, or to late endosomes/multivesicular bodies (MVBs) en route to the lysosome/vacuole for degradation [1,2]. While the role of the recycling endosome in protein recycling has been extensively clarified, another route that involves retrograde transport from the endosome to the trans-Golgi network (TGN), resulting in access to the secretory pathways, has also been characterized in recent years [3].

Cell wall integrity (CWI) signaling is known to be regulated by constitutive endocytosis and recycling of cell wall sensor proteins that detect and transmit information on cell wall status to the Rho1-mediated CWI signaling pathway [4]. In yeast, the cell wall sensor proteins include Wsc1p with the homologous proteins Wsc2-4p and Mid2p, and its homologue Mtl1p [4]. Wsc1p is normally localized to the polarized cell surface at 24 °C, but after cell wall stress imposed by heat shock (shift to 37 °C), the principal site of Wsc1p localization changes from the plasma membrane to vacuoles to down-regulate the CWI signaling pathway [4,5]. Deletion of *SLA1*, or expression of a mutant form of Sla1p lacking the SHD1 domain, which acts as an adaptor for the $NPFX_{(1,2)}D$ endocytic targeting signal, blocks Wsc1p internalization and results in defective polarized deposition of the cell wall with increased sensitivity to perturbation of cell wall synthesis [5]. These findings show that $NPFX_{(1,2)}D$-mediated endocytosis is responsible for directing Wsc1p into an endocytosis and recycling pathway necessary for maintaining cell wall polarity in yeast. However, the mechanism of temperature-regulated Wsc1p sorting is little understood.

Abbreviations: VPS, vacuolar protein sorting; PVC, prevacuolar compartment; MVB, multivesicular body; ESCRT, endosomal sorting complex required for transport; GARP, Golgi-associated retrograde protein.

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Vacuolar-type H^+-ATPase (V-ATPase) is a highly conserved proton pump responsible for acidification of intracellular organelles, such as the lysosome/vacuole, Golgi apparatus, and endosomes, and works together with other ion channels and transporters to maintain pH homeostasis in all eukaryotic cells [6,7]. Eukaryotic V-ATPase is a multi-subunit complex composed of an integral membrane protein, V_o, and a peripheral membrane protein, V_1. Deletion of genes encoding any V-ATPase subunit (*vma* mutants) results in a well defined set of growth defects in yeast, including sensitivity to elevated pH and calcium concentration, inability to grow on non-fermentable carbon sources, and sensitivity to a variety of heavy metals [8]. Previous studies have also suggested a requirement for V-ATPase activity in the intracellular protein trafficking pathway, including endocytosis, vacuolar protein sorting and protein recycling [9,10], but the role of V-ATPase in protein trafficking is still largely unclear.

In this study we screened yeast single-gene deletion mutants exhibiting defects in Wsc1p trafficking and identified the *VPH1* gene, encoding the V_o subunit of vacuolar-type H^+-ATPase (V-ATPase), deletion of which leads to mis-localization of Wsc1p from the plasma membrane to the vacuole. Other V-ATPase gene mutants (*vma* mutants) also exhibited a phenotype similar to that of the *vph1Δ* mutant. We further demonstrate the functional interaction between the retromer complex and V-ATPase-dependent luminal acidification in the Wsc1p recycling pathway.

2. Materials and methods

2.1. Yeast strains and growth conditions

The yeast strains used in this study are listed in Supplementary Table 2. All strains were grown in standard rich medium (YPD) or synthetic medium (SM) supplemented with 2% glucose and appropriate amino acids.

2.2. Plasmids and strain construction

The triple GFP was integrated at the C terminus of the *WSC1* gene as follows: The 3GFP fragment was subcloned into BamHI- and NotI-digested pBlueScript II SK (pBS-3GFP), and the HIS3MX6 was amplified by PCR using pFA6a-GFP (S65T)-HIS3MX6 as a template, and inserted into the NotI and SacII sites of pBS-3GFP to give pBS-3GFP-HIS3 (pBS-3GFP-HIS3). To create an integration plasmid, a fragment of the *WSC1* ORF (nt 721-1134) was generated by PCR and cloned into the BamHI site of pBS-3GFP-HIS3. To integrate 3GFP at the C-terminus of the *WSC1* gene, the integration plasmid was linearized by ApaI and transformed into yeast. GFP, mCherry, and pHluorin were introduced by standard PCR-based method.

2.3. Fluorescence labeling of α-factor and endocytosis assays

Fluorescence labeling of α-factor was performed as described previously [11]. For endocytosis assays, cells were grown to an OD600 of ~0.5 in 0.5 ml YPD, briefly centrifuged, resuspended in 100 μl SM containing 2% glucose and amino acids, and then added 5 μM Alexa Fluor 594-α-factor. After incubation for 20 min at 25 °C, the cells were washed three time with SM, resuspended in 20 μl SM containing 2% glucose and amino acids and observed by microscopy.

2.4. Fluorescence microscopy

Fluorescence microscopy was performed using an Olympus IX81 microscope equipped with a ×100/NA 1.40 (Olympus) objective and Orca-AG cooled CCD camera (Hamamatsu), using Metamorph software (Universal Imaging).

3. Results

3.1. Screening for yeast mutants defective in Wsc1p trafficking

Wsc1p is a cell wall stress sensor and a putative upstream regulator of the CWI signaling pathway [4]. At 24 °C, Wsc1p is localized primarily at the plasma membrane, but changes its localization to the vacuole when the cell wall is subjected to heat shock stress (a shift to 37 °C) [4,5]. To perform in-depth characterization of Wsc1p localization, we tagged endogenous Wsc1p with three tandem copies of GFP (3GFP) at the C terminus, and examined their localization in living cells. Consistent with prior studies using Wsc1-GFP [5], Wsc1-3GFP displayed a mostly polarized cell surface distribution along with some intracellular localization at 25 °C, and changed its distribution to the vacuole upon a temperature shift to 37 °C (Fig. 1A) [5]. To identify proteins required for Wsc1p trafficking, we extracted 251 genes that were possibly related to intracellular protein trafficking from the *Saccharomyces* Genome Database, and expressed Wsc1-3GFP in cells lacking each of the individual genes (Supplemental Table 1). Each of the mutant cells expressing Wsc1-3GFP was grown to early logarithmic phase in YPD medium at 25 °C, exposed or unexposed to heat shock at 37 °C for 2 h, and then studied for Wsc1p localization using epifluorescence and differential interference contrast (DIC) microscopy. This screening identified 24 mutants that exhibited differences in Wsc1-3GFP localization from wild-type cells at 25 and/or 37 °C. These genes were categorized into at least five groups (Table 1). Six of these genes (Group A in Table 1) – *END3*, *RVS161*, *SAC6*, *SLA1*, *SLA2*, and *VRP1* – encoded proteins that have been implicated in endocytic internalization [12]. In cells depleted of these genes, Wsc1-3GFP was localized to the plasma membrane at both 25 and 37 °C (Fig. 1B and Table 1), suggesting defective internalization of Wsc1p in these strains. Another six genes (Group B in Table 1) – *VPS20*, *VPS25*, *VPS27*, *VPS32*, *VPS36*, and *SNF8* – belonged to the class E *vps* (vacuolar protein sorting) family, one of the *vps* mutant subgroups, which exhibits a modest degree of secretion of newly synthesized carboxypeptidase Y (CPY) [13]. The class E *vps* mutants accumulate an exaggerated endosomal/prevacuolar compartment (class E compartment/PVC) that contains endocytosed markers, such as FM4-64 and Alexa Fluor 594-α-factor (A594-α-factor), as well as vacuolar proteins and Golgi membrane proteins that are unable to recycle back to the Golgi complex [13,14]. Wsc1-3GFP also accumulated in the class E compartments, labeled by A594-α-factor, adjacent to the vacuole in these mutants at both 25 and 37 °C (Figs. 1C, 2A, and Table 1). Three genes (Group C in Table 1) – *VPS51*, *VPS52*, and *VPS53* – encoded subunits of the GARP (Golgi-associated retrograde protein) complex, which is a protein complex involved in the recycling of proteins from endosomes to the late Golgi [15]. In the *vps51Δ*, *vps52Δ*, and *vps53Δ* mutants, Wsc1-3GFP showed punctate localization in the cytosol (Fig. 1D and Table 1). Partial co-localization with mCherry-tagged Pep4p, a vacuolar aspartyl protease, and previous observations indicating that the *vps51Δ*, *vps52Δ*, and *vps53Δ* mutants have fragmented vacuoles suggest that some of the Wsc1p is localized to the fragmented vacuoles (Fig. 2B) [16,17]. In mutants with deletion of eight genes (Group D in Table 1), including *VPS11*, *VPS33*, *VPS41*, *VAM3*, *CCZ1*, and *MON1*, which encode proteins required for endosomal maturation and vacuolar fusion processes [18], Wsc1-3GFP displayed a punctate distribution in the cytosol (Figs. 1E and 2C). Taken together, these results demonstrate that Wsc1p is recycled by the GARP complex-mediated endocytic recycling pathway in which endocytosed proteins