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Pil1, an eisosome organizer, plays an important role in the recruitment of synaptojanins and amphiphysins to facilitate receptor-mediated endocytosis in yeast

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ABSTRACT

The eisosome protein Pil1 is known to be implicated in the endocytosis of Ste3, but the precise biological function of it during endocytosis is poorly understood. Here, we present data to reveal Pil1's role in receptor-mediated endocytosis. Using live cell imaging, we show that endocytic patches carrying Abp1 and Las17 persisted much longer in *PIL1*-deficient cells. The loss of Pil1 also greatly affected both the scission efficiency and the frequency of formation of endocytic sites carrying Rvs161- and Rvs167-GFP. Furthermore, the mistargeting of the synaptojanins, Sjl1 and Sjl2, to the cytoplasm in *pil1*Δ cells suggests that Pil1 is required for the proper recruitment of the synaptojanins to endocytic sites. A severe motility defect of Abp1-GFP during its internalization in a codeletant of *PIL1* and *SJL2* indicates a functional interplay between them in endocytosis. Together, these results establish that Pil1 is involved in the recruitment of endocytic proteins to optimize endocytosis.

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Introduction

Endocytosis is the process in which the plasma membrane invaginates to take up essential nutrients, lipids, and surface receptors. At the molecular level, the mechanism by which endocytosis initiates at particular sites on the plasma membrane is poorly understood. However, previous studies have suggested that large immobile protein assemblies called eisosomes not only mark the site of endocytosis but also ensure an even distribution of potential endocytic sites (Moreira et al., 2009; Walther et al., 2006). It appears that Pil1 plays a more important role than other components of the eisosome in maintaining the structural integrity of it since loss of Pil1 leads to a severe disruption of eisosome organization (Grossmann et al., 2007; Walther et al., 2006). Two functionally redundant serine/threonine protein kinases Pkh1/2, which physically associate with the eisosome, have been shown to phosphorylate Pil1 *in vitro*, and perturbation of Pil1 phosphorylation affects Pil1 assembly into eisosomes (Walther et al., 2007; Zhang et al., 2004): the dephosphorylated form of Pil1 preferentially assembles into eisosomes, and hyperphosphorylation of Pil1 causes its disassembly from eisosomes. Besides Pil1 and Pkh1/2, many more genes (88) appear to be involved in the organization of the eisosome according to a genome-wide screen for genes affecting eisosomes (Frohlich et al., 2009).

Membrane invagination and its subsequent pinch-off event are followed by spatiotemporal recruitment of endocytic proteins. Once an endocytic site is selected, actin-independent endocytic proteins, such as clathrin, Las17, and Pan1, are recruited to the site (Jonsdottir and Li, 2004; Kaksonen et al., 2003, 2005; Newpher et al., 2005). Actin and actin binding proteins, including Abp1 and Arp2/3 complex, then arrive at the site, and initiate rapid polymerization of actin that drives the formation of the invaginated membrane (Merrifield et al., 2004). Membrane fission must then occur to release an endocytic vesicle, perhaps by a rapid burst of actin polymerization and the membrane tubulating activity of the amphiphysins, Rvs161 and Rvs167 (Galletta and Cooper, 2009; Kaksonen et al., 2005; Youn et al., 2010).

A recent yeast endocytic fission model emphasizes the role of the amphiphysins for proper scission (Liu et al., 2009): based on the finding by a previous EM study that Rvs167 localizes at the intermediate area (or neck) of invaginated pits (Idrissi et al., 2008), Liu et al. (2009) proposed that the amphiphysins bind to the membrane curvature at the neck and protect the underlying PIP_2 from hydrolysis mediated by the access of the synaptojanins, Sjl1 and Sjl2. In contrast, they predicted that depletion of PIP_2 occurs rapidly at the tip of the invagination due to the full access of the synaptojanins. The unequal levels of PIP_2 across the endocytic invagination appear to generate an interfacial force which squeezes the bud neck and as a result vesicle scission occurs.

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Although previous evidence has suggested that Pil1 is implicated in yeast endocytosis (Walther et al., 2006), the precise function of it during the endocytic process remains undiscovered. In this study, we provide evidence that Pil1 functions in proper recruitment of the synaptojanins and the amphiphysins to endocytic sites, required for efficient receptor-mediated endocytosis. Importantly, we show that the deletion of *SJL2* in the background of *pil1*Δ further perturbs endocytosis, most likely due to the disruption in the actin cytoskeleton.

Methods and materials

Yeast strain construction and media

Yeast strains used in this study are listed in Table 1. Strains expressing GFP- or RFP-fused proteins were constructed by integrating respective GFP or RFP sequence at the 3′end of the gene of interest as described previously (Kim et al., 2006; Longtine et al., 1998; Nannapaneni et al., 2010). Cells expressing both GFP- and RFP-fused proteins were constructed by the same method. To generate gene deletion mutants, WT cells were transformed with PCR product carrying a disruption construct as described before (Longtine et al., 1998). Double deletion mutant strains (*pil1*Δ*sjl2*Δ, *pil1*Δ*rvs161*Δ, and *pil1*Δ*rvs167*Δ) were prepared by crossing various strains of haploid *pil1*Δ cells with *sjl2*Δ, *rvs161*Δ, or *rvs167*Δ, followed by dissecting haploid seeds and by screening the genotypes of them. One deletion strain (*sjl2*Δ) was obtained from Scott Emr's lab. All yeast strains were grown in standard yeast peptone dextrose (YPD) medium and cultured at 30 °C, unless otherwise stated.

Spinning confocal microscopy

Time-lapse movies and still images of GFP or RFP labeled cells were made with a spinning disk confocal system that includes an inverted Olympus IX81 microscope, a Yokogawa CSUX1 spinning disk head, a 100× numerical aperture (NA) 1.4 PlanApo oil objective, and an Electron Amplified CCD (ImagEM, Hamamatsu). The temperature of the specimen and stage was maintained at 30 °C. The image was focused at an equatorial plane of the cells. For time-lapse movies of GFP- or RFP-fused proteins, a single channel was utilized, and movies were captured at 2 frames/s for movie duration of 1 min. Simultaneous two-color imaging was done using an image splitter to separate red and green emission signals.

Measurement of patch lifetime at the membrane and kymograph

To examine patch lifetime at the membrane we used the following 3 independent microscopic analyses. First, the time spent by a GFP-fused protein at the membrane (from the time of its appearance to the time at which it moves away from its origin or disappears) was manually determined as described previously (Nannapaneni et al., 2010). For example, a total of 15–30 newly forming patches at the membrane were used to determine the mean membrane lifespan of Abp1-GFP patches. Patches that at any point in their lifetime are too close to another patch to be clearly resolved were excluded from our analysis. Second, to directly visualize the duration of time spent by a patch on the membrane, a kymographic representation of GFP- or RFP-fused proteins in a single patch over time was made using SlideBook (v.5). Third, the fluorescence intensity profile of endocytic patches over time was also made using SlideBook (v.5).

Computer-assisted tracking of patch movement

Time-lapse movies of GFP- or RFP-fused proteins in WT and *pil1*Δ cells were analyzed with the aid of a patch tracking software to quantitate the behavior and degree of patch motion. As described previously (Carlsson et al., 2002; Kim et al., 2006; Nannapaneni et al., 2010), the square of the patch displacement (μm^2) from the origin over time was determined. The mean squared displacement (MSD, μm^2) of Abp1-GFP patches was calculated by analyzing 15–30 newly forming patches at the cell cortex.

Actin staining with fluorescein isothiocyanate (FITC)-Phalloidin

Yeast actin cytoskeleton was stained with FITC-Phalloidin using the method described previously (Kamble et al., 2011). Cells were fixed by formaldehyde (5% final concentration) and sonicated briefly. F-actin was stained by 1 μl of a 3.3 μM stock of FITC-Phalloidin for 15 min in dark. Cells were visualized under the spinning confocal microscope equipped with Orca-R2 camera. To determine the percentage of actin polarization, fluorescence images were quantified by counting 50 cells from each strain. Cells were classified as polarized when fewer than 5 actin patches were present in the mother portion of small budded cells. In particular, to measure the amount of F-actin at the cortical patches, images were taken at the same exposure rate of 100 ms, followed by measuring the fluorescence intensity of the cortical patches using ImageJ software.

Cell growth assay

A spotting assay was performed to dilute cells by a factor of 5 onto YPD plates. The plates were then grown for 2 days at 30 °C or 37 °C. A liquid growth assay was performed to determine doubling time of cells.

Results

Loss of Pil1 affects receptor-mediated endocytosis

Consistent with the previous findings that Pil1 plays a role in endocytosis (Walther et al., 2006), we found a delay in the trafficking of FM4-64 dye, which follows liquid-phase endocytosis, to the vacuole in *pil1*Δ cells (Data not shown). In order to investigate whether Pil1 plays a role in receptor-mediated endocytosis (RME), we tagged two well-validated RME markers Las17 and Abp1 with GFP at their C-termini, and recorded time-lapse fluorescence images. Las17, an Arp2/3 complex activator, arrives at endocytic sites and stays immobile prior to its disassembly (Kaksonen et al., 2003). To characterize any effects caused by loss of Pil1 on the assembly dynamics of Las17-GFP, we measured the membrane lifespan of Las17-GFP in both WT and *pil1*Δ cells by calculating the time spent by Las17-GFP at the endocytic site. The mean membrane lifespan of Las17-GFP in WT cells (28.8 ± 3.4 s) was in agreement with that of previous studies (Kaksonen et al., 2003; Kim et al., 2006; Nannapaneni et al., 2010), whereas Las17-GFP in *pil1*Δ cells persisted much longer at its origin with the mean lifespan of 51.5 ± 17.3 s (Fig. 1A). Actin binding protein Abp1 is a well-characterized late marker of RME. It arrives at endocytic sites (hitherto, actin patch) approximately 5–8 s before pinch-off process, moves away from the site in a directed manner, and departs from the postinternalized actin patches to be reused for the next round of endocytosis (Kim et al., 2006; Toret et al., 2008). The membrane lifespan of Abp1-GFP (17.2 ± 3.2 s) in *pil1*Δ cells was about two times longer than in WT cells (8.2 ± 1.7 s) (Fig. 1B). As shown in Fig. 1C and D, our kymograph and fluorescence intensity profile