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The yeast cell wall integrity pathway signals from recycling endosomes upon elimination of phosphatidylinositol (4,5)-*bis*phosphate by mammalian phosphatidylinositol 3-kinase

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ABSTRACT

Phosphatidylinositol (4,5)-*bis*phosphate [PtdIns(4,5)P_2] is essential for recognition of the plasma membrane inner leaf by protein complexes. We expressed mammalian class I phosphoinositide 3-kinase (PI3K) in *Saccharomyces cerevisiae* to eliminate PtdIns(4,5)P_2 by its conversion into PtdIns(3,4,5)P_3, a lipid naturally missing in this yeast. This led to loss of actin function and endocytosis defects, causing a blockage in polarized secretion. Also, the cell wall integrity (CWI) mitogen-activated protein kinase (MAPK) pathway was activated, triggering a typical transcriptional response. In the absence of PtdIns(4,5)P_2 at the plasma membrane, the Pkc1 protein kinase upstream the CWI MAPK module localized to post-Golgi endosomes marked by SNARE Snc1 and Rab GTPases Ypt31 and Ypt32. Other components at the head of the pathway, like the mechanosensor Wsc1, the GTPase Rho1 and its activator the GDP/GTP exchange factor Rom2, co-localized with Pkc1 in these compartments. Chemical inhibition of PI3K proved that both CWI activation and Pkc1 relocation to endosomes are reversible. These results suggest that the CWI pathway is able to respond to loss of plasma membrane identity from recycling endosomes.

1. Introduction

Phosphoinositides are lipid molecules, generated by the phosphorylation of phosphatidylinositol (PtdIns), that regulate signaling and membrane trafficking in all eukaryotic cells [1]. They operate by recruiting intracellular protein effectors through specific interaction domains, thus favoring the dynamic formation of binary and ternary signaling complexes, often in collaboration with small GTPases. There are seven species of phoshoinositides, depending on which positions are phosphorylated in the inositol ring. Each of them presents a limited subcellular distribution that provides identity to the membrane in which they are enriched, thus coordinating a particular local function. The conversion among these species is carried out by kinases and phosphatases that act precisely to keep the appropriate balance [2,3]. PtdIns(4,5)P_2 represents less than 1% of the total cellular phospholipids, but regulates important functions like migration, adhesion, cell polarity, actin cytoskeleton organization, clathrin-mediated endocytosis, lipid homeostasis and signal transduction [4]. In mammalian cells, PtdIns(4,5)P_2 is converted by class I phosphatidylinositol 3-kinase (PI3K) into PtdIns(3,4,5)P_3, a phosphoinositide that is known to promote proliferative and anti-apoptotic pathways [5–7].

The conservation of basic cellular processes between the budding yeast *Saccharomyces cerevisiae* and higher eukaryotes has universalized its use as a model organism. However, class I PI3K and subsequent PtdIns(3,4,5)P_3-dependent signaling is absent in *S. cerevisiae*. Interestingly, heterologous expression of a plasma membrane-targeted version of class I PI3K mammalian catalytic subunit p110α leads to severe growth inhibition that is dependent on PI3K catalytic activity, due to depletion of the essential pools of PtdIns(4,5)P_2 [8]. This "humanized" yeast model has been proven to be not only useful for the realization of both genetic studies on the PI3K pathway [9] and *in vivo* inhibition bioassays in search for PI3K inhibitors [10], but it is also valuable for exploring the function and relevance of PtdIns(4,5)P_2 in this organism.

In *S. cerevisiae*, PtdIns(4,5)P_2 is synthesized at the plasma membrane, where the PtdIns(4)P 5-kinase Mss4 phosphorylates PtdIns(4)P, in turn generated from PtdIns by the Stt4 kinase. *MSS4* is an essential gene, but genetic studies based on thermosensitive mutant versions of the kinase have solidly established the involvement of PtdIns(4,5)P_2 in the regulation of actin cytoskeleton, as in higher eukaryotes [11–14]. Also this phosphoinositide directly regulates clathrin-mediated endocytosis, namely at the stages of cytoplasmic internalization and vesicle scission [15]. Several endocytic patch adaptors contain specific protein domains which mediate interaction with PtdIns(4,5)-P_2 [16,17]. In

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addition, this molecule carries out a very important function as a cell polarity marker, directing the attachment of exocyst components to the plasma membrane at the final stage of the secretory pathway [18,19]. In a nutshell, all trafficking events at the level of the plasma membrane that determine its function, protein composition and polarized growth rely on the presence of this phosphoinositide. Still, many functional aspects of this lipid and the regulation of its levels are largely unknown.

Our previous work showed that conversion of PtdIns(4,5)P_2 into PtdIns(3,4,5)P_3 by expression of heterologous PI3K in *S. cerevisiae*, in agreement with the reported roles for PtdIns(4,5)P_2, caused loss of actin cytoskeleton polarity and alterations in the endocytic transport. Interestingly, it also led to phosphorylation of the Slt2 MAPK of the cell wall integrity (CWI) pathway [8]. This pathway is activated in response to cell wall stress and leads to the transcriptional regulation of genes involved in cell wall remodeling. At particular cell cycle stages, such as budding and cytokinesis, CWI activation takes place in polarized growth sites, where the localized synthesis of new cell wall polymers is important for cell growth or separation [20]. The stress is sensed by transmembrane mechanosensors (Wsc1/2/3, Mid2 and Mtl1) which transmit the signal to the GTPase GEFs (GDP/GTP Exchange factors) Rom1/2. These proteins promote the activation of the small GTPase Rho1, which in turn activates protein kinase C (Pkc1), which is directly upstream the CWI MAPK cascade. Pkc1 phosphorylates the MAPKKK Bck1, which activates the MAPKKs Mkk1 and Mkk2 that finally phosphorylate the MAPK Slt2/Mpk1. Once activated, Slt2 phosphorylates the transcription factor Rlm1, among other substrates. Rlm1 is responsible for the major transcriptional response to CWI pathway activation [21], aimed to synthesize and repair cell wall, reorganize actin cytoskeleton and repolarize secretion to growth sites. PtdIns(4,5)P_2 has been reported to be involved in signal transduction through the CWI pathway, likely through interaction with the Pleckstrin Homology (PH) domain of Rom2 [22,23].

Here, to gain further insight into the role of this phosphoinositide in cell signaling, we take advantage of heterologous expression of PI3K to study the effects of its conversion to futile PtdIns(3,4,5)P_3 in yeast cells. We show that PtdIns(4,5)P_2 depletion activates the CWI pathway and generates a transcriptional profile reminiscent of that induced by cell wall aggressions. However, the components at the head of the pathway, instead of being recruited to the plasma membrane as in the case of other stimuli, are concentrated at cytoplasmic compartments which are consistent with recycling post-Golgi endosomes (PGEs). This is the first evidence to our knowledge of a yeast MAPK pathway operating from internal membranes, a mechanism that might allow the cell to restore polarized secretion upon severe loss of plasma membrane special cues.

2. Materials and methods

2.1. Plasmids

YCpLG-Myr-PI3KαQ was generated by PCR amplification of nucleotides 1–1900 of human *PIK3CA* ORF with primers containing *Bam*HI/*Xba*I sites at 5′ tails and subcloned into plasmid YCpLG-PI3Kα [8]. Plasmid YEplac112-mCherry was constructed by PCR amplification of the sequence of mCherry with primers containing *Bam*HI/*Eco*RI sites at 5′ tails and subcloned into plasmid YEPlac112 [24]. YEPlac112-Anp1-mCherry, YEPlac112-Snf7-mCherry and YEPlac112-Ste2-mCherry were constructed by PCR amplification of *STE2*, *ANP1* and *SNF7* coding sequence with primers containing *Sph*I/*Bam*HI sites at 5′ tails and subcloned into plasmid YEPlac112-mCherry. Plasmid YCplac22-GAL-PI3Kα-CAAX was constructed by introducing the *GAL1* promoter sequence, flanked by *Eco*RI/*Bam*HI sites, into the corresponding sites in plasmid YCplac22, giving raise to YCplac22-GAL1. The PI3Kα-CAAX-coding sequence was amplified by PCR from plasmid YCpLG-PI3Kα-CAAX and subcloned into *Bam*H1 site in YCplac22-GAL1. pVD67-Pkc1-mCherry was constructed by PCR amplification of mCherry sequence with primers containing *Pst*I sites at 5′ tails and introduction into pGEM-T (Promega, Fitchburg, WI, USA). By site directed mutagenesis, an internal *Pst*I site at mCherry was eliminated, and then it was subcloned into the *Pst*I site of pVD67. For the construction of YCplac111-mCherry-Rho1, the *RHO1* gene (ORF plus 500 bps upstream) was amplified by PCR with primers containing *Hind*III/*Bam*HI sites at 5′ tails and subcloned into pGEM-T. Then, site directed mutagenesis was performed to generate an *Xba*I site just upstream the *RHO1* coding sequence. Modified *RHO1* was then subcloned into YCplac111 [24] at *Hind*III/*Bam*HI sites. Finally, the mCherry sequence was amplified by PCR with primers containing *Xba*I sites at 5′ tails and subcloned into YCplac111-Rho1. *E. coli* DH5α and yeast transformation as well as other basic molecular biology methods were carried out by standard procedures.

YCpLG-PI3Kα-CAAX, YCpLG-PI3K (K802R)-CAAX [8], pRS315-HA-GFP-cSNC1 [25], pMLP1-lacZ [26], pRS426-GFP-2XPH(PLCδ) [27], pVD67 [28], pRS414-dsRED-Vps21, pRS415-Vps3-GFP, pRS415-Vps8-GFP [29], pRS414-mCherry-Ypt7, pRS414-dsRED-Pep12 [30] and pRC647 [31] were already described. These reagents were kindly donated by J. Arroyo, S. Emr, M. Cyert, M. Cabrera, C. Ungermann and R. Collins. Plasmid pRC630, expressing GFP-Ypt32, was kindly provided by R. Collins.

2.2. Strains, culture media and growth conditions

Yeast strains used in this work are listed in Table S1.

YPD [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose/glucose] broth or agar was the general non-selective medium used for yeast cell growth. Synthetic dextrose (SD) medium contained 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate and 2% glucose and 1.24 g/l synthetic complete mixture drop out mix lacking the appropriate amino acids and nucleic acid bases to maintain plasmid selection. In SG (synthetic galactose) and SR (synthetic raffinose) media, glucose was replaced with 2% (w/v) galactose or raffinose.

For *GAL1* promoter induction yeast cells were cultured in SR medium for 18 h, then fresh SR media was added to a final OD_{600} of 0.3 and galactose was added to a final concentration of 2%. Cultures were incubated for 4–6 h at 30 °C. Spot growth assays on agar were performed as described [8].

2.3. DNA microarray transcriptomic analysis

For RNA extraction, 5 ml of the cultures were collected by centrifugation at 3000 rpm at 4 °C and RNA was extracted by mechanical breakage using a kit RNeasy MIDI kit (Qiagen, Venlo, Netherlands). RNA concentration was measured at 260 nm in a ND-1000 spectrophotometer and its quality and size profile was checked by capillary chromatography in a Bioanalyzer 2100B (Agilent Technologies, Santa Clara, CA, USA) chromatographer. For each condition RNA from cultures from three different transformants was extracted.

One-cycle cDNA Synthesis and GeneChip Sample Cleanup Module kits (Affymetrix, Santa Clara, CA, USA) were used for reverse transcription and subsequent purification. Template transcription was carried out by using biotinylated ribonucleotide analogs and the cRNA GeneChip IVT Labeling Kit, purified as above and then hydrolyzed onto 35 to 200 pb fragments, which contains primers representing approximately every ORF from *S. cerevisiae*. The biotinylated cRNA hybridization cocktail was incubated for 16 h at 45 °C on an Affymetrix platform, then washed in a GeneChip Fluidics Station 450 and stained with streptavidin-phycoerythrin biotinylated anti-streptavidin fluorescent antibodies. GCRMA software [32] was used for image analysis and data processing. Differential gene expression was expressed as a ratio obtained by dividing the average fluorescence signal of PI3K-expressing samples by that of the control. It was considered gene induction when ratio was ≥1.7 and gene repression when ratio was below ≤0.6. Statistical analysis was performed by Cyber-t program (http://