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Role of Plc1p in regulation of Mcm1p-dependent genes

Katarzyna Guzinska, **Roger Varghese**, and **Ales Vancura***
Department of Biological Sciences, St. John's University, Queens, New York 11439

Abstract

In budding yeast, phosphoinositide-specific phospholipase C (Plc1p encoded by *PLC1* gene) and several inositol polyphosphate kinases represent a nuclear pathway for synthesis of inositol polyphosphates (InsPs) that are involved in several aspects of DNA and RNA metabolism, including transcriptional regulation. Plc1p-produced $InsP_3$ is phosphorylated by Ipk2p/Arg82p to yield $InsP_4$/$InsP_5$. Ipk2p/Arg82p is also a component of ArgR-Mcm1p complex that regulates transcription of genes involved in arginine metabolism. The role of Ipk2p/Arg82p in this complex is to stabilize the essential MADS box protein Mcm1p. Consequently, *ipk2*Δ cells display reduced level of Mcm1p and attenuated expression of Mcm1p-dependent genes. Since *plc1*Δ cells display aberrant expression of several groups of genes, including genes involved in stress response, the objective of this study was to determine whether Plc1p also affects expression of Mcm1p-dependent genes. We report here that not only *ipk2*Δ, but also *plc1*Δ cells display decreased expression of Mcm1p-dependent genes. However, Plc1p is not involved in stabilization of Mcm1p and affects transcription of Mcm1p-dependent genes by a different mechanism, probably involving regulation of chromatin remodeling complexes.

Keywords

phospholipase C; *MCM1*; transcriptional regulation; inositol polyphosphates

Introduction

In budding yeast *Saccharomyces cerevisiae*, phospholipase C (Plc1p encoded by *PLC1* gene) and four inositol polyphosphate kinases (Ipk2p/Arg82p, Ipk1p, Kcs1p, and Vip1p) constitute a nuclear signaling pathway that is responsible for synthesis of inositol polyphosphates (InsPs) and affects transcriptional control (Odom, *et al.*, 2000), export of mRNA from the nucleus (York, *et al.*, 1999), homologous DNA recombination (Luo et al., 2002), cell death, and telomere length (Saiardi et al., 2005; York et al., 2005). Hydrolysis of phosphatidylinositol-4,5-bisphosphate by phospholipase C yields inositol trisphosphate ($InsP_3$) and diacylglycerol, and is the only pathway for InsPs synthesis in budding yeast cells. Ipk2p/Arg82p is a dual specificity kinase that converts Plc1p-generated $InsP_3$ into $InsP_5$ via $InsP_4$ (Odom et al., 2000). $InsP_4$ and $InsP_5$ are involved in transcription by regulating chromatin remodeling complexes (Shen et al., 2003; Steger et al., 2003). $InsP_5$ is subsequently converted to $InsP_6$ by Ipk1p. $InsP_6$ is an effector molecule that regulates mRNA export from the nucleus (York et al., 1999). The mechanism involves binding of $InsP_6$ by nuclear pore protein Gle1p and stimulation of RNA-dependent ATPase activity of Dbp1p, which is essential for nuclear mRNA export (Weirich et al., 2006; Alcazar-Roman et al., 2006). Kcs1p and Vip1p are $InsP_6$ and $InsP_7$ kinases responsible for synthesis of 5-PP-$InsP_5$ and 4-PP-$InsP_5$/6-PP-$InsP_5$, respectively (Saiardi et al., 1999, 2000; Mulugu et al.,

*Corresponding author. Mailing address: Department of Biological Sciences, St. John's University, 8000 Utopia Parkway, Queens, NY 11439. Phone: +1 718 990-6287. Fax: +1 718 990-5958. vancuraa@stjohns.edu.

2007; Lee et al., 2007). Ultimately, Kc1p and Vip1p can produce $InsP_8$ molecules 4,5-PP_2-$InsP_4$ and 5,6-PP_2-$InsP_4$. It appears that inositol pyrophosphates are required for number of cellular functions, including inhibition of Pho80p-Pho85p cyclin-CDK complex by the Pho81p inhibitor (Lee et al., 2007; Luo et al., 2002; Saiardi et al., 2005; York et al., 2005).

Ipk2p/Arg82p is together with Arg80p, Arg81p, and Mcm1p a component of the transcriptional complex ArgR-Mcm1 that regulates transcription of genes involved in arginine metabolism (El Bakkoury et al., 2000). When arginine is present, these four proteins repress synthesis of arginine biosynthetic enzymes and induce synthesis of catabolic enzymes. Arg80p and Arg81p are specific regulators of the arginine system, while Ipk2p/Arg82p and Mcm1p are global regulators involved in other processes as well (Dubois & Messenguy, 1991, Messenguy & Dubois, 1993). Arg81p is the sensor of arginine that interacts with the two MADS box proteins Arg80p and Mcm1p to form a complex at the promoters of arginine regulated genes (El Bakkoury et al., 2000; Messenguy and Dubois, 2003). Mcm1p is an essential protein that plays a role in transcription of genes involved in M/G1 and G2/M cell-cycle progression, mating, recombination, and stress tolerance (Messenguy and Dubois, 2003). The role of Ipk2p/Arg82p in the regulation of arginine-responsive genes consists in binding and stabilizing both Arg80p and Mcm1p but does not involve its $InsP_3$ kinase activity. However, the kinase activity of Arg82p is required for proper expression of genes regulated by phosphate and nitrogen (El Alami et al., 2003).

Using genome-wide expression analysis our laboratory found previously that not only *ipk2*Δ, but also *plc1*Δ cells display decreased expression of Mcm1-dependent genes (Demczuk, *et al.*, 2008). Hence, in this study our objective was to determine whether Plc1p in addition to Ipk2p is also required for stabilization of Mcm1p and thus for expression of Mcm1p-dependent genes. In this report we demonstrate that Plc1p is required for expression of Mcm1-depended genes, however, it does not affect the stability of Mcm1p. Therefore, Plc1p affects expression of Mcm1-depended genes by a different mechanism, probably by affecting the activity of chromatin remodeling complexes.

Materials and Methods

Strains and media

All yeast strains are listed in Table 1. Standard genetic techniques were used to manipulate yeast strains and to introduce mutations from non-W303 strains into the W303 background (Sherman, 1991). Cells were grown in rich medium (YPD; 1% yeast extract, 2% Bacto-peptone, 2% glucose) or under selection in synthetic complete medium (CSM) containing 2% glucose and, when appropriate, lacking specific nutrients in order to select for a plasmid or strain with a particular genotype. Meiosis was induced in diploid cells by incubation in 1% potassium acetate.

B-Galactosidase Assays

Wild-type, *plc1*Δ, and *ipk2*Δ strains were transformed with the following plasmids: pFV55 (*CLN3-LacZ*), pFV56 (*FAR1-LacZ*), pFV57 (*PIS1-LacZ*), pFV58 (*CDC6- LacZ*), and pFV60 (*PMA1-LacZ*) (El Bakkoury, *et al.*, 2000). The transformants were grown under selection in CSM-Ura medium at 30°C and subsequently diluted in YPD medium to an $A_{600\ nm}$ of 0.2 and grown until the culture reached $A_{600\ nm} \sim 1.0$. Cells from 10 ml culture were harvested by centrifugation and resuspended in β-galactosidase breaking buffer (100 mM Tris-HCl pH 8.0, 1 mM DTT, 20 % glycerol) containing protease inhibitors (Roche; Complete protease inhibitors) and 2 mM PMSF (phenylmethylsulfonyl fluoride). Samples were disrupted by vortexing with glass beads, and 10-100 μl of the collected supernatant was added to 0.9 ml of Z buffer (60 mM $Na_2HPO_4.7H_2O$; 40 mM $NaH_2PO_4.H_2O$; 10 mM

KCl; 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0). The volume was adjusted up to 1 ml with β-galactosidase breaking buffer and the assay mixture incubated in water bath at 28°C for 5 min with moderate shaking. Reaction was initiated by adding 0.2 ml of *o*-nitrophenyl-β-D-galactopyranoside (ONPG; 4 mg/ml in Z buffer) and continued at 28°C until mixture turned pale yellow and terminated by addition of 0.5 ml of 1 M Na_2CO_3 (Choi et al., 1998). Protein concentration was determined using the Coomassie Plus protein assay kit (Pierce). The specific activity was expressed in terms of nanomoles of ONPG hydrolyzed per minute per mg of protein.

Real-time RT- PCR analysis

Total RNA was isolated from cultures grown in YPD medium to optical density A_{600nm} ~ 1.0 by the hot phenol method as described previously (Iyer & Struhl, 1996), treated with RNase-free DNase (Qiagen), and purified with an RNeasy mini kit (Qiagen). RT and real-time PCR amplification using BioRad MyIQ single color real-time PCR detection system (Bio-Rad) were performed with iScript kit (BioRad), 100 ng of RNA, and the following primers: *ACT1* (5′-TATGTGTAAAGCCGGTTTTGC-3′and 5′-GACAATACCGTGTTCAATTGGG-3′), *STE2* (5′-ACCATCACTTTCGATGAGT TGC-3′ and 5′-GGTTGATAATGAAAATCGGCG-3′), *STE3 (*5′-CCTTTAGCAT GGCATTCACATAC-3′ and 5′-GATATGCCAATATTCGCACCAAC-3′), *BAR1* (5′-ACGAAGAGGAGATGTATTACGCAAC-3′and 5′-ACCTGCAATCAATTGAAGGC-3′), *MFalpha1* (5′-GCTGAAGCTGTCATCGGTTACTTAG-3′ and CCAGGTTTT AGTTGCAACCAATG-3′).

Western blotting

Yeast cultures were grown in 200 ml YPD to an optical density A_{600nm} =1.0. To determine the Mcm1p stability, 20 μg/ml cycloheximide (CYH) was added at time zero. At different time points after the treatment, cells from 30 ml of the culture were harvested by centrifugation and subsequently converted to spheroplasts using yeast lytic enzyme (Sigma; 700 U/ml in 10 mM Tris-HCl buffer, pH 7.5, containing10 % sucrose). Protein concentration of the samples was determined using Coomassie Plus protein assay kit (Pierce). Denatured proteins were separated on 10% denaturing polyacrylamide gel and Western blotting with 0.5 μg/ml of anti-TAP antibody (Open Biosystems) was carried out as described previously (Demczuk et al., 2008) and the blots were visualized using GeneGnome Bioimaging System (Syngene). To confirm equivalent amounts of loaded proteins, the membrane was stripped and incubated with anti-3-phosphoglycerate kinase (PGK) antibody (Molecular Probes).

Results and Discussion

To identify transcriptional targets of InPs, our laboratory performed genome-wide expression analysis with wild-type, *plc1*Δ, *ipk2*Δ, and *ipk1*Δ strains (Demczuk et al., 2008). In addition to increased expression of Msn2p-dependent stress-responsive genes in *plc1*Δ cells, we also observed decreased expression of cell-type-specific and Mcm1p - dependent genes such as *BAR1*, *MFA1* and *STE2* in *ipk2*Δ strain (Demczuk et al., 2008). This was not entirely surprising, since *ipk2*Δ cells display decreased stability of Mcm1p (El Bakhoury et al., 2000). However, several of the Mcm1p-dependent genes were expressed at a lower level also in the *plc1*Δ cells (Demczuk et al., 2008). These results suggested that Plc1p and/or synthesis of InsPs may be required for full expression of Mcm1p-dependent genes, perhaps also by affecting the stability of Mcm1p. One possible model that could account for the stabilizing effect of $InsP_3$ on Mcm1p would involve assumption that Ipk2p/Arg82 must bind InsPs in order to stabilize Mcm1p.

To analyze the involvement of Plc1p and $InsP_3$ in the control of Mcm1-dependent genes, wild-type, *plc1*Δ, and *ipk2*Δ strains were transformed with plasmids containing *lacZ* reporter gene under the control of Mcm1p-regulated promoters of *CLN3, FAR1, PIS1, CDC6* and *PMA1* (El Bakkoury, *et al.*, 2000). As reported previously (El Bakkoury, et al., 2000), β-galactosidase activities were strongly reduced in *ipk2*Δ strain in comparison to wild-type strain. In *plc1*Δ strain the activities were also reduced, however to a lesser extent than in the *ipk2*Δ strain (Fig. 1). These results thus suggest that Plc1p is also required for full expression of Mcm1p-dependent genes, however, Plc1p appears to be less important than Ipk2p.

Since the expression of Mcm1p-dependent genes might be affected by promoter chromatin structure that may not be faithfully reconstituted in the context of reporter plasmids, we determined expression of several Mcm1p-dependent genes in their normal chromosomal locations. RNA was isolated from both *MATa* and *MATα* cells of wild-type, *plc1*Δ, and *ipk2*Δ strains, and the relative transcript levels of a-specific genes (*BAR1*, *STE2*) and α-specific genes (*STE3* and *MFα1*) were determined. In *MATα* cells, cell-type specific transcriptional factor Matα1p and Mcm1p bind cooperatively to promoters of corresponding genes, thus activating α -cell-type-specific gene expression. In a cells, Mcm1p activates transcription of a - specific genes by binding to the Mcm1-binding site found in promoter regions of a - specific genes. Thus, expression of α – specific genes in *MATα* cells as well as expression of a – specific genes in *MATa* cells requires Mcm1p. Previous studies have shown that Ipk2p is required for the expression of certain a - and α - specific genes that are also controlled by Mcm1p (Dubois and Messenguy, 1994). As shown in Figure 2A, the transcription levels of a - specific genes *BAR1* and *STE2* in *ipk2*Δ mutant were significantly lower than those found in wild-type cells. The same pattern was observed in *plc1*Δ strain, however, the decrease was not as dramatic as in *ipk2*Δ strain (Fig. 2A). The transcript levels of α - specific genes *STE3* and *MFα1* in *ipk2*Δ strain were also significantly reduced as compared to wild-type. Again, the pattern observed in *plc1*Δ strain was similar but the decrease was not as dramatic as in *ipk2*Δ strain (Fig 2B).

The above results support the conclusion that not only Ipk2p, but also Plc1p is important for full expression of Mcm1p-dependent genes. However, the *ipk2*Δ mutation affects expression of Mcm1p-dependent genes more significantly than *plc1*Δ mutation. Since Ipk2p affects expression of Mcm1p-dependent genes by physically interacting with and stabilizing Mcm1p (El Bakkoury et al., 2000; El Alami et al., 2003), we considered possibility that Plc1p also affects intracellular level of Mcm1p. There are several indications that Plc1p may be involved in regulation of stability of certain proteins. First, 26S proteasome-mediated destruction of C-type cyclin Ume3p/Srb11p/Ssn3p upon oxidative stress requires Plc1p (Cooper et al., 1999). Second, genome-wide identification of protein complexes revealed that Plc1p interacts with Caf130p (Krogan et al., 2006), a component of Ccr4/Not transcriptional regulatory complex. One of the subunits of the Ccr4-Not complex is Not4p, an ubiquitin E3 ligase (Albert et al., 2002; Collart, 2003) that interacts with Ubc4p, another ubiquitin-conjugating enzyme. In addition, the Ccr4-Not complex associates with the proteasome (Laribee et al., 2007). These findings suggest that at least fraction of Plc1p is found in a molecular complex with ubiquitin-conjugating enzymes and proteasome. In addition, we have described previously that Plc1p is required for recruitment of histone aceryltransferase complex SAGA to Sko1p-regulated promoters (Guha et al., 2007). Coincidentaly, the proteasome 19S regulatory particle was found to facilitate loading of SAGA onto chromatin (Lee et al., 2005). These findings prompted us to test whether Plc1p, similarly to Ipk2p, affects stability of Mcm1p. We examined stability of TAP-tagged Mcm1p in WT, *plc1*Δ, and *ipk2*Δ strains. The cells were grown in YPD medium to early exponential phase and subsequently treated with cycloheximide (CYH) to block protein synthesis. Mcm1p levels were determined by Western blotting using anti-TAP antibodies. In

comparison with wild-type cells, the amount of Mcm1p in *ipk2*Δ strains is reduced to about 60% before treatment with CYH and to about 20% after 3 h treatment with CYH (Fig. 3). This result demonstrates that Mcm1p is less stable in *ipk2*Δ strain and agrees with previous results that showed that Ipk2p is required for Mcm1p stability (El Bakkoury, *et al.*, 2000). In contrast, 3 h treatment with CYH in wild-type as well as *plc1*Δ cells reduced Mcm1p level only to 90% (Fig. 3). Thus, Plc1p is not required for Mcm1p stability and Plc1p and/or synthesis of $InsP_3$ affect expression of Mcm1p-dependent genes by a different mechanism. Since InsPs regulate promoter recruitment and activity of chromatin remodeling complexes such as Swi/Snf and Ino80 (Shen et al., 2003; Steger et al., 2003), it is likely that reduced recruitment and/or activity of chromatin remodeling complexes in *plc1*Δ cells is responsible for decreased expression of Mcm1p-dependent genes. Future experiments will address the role of InsPs in regulation of chromatin remodeling complexes and expression of Mcm1p-dependent genes.

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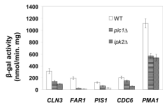

Figure 1. *plc1*Δ cells display reduced expression of Mcm1p-dependent genes

Wild-type, *plc1*Δ, and *ipk2*Δ strains were transformed with plasmids containing *lacZ* reporter genes under the control of the indicated Mcm1p-regulated promoters (El Bakkoury et al., 2000). The β-galactosidase assays were carried out as described previously (Choi et al., 1998) and the values were calculated from three independent experiments and represent means ± SD.

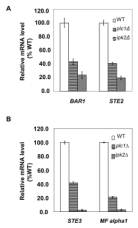

Figure 2. *plc1*Δ cells display reduced expression of cell-type-specific genes

The indicated strains were grown in YPD medium at 30°C to A_{600nm}=1.0 and the total RNA was isolated and assayed for *ACT1*, *BAR1*, *STE2*, *STE3*, and *MF alpha 1* transcripts by real time RT-PCR. The results were normalized to *ACT1* RNA and expressed as relative values in comparison to corresponding WT strain. The experiment was repeated three times and the results represent means ± S.D. (A) Expression of a – specific genes *BAR1* and *STE2* in *Mata* cells. (B) Expression of α – specific genes *STE3* and *MF alpha1* in *MAT*α cells.

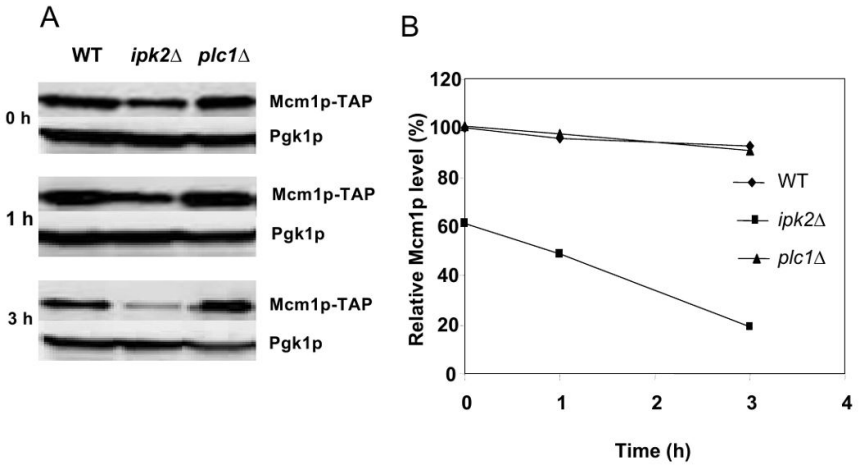

Figure 3. Plc1p is not required for Mcm1p stability

(A) Wild-type, *ipk2*Δ, and *plc1*Δ cells expressing TAP-tagged Mcm1p were grown in YPD medium to early exponential phase (A_{600nm} = 1.0) and subsequently treated with cycloheximide to block protein synthesis. Mcm1p-TAP levels were determined by Western blotting using anti-TAP antiobody before addition of cycloheximide and after 1 and 3 h. Even loading of protein samples was confirmed with anti-3-phosphoglycerate (Pgk1p) antibody. The experiment was performed three times, and representative results are shown. (B) Densitometric evaluation of the representative Western blot.

Table 1

Yeast strains used in this study

Strain	Genotype	Source/ reference
W303-1a	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 ssd1-d2 can1-100*	R. Rothstein
W303-1α	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 ssd1-d2 can1-100*	R. Rothstein
HL1-1	W303-1α *plc1::URA3*	Lin, et al. (2000)
HL1-3	W303-1*a plc1::URA3*	De Lillo et al. (2003)
AOY138	W303-1α *ipk2::kanMX*	York et al. (2005)
A0004	W303-1α *ipk1::kanMX*	York et al. (1999)
YSC1178-7502110	*MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 MCM1-TAP::HIS3*	Open Biosystems
KG026	W303-1*a MCM1-TAP::HIS3*	This study
KG002	W303-1*a plc1::URA3 MCM1-TAP::HIS3*	This study
KG022	W303-1*a ipk2::kanMX MCM1-TAP::HIS3*	This study