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Phosphatidic acid and phosphoinositides facilitate liposome association of Yas3p and potentiate derepression of ARE1 (alkane-responsive element one)-mediated transcription control ☆

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ABSTRACT

In the *n*-alkane assimilating yeast *Yarrowia lipolytica*, the expression of *ALK1*, encoding a cytochrome P450 that catalyzes terminal mono-oxygenation of *n*-alkanes, is induced by *n*-alkanes. The transcription of *ALK1* is regulated by a heterocomplex that comprises the basic helix-loop-helix transcription activators, Yas1p and Yas2p, and binds to alkane-responsive element 1 (ARE1) in the *ALK1* promoter. An Opi1 family transcription repressor, Yas3p, represses transcription by binding to Yas2p. Yas3p localizes in the nucleus when *Y. lipolytica* is grown on glucose but localizes to the endoplasmic reticulum (ER) upon the addition of *n*-alkanes. In this study, we showed that recombinant Yas3p binds to the acidic phospholipids, phosphatidic acid (PA) and phosphoinositides (PIPs), *in vitro*. The ARE1-mediated transcription was enhanced *in vivo* in mutants defective in an ortholog of the *Saccharomyces cerevisiae* gene *PAH1*, encoding PA phosphatase, and in an ortholog of *SAC1*, encoding PIP phosphatase in the ER. Truncation mutation analyses for Yas3p revealed two regions that bound to PA and PIPs. These results suggest that the interaction with acidic phospholipids is important for the *n*-alkane-induced association of Yas3p with the ER membrane.

1. Introduction

Many microorganisms are challenged by rapid changes in their environment and must adapt to survive. They have developed various systems for sensing environmental conditions and for adaptation. For example, microbial cells respond to nutrient resources and inductively express genes involved in the metabolism of nutrient compounds (Gurvitz and Rottensteiner, 2006). Some microbial cells respond to xenobiotic compounds via direct interactions with sensor proteins, resulting in the expression of genes responsible for detoxification (Näär and Thakur, 2009).

Alkanes are ubiquitous high-energy carbon sources. Many microorganisms, including bacteria and fungi, can assimilate *n*-alkanes by using cytochromes P450 (P450s), which are heme-containing monooxygenases (Van Bogaert et al., 2011). In alkane-assimilating yeasts, such as *Candida tropicalis* (Sanglard et al., 1987; Seghezzi et al., 1992; Seghezzi et al., 1991), *Candida maltosa* (Ohkuma et al., 1995; Ohkuma et al., 1991), and *Yarrowia lipolytica* (Iida et al., 1998, 2000), terminal mono-oxygenation of *n*-alkanes is catalyzed by the cytochromes P450ALKs that are classified into the CYP52 family. In these yeasts, the expression of genes encoding P450ALKs is induced in the presence of *n*-alkanes (Van Bogaert et al., 2011). However, little is known about the molecular mechanisms how *n*-alkanes are recognized by cells and cause the transcriptional induction of P450ALK genes.

Y. lipolytica can utilize various hydrophobic compounds, including *n*-alkanes and fatty acids, as carbon sources (Fickers et al., 2005; Fukuda, 2013). Because of this property, *Y. lipolytica* has been intensively studied with respect to both fundamental and application interests (Bankar et al., 2009; Nicaud, 2012; Sabirova et al., 2011). Eight *ALK* genes (*ALK1* to *ALK8*) that were deduced to encode P450ALKs in *Y.*

Abbreviations: AID, auxin-inducible degron; ARE1, alkane-responsive element 1; bHLH, basic helix-loop-helix; DAG, diacylglycerol; DAGPP, diacylglycerol pyrophosphate; DTT, dithiothreitol; 2D-TLC, two-dimensional thin layer chromatography; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FFAT, two phenylalanines in an acidic tract; GST, glutathione *S*-transferase; IPTG, isopropyl β-D-1-thiogalactopyranoside; NAA, 1-naphthaleneacetic acid; NLS, nuclear localization signal; ORF, open reading frame; P450, cytochrome P450; PA, phosphatidic acid; PC, phosphatidylcholine; PCR, polymerase chain reaction; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphoinositide; PI(4)P, phosphatidylinositol 4-phosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; PLO assay, protein lipid overlay assay; PS, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate-poly acrylamide gel electrophoresis; RLM-RACE, RNA ligase-mediated rapid amplification of cDNA ends.

☆ The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank™/EMBL Data Bank with accession numbers, AB795935, AB795936, AB795937, and AB795938.

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lipolytica have been isolated earlier, and additional four *ALK* genes (*ALK9* to *ALK12*) were suggested from its genome information (Dujon et al., 2004; Fickers et al., 2005; Fukuda, 2013; Iida et al., 1998, 2000). The expression of most of these *ALK* genes was induced in the presence of *n*-alkanes (Hirakawa et al., 2009). Mutant cells lacking all of twelve *ALK* genes could not grow on *n*-alkanes, but could grow almost normally with the expression of *ALK1*, indicating that the product of *ALK1* plays a major role in the metabolism of *n*-alkanes (Takai et al., 2012).

Among the twelve *ALK* genes, *ALK1* is highly induced in the presence of *n*-alkanes such as *n*-decane and *n*-hexadecane (Hirakawa et al., 2009; Iida et al., 2000). We identified an upstream activating sequence that mediates transcriptional activation in response to *n*-alkane in the *ALK1* promoter and named it alkane-responsive element 1 (ARE1) (Sumita et al., 2002; Yamagami et al., 2004). ARE1-like sequences were found in the promoters of other genes involved in *n*-alkane assimilation. We also identified two basic helix-loop-helix (bHLH) transcription factors, Yas1p and Yas2p, that were necessary for the transcriptional induction of *ALK1* in response to *n*-alkane (Endoh-Yamagami et al., 2007; Yamagami et al., 2004). Yas1p and Yas2p form a heterocomplex and bind to ARE1.

In addition, we found that Yas3p, an Opi1 family transcription repressor, is involved in the regulation of ARE1-mediated transcription through interaction with Yas2p (Hirakawa et al., 2009). Two types of *YAS3* transcripts with different transcription start sites exist: one, encoding a long form of Yas3p (l-Yas3p) and the other, encoding a short form of Yas3p (s-Yas3p). Although l-Yas3p includes the entire amino acid sequence of s-Yas3p, our data suggested that s-Yas3p plays a pivotal role in the transcriptional regulation of *ALK1*. Yas1p and Yas2p localize in the nucleus independent of the presence of *n*-alkane (Hirakawa et al., 2009; Yamagami et al., 2004). In contrast, Yas3p localizes to the endoplasmic reticulum (ER) in the presence of *n*-alkane, while it localizes in the nucleus in the absence of *n*-alkane, suggesting that Yas3p regulates ARE1-mediated transcription by changing its subcellular localization between the nucleus and the ER (Hirakawa et al., 2009). However, it is not clear how the localization of Yas3p is regulated.

In *Saccharomyces cerevisiae*, a transcriptional repressor Opi1p regulates the expression of phospholipid biosynthetic genes, containing an upstream activating sequence (UAS_{INO}) in their promoters, in response to *myo*-inositol (Henry et al., 2012). Loewen et al. proposed a model in which Opi1p is anchored to the ER membrane through interactions with phosphatidic acid (PA) and with the integral membrane protein, Scs2p, in the absence of *myo*-inositol, resulting in the transcriptional activation of target genes by the activator complex composed of two bHLH proteins, Ino2p and Ino4p (Loewen et al., 2004). Addition of *myo*-inositol causes the consumption of PA in the ER membrane through the synthesis of phosphatidylinositol (PI), leading to the release of Opi1p from the ER. Then, Opi1p translocates into the nucleus where it represses transcription by interacting with Ino2p (Wagner et al., 2001). Opi1 family proteins are encoded in genomes of a wide variety of yeasts and fungi. Functional characterizations of Opi1p orthologs have been performed for *Candida albicans*, *Candida glabrata*, and *Y. lipolytica* (Bethea et al., 2010; Henry et al., 2012; Heyken et al., 2003; Hirakawa et al., 2009), but it has remained unclear whether these Opi1p orthologs bind to PA. Yas3p and Opi1p share significant sequence similarities in their leucine zipper domain, uncharacterized conserved domain, and activator interaction domain responsible for the interaction with Ino2p (Hirakawa et al., 2009). In contrast, the PA-binding region of Opi1p is only partially conserved in Yas3p. In addition, Yas3p does not have two phenylalanines in an acidic tract (FFAT) motif that is necessary for Opi1p to interact with Scs2p (Loewen et al., 2003), and the orthologs of Scs2p in *Y. lipolytica* are dispensable for the transcriptional induction of *ALK1* in response to *n*-alkane (Kobayashi et al., 2008). These results raise an important question how Yas3p is localized to the ER membrane in the presence of *n*-alkane.

In this study, we have demonstrated that Yas3p binds to PA and phosphoinositides (PIPs) and that association of Yas3p with liposomes was facilitated by these phospholipids. In addition, genetic manipulations of metabolism of these phospholipids derepressed ARE1-mediated transcription, suggesting their importance in the *n*-alkane-responsive transcription regulation.

2. Materials and methods

2.1. Yeast strains and growth condition

Yeast strains used in this study are shown in Table 1. *Y. lipolytica* strain CXAU/A1 was used as a wild-type strain.

The Δ*pah1* strain was obtained by replacing the *YlPAH1* ORF with *ADE1*. The *YlPAH1* deletion cassette liberated by digestion of pBpah1-ADE1 with EcoRV and XbaI was introduced into CXAU1 strain. REA1 strain was obtained by replacing *ade1* in RE1 strain with *ADE1*. The *ADE1*-carrying BamHI fragment of pSAT4 was introduced into RE1 strain. pBura3-TIR1-ADE1 was digested with ApaI and XbaI, and *ADE1*-carrying fragment was introduced into CXAU1 to obtain TIR1 strain. Ade^+ transformants were selected, and correct recombination was confirmed by PCR and Southern blot analysis. *AID-SAC1* strain was obtained with pop-in-pop-out method as described previously (Takai et al., 2012). Briefly, pBU-AID-SAC1 digested with SphI was introduced into TIR1 strain. Ura^+ transformants were selected. Then the cells, in which the DNA region corresponding to intact *YlSAC1*, *URA3* and the vector was popped out, were selected on YPD solid medium containing 1 mg/ml 5-fluoroorotic acid (5-FOA). Correct integration and recombination was confirmed by PCR and Southern blot analysis.

An appropriate carbon source was added to YNB [0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.5% ammonium sulfate] as follows: 2% (w/v) glucose (SD medium); 2% (w/v) glycerol; 2% (v/v) *n*-decane; 2% (v/v) *n*-hexadecane; and 2% (v/v) oleic acid. Uracil (24 mg/l) was added, if necessary. For solid medium, 2% agar was added. *n*-Alkanes were supplied in the vapor phase to YNB solid media as described previously (Endoh-Yamagami et al., 2007). 1-Naphthaleneacetic acid (NAA) was added at the concentration of 500 μM. Yeast cells were grown at 30 °C.

2.2. Plasmids

Plasmids used in this study are shown in Table 2. Sequences of used primers and oligonucleotides are shown in Table 3.

The *YlPAH1* 5′-flanking region was amplified from CXAU1 total DNA by PCR using primers EcoRI-pah1P-F and pah1P-R-BamHI, and the amplified fragment was digested with EcoRI and BamHI. The *YlPAH1* 3′-flanking region was also amplified by PCR using

Table 1
Yeast strains used in this study.

Strain	Genotype	Source or reference
CXAU1	*MATA ade1 ura3*	Iida et al. (1998)
CXAU/A1	CXAU1 *ade1*::*ADE1*	Yamagami et al. (2004)
RE1	CXAU1 *sec61*::*SEC61-DsRed*	Hirakawa et al. (2009)
Δpah1	CXAU1 *pah1*::*ADE1*	This study
TIR1	CXAU1 *ura3*:: :: *pRPS7-OsTIR1-myc-ADE1*	This study
AID-SAC1	*TIR1 sac1*::*AID-HA-SAC1*	This study
REA1	RE1 *ade1*::*ADE1*	This study
Δyas3LZ	CXU3xLZ1 *yas3*	This study