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Ylpex5 mutation partially suppresses the defective hyphal growth of a *Yarrowia lipolytica* ceramide synthase mutant, *Yllac1*, by recovering lipid raft polarization and vacuole morphogenesis

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ABSTRACT

Sphingolipids are involved in cell differentiation and morphogenesis in eukaryotic cells. In this study, YlLac1p, a ceramide synthase required for glucosylceramide (GlcCer) synthesis, was found to be essential for hyphal growth in *Yarrowia lipolytica*. *Y. lipolytica* GlcCer was shown to be composed of a C16:0 fatty acid, which is hydroxylated at C2, and a C18:2 long chain base, which is unsaturated at both C4 and C8 and methylated at C9. Domain swapping analysis revealed that the entire TRAM/Lag1/CLN8 (TLC) domain, not the Lag1 motif, is crucial for the function of YlLac1p. YlDes1p, the C4 desaturase of the ceramide synthesized by YlLac1p, was also required for *Y. lipolytica* morphogenesis. Both *Yllac1*Δ and *Yldes1*Δ mutants neither polarize lipid rafts nor form normal vacuoles. Interestingly, mutation in *YlPEX5*, which encode a peroxisomal targeting signal receptor, partially suppressed the defective hyphal growth of *Yllac1*Δ. The *Yllac1*Δ *Ylpex5*Δ mutant restored the ability to polarize lipid rafts and to form normal vacuoles, although it could not synthesize GlcCer. Taken together, our results suggest that GlcCer or GlcCer derivatives may be involved in hyphal morphogenesis in *Y. lipolytica*, at least in part, by affecting polarization of lipid rafts and vacuole morphogenesis.

1. Introduction

Sphingolipids are important bioactive molecules as well as critical structural components of cellular membranes. They play major roles in regulating diverse signaling pathways for essential cellular processes, including growth, differentiation, migration, apoptosis, stress response, and cell polarity (Bagnat and Simons, 2002; Cheng et al., 2001; Dickson et al., 2006; Krishnamurthy et al., 2007; Obeid et al., 2002; Ogretmen and Hannun, 2004; Savtchouk et al., 2007). Ceramide is a key molecule among the sphingolipid species.

As an initial step to synthesize ceramide, serine palmitoyltransferase condenses palmitoyl-CoA and serine to yield 3-ketosphinganine, which is used to form sphinganine through the activity of 3-ketosphinganine reductase. Sphinganine is N-acylated with fatty acids to produce ceramide, a reaction catalyzed by ceramide synthase. Depending on the substrate specificity of ceramide synthase for the hydroxylation level of sphinganine (dihydroxy or trihydroxy) and the fatty acid chain length (C16–C18 or C24–C26), two distinct pools of ceramide are generated and serve as building blocks in some yeast and fungal species for two major fungal sphingolipids, inositol-containing sphingolipids and glucosylceramides (GlcCers) (Fig. S1) (Rittenour et al., 2011; Ternes et al., 2011; Cheon et al., 2012).

In *Saccharomyces cerevisiae*, ceramide biosynthesis relies on the longevity assurance gene (*LAG1*) and its close homologue *LAC1* (D'Mello et al., 1994; Guillas et al., 2001; Schorling et al., 2001), which are the founding members of the Lag1 family present in eukaryotic cells. At least two Lag1 family genes have been found in all species studied to date, and six Lag1 homologues, longevity assurance genes (Lass), are known in human and mouse (Pewzner-Jung et al., 2006; Teufel et al., 2009). The two homologous genes found in yeast and filamentous fungi can be classified into two distinct groups according to their phylogenic relationship (Pewzner-Jung et al., 2006; Takakuwa et al., 2008) and may have different substrate specificities responsible for the generation of different types of ceramides, inositol-containing sphingolipids and GlcCers (Cheon et al., 2012; Li et al., 2006; Rittenour et al., 2011; Ternes et al., 2011).

Yarrowia lipolytica can switch from yeast to hyphae in response to a number of stimuli, including N-acetylglucosamine and serum (Kim et al., 2000; Perez-Campo and Dominguez, 2001; Ruiz-Herrera and Sentandreu, 2002; Rodriguez and Domínguez, 1984). *Y. lipolytica* is more amenable to genetic manipulation than

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Candida albicans. Thus, *Y. lipolytica* has an advantage over *C. albicans* for genetic studies relating to hyphal morphogenesis.

Recently, GlcCer was reported to be involved in growth, differentiation, virulence, immunogenicity, and lipid raft architecture (Noble et al., 2004; Rhome et al., 2011; Rittershaus et al., 2006). However, the underlying mechanisms have not yet been fully elucidated. In this study, we show that deletion of the ceramide synthase *YlLAC1* gene responsible for GlcCer synthesis resulted in a defect in hyphal growth in *Y. lipolytica* and introduction of an *Ylpex5* mutation into the *Yllac1Δ* mutant moderately recovered the defective hyphal growth.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The strains and plasmids used in this study are summarized in Tables 1 and 2, respectively. *Y. lipolytica* strains were typically grown at 28 °C in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) or synthetic complete medium (SC; 0.67% yeast nitrogen base without amino acids, 2% glucose, and drop-out amino acid mixture) supplemented with the required amino acids. To induce filamentous growth in *Y. lipolytica*, 10% fetal bovine serum was added to SC medium buffered with 50 mM citrate buffer (pH 6.0).

2.2. Cloning, genomic library construction and sequence analysis

General DNA manipulations were performed as described by Sambrook and Russell (Sambrook and Russell, 2001). To construct the *Y. lipolytica* genomic library of a *Yllac1* mutant strain, Sau3AI-digested DNA fragments corresponding to 5–7 kbp in size were ligated into the unique *Bam*HI site of the *Y. lipolytica*/*Escherichia coli* shuttle vector, pINATX1 (Cheon, 2007). The ligated plasmids were then transformed into *E. coli DH5α* by electroporation. Sequence analysis was performed using Lasergene 5.06 (DNASTAR, Inc.) and Vector NTI (Invitrogen) software. DNA sequences were retrieved from the *Y. lipolytica* genome sequencing consortium database (http://genome.jouy.inra.fr/clib/consortium/) and compared using the BLAST algorithm.

2.3. Complementation analysis

For complementation analysis in *Y. lipolytica*, *LAG1* homologous genes controlled by the constitutive *Y. lipolytica TEF* promoter were cloned into pINATX1. *Yllac1Δ* mutants transformed with vectors carrying each *LAG1* homologue were selected on solid synthetic medium lacking leucine and tested for hyphal growth on SC medium containing 10% fetal bovine sserum.

2.4. Thin layer chromatography and gas chromatography mass spectrometry (GC–MS) analysis of GlcCer

GlcCer was analyzed by thin layer chromatography as previously described (Cheon et al., 2012). The type and length of fatty acid (FA) chain present in the *Y. lipolytica* GlcCer were examined by GC–MS after conversion of FAs to FA methyl esters. The GlcCers scraped from the silica gel thin layer chromatography plate were eluted in chloroform:methanol (2:1, v/v), resuspended in 3 mL of 1.37 M methanolic-HCl and 5% 2,2-dimethoxypropane, and incubated for 5 h at 100 °C. After addition of 6 mL 1% KCl, FA methyl esters were extracted twice with 3 mL hexane, dried, suspended in chloroform, and subjected to GC separation performed on a Agilent 6890N Network GC System (Agilent Technologies) equipped with a HP-5MS capillary column (30 m × 0.25 mm) using helium as the carrier gas. The injector temperature was 250 °C, and the oven temperature was increased from 60 to 280 °C. Mass spectra data were collected and quantified on a 5973 Network Mass Selective Detector (Agilent Technologies). The samples were ionized by the electron ionization method (ionization energy 70 eV and source temperature at 240 °C) and were analyzed in positive ion mode.

2.5. Matrix-assisted laser desorption ionization-time of flight (MALDI–TOF) analysis of GlcCer

GlcCer eluted from the thin layer chromatography plate in chloroform:methanol (2:1, v/v) were mixed with an equal volume of the 7 mg/ml 2,5-dihydroxybenzoic acid matrix dissolved in methanol. MALDI–TOF MS was performed in the reflector positive ion mode using a Microflex TOF (Bruker Daltonik GmbH, Bremen, Germany). All spectra were acquired with the manufacturer's rec-

Table 1
Strains used in this study.

Strain	Parental Strain	Genotype	Source
SMS397A		*MatAade1ura3xpr2*	Park et al., 1997
IL3	SMS397A	As SMS397A, but *leu2::tc*	Cheon, 2007
Yllac1Δ	IL3	As SMS397A, *leu2::tc Yllac1::tc-URA3-tc*	Cheon, 2007
Yllac1Δ[*YlLAG1*]	*Yllac1Δ*	As *Yllac1Δ*, pINATX1-*YlLAG1*	This study
Yllac1Δ[*YlLAC1*]	*Yllac1Δ*	As *Yllac1Δ*, pINATX1-*YlLAC1*	This study
Yllac1Δ[*AnLagA*]	*Yllac1Δ*	As *Yllac1Δ*, pINATX1-*AnLagA*	This study
Yllac1Δ[*AnBarA*]	*Yllac1Δ*	As *Yllac1Δ*, pINATX1-*AnBarA*	This study
Yllac1Δ[*CaLAG1*]	*Yllac1Δ*	As *Yllac1Δ*, pINATX1-*CaLAG1*	This study
Yllac1Δ[*CaLAC1*]	*Yllac1Δ*	As *Yllac1Δ*, pINATX1-*CaLAC1*	This study
Yllac1Δ [*YlLAC1*-YlLag1 motif]	*Yllac1Δ*	As *Yllac1Δ*, pINATX1-*YlLAC1*-YlLag1 motif	This study
Yllac1Δ [*YlLAG1*-YlLac1 motif]	*Yllac1Δ*	As *Yllac1Δ*, pINATX1-*YlLAG1*-YlLac1 motif	This study
Yllac1Δ [*YlLAG1*-YlLac1 LC]	*Yllac1Δ*	As *Yllac1Δ*, pINATX1-*YlLAG1*-YlLac1 LC	This study
Yllac1Δ [*YlLAG1*-YlLac1 TL]	*Yllac1Δ*	As *Yllac1Δ*, pINATX1-*YlLAG1*-YlLac1 TL	This study
Yllac1Δ [*YlLAG1*-YlLac1 TLC]	*Yllac1Δ*	As *Yllac1Δ*, pINATX1-*YlLAG1*-YlLac1 TLC	This study
*Yllac1Δ*l [*AnLagA*-AnBarA motif]	*Yllac1Δ*	As *Yllac1Δ*, pINATX1-*AnLagA*-AnBarA motif	This study
*Yllac1Δ*l [*AnLagA*-AnBarA TLC]	*Yllac1Δ*	As *Yllac1Δ*, pINATX1-*AnLagA*-AnBarA TLC	This study
Yldes1Δ	IL3	As SMS397A, *leu2::tc Yldes1::tc-URA3-tc*	Cheon, 2007
Yldes1Δ [*YlDES1*]	*Yldes1Δ*	As *Yldes1Δ*, pINATX1-*YlDES1*	This study
Ylpex5Δ	IL3	As SMS397A, *leu2::tc Ylpex5::tc-URA3-tc*	This study
Ylpex5Δ [*YlPEX5*]	*Ylpex5Δ*	As *Ylpex5Δ*, pINATX1-*YlPEX5*	This study
Yllac1Δ Ylpex5Δ	*Yllac1Δ*	As *Yllac1Δ*, *Ylpex5::tc-URA3-tc*	This study
Yllac1Δ Ylpex5Δ [*YlPEX5*]	*Yllac1Δ Ylpex5Δ*	As *Yllac1Δ Ylpex5Δ*, pINATX1-*Ylpex5*	This study