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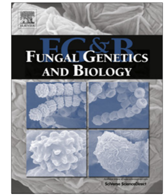

Candida albicans cis-prenyltransferase Rer2 is required for protein glycosylation, cell wall integrity and hypha formation

Mateusz Juchimiuk[a,b,*,1], Jacek Orłowski[a,b,1], Katarzyna Gawarecka[a], Ewa Świeżewska[a], Joachim F. Ernst[b], Grażyna Palamarczyk[a]

[a] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02 106 Warsaw, Poland
[b] Department Biologie, Molekulare Mykologie, Heinrich-Heine-Universität, Düsseldorf, Germany

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ABSTRACT

cis-Prenyltransferase is the first enzyme of the mevalonate pathway committed to the biosynthesis of dolichol in eukaryotes. The *RER2* gene encoding *cis*-prenyltransferase (Rer2p) in the human fungal pathogen *Candida albicans* was characterized. In addition, the *ORF19.5236* encoding the second *cis*-prenyltransferase, which putatively is responsible for the synthesis of longer polyisoprenoids chains, was identified. When cultivated under repressive conditions, the conditional mutant strain expressing the *RER2* gene from the regulatable *MET3* promoter contained only 4% of *cis*-prenyltransferase activity and markedly diminished amounts of dolichols, as compared to the wild-type strain. Moreover, transcriptomal analyses revealed changes in the expression of 300 genes, mainly involved in transport, response to stress, filamentous growth and organelle organization. Growth of the conditional strain was blocked completely at 37 °C. The strain was hypersensitive to a wide range of inhibitors, which suggested glycosylation defects and compromised cell wall integrity. Moreover, the *rer2* conditional mutant grown in the repressive conditions, unlike the same strain in the absence of repressor, failed to form hyphae. The results indicate that dolichols are essential not only for protein glycosylation and cell wall integrity but also for growth and development of *C. albicans*.

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1. Introduction

The human fungal pathogen *Candida albicans* and the non-pathogenic budding yeast *Saccharomyces cerevisiae* belong to the same taxonomic family *Saccharomycetaceae*. However, the two species have distinct life styles and highly divergent genomes. Normally, *C. albicans* can live as a harmless commensal in many different body locations including gastrointestinal and genitourinary tracts (Odds, 1987), but it can also cause tenacious superficial and life-threatening systemic infections (Edmond et al., 1999; Ranquel-Frants et al., 1999). The virulence of *C. albicans* is correlated with its polymorphism, i.e. the ability to grow as a unicellular budding yeast or in the form of pseudohyphae or true hyphae (Jacobsen et al., 2012).

Recent studies demonstrated a crucial role of the *C. albicans* cell wall mannoproteins in its virulence (Buurman et al., 1998; Gow, 2004; Hobson et al., 2004; Masuoka, 2004; Munro et al., 2005; Bates et al., 2006). Functions of many *C. albicans* genes involved in the elaboration of highly branched N-linked mannan and short linear O-mannan were clarified using specific mutants (reviewed by Hall and Gow, 2013). Typically, glycosylation-deficient phenotypes were reported to include inefficient cell separation, impaired bud growth, clumping and flocculation, as well as increased sensitivity to a wide range of antifungal drugs. Moreover, many glycosylation mutants altered the immune response patterns of the host, which is known to depend on fungal glycan epitopes.

On the other hand, there is little information on early glycosylation events in *C. albicans* such as the formation of the lipid intermediate, dolichyl phosphate (DolP), and the assembly of the DolP-linked oligosaccharide (DolPP-$GlcNAc_2Man_9Glc_3$), which is the substrate for *N*-glycosylation. The only available data describe *in vitro* formation of DolP-Man (required for *N*- and *O*-glycosylation and for the synthesis of GPI anchors (Orlean, 1990)) and DolP-Glc from GDP-Man and UDP-Glc and exogenous dolichyl phosphate, a reaction that was catalyzed by a crude membrane fraction (Arroyo-Flores et al., 1995; Rodríguez-Bonilla et al., 1998). Here we report that the *C. albicans* genome sequence contains open reading frames *ORF19.4028* and *ORF19.5236*, which encode proteins with significant similarity to the *S. cerevisiae* Rer2 and Srt1

* Corresponding author. Address: Department Biologie, Molekulare Mykologie, Heinrich-Heine-Universität, Universitätsstraße 1, 40225 Düsseldorf, Germany. Fax: +49 211 81 15176.
[1] These authors contributed equally.

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proteins, respectively. These proteins represent the *cis*-prenyltransferases, which are responsible for the synthesis of the dolichol backbone (α-dihydro-*cis*-polyprenol-diphosphate). Prenyltransferases catalyze the reaction of 1′-4 condensation of isopentenyl pyrophosphate (IPP) molecules, which leads to isoprenoid chain elongation. Based on the stereochemistry of the new double bonds formed during condensation, prenyltransferases are classified as *trans*- and *cis*-types (Kellogg and Poulter, 1997). The enzymes of the former class generate products with chain lengths ranging from C-10 to C-50, which are used for modifications of some proteins (e.g. farnesylation of yeast RAS proteins) (Clarke, 1992) or build the side chains of ubiquinones (Okada et al., 1996). Products of *cis*-prenyltransferases usually consist of >10 isoprenoid units and their main biological functions are related to protein glycosylation, or to the bacterial peptidoglycan biosynthesis (Allen, 1985; Sato et al., 1999). In this work we cloned and characterized the *C. albicans RER2* gene and showed that its mutation hampers dolichol synthesis and leads to various defects in growth, hyphal differentiation, cell wall formation and sensitivity to antifungal agents.

2. Materials and methods

2.1. Growth media and strains

C. albicans and *S. cerevisiae* strains used in the present study are described in Table 1.

C. albicans strains were grown in yeast extract–peptone–dextrose (YPD) medium or supplemented synthetic dextrose (SD) (Sherman et al., 1986). Solid media were prepared with 2% Bacto agar. To repress the *MET3* promoter, media were supplemented with 2.5 mM of methionine and cysteine (Met/Cys) (Care et al., 1999). Doxycycline (Sigma) or anhydrotetracycline (IBA-Lifescience) at different concentrations were used to repress the tetracycline-regulatable promoter (Tet-OFF system). The *GAL1* promoter was induced by cultivating cells on YPGal plates (1% yeast extract, 0.5% peptone, 2% galactose).

To test the sensitivity to various chemical agents, mid-log phase cells in serial 1:10 dilutions were spotted either onto YPD agar plates containing the indicated amounts of the chemicals or onto selective medium buffered with 50 mM MOPS and adjusted to pH 6.2. Plates were incubated for 48–72 h at the permissive temperature of 28 °C.

To induce *S. cerevisiae* sporulation the cells were starved on sporulation medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose) and tetrads were dissected on YPD or YPGal plates using a Singer MSM 200 System micromanipulator.

Chlamydospore formation was tested on corn meal agar with 0.5% Tween 80 (Difco). Cultures were streaked on the agar, covered with a microscopic cover slip and incubated in darkness at 25 °C for 7 days.

Hyphal growth was induced on Spider plates (1% mannitol, 1% nutrient broth, 0.2% K_2HPO_4, pH 7.2, 2% agar) and in/on YPSerum (1% yeast extract, 0.5% peptone, 10% horse serum) liquid/solid medium. Due to the thermosensitive phenotype of the conditional mutant, growth in repressive conditions was performed at 30 °C. Hypha formation was monitored by light microscopy.

2.2. Plasmid and strain construction

Plasmids are listed in Table 2 and primers in supplementary material (Table S1).

Total DNA from yeast cells was isolated as described (Sambrook et al., 1989). *S. cerevisiae* and *C. albicans* transformation was done according to the lithium acetate/single-stranded DNA/polyethylene glycol protocol (Gietz et al., 1995).

For yeast transformation the *RER2* ORF was cloned into the *S. cerevisiae* pNEV vector. To this end, primers 19.4028NotIF and 19.4028NotIR were used to amplify the 987 bp coding region of *ORF19.4028*. The resulting PCR product was subcloned into pMOS-Blue and sequenced. Subsequently, the *Not*I insert was cloned into the *Not*I-digested pNEV and the obtained plasmid was used to transform the KG219 strain. Primers SRT1-F and SRT1-R, both introducing *Bam*HI restriction sites, were used to PCR-amplify the coding region of *ORF19.5236* from cDNA of CAI4 strain. After cloning into vector pMOS-Blue, the PCR-product was sequenced and cloned into the *Bam*HI site downstream of the *GAL1* promoter of plasmid p426.

Construction of the P_{MET3}*RER2/rer2Δ* mutant (strain JOS18) and results of diagnostic PCR is illustrated in Supplementary Fig. S1.

The coding region of *RER2* was disrupted by the Ura-blaster method (Fonzi and Irwin, 1993), resulting in the deletion of nucleotides 110–981 from the 987-nucleotide ORF. A cassette for disruption of Ca*RER2* was constructed in several steps. A 650 bp sequence 5′ to the start of the *CaRER2* ORF was amplified using primers *RER2*F1F and *RER2*F1R (Table S.1). Similarly, 570 bp of the 3′ sequences flanking the stop codon were amplified using primers *RER2*F2F and *RER2*F2R. The obtained PCR fragments were subcloned into pMOS-Blue, which resulted in plasmids pJO22 and pJO23, respectively. The *Sac*I/*Bgl*II fragment of pJO22 and the *Bam*HI/*Sal*I fragment of pJO23 were inserted into the respective sites of the plasmid p5921 resulting in plasmid pJO24. The *Sac*I/*Sal*I fragment of pJO24 containing the Ca*RER2* disruption cassette was used to transform strain CAI4.

To place *CaRER2* under control of the *MET3* promoter we followed a previously described strategy (Care et al., 1999). First, a 415 bp fragment corresponding to the 5′ end of the *CaRER2* ORF was amplified using primers RER2FMet and RER2RMet and the PCR fragment was cloned into pMOS-Blue. The *Bam*HI/*Sph*I fragment of the resulting plasmid was inserted into the *Bam*HI/*Sph*I sites of pCaDis, downstream of the *MET3* promoter. The resulting plasmid pJO27 was linearized within the inserted fragment with *Pst*I and used to transform the heterozygous strain JOS14.

In parallel, we constructed another conditional strain with the *CaRER2* ORF placed under control of a tetracycline-regulatable promoter. To this end, the first 645 bp of *ORF19.4028* were amplified by PCR using RER-fr-F and RER-fr-R primers, both introducing *Spe*I sites. The PCR product was subcloned into pGEM T-Easy,

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Table 1
C. albicans and *S. cerevisiae* strains.

Strain	Relevant genotype	Parent	Reference
CAF2-1	*ura3Δ::imm434/URA3*	SC5314	Fonzi and Irwin (1993)
CAI4	*ura3Δ::imm434/ura3Δ::imm434*	CAF2-1	Fonzi and Irwin (1993)
JOS13	as CAI4 but *RER2/rer2Δ::hisG-URA3-hisG*	CAI4	This work
JOS14	as CAI4 but *RER2/rer2Δ::hisG*	JOS13	This work
JOS18	as CAI4 but P_{MET3}-*RER2/rer2Δ::hisG*	JOS14	This work
CMJ3	as CAI4 but $P_{tetO\text{-}ScHOP1}$-*RER2/rer2Δ::hisG*	JOS14	This work
KG219	*MATa/MATα* his3Δ1/his3Δ1 *leu2Δ0*/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 rer2::*kanMX4*/RER2 srt1:: *his3MX6loxP*/SRT1		Grabińska et al. (2010)

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