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The mannoprotein *TIR3* (CAGL0C03872g) is required for sterol uptake in *Candida glabrata*

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ABSTRACT

Sterol uptake in the pathogenic fungus, *Candida glabrata*, occurs via the sterol transporter, CgAus1p. Azole inhibition of sterol biosynthesis can under certain circumstances be reversed by adding exogenously sterol. Here we demonstrate that the *CgTIR3* (CAGL0C03872g) gene product is also required for sterol uptake, since *Cgtir3*Δ strains fail to take up sterol both aerobically and under hypoxic conditions. Western analysis using an HA-tagged *TIR3* strain showed that CgTir3p localizes to the cell wall, and its expression is induced by serum. Semi-quantitative reverse transcriptase-PCR also showed that two transcription regulatory genes, *CgUPC2A* and *CgUPC2B*, control *CgTIR3* as well as *CgAUS1* gene expression. Interestingly, complementation studies using *Cgtir3*Δ showed that *ScDAN1*, a mannoprotein required for sterol uptake in *Saccharomyces cerevisiae*, could not complement the *C. glabrata TIR3* function. Furthermore, sterol analyses, in which both the *CgAUS1* and *CgTIR3* genes were constitutively expressed, resulted in aerobic sterol uptake although the amount of uptake was considerably less than that of cells cultured aerobically with serum. These results suggest that additional factors other than *CgAUS1* and *CgTIR3* are required for sterol uptake in *C. glabrata*.

1. Introduction

Ergosterol, the predominant fungal sterol, is required for normal membrane function in both pathogenic and non-pathogenic fungi. Various antifungal agents, including the azoles, amphotericin B, zaragozic acid, and terbinafine inhibit ergosterol biosynthesis at various steps of the pathway [1]. Ergosterol is most abundant in the plasma membrane but is synthesized in the endoplasmic reticulum (ER), the membrane system with the lowest level of this sterol. Sterol metabolism in the non-pathogenic yeast, *Saccharomyces cerevisiae*, has been intensively studied [reviewed in 2–4]. Under anaerobic conditions, or in certain mutant backgrounds, *S. cerevisiae* requires exogenous methionine, sterols, and unsaturated fatty acids, since the synthesis of these requires both molecular oxygen and heme [5–8]. Therefore, this yeast can take up exogenous sterol required for growth under anaerobic, but not aerobic conditions, a phenomenon known as "aerobic sterol exclusion" [9]. Sterol uptake in this yeast is mediated by two ATP-binding cassette transporters, Aus1p or Pdr11p, and the mannoprotein, Dan1p, which is a member of *DAN/TIR* (member of Srp1p/Tip1p family of serine–alanine-rich proteins) [10]. The exact role of mannoproteins in sterol uptake has not been elucidated.

The *S. cerevisiae DAN/TIR* genes are among a large group of genes that are upregulated during adaptation to anaerobic growth [11,12], and function as anaerobic counterparts of the major aerobic mannoproteins, *CWP1* and *CWP2*. The latter are abundant constituents of the cell wall and are believed to play a role in cell wall stabilization [13]. In addition, the *S. cerevisiae DAN/TIR* gene products are also expressed under low temperature or high hydrostatic pressure as adaptation mechanisms to such severe conditions [14]. Although the specific mechanism(s) by which *DAN/TIR* genes are regulated have not been completely elucidated, these genes are regulated by heme and three transcription factors: ScUpc2p (an activator) and ScMot3p and ScRox1p (repressors) [15,16].

Through a consensus site termed AR1, the Zn[2]Cys[6] binuclear transcription factor ScUpc2p regulates the expression of genes involved

Abbreviations: YPD, Yeast–Peptone–Dextrose; CSM, complete synthetic medium; ORF, open reading frame; HA, hemagglutinin; GFP, green fluorescent protein; MIC, minimum inhibitory concentration; RPMI, Roswell Park Memorial Institute medium; HPLC, high-performance liquid chromatography; 7-DHC, 7-dehydrocholesterol; RT-PCR, reverse transcriptase-polymerase chain reaction; GPI, glycosylphosphatidylinositol

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in sterol biosynthesis and import, including upregulation of *ScAUS1*, *ScPDR11* and *ScDAN1*, and up to one-third of all anaerobically expressed *S. cerevisiae* genes [10,15]. Thus, ScUpc2p is critically important for adaptation to hypoxia [17]. Constitutively active mutants of ScUpc2p, or its paralog, ScEcm22p, induce sterol uptake under aerobic conditions, overcoming the phenomenon of aerobic sterol exclusion [18,19].

Fungal species of the genus *Candida* generally live communally on and in the human body, yet *Candida* infections can become systemic and lethal in up to 60% of immunocompromised patients [20]. The pathogenic fungus, *Candida glabrata* is the second most common causative agent of candidiasis, and systemic infections have been linked to the death of immunocompromised and immunosuppressed patients [20]. Sustained use of azole antifungals to which *C. glabrata* is somewhat resistant, steroid and immunosuppressive agents, as well as use of other antibiotics which collapse microbial flora, have led to the emergence of *C. glabrata* as an important opportunistic fungus [21–23]. In addition, recent surveillance data have revealed an increase of *C. glabrata* clinical isolates that display resistance to not only azoles, but also to the echinocandin-class of antifungals which inhibit cell wall biosynthesis [24,25].

Since *C. glabrata* is a genetically close relative of *S. cerevisiae*, some of the factors involved in sterol uptake are conserved. Previous studies have identified the *C. glabrata CgAUS1* as orthologous to the *S. cerevisiae ScAUS1* and two genes, *CgUPC2A* and *CgUPC2B*, as orthologous to *S. cerevisiae ScUPC2* [26,27]. Like *S. cerevisiae*, the expression of *CgAUS1* is strongly upregulated by CgUpc2Ap and CgUpc2Bp [27]. Despite sharing these common gene products, *C. glabrata* differs in several important aspects relating to sterol import: *C. glabrata* wildtype strains can import sterols aerobically in the presence of serum or aerobically under iron poor conditions during azole treatment [28,29]. However, *S. cerevisiae* does not take up sterol aerobically unless a mutation in either the *UPC2* gene or various heme biosynthetic genes occurs. Therefore, it has been suggested that sterol uptake in *C. glabrata* may also partly explain the poor response of this yeast to azole antifungals [21].

Understanding sterol metabolism and uptake, in medically important pathogenic fungi such as *C. glabrata* will help to elucidate growth responses during infection particularly in hypoxic host environments as well as in tissues that have a rich supply of cholesterol. In this study, we identified the mannoprotein required for sterol uptake in *C. glabrata* and elucidated its cellular location and means by which it is regulated.

2. Materials and methods

2.1. Strains and media

Escherichia coli DH5α [F-, φ80, *lacZ*ΔM15, Δ(l*acZYA-argF*)U169, *hsdR17*(rk-mk+), *recA*1, *endA*1, *deoR*, *thi*-1, *supE*44, *gyrA*96, *relA*1 l2] was used in plasmid propagation. Bacterial strains were grown in a Luria–Bertani medium (LB) with ampicillin. Strains used in this study (Table 1) were grown on Sabouraud agar (2% Bacto peptone (Difco) and 2% glucose), in YPD (Yeast–Peptone–Dextrose) medium (1% Bacto yeast extract (Difco), 2% Bacto peptone (Difco) and 2% glucose) or in CSM (complete synthetic medium) medium (pH 5.8) (0.67% Bacto yeast nitrogen base without amino acids (Difco), 0.079% CSM complete supplement mixture (MP Biomedicals, Inc., OH, USA), 2% glucose and appropriate amino acids). Solid medium contained 2% agar (Nacalai Tesque, Kyoto, Japan). When cholesterol or 7-DHC (7-dehydrocholesterol) was added to the growth medium, sterols from a stock solution [5 mg/ml, dissolved in EtOH/Tween 80 (1:1)] were added to a final concentration of 50 μg/ml for agar medium or 25 μg/ml for liquid medium. Hypoxic conditions were generated using AnaeroPack-Anaero (Mitsubishi Gas Chemical Inc., Tokyo, Japan) according to the manufacturer's instructions.

2.2. Plasmid construction

Plasmids used for the transformation were constructed as follows.

pZeoi_CgTIR3: The CAGL0C03872g (*CgTIR3*) ORF (open reading frame) with its 985-bp promoter and 545-bp terminator was amplified using the primers TIR3-comp-F and TIR3-comp-R with wildtype genomic CBS138 DNA as template. Amplified products and the parent vector pZeoi_comp606 were digested with *Eco* RV and *Xho* I. Insert and vector (pZeoi_comp606) digested with *Pvu* II and *Sal* I were ligated to construct pZeoi_CgTIR3 [30].

p916TRP-CgTIR3, p916TRP-ScTIR3, and p916TRP-ScDAN1: The *CgTIR3* ORF was amplified using the primers TIR3-ORF-F and TIR3-ORF-R and *C. glabrata* wildtype CBS138 genomic DNA as template. The *ScTIR3* and *ScDAN1* ORFs were amplified using *S. cerevisiae* laboratory strain W303-1A genomic DNA as template [31], and primer pairs, ScTIR3-ORF-F and ScTIR3-ORF-R, or ScDAN1-ORF-F and ScDAN1-ORF-R, respectively. The amplified fragments and parent vector p916TRP were digested with *Apa* I and *Xho* I [32], and digested fragments were then ligated to obtain p916TRP-CgTIR3, p916TRP-ScTIR3, and p916TRP-ScDAN1, respectively. Primer sequences used in this study are provided in Table S1.

2.3. Strain construction

Deletion of the CAGL0C03872g (*CgTIR3*) gene was carried out in the parental strain KUE200, using a method described by Ueno et al. [32]. The target gene, *CgTIR3* was replaced by a DNA fragment harboring the *CgHIS3* gene, and after homologous recombination, generating strain K2tir3Δ. The replacement cassette was prepared by PCR using a pHIS906 plasmid containing *CgHIS3* and primers C03872KOF and C03872KOR [30] The recombination locus and deleted sequences were verified using PCR primers C03872CHF and C03872CHR (data not shown).

The strain in which *CgTIR3* is reintroduced into a *Cgtir3* deletant is designated, K2TIR3-re, and was generated using the method described by Ueno et al. [30]. The DNA fragment harboring the *CgTIR3*-ORF and *Zeo* R (Resistance to Zeocin™ is conferred by the *Sh ble* gene from *Streptoalloteichus hindustanus*) genes were amplified using primers pChr606 F1 and pChr606 R1 with pZeoi_CgTIR3 DNA as template. The amplified fragment was transformed into K2tir3Δ, resulting in the reintegration of *CgTIR3* and *Zeo*R genes into the non-coding region on chromosome F (positions 605,901–606,015). The site of integration was verified using PCR primers pZeoORFcheckF1 and pchr606check (data not shown).

Strains containing the triple HA (hemagglutinin) (3× HA), TIR3-HA DNA fragment were generated using methods described by Schneider et al. [33]. DNA fragments comprising 3× HA–ScURA3–3× HA with homologous regions of the CgTIR3-ORF (+16 to +75 and +76 to +135) gene were amplified using primers TIR3-HA-F and TIR3-HA-R and pMPY–3× HA as template DNA. The amplified fragments were transformed into the uracil auxotroph strain NAU3. After confirming the integration, using PCR primers C03872CHF and HA-check-Rev (data not shown), the *ScURA3* gene was removed using 5-FOA (5-Fluoroorotic acid, Wako Pure Chemical Industries, Ltd. Osaka, Japan), resulting in strain, TIR3-HA. Removal of the *ScURA3* gene from *TIR3* locus was confirmed using PCR primers C03872CHF and HA-check-Rev (data not shown).

To introduce GFP (green fluorescent protein) into the carboxyl terminus of CgAus1p, a DNA fragment, containing GFP-*ScURA3* harboring the *CgAUS1* carboxyl terminal portion (2877–4697 bp) and 3′ flanking region (894 bp), was amplified using PCR primers AUS1-C5 and CgAUS1DBR and AD/CgAUS1-GFP genomic DNA as template [29]. The amplified fragment was transformed into 2001UTH, TIR3-HA or 99AUS1