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ORIGINAL ARTICLE

Functional analysis of genes involved in beta-1, 2-Mannosylation of glycans and putative cell wall proteins in *Candida albicans* during hyphae transition

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Abstract Structural studies of cell wall components of the pathogenic yeast *Candida albicans* revealed a family of genes involved in beta-mannosyltransferase associated with the addition of beta-mannose to the acid-labile fraction of cell wall phosphopeptidomannan. Despite the importance of beta-1, 2-oligomannosides in virulence, the gene expression pattern of this family during hyphae transition have not been identified in *C. albicans*. We investigated using RT-qPCR whether genes encoding beta-1, 2-mannosylation of glycans and putative cell wall proteins are expressed during hyphae induction. Here, we identify four out of nine putative genes encoding beta-1, 2-mannosylation of glycans, *BMT2*, *BMT4*, *BMT5*, and *BMT6* were downregulated during 30, 60, and 180 min of hyphae transition. We also observed that expression of Candida putative GPI-anchored protein coding genes *PGA6*, *PGA19*, *PGA54*, *PGA56*, *PGA58*, *PGA59*, and *PGA13* were upregulated after 60 min of hyphae transition, while the levels of expression of these genes showed moderately changes during 15 and 180 min of hyphae transition. In either case, the involvement of these genes in hyphal growth makes them putative targets for new antifungal drugs aimed at inhibiting hyphae formation in *C. albicans*.

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Introduction

Candida albicans is a major human pathogen and the number one cause of fungal infection in human. Unlike other pathogens, it can be found in skin and the gastrointestinal tract as a harmless commensal organism, producing serious disease in people with weakened immune systems (Zaoutis et al. 2005; Shorr et al. 2009). *C. albicans* is a truly polymorphic organism; it has the ability to undergo morphological changes between the yeast form (with rounded cells and daughter buds that physically separate from the mother cell), the pseudohyphal form (which consists of chains of cells with various degrees of elongation that still show constrictions between adjacent cells), and the true hyphal form (which consists of long tubes with parallel sides and no constrictions) (Sudbery et al. 2004; Rashki et al. 2012). Yeast cells disseminate more easily in the bloodstream, while hyphae are invasive and can penetrate host tissues during the early stages of infections (Sudbery et al. 2004; Shen et al. 2008; Rashki et al. 2012). Furthermore, switching of *C. albicans* to the hyphal form in the host has long been considered to be important for pathogenesis, since mutants defective in hyphal growth are known to be less virulent (Lo et al. 1997; Lermann and Morschhauser 2008). Thus, identification of genes involved in the yeast-to-hyphae transition is important for the development of new antifungal agents (Ghalehnoo et al. 2010). The cell wall proteins of *C. albicans* play a key role in morphogenesis and pathogenesis and might be potential target sites for new specific antifungal drugs. However, these proteins are

difficult to analyze because of their high heterogeneity, interconnections with wall polysaccharides (mannan, glucan, and chitin), low abundance, low solubility, and hydrophobic nature. Here, we report a functional analytic approach to the study of some selected genes coding for cell wall proteins during *C. albicans* yeast and hyphal forms. We also highlight the expression pattern for a new family of genes involved in beta-1, 2-Mannosylation of glycans during yeast-to-hyphae transition in *C. albicans*.

Materials and methods

Strains, media, and growth conditions

The *C. albicans* strains used were SC5314 (prototrophic), its Ura− derivative, CAI4 (Δ*ura3*::*imm434*/Δ*ura3*::*imm434*) (Fonzi and Irwin 1993). Strains were grown in yeast extract-peptone-dextrose or YNB liquid and solid media (Ghalehnoo et al. 2010; Sherman 2005), which for Ura− strains was supplemented with 50 μg of uridine/ml. The dimorphic transition in liquid medium was induced by growing cells in YNB at 28 °C and changing them at a density of 1×10^7 cells/ml in Lee medium plus 10 % BCS (Invitrogen) or to Lee medium with 1.25 % N-acetylglucosamine (GlcNAc) instead of glucose and incubated at 37 °C. For RNA isolation, cDNA preparation and for RT-qPCR experiments, C. *albicans* strain CAI4 and SC5314 was grown in YNB or under our condition for the yeast-to-hyphae transition. Cells were pregrown in Lee medium up to the exponential phase at 28 °C, washed, starved in water for 24 h at 4 °C, and then incubated in Lee plus 10 % BSC medium at 37 °C. Samples were collected at 15, 60, and 180 min harvested at room temperature, re-suspended in a very small volume of the supernatant, and immediately frozen by releasing small drops of cell samples into liquid nitrogen. Cells were then stored at −80 °C until RNA extraction.

RNA extraction and real-time quantitative PCR (RT-qPCR

Total RNA was isolated by breaking cells in a microdismembrator (Braun, Melsungen), followed by the trizol extraction method, as described in the Galar Fungal standard operating procedures for RNA extraction (http://www.pasteur.fr/recherche/unites/Galar_Fungail/), keeping the cells frozen in liquid nitrogen at all times. RNA quality was controlled by gel electrophoresis, and RNA concentrations were measured by determination of the absorbance at 260 nm (one unit of the optical density at 260 nm is equal to 40 μg of RNA/ml of DEPC treated H_2O, based on the extinction coefficient of RNA in H_2O). One microgram of total RNA was treated with DNase, and cDNA was synthesized as described previously (Ghalehnoo et al. 2010; Felk et al. 2002). Quantitative real-time PCRs were performed in triplicate using the 7,900 sequence detection system (Applied Biosystems, Foster City, CA). Primers were designed using primer express software (Table 1) and the housekeeping gene *ACT1* (Losberger and Ernst 1989; Sandovsky-Losica et al. 2006), which was expressed to a similar extent under all conditions investigated, was used as a standard control. The PCR conditions consisted of Taqman Gold PCR kit activation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 s, and annealing/extension at 60 °C for 1 min. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified using software provided with the 7,900

Table 1 *C. albicans* PCR primers used in this study

Primer	Primer Sequence
GSL1-F	TGGTTAAGCTCTTGTCGGCT
GSL1-R	TTTGGGAAACAACGTGAACA
IFF5-F	ACCAATTCCAAAAGCACCAG
IFF5-R	GGTTGGGAAAATTGATGTGG
DSE1-F	AGTAGTGCCAATACCGTCGC
DSE1-R	ACCATGTCCTGCGGCTAATA
CHT2-F	CGATGTTGCTTCTGCCACTA
CHT2-R	TCAGGGTACCCAGTAGCACC
BMT5-F	TAGGCTCGTCTGTTTGGCTT
BMT5-R	CCAATTACGATCAAACGCCT
BMT6-F	ATATCGCTGTTCCTGGTTGG
BMT6-R	ACGTCTCGCTCAATTCCATC
BMT4-F	GAAAGAAAAGGCGGAAAAGG
BMT4-R	TCACCATCACCATCACCATC
BMT2-F	GAGAAATGTGGCTGTGGTGA
BMT2-R	ATCAGGCCACCATGGTAAAA
PGA13-F	ATTCCTGTGGAAACAGCCAC
PGA13-R	TCAATAGTGGTTGGATGGCA
PGA62-F	CTGAATCTTCCCCAGTTCCA
PGA62-R	AGATTCAGCAGCAACGGTCT
PGA59-F	CACTGACATTGCTACCACCG
PGA59-R	GGGTGGTGGTGGAGTTAGAA
PGA54-F	CAGCCGAAGCTACTGAATCC
PGA54-R	GGCAGAAACGTAGGTGGTGT
PGA6-F	TCGCTGGTTCTTTAGTTGCC
PGA6-R	TTGAACAGCTGGAGCAACAC
PGA19-F	ACACGTTGAGCCAAATGACA
PGA19-R	AGCACTGATCTGCCAACCTT
PGA56-F	GTGGTGCTGGTTCCCATAAC
PGA56-R	CAGCACCAACAGCAGCTAAA
PGA58-F	TACACCAGCTCCAGCTCCTT
PGA58-R	GAAGCTGGTTGTTCTGGAGC
SCW11-F	GATTGTGGATCATTAACCA
SCW11-R	CCCAACCATTGGATTGTCCAT

sequence detection system. The results are shown as relative expression compared to *ACT1*.

Results

Morphology of the cultures

The yeast-to-hyphae transition was induced from cells pregrown in Lee medium up to the exponential phase at 28 °C, washed, starved in water for 24 h at 4 °C, and then incubated in Lee medium at 37 °C. Under these conditions, the growth of most of the cells was arrested in the G_1 phase of the cell cycle, and a reproducible and homogeneous hyphal formation was obtained (Fig. 1).

Expression pattern of genes involved in beta-1, 2-Mannosylation of glycans and putative cell wall proteins

We assumed that growth during yeast-to-hyphae transition may directly or indirectly influence the gene expression patterns of cell wall proteins. For analysis of gene expression patterns during yeast-to-hyphae transition, Candida cells were grown in Lee liquid medium for 15, 60, and 180 min, harvested by centrifugation and stored at −80 °C.

Total RNA was isolated using the trizol reagent (Invitrogen), and cDNA was synthesized as previously described (Ghalehnoo et al. 2010). The resulting ss cDNA was used as the template for a series of RT-qPCRs with 17 selected genes of interest. The expression data observed by RT-qPCR from three independent experiments are summarized in Fig. 2. First, the levels of expression of BMT genes form a novel family of fungal genes were determined. Of nine BMT genes, expression level of only *BMT2*, *BMT4*, *BMT5*, and *BMT6* were decreased during 15, 60, and 180 min of hyphae transition (Fig. 2). The levels of expression of other BMT family genes, *BMT1*, *BMT3*, *BMT7*, *BMT8*, and *BMT9* were either not affected or moderately decreased during hyphae transition in *C. albicans* (data not shown). Furthermore, some expression level of genes encode putative GPI-anchored proteins were investigated during hyphae transition. One gene known as an essential cell wall protein involved in cell wall integrity and rigidity (*DSE1*) showed a moderate downregulation of expression. Similarly, the levels of expression of one gene known to be induced during planktonic growth (*IFF5*) which encodes an adhesin-like protein (Terashima et al. 2000; De Groot et al. 2003), gene *SCW11* which encodes a cell wall protein (Bensen et al. 2002; de Groot et al. 2004), gene *CHT2* which encodes a GPI-linked chitinase that induced in yeast form cells (Harcus et al. 2004), and *GSL1* which encodes a beta-1,3-glucan synthase (Mio et al. 1997; Liu et al. 2005) were also decreased. Since the yeast-to-hyphae transition of *C. albicans* cells is known to cause upregulation of a number of cell wall encoding genes, we also investigated the expression patterns of some selected genes coding for putative GPI-anchored proteins during time point of yeast-to-hyphae transition. The expression of Candida putative GPI-anchored protein coding genes *PGA6*, *PGA19*,

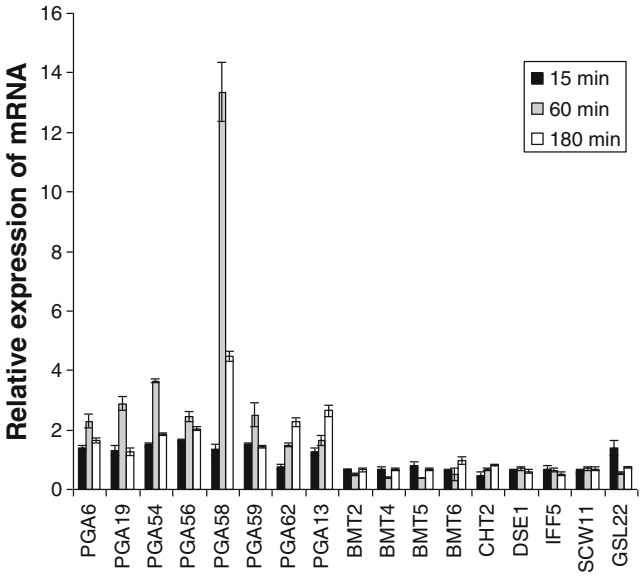

Fig. 2 Relative expressions of mRNAs in *C. albicans* during hyphae transition. *C. albicans* (1×10^7 cells/ml in Lee's medium containing 10 % FBS) was incubated at 37 °C for 15, 60, and 180 min. After incubation and extraction of RNA, the relative expressions of selected genes were measured as described in materials and methods. Results are means +/− standard errors of the means of triplicate determinations

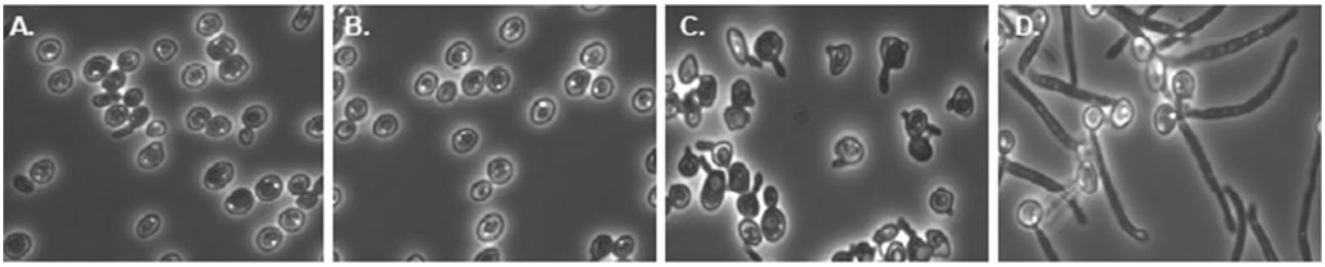

Fig. 1 Morphology of *Candida albicans*. Yeast phenotype of the cells of the inoculum incubated in Lee medium up to the exponential phase at 28 °C, washed and starved in water for 24 h at 4 °C (**a**). Yeast-hyphal transition after 15 (**b**), 60 (**c**), and 180 min (**d**)

PGA54, *PGA56*, *PGA58*, *PGA59*, and *PGA13* were upregulated after 60 min of hyphae transition, while the levels of expression of these genes showed moderately changes during 15 and 180 min of hyphae transition.

Discussion

The significant increase in opportunistic fungal infections observed over the last two decades represents an important medical challenge. In particular, *C. albicans* is one of the most frequent causes of nosocomial bloodstream infections and is associated with high mortality rates (Wisplinghoff et al. 2004; Trofa et al. 2008). Despite increased investments in antifungal therapies, only limited progress has been achieved in controlling nosocomial candidiasis (Abrams et al. 1991). *C. albicans* exists as a harmless, commensal microorganism in the majority of immunocompetent people. However, the increase in number of patients with compromised immunity has contributed to the observed rise in opportunistic fungal infections. Several traits, including differential expression of adhesins (Ramanan and Wang 2000; Mille et al. 2008) and lytic enzymes morphological changes, stress response, and changes in the glyoxylate cycle have been linked to pathogenicity of *C. albicans* (Kniemeyer et al. 2011). However, the way in which *C. albicans* coordinates these factors in its continuous adaptation to host conditions and yeast-to-hyphae transition is still poorly understood. Among cell wall proteins contributing to *C. albicans* virulence, which still pose unresolved questions, are putative beta-1, 2-mannosyltransferases, and GPI-anchored proteins. However, they may also act directly against virulence attributes which are not important for in vitro cell culture growth. In order to elucidate the mode of action of these cell wall proteins during yeast-to-hyphae transition, we investigated the expression profiles of selected genes encoding known or putative cell wall proteins in cells exposed to Lee medium supplemented with 10 % bovine calf serum at 37 °C in *C. albicans*, a commensal fungus and an opportunistic pathogen that can cause severe infection in immunocompromised humans (Elson et al. 2009). In the host, *C. albicans* exists in a variety of morphological forms, including budding yeast, pseudohyphae (chains of elongated ellipsoidal cells), and hyphae (chains of long, cylindrical cells with parallel cell walls) (Sudbery et al. 2004). The ability to rapidly switch among these forms in response to external cues is one of numerous factors contributing to virulence. The hyphal form in particular has been associated with numerous virulence attributes such as passage through endothelial and epithelial barriers and host tissue damage. In this study, we selected at least 17 genes corresponding to putative and well-characterized cell wall proteins. Four of repressed genes *BMT2*, *BMT4*, *BMT5*, and *BMT6* are classified in the same subfamily. In *C. albicans*, *BMT2*, *BMT5*, and *BMT8* are in one chromosome. Whether an independent induction or a joint induction occurs must be clarified. Although the genes encoding these proteins share a "DUF" families that are annotated with the domain of unknown function, our data suggest that each protein has a different function, and they are in agreement with those described by Dominguez and Nantel's group (Nantel et al. 2002), who observe the downregulation of four putative beta-mannosyltransferases during yeast-to-hyphae transition. Our data and those reported previously (Nantel et al. 2002) point to a different type of regulation for some BMT genes (*BMT1*, *BMT3*, *BMT7*, *BMT8*, and *BMT9*) in *C. albicans* and suggest a role of the BMT genes in cell wall remodeling per se during the yeast–hyphae transition, independently of virulence or any other factors. It may be predicted that this group of genes, together with those involved in O-mannosylation, will be essential for understanding why *C. albicans* is virulent. Upon comparing our results concerning PGA genes with those described by Sentandreu's group (Castillo et al. 2006) on the regeneration/formation of cell wall after *C. albicans* protoplasting, we also observed the upregulation of *PGA6*, *13*, *54*, *56*, *59*, and 62 (although slightly earlier). Another interesting fact is that unlike the upregulation found in most of the cell wall genes, we observed the downregulation of five cell wall coding proteins genes, *DSE1*, *IFF5*, *SCW11*, *CHT2*, and *GSL1* during time point of hyphae transition. This implies role of these proteins in cell wall remodeling in the yeast-to-hyphae transition in *C. albicans*.

References

Abrams BB, Hanel H, Hoehler T (1991) Ciclopirox olamine: a hydroxypyridone antifungal agent. Clin Dermatol 9:471–477

Bensen ES, Filler SG, Berman J (2002) A forkhead transcription factor is important for true hyphal as well as yeast morphogenesis in *Candida albicans*. Eukaryot Cell 1:787–798

Castillo L, Martinez AI, Garcera A, Garcia-Martinez J, Ruiz-Herrera J, Valentin E, Sentandreu R (2006) Genomic response programs of *Candida albicans* following protoplasting and regeneration. Fungal Genet Biol 43:124–134

De Groot PW, de Boer AD, Cunningham J, Dekker HL, de Jong L, Hellingwerf KJ, de Koster C, Klis FM (2004) Proteomic analysis of *Candida albicans* cell walls reveals covalently bound carbohydrate-active enzymes and adhesins. Eukaryot Cell 3:955–965

De Groot PW, Hellingwerf KJ, Klis FM (2003) Genome-wide identification of fungal GPI proteins. Yeast 20:781–796

Elson SL, Noble SM, Solis NV, Filler SG, Johnson AD (2009) An RNA transport system in *Candida albicans* regulates hyphal morphology and invasive growth. PLoS Genet 5:e1000664

Felk A, Kretschmar M, Albrecht A, Schaller M, Beinhauer S, Nichterlein T, Sanglard D, Korting HC, Schafer W, Hube B (2002) *Candida albicans* hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. Infect Immun 70:3689–3700

Fonzi WA, Irwin MY (1993) Isogenic strain construction and gene mapping in *Candida albicans*. Genetics 134:717–728

Ghalehnoo ZR, Rashki A, Najimi M, Dominguez A (2010) The role of diclofenac sodium in the dimorphic transition in *Candida albicans*. Microb Pathog 48:110–115

Harcus D, Nantel A, Marcil A, Rigby T, Whiteway M (2004) Transcription profiling of cyclic AMP signaling in *Candida albicans*. Mol Biol Cell 15:4490–4499

Kniemeyer O, Schmidt AD, Vodisch M, Wartenberg D, Brakhage AA (2011) Identification of virulence determinants of the human pathogenic fungi *Aspergillus fumigatus* and *Candida albicans* by proteomics. Int J Med Microbiol 301(5):368–377

Lermann U, Morschhauser J (2008) Secreted aspartic proteases are not required for invasion of reconstituted human epithelia by *Candida albicans*. Microbiology 154:3281–3295

Liu TT, Lee RE, Barker KS, Lee RE, Wei L, Homayouni R, Rogers PD (2005) Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. Antimicrob Agents Chemother 49:2226–2236

Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR (1997) Nonfilamentous *C. albicans* mutants are avirulent. Cell 90:939–949

Losberger C, Ernst JF (1989) Sequence of the *Candida albicans* gene encoding actin. Nucleic Acids Res 17:9488

Mille C, Bobrowicz P, Trinel PA, Li H, Maes E, Guerardel Y, Fradin C, Martinez-Esparza M, Davidson RC, Janbon G, Poulain D, Wildt S (2008) Identification of a new family of genes involved in beta-1, 2-mannosylation of glycans in Pichia pastoris, and *Candida albicans*. J Biol Chem 283:9724–9736

Mio T, Adachi-Shimizu M, Tachibana Y, Tabuchi H, Inoue SB, Yabe T, Yamada-Okabe T, Arisawa M, Watanabe T, Yamada-Okabe H (1997) Cloning of the *Candida albicans* homolog of Saccharomyces cerevisiae GSC1/FKS1 and its involvement in beta-1, 3-glucan synthesis. J Bacteriol 179:4096–4105

Nantel A, Dignard D, Bachewich C, Harcus D, Marcil A, Bouin AP, Sensen CW, Hogues H, van het Hoog M, Gordon P, Rigby T, Benoit F, Tessier DC, Thomas DY, Whiteway M (2002) Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. Mol Biol Cell 13:3452–3465

Ramanan N, Wang Y (2000) A high-affinity iron permease essential for *Candida albicans* virulence. Science 288:1062–1064

Rashki A, Ghalehnoo ZR, Dominguez A (2012) The early response of *Candida albicans* filament induction is coupled with wholesale expression of the translation machinery. Comp Clin Pathol 21:1533–1545

Sandovsky-Losica H, Chauhan N, Calderone R, Segal E (2006) Gene transcription studies of *Candida albicans* following infection of HEp2 epithelial cells. Med Mycol 44:329–334

Shen J, Cowen LE, Griffin AM, Chan L, Kohler JR (2008) The *Candida albicans* pescadillo homolog is required for normal hyphal-to-yeast morphogenesis and yeast proliferation. Proc Natl Acad Sci U S A 105:20918–20923

Sherman F (2005) The importance of mutation, then and now: studies with yeast cytochrome c. Mutat Res 589:1–16

Shorr AF, Gupta V, Sun X, Johannes RS, Spalding J, Tabak YP (2009) Burden of early-onset candidemia: analysis of culture-positive bloodstream infections from a large U.S. database. Crit Care Med 37:2519–2526, quiz 2535

Sudbery P, Gow N, Berman J (2004) The distinct morphogenic states of *Candida albicans*. Trends Microbiol 12:317–324

Terashima H, Yabuki N, Arisawa M, Hamada K, Kitada K (2000) Upregulation of genes encoding glycosylphosphatidylinositol (GPI)-attached proteins in response to cell wall damage caused by disruption of FKS1 in *Saccharomyces cerevisiae*. Mol Gen Genet 264:64–74

Trofa D, Gacser A, Nosanchuk JD (2008) *Candida parapsilosis*, an emerging fungal pathogen. Clin Microbiol Rev 21:606–625

Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB (2004) Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis 39:309–317

Zaoutis TE, Argon J, Chu J, Berlin JA, Walsh TJ, Feudtner C (2005) The epidemiology and attributable outcomes of candidemia in adults and children hospitalized in the United States: a propensity analysis. Clin Infect Dis 41:1232–1239