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The role of Alg13 *N*-acetylglucosaminyl transferase in the expression of pathogenic features of *Candida albicans*

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ABSTRACT

Background: The pathogenic potential of *Candida albicans* depends on adhesion to the host cells mediated by highly glycosylated adhesins, hyphae formation and growth of biofilm. These factors require effective *N*-glycosylation of proteins.
Here, we present consequences of up- and down-regulation of the newly identified *ALG13* gene encoding *N*-acetylglucosaminyl transferase, a potential member of the Alg7p/Alg13p/Alg14p complex catalyzing the first two initial reactions in the *N*-glycosylation process.
Methods: We constructed *C. albicans* strain *alg13*Δ*::hisG/TR*p-*ALG13* with one allele of *ALG13* disrupted and the other under the control of a regulatable promoter, TRp. Gene expression and enzyme activity were measured using RT-qPCR and radioactive substrate. Cell wall composition was estimated by HPLC DIONEX. Protein glycosylation status was analyzed by electrophoresis of HexNAcase, a model *N*-glycosylated protein in *C. albicans*.
Results: Both decreased and elevated expression of *ALG13* changed expression of all members of the complex and resulted in a decreased activity of Alg7p and Alg13p and under-glycosylation of HexNAcase. The *alg13* strain was also defective in hyphae formation and growth of biofilm. These defects could result from altered expression of genes encoding adhesins and from changes in the carbohydrate content of the cell wall of the mutant.
General significance: This work confirms the important role of protein *N*-glycosylation in the pathogenic potential of *C. albicans*.

1. Introduction

Candida albicans is a common component of human microflora and the most common cause of opportunistic fungal infections of immunocompromised patients, with a mortality rate around 30–50% [1,2]. The pathogenic potential of *C. albicans* is attributed to several factors including expression of adhesins, the yeast-to-hyphae transition and biofilm formation. All these factors can be affected by changes in glycosylation of proteins which therefore plays an important role in *Candida* virulence [3–6].

N-glycosylation is an essential protein modification highly conserved in evolution. In all eukaryotes, *N*-glycosylation is obligatory for viability since glycans have a common role in promoting protein folding, quality control, and certain sorting events and, finally, determination of protein activity [7,8]. *N*-glycosylation can be divided into two phases: the first is the assembling of the core polysaccharide containing 14 monosaccharide residues which is then transferred *en bloc* to a growing polypeptide chain. The second step of *N*-glycosylation is further processing of the polycarbohydrate *N*-linked to the protein to the mature structures characteristic for the host [2,9–13]. Glycosylation requires a phosphorylated isoprenoid lipid, dolichyl phosphate (Dol-P), as a carrier of the carbohydrates. During *N*-glycosylation the whole core polysaccharide is assembled on Dol-P [10]. Biosynthesis of the lipid-linked oligosaccharide (LLO) begins at the cytosolic side of the endoplasmic reticulum (ER) with a sequential addition of two *N*-acetylglucosamine (GlcNAc) residues and five mannoses to Dol-P with nucleotide diphosphate sugars, UDP-GlcNAc and GDP-mannose, as donors [14]. The resulting oligosaccharide, Dol-PP-$GlcNAc_2$ Man_5, is then flipped to the lumen of the ER and four mannosyl and three glucosyl residues from Dol-P-mannose and Dol-P-glucose are added to form Dol-PP-$GlcNAc_2$ Man_9 Glc_3. The assembled core oligosaccharide is transferred to the γ-amido group of asparagine residues located in a highly conserved motif Asn-X-Ser/Thr (X can be any amino acid except proline) of the modified protein.

The addition of the second GlcNAc residue to Dol-PP-GlcNAc is catalyzed by a hetero-oligomeric GlcNAc transferase that in most eukaryotes comprises the Alg13p and Alg14p subunits. Alg13p is the catalytic subunit recruited to the ER by the membrane protein Alg14p [15–17].

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Bickel et al. [15] have demonstrated that yeast membranes depleted of Alg13p or Alg14p lack GlcNAc transferase activity *in vitro* and accumulate Dol-PP-GlcNAc *in vivo*. This activity is also present in bacteria, however, in *E. coli* the GlcNAc transferase (MurG) catalyzing peptidoglycan biosynthesis is a single protein with Alg13/Alg14 homologous domains. A structural comparison of Alg13p, Alg14p and MurGp based on the crystal structure of the latter has revealed that Alg13p corresponds to the C-terminal part of MurG thought to bind the UDP-GlcNAc donor, and Alg14p to the N-terminal part containing a glycine-rich motif postulated to be a membrane association site involved in Dol-P recognition [18]. The interaction between the two Alg13p/Alg14p subunits of UDP-GlcNAc transferase in yeast is limited to a C-terminal α-helix (comprised of fifteen amino acids) of Alg13p and three amino acids of Alg14p [16,17,19]. Furthermore, the N-terminal region of Alg14p interacts directly with one more protein, Alg7p. This enzymatic protein catalyzes the formation of Dol-PP-GlcNAc, the acceptor of the second GlcNAc added by Alg13p [17,20]. Thus in *S. cerevisiae* Alg7p is, in fact, the third member of the Alg7p/Alg13p/Alg14p (Alg7/13/14) complex indispensable for the initial reaction in LLO biosynthesis. The genes coding for the proteins forming this complex are essential.

Here, we cloned and analyzed a previously uncharacterized *orf19.6025* from *C. albicans*. This ORF was found by searching a *C. albicans* genomic data base with the Alg13p sequence from *S. cerevisiae* as probe.

To analyze the function of this hypothetical Alg13 protein we constructed a *C. albicans* strain *alg13Δ::hisG/TR*p-*ALG13* with one allele of *ALG13* disrupted and the other under the control of a regulatable promoter, TRp. TRp is a strong promoter which can be repressed by doxycycline. Thus, depending on the composition of medium the constructed strain expressed *ALG13* at a level exceeding that of the wild type (without doxycycline added) or markedly lower (with doxycycline).

The changes in expression of *ALG13* influenced the expression of *ALG7* and *ALG14* and altered Alg7p and Alg13p activities causing under-*N*-glycosylation of the model *N*-glycosylated protein HexNAcase.

The impaired glycosylation resulted in defects in hyphae formation and biofilm growth — the two invasive forms of *Candida*. The background of these changes included altered expression of *HWP1* (hyphae wall protein) and *ALS1* (agglutinin-like sequence protein) genes encoding factors contributing to biofilm formation. Changes in the cell wall composition and in the composition of the extracellular matrix of biofilm were also observed.

2. Materials and methods

2.1. Strains and growth conditions

C. albicans strain CAI4 (genotype: *ura3 Δ::imm434/ura3 Δ::imm434*), an uridine auxotroph was used for deletion of *ALG13* gene (Table 1).

E. coli strain DH5α F′ (genotype: *F′ supE44 ΔlacU169 {ϕ80 lacZ ΔM15} hsdR17 recA1 endA1 ngyrA96 thi-1 relA1*) [21] was used for plasmid propagation.

C. albicans strains were routinely grown at 30 °C in YPD medium (1% yeast extract, 1% Bacto-peptone, 2% glucose) or SD medium (0.67% yeast nitrogen base, 2% glucose). Uridine auxotrophic strains were grown on media supplemented with uridine (20 μg/ml). To repress the tetracycline promoter, doxycycline was added to the medium at concentrations from 10 to 100 μg/ml; 40 μg/ml was established as the optimal concentration and was then used throughout the study.

The ability to form hyphae was tested on Spider medium (1% nutrient broth, 1% mannitol, 0.2% K_2HPO_4 and 1.35% agar), YPSerum (1% yeast extract, 0.5% peptone, 10% horse serum and 2% agar) or YPD [22].

For sensitivity assays solid YPD medium (1.5% agar) was supplemented with uridine (20 μg/ml), doxycycline (40 μg/ml), tunicamycin (1, 1.5, 2 μg/ml) or Congo Red (5, 10, 15 μg/ml) or Calcofluor White (5, 10, 15 μg/ml). To induce the excision of *URA3* gene, *C. albicans* transformants were grown on FOA plates (0.67% yeast nitrogen base, 2% glucose, 0.3% 5-fluoroorotic acid, 40 μg/ml uridine, 2% agar).

Table 1
Candida albicans strains used in this study.

Name	Genotype	Source
C. albicans CAI4	*ura3 Δ::imm434/ ura3 Δ::imm434*	Fonzi and Irwin [23]
alg13Δ::hisG/ALG13	CAI4; *alg13Δ::hisG/ALG13*	This study
*alg13Δ::hisG/TR*p-*ALG13*	CAI4; *alg13Δ::hisG/TR*p-*ALG13*	This study

2.2. Construction of C. albicans strain

One copy of *ALG13* (*orf19.6025*) was deleted using the "URA-Blaster" method [23]. For the construction of the deletion cassette the following primers were used: ALG13-flank1-F/ALG13-flank1-R for amplification of the 5′ region of homology (−494 to −46 upstream of the ORF) and ALG13-flank2-F/ALG13flank2-R for amplification of the 3′ region of homology (+3 to +425 downstream of the AUG start codon) (Table 2). The obtained fragments were cloned to the p5921 plasmid in the *Sac*I/*Bgl*II and *Bam*HI/*Sal*I sites, respectively. The *Sac*I/*Sal*I fragment was then excised and used for gene replacement in the CAI4 strain to obtain the *alg13Δ::hisG-URA3-hisG/ALG13* strain. The *URA3* selective marker was removed as above to obtain the *alg13Δ:hisG/ALG13* hemizygote [23].

To put the second copy of *ALG13* under the control of tetracycline promoter (TRp), primers ALG13-TRp-F and ALG13-TRp-R were used to amplify the cassette on the template of p2151c plasmid. The 4067-bp fragment contained: a 55-bp fragment homologous to region −162 to −107 bp upstream of the *ALG13* start codon, the *URA3* selection marker, the fusion transactivator — *tetR-ScHAP4AD*, the regulatable *tetO-ScHOP1* promoter and a 53-bp sequence homologous to region −4 to +49 bp of *ALG13*. The cassette was used for transformation of the *alg13Δ::hisG/ALG13* strain.

Proper construction of the strains was confirmed by Southern blot analysis (Fig. 1S). DNA was isolated from the *alg13Δ::hisG/ALG13*, *alg13Δ::hisG/TR*p-*ALG13* and the CAI4 control strain, digested with *Ban*I, *Bgl*II, or *Hin*dIII, electrophoresed, transferred to Hybond-N membrane and hybridized with a DIG-labelled 445-bp probe homologous to the coding region of *ALG13*. The probe was amplified by PCR with ALG13-flank2-F and ALG13-flank2-R primers on *C. albicans* genomic DNA and visualized with the NBT/BCIP system (Promega).

2.3. Molecular biology methods

Chromosomal DNA was isolated from *C. albicans* using the Promega Wizard Genomic DNA Purification kit. Total RNA was isolated using the single-step method described by Chomczynski and Sacchi [24]. Other molecular biology procedures were performed according to standard protocols [25].

2.4. Quantitative reverse transcription PCR (RT-qPCR)

Reverse transcription was performed using the Advantage RT-for-PCR Kit (Clontech Laboratories) with 1 μg of total RNA as a template. Gene-specific primers designed using Clone Manager 6 software, their calculated Tm and amplicon sizes are shown in Table 3. qPCR assays were performed in a Light Cycler 1.6 Instrument (Roche Life Science). For the amplification Light Cycler Fast Start DNA Master PLUS SYBR Green (Roche Life Science) mix was combined with 0.2 μM forward and reverse primers and cDNA diluted 1:5 with nuclease-free water. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at appropriate temperature (Table 3) for 10 s and elongation at 72 °C for 1 s per 25 bp. All primer pairs produced a single