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Role of N-Acetylglucosamine in Ca2+ and Mg2+ ions- regulated Filamentous Growth and Protein Glycosylation in C. albicans.

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Abstract:

Glucose or N-Acetylglucosamine induce similar filamentous growth and a specific calcium and magnesium-dependent intracellular glycoprotein pattern in C. albicans at 37 °C in vitro. Both filament growth and its associated glycoprotein pattern are inhibited by calcium ions. The above inhibition can be reversed by magnesium ions, though only in N-Acetylglucosamine- but not in glucose-treated cells. Our data show that in contrast to glucose, N-Acetylglucosamine more than being only a carbon source, it is a key factor for filamentous growth and intracellular protein glycosylation to occur, and thus, it may effect the interaction of C. albicans with its host in a calcium- and magnesium-dependent manner.

Key Words: C.albicans, filamentous growth, glycosylation, mannoproteins, N-Acetyl Glucosamine.

Introduction:

Filament growth of Candida albicans is thought to be one of its virulence factors[1]. This pathogen is widely found in blood-wormed species and human[2]. Attempts to understand how C.albicans undergoes yeast-to-filament growth transition may help developing new therapeutic tools. Environmental growth conditions such as pH and temperature are key factors for filamentous growth to occur[3-5], and many genetic and molecular biology studies have shed light on a number of genes involved in the process of filamentation and pH-response[6]. According to the literature, there exist some signal transduction pathways that control filament growth in C.albicans, including the MAP kinase, cAMP/PKA and TUP1 pathways [7-14]. Mutations of many genes [15-19] were found to cause growth defect and/or inhibition of filament growth and loss of virulence of C.albicans. However, many of the mutation studies [20-22] showed a growth defect in a medium-dependent manner, which indicates that triggering yeast-to-filament growth transition may involve, yet unknown pathways and/or factors in the growth medium. In a previous work, we have shown that filament growth of C.albicans is associated with an increase of the intracellular pattern of glycoproteins, among which two 52/42 kDa mannoproteins occurred in the soluble subcellular fraction of filament cells [23]. In this study, we show that microscopically similar filament growth of C. albicans can be associated with different glycoprotein patterns, and that calcium ions inhibit filamentous growth and its associated glycoprotein pattern. Interestingly, in our cell growth conditions, in contrast to glucose, N-Acetylglucosamine in the presence of magnesium play a key role in filamentous growth.

Materials and Methods:

Cell growth conditions: C. albicans (ATCC 26555, serotype A) was employed through this study. It was subcultured every 2-3 weeks on 1.5 % (w/v) Bacto-Agar Difco) slopes of Sabouraud-dextrose medium. An Erlenmeyer flask (500 ml) containing 150 ml of Lee's medium was inoculated with a loopful of cells from 12-18 hours culture and incubated in a gyratory incubator (200 rpm) at 28°C for 15 hours. Cells were then collected by centrifugation at 3000xg during 10 minutes, washed twice with sterile distilled water and recovered at a concentration of approximately 1mg cells (dry weight) per ml in sterile glass-distilled water. The cells were then incubated at 28°C for 2 hours with shaking and were then stored at 4°C at least 24 hours for starvation before use. For growth experiments, a sample of the above starved blastoconidia cells was inoculated in 50-150 ml (at approximately 200 µg cell dry weight ml-1) of fresh minimum medium (MM) that contains in g l-1: KH2PO4, 2,5; (NH4)2SO4, 5; NaCl, 5; pH, 6,8. MgSO4, CaCl2, glucose or N-Acetylglucosamine were added where indi-

cated. Cells were incubated in a gyratory incubator at 28°C or 37°C, in order to grow as yeast or filament, respectively.

Preparation of cell-free homogenate: Blastoconidia starved cells were inoculated into 50 ml of fresh MM medium in the presence of glucose (40 mM) or N-Acetylglucosamine (40 mM). MgSO4 and CaCl2 were added where indicated. The cell suspension was incubated in a gyratory incubator for a given time period at 28°C or 37°C. Cells were then recovered in a cold 50 ml polypropylene tube and centrifuged at 5000 rpm (Sorvall centrifuge, rotor SS-34) for twenty min at 4°C. The cell pellet was resuspended in 4 to 5 ml of detergent-containing lysis buffer A (20 mM Hepes, 135 mM NaCl, 1 mM EGTA, 10 % (v/v) glycerol, 1 % (v/v) Triton x-100, 0.1 mM PMSF, 1 pg ml-1 aprotinin, 1 pg ml-1 leupeptin, 0.1 mM sodium orthovanadate, 20 mM NaF, pH 7.3), and transferred to a 10 ml polypropylene tube, to which glass beads (450-600 mesh, Sigma) were added (3 vol cell suspension per 1 vol glass beads). Tubes were kept at ice-cold temperature, and cells were broken by 8x1 min vortex. Glass beads were allowed to sediment, and cell lysate was recovered and centrifuged five min at 14000 rpm in an Eppendorf microcentrifuge. The supernatant (cell-free homogenate) was recovered for further analysis (see below).

Analysis of glycoproteins extracted with Concanavalin A-Sepharose: Glycoproteins were characterized by their interaction with two lectins, Concanavalin A (ConA) and wheat germ agglutinin (WGA)[24, 25]. ConA is a Jack bean protein that binds to certain high mannose type and biantennary type N-linked oligosaccharids, while WGA recognizes N-acetylglucosamine and sialic acid residues. Samples of cell-free homogenate obtained as described above were matched for protein content using the BioRad assay for protein measurement[26]. Aliquots of ConA-Sepharose beads (Sigma) (0.25 mg) were added to 0.5-1 mg of protein sample from each cell-free homogenate in a final volume of 1ml in Eppendorf tubes. After an incubation of 4 hours at 4°C under shaking, Sepharose beads were pelleted (14000 rpm, 1min, Eppendorf Centrifuge) and washed three times with 1 ml of HNT buffer (20 mM Hepes, 0.2M NaCl, 0.1 % (v/v) Triton x-100, pH 7.4), and were resuspended in 100 to 200 µl of 20 mM Hepes, pH 7.4 and kept at 4°C until analysis.

SDS-PAGE and Western Blot analysis of glycoproteins: SDS-PAGE and Western Blot methods were performed according to [27] and [28], respectively. An aliquot of protein extract prepared either as cell-free homogenate or as ConA-Sepharose extracted glycoproteins was mixed to an equal volume of 2x Laemmli sample buffer, boiled for five min and centrifuged. An aliquot was loaded onto a 10 to 15 % SDS-PAGE. The gel was stained by Coomassie blue, and other equivalent gels were used for transferring proteins onto a nitrocellulose filter by mean of a Bio-Rad transblot system. The filter was then washed in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1

% (v/v) Triton x-100, pH 7.6) and blocked at 37°C in 5 % (w/v) BSA (in TBST) for one hour. The filter was then incubated with 1 pg ml-1 of peroxidase-labeled ConA (Sigma) or peroxidase-labeled WGA (Sigma) in TBST at room temperature during forty min. The filter was washed extensively with TBST containing 1 M NaCl. A last wash was carried out in TBS. WGA-blotted filter was incubated for one min in the Amersham ECL solution (according to the method described by the manufacturer) then it was dried. A Kodak film was then exposed to the filter for an appropriate time (l0 to 60 seconds) and then developed. ConA-blotted filter was incubated for ten to fifteen min in TBS containing 25 % (v/v) methanol, 3.3 mM 4chloro-l-naphtol and 5.2 mM H202 until the bands appeared.

Drugs and chemicals were from Sigma, Boehringer and Merck.

Results:

Effect of calcium and magnesium onfilamentous growth and intracellular glycoprotein pattern of C.albicans:

Starved blastoconidia cells were inoculated in 50 ml MM medium containing either CaCl2 (1 mM) and MgSO4 (1 mM); EGTA (5 mM) alone; EGTA (5 mM) and MgSO4 (8 mM); EGTA (5 mM) and CaCl2 (8 mM). Cells then received N-acetylglucosamine (40 mM) or glucose (40 mM) and were incubated at 37 °C during 15 hours. Under the above growth conditions, filament growth was obtained in all but EGTA/calcium-treated cells, which grew in the yeast form (Figure 1, lane 4). Cell-free homogenate were prepared from each growth condition and were subjected to SDS-PAGE followed by western blot analysis with lectin bound peroxidase. As shown in figure 1 (panel A, Con A Blot, lanes 1 through 3), ConA blot shows a glycoprotein pattern characterized by the presence of a main 52 kDa band in filaments obtained in the presence of calcium and magnesium ions (lane 1). The main 52 kDa band was the major glycoprotein recognized in WGA blot along with a minor 42 kDa band (Panel A, WGA Blot, lane 1, see arrows). The above glycoprotein pattern was diminished in EGTA- and EGTA/magnesium-treated cells (lanes 2 and 3, respectively), however, a common feature of these two lanes with lane 1 is that two minor high MW bands (see arrows, between 79 kDa and 115 kDa) and the 52 kDa band seem to be shared by the three growth conditions, which may indicates that these three glycoproteins are probably associated with filament growth under our growth conditions, with either glucose or N-Acetylglucosamine as a carbon source. Instead, in the presence of EGTA/calcium (Figure 1, panel A, Con A Blot, lane 4), a polydispersed material was detected in ConA blotting, whereas in WGA blotting, the 52 kDa band was less detected in N-aceytlglucosamine-treated cells and totally absent in glucose-treated cells (figure 1, panel A, WGA Blot, lane 4). This result shows that filament growth is inhibited by calcium ions and that the pattern of glycoproteins is very compromised under these

conditions. The same experiment as shown above was performed in the presence of N-Acetylglucosamine as a carbon source. At the end of cell growth, intracellular glycoproteins were first extracted from cell free homogenate with Concanavalin A-Sepharose and then analysed by SDS-PAGE and Western Blot with lectin-bound peroxidase as indicated in Methods. As shown in figure 1 (Panel B, Coomassie Blue, lane1), Coomassie Blue staining revealed the presence of the main 52 kDa glycoprotein in filament growth obtained in the presence of calcium and magnesium, whereas this band was less detected in filaments of EGTA- and EGTA/magnesium-treated cells (lanes 2 and 3, respectively), as well as in yeast growth obtained in EGTA/calcium-treated cells (lane 4). ConA blotting (figure 1, panel B, Con A Blot) showed that filament growth in the presence of EGTA or EGTA/magnesium is associated with a reduced glycoprotein pattern (lanes 2 and 3, respectively) although the major bands (52 kDa, and bands between 79 kDa and 115 kDa) were present, whereas in the yeast form obtained in EGTA/calcium-treated cells, the glycoprotein pattern was very compromised and only a polydispersed glycoprotein pattern was obtained in Con A blot (lane 4). In WGA blotting (Figure 1, panel B, WGA Blot), the main 52 kDa glycoprotein band was obtained only in filament form obtained in calcium and magnesium-treated cells (lane 1), whereas it was lacking in all other growth conditions (lane 2 trough 4).

Filament growth and protein glycosylation are differentially modulated by calcium and magnesium:

In this experiment, we sought to investigate whether magnesium could reverse the inhibitory effect of calcium on filament growth and protein glycosylation. Starved blastoconidia cells were inoculated in 50 ml MM medium containing either CaCl2 (1 mM) and MgSO4 (1 mM); EGTA (5 mM) and MgSO4 (8 mM); EGTA (5 mM) and CaCl2 (8 mM). Cells then received N-acetylglucosamine (40 mM) or glucose (40 mM) and were incubated at 37°C. Three hours later, a set of EGTA/calcium-treated cells received 3 mM of MgSO4. For comparison to yeast growth, a set of cells were treated with CaCl2 (1 mM) and MgSO4 (1 mM) and incubated at 28 °C. Following 15 hours of growth, it was found that magnesium have induced a recovery of filamentous growth in EGTA/calcium-inhibited cells, and thus, in N-Acetylglucosamine- but not in glucose-treated cells (Figure 2, lane 4). The recovery of filamentous growth in N-Acetylglucosamine-treated cells was associated with a recovery of a full glycoprotein pattern as well, as evidenced by glycoprotein analysis in Coomassie Blue staining and blotting with peroxidase-labeled lectins (Figure 2, N-Acetylglucosamine, panels Coomassie Blue, Con A Blot and WGA Blot, lane 4). Interestingly, in glucose-treated cells, when filament growth was inhibited by EGTA/calcium, addition of magnesium back into the growth medium could induce a recovery of both filament growth and its

associated glycoprotein pattern when N-Acetylglucosamine was added as well (data not shown). For comparison to the inhibitory effect of calcium, we used tunicamycin as an antibiotic that is known to interfere with N-glycosylation process and cell growth, and we have found that the inhibitory effect of tunicamycin on filament growth is associated with a strong inhibition of protein glycosylation as shown by the analysis of intracellular glycoproteins in both cell free homogenate (Figure 3, panel A) and glycoprotein extract with Con A-Sepharose (Figure 3, panel B), and thus, in both N-Acetylglucosamine and glucose-treated cells.

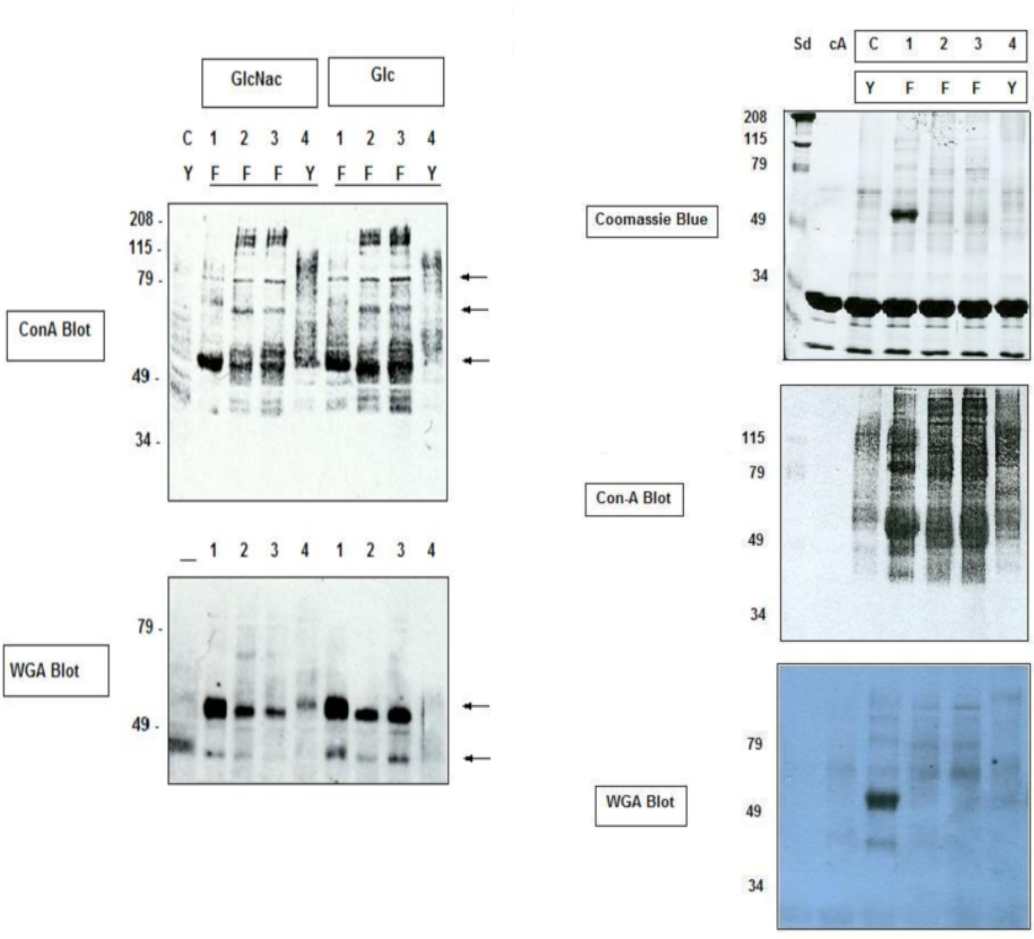

Figure 1: Effect of calcium and magnesium on the pattern of glycoproteins in total cell free homogenate Starved cells of C. albicans were inoculated in 50 ml of fresh MM medium containing either CaCl2 (1mM) and MgS04 (l mM) (lane 1), EGTA (5 mM) (lane 2), 5 mM EGTA followed by addition of either 8 mM of MgS04 (lane 3) or CaCl2 (lane 4). 40 mM of N-Acetylglucosamine (GlcNAc) or glucose (Glc) were then added where indicated, and cells were incubated in gyratory incubator at 37°C. Control cells (lane C) received CaCl2 (1mM) and MgS04 (l mM) only. 15 hours latter, cells were collected and cell free homogenate was prepared in order to analyse glycoprotein as indicated in Methods, either in cell free homogenates (panel A, Left Picture) or following their extraction with Con A-Sepharose (panel B, data correspond to N-Acetylglucosamine-treated cells). Proteins were separated by SDS-PAGE (10 %). Standard molecular weight markers

(kDa) are indicated on the left of figure. Filament (F) form was obtained in lanes 1, 2 and 3. Yeast (Y) growth was obtained in lane 4. A sample of ConA Sepharose (cA) alone is included in panel B (Right picture).

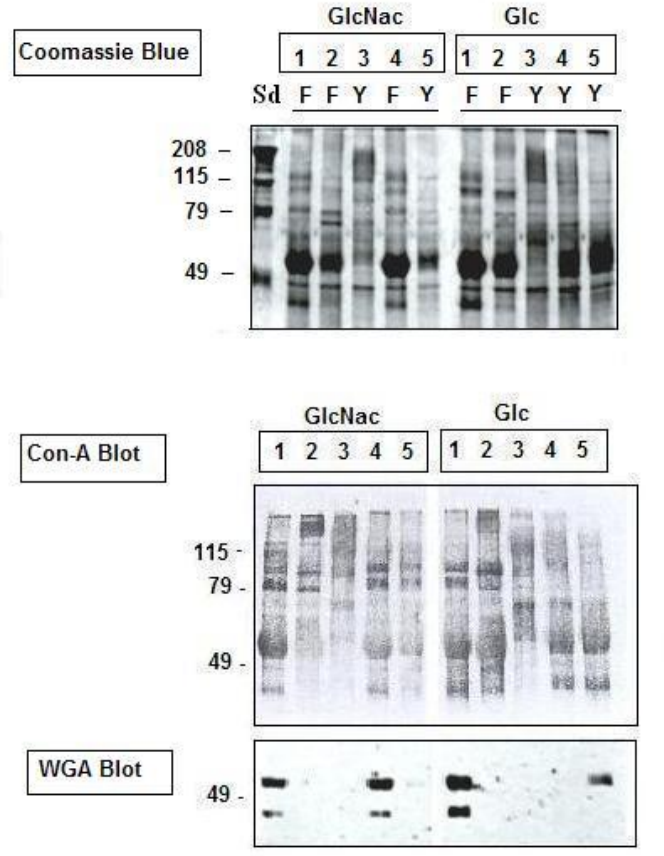

Figure 2: Filament growth and protein glycosylation obtained with glucose or N-Acetylglucosamine are differentially modulated by calcium and magnesium Starved cells of C. albicans were inoculated in 50 ml of fresh MM medium containing either CaCl2 (1mM) and MgS04 (l mM) (lane 1), 5 mM EGTA followed by addition of either 8 mM of MgS04 (lane 2) or CaCl2 (lane 3 and 4). 40 mM of N-Acetylglucosamine or glucose were then added, and cells were incubated in gyratory incubator at 37°C. Control yeast cells received CaCl2 (1mM) and MgS04 (l mM) and were incubated at 28°C (lane 5). Three hours latter, a set of EGTA/CaCl2 – treated cells (lane 4) received MgS04 (3 mM). After 15 hours of growth, cells were collected and cell free homogenate was prepared as indicated in Methods. Cell free homogenates from each growth condition were matched for proteins and added to ConA-Sepharose beads for glycoprotein extraction as mentioned in Methods. ConA-Sepharose extracted glycoproteins were loaded onto SDS-PAGE (10 %). Proteins were then stained with Coomassie Blue (panel Coomassie Blue), and analysed by Western blotting with peroxidase-labeled ConA (panel Con-A Blot) or –WGA (panel WGA Blot). Standard (Sd) molecular weight markers (kDa) are indicated on the left of each figure.

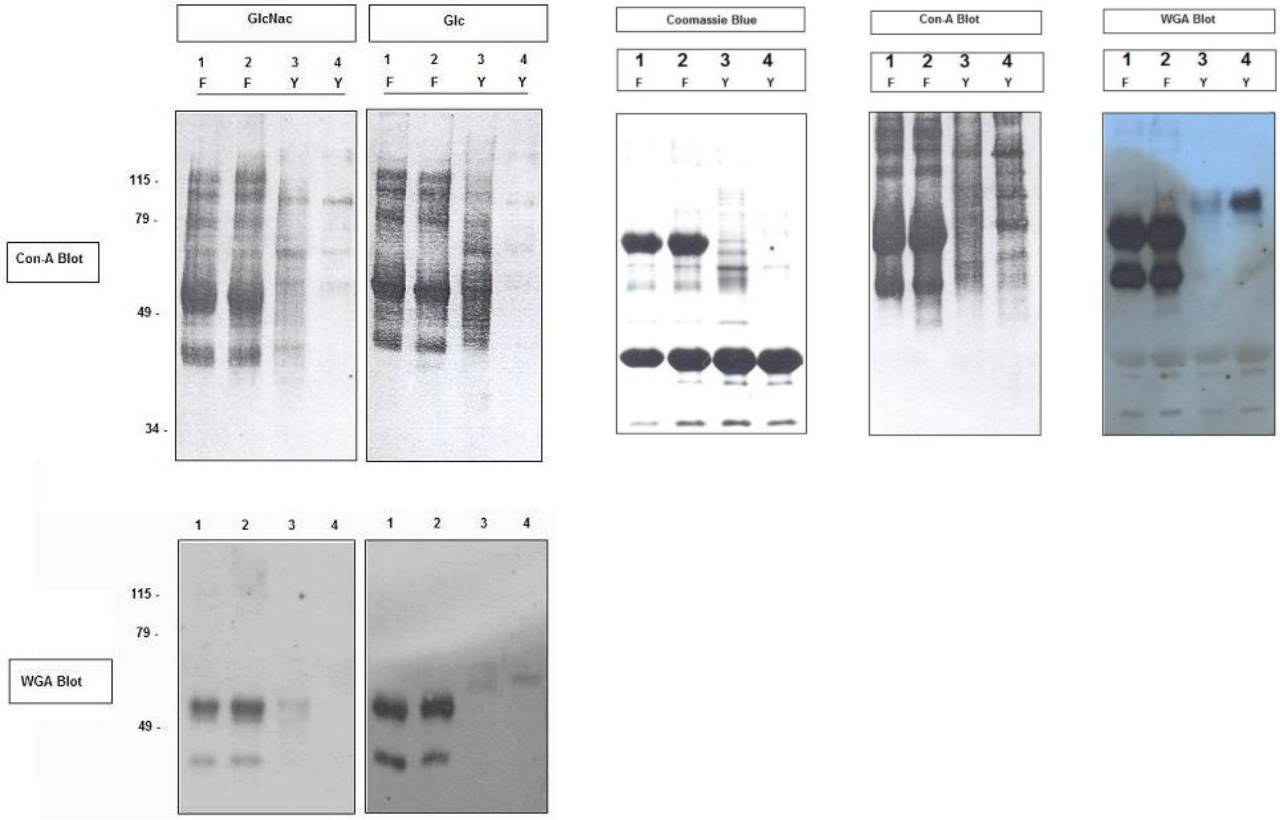

Figure 3: Inhibitory effect of tunicamycin on filament growth and its associated protein glycosylation. Starved cells of C. albicans were inoculated in 100 ml of fresh MM medium containing either CaCl2 (1mM) and MgS04 (l mM) (lane 1), followed by addition of increasing concentrations of tunicamycin. Cells received 40 mM of either glucose (Glc) or N-acetylglucosamine (GlcNAc) where indicated. 15 hours latter, cells were collected for cell free homogenate preparation, then samples were matched for proteins and analysed as such (panel A, left picture) by Western blot, or used for glycoprotein extraction by Con A-Sepharose followed by Coomassie Blue staining and Western Blotting (panel B, data correspond to glucose-treated cells are shown). Proteins were resolved on SDS-PAGE (10 %). Standard molecular weight markers (kDa) are indicated on the left of each figure. Lanes 1, 2, 3 and 4 correspond to tunicamycin concentrations of 0; 0,1; 1 and 10 µg in the assay. F and Y refer to filament and yeast growth, respectively.

Discussion:

Many studies on morphogenesis of C.albicans questioned the molecular mechanisms involved in its filamentous growth and its relationship to virulence. Genetic and mutation studies have shed light on numerous genes whose products are essential for virulence and filament growth of C.albicans, though, many of these studies have found that gene mutation studies usually lead to filament growth defect in a medium-dependent manner[20-

[22], and even non filamentous mutants in a growth medium can give filamentous cells in an animal [model] of infection[29], whereas other studies demonstrated that non filamentous C.albicans mutants were avirulent[18-21]. The above reports indicate that yet unknown pathways and nutrients factors could be involved in the induction of filamentous process, and virulence of C.albicans may not exclusively be linked to filament form[21,30], instead, N-Acetylglucosamine sensing and/or catabolism may directly be associated with filamentous process and/or virulence[21,22,31,32].

In our study, we show that some filament form can be obtained with different medium composition, and although they look similar under the microscope, they have different intracellular glycoprotein pattern, but some of the glycoproteins were shared by all filaments (Con A-interacting 52 kDa band and two bands between 79 kDa and 115 kDa). We have shown previously that the 52 kDa glycoprotein is highly N-glycosylated and O-mannosylated, whereas the 42 kDa is only N-glycosylated[23]. The glycosylation process[33] could be one of the signalling mechanisms involved in the process of filamentous growth of C.albicans. On the other hand, our study confirms the inhibitory effect of calcium on filament growth of C.albicans[34], and shows that filaments can be obtained in a magnesium-free medium, although this cation was shown to maintain filament growth by others [35]. This hypothesis is in accordance with our present data, since in our experimental conditions, EGTA/calcium-inhibited cells could recover a regular filament growth and its associated glycoprotein pattern upon magnesium addition when N-Acetylglucosamine is used as a carbon source (Figure 3). The above result revealed an important difference between N-Acetylglucosamine and glucose in relationship to the induction of filament growth and protein glycosylation by glucose, and we can hypothesize that the effect of glucose might be mediated by some glucose metabolism-dependent pathway that involves both N-Acetylglucosamine as well as calcium and magnesium ions as regulatory factors. In conclusion, we propose that filament form and its glycoprotein pattern in C.albicans is under the control of calcium and magnesium ions, and that N-Acetylglucosamine may play a key role in the induction of filament growth more than being merely a carbon source, and this could have a significant relevance in the interaction of C.albicans with its host.

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