Characterization Of Two Soluble (52 / 42 Kda) Glycoproteins Whose Pattern Is Up-Regulated During Yeast-To-Filament Growth Transition Of C. Albicans.

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Abstract:

In this study, we sought to investigate the pattern of cellular glycoproteins during yeast-to-filament growth transition in Candida albicans, in vitro. By mean of glycoprotein extraction with Concanavalin A-Sepharose, and Western Blot analysis with peroxidase-labeled lectins (concanavalin A, wheat germ agglutinin), we have characterized two glycoproteins (52 kDa and 42 kDa) whose pattern is increased in the filamentous form of C.albicans. Analysis of subcellular fractions of C.albicans showed that the 52/42 kDa glycoproteins are located in the soluble fraction. in vitro treatment of concanavalin A-Sepharose extracted 52/42 kDa glycoproteins with the peptide N-linked glycosidase F showed that the 52 kDa protein is highly N-glycosylated and mannose O-glycosylated, whereas the 42 kDa is N-glycosylated. Regulation of synthesis and / or glycosylation of the 52/42 kDa glycoproteins could be associated with yeast-to-filament growth transition of C.albicans.

Introduction:

Candida albicans, is an important fungal pathogen found in human [1]. Its filamentous growth is thought to be one of its virulence factors [2]. Morphogenesis requires the integration of many cellular factors that extend from reception of environmental signals to assembly of macromolecules to produce the final molecular architecture of the cell wall responsible of C. albicans morphology [3]. Many research works on environmental conditions of growth have demonstrated that pH and temperature of the growth medium are two main variables that modulate filamentous growth [4-6]. Genetic and molecular biology studies have shed light on signal transduction in C.albicans. Cph1 was shown as part of the MAP kinase pathway [7-9], and Efg1 was shown as part of the cAMP/PKA signal transduction pathway [10]. In the same line of research, RAS1 gene was shown to be upstream of Efg1 and Cph1 signalling pathways [11]. A TUP1 signalling mechanism controlling filament growth was proposed as a third pathway [12]. A pH-dependent signal pathway that regulates filament growth and other gene expression was described in C.albicans [13] and A.nidulans [14]. According to the literature, many environment factors could modulate filament growth and virulence factors of C.albicans [15-18]. Research on biochemical events that take place during yeast-to-filament transition may help to unravel yet unknown molecular mechanisms of morphogenesis in C. albicans and possibly to characterization of new targets for therapeutic application. In this study, we have characterised two glycoproteins (52 / 42 kDa) whose pattern is up-regulated in C.albicans filaments at 37°C in vitro.

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 indicated. Cells were incubated in a gyratory incubator at 28°C or 37°C, in order to grow as yeast or filament, respectively. Drugs and chemicals were from Sigma, Boehringer and Merck.

Preparation of cell free homogenate: Blastoconidia starved cells were inoculated into 50 ml 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 polypropyl-

ene tube and centrifuged at 5000 rpm (Sorvall centrifuge, rotor SS-34) for twenty min at 4°C. The cell pellet was recovered in 4 to 5 ml of detergent-containing lysis buffer A (20 mM Hepes, 135 mM NaCl, 1 mM ethylene glycol bis-(5-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 10 % (v/v) glycerol, 1 % (v/v) Triton x-100, 0.1 mM phenylmethylsulfonylfluoride (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).

Preparation of soluble and particulate fractions of C. albicans filament: Blastoconidia starved cells were inoculated into 250 ml of fresh MM medium containing glucose (40 mM) or N-Acetylglucosamine (40 mM), MgSO4 (1 mM) and CaCl2 (1mM). Following a filamentous growth at 37°C during 15 hours in a gyratory incubator, cells were recovered into 50 ml polypropylene tubes and centrifuged. Cell pellets were recovered in detergent-free lysis buffer B (20 mM Hepes, 340 mM sucrose, 150 mM KCl, 3 mM MgCl2, 1 mM EGTA, 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 cell-free homogenate was prepared as described above. The later was centrifuged for one hour at 35000 rpm in a Sorvall ultracentrifuge (rotor SW60). The

supernatant that represents the soluble fraction was recovered, and the corresponding pellet (particulate fraction) was recovered in buffer B and sonicated. Triton x-100 was added (final concentration 1 % (v/v)) to both soluble and particulate fraction. Samples were kept at ice cold temperature until use.

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) (19,20). 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 Bio-Rad assay for protein measurement [21]. 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 recovered 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 (22) and (23), 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 4-chloro-l-naphtol and 5.2 mM H_2O_2 until the bands appeared.

In vitro enzymatic digestion of soluble glycoproteins of C.albicans filaments by PNGase F: Starved blastoconidia cells were inoculated into 250 ml of MM medium containing glucose (40 mM), $CaCl_2$ (1mM) and $MgSO_4$ (1mM). Following 15 hours of filamentous growth at 37°C in a gyratory incubator, cells were collected to prepare cell-free homogenate in detergent-containing buffer A. Cell-free homogenate of C.albicans filaments was used for glycoprotein extraction by mean of ConA-Sepharose beads as indicated above. ConA-Sepharose beads

were washed twice in HNT and twice in buffer C (Na_2PO_4 100 mM, EDTA 50mM, pH 7.5), and finally were recovered in 1ml of buffer C, to which SDS (0.5 %, w/v) and ß-mercaptoethanol (1 mM) was added. The mixture was boiled five min in a water bath, then Triton x-100 (3%, v/v) was added to the stock of glycoprotein preparation. Aliquots of the above ConA-Sepharose-glycoproteins mixture were distributed in Eppendorf tubes to which 0.1 to 1 Unit of PNGase F (Peptide N-linked glycosidase F, from Boehringer) was added in a final volume reaction of 100 ml. Reaction was started by incubating samples at 37°C. Four hours later, enzymatic digestion was terminated by adding an equal volume of 2x Laemmli sample buffer. Samples were boiled for 5 min then centrifuged and aliquots from each reaction condition was subjected to a 10 % SDS-PAGE, followed by Western Blot analyses with peroxidase-labeled ConA or peroxidase-labeled WGA, as described above.

Results:

Glycoprotein analysis in cell-free homogenate of yeast and filamentous forms of C.albicans

Starved cells of C.albicans were inoculated into fresh MM medium containing glucose (40mM), $CaCl_2$ (1mM) and $MgSO_4$ (1mM), and were incubated for 15 hours at 28°C or 37°C for growth as yeast or filaments, respectively. A control cell suspension was left at 4°C. At the end of incubation period, cell-free homogenate from each growth condition was prepared for glycoprotein extraction by ConA-Sepharose affinity, and analysis of glycoproteins with SDS-PAGE and Western Blot techniques using peroxidase-

labeled lectins, as described in Methods. Samples from both whole cell-free homogenate and ConA-Sepharose extracted glycoproteins were analysed. As shown in figure 1A (panel "Con A-S", lane 3), Coomassie Blue staining of the gel showed the presence of a 52kDa protein corresponding to filament growth. In the Western Blot analysis with peroxidase-labeled WGA, the 52kDa protein was recognized along with a 42 kDa protein, and both of the two proteins were enriched in the filamentous form when compared to the yeast form (Figure 1B, panel "Con A-S", lanes 2 and 3). Western Blot with WGA with whole cell-free homogenate (Figure 1B, panel "Homogenate", lane 3) detected only the 52 kDa protein corresponding to filamentous growth of C.albicans. A sample from cells that were kept at ice cold temperature is included for comparison (Figure 1, lane 1), as well as a sample corresponding to ConA-Sepharose alone (Figure 1, lane c). The above result shows that cell content of the 52/42 kDa glycoproteins is up-regulated in C.albicans filaments at 37°C.

Subcellular location of the 52/42 kDa glycoproteins

Starved cells of C.albicans were inoculated in 250 ml of fresh MM medium containing glucose (40mM) or N-Acetylglucosamine (40 mM), CaCl2 (1mM) and MgSO4 (1mM). Following 15 hours of growth at 37°C as filaments, cells were collected and cell-free homogenate was prepared to obtain soluble and particulate fractions, as described in Methods. Cell-free homogenate, as well as soluble and particulate fraction were incubated with ConA-Sepharose beads for glycoprotein extraction, followed by SDS-PAGE, and Western Blot analyses with peroxidase-

labeled WGA and peroxidase-labeled ConA. As shown in figure 2 (lane 2: soluble fraction), both the 52 kDa and the 42 kDa glycoproteins are located in the soluble fraction of C.albicans, whether glucose or N-Acetylglucosamine was used as a carbon source, as evidenced by Coomassie Blue staining (figure 2A) and interaction with WGA lectin (Figure 2B). On the other hand, Western Blot analysis with peroxidase-labeled ConA detected in the particulate fraction (Figure 2C, lane 3) some high MW glycoproteins above 50kDa which were not recognized by WGA. The above subcellular study of glycoproteins shows that the 52/42 kDa glycoproteins are two main cellular mannoproteins that are specifically recognized by ConA on one hand, on the other hand, these two proteins have substantial amount of glycosidic motifs (N-acetylglucosamine) that react with WGA, and are located in the cytosol-enriched fraction of C.albicans. Glucose and N-Acetylglucosamine gave very similar patterns of glycoproteins according the above subcellular analysis.

Enzymatic digestion of the 52/42 kDa glycoproteins of C.albicans filaments by PNGase F

Stock of glycosylated proteins was prepared from the cytosol-enriched fraction of C.albicans filament that grew in the presence of glucose (40 mM), as described in Method section. This glycoprotein preparation contains substantial amount of the 52/42 kDa proteins which is used in the assay of PNGase F digestion of glycoproteins. As shown in figure 3A (Coomassie Blue staining), incubation of ConA-Sepharose extracted glycoproteins with PNGase F resulted in a shift of the 52 kDa band (in-

creased mobility) in a concentration-dependent manner of the enzyme. The 52 kDa glycoprotein ended as a newly generated band of 42 kDa upon PNGase F treatment (Figure 3A, lanes 2 through 4). The newly generated 42 kDa protein was recognized in the Western Blot analysis with peroxidase labelled ConA (Figure 3B, lanes 2 through 4), indicating that it is a mannose glycosylated protein, on the other hand, the above 42 kDa glycoprotein was not detected with Western Blot analysis using peroxidase-labeled WGA (Figure 3C, lanes 3 and 4), which indicates that the original 52kDa has totally lost WGA-reacting motifs upon 0.1 Unit and 1.0 Unit of PNGase F treat-

ment. This later result is an evidence that the 52 kDa glycoprotein is highly N-glycosylated, and mannose O-glycosylated. The 42 kDa glycoprotein that is co-extracted with the 52 kDa glycoprotein by ConA-Sepharose beads from soluble fraction C.albicans filaments was not detected upon PNGase F treatment, as evidenced from the analysis of proteins by Coomassie Blue staining and Western Blot with peroxidase-labeled ConA and peroxidase-labeled WGA (Figure 3, A-C, lanes 2 through 4). This result shows that the 42 kDa glycoprotein has only N-glycosylation moities (mannose and N-acetylglucosamine).
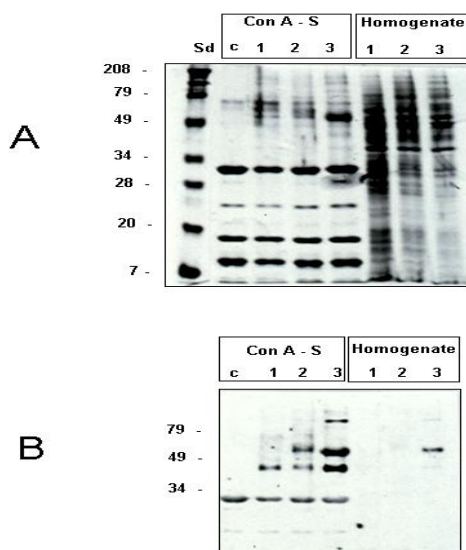

Figure 1: Analysis of glycoproteins in cell-free homogenate of C. aIbicans after yeast or filament growth. Starved cells of C. albicans were inoculated in 50 ml of fresh MM medium containing CaCl2 (1mM), MgS04 (l mM) and glucose (40 mM), and incubated in gyratory incubator at 28°C or at 37°C, in order to grow in a yeast (lane 2) or filamentous form (lane 3), respectively. Control cells were left at 4°C (lane 1). 15 hours latter, 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 (panel "Con A-S") and proteins from whole cell-free homogenate (panel "Homogenate") were loaded onto SDS-PAGE (15 %). A sample corresponding to ConA-Sepharose beads that were incubated with detergent-containing buffer A alone was run as a control (lane c). Proteins were then analysed by Coomassie Blue staining (A) and Western blotting with peroxidase-labeled WGA (B). Standard (Sd) molecular weight markers (kDa) are indicated on the left of each figure.
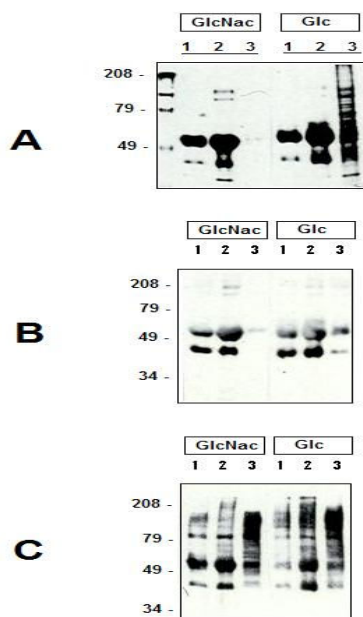
Figure 2: Subcellular location of the 52/42 kDa glycoproteins of C. albicans filaments.
Starved cells of C. albicans were inoculated into 250 ml of fresh MM medium containing CaC12 (1mM), MgS04 (l mM), and N-Acetylglucosamine (40 mM) or glucose (40 mM), and incubated at 37°C. 15 hours later, cells were collected and broken in detergent-free lysis buffer B for cell free homogenate preparation, followed by ultracentrifugation to obtain a soluble cytosol-enriched fraction and a particulate membrane-enriched fraction, as described in "Methods". Whole cell-free homogenate, as well as soluble and particulate fractions of C.albicans filaments were added to ConA-Sepharose beads for glycoprotein extraction followed by SDS-PAGE analysis as described in Methods. Glycoproteins were analysed by Coomassie Blue staining (A) and Western Blot with peroxidase-labeled WGA (B) and peroxidase-labeled ConA (C). Glycoproteins extracted from whole cell free homogenate, soluble and particulate fractions of C.albicans filaments are referred to as lane 1, 2 and 3, respectively. GlcNac: N-Acetylglucosamine, Glc: glucose.
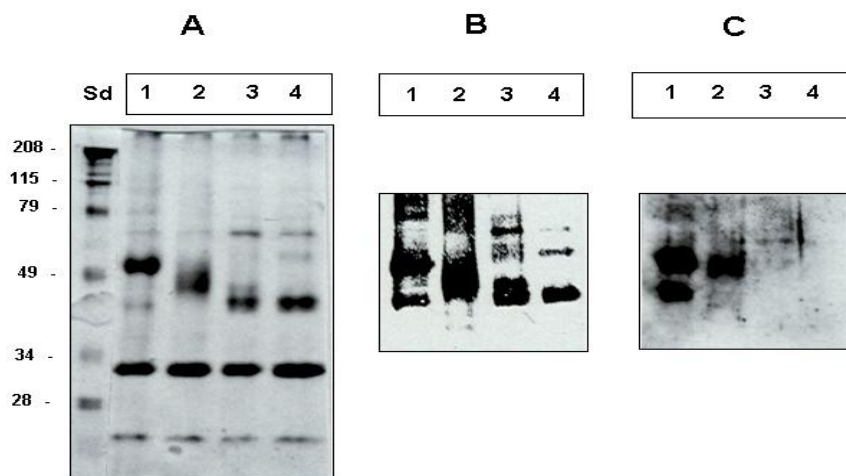

Figure 3: Digestion of the 52/42 kDa glycoproteins with PNGase F.
Following a filamentous growth of C.albicans during 15 hours at 37°C, cells were used for the preparation of a 52/42 kDa glycoproteins enriched sample that will be digested with increasing concentration of PNGase F in vitro, as described in Methods. Enzymatic reaction was terminated by adding one volume of 2x Laemmli sample buffer , and the mixture was boiled for 5 min. Tubes were centrifuged and aliquots from each reaction condition were resolved by SDS-PAGE (10 %) and analysed by Coomassie blue staining (A), Western blotting with peroxidase-labeled ConA (B) and peroxidase-labeled WGA (C). PNGase F concentrations were 0, 0.01, 0.1 and 1 Unit in lanes 1,2,3, and 4, respectively. Molecular weight markers (kDa) are shown on the left of figure.

Discussion:

Early studies on morphogenesis in C.albicans pointed out to the importance of both temperature and pH of the culture media as key environmental factors for triggering filament development [1-6]. Substantial genetic studies in C.albicans in the field of signal transduction have described at least three signalling pathways that control cell morphogenesis, MAP kinase pathway, cAMP/PKA pathway, and TUP1 pathway [7-15]. In relation to cell wall biogenesis, several studies pointed out to the role of many gene products implicated in protein glycosylation in the cell wall of C.albicans [24-26]. On the other hand, growing evidence is brought about the involvement of protein glycosylation in cell signal transduction [27,28]. In this line of research, we attempted to investigate possible intracellular glycosylation process that could take place in the transition of C.albicans from yeast to filament form. In our present study, we took advantage of concanavalin A properties for the extraction of glycoproteins from a cell free homogenate of C. albicans. We made similar attempts with wheat germ agglutinin-bound Sepharose but no glycoproteins were extracted by this method (data not shown). Concanavalin A-Sepharose beads allowed the extraction of many glycoproteins from cell free homogenate of C. albicans filaments. Among these glycoproteins, we have found that two glycoproteins (52/42 kDa) were up-regulated in the filamentous form at 37°C (Figure 1). The two 52/42 kDa glycoproteins were found in the soluble enriched fraction of C.albicans filaments as evidenced by subcelluar fractionation and glycoproteins analysis, when glucose or N-Acetylglucosamine were used as a carbon source (Figure 2). On the basis of mannose-binding property of ConA, the two 52/42 kDa glycoproteins can be considered as mannoproteins. On the other hand, their high interaction with WGA as evidenced by Western Blotting with this lectin indicates that they probably are highly enriched with N-acetylglucosamine moieties and possibly with sialic acid as well. Upon PNGase F treatment, the 52 kDa glycoprotein ended as a 42 kDa glycoprotein that still interacted with concanavalin A in the blot assay, and thus, which is an evidence for mannose O-glycosylation (Figure 3B, lanes 3 and 4). PNGase F treatment resulted in total deglycosylation of the 42 kDa glycoprotein (Figure 3, B,C), and therefore, this glycoprotein might only bee N-glycosylated.

The fact that the 52/42 kDa glycoprotein pattern was increased in filament compared to yeast growth (Figure 1) may be considered as an up-regulation of the two proteins during yeast to filament growth transition, which could have a possible role during morphogenesis process. It is likely that the 52/42 kDa glycoproteins are modulated by a process that involve protein glycosylation (N- and / or O-glycosylation for the 52 kDa protein; N-glycosylation for the 42 kDa protein) that could be induced during filamentation at 37°C. Similar results were reported for PRA1 and TSA1 where a post-translational glycosylation occured during filament formation of C. albicans [29]. Further studies will elucidate the relevance of the 52/42 kDa glycoproteins up-regulation in the process of filamentation of C.albicans.

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