Journal of Proteomics 127 (2015) 340–351

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Candida albicans cell shaving uncovers new proteins involved in cell wall integrity, yeast to hypha transition, stress response and host–pathogen interaction☆

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ARTICLE INFO

Article history:
Received 24 March 2015
Received in revised form 25 May 2015
Accepted 9 June 2015
Available online 15 June 2015

Keywords:
Candida albicans
Trypsin digestion
Surface proteins
Cell wall integrity
Osmotic stress
Oxidative stress

ABSTRACT

The ability to switch from yeast to hyphal growth is essential for virulence in *Candida albicans*. The cell surface is the initial point of contact between the fungus and the host. In this work, a free-gel proteomic strategy based on tryptic digestion of live yeast and hyphae cells and protein identification using LC–MS/MS methodology was used to identify cell surface proteins. Using this strategy, a total of 943 proteins were identified, of which 438 were in yeast and 928 were in hyphae. Of these proteins, 79 were closely related to the organization and biogenesis of the cell wall, including 28 GPI-anchored proteins, such as Hyr1 and Sod5 which were detected exclusively in hyphae, and Als2 and Sap10which were detected only in yeast. A group of 17 proteins of unknown function were subsequently studied by analysis of the corresponding deletion mutants. We found that four new proteins, Pst3, Tos1, Orf19.3060 and Orf19.5352 are involved in cell wall integrity and in *C. albicans*' engulfment by macrophages. Moreover, the putative NADH-ubiquinone-related proteins, Ali1, Mci4, Orf19.287 and Orf19.7590, are also involved in osmotic and oxidative resistance, yeast to hypha transition and the ability to damage and invade oral epithelial cells. This article is part of a Special Issue entitled: HUPO 2014.

1. Introduction

The cell wall is the most external structure in fungi, confers organization and form to the cell and protects the organism from physical and osmotic damage. Surface proteins play an important role in the pathogenic process as they are the initial point of contact between the cell and the environment. *Candida albicans* is an important opportunistic fungus that causes a wide variety of diseases in patients, ranging from the superficial mucocutaneous candidiasis (affecting the nails, skin, and oral and genital mucosae) to life-threatening disseminated infections [1,2]. In patient in critical care, candidemia is the most important fungi disease with a 30% mortality rate [3]. The *C. albicans*' cell wall maintains structural integrity and acts as intermediate between the cell and the environment. As the initial point of contact with host cells, the cell wall is an obvious target for development of antifungals and vaccines. It is composed of β-1,3-glucan, β-1,6-glucan, a small percentage of chitin and different wall proteins, most of them covalently attached to β-1,6-glucan linkage through a remnant of glycosylphosphatidylinositol (GPI) anchors [4–6]. The non-glucan-linked proteins traffic to the cell surface by either the classical or alternative secretory pathway [7,8]. These cell wall proteins maintain structural integrity, mediate adherence and/or invasion of host cells, or function as enzymes [5,8]. One of these proteins is Ecm33p, a GPI-linked cell wall protein whose absence affects both yeast cells and hyphal morphology and results in an aberrant wall structure and reduced virulence *in vitro* and *in vivo* [9,10]. Another GPI-linked cell wall protein is the secreted yeast wall protein Ywp1p, which is covalently linked to glucans of the wall matrix and has the highest expression during yeast exponential growth. The *ywp1*Δ/Δ mutant has increased adhesiveness and biofilm formation but no obvious change in growth, morphology or virulence, suggesting that Ywp1p promotes dispersal of yeast form cells in *C. albicans* [11]. Pir proteins (proteins with internal repeats) are an additional group of *C. albicans*' cell wall proteins and are linked directly to β-1,3-glucan [12,13]. *PIR1* is an essential gene and its abundance changes in response to environmental conditions [13–15]. Among no covalent attachment proteins, Bgl2p is involved in cell wall biogenesis [16]. It is the major β-1,3-glycosyltransferase and *bgl2*Δ/Δ mutants have attenuated virulence in mice. Furthermore, Bgl2 is recognized by IgG antibodies from patients with invasive candidiasis, which has diagnostic and prognostic usefulness [17]. Some

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http://dx.doi.org/10.1016/j.jprot.2015.06.006
1874-3919/

secreted proteins, such as secreted aspartyl proteinase (SAP) and phospholipase B (PLB) families, must pass through the cell wall and have been detected there [18]. These proteins are hydrolytic enzymes which enable the organism to break down proteins for nutrition, but their relative contribution to *C. albicans*' pathogenicity is controversial [19–23]. In addition, many proteins identified on the surface of *C. albicans* lack classical secretion signal peptides and are dual function proteins, that function as enzymes or chaperones in the cytoplasm and as adhesins, invasins, or immunogens when expressed on the cell surface. These proteins include glyceraldehyde-3-phosphate dehydrogenase (Tdh3), enolase (Eno1) and heat shock proteins such as Hsp70 [24–26].

C. albicans is able to grow in different morphological forms. The ability to switch between yeast and hypha is necessary for virulence [23,27,28]. Both morphological forms are important during infection. The yeast form probably disseminates *via* the bloodstream, spreading the organism to different host niches, while the hyphal form is invasive and enables the organism to evade phagocytic cells [29,30]. Consequently, *C. albicans* expresses distinct cell surface proteins in these stages.

The study of cell surface protein composition of yeast and hypha morphologies and their differences will help to find novel therapeutic targets. In recent years, the response of the cell wall proteome to changes in ambient pH and with respect to yeast to hyphal transition has been investigated [31–35]. These classical proteomic approaches involve several steps based on subcellular fractionation which are time-consuming and laborious. Hernáez et al. [36] and Vialás et al. [35] used a proteomic strategy based on cell shaving of extracellular peptides to identify surface proteins in *C. albicans* yeast and hyphae forms, using Nano-LC followed by off-line MS/MS for peptide separation and identification. By this method, many novel surface proteins were identified that had not previously been reported as being on the cell surface. These proteins included some with unknown functions and aerobic respiration-related and ribosomal proteins, such as Rpl15A, Rps16A and Rps4A. In the present work, growing yeast and hypha cells were analyzed using the same strategy and more sensitive separation and identification equipment, enabling the identification of a larger number of proteins in each sample. In addition, a phenotypic analysis *in vitro* and/or *in vivo* of mutants with undescribed function of 17 identified proteins was performed to investigate their role in cell wall biogenesis, stress and virulence.

2. Materials and methods

2.1. Strains and growth conditions

C. albicans SC5314 [37] was used as wild type in this work. *C. albicans* mutant strains used in the *in vitro* and *in vivo* phenotypic studies were acquired from Noble collection [38] stored in the Fungal Genetics Stock Center (Kansas City, Missouri USA) [39]. *C. albicans* strains were maintained on YPD (1% yeast extract, 2% peptone, and 2% glucose) agar plates at 30 °C. Yeast cells were pre-cultured in liquid YPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) in a rotary shaker at 200 rpm and 30 °C overnight. For the cell surface study, yeast cells were collected at an OD of 0.6 ± 0.2, which corresponds to logarithmic growth phase, and washed. A total of 5×10^6 cells/mL were resuspended in Lee's medium [40] (0.2 g/l magnesium sulfate, 2.5 g/l dipotassium phosphate, 5 g/l sodium chloride, 5 g/l ammonium sulfate, 0.5 g/l L-alanine, 1.3 g/l Leucine, 1 g/l L-lysine, 0.1 g/l L-methionine, 0.07 g/l L-ornithine, 0.5 g/l L-phenylalanine, 0.5 g/l L-proline, 0.5 g/l L-threonine, 25 ml/l 50% glucose and 1 ml/l 0.1% biotin) at two different pHs, pH 4.2 for yeast growth and pH 6.7 for hyhae growth, and incubated at 37 °C for 6 h. At the end of the incubation period, cells were analyzed by light microscopy showing yeast shape at pH 4.2 and *C. albicans* hyphae at pH 6.7. To determine the generation time, strains were cultured in liquid YPD at 30 °C with OD_{600} 0.1. Absorbance of the cultures was measured at 600 nm every hour. Linear regression was used to calculate specific growth rate and generation time. For phagocytosis and cytotoxicity assays, RAW 264.7 murine macrophages were cultured in RPMI 1640 medium supplemented with antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml), L-Glutamine (2 mM) and 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. The FaDu oral epithelial cell line was obtained from the American Type Culture Collection and maintained in Eagle's minimum essential medium with Earle's balanced salt solution (Irvine Scientific) supplemented with 10% fetal bovine serum, 1 mM pyruvic acid 2 mM L-glutamine and 0.1 mM non essential amino acids.

2.2. Surface shaving

For surface protein identification, Hernáez et al. [36] method was used. Briefly, after 6 h of culture under different conditions, cells were collected and washed several times. Subsequently, cells were resuspended in 800 μl of 25 mM ammonium bicarbonate buffer (pH 8.0). A total amount of 5 μg of trypsin (Roche) in the presence of 0.1 mM DTT was added to the cell suspensions. After incubation at 37 °C for 5 min in a rotary shaker at 600 rpm, proteolytic reactions were stopped by adding 0.1% trifluoroacetic acid (TFA) v/v. Samples were centrifuged and supernatants were collected. The peptide supernatants were filtered through 0.22 μm pore-size filters (Millipore). Cell lysis was controlled by propidium iodide (PI) staining using flow cytometry in case of yeast and by fluorescence microscopy in case of hyphae. Before mass spectrometry analysis, the peptide supernatants were cleaned up with a microcolumn filled with Poros 50 R2 packing (PerSeptive Biosystems). Peptides were eluted with 80% acetonitrile (ACN) in 0.1% TFA, dried in a Speed-vac and resuspended in 0.1% formic acid. The samples were stored at −20 °C before nano-LC–MS/MS analysis.

2.3. LTQ-Orbitrap Velos analysis and protein identification

Prior to nano-LC–MS/MS analysis, all peptide samples were purified and desalted using C18-A1 ASY-column 2 cm pre-column (Thermo Scientific) and then eluted onto a Biosphere C18 column (Nano-Separations). Peptides were separated with a 140 min gradient (110 min from 0 to 40% Buffer B; Buffer A: 0.1% formic acid/2% acetonitrile; Buffer B: 0.1% formic acid in acetonitrile) at a flow-rate of 250 nl/min on a nano-Easy HPLC (Proxeon) coupled to a nano-electrospray ion source (Proxeon). Mass spectra were acquired on the LTQ-Orbitrap Velos (Thermo Scientific) in the positive ion mode. Full-scan MS spectra (m/z 400/1400) were acquired in the Orbitrap with a target value of 1,000,000 at a resolution of 60,000 at m/z 400 and the 15 most intense ions were selected for collision induced dissociation (CID) fragmentation in the LTQ with a target value of 10,000 and normalized collision energy of 38%. Precursor ion charge state screening and monoisotopic precursor selection were enabled. Singly charged ions and unassigned charge states were rejected. Dynamic exclusion was enabled with a repeat count of 1 and exclusion duration of 30 s.

2.4. Protein identification and analysis

Protein identification from raw data was carried out using a licensed version of search engine MASCOT 2.3.0 with Proteome Discoverer software version 1.4.1.14 (Thermo Scientific). A database search was performed against the CGD21 database (6221 sequences). Search parameters were oxidized methionine as variable modification, peptide mass tolerance 10 ppm, 1 missed trypsin cleavage site and MS/MS fragment mass tolerance of 0.8 Da. In all protein identification, the FDR was <1%, using a Mascot Percolator [41], with a q-value of 0.01. As an estimation of the relative protein abundances the normalized spectral abundance factor (NSAF) was used [42], and the average of the normalized values was calculated. The MS output files have been submitted to PeptideAtlas through the PeptideAtlas Submission System (PASS) with identifier PASS00446.