

Interaction of human fibronectin with *Candida glabrata* epithelial adhesin 6 (Epa6)*

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Adherence of pathogens to extracellular matrix proteins and host cells is one of the essential steps in the microbial colonization of the human organism. The adhesion of *C. glabrata*, i.e. the second major causative agent of human disseminated candidiasis after *C. albicans*, to the host epithelium mainly engages specific fungal cell wall proteins – epithelial adhesins (Epa) – in particular, Epa1, Epa6 and Epa7. The aim of the present study was to identify the major Epa protein involved in the interactions with the human extracellular matrix protein – fibronectin – and to present the kinetic and thermodynamic characteristics of these interactions. A relatively novel gel-free approach, i.e. the “cell surface shaving” that consists in short treatment of fungal cells with trypsin was employed to identify the *C. glabrata* surfaceome. Epa6 was purified, and the isolated protein was characterized in terms of its affinity to human fibronectin using a microplate ligand-binding assay and surface plasmon resonance measurements. The dissociation constants for the binding of Epa6 to fibronectin were determined to range between 9.03×10^{-9} M and 7.22×10^{-8} M, depending on the method used (surface plasmon resonance measurements versus the microplate ligand-binding assay, respectively). The identified fungal pathogen-human host protein-protein interactions might become a potential target for novel anticandidal therapeutic approaches.

Key words: *Candida glabrata*, epithelial adhesins, fibronectin, surface plasmon resonance

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INTRODUCTION

In clinical practice, a continuously increasing incidence of severe fungal diseases has been recorded over the last few decades, which has now become a serious medical problem. *Candida albicans* is still diagnosed as one of the major fungal pathogens of humans; however, the frequency of severe candidiasis caused by other *Candida* species has alarmingly increased (Tadec *et al.*, 2016). Currently, *C. glabrata* is considered to be the second major causative agent of fungal diseases after *C. albicans*, responsible for almost 22% of superficial and systemic candidal infections in North America and for 10% of candidiasis in Europe (Perlroth *et al.*, 2007; Bassetti *et al.*, 2015; Gupta *et al.*, 2015). Interestingly, *C. glabrata* is more closely related to baker's yeast *Saccharomyces cerevisiae* than to other *Candida* species (Dujon *et al.*, 2004). In

contrast to *C. albicans*, *C. glabrata* is strictly haploid and normally grows only in the yeast-like form (Kaur *et al.*, 2005), although it still possesses mechanisms appropriate both for a commensal lifestyle within the human organism and for the host infection.

One of the critical steps in the infectious process, essential both for the initial stages of host colonization and for further development of disease, is the adherence of the pathogen to the host epithelial cells. Thus, many bacterial species possess complex systems, mainly localized on the cell surface, involved in detection of host ligands for further induction of the expression of specific bacterial adhesins, which allow the pathogen to attach firmly to host tissues (Kline *et al.*, 2009). As in bacteria, the cell surface of *Candida* spp. is involved in adhesion of these fungi to host cells and proteins during infection. Among all cell wall components, proteins exposed on the cell surface are considered to play a key role in candidal pathogenicity (Chaffin *et al.*, 2008; Heilmann *et al.*, 2012). Accordingly, the interactions between proteins exposed at *C. glabrata* cell surface and human epithelial cells were characterized and shown mainly to engage Epa1, Epa6 and Epa7 (Castano *et al.*, 2005), i.e. proteins belonging to the epithelial adhesin (Epa) family (Kaur *et al.*, 2005; Roetzer *et al.*, 2011). Moreover, Epa6 and Epa7 were found to contribute to strong hydrophobic interactions with various abiotic surfaces and to participate in biofilm formation on the surface of medical devices such as vascular and urinary catheters, artificial joints, stents, or implants (El-Kirat-Chatel *et al.*, 2015; d'Enfert & Janbon, 2016). Epa1 and Epa7 were shown to be involved in interactions with endothelial cells (Zupancic *et al.*, 2008), whereas Epa1 could also mediate adherence to macrophages and mononuclear cells from peripheral blood (Kuhn & Vyas, 2012).

One of the most important proteinaceous components of the extracellular matrix (ECM) is fibronectin (FN), which is a multifunctional protein involved in

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Abbreviations: BSA, bovine serum albumin; CGD, *Candida* Genome Database; DTT, dithiothreitol; ECM, extracellular matrix; Epa, epithelial adhesin; ESI, electrospray ionization; FN, fibronectin; GPI, glycosylphosphatidylinositol; LC, liquid chromatography; LC-MS/MS, liquid chromatography-coupled tandem mass spectrometry; MS/MS, tandem mass spectrometry; NHS, N-hydroxysuccinimide; PBS, phosphate buffered saline; SA-HRP, streptavidin-conjugated horseradish peroxidase; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SPR, surface plasmon resonance

clotting, wound repair, and host cell adhesion and migration (Tate *et al.*, 2002; Ghost *et al.*, 2006). Binding of this high-molecular-mass glycoprotein on the fungal cell surface can greatly enhance the virulence of *Candida* species, because adsorption thereof provides a bridge to deeper tissues, thereby facilitating colonization and dissemination of pathogens within the human organism (Sturtevant & Calderone, 1997; Nett *et al.*, 2016). Certain proteins exposed on the surface of *C. albicans* cells, such as a typical adhesin Als1 (agglutinin-like sequence), are known to interact with FN (Donohue *et al.*, 2011; Jordan *et al.*, 2014). Moreover, some of the cell wall-associated proteins of non-*albicans* *Candida* species, such as *C. tropicalis* and *C. parapsilosis*, play an important role in adherence to ECM proteins (Kozik *et al.*, 2015). For *C. glabrata*, the binding of N-terminal part of Epa1 to FN was only demonstrated after overexpression of this fragmental fungal protein in *Escherichia coli* and its purification (Iclais *et al.*, 2014).

Since the phenomenon of fungal adhesion is based on interactions of candidal surface-exposed proteins with cells and soluble proteins of the host, in order to effectively prevent such infections, it is necessary to recognize in details the structure of the pathogen's cell wall and to understand the mechanisms of adhesion (Brunke & Hube, 2013). In the present study, we analyzed the *C. glabrata* surfaceome and identified Epa6 as one of the major factors capable of binding to human FN. Furthermore, the interaction of FN with isolated and purified Epa6 was characterized in terms of kinetic and thermodynamic parameters.

MATERIALS AND METHODS

Yeast strain and culturing conditions. *C. glabrata* strain CBS138 (ATCC® 2001™) was purchased from the American Type Culture Collection (Manassas, VA, USA). Yeast cells were grown in YPD medium, pH 6.0 (1% yeast extract, 2% soybean peptone and 2% glucose) (Sigma, St. Louis, MO, USA) at 37°C for 17 h. To induce Epa6 surface exposition, the cells were cultured in the defined medium RPMI 1640, pH 7.4 (PAA Laboratories GmbH, Pasching, Austria) at 37°C for 72 h. In this particular case, human FN (R&D Systems, Minneapolis, MN, USA) was added to the YPD medium to a final concentration of 0.2 mg/ml.

Experimental strategy. We used four sets of experiments aimed to identify major FN-binding proteins of *C. glabrata* cell wall that finally allowed to assign this activity to Epa6 protein:

(i) the extraction of cell wall-associated proteins with β -1,6-glucanase from *C. glabrata* cells cultured in YPD and RPMI media and the analysis of these extracts for FN binding;

(ii) Epa6 purification, using a protocol including: ion exchange chromatography, selection of fractions with FN-binding activity, gel filtration;

(iii) kinetic and thermodynamic characterization of the interactions between purified Epa6 and FN, using a microplate ligand-binding assay and SPR measurements;

(iv) tryptic "shaving" of *C. glabrata* cells cultured in the presence of FN, followed by the identification of cell surface-exposed proteins.

Extraction of cell wall-associated proteins. The cell wall-associated proteins were extracted from both biotinylated and non-biotinylated cells, using the previously published protocols (Kozik *et al.*, 2015). Briefly, to biotinylate the proteins associated with the fungal cell wall,

1 g (wet weight) of the *C. glabrata* cells (YPD-cultured at 37°C for 17 h or RPMI 1640-cultured at 37°C for 72 h) was suspended in 0.1 M bicarbonate buffer, pH 8.3 and a solution of N-hydroxysuccinimide-biotin (NHS-biotin, Sigma) in dimethylformamide (1 mg/50 μ l) was added, followed by sample incubation for 1 h at room temperature in the dark. After incubation, the cells were extensively washed with 50 mM phosphate buffer (pH 6.0) in order to remove the excess reagent. Next, the fungal cells were placed in 1 ml of McIlvaine buffer (a mixture of 0.1 M citric acid and 0.2 M disodium phosphate, pH 6.0, with 0.5 M sodium tartrate as an osmotic stabilizer) and treated with 2 U of β -1,6-glucanase for 24 h at 37°C. After extraction, the supernatants were collected and dialyzed against phosphate buffered saline (PBS), pH 7.4, at 4°C for 48 h. Cell membrane integrity was tested by staining with Trypan Blue (Sigma). The protein mixtures obtained were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the Laemmli system (Laemmli, 1970).

Analysis of binding of biotinylated cell wall-associated proteins to microplate-immobilized FN. FN was immobilized in the wells of the MaxiSorp 96-well microplate (Sarstedt, Nümbrecht, Germany) by overnight incubation at 4°C (5 pmol of protein per well). The unoccupied surface in each well was blocked with 3% BSA in PBS at 4°C overnight. Solutions of biotinylated fungal protein in PBS with 10 mM calcium chloride (50 μ l) were added to the wells and then the microplate was incubated at 37°C for 1.5 h. The amounts of bound biotinylated proteins were determined with the use of a SA-HRP/TMB detection system (Rapala-Kozik *et al.*, 2008).

Purification of Epa6. Ion-exchange chromatography. The mixture of the extracted cell wall-associated proteins was dialyzed against 20 mM Tris-HCl buffer for 48 h and then applied to a Resource Q column (6.4 mm \times 30 mm, particle size 15 μ m) (Pharmacia Biotech, Uppsala, Sweden). The proteins were eluted using a 20 ml linear gradient of 0–0.5 M NaCl at a flow rate of 1 ml/min. Fractions (1 min) were collected and analyzed for: (i) SDS-PAGE characteristics; (ii) FN-binding activity, and (iii) the presence of Epa proteins (identified by mass spectrometry).

Competitive FN-binding assay of fractions obtained from ion-exchange chromatography. Fractions eluted from the Resource Q column were analyzed for a competition with biotinylated cell wall proteins for binding to the microplate-adsorbed FN. For this assay, FN was immobilized in the wells of the MaxiSorp 96-well microplate (Sarstedt) by overnight incubation at 4°C (5 pmol protein per well). After this and all following steps, the wells were washed three times with 1% BSA in PBS buffer. The unoccupied surface in each well was blocked with 3% BSA in PBS at 4°C by overnight incubation. The mixture of biotinylated cell wall-extracted proteins at a concentration of 20 μ g/ml (25 μ l) with a given fraction from Resource Q chromatography (25 μ l) was added to the wells and the plate was incubated at 37°C for 1.5 h. The wells with biotinylated cell wall-extracted proteins, diluted two-fold with PBS (50 μ l), represented the maximal binding (taken as 100%). The amounts of bound proteins were determined with the use of the SA-HRP/TMB detection system. The wells without FN, which were surface-blocked with 3% BSA, served as a control. The values obtained for the control samples were subtracted from the total binding.

Selection of Epa6-containing fractions. To identify the content of protein bands on SDS-PAGE gels, the bands were manually excised and destained in 25% acetonitrile

(ACN) and then in 50% ACN in 25 mM NH_4HCO_3 . The next step was reduction with 50 mM DTT in 25 mM NH_4HCO_3 at 37°C for 45 min, followed by alkylation with 55 mM iodoacetamide in 25 mM NH_4HCO_3 for 90 min at room temperature in the dark. The excess reagents were washed out with 50% ACN. The gel pieces were then dehydrated in 100% ACN and dissolved in 13 μl of a trypsin (Promega, Madison, WI, USA) solution (0.2 $\mu\text{g}/\mu\text{l}$ in 25 mM NH_4HCO_3), followed by the incubation of the sample for 15 min at room temperature. After addition of 20 μl of 25 mM NH_4HCO_3 , the digestion was continued at 37°C overnight. Peptides were extracted by sonication in 50% ACN and 0.5% formic acid at 37°C. The peptides obtained were separated and analyzed by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS), following protocols described previously (Karkowska-Kuleta *et al.*, 2015).

Gel filtration. After separation on the Resource Q column, Epa6-containing fractions were injected onto a TSK G 3000 SW column (21.5 mm \times 30 cm, particle size 13 μm) (Tosoh Bioscience, USA). The column was eluted with 0.1 M sodium sulfate and 0.1 M sodium dihydrogen phosphate, pH 6.7. Proteins were separated at a flow rate of 2 ml/min. Fractions were collected, characterized by SDS-PAGE and analyzed by LC-MS/MS.

Labeling of Epa6 with fluorescein. A solution (1 mg/100 μl) of NHS-fluorescein (Thermo Fisher Scientific, Waltham, MA, USA) in dimethyl sulfoxide was added to purified Epa6 (100 μg) in 200 μl of 0.1 M bicarbonate buffer, pH 8.3, and the mixture was incubated at 4°C for 4 h, followed by dialysis against PBS at 4°C for 48 h.

Binding of fluorescein-labeled Epa6 to microplate-immobilized FN. For this version of the FN-binding test, FN was immobilized in the wells of the MaxiSorp 96-well microplate (Sarsted) by overnight incubation at 4°C (3 pmol protein per well). The unoccupied surface in each well was blocked with 0.5% BSA in PBS at 37°C for 3 h. Solutions (50 μl) of fluorescein-labeled proteins at increasing concentrations in PBS with 10 mM calcium chloride were added to the wells and the plate was incubated at 37°C for 1.5 h. The amounts of bound proteins were determined with the use of a fluorometric microplate reader model Synergy H1 (BioTek, Winooski, VT, USA). The wells with immobilized FN and filled with solutions containing fluorescein-labeled Epa6 (at the given concentration) and ten-fold molar excess of unlabeled Epa6 served as controls for “non-specific” binding. The signals for non-specific binding were subtracted from the total binding signals. A one-site binding model was fitted to the experimental data with the use of GraphPad Prism software.

Quantitative surface plasmon resonance (SPR) analysis of Epa6 binding to FN. FN was immobilized on CM5 chips of the BIACORE 3000 system (GE Healthcare, Uppsala, Sweden) in 10 mM sodium acetate buffer, pH 4.5. The immobilization was performed at 25°C with a flow rate of 10 $\mu\text{l}/\text{min}$ for 7 min using an Amine Coupling Kit (GE Healthcare), i.e. the mixture of 50 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 200 mM N-hydroxysuccinimide (NHS). The remaining activated ester groups were blocked with ethanolamine. The immobilization level reached ca. 280 resonance units (RU). The Epa6 solution was pre-dialyzed against a running buffer (10 mM HEPES, 150 mM sodium chloride, 10 mM calcium chloride, 0.005%, w/v, surfactant P20, pH 7.4) and then injected (a concentration range of 5–125 nM) through the chip flow cells with immobilized FN (and a control cell without FN) at a flow rate of 30 $\mu\text{l}/\text{min}$. The association and

dissociation time was 120 s. The chip surface was regenerated between binding cycles by injection of 1 M NaCl for 30 seconds. The binding parameters were obtained by the analysis of sensograms using BIAevaluation 4.1 software (GE Healthcare). The dissociation and association rate constants (k_d and k_a) were obtained on the basis of a global fit with a simple Langmuir model (1:1) with a drifting baseline, and the K_d values were calculated as the ratios of the rate constants.

Cell surface shaving with trypsin. Protocols described previously (Karkowska-Kuleta *et al.*, 2015) were used in the analysis. Briefly, after culturing *C. glabrata* yeast in YPD medium, 2×10^7 cells were washed with 25 mM ammonium bicarbonate buffer (NH_4HCO_3), suspended in 100 μl of the same buffer with 5 mM dithiothreitol (DTT), and treated with sequencing-grade trypsin (10 μg) (Promega, Madison, WI, USA) at 37°C for 5 min. After centrifugation (5 minutes, 6000 rpm) and filtration through a filter with 0.22 μm -diameter pores, the supernatant was subjected to further incubation with trypsin for 5 h at the same temperature. To stop the enzymatic reaction, trifluoroacetic acid (TFA) (Sigma) was added to the supernatant (final concentration of 0.1%). After incubation on ice for 15 min, the sample was centrifuged (15 min, 12000 rpm), dried in a Speed-Vac (Martin Christ, Osterode am Harz, Germany) and frozen until further use. The cells that remained in the pellet were tested for cell membrane integrity by Trypan Blue (Sigma) staining.

Protein identification with LC-MS/MS. The peptides were analyzed with a HCTUltra ETDII ion-trap mass spectrometer equipped with an electrospray ionization ion source (Bruker, Bremen, Germany) and coupled to an ultra-high-performance liquid chromatograph Dionex Ultimate 3000 system (Thermo Scientific, Waltham, MA, USA). Protocols described previously (Karkowska-Kuleta *et al.*, 2015) were used in the analysis. Briefly, after dissolution in 100 μl loading buffer (10% ACN with 0.1% formic acid), all peptide samples were separated on an Accucore C18 column (100 mm \times 2.1 mm, particle size 2.6 μm) (Thermo Fisher Scientific) with a 60 min gradient (from 10 to 60% of phase B) at a flow-rate of 0.2 $\mu\text{l}/\text{min}$. Mascot Generic format (.mgf) files were generated by Data Analysis 4.0 software (Bruker). The lists of the peaks obtained were searched against the NCBI protein database with taxonomy restriction to the Fungi or SwissProt protein database with taxonomy restriction to *Homo sapiens*, using an in-house Mascot server (v.2.3.0; Matrix Science, London, UK). The following search parameters were applied: enzyme specificity – trypsin; permitted number of missed cleavages – 2; fixed modification – carbamidomethylation (C); variable modifications – oxidation (M); protein mass – unrestricted; peptide mass tolerance of ± 0.3 Da and fragment mass tolerance of ± 0.5 Da.

RESULTS

Binding of immobilized FN by *C. glabrata* cell wall-extracted proteins

After culturing the yeast cells in the YPD or RPMI 1640 media, *C. glabrata* cell wall proteome was revealed and found to differ to some extent depending on growth conditions. For instance, after “cell surface shaving”, the typical adhesin Epa6 was found only on the cell surface after growth in the RPMI 1640 medium (data not shown). A mixture of cell wall-associated proteins, used in further studies was obtained after releasing proteins

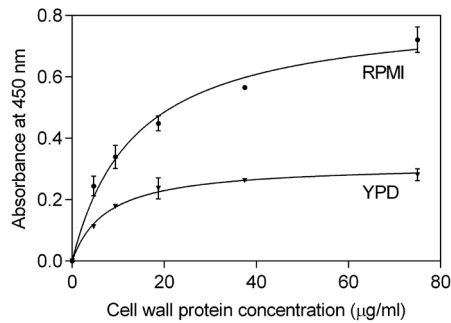


Figure 1. Binding of biotinylated *C. glabrata* cell wall proteins to microplate-immobilized FN.

Before extraction with β -1,6-glucanase, the cells were cultured at different conditions (17 h at 30°C in the YPD medium or 72 h at 37°C in the RPMI medium). Cell wall-extracted proteins at a concentration in the range of 1.5–75 μ g/ml were added to immobilized FN. The wells without FN, which were surface-blocked with 3% BSA, served as a control. The non-specific binding level was estimated from the readings of wells without immobilized FN and this value was subtracted from the total binding. Data points represent mean values from three determinations (three wells) \pm standard deviation.

from the cell wall with β -1,6-glucanase. After this treatment, more than 95% of yeast cells remained viable. Plots for saturable binding of biotin-labelled cell wall-extracted proteins to microplate-immobilized FN are shown in Fig. 1. The FN-binding capacity of the proteins isolated from yeasts grown in the RPMI 1640 medium was three-fold higher than that of proteins from YPD-cultured yeasts.

Purification of Epa6 as a major FN-binding protein of the *C. glabrata* cell wall

The whole mixture of proteins obtained by β -1,6-glucanase treatment of *C. glabrata* cells cultured in the RPMI 1640 medium were subjected to ion-exchange chromatography on Resource Q column (Fig. 2). A competitive ligand-binding assay was applied in order to identify FN-binding proteins in fractions obtained during

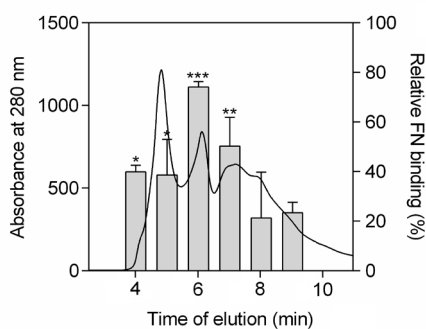


Figure 2. Separation of *C. glabrata* cell wall proteins by ion-exchange chromatography on the Resource Q column, with monitoring FN-binding activity.

The mixture of extracted cell wall proteins were separated as described in the text. A representative chromatogram is presented as a solid line. Fractions (1 min) were collected and analyzed for FN-binding activity (bars) using a competitive assay. To this end, biotinylated cell wall proteins (final concentration of 20 μ g/ml), mixed with chromatographic fractions (in the figure marked with the corresponding elution time) were added to immobilized FN. The statistical significance of the signals obtained against the signal from the control sample containing the biotinylated proteins without any competitor is indicated by asterisks: * p <0.05, ** p <0.005, *** p <0.001.

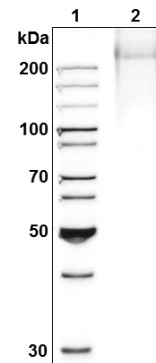


Figure 3. SDS-PAGE analysis of purified *C. glabrata* epithelial adhesin 6.

A sample of purified Epa6 (3 μ g) was analyzed on 10% gel in the Laemmli system under reducing conditions. After SDS-PAGE the protein bands were visualized by silver staining. **Lane 1:** molecular mass standard; **lane 2:** purified Epa6.

the protein separation procedure. The assay was based on the use of microplate-immobilized FN and the analysis of the competition between the proteins contained in the collected fractions and the whole mixture of biotinylated fungal cell wall-associated proteins isolated with β -1,6-glucanase. Several fractions were found to exhibit a detectable FN-binding ability (up to 70% displacement in the competitive assay). These fractions were analyzed by SDS-PAGE, and the proteins, contained in the individual bands visualized on the gel were identified by LC-MS/MS (Table 1). Three fractions (elution times of 5–7 min), representing the maximal FN-binding ability in the competitive assay, were found to contain epithelial adhesins, Epa3 and Epa6. The Epa6-containing fraction (eluted at 7 min) was subjected to high-performance gel filtration on a TSK 3000 G SW column, yielding a reasonably pure protein, represented by a single although slightly diffused band in SDS-PAGE (Fig. 3). It was unequivocally confirmed by LC-MS/MS to contain Epa6 as the only detectable component (in-gel digestion with trypsin, Mascot score of 312, 34% sequence coverage). However, the molecular mass of this protein was much higher than that calculated from Epa6 amino acid sequence (78 kDa), reflecting the heavily mannosylated status of Epa molecules. A slight heterogeneity of the

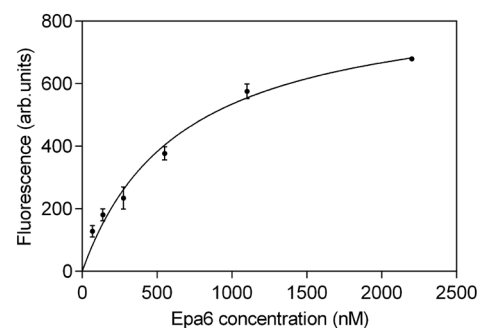


Figure 4. A microplate assay for testing the interactions of purified Epa6 with fibronectin.

The binding plot was obtained after incubation of the fluorescein-labeled Epa6 (concentration range of 6–220 nM) with immobilized FN. The applied experimental protocol is described under Methods section. The values obtained for control samples (the mixtures of fluorescein-labeled Epa6 with 10-fold molar excess of unlabeled Epa6) were subtracted from the total binding. Data points represent mean values from three determinations (three wells) \pm standard deviation.

Table 1. Mass spectrometry identification of proteins present in fractions obtained during the separation of *C. glabrata* cell wall proteins on Resource Q column.

After SDS-PAGE analysis, major protein bands were excised from the gel and subjected to digestion with trypsin. The peptides obtained were analyzed by LC-MS/MS.

Fraction (min)*	Major proteins identified	Protein concentration (mg/ml)
4	hypothetical protein [<i>Candida glabrata</i> CBS 138] similar to <i>Saccharomyces cerevisiae</i> putative GPI-linked cell wall mannoprotein of the Srp1p/Tip1p family	0.11
5	epithelial adhesin 3 [<i>Candida glabrata</i> CBS 138], hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> enolase I (Eno1), hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> transaldolase (Tal1), hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> cobalamin-independent methionine synthase (Met6)	0.18
6	epithelial adhesin 3 [<i>Candida glabrata</i> CBS 138], epithelial adhesin 6 [<i>Candida glabrata</i> CBS 138], hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> glucose-6-phosphate isomerase (Pgi)	0.15
7	epithelial adhesin 6 [<i>Candida glabrata</i> CBS 138], hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> triose phosphate isomerase (Tpi), hypothetical protein [<i>Candida glabrata</i> CBS 138] similar to <i>Saccharomyces cerevisiae</i> cell wall mannoprotein (Cwp1), hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> endo-beta-1,3-glucanase (Bgl2), hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> glyceraldehyde-3-phosphate dehydrogenase (Tdh3)	0.36
8	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> extracellular mutant (Ecm4), hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> triose phosphate isomerase (Tpi)	0.11
9	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> haze protective factor (Hpf1), hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> member of the FLO family of cell wall flocculation proteins (Flo10)	0.10

*1-ml fraction collected at the indicated elution time

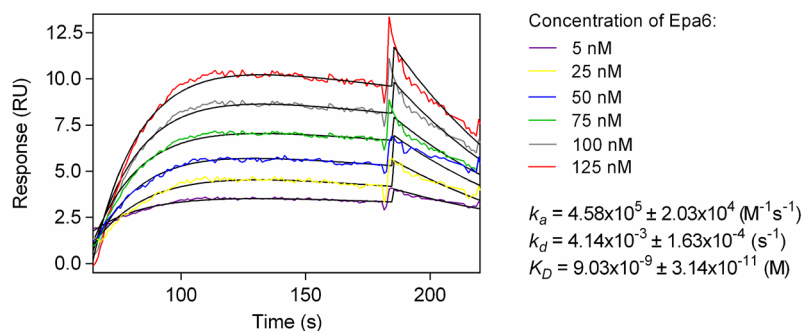
polysaccharide moiety could account for the diffused appearance of the electrophoretic band.

Kinetic and thermodynamic characterization of the interaction between FN and purified *C. glabrata* Epa6

First, the interaction of purified Epa6 with FN was directly demonstrated by the semi-quantitative microplate ligand-binding assay (Fig. 4). Through fitting a simple model, i.e. one-site binding (hyperbola), to the experimental data, the $K_{0.5}$ parameter, which is a rough approximation of the dissociation constant (K_D) for the binding

of fluorescein-labeled Epa6 to microplate-immobilized FN, was estimated at 7.22×10^{-8} M.

The quantitative SPR method was then applied to characterize the Epa6-FN interaction in terms of kinetics and thermodynamics. To this end, FN was immobilized on the CM5 chips of the BIACORE 3000 system. The sensograms obtained for binding of Epa6 to chip-immobilized FN are presented in Fig. 5. A good global fit to the sensograms was obtained with the Langmuir 1:1 binding model with a drifting baseline, resulting in the binding parameters specified in Fig. 5.

**Figure 5. SPR analysis of the interaction of Epa6 with FN.**

Sensograms were obtained after injection of Epa6 (concentration of the analyte within a range of 5–125 nM) over a CM5 chip containing immobilized FN (level: 280 RU) at a flow rate of 30 $\mu\text{l}/\text{min}$. A Langmuir 1:1 binding model with a drifting baseline was well fitted to the sensograms, as shown by the black lines.

Identification of *C. glabrata* cell surface-exposed proteins

A gel-free approach, i.e. the “cell surface shaving”, consisting in short treatment of fungal cells with trypsin, was used to identify proteins that were exposed on the surface of yeast cells cultured under different environmental conditions (YPD medium vs. YPD supplemented with FN). A procedure that had been previously applied to analyze *C. tropicalis* and *C. parapsilosis* “surfaceomes” (Karkowska-Kuleta *et al.*, 2015) was used in this study. The yeast cell integrity was checked in each experiment with the use of Trypan Blue staining, because the crucial requirement for

Table 2. Mass spectrometry analysis of proteins identified at the cell surface of *C. glabrata*.

The yeast cells were cultured at 37°C for 17 h in YPD medium or in the presence of FN in the same medium. After “cell surface shaving” with trypsin and additional digestion of obtained proteins for 5 hours, peptides were analyzed by LC-MS/MS. The obtained lists of peaks were searched against the NCBI protein database with taxonomy restriction – Fungi and SwissProt protein database with taxonomy restriction – Human, using in-house Mascot server (SC – sequence coverage).

Surface-exposed fungal proteins									
Accession	Protein description	Molecular mass [kDa]	pI	Score	Number of peptides	SC [%]	YPD	YPD + FN	
gij50289857	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> enolase I (Eno1)	46.8	4.23	1052	19	50	+	+	
gij50288681	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> glyceraldehyde-3-phosphate dehydrogenase (Tdh3)	36.0	6.19	727	17	59	+	+	
gij25992752	pyruvate decarboxylase (Pdc) [<i>Candida glabrata</i> CBS 138]	62.0	4.57	514	12	29	+	+	
gij50290317	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> alcohol dehydrogenase I (Adh1)	37.9	4.60	444	9	37	+	+	
gij50291073	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> secreted glycoprotein (Ygp1)	37.8	4.24	392	10	30	+	+	
gij50288201	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> stress-seventy subfamily A (Ssa2)	69.6	4.33	388	10	27	+	+	
gij50292893	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> fructose-bisphosphate aldolase (Fba1)	39.5	4.75	292	6	18	+	+	
gij50285959	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> stress-seventy subfamily B (Ssb2)	66.5	5.14	280	5	13	+	+	
gij50293403	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> phosphoglycerate kinase (Pgk1)	44.7	7.80	209	6	18	+	+	
gij50292725	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> heat shock protein (Hsc82)	81.0	4.48	165	4	10	+	+	
gij50295024	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> 6-phosphogluconate dehydrogenase (Gnd1)	53.9	6.60	121	3	10	+	+	
gij50284959	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> elongation factor 2 (Ef2)	93.8	4.72	113	4	8	+	+	
gij50286075	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> translation elongation factor (Tef1)	50.2	9.58	155	4	14	+		
gij50287821	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> elongation factor beta (Efb1)	23.0	4.05	152	3	32	+		
gij50284963	60S ribosomal protein L19 [<i>Candida glabrata</i> CBS 138]	21.7	11.9	115	3	14	+		
gij3786314	translation elongation factor3 [<i>Candida glabrata</i> CBS 138]	116.8	5.91	109	2	2	+		
gij32563290	translation elongation factor 1-alpha, partial (Tef1) [<i>Candida glabrata</i> CBS 138]	50.2		270	7	20			+
gij50293029	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> malate synthase (Mls1)	63.5	7.20	207	6	15			+
gij50285355	hypothetical protein [<i>Candida glabrata</i> CBS 138] highly similar to <i>Saccharomyces cerevisiae</i> transaldolase (Tal1)	36.8	6.33	198	3	14			+

gi 50285407	hypothetical protein [<i>Candida glabrata</i> CBS 138] similar to <i>Saccharomyces cerevisiae</i> protein with similarity to a family of flavodoxin-like proteins (Pst2)	29.9	5.55	169	3	24	+
gi 49617210	epithelial adhesin 6 [<i>Candida glabrata</i> CBS 138]	78.6	5.28	100	2	4	+
Surface-bound human proteins							
FINC_HUMAN	fibronectin OS=homo sapiens GN=FN1 PE=1 SV=4	266		249	14	12	+

the whole treatment was that cell membranes remain intact, i.e., no cell lysis could occur.

This method allowed identification of 16 and 17 proteins, assigned to cells grown in the YPD medium and the YPD medium with FN, respectively. A list of the identified proteins is presented in Table 2. As shown, 12 of the identified proteins were common in yeasts cultured in either conditions, but some proteins were unique, as for example Epa6, which was found on the cell surface only if FN was added to the cultivation medium. All abbreviated names and protein descriptions included in this study were taken from the NCBI protein database and the *Candida* Genome Database (CGD). Because the genome sequence of *C. glabrata* is still poorly described, the identified proteins were compared and assigned to orthologous genes/proteins from baker's yeast *S. cerevisiae*.

DISCUSSION

Despite its close relatedness to the non-pathogenic yeast *S. cerevisiae*, *C. glabrata* has become the second fungal pathogen in the USA and the third in Europe in terms of the frequency of infections caused (Mikulska *et al.*, 2012; Pfaller *et al.*, 2012). Admittedly, issues concerning *C. glabrata* pathogenicity have been the subject of quite extensive studies in recent years; however, disseminated infections caused by this *Candida* species, partly due to the high drug resistance of this fungus, are a serious problem in the current clinical practice.

Although *C. albicans* and *C. glabrata* are considered as two *Candida* species that are most commonly distributed in the human population, they markedly differ in terms of their phylogenetic, genetic and phenotypic features (Polke *et al.*, 2015). Nevertheless, the main goals during the invasion of host cells – nutrient acquisition and immune evasion – are common for both pathogens. It is also interesting that the strategy to achieve these common goals is quite different for each of the *Candida* species (Brunke & Hube, 2013). A critical step for the development of fungal infection is adhesion to host cells and proteins through molecules exposed on the surface of *Candida* cells. It is well known that hyphal forms are mainly involved in this process in *C. albicans*. Since *C. glabrata* only exists in the yeast-like form, it had to develop a different strategy required for adhesion and invasion of host cells.

The genome of *C. glabrata* contains genes that encode 67 adhesin-like glycosylphosphatidylinositol(GPI)-anchored cell-wall proteins, which are classified into seven groups. The largest and best characterized is the Epa family, with Epa1, Epa6 and Epa7 being the most notable members. This group of proteins exhibiting diverse ligand-binding characteristics is involved in the interactions with epithelial and endothelial cells (de Groot *et al.*, 2008; Diderrich *et al.*, 2015). In terms of primary structures, Epas are similar to *S. cerevisiae* Flo proteins and the

members of the *C. albicans* Als family of typical adhesins that comprise three regions: a C-terminal domain with a GPI-anchor, a central domain with a heavily glycosylated Ser/Thr-rich region, and an N-terminal domain required for ligand recognition (Sheppard *et al.*, 2004).

For all pathogens, the interplay between surface-exposed proteins and human extracellular matrix proteins is an essential function that facilitates development of infection. FN has been proven to be important for tissue-attachment of many prokaryotic and eukaryotic pathogens such as *Staphylococcus* spp., *Streptococcus* spp. (Schwarz-Linek *et al.*, 2004), *Treponema pallidum* (Dickerson *et al.*, 2012), *Trypanosoma cruzi* (Pinho *et al.*, 2002) and *Candida* spp. (Nett *et al.*, 2006; Kozik *et al.*, 2015). The knowledge on the interactions of *C. glabrata* adhesins with human FN is essentially limited to data on FN binding by an N-terminal fragment of Epa1, heterologously overproduced in *E. coli*. In the present study, we found a substantial body of evidence for the predominant role of Epa6 in the FN binding on the *C. glabrata* cell surface.

The proteins extracted from the cell wall of *C. glabrata* cultured in the RPMI 1640 medium were found to contain Epa6 and at the same time, to exhibit high FN binding activity, considerably higher than that of proteins isolated from the YPD-cultured yeast, among which no Epa6 could be detected. In contrast to *C. albicans*, *C. glabrata* does not produce hyphal or pseudohyphal forms in the RPMI 1640 medium (Hoyer *et al.*, 1995). This implies that these two fungal species have independently evolved different strategies to detect the presence of host proteins. While the details differ, the basic principle is the same – upon detection of the host environment, adhesins are expressed to attach to the host cells (Brunke & Hube, 2013).

In the fractions obtained from ion-exchange chromatography with the highest FN-binding activity, two members of the Epa family, i.e. Epa6 and Epa3, were identified by LC-MS/MS. Interestingly, in other studies, these particular adhesins were isolated from biofilm-forming cells, and a higher mRNA level for *EPA6* was observed and correlated with increased virulence in hyper-adhesive clinical isolates (Gomez-Moler *et al.*, 2015). The purification of Epa6 was completed using high performance gel filtration on the TSK G 3000 SW column, which yielded an acceptably pure protein preparation. It was subjected to direct FN-binding tests to confirm the actual formation of the complex of these two proteins and to determine the basic thermodynamic and kinetic parameters for this protein-protein interaction. The first method, i.e. the microplate ligand binding assay, is useful only for rough estimation of the dissociation constant; the second method, SPR measurements, facilitates precise determination of the kinetic constants for the complex formation and dissociation, as well as the thermodynamic dissociation constant. Only a few similar studies based on application of SPR measurements and performed for

C. albicans have been reported so far and presented reports on binding of candidal cell wall proteins with proteinaceous components of the human extracellular matrix (Donohue *et al.*, 2011; Jordan *et al.*, 2014) and the plasma kinin-forming system (Seweryn *et al.*, 2015). The interaction between the N-terminal domain of Epa1 and FN was demonstrated in one report on *C. glabrata*, with an apparent K_D estimated at 9.11×10^{-7} M (Ielasi *et al.*, 2014). In the present study, the dissociation constant for Epa6 binding to FN was determined to be lower by 1–2 orders of magnitude (9.03×10^{-9} M or 7.2×10^{-8} M by SPR or the microplate ligand-binding assay, respectively). These values should be classified as having moderate strength (Tudos & Schasfoort, 2008). Comparable values of K_D , in a range of 3.16×10^{-9} M, were obtained using the SPR method for the interaction between FN and the recombinant domains of streptococcal and staphylococcal adhesins (Joh *et al.*, 1999; Allignet *et al.*, 2012).

Our findings suggest that Epa6 is an effective adhesin, capable of strong interaction with extracellular matrix. However, it is difficult to conclude about the relative FN-binding strength between different Epas, because the only reference SPR study (Ielasi *et al.*, 2014) was performed on Epa1 N-terminal fragment heterologously expressed in bacteria and devoid of sugar elements which could be involved in FN binding. Our study aimed at purification of the natural, full length Epa6. During this purification procedure, Epa3 appeared in some fractions (Table 1) and, although not further purified, was determined by SPR to bind FN with the dissociation constant in micromolar range (results not presented). One should also bear in mind that the specificity of ligand binding demonstrated by these particular Epa proteins might be quite different. Previous studies showed that most Epas, including Epa1, Epa6 and Epa7 bind to galactose-containing oligosaccharides which occur, for instance, in mucin-type O-glycans (Zupancic *et al.*, 2008; Maestre-Reyna *et al.*, 2012; Diderrich *et al.*, 2015).

The “cell surface shaving” approach has often been used for rapid analysis of changes occurring within the cell wall of live cells. The major advantage of this method is that, after a short incubation with trypsin, the cells remain viable and their membrane is not damaged (Olaya-Abril *et al.*, 2014). This novel approach that allows identification of a number of surface-exposed proteins was successfully used for studying the cell surface of several bacterial species and, more recently, fungal opportunistic pathogens such as *C. albicans* (Hernández *et al.*, 2010; Vialás *et al.*, 2012; Gil-Bona *et al.*, 2015), *C. parapsilosis* and *C. tropicalis* (Karkowska-Kuleta *et al.*, 2015). In the present study, the comparative analysis of the proteinaceous components of the *C. glabrata* cell wall demonstrated some changes on the fungal cell surface after 17 hours of growth in the presence of human FN. In these experiments, FN was used at a concentration of 200 µg/ml, comparable to the physiological concentrations in human plasma (a range of 150–800 µg/ml) (Garat *et al.*, 1996).

Adhesive proteins exposed on the candidal cell surface might be classified into two groups. The first group contains classical adhesins equipped with a N-terminal signal peptide and exported outside the cell through the classical secretory pathway *via* the endoplasmic reticulum and Golgi apparatus (Pitarch *et al.*, 2002; Klis *et al.*, 2006). The second group of proteins often termed as ‘atypical’, consists of mainly cytoplasmic proteins with evolutionary conserved enzymatic functions whose exposition at the cell wall starts to be considered as a rule rather than an exception (Karkowska-Kuleta & Kozik,

2014). In our work, besides the occurrence of several ‘atypical’ proteins, the presentation of a classical adhesin, Epa6, on the cell surface after addition of human FN to the culturing medium was the most important change observed in the cell wall proteome. Furthermore, while searching the data obtained against the SwissProt database with restriction to human proteins, we found FN to be adsorbed on the fungal cell surface. These findings support the hypothesis that FN binding to *C. glabrata* cell surfaces mainly depends on Epa6. On the other hand, several ‘atypical’ proteins that were identified could also play a role in this interaction, consistently with recent suggestions that *C. parapsilosis* elongation factor 2, malate synthase, 6-phosphogluconate dehydrogenase and *C. tropicalis* enolase, fructose-bisphosphate aldolase, and transaldolase are involved in the interactions with human FN (Kozik *et al.*, 2015).

Human FN is locally produced by fibroblasts, lymphocytes, macrophages, epithelial cells and vascular myocytes, and is present at a relatively high concentration in plasma (Alitalo *et al.*, 1980; Magnuson *et al.*, 1998; Lenseink, 2013). FN binding by *C. glabrata* may facilitate colonization of the host by preventing pathogen detachment, as reported for pathogenic bacteria, *Streptococcus pyogenes* or *Staphylococcus aureus*, which attached to epithelial cells throughout interactions with FN (Dziewanowska *et al.*, 1999; Cue *et al.*, 2000). In contrast to active, hypha-mediated penetration of epithelial and endothelial cells by *C. albicans*, *C. glabrata* is able to enter the bloodstream and spread relatively easily, even without damage to host cells, as shown in a chicken embryo model (Jacobsen *et al.*, 2011) or a mouse model (Westwatre *et al.*, 2007) of candidal infection. Therefore, it was postulated that instead of active penetration, the mechanism of *C. glabrata* virulence and invasion may be based on endocytosis without damaging the host cell (Li *et al.*, 2007). It is also well known that *C. glabrata* may exist for a long time within macrophages without inducing inflammation; therefore, the interactions with FN might facilitate phagocytosis of *C. glabrata* cells by macrophages in order to enter these host cells and survive within them, as reported for *S. aureus* (Shinji *et al.*, 2003).

In conclusion, the present study clearly showed an interaction between Epa6 and FN that might be significant in the process of fungal adhesion to the proteins, cells, and tissues of the human host. As regards adhesins, which target host proteins as their binding ligands, the knowledge in this area is still insufficient, in contrast to the well-described lectin-type activity of Epa proteins. Investigations in this field would considerably improve our understanding of how this pathogenic yeast can cause a vast range of invasive as well as superficial infections in humans.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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