

Binding of human plasminogen and high-molecular-mass kininogen by cell surface-exposed proteins of *Candida parapsilosis**

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Pathogenic microbes can recruit to their cell surface human proteins that are components of important proteolytic cascades involved in coagulation, fibrinolysis and innate immune response. Once located at the bacterial or fungal surface, such deployed proteins might be utilized by pathogens to facilitate invasion and dissemination within the host organism by interfering with functionality of these systems or by exploiting specific activity of the bound enzymes. Aim of the study presented here was to characterize this phenomenon in *Candida parapsilosis* (Ashford) Langeron et Talice – an important causative agent of systemic fungal infections (candidiasis and candidemias) in humans. We have investigated the interactions of fungal surface-exposed proteins with plasminogen (HPG) and high-molecular-mass kininogen (HK) – the crucial components of human fibrinolytic system and proinflammatory/procoagulant contact-activated kinin-forming system, respectively. After confirming ability of the fungal surface-exposed proteins to bind HPG and HK, four of them – two agglutinin-like sequence (Als) proteins CPAR2_404780 and CPAR2_404800, a heat shock protein Ssa2 and a moonlighting protein 6-phosphogluconate dehydrogenase 1 – were purified using ion-exchange chromatography, gel filtration and chromatofocusing. Then, their affinities to HPG and HK were characterized with surface plasmon resonance measurements. The determined dissociation constants for the investigated protein-protein complexes were within a 10^{-7} M order for the HPG binding and in a range of 10^{-8} – 10^{-9} M for the HK binding. Detailed characterization of adsorption of these two important plasma proteins on the fungal cell surface may help to increase our understanding of molecular mechanisms of *C. parapsilosis*-dependent candidiasis.

Key words: candidiasis, cell wall proteins, fibrinolysis, contact system

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Abbreviations: Als, agglutinin-like sequence; BSA, bovine serum albumin; GPI, glycosylphosphatidylinositol; HK, high-molecular-mass kininogen; HPG, human plasminogen; Hyr, hyphally regulated protein; LC-MS/MS, liquid chromatography-coupled tandem mass spectrometry; PBS, phosphate buffered saline; RU, resonance units; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

INTRODUCTION

The development of infections caused by pathogenic microorganisms affects the action of major systems involved in maintaining the biochemical homeostasis of the human organism. This phenomenon is related to both, triggering of the host immune responses and engagement and activation of the pathogens' virulence factors. To efficiently disseminate with the bloodstream, colonize new niches of the human body and to successfully evade the host immune system, microorganisms can take control over several plasma proteolytic cascades involved in the coagulation and subsequent degradation of fibrin clots, or in the development of the inflammatory state during infection (Sun, 2006; Shannon *et al.*, 2013).

Secreted or surface-located microbial proteases often possess an ability to activate or inactivate particular enzymatic components of these proteolytic cascades or to degrade dedicated proteinase inhibitors, thus leading to deregulation of the physiological functionality of such systems (Maeda & Yamamoto, 1996; Potempa *et al.*, 2000; Kozik *et al.*, 2015a; Gogol *et al.*, 2016; Huish *et al.*, 2017). Moreover, a great variety of bacterial or fungal surface-exposed proteins were repeatedly reported to be involved in the interactions with human plasma proteins (Smeesters *et al.*, 2010; Rapala-Kozik *et al.*, 2011; Wollein Waldetoft *et al.*, 2012; Castiblanco-Valencia *et al.*, 2016).

Gathering the components of plasma kinin-generating system (“contact system”) – prekallikrein (pHPK), coagulation factor XII (FXII) and high-molecular-mass kininogen (HK) – at the microbial cell surface can result in this system's activation. This might be related not only to the final formation of fibrin clots or to the complement activation, but, most importantly, also to the release from HK biologically active peptides – kinins – that are potent proinflammatory mediators responsible for increase in the vascular permeability and vasodilatation (Holm *et al.*, 2011; Long *et al.*, 2016; Vieira *et al.*, 2016). Although activation of the contact system is one of the mechanisms contributing to the mobilization of human defense against pathogens, the increased inflow of plasma to the site of infection greatly facilitates microbial dissemination and helps them to acquire nutrients, thereby becoming a beneficial phenomenon for invading bacteria and fungi (Frick *et al.*, 2007; Nickel & Renné, 2012).

Additionally, capturing a key component of fibrinolytic system – plasminogen (HPG) – by the surface-associated proteins allows microorganisms to utilize its zymogen

activity. After conversion of plasminogen to plasmin, the latter surface-bound active proteinase can be employed to hydrolyze a fibrin clot, in which the pathogens are entrapped, or to degrade extracellular matrix proteins and penetrate the surrounding tissues (Herwald *et al.*, 2003; Loof *et al.*, 2014).

In addition to many reports concerning the binding of human HPG and HK by the cell wall proteins of the most important fungal human pathogen, *Candida albicans* (Robin) Berkhout (Crowe *et al.*, 2003; Karkowska-Kuleta *et al.*, 2011; Marín *et al.*, 2015), still relatively little is known about this phenomenon in other emerging pathogens from the *Candida* genus — the so-called “non-albicans” *Candida* species. One of them is *C. parapsilosis* (Ashford) Langeron et Talice, which has been recognized as a commensal strain widely distributed in the environment and colonizing human skin and nails (Bonassoli *et al.*, 2005; Yang *et al.*, 2011); however, currently *C. parapsilosis* is frequently reported to be the third or even the second, right after *C. albicans*, causative agent of invasive candidiasis in humans (Trofa *et al.*, 2008; Pfaller *et al.*, 2014; Fathi *et al.*, 2016; Yilmaz *et al.*, 2016). *C. parapsilosis* is often isolated from hands of the health personnel and from environmental surfaces in hospitals (Sanchez *et al.*, 1993; Sabino *et al.*, 2011). Furthermore, it possesses a great ability to adhere to and form biofilms on the surface of implanted medical devices (Diekema *et al.*, 1997; Pongrácz *et al.*, 2016). Therefore, this species is often responsible for serious nosocomial infections, including life-threatening candidemia and infectious endocarditis, diagnosed predominantly in immunocompromised individuals and among critically ill neonates (Garzoni *et al.*, 2007; Juyal *et al.*, 2013; Lovero *et al.*, 2016). In the case of premature infants and newborns with birth weight lower than 1500 g, the mortality rates of about 10% related to invasive infections caused by *C. parapsilosis* have now become an emerging problem (Benjamin *et al.*, 2006). One of the important factors that is conducive to such infections is the presence of *C. parapsilosis* on the skin that facilitates horizontal transmissions of the fungus in hospitals (Lupetti *et al.*, 2002). Another important problem is a significant predisposition of this species to adhere to vascular catheters used for parenteral nutrition of neonates, which is often followed by formation of biofilm and further development of disseminated infection and antifungal drug resistance (Chow *et al.*, 2012). Expanding the knowledge on *C. parapsilosis* virulence factors, especially in terms of fungal adhesion to host proteins and cells may be very helpful to prevent such infections in the future (Pammi *et al.*, 2013).

In the *C. parapsilosis* genome many gene families have been annotated as encoding proteins likely to be located within the fungal cell wall — an important part of the cell that participates in ceaseless and interactive contact with the host during infection. Examples include families of predicted proteins equipped with glycosylphosphatidylinositol (GPI) anchors, such as chitinases, Ecm33-like proteins, Sod-like (superoxide dismutase) proteins, yapsins, Hwp-like (hyphal wall protein) proteins, Rbt-like (repressed by TUP1) proteins, Pga24/59/62- and Pga30-like (predicted GPI-anchored) proteins, as well as large families of Als-like (agglutinin-like sequence) and Hyr/Iff-like (hyphally regulated) proteins, and others (Butler *et al.*, 2009). Moreover, a number of proteins without predicted GPI anchor are present at the cell surface of *C. parapsilosis* cells, as indicated by the results obtained with the method of cell surface shaving with trypsin (Karkowska-Kuleta *et al.*, 2015), including heat shock protein Ssa2, enolase, phosphoglycerate mutase and al-

cohol dehydrogenase. Proteins representing both groups have been shown recently to play an important role in adhesion of *C. parapsilosis* cells to biotic and abiotic surfaces. Bertini *et al.* (2016) demonstrated involvement of the *C. parapsilosis* Als-like protein CPAR2_404800 in interactions with human epithelial cells, whereas Núñez-Beltrán *et al.* (2017) confirmed the contribution of surface-localized phosphoglycerate kinase and enolase in adherence of the *C. parapsilosis* cells to silicone implants.

Since sequestration of the host plasma proteins at the pathogen cell surface might be an efficient mechanism contributing to increased pathogenicity of *Candida* spp. (Jong *et al.*, 2003; Rapala-Kozik *et al.*, 2008; Karkowska-Kuleta *et al.*, 2010; Funk *et al.*, 2016), our current work aimed at a detailed characterization of the interactions between human plasma proteins important for maintaining the hemostatic balance and the development of inflammation — HPG and HK — and selected fungal proteins known to be present at the *C. parapsilosis* cell surface. A subset of *C. parapsilosis* cell wall proteins potentially capable of interacting with HPG and HK was first identified using an affinity chromatography on agarose gels coupled with these human proteins. Then, the whole mixture of native cell wall proteins was subjected to protein purification procedures, searching for the pre-assumed HPG- and HK-binding candidates in the chromatographic fractions. Four confirmed HPG- and HK-binding proteins were finally isolated and purified, including two GPI-anchored putative adhesins from the Als-like protein family (CPAR2_404800 and CPAR2_404780) and two cell surface proteins that are not equipped with GPI-anchor but for which there is evidence for involvement in interactions with the host proteins and peptides — heat shock protein Ssa2 (Sun *et al.*, 2008) and 6-phosphogluconate dehydrogenase (Gnd1) (Karkowska-Kuleta *et al.*, 2011). The interactions of these proteins with HPG and HK were then characterized with the use of surface plasmon resonance (SPR) measurements.

MATERIALS AND METHODS

Proteins. Human plasma HK was purchased from Enzyme Research Laboratories (South Bend, IN, USA). Human plasma HPG and β -1,3-glucanase were from Sigma (St. Louis, MO, USA), β -1,6-glucanase from Takara Bio Inc. (Otsu, Shiga, Japan) and trypsin was from Promega (Madison, WI, USA). Bovine serum albumin (BSA) was obtained from BioShop Canada Inc (Burlington, Ontario, Canada) and the horseradish peroxidase-conjugated streptavidin solution (SA-HRP) from MP Biomedicals (Solon, OH, USA). Biotin-labeled HPG (HPG-Bt) and HK (HK-Bt) were obtained using biotin N-hydroxysuccinimide ester (Sigma) and a procedure described previously (Rapala-Kozik *et al.*, 2008).

Yeast strain and culture conditions. *C. parapsilosis* strain CDC 317 (ATCC[®] MYA-4646[™]) was purchased from American Type Culture Collection (Manassas, VA, USA). Fungal pseudohyphal forms were obtained after inoculating of yeast forms that were previously grown to the stationary phase for 16 hours at 30°C in YPD medium (1% yeast extract, 2% soybean peptone and 2% glucose) (Sigma), to RPMI 1640 medium (PAA Laboratories GmbH, Pasching, Austria) and further culturing for 24 hours at 37°C in the wells of microtiter plates without shaking or for 72 hours at 37°C in flasks on an orbital rotary shaker MaxQ 4000 (Thermo Fisher Scientific, Waltham, MA, USA).

Competition between HPG and HK for binding to the cell surface of *C. parapsilosis* pseudohyphae. The competition assay was performed on MaxiSorp 96-well microtiter plates (Nunc, Roskilde, Denmark) where 1×10^6 of *C. parapsilosis* cells were grown in 150 μ l of RPMI 1640 medium for 24 hours. After each stage of the assay, the cells were gently washed three times with 200 μ l of phosphate buffered saline (PBS), pH 7.4, additionally containing 1% BSA. The unoccupied well surface was blocked for 1 hour at 37°C with 300 μ l of 3% BSA in PBS. Then, 40 μ l of 50 nM HPG-Bt or HK-Bt solution were added to the wells together with 10 μ l of a competitor solution – unlabeled HK or HPG, respectively – used at a variable molar excess (the final concentrations were within a range of 50–800 nM). After incubation for 1.5 hours at 37°C, the bound labeled protein was detected with a solution of horseradish peroxidase-conjugated streptavidin (SA-HRP) and the substrate for HRP, 3,3',5,5' tetramethylbenzidine (TMB) (Sigma) as described previously (Rapala-Kozik *et al.*, 2008).

Binding of biotinylated HPG and HK to the surface of *C. parapsilosis* pseudohyphae pretreated with β -1,3-glucanase, β -1,6-glucanase, heat or trypsin. HPG- and HK-binding capacities of fungal cells after treatment with β -1,3-glucanase and β -1,6-glucanase were tested with the use of pseudohyphal forms of *C. parapsilosis* pretreated with both enzymes separately. β -1,3- and β -1,6-glucanase release cell wall proteins covalently attached to glucans by hydrolyzing the glucan network in which the surface proteins are embedded, but the latter enzyme does it without destroying the β -1,3-glucan layer of the cell wall (Kapteyn *et al.*, 2000). Moreover, in an additional approach, the cell wall-associated proteins were also removed by trypsin hydrolysis and such cells were also subjected to further binding analysis. *C. parapsilosis* cells (5×10^8) were incubated in Eppendorf tubes for 1.5 hours at 37°C with: (i) 100 U of β -1,3-glucanase in 100 μ l of 50 mM Tris buffer (pH 7.5); (ii) 0.2 U of β -1,6-glucanase in 100 μ l of McIlvaine buffer (pH 6.0), or (iii) 10 U of trypsin in 100 μ l of 25 mM ammonium bicarbonate buffer (pH 8.0). Separate portions of cells suspended in 100 μ l of PBS were heated at 80°C for 30 minutes and then cooled. Next, the fungal cells were washed three times with 1 ml of PBS and incubated with 100 μ l of 50 nM solution of HPG-Bt or HK-Bt in PBS for 1.5 hours at 37°C. The amount of bound biotinylated protein was determined with the SA-HRP/TMB detection system after transferring cells to new Eppendorf tubes and extensive washing out of unbound proteins.

Affinity chromatographic identification of HPG- and HK-binding *C. parapsilosis* proteins. The method described previously (Karkowska-Kuleta *et al.*, 2011) was followed with some modifications. Briefly, Affi Gel-10 (Bio-Rad, Herkules, CA, USA) containing covalently coupled HPG or HK (500 μ g per 1 ml of gel) was incubated with the whole mixture of *C. parapsilosis* cell wall proteins isolated with β -1,6-glucanase. After washing out of the unbound proteins, the adsorbed putative HPG- and HK-binding proteins were eluted by boiling the gel in SDS. The isolated proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the Laemmli system (Laemmli, 1970) and stained with Coomassie Brilliant Blue R-250. The proteins were then identified with liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) (see below).

Purification of heat shock protein Ssa2, 6-phosphogluconate dehydrogenase 1 (Gnd1) and agglutinin-like sequence proteins, CPAR2_404780 and

CPAR2_404800, from the whole mixture of proteins isolated from *C. parapsilosis* cell wall with β -1,6-glucanase. *C. parapsilosis* pseudohyphae obtained after growth in RPMI 1640 medium for 72 hours at 37°C were centrifuged and washed three times with 1 ml of PBS. Then, the fungal cells (0.4 g of wet weight) were incubated for 24 hours at 37°C in 1 ml of McIlvaine buffer, pH 6.0 with 2 U of β -1,6-glucanase. Next, the supernatant was collected and dialyzed against 20 mM Tris-HCl buffer, pH 8.0 for 48 hours at 4°C. The integrity of cell membrane was tested with Trypan Blue (Sigma) staining. The quality of the obtained protein extracts was tested with SDS-PAGE in the Laemmli system and the protein concentration was assessed with the Bradford method (Bradford, 1976).

To purify selected *C. parapsilosis* surface-exposed proteins, the purification strategy similar to that described previously for *C. tropicalis* (Castellani) Berkhout cell wall proteins (Karkowska-Kuleta *et al.*, 2016) was applied. During the first step of the purification procedure – ion-exchange chromatography on Resource™ Q column (GE Healthcare, Uppsala, Sweden) – we searched for fractions that on SDS-PAGE showed strong major band(s) which were found by LC-MS/MS to contain one of the candidate proteins, belonging to pre-identified (by affinity chromatography on immobilized HPG or HK, see above) subsets of putative HPG- or HK-binding cell wall proteins. Only those fractions were subjected to the next purification step – gel filtration on Superdex 200 HR column (GE Healthcare/Amersham Biosciences, Little Chalfont, UK). However, for the purification of Gnd1 and Ssa2, chromatofocusing was additionally used after gel filtration. For that step, the Ssa2- and Gnd1-containing fractions after gel filtration were dialyzed against 25 mM Bis-Tris-iminodiacetic acid buffer, pH 7.1, for 48 hours and then applied to a MonoP HR 5/20 column (GE Healthcare) pre-equilibrated with the same buffer. In order to elute proteins, a 20 ml linear gradient of 10% Polybuffer 74 in 20 mM Bis-Tris-iminodiacetic acid buffer pH 7.1 (Sigma) was used at a flow rate of 0.5 ml/min (Bras *et al.*, 2013). The effluent absorbance at 280 nm was continuously monitored and pH of the eluted fractions was checked with laboratory pH-meter every three minutes during the separation to ensure the linearity of the pH gradient. After observing an increase in the absorbance, the fractions containing proteins with isoelectric point (pI) values close to 5.0 and 6.0 were collected and then individually applied to the Superdex 200 HR 10/50 column in the 20 mM Tris-HCl pH 6.7 at a flow rate of 0.5 ml/min in order to separate proteins from ampholytes. After each purification step, the selected fractions were characterized by SDS-PAGE and particular proteins were identified with LC-MS/MS, as described in detail below.

Protein identification with LC-MS/MS. To identify the content of the protein bands on the electrophoretic gels, the previously published protocol was used (Seweryn *et al.*, 2015), with minor modifications. Briefly, the bands were manually excised and destained by an extensive washing with 100 μ l of 50% acetonitrile (ACN) and 25% ACN in 25 mM ammonium bicarbonate (NH_4HCO_3). After that, they were subjected to reduction with 100 μ l of 10 mM dithiothreitol in 25 mM NH_4HCO_3 at 37°C for 45 minutes and then alkylation with 100 μ l of 50 mM iodoacetamide in 25 mM NH_4HCO_3 for 1.5 hours at room temperature in the dark. The excess reagents were washed out with 200 μ l of 50% ACN in 25 mM NH_4HCO_3 and protein bands were treated with 50 μ l of 100% ACN and then air-dried. In the next

step, the proteins were treated with 15 μl of trypsin (0.1 $\mu\text{g}/10 \mu\text{l}$ in 25 mM NH_4HCO_3) overnight at 37°C. The peptides were extracted with 50 μl of 50% ACN and 0.5% formic acid and dried in an Alpha 1-2 lyophilizer (Christ, Osterode, Germany). After dissolution in 35 μl of 10% ACN with 0.1% formic acid, the obtained peptides were separated and analyzed by the LC-MS/MS technique carried out with an HCT Ultra ion-trap mass spectrometer equipped with an electrospray ionization ion source and an electron-transfer dissociation II fragmentation module (Bruker, Bremen, Germany) and coupled to an ultrahigh-performance liquid chromatography Dionex Ultimate 3000 system (Carlsbad, CA, USA). The protein identification was performed through the National Center for Biotechnology Information (NCBI) protein database search with an in-house Mascot server (v.3.0, Matrix Science, London, UK).

Binding of HPG-Bt or HK-Bt to *C. parapsilosis* pseudohyphae in the presence of purified CPAR2_404780, CPAR2_404800, Ssa2 and Gnd1.

C. parapsilosis pseudohyphae were obtained after growth of 1×10^6 cells in RPMI 1640 medium in the wells of MaxiSorp microtiter plates for 24 hours at 37°C. After each step of the competition assay, the cells were washed with 300 μl of PBS with 1% BSA. The unoccupied well surface was blocked for 1 hour at 37°C with 300 μl of 3% BSA in PBS. Then, 40 μl of the HPG-Bt or HK-Bt (50 nM) solutions, prepared in PBS, were added to the wells together with 10 μl of the 1.2 μM solutions of a particular purified fungal protein, CPAR2_404780, CPAR2_404800, Ssa2 or Gnd1 (final concentration: 240 nM), also prepared in PBS, and gently mixed. After incubation for 1.5 hours at 37°C and washing out of the unbound proteins, the amount of bound biotinylated protein was detected with SA-HRP/TMB detection system.

Characterization of binding of *C. parapsilosis* proteins to HPG and HK with SPR measurements.

The kinetic and thermodynamic analyses of binding of CPAR2_404780, CPAR2_404800, Ssa2 and Gnd1 to HPG or HK were carried out with a BIACORE 3000 system (GE Healthcare). For those measurements, the fungal protein was immobilized onto a surface of CM5 *via* amine groups and the solutions of human proteins, used at a sufficiently wide range of concentrations, flowed over this surface. In detail, the fungal proteins were dialyzed against 10 mM HEPES buffer, 150 mM NaCl, pH 7.4 and immobilized onto a CM5 sensor chips with the use of the Amine Coupling Kit (GE Healthcare). The surface of sensor chip was first activated by injection of 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 200 mM N-hydroxysuccinimide (NHS), then the particular protein of interest was injected over the chip surface with a flow rate of 10 $\mu\text{l}/\text{min}$ for 7 minutes. The immobilization was carried out in 10 mM sodium acetate buffer, pH 4.0 for CPAR2_404780 and CPAR2_404800, pH 4.5 for Ssa2 and pH 5.0 for Gnd1, and the obtained immobilization level was 300 resonance units (RU) for CPAR2_404780, 310 for CPAR2_404800, 430 for Ssa2 and 300 for Gnd1. Next, the solutions of HPG or HK in 10 mM HEPES buffer with 150 mM NaCl and 0.005% surfactant P20, which was also used as the running buffer, were injected at a flow rate of 30 $\mu\text{l}/\text{min}$ at 25°C. The association and dissociation time was 120 seconds and the regeneration of chip surface was carried out with a single injection of 1 M NaCl at a flow rate of 30 $\mu\text{l}/\text{min}$ for 30 seconds. The obtained results were analyzed with a BIAevaluation 4.1 software (GE Healthcare). The dissociation and association rate constants (k_d and k_a) and the equilibrium dis-

sociation constants (K_D) were calculated with the global fit of a simple (1:1) Langmuir model with a baseline drift to the complete set of obtained sensograms.

Statistical analysis. The data presented as the displacement plots were fitted using GraphPad Prism software with the nonlinear-regression one-phase exponential decay, and the particular data points are reported as the mean \pm standard deviation (S.D.). All results presented as the graph bars correspond to means \pm S.D. and after performing the Student's *t*-test it was assumed that the differences between values were statistically significant when $p < 0.05$. The kinetic and thermodynamic binding parameters included in Table 2 and determined after global fitting analysis with the use of BIAevaluation 4.1 software, are presented with the standard errors (S.E.) of the obtained values.

RESULTS

The involvement of *C. parapsilosis* surface-exposed proteins in the fungal pseudohyphae interactions with human HPG and HK

It was confirmed that both investigated human plasma proteins — HPG and HK — could bind to the cell surface of *C. parapsilosis* pseudohyphae. Moreover, the displacement plots presented in Fig. 1 show that some binding sites located on the fungal cell wall might be shared in common by these two human proteins. When biotinylated HK competed with twenty-fold molar excess of non-biotinylated HPG for binding to *C. parapsilosis* cells grown in the wells of MaxiSorp microplate, its binding level decreased only by about 20%. In an opposite situation, when HPG-Bt competed with HK added at the same molar excess, about 40% of the initial level of binding was retained. This result suggested a higher binding level for HK than for HPG.

The treatment of *C. parapsilosis* pseudohyphae with β -1,3-glucanase or β -1,6-glucanase, resulting in the release of the substantial part of cell wall-associated proteins (Kapteyn *et al.*, 1995; Rapala-Kozik *et al.*, 2008; Karkowska-Kuleta & Kozik, 2015) led to a decrease in the binding level of both, HPG and HK, to approximately 20–25% in comparison

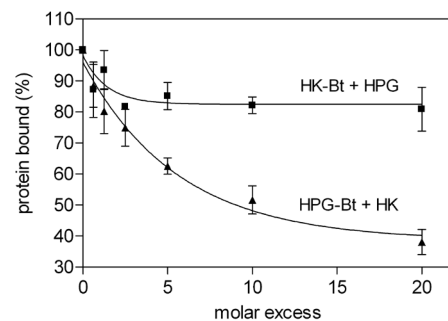


Figure 1. The displacement plots for competition between biotinylated HPG (▲) or HK (■) and an unlabeled respective protein for binding to *C. parapsilosis* pseudohyphae.

Pseudohyphal forms (1×10^6) of *C. parapsilosis* grown in the wells of MaxiSorp microplate were incubated with the mixture of 40 μl of 50 nM HPG-Bt or HK-Bt prepared in PBS (the final concentration was 40 nM) and 10 μl of HK or HPG as a competitor added in the final concentration range of 50–800 nM. After incubation at 37°C for 1.5 hours, the amount of bound biotinylated protein was determined with SA-HRP/TMB detection system. The displacement plots represent data fits with the nonlinear-regression for one phase exponential decay, obtained with the use of GraphPad Prism software for data points reported as the mean \pm standard deviation (S.D.).

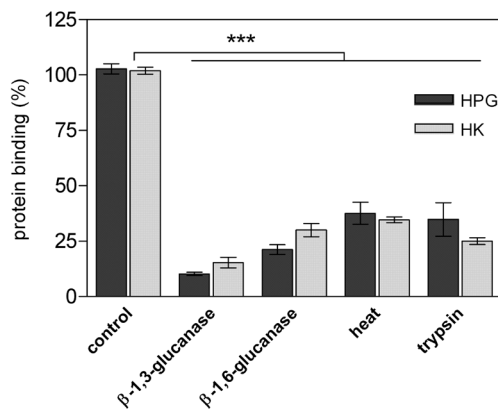


Figure 2. Binding of HPG-Bt and HK-Bt to the *C. parapsilosis* pseudohyphal forms that were treated with β -1,3-glucanase, β -1,6-glucanase, heat, and trypsin.

C. parapsilosis pseudohyphae (5×10^8) were incubated for 1.5 hours at 37°C with β -1,3-glucanase (100 U in 100 μ l), β -1,6-glucanase (0.2 U in 100 μ l), trypsin (10 U in 100 μ l) or were heated at 80°C for 30 minutes in 100 μ l of PBS. Non-treated cells in PBS acted as a control of 100% binding. Then, 50 nM solution of HPG-Bt or HK-Bt prepared in 100 μ l of PBS was incubated with fungal cells for 1.5 hours at 37°C. After washing out the unbound proteins, the bound HPG-Bt or HK-Bt was detected with the SA-HRP/TMB system. The results are presented as the mean \pm standard deviation of three independent determinations. The statistical significance for each protein between values obtained for non-treated and treated cells was determined after performing the Student *t*-test and indicated as ***for $p < 0.001$.

Table 1. Mass spectrometric identification of HPG-binding *C. parapsilosis* cell wall proteins.

β -1,6-glucanase was used for extraction of *C. parapsilosis* proteins. Particular HPG-binders were isolated with affinity chromatography and specific protein bands were excised after SDS-PAGE electrophoresis and digested using trypsin. Peptides were analyzed using the Dionex Ultimate 3000 ultrahigh-performance liquid chromatography system coupled to an HCT Ultra ETDII mass spectrometer equipped with an electrospray ionization ion source. The obtained lists of peaks were searched against the NCBI protein database (SC – sequence coverage).

Accession	Protein	Molecular mass [kDa]	pI	Scores	Number of peptides	SC [%]
gi 354544475	hypothetical protein CPAR2_301880, amidase [<i>Candida parapsilosis</i>]	62.1	5.71	236	6	11
gi 354547939	protein CPAR2_404780, cell-wall agglutinin, N-terminal, ligand-sugar binding [<i>Candida parapsilosis</i>]	114.0	3.80	96	3	4
gi 354547941	hypothetical protein CPAR2_404800, cell-wall agglutinin, N-terminal, ligand-sugar binding [<i>Candida parapsilosis</i>]	144.0	4.19	118	4	3
gi 8927048	elongation factor 2 [<i>Candida parapsilosis</i>]	90.3	6.57	130	4	4
gi 354548043	hypothetical protein CPAR2_405810, similar to <i>C. albicans</i> ACS1, putative acetyl-CoA synthetase [<i>Candida parapsilosis</i>]	75.0	5.63	281	7	11
gi 354544776	hypothetical protein CPAR2_800530, similar to <i>C. albicans</i> PCK1, phosphoenolpyruvate carboxykinase [<i>Candida parapsilosis</i>]	61.9	6.05	148	4	8
gi 354545113	hypothetical protein CPAR2_803890, similar to <i>C. albicans</i> MLS1, malate synthase [<i>Candida parapsilosis</i>]	62.7	8.73	396	23	14
gi 354546116	hypothetical protein CPAR2_204880, similar to <i>C. albicans</i> PGI1, putative glucose-6-phosphate isomerase [<i>Candida parapsilosis</i>]	61.5	5.97	110	4	8
gi 354545980	hypothetical protein CPAR2_203520, similar to <i>C. albicans</i> GND1, putative 6-phosphogluconate dehydrogenase [<i>Candida parapsilosis</i>]	55.4	5.94	270	6	8
gi 354546845	hypothetical protein CPAR2_212210, similar to <i>C. albicans</i> ALD5, NAD-aldehyde dehydrogenase [<i>Candida parapsilosis</i>]	54.0	5.71	671	17	38
gi 354545888	hypothetical protein CPAR2_202600, similar to <i>C. albicans</i> TAL1, putative transaldolase [<i>Candida parapsilosis</i>]	35.9	5.22	296	7	22

to the non-treated cells (Fig. 2). Therefore, these fractions were considered as useful for further procedure of protein purification. However, because the protein isolation with β -1,3-glucanase might result in slightly larger quantities of damaged cells as the deeper layer of the cell wall is affected (Klis *et al.*, 2009), the milder protein isolation with β -1,6-glucanase was further used, due to a reduced risk of potential contamination of the obtained extracts by cytoplasmic proteins derived from broken cells.

Moreover, fungal cells treated with heat or trypsin in order to deprive them of surface protein-dependent HPG- and HK-binding capacity (Bouchara *et al.*, 1990), demonstrated a decrease in binding level to approximately 35–40% of the initial binding level, thus confirming a predominant role of the surface-located proteins, in comparison to other cell surface components, in the phenomenon of HPG and HK binding.

The kinetic and thermodynamic studies on interactions between purified *C. parapsilosis* proteins and HPG or HK

The next step was to isolate and purify particular HPG- or HK-binding *C. parapsilosis* cell wall proteins. Our purification strategy (see Materials and Methods section) required that a subset of candidate HPG- or HK-binding proteins be pre-determined. For that purpose, an affinity chromatography on agarose-immobilized HPG or HK was applied. As an example, a list of putative HPG-binding proteins is presented in Table 1; similar results were obtained for HK-coupled gel (data not

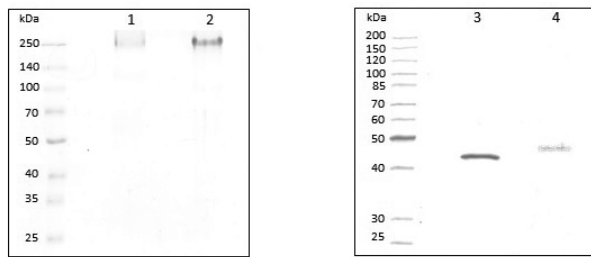


Figure 3. Electrophoretic characteristics of *C. parapsilosis* purified proteins: (1) CPAR2_404780, (2) CPAR2_404800, (3) Ssa2 and (4) Gnd1.

Purified *C. parapsilosis* proteins were separated by SDS-PAGE under reducing conditions in the Laemmli system using 10% (1, 2) or 12% (3, 4) separating gel and then stained with Coomassie Brilliant Blue R-250. A pattern for molecular-mass marker mixture is shown in the leftmost lane separately for each gel.

shown). Additionally, literature data on the involvement of particular *C. parapsilosis* proteins or their orthologs in the binding of various host proteins and peptides, such as histatin 5, HK, coagulation factor XII, plasma prekallikrein, fibronectin, vitronectin and laminin (López-Ribot *et al.*, 1996; Karkowska-Kuleta *et al.*, 2011; 2016; Kozik *et al.*, 2015b; Seweryn *et al.*, 2015) were taken into account.

Starting from the whole mixture of native, β -1,6-glucanase-extractable fungal proteins, with the use of a combination of ion-exchange chromatography, gel filtration and chromatofocusing, we successfully isolated four HPG- and HK-binding proteins in SDS-PAGE pure forms (Fig. 3). These included two proteins similar to the Als family proteins (CPAR2_404780 and CPAR2_404800), heat shock protein Ssa2 and 6-phosphogluconate dehydrogenase (Gnd1). They also belong to two distinct groups of cell wall proteins distinguished above, thus ensuring a diverse look at the participation of these groups to the adhesion processes. An ability of these four *C. parapsilosis* purified proteins to directly compete with surface-located proteins for binding of HK or HPG was tested with a displacement assay using *C. parapsilosis* pseudohyphal forms (Fig. 4). Biotinylated human protein interacted at the same time with the surface of immobilized fungal cells and soluble individual purified protein in solution used at six-fold molar excess relative to HPG-Bt and HK-Bt. In the case of HK, presence of

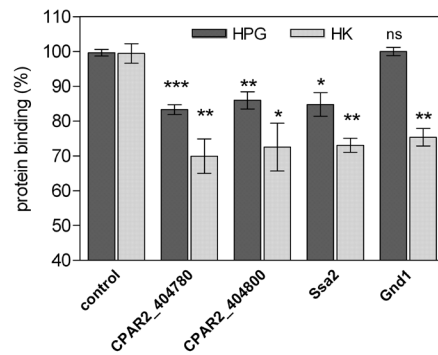


Figure 4. The displacement of HPG-Bt or HK-Bt by purified, soluble CPAR2_404780, CPAR2_404800, Ssa2 and Gnd1 from binding to *C. parapsilosis* pseudohyphae.

40 μ l of 50 nM solutions of HPG-Bt or HK-Bt were added to the microplate wells with 1×10^6 fungal cells grown as pseudohyphae for 24 hours at 37°C in RPMI 1640 medium, and mixed with 10 μ l of the solution of a particular soluble fungal protein: CPAR2_404780, CPAR2_404800, Ssa2 or Gnd1 prepared in 6-fold molar excess in PBS. After incubation for 1.5 hours at 37°C and washing out of the unbound proteins, the amount of bound biotinylated HPG or HK was determined with the SA-HRP/TMB detection system. The level of binding of the biotinylated protein to the fungal cells without competitor added was considered as 100%. Bars on graph represent the mean values of 3 determinations \pm standard deviation. Statistical significance levels against the controls were determined for each human protein separately and are marked with * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$ and ns for "not significant".

all four purified proteins resulted in the decrease of HK binding level by about 25–30%. Gnd1 was the only fungal protein that did not decrease the HPG binding level, suggesting that these two proteins did not interact with each other. However, the other three proteins caused a decrease in the HPG-binding level to approximately 85% of the initial level of HPG binding, as determined for the cells incubated with labeled human protein without any competitor added.

The SPR measurements were used to characterize, in kinetic and thermodynamic terms, the interactions of individual fungal protein-HPG or fungal protein-HK pairs. The sensograms for HPG and HK, confirming these interactions are shown in Fig. 5 and Fig. 6, respectively. As indicated in the Table 2, K_D for both Als proteins – CPAR2_404780 and CPAR2_404800 – were of an order of 10^{-7} M when interacting with HPG or 10^{-9} M when interacting with HK, indicating that the binding affinity was higher for the Als protein-HK interaction. Moreover, K_D determined for the interacting pair Ssa2-HK was by one order smaller (10^{-8} M) than that obtained for pair Ssa2-HPG (10^{-7} M). This observation again indicates a stronger interaction of the fungal protein with HK than with HPG. Interestingly, *C. parapsilosis* Gnd1 interacts only with HK, giving a K_D value within a nanomolar range. No Gnd1 binding to HPG was observed with the SPR measurements. This also confirms the results described above, indicating the lack of direct competition of soluble

Table 2. The kinetic and thermodynamic parameters for interactions between CPAR2_404780, CPAR2_404800, Ssa2 and Gnd1 and HPG or HK, determined by SPR measurements.

A simple Langmuir model (1:1) with a baseline drift was globally fitted to the sensograms presenting the interactions between a given fungal protein as a ligand and human proteins as an analyte. All the parameters are presented with the standard error (S.E.) indicating the confidence in the reported value for the particular parameter.

Protein	K_D (M)	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})
HPG binding			
CPAR2_404780	$3.57 \times 10^{-7} \pm 1.66 \times 10^{-8}$	$5.09 \times 10^4 \pm 4.21 \times 10^3$	$1.82 \times 10^{-2} \pm 4.94 \times 10^{-4}$
CPAR2_404800	$4.52 \times 10^{-7} \pm 1.61 \times 10^{-8}$	$6.12 \times 10^3 \pm 6.62 \times 10^2$	$2.77 \times 10^{-3} \pm 2.60 \times 10^{-4}$
Ssa2	$4.03 \times 10^{-7} \pm 1.91 \times 10^{-8}$	$4.09 \times 10^4 \pm 2.75 \times 10^3$	$1.65 \times 10^{-2} \pm 2.71 \times 10^{-4}$
HK binding			
CPAR2_404780	$5.82 \times 10^{-9} \pm 1.14 \times 10^{-10}$	$4.16 \times 10^5 \pm 8.83 \times 10^3$	$2.42 \times 10^{-3} \pm 6.97 \times 10^{-5}$
CPAR2_404800	$6.27 \times 10^{-9} \pm 3.81 \times 10^{-10}$	$1.77 \times 10^6 \pm 1.17 \times 10^5$	$1.11 \times 10^{-2} \pm 4.69 \times 10^{-4}$
Ssa2	$6.93 \times 10^{-8} \pm 4.51 \times 10^{-9}$	$1.31 \times 10^5 \pm 2.76 \times 10^4$	$9.08 \times 10^{-3} \pm 1.92 \times 10^{-4}$
Gnd1	$7.22 \times 10^{-9} \pm 2.06 \times 10^{-10}$	$4.89 \times 10^5 \pm 3.99 \times 10^4$	$3.53 \times 10^{-3} \pm 1.50 \times 10^{-4}$

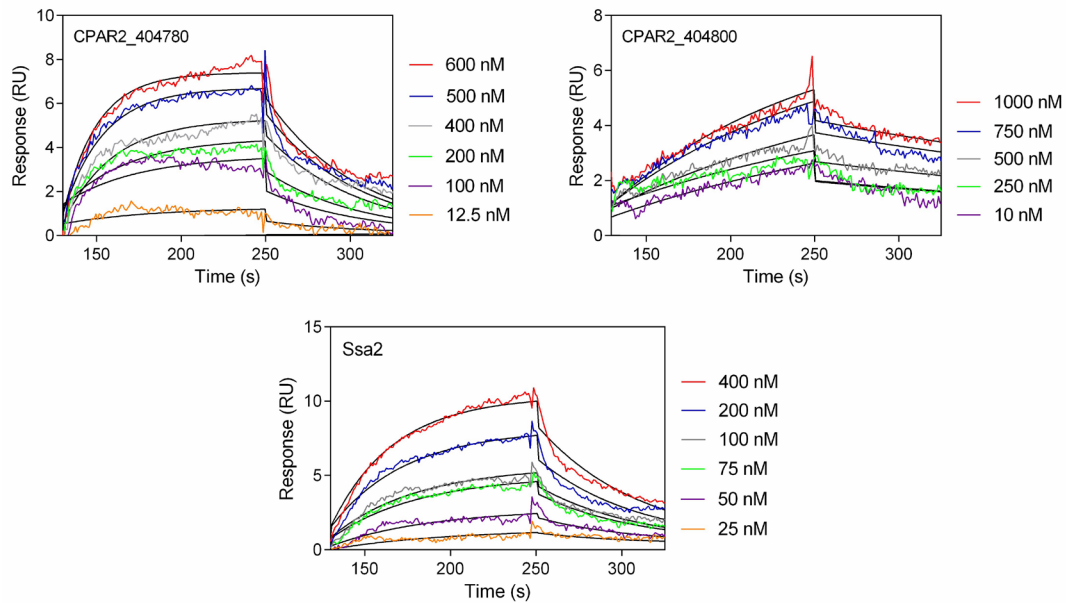


Figure 5. SPR sensograms representing interactions between HPG and CPAR2_404780, CPAR2_404800 and Ssa2. SPR characteristics of the interaction between the HPG and a particular fungal protein immobilized onto a surface of the CM5 chip. The global data fits with the Langmuir 1:1 binding model (with a baseline drift) are shown as black lines. The HPG solutions at a concentration range of 10–1000 nM were injected at a flow rate of 30 μ l/min for 120 seconds.

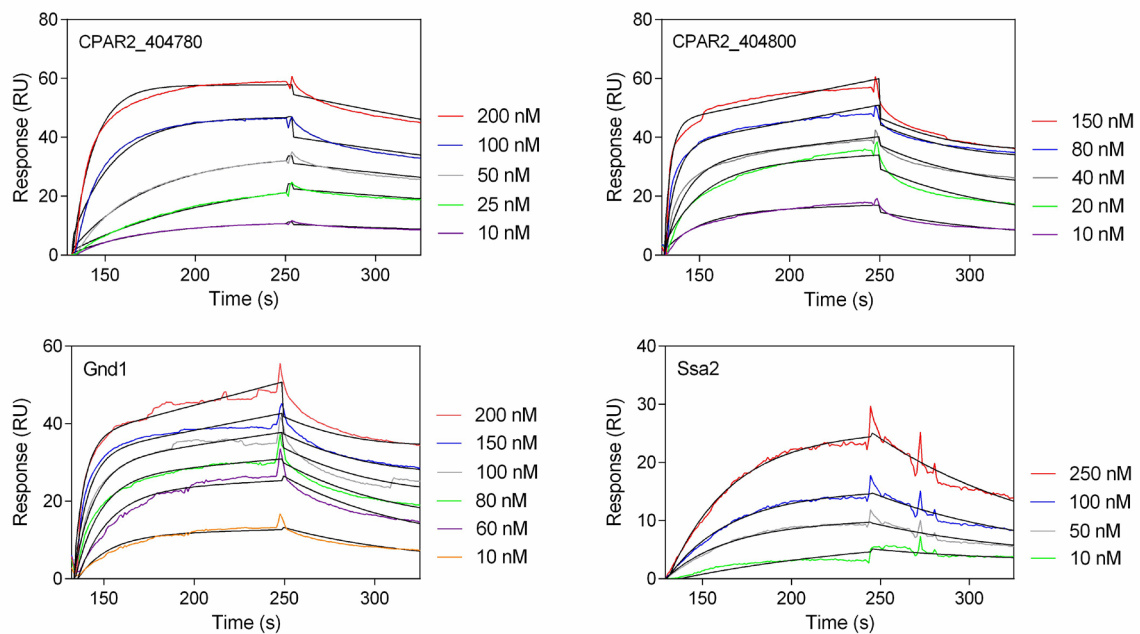


Figure 6. HK binding by CPAR2_404780, CPAR2_404800, Ssa2 and Gnd1 investigated by SPR measurements. Interactions between HK and individual fungal protein presented as the entire sets of SPR sensograms obtained during the injection of HK solutions at a concentration range of 10–250 nM at a flow rate of 30 μ l/min for 120 seconds over the surface of the CM5 chip with immobilized *C. parapsilosis* individual protein. Black lines illustrate the global data fits with the Langmuir 1:1 binding model (with a baseline drift).

Gnd1 and surface-located proteins for binding to HPG (Fig. 4).

DISCUSSION

Invasive bacterial or fungal infections related to the dissemination of microbial cells with the bloodstream always carry a risk of uncontrolled activation of the human plasma proteolytic cascades involved in the maintenance of hemostatic balance (Degen *et al.*, 2007; Oe-

hmcke & Herwald, 2010; Loof *et al.*, 2014). Such complete deregulation of the fundamental processes of coagulation and fibrinolysis can lead to the development of systemic inflammatory response syndrome (SIRS), septic shock and severe sepsis associated with high morbidity and mortality rates (Jacobi, 2002; Duggan *et al.*, 2015; Ng *et al.*, 2015). The process of sequestration of the key components of these systems, such as HPG and HK, at the pathogens' cell surface, often correlated with the acquisition of additional proteolytic activity, greatly fa-

Table 3. The K_D values (M) previously obtained for interactions between *C. albicans* (Seweryn *et al.*, 2015) and *C. tropicalis* (Karkowska-Kuleta *et al.*, 2016) cell wall-associated proteins and HK, as determined by SPR measurements (ND – not determined).

Protein name	<i>C. albicans</i>	<i>C. tropicalis</i>
Agglutinin like sequence protein 3	2.13×10^{-7}	ND
Triosephosphate isomerase 1	7.87×10^{-8}	ND
Enolase 1	2.25×10^{-7}	1.42×10^{-7}
Phosphoglycerate mutase 1	4.79×10^{-7}	5.81×10^{-7}
Hyphally regulated protein	ND	2.20×10^{-7}

cilitates invasion of the host organism. Therefore, it has been currently extensively studied for pathogenic bacteria (Bhattacharya *et al.*, 2012; Nickel & Renné, 2012; Peetermans *et al.*, 2016); however, for fungi from the *Candida* genus, important fungal pathogens for humans, this problem has not been sufficiently addressed. The results presented in this work suggest that such potential binding phenomenon might also result in the activation of the contact system or fibrinolytic system during the development of infections caused by *C. parapsilosis*.

There were reports concerning an ability of the cell wall proteins of *C. albicans* to bind host plasminogen (Funk *et al.*, 2016) and several HPG-binding proteins were identified for this species, including atypical cell wall proteins phosphoglycerate mutase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, fructose biphosphate aldolase, alcohol dehydrogenase, thioredoxin peroxidase, catalase and transcription elongation factor (Crowe *et al.*, 2003; Poltermann *et al.*, 2007), glycerol-3-phosphate dehydrogenase 2 (Luo *et al.*, 2013) and pH-regulated antigen 1 (Luo *et al.*, 2009). Moreover, it was suggested that binding of the *C. albicans* surface-associated enolase to HPG after its activation to plasmin might be beneficial for the pathogen during crossing of the layer of brain microvascular endothelial cells (Jong *et al.*, 2003). Furthermore, in our previous studies carried out for HK, not only the particular binding proteins were identified at *C. albicans* and *C. tropicalis* cell surface, but also the binding parameters were determined by SPR measurements performed for the interactions between this key component of the contact system and selected, purified *C. albicans* proteins (Table 3) (Seweryn *et al.*, 2015; Karkowska-Kuleta *et al.*, 2016).

However, in the case of *C. parapsilosis*, data on the interactions with these two plasma proteins are missing, especially if one takes into account the risk associated with the increasing frequency of invasive infections and candidemia caused by this species among neonates, patients with impaired immunity and critically ill individuals (Chatzinikolaou & Raad, 2000; Pfaller *et al.*, 2014; Wadile & Bhate, 2015). It was only proven, so far, that HK could be adsorbed at the cell surface of *C. parapsilosis* pseudohyphal forms (Rapala-Kozik *et al.*, 2008) and pro-inflammatory kinins might be generated from their precursors by *C. parapsilosis* secreted aspartic proteases (Bras *et al.*, 2013). Therefore, in this work, for the first time, we characterized in details the interactions of HPG/HK and the cell surface of *C. parapsilosis* pseudohyphae.

At first, the involvement of surface-exposed fungal proteins in the interactions with HPG and HK was confirmed (Fig. 2) and then four *C. parapsilosis* surface-associated proteins were purified. These proteins belonged to three diverse groups of cell wall proteins: (i) typical cell wall proteins, highly glycosylated adhesins covalently bound to

the cell wall glucans, represented by CPAR2_404780 and CPAR2_404800 (Butler *et al.*, 2009; Gacser, 2016), (ii) heat shock protein Ssa2, a chaperone putatively non-covalently associated with the cell wall although missing a signal peptide for classical secretion (López-Ribot *et al.*, 1996), and (iii) an atypical cell wall-associated protein, Gnd1, a role of which at the fungal cell surface, as well its secretion pathway, have not been fully understood so far – it is primarily a cytoplasmic enzyme involved in the pentose phosphate pathway, but its confirmed presence at the microbe cell surface classifies it to an enigmatic group of “moonlighting proteins” (Karkowska-Kuleta & Kozik, 2014).

In the case of *C. albicans*, the importance of Als proteins in the pathogenesis of infections was emphasized (Hoyer & Cota, 2016), and that is why two *C. parapsilosis* proteins similar to *C. albicans* Als proteins were selected for further investigation of their involvement in the HPG- and HK-binding. Heat shock protein Ssa2, the surface-located chaperone involved in protein folding, was also reported to be responsible for binding of salivary antimicrobial peptide, histatin 5 (López-Ribot *et al.*, 1996; Li *et al.*, 2003; Sun *et al.*, 2008). Gnd1, although an atypical cell wall protein, was reported to be a *C. parapsilosis* binding protein for extracellular matrix proteins, fibronectin and vitronectin (Kozik *et al.*, 2015b). What is more, *C. albicans* Gnd1 was identified as a HK- and plasma prekallikrein-binding protein (Karkowska-Kuleta *et al.*, 2011; Seweryn *et al.*, 2015).

In the study presented here, the competition analyses had shown that all four investigated *C. parapsilosis* proteins might be responsible for attracting HK to the surface of pseudohyphae, and additionally three of them, excluding Gnd1, possess a HPG binding capacity. However, the incomplete displacement of binding of biotinylated human protein from binding to pseudohyphae by individual soluble fungal protein (Fig. 4) indicates a potential involvement of additional fungal proteins in these phenomena. Of the two human proteins investigated, HK bound more strongly to the tested fungal proteins, as judged by K_D values that were lower by one (for Ssa2) or two (for Als-like proteins) orders of magnitude than those determined for HPG under the same conditions. A comparison of the binding parameters, summarized in Table 3, that were previously obtained for HK-binding proteins isolated from the cell walls of *C. albicans* and *C. tropicalis*, suggests that *C. parapsilosis* proteins studied in this work showed significantly stronger affinities to HK. The proteins studied previously are both GPI-anchored cell wall proteins such as Als3 and Hyr, and atypical proteins: enolase, phosphoglycerate mutase and triosephosphate isomerase. Similarly to *C. albicans*, in the current research, *C. parapsilosis* Als proteins have been identified as strong HK-binders. For *C. albicans* and *C. tropicalis*, two HK-binding proteins were common for both species – enolase and phosphoglycerate mutase – whereas for *C. parapsilosis* two other proteins were characterized in terms of interactions with this particular human protein. Such observation of the differences between *Candida* species may be of great importance in the design of possible inhibitory agents. This is a particularly important issue in the case of premature newborns suffering from candidiasis. The involvement of surface-exposed adhesins in the interactions with human epithelial cells can greatly increase the risk of *Candida* invasion and development of oral infections in children (Pammi *et al.*, 2013). As *C. parapsilosis* is still considered as one of the most frequently isolated species from the *Candida* genus causing serious, life-threatening infections in neonates, investigating the role of adhesive, surface proteins of this species might be useful for prevention of such cases.

In conclusion, the detailed kinetic and thermodynamic characterization of HPG and HK binding was performed for the first time for a set of four *C. parapsilosis* proteins including typical adhesins and atypical cell wall proteins. Such diversity of investigated HPG- and HK-binding proteins with regard to their functional and structural class indicates a high complexity of the mechanisms of interactions between the pathogen cell surface and the human host that can significantly hinder a full understanding of the processes that contribute to the pathogenicity of *C. parapsilosis*. Recently, an important role of CPAR2_404800 in the adhesion of *C. parapsilosis* to buccal epithelial cells and its involvement in the murine urinary candidiasis was reported (Bertini *et al.*, 2016), demonstrating an urgent need to further investigate function of the surface proteins in the *C. parapsilosis* virulence mechanisms. Therefore, the detailed description of the adsorption phenomenon of these two important plasma proteins on the fungal cell surface sheds new light on molecular mechanisms of *C. parapsilosis*-dependent life-threatening invasive infections.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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