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Regular paper

Towards understanding the novel adhesin function of *Candida albicans* phosphoglycerate mutase at the pathogen cell surface: some structural analysis of the interactions with human host extracellular matrix proteins*

Dorota Satala[1], Aleksandra Zelazna[2], Grzegorz Satala[3], Michal Bukowski[2], Marcin Zawrotniak[1], Maria Rapala-Kozik[1] and Andrzej Kozik[2]✉

[1]Department of Comparative Biochemistry and Bioanalytics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Krakow, Kraków, Poland; [2]Department of Analytical Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Krakow, Kraków, Poland; [3]Department of Medicinal Chemistry, Maj Institute of Pharmacology, Polish Academy of Sciences, Kraków, Poland

Although many atypical proteinaceous cell wall components that belong to a group of multitasking, "moonlighting" proteins, have been repeatedly identified in numerous pathogenic microorganisms, their novel extracellular functions and secretion mechanisms remain largely unrecognized. In *Candida albicans*, one of the most common fungal pathogens in humans, phosphoglycerate mutase (Gpm1) – a cytoplasmic enzyme involved in the glycolysis pathway – has been shown to occur on the cell surface and has been identified as a potentially important virulence factor. In this study, we demonstrated tight binding of *C. albicans* Gpm1 to the candidal cell surface, thus suggesting that the readsorption of soluble Gpm1 from the external environment could be a likely mechanism leading to the presence of this moonlighting protein on the pathogen surface. Several putative Gpm1-binding receptors on the yeast surface were identified. The affinities of Gpm1 to human vitronectin (VTR) and fibronectin (FN) were characterized with surface plasmon resonance measurements, and the dissociation constants of the complexes formed were determined to be in the order of 10^{-8} M. The internal Gpm1 sequence motifs, directly interacting with VTR (aa 116-158) and FN (aa 138-175) were mapped using chemical crosslinking and mass spectrometry. Synthetic peptides with matching sequences significantly inhibited formation of the Gpm1-VTR and Gpm1-FN complexes. A molecular model of the Gpm1-VTR complex was developed. These results provide the first structural insights into the adhesin function of candidal surface-exposed Gpm1.

Keywords: *Candida albicans*, phosphoglycerate mutase, moonlighting proteins, extracellular matrix, vitronectin, fibronectin

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✉e-mail: andrzej.kozik@uj.edu.pl

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Abbreviations: BSA, bovine serum albumin; CWP, cell wall proteins; ECM, extracellular matrix; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; FN, fibronectin; GPI, glycosylphosphatidylinositol; Gpm1, phosphoglycerate mutase 1; Gpm1-Bt, biotin-labelled Gpm1; Gpm1-F, fluorescein-labelled Gpm1; HaCaT, an immortalized human keratinocyte line; HUVEC, human umbilical vein endothelial cells; PAI-1, plasminogen activator inhibitor-1; IPTG, isopropyl-β-D-thiogalactopyranoside; LC-MS/MS, liquid chromatography-coupled tandem mass spectrometry; micro-RDA, micro-representation difference analysis; MLV, moloney murine leukemia virus reverse transcriptase; NHS, N-hydroxysuccinimide; PBS, phosphate buffered saline; PCR, polymerase chain reaction; R-Gpm1; recombinant phosphoglycerate mutase 1; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SA-HRP, streptavidin-horseradish peroxidase adduct; SPR, surface plasmon resonance; TMB, 3,3′,5,5′ tetramethylbenzidine; Tpi, triosephosphate isomerase; TFIIIC, transcription factor IIIC; VTR, vitronectin

INTRODUCTION

The yeast-like fungus *Candida albicans* is a commensal inhabitant of the skin and mucous membranes of most healthy individuals in the human population but also one of the most common causes of fungal infections (Guinea, 2014; Ricotta *et al.*, 2021). Candidal infections can proceed in various forms, from relatively harmless surface lesions to multi-organ and systemic candidiasis, the latter often difficult to cure and thus directly life-threatening. A large body of evidence has proven that the pathogenic nature of this yeast is primarily a consequence of significant impairment of the host immune mechanisms (Bongomin *et al.*, 2017; Ho *et al.*, 2021).

The outermost layer of candidal cells is the cell wall. As a dynamic structure, it is continuously remodelled in response to changes in environmental conditions (Sosinska *et al.*, 2011; Vialás *et al.*, 2012; Garcia-Rubio *et al.*, 2020). Among its components, the cell wall proteins (CWP), capable of adhering to host proteins and cells, play a crucial role in colonization of new niches in the host organism and formation of the biofilm structures (Chaffin, 2008; Klis *et al.*, 2009; Höfs *et al.*, 2016; Hoyer & Cota, 2016). Comparative analyses of the proteinaceous components of the cell wall have shown that the adhesion to the host depends not only on strictly dedicated adhesins, covalently bound to the yeast cell wall mainly through glycosylphosphatidylinositol (GPI) anchors, but also on multiple other proteins, more loosely bound to the cell surface and hence considered as "atypical" cell wall components (Chaffin, 2008). Many of them are multitasking proteins that perform major, evolutionarily conserved functions inside the cell, such as critical involvement in major metabolic pathways. Due to their additional roles, manifested once these proteins appear on the cell surface, they are also known as "moonlighting" proteins (Jeffery, 2003; Karkowska-Ku-

leta & Kozik, 2014; Karkowska-Kuleta & Kozik, 2015; Jeffery, 2019; Satala *et al*., 2020). The apparent adhesin functions of numerous moonlighting proteins on the cell surface of pathogenic microorganisms determine their contribution to microbial pathogenensis (for reviews see: Karkowska-Kuleta & Kozik, 2014; Satala *et al*., 2020). Since these proteins do not have a secretion signal, the mechanism of their appearance on the cell surface remains poorly understood. Also, the biological and structural backgrounds of their novel functions at the new location are unsatisfactorily explained. While enolase is the most widely described moonlighting protein in numerous prokaryotic and eukaryotic organisms (Díaz-Ramos *et al*., 2012; Henderson & Martin, 2011), there are also reports of a newly uncovered adhesive function of phosphoglycerate mutase (Gpm1) in pathogenic microorganisms (Poltermann *et al*., 2007; Zhang *et al*., 2014; Lopez *et al*., 2014; Seweryn *et al*., 2015; Ramos-Hegazy *et al*., 2020). A primary function of Gpm1 (EC 5.4.2.11) is to convert 3-phosphoglycerate to 2-phosphoglycerate in the glycolysis pathway and reverse the reaction during gluconeogenesis. In *Streptococcus suis*, Gpm1 was present in the mixture of cell wall proteins and secretory proteins that additionally possessed FN- and type I collagen-binding activity (Zhang *et al*., 2014). A micro-representation difference analysis (micro-RDA) combined with Northern blotting showed that *GPM1* expression increased during biofilm formation by *Staphylococcus aureus*, when compared with planktonic cultures (Becker *et al*., 2001), possibly due to oxygen deprivation in biofilm, but also suggesting a potential role of Gpm in bacterial pathogenicity. Although surface exposure of Gpm1 was not confirmed for another opportunistic bacterium – *Stenotrophomonas maltophilia* – the results obtained for a *GPM1*-deleted mutant with growth kinetics similar to that of the wild-type showed a reduction in the attachment to CF-derived bronchial epithelial cells, and also to a polystyrene surface (Ramos-Hegazy *et al*., 2020), suggesting that Gpm1 has a role in the adhesion phenomenon. Similarly, in *C. albicans* yeast, based on immunodetection analyses, Gpm1 was shown to localize to the cell wall of both, the blastospores and hyphae (Poltermann *et al*., 2007; Vialás *et al*., 2012). After being exposed on the fungal surface, it could interact with components of the alternative complement pathway – factor H and factor H-binding protein-like 1, a component of the fibrinolytic system – plasminogen, and the proteins of plasma kinin-generation system (the contact system) – kininogen, prekallikrein and factor XII (Poltermann *et al*., 2007; Lopez *et al*., 2014; Seweryn *et al*., 2015).

A common strategy of pathogenic microbes for initiating infection is recognition and interaction with the host extracellular matrix (ECM) a highly complex structure that provides many sites for microbial attachment. To date, a recombinant *C. albicans* Gpm1 protein was shown in an ELISA test to bind to human VTR and FN (Lopez *et al*., 2014). Moreover, the use of recombinant Gpm1-coated latex beads and the *GPM1* deletion mutant confirmed the role of this protein in the attachment of yeast to human endothelial cells (HUVEC) and keratinocytes (HaCaT) (Lopez *et al*., 2014). It was also shown that VTR, present on both types of tested cells, while recognized by Gpm1, provided a bridge for yeast interactions with human cells and possibly ECM (Lopez *et al*., 2014).

Since information on the transport of moonlighting proteins to the surface of fungal cells is very limited, in the first part of the hereby reported study we tested one possible mechanism – a re-adsorption of soluble Gpm1 from the external environment to the cell surface. It has been reported that the ability of pathogenic cells to bind ECM components might be an effective mechanism contributing to the increased pathogenicity of *C. albicans* (Klotz, 1994); furthermore, in a previous ELISA-based study, Gpm1 has been indicated as a candidal cell surface partner for the interaction with human VTR and FN (Lopez *et al*., 2014). Therefore, in the second part of this study we characterized for the first time the interactions of Gpm1 with human ECM proteins in physicochemical and structural terms, based on SPR measurements and chemical cross-linking combined with molecular modeling.

MATERIALS AND METHODS

Isolation and purification of Gpm1 from *C. albicans* cell wall

For yeast culturing and CWP isolation, previously described methods were used (Karkowska-Kuleta *et al*., 2017). *C. albicans* ATCC®10231™ strain (American Type Culture Collection, Manassas, VA, USA) was cultured in the YPD medium (1% yeast extract, 2% soybean peptone and 2% glucose) for 16 h at 30°C with shaking (170 rpm), then yeasts were cultured in RPMI 1640 (PAA Laboratories GmbH, Pasching, Austria) for 72 h at 37°C with shaking (170 rpm). CWP were extracted by treating cells with β-1,6-glucanase (TeKaRa Bio Inc., 2 U of enzyme per 1 g of wet cell mass), diluted in McIlvaine buffer (0.2 M disodium hydrogen phosphate in 0.1 M citric acid, 63.2:36.8 (v/v) pH 6.0, with 0.5 M potassium sodium tartrate), for 4 h at 37°C with gentle shaking. Released proteins were separated from cells by three-step centrifugation (3000 rpm for 3 min, 6000 rpm for 6 min, and 10 000 rpm for 10 min). After each step, the supernatant was collected into new eppendorf tubes. Harvested supernatants with a CWP mixture were dialyzed for 48 h against 20 mM Tris buffer pH 8.0 at 4°C, with a buffer change after 24 h. CWP were first separated on a ResourceQ column (GE Healthcare, Uppsala, Sweden) with an ÄKTA Pure protein chromatography system (GE Healthcare), with 20 mM Tris, pH 8.0, as the binding buffer and 20 mM Tris with 500 mM NaCl, pH 8.0, as the elution buffer, in a 20-min linear gradient at a flow rate of 1 ml/min. Gpm1-containing samples were applied onto a gel filtration TSK G3000SW column (Tosoh Bioscience, PA, USA), eluted with 0.1 M Na_2SO_4, 0.1 M NaH_2PO_4, pH 6.7, buffer with a flow rate of 2 ml/min. After both purification steps, the fractions collected were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in the Laemmli system (Laemmli, 1970) with Coomassie Brilliant Blue R-250 staining, and analyzed with liquid-chromatography-coupled tandem mass spectrometry (LC-MS/MS) (see below).

Expression and purification of recombinant *C. albicans* Gpm1

Expression and purification of recombinant Gpm1 (R-Gpm1) of *C. albicans* were carried out based on the method described in our previous article (Satala *et al*., 2021). Collected *C. albicans* cells were suspended in the TRI-Reagent (Sigma Aldrich, St. Louis, MO, USA) and ruptured with the use of glass beads (425–600 μm, Sigma Aldrich) and FastPrep Precellys Evolution device (Bertin Technology, Montigny-le-Bretonneu, France)

with 2 cycles at 45 s and 6.0 rpm. Extraction of *C. albicans* total RNA was performed according to the standard TRI-Reagent protocol. cDNA was obtained by the use of 2 μg RNA, 0.5 μg of oligonucleotide (dT)18 primer and 200 U of moloney murine leukemia virus reverse transcriptase (MLV) (Promega, Madison, WI, USA). Amplification of the *C. albicans GPM1*-coding region was performed by PCR in a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) at specific conditions: 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s, 74°C for 3 min and 74°C for 10 min, with the use of primers: forward 5'GGTGATGATGATGACAAGATGCCAAAGTTAGTTTTAGTTAGACACG3' and reverse 5'GGAGATGGGAAGTCATTATTATTTCTTTTGACCTTGAGCAGCAAC3'. Quality of performed PCR was assessed by 1% agar electrophoresis, and cDNA was purified with Gel-Out Concentrator Kit (A&A Biotechnology, Gdynia, Poland). Released cDNA was further processed with aLICator LIC Cloning and Expression Kit (Thermo Scientific Fischer, Waltham, MA, USA). First, it was treated with T4 polymerase and LIC buffer for 5 min, and then the reaction was stopped with 30 mM EDTA. Next, the pLATE51 vector encoding a 6-His tag N-terminal anchor and enterokinase cleavage site was added to the mixture and incubation was continued at room temperature for 5 min. Transformation was carried out with *E. coli* TOP10 (Thermo Scientific Fischer), followed by a colony PCR, culturing positive colonies and isolation of plasmid DNA with Plasmid Mini Kit (A&A Biotechnology). Then, plasmids with correct inserts verified by Sanger sequencing were used for transformation of Rosetta 2™ (DE3) *E. coli* by the heat-freeze method. Cultures of Rosetta 2 were conducted with the use of antibiotics: ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). After cultures reached OD_{600} 0.6–0.8, expression of Gpm1 was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG), and incubation was continued at 30°C for 3 h with shaking. The cells were collected by centrifugation (6500 rpm, 30 min, 4°C), suspended in a lysis buffer (50 mM NaH_2PO_4, pH 8.0, 10 mM imidazole, 300 mM NaCl, 20% glycerol) and freezed. The lysis of *E. coli* cells was performed by sonication to release all of the proteins and centrifuged (13 500 rpm, 30 min, 4°C) to harvest supernatants. Supernatants were purified with Ni-NTA Sepharose High Performance affinity matrix at 4°C with four buffers: lysis buffer (50 mM NaH_2PO_4, pH 8.0, 10 mM imidazole, 300 mM NaCl, 20% glycerol), two wash buffers (50 mM NaH_2PO_4, pH 8.0, 20 mM imidazole, 1 M NaCl, 20% glycerol; and 50 mM NaH_2PO_4, pH 8.0, 20 mM imidazole, 300 mM NaCl, 20% glycerol), and elution buffer (50 mM NaH_2PO_4, pH 8.0, 250 mM imidazole, 300 mM NaCl, 20% glycerol). Selected fractions were dialyzed against 20 mM Tris, 50 mM NaCl, 250 mM imidazole, 20% glycerol, pH 8.0, followed by removal of His-Tag by addition of enterokinase (10 U/μg) and incubation for 16 h at room temperature. The obtained non-tagged protein was purified with Mono Q HR 5/5 1 ml column (GE Healthcare) with an ÄKTA Pure system at a flow rate of 0.5 ml/min in a linear gradient between 50 mM NaH_2PO_4, 100 mM NaCl, pH 8.0, and 50 mM NaH_2PO_4, 1 M NaCl for 130 min.

Analysis of binding of biotin- and fluorescein-labelled Gpm1 to *C. albicans* cell surface

For obtaining biotin- and fluorescein-labelled Gpm1 (Gpm1-Bt and Gpm1-F, respectively), R-Gpm1, pre-dialyzed against 0.1 M bicarbonate buffer, pH 8.3, was treated with biotin N-hydroxysuccinimide ester (1 mg/100 μl of dimethylformamide) (Sigma-Aldrich) or fluorescein N-hydroxysuccinimide ester (1 mg/100 μl of dimethyl sulfoxide) (Thermo Fisher Scientific), with a 20-fold molar excess of the labelling reagent. After 4 h (Gpm1-Bt) or 1 h (Gpm1-F) incubation at 4°C, the labelled proteins were dialyzed against phosphate buffered saline (PBS) pH 7.4, for 48 h with four buffer changes, at 4°C.

C. albicans cells were cultured in the YPD medium for 16 h at 30°C with 170 rpm shaking, then washed cells were suspended in RPMI 1640 and placed in wells of a MaxiSorp 96-well plate (Sarstedt, Nümbrecht, Germany) (10^6 cells per well) for Gpm1-Bt experiment, or on a special glass-like polymer 96-well plate (CellVis, Mountain View, CA, USA) (10^4 cells per well) for Gpm1-F, and cultured at 37°C for 4 h. In the next step, cells (in the pseudohyphal forms) were washed with 1% bovine serum albumin (BSA) in PBS (used for washes between each step), and then wells were blocked with 3% BSA in PBS for 1 h at 37°C. Subsequently, washed wells were treated with 50 μl of labelled proteins and incubated for 1.5 h at 37°C. Then, the wells with bound Gpm1-Bt were incubated with streptavidin-horseradish peroxidase (SA-HRP, MP Biomedicals) for 1 h at room temperature in the dark. After final washing, the amount of bound protein to candidal cell surface was determined based on SA-HRP/TMB (Sigma Aldrich) detection by addition of 50 μl of 3,3',5,5'-tetramethylbenzidine (TMB). The reaction was stopped with 50 μl of 2 M HCl, followed by absorbance measurements at 450 nm (Rapala-Kozik *et al.*, 2008). In experiments with Gpm1-F, after incubation with this protein, the wells were washed and observed under an Olympus IX73 microscope.

Yeast-like cells were obtained as described above, but culturing in RPMI 1640 was omitted. Cells, washed three times with PBS, were placed in eppendorf tubes (3×10^7 cells per tube). The blocking step was skipped; a removal of background signal was performed by tube change before TMB addition. Cells were incubated with 50 μl of Gpm1-Bt for 1.5 h at 37°C with gentle shaking, then washed three times with PBS, pH 7.4. Tube contents were treated with 50 μl of SA-HRP (1:4000 in PBS, pH 7.4) and incubated at room temperature for 1 h in the dark. After washing, cells were transferred to new eppendorf tubes to which 50 μl of TMB were then added to start the enzymatic reaction.

Identification of Gpm1-binding candidal cell surface proteins

Identification of surface proteins that apparently interact with Gpm1 was performed by chemical crosslinking, following methods previously described (Kozik *et al.,* 2015). A photoactivatable, cleavable crosslinker – sulfo-succinimidyl 2-([4,4'-azipentanamido]ethyl)-1,3'-dithiopropionate (sulfo-SDAD) (Thermo Fisher Scientific) was used. Sulfo-SDAD (0.5 mM) was incubated with 30 μg of Gpm1-Bt in 100 μl of PBS for 2 h in the dark at 4°C. After stopping the reaction with 50 mM Tris pH 7.4, the sample was dialyzed against PBS, pH 7.4, at 4°C overnight to remove excess of the crosslinker. *C. albicans* CWP were added to crosslinker-bearing Gpm1-Bt, followed by sample incubation for 1.5 h at 37°C with gentle shaking. Next, the sample was exposed to UV light (365 nm) (6 W, Vilber Lourmat) for 15 min. The samples containing CWP-Gpm1-Bt adducts were incubated with MagnaBind Streptavidin Beads M280 (Thermo Fisher Scientific) for 1 h at room temperature

with shaking. A control sample was prepared by addition of CWP to washed streptavidin beads. Unbound and non-biotinylated proteins were removed by washing five times with PBS, pH 7.4. The dissociation of bound CWP were conducted by boiling at 95°C for 30 min in 30 µl of 2.5% β-mercaptoethanol and 2% SDS. Boiled samples were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Visualized bands of bound proteins were excised, extracted and analyzed by LC-MS/MS, using a method described in detail below.

Mapping the interaction sites between Gpm1 and VTR or FN molecules

Identification of interaction sites between human ECM proteins and Gpm1 was performed based on our previous publication (Satala *et al.,* 2021). Experiments began with incubations of 0.5 mM sulfo-SDAD with Gpm1 (30 µg) and either 30 µg of VTR (R&D Systems, Minneapolis, MN, USA) or 30 µg of FN (Sigma Aldrich) in 100 µl of PBS in the dark for 2 h at 4°C. The order of incubation of Gpm1 or ECM protein with the crosslinker determined which protein could be mapped for spots involved in the other protein-binding. If ECM proteins were first incubated with sulfo-SDAD, the Gpm1 molecule was probed for binding sites. In contrast, to map the binding sites on the ECM protein, initial incubation of the crosslinker with Gpm1 was necessary. Thus, after incubation of the first protein with sulfo-SDAD in the dark, the reaction was stopped with 50 mM Tris and the sample was dialyzed against PBS at 4°C overnight in the dark. Still in the dark, the second protein was added, followed by sample incubation for 1.5 h at 37°C with gentle shaking. The sample was then exposed to UV light (365 nm) for 15 min and then dialyzed against 25 mM ammonium bicarbonate buffer (NH_4HCO_3) at 4°C overnight. To cleave the crosslinker arm, 10 mM $CaCl_2$ and 5 mM dithiothreitol were added to the sample, followed by incubation at 60°C for 1 h. The next incubation proceeded for 45 min with 0.1% iodoacetamide in the dark at room temperature. Proteins were then hydrolyzed by trypsin (10 ng/µl in 25 mM NH_4HCO_3, pH 8.0) at 37°C overnight. The reaction was stopped with 7 µl of 2 M HCl for 10 min on ice, and then the sample was evaporated for 2 h, re-dissolved in 10% acetonitrile with 0.1% formic acid and analyzed by LC-MS/MS as is described below.

Analysis of protein-protein interactions by surface plasmon resonance (SPR) measurements

Proteins were immobilized on CM5 chips of the BIACORE 3000 system (GE Healthcare) as previously described (Karkowska-Kuleta *et al.,* 2017). Briefly, immobilization of Gpm1 was performed *via* amine groups using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS) and ethanolamine (Amine Coupling Kit, GE Healthcare) in an acetate buffer at pH 4.0, at 25°C with a flow rate of 10 µl/min. The resonance units (RU) of bound R-Gpm1 and Gpm1 reached about 300. Human ECM proteins were dialyzed against 10 mM HEPES buffer with 150 mM NaCl, pH 7.4. Injections of ECM proteins at various concentrations over immobilized Gpm1 were carried out at a 30 µl/min flow rate, with the association and dissociation times of 180 sec. The level of bound ECM proteins to Gpm1 was obtained by SPR signal expressed in RU. A regeneration of the CM5 chip was conducted by the addition of 1 M HCl with a flow rate of 30 µl/min for 30 sec.

Peptide inhibition of VTR- or FN-binding with Gpm1

Selected peptides were tested for inhibition of the Gpm1-ECM protein interactions in an ELISA-like competitive microplate-based ligand binding assay with final SA-HRP/TMB detection. In experiments on VTR, Gpm1 (10 pmoles per well) was immobilized in the wells of a MaxiSorp 96-well plate (Sarstedt) overnight, at 4°C. For study of FN, the human protein was immobilized in the microplate (2 pmoles per well). The first step after protein immobilization was washing wells with 1% BSA in PBS, pH 7.4, and blocking unoccupied binding sites within the well with 3% BSA in PBS, pH 7.4. The wash solution (1% BSA in PBS, pH 7.4) was used three times before each experimental step. Blocking of wells lasts 1.5 h at 37°C. Then, to the washed wells, a mixture of protein and the competitive peptide was added. In the Gpm1-VTR experiment, the added protein was 100 nM biotin-labelled VTR (final concentration). In contrast, the FN-covered plate was incubated with Gpm1-Bt (at a final concentration of 150 nM). Peptides were added to wells at various amounts per well. The plates were incubated for 1.5 h at 37°C. Finally, 50 µl of SA-HRP (1:4000 in PBS, pH 7.4) were added, followed by microplate incubation for 1 h at room temperature in the dark. The binding level was calculated based on the SA-HRP/TMB detection.

Identification of proteins and mapping interaction sites by mass spectrometry

The protocol for identification of the electrophoretic band contents was based on a modification of previously described method (Karkowska-Kuleta *et al.,* 2017). In the first step, bands, excised from the electrophoresis gel, were washed twice with 25% acetonitrile in 25 mM NH_4HCO_3, and twice with 50% acetonitrile in 25 mM NH_4HCO_3. Next, to reduce the disulfide bonds, bands were incubated with 10 mM dithiothreitol in 25 mM NH_4HCO_3 at 37°C for 45 minutes, and then with 50 mM iodoacetamide in 25 mM NH_4HCO_3 for 1.5 h at room temperature in the dark for sulfhydryl groups' alkylation. Preparation of samples for trypsin digestion required double wash with 50% acetonitrile, followed by incubation in 100% acetonitrile for 10 min and drying of bands under fume hood for 15 min. Proteins in the gel bands were hydrolyzed with 13 µl of trypsin (Promega) (0.1 µg/10 µl in 25 mM NH_4HCO_3) overnight at 37°C. Afterwards, peptides were released to solution by sonication and dryed in SpeedVac. Dried samples were dissolved in 10% acetonitrile with 0.1% formic acid and analyzed by LC-MS/MS. Peptides were separated on Aeris 3.6 um PEPTIDE XB C18 column (Phenomenex) in 10–60% gradient of 0.1% formic acid in 80% acetonitrile with a flow rate of 0.2 µl/min in an ultrahigh-performance liquid chromatography Dionex Ultimate 3000 system (Carlsbad, CA, USA) coupled with a HCT Ultra ion-trap mass spectrometer equipped with an electrospray ionization ion source and an electron-transfer dissociation II fragmentation module (Bruker, Bremen, Germany). Results were analyzed with the Mascot server (v.3.0, Matrix Science, London, UK).

Further steps were made based on a previously described method (Satala *et al.,* 2021). In brief, samples for interaction-site mapping were suspended in 10% acetonitrile with 0.1% formic acid and analyzed with the same mass spectrometer as mentioned above. After LC-MS/MS analysis, the obtained data were checked for sequence coverage and score with Swiss Prot database for both, the human and candidal proteins. The site-map-

ping based on the closest region to anchored crosslinker was obtained by Mass Matrix PC server Version 4.3 (Xu & Freitas, 2009)

Molecular modelling of the interaction between *C. albicans* Gpm1 and human VTR

Molecular modelling was performed in accordance with a previously described method (Satala *et al.,* 2021). Briefly, the *C. albicans* Gpm1 crystal structure (SWISS-MODEL repository accession number: P82612) and the VTR structure (uniport ID: P04004, vitronectin V65 subunit) previously obtained with the Prime software (Schrödinger Release 2019-1, Schrödinger LLC, New York, NY) were docked using ClusPro 2.0: protein-protein docking software (Boston University), the server version of which is available at https://cluspro.bu.edu. The resulting fungal protein-human protein complex was analyzed by comparing the distances between experimentally selected Gpm1 amino acid residues and the docked VTR using PyMOL Molecular Graphics System software (version 1.7.2.1; Schrödinger, LLC).

RESULTS

Exposition of multiple moonlighting proteins on candidal cell surface has been demonstrated by many research group (Chaffin, 2008; Karkowska-Kuleta & Kozik, 2015; Jeffery *et al*., 2019; Satala *et al*., 2021), but the mechanisms of non-classic transport of these cytoplasmic proteins to the cell surface remain mysterious. In this study we present data supporting a hypothesis that significant amounts of moonlighting proteins, such as Gpm1, occur at the infections foci in a fluid phase due to release from extracellular vesicles and/or cytoplasm leakage from damaged or dead cells, and subsequently adsorb on the surface of living cells and moonlight there as supplementary adhesins. Such a mechanism has been recently suggested for candidal enolase (Karkowska-Kuleta *et al*., 2021). Experiments reported here were carried out on both, isolated natural Gpm1 and R-Gpm1, obtained as electrophoretically pure preparations (Supplementary Fig. S1 at https://ojs.ptbioch.edu.pl/index.php/abp/). First, we used biotin-labelled fungal protein and the SA-HRP/TMB system to detect the association of exogenous Gpm1 with living candidal cells (Fig. 1). Both, the yeast-like cells (3×10^7 per tube) and the filamentous forms (generated from 10^6 cells per well), were found to bind Gpm1 in a ligand concentration-dependent manner (Figs 1A and 1B, respectively), the latter to a capacity higher by two orders of magnitude than the unicellular form. To confirm these findings, we also performed fluorescence-microscopic observations of live filamentous forms of *C. albicans* after coating with Gpm1-F (Fig. 1C).

The next step in current characterization of Gpm1 re-adsorption onto candidal cell surface aimed at identification of specific CWP that possibly serve as docking platforms for Gpm1. Samples containing R-Gpm1 and the mixture of CWP were subjected to crosslinking with sulfo-SDAD – a photoactivatable, heterobifunctional cross-linker with cleavable disulfide bond in its long arm. Putative Gpm1 receptors identified by this method are listed in Table 1. Multiple moonlighting proteins are on this list, with the highest score noted for phosphoglycerate kinase and elongation factor 2. Additionally, we observed peroxiredoxin, transketolase, 60S ribosomal protein L10a, pyruvate kinase and enolase. The existence of the latter on candidal cell surface was confirmed in

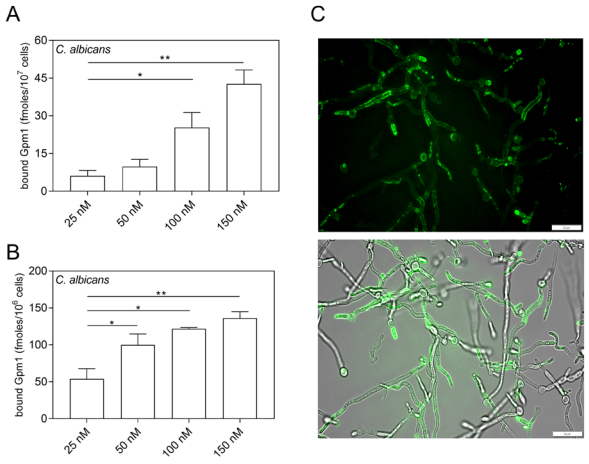

Figure 1. Binding of purified Gpm1 to the surface of the yeast-like and filamentous forms of *C. albicans* cells.
C. albicans blastospores (**A**) and hyphae (**B**) were incubated with Gpm1-Bt at a concentration range of 25-150 nM in PBS, pH 7.4, at 37°C for 1.5 h. After washing out the unbound protein, the bound Gpm1 was determined with the SA-HRP/TMB system. Statistical significance levels were determined using Student's t test and are marked with * for $p<0.05$ and ** for $p<0.01$. (**C**) *C. albicans* filamentous forms, obtained from 10^4 yeast-like cells cultured for 3 h in the wells of glass microplates in RPMI 1640 at 37°C, were incubated with 50 µl of fluorescein-labeled purified Gpm1 (100 nM) for 1 h at 37°C in PBS buffer, pH 7.4. After removal of unbound Gpm1, cells were visualized in transmitted light (bottom panel) and green fluorescence (top panel) using an Olympus IX73 microscope.

many reports (Martin *et al.,* 2015; Gil-Bona *et al.,* 2018; Karkowska-Kuleta *et al.,* 2015; Lee *et al.,* 2014; Vialás *et al.,* 2012). Moreover, typical cell surface proteins, such as Als3 – a member of the agglutinin-like sequence protein family, and cell wall protein Rbt1 were also found in our analysis. Interestingly, one of the proteins identified, multifunctional adhesin Als3, has been previously identified as a molecule that mediates binding of another moonlighting protein – enolase to the candidal cell wall (Karkowska-Kuleta *et al*., 2021). A low identification score for typical adhesins in comparison with moonlighting proteins can result from the particle size and limitations of the method used. Chemical crosslinking requires the availability of reactive sites on the molecule, which in the case of classic proteins of the cell wall is problematic due to strong glycosylation and hydrophobic nature (de Groot *et al.,* 2013). Besides, the chemical crosslinking experiments suggested that Gpm1-Gpm1 interactions, with a high score in Table 1, might be a kind of stacking of these protein molecules on the cell surface. The raw data from these experiments are presented in supplementary Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/.

The presence of Gpm1 on the cell wall of *C. albicans* cells implies some moonlighting functions of this protein on the candidal surface. It has been proven that many moonlighting proteins bind human ECM, an activity important for a successful invasion of the host (Gründel *et al.,* 2016; Kozik *et al.,* 2015; Satala *et al.,* 2020). In the current study, SPR analyses showed a strong interaction of Gpm1 with ECM proteins (Fig. 2). For both Gpm1 forms, the resonance signal increased with concentration of the ECM protein, injected over the CM5 chip surface with immobilized Gpm1. The equilibrium dissociation constants (*KD*) for the interactions were found to be comparable between both forms of the fungal protein and were in the order

Table 1. Identification of *C. albicans* cell wall proteins that putatively bind candidal Gpm1.
Results were obtained by crosslinking between R-Gpm1-Bt and the mixture of *C. albicans* CWP, followed by LC-MS/MS analysis.

SwissProt protein database accession	Protein name	Molecular mass (kDa)	Sample score
PGK_CANAL	Phosphoglycerate kinase	45.27	867
EF2_CANAL	Elongation factor 2	93.87	513
PMGY_CANAL	Phosphoglycerate mutase	27.44	448
METE_CANAL	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	85.76	371
TSA1B_CANAL	Peroxiredoxin TSA1-B	21.96	353
ALS3_CANAL	Agglutinin-like protein 3	124.31	346
SAHH_CANAL	Adenosylhomocysteinase	49.67	230
G6PI_CANAL	Glucose-6-phosphate isomerase	61.15	222
TKT1_CANAX	Transketolase	73.84	210
RL10A_CANAL	60S ribosomal protein L10a	24.48	204
NNRD_CANAL	ATP-dependent (S)-NAD(P)H-hydrate dehydratase	42.10	137
ENO1_CANAL	Enolase 1	47.20	114
SODM_CANAX	Superoxide dismutase [Mn], mitochondrial	26.21	91
KPYK_CANAL	Pyruvate kinase	55.75	90
RBT1_CANAL	Cell wall protein RTB1	74.49	68

of 10^{-8} M, while some variations in the rate constants for various fungal protein-ECM protein pairs were noted (Table 2).

Crosslinking with sulfo-SDAD was also used to map the sites on fungal and human proteins that seem to be involved in these protein-protein interactions. The R-Gpm1-VTR and R-Gpm1-FN were crosslinked and analyzed by LC-MS/MS. The internal motifs of Gpm1, involved in VTR binding included the following sequences: aa 61-80, aa 116-136, aa 138-158 (Table 3). From the VTR side, the aa 354-367 sequence was probably critical for interaction with Gpm1. For binding of FN, Gpm1 was suggested to engage the aa 138-175 motif. Its N-terminal 11-amino acid sequence (aa 138-158) was identical to that involved in VTR binding, indicating that the regions of Gpm1 molecule contributing to the binding of both ECM proteins significantly overlap. On FN, two Gpm1-binding sequences – aa 904-922 and aa 1117-1129 – were identified. All fitting parameters obtained by the MassMatrix analysis sum-

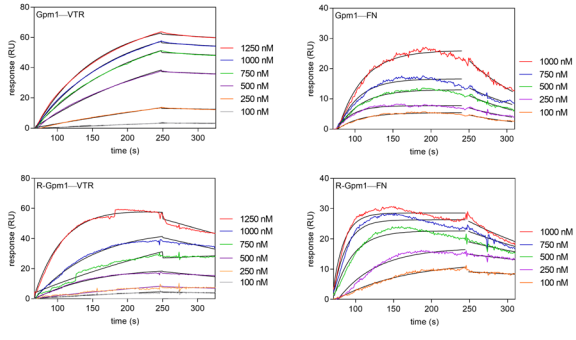

Figure 2. SPR sensograms for interactions between Gpm1 and human VTR or FN.
The upper panels show the results for purified Gpm1 and the bottom panels for R-Gpm1. Sensograms were obtained after injection of VTR or FN at a concentration within a range of 100–1250 nM and at a flow rate of 30 µl/min over a CM5 chip containing Gpm1, immobilized to a level of 300 RU.

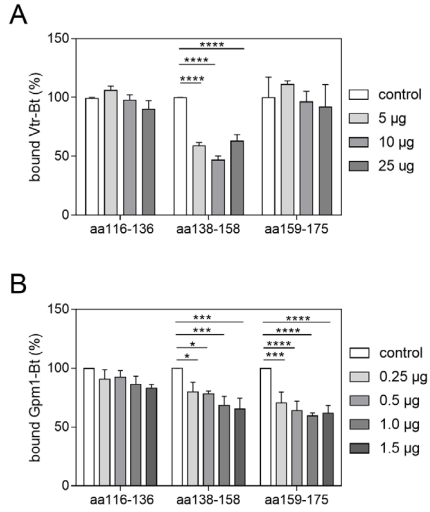

Figure 3. Effects of Gpm1-derived synthetic peptides on binding of VTR (A) or FN (B) to *C. albicans* R-Gpm1.
(**A**) The wells of a 96-well microplate were coated with 10 pmoles of R-Gpm1 at 4°C overnight. Peptides with specified Gpm1-derived amino acid sequences were added at amounts of 5, 10 and 25 µg per well with VTR-Bt (100 nM) in 50 µl of PBS. (**B**) The wells were coated with 2 pmoles of FN at 4°C overnight. Gpm1-derived peptides at amounts of 0.25, 0.5, 1.0 and 1.5 µg per ml were added with R-Gpm1-Bt (150 nM) in 50 µl of PBS. In both cases, a peptide-free well served as a control. Statistical significance levels were determined using Student's t test and are marked with * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$ and **** for $p<0.0001$.

Table 2. Kinetic and thermodynamic parameters for the interactions of human VTR or FN with candidal Gpm1, determined by SPR measurements.
The binding parameters for interactions between *C. albicans* Gpm1 and human VTR or FN were obtained after fitting the data with a 1:1 Langmuir binding model.

Fungal protein	*ka* (1/Ms)	*kd* (1/s)	*KD* (M)
VTR-binding			
C. albicans Gpm1	$1.83x10^{4}\pm2.20x10^{3}$	$4.99x10^{-4}\pm4.99x10^{-5}$	$2.73x10^{-8}\pm2.35x10^{-9}$
C. albicans R-Gpm1	$1.24x10^{5}\pm3.89x10^{3}$	$2.70x10^{-3}\pm7.66x10^{-5}$	$2.18x10^{-8}\pm6.05x10^{-9}$
FN-binding			
C. albicans Gpm1	$8.23x10^{4}\pm6.91x10^{3}$	$1.66x10^{-3}\pm9.89x10^{-5}$	$2.02x10^{-8}\pm1.08x10^{-9}$
C. albicans R-Gpm1	$1.92x10^{5}\pm7.65x10^{3}$	$6.38x10^{-3}\pm5.65x10^{-4}$	$3.32x10^{-8}\pm7.36x10^{-9}$

Table 3. Mass spectrometry identification of internal sequence motifs in *C. albicans* Gpm1 and human VTR and FN molecules involved in R-Gpm1-ECM protein interactions.
Peptides were identified by cross-linking analyses of the binding between recombined yeast Gpm1 and human proteins.

Peptides of *C. albicans* R-Gpm1	
VTR-binding	FN-binding
$_{61}$AIQTANIALDAADQLYVPVK$_{80}$ $_{116}$SFDVPPPKIDPKDEYSQVGDR$_{136}$ $_{138}$YADVDPAVVPLTESLALVIDR$_{158}$	$_{138}$YADVDPAVVPLTESLALVIDR$_{158}$ $_{159}$LLPYWQDEIAGDLLAGK$_{175}$
R-GpmPeptides of ECM proteins	
C. albicans Gpm1-binding VTR peptides	*C. albicans* Gpm1-binding FN peptides
$_{354}$IYISGMAPRPSLAK$_{367}$	$_{904}$SDTVPSPRDLQFVEVTDVK$_{922}$ $_{1117}$LGVRPSQGGEAPR$_{1129}$

marized above are presented in supplementary Table S2 at https://ojs.ptbioch.edu.pl/index.php/abp/.

To further verify the results obtained by the crosslinking method, synthetic peptides with sequences matching those of VTR- and FN-binding Gpm1 motifs listed above were tested for possible inhibitory effects on Gpm1 binding to both ECM proteins (Fig. 3). The results of this set of experiments strongly confirmed that aa 138-158 peptide is involved in interaction with both, the VTR and FN. Addition of 80 pmoles (10 μg) per well of aa 138-158 peptide resulted in 60% decrease of VTR-Bt binding, and a 40% decrease of FN binding was observed after addition of 12 pmoles (1.5 μg) of this peptide per well. In this competitive binding assay, we were not able to confirm comparable effects of aa 116-136 and aa 159-175 peptides on VTR binding to Gpm1, while the latter peptide strongly, down to 40%, displaced FN from the complex with Gpm1. Similar tests with the aa 61-81 peptide showed no displacement effect for both ECM proteins (not shown).

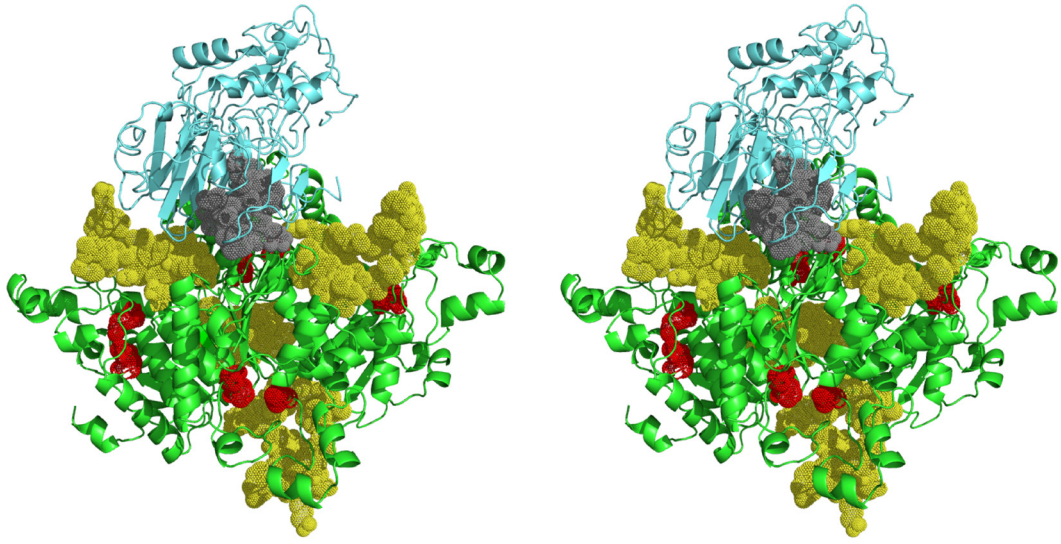

Figure 4. Proposed model of the interaction between *C. albicans* Gpm1 (green) and human VTR (cyan).
The Gpm1 and VTR peptides identified in the chemical cross-linking experiments are indicated in yellow and gray, respectively. The residues of the catalytic center of Gpm1 are indicated in red. The 3D molecular model is presented in a wall-eyed stereo view.

Peptides proposed by chemical crosslinking were fitted into the interaction model of *C. albicans* Gpm1. Based on performed analysis, two peptides (aa 116-136, aa 138-158) appeared to be involved in a direct interaction with VTR (Fig. 4). In the case of the third peptide (aa 61-80), for which an involvement in the interactions with human proteins could not be confirmed in the inhibition tests with synthetic peptides, this fragment is seen to be involved in interaction between the Gpm1 subunits. In the presented molecular model, the active site residues of the catalytic center in the tetrameric form of Gpm1 were also indicated. Both interaction sites with human protein are distant from the Gpm1 catalytic center, indicative of undisturbed enzymatic activity of Gpm1 during VTR-Gpm1 interaction.

DISCUSSION

The primary intracellular function of Gpm1 is to catalyze the eighth reaction in the glycolysis pathway. A lack of this enzyme in model organisms, such as *S. cerevisae,* results in inhibition of growth and development, and disturbs gene transcription (Dumay-Odelot *et al.,* 2007; Manaud *et al.,* 1998). Studies on *S. cerevisiae* mutants with deletion of *GPM1* demonstrated a difficulty in the transcription of factor IIIC (TFIIIC) DNA, associated with the polypeptide τ55, which is partially composed of Gpm1 (Dumay-Odelot *et al.,* 2007; Manaud *et al.,* 1998; Rigden, 2008). Lack of τ55 or Gpm1 results in instability of the TFIIIC complex structure and in inability to bind DNA. Additionally, *C. albicans* TFIIIC is known to contain polypeptide τ55 in its structure and requires Gpm1 for proper function (Dumay-Odelot *et al.,* 2007).

In recent years, numerous studies have demonstrated the presence of Gpm in the cell wall of many microorganisms, including such bacteria as *S. aureus*, *Lactobacillus* spp. and *Stenotrophomonas maltophilia* (Becker *et al.*, 2001; Pérez Montoro *et al.*, 2018; Ramos-Hegazy *et al.*, 2020), and yeast-like fungi such as *C. parapsilosis*, *C. tropicalis*, *C. glabrata* and *S. cerevisae* (Karkowska-Kuleta *et al.,* 2019; Kozik *et al.,* 2015; Motshwene *et al.,* 2003). Nevertheless, the mechanisms of its emergence on the cell surface have not yet been satisfactorily investigated. In our current study, we observed a concentration-dependent deposition of externally added labelled Gpm1 on the surface of living candidal cells, supporting a hypothesis that *in vivo* Gpm1 may appear on the fungal surface due to re-adsorption of the external soluble protein, constantly present in the surroundings, to some proteinaceous receptors on the living fungal cells. Similar observations have been reported for another moonlighting protein – enolase from *C. albicans* and *Streptococcus pneumoniae* (Karkowska-Kuleta *et al.,* 2021; Bergmann *et al.,* 2001). Studies on *S. aureus* grown under silver-ion stress conditions have shown that the appearance of cytoplasmic proteins outside the cell can be a result of leakage or controlled release (Smith *et al.*, 2013). As has been demonstrated in other proteomic studies, *C. albicans* and *C. glabrata* Gpm1 were variably regulated during response to oxidative stress (Ramírez-Quijas *et al.,* 2015). The authors suggested that Gpm1 is required for their survival in the presence of reactive oxygen forms (Ramírez-Quijas *et al.,* 2015). These findings support a hypothesis that the appearance of many moonlighting proteins on the microbial cell surface is not just accidental, but may be a part of cellular response to changes in the external environment. During colonization and infection of the host organism, one of the major mechanisms to cause changes in the external environment for invading microbes is the influx of the host immune cells to the site of infection. An important part of the host immunity is also activation of the complement system. Two human factors – H and FHL-1 – are responsible for preventing the complement system from activating in an alternative pathway. Activation of this path results in pore production through the microbial cell membrane, disrupting and killing the pathogen cell (Harpf *et al.,* 2020). Previous studies demonstrated that *C. albicans* Gpm1 was a factor H- and FHL-1 binding protein, which inhibited activation of the alternative pathway of the complement system (Poltermann *et al.*, 2007). For bacteria, it was shown that *S. aureus* cells could respond to silver ion stress conditions with overexpression of proteins involved in the central metabolism, such as Gpm1, virulence and stress response, followed by leakage or release of cytoplasmic proteins (Smith *et al.*, 2013). Pore formation, leakage of cytoplasmic content and eventually death was observed for *C. albicans* during silver ions stress (Kim *et al.,* 2009). Possibly, similar phenomena underlie the Gpm1 appearance on the candidal cell surface, consequently suggesting that Gpm1's ability to bind various host proteins can help microbes to survive and effectively counteract the host defense during infection. Except for such a hypothetical defense role, another major function of Gpm1 on the microbial cell surface should be contribution to adhesion to the host cells and proteins, as was presented for many bacterial species, for example the lactobacilli. Bacteria from *Lactobacillus* spp. mainly colonize the urogenital tract, at a low pH environment. Gpm1 was detected on the *Lactobacillus* cell surface and was shown to be an adhesion supporting factor under those unfavorable conditions (Pérez Montoro *et al.*, 2018). Thus, *GPM1*-deficient mutants presented a definitely lower adhesion ability in acidic environments. Moreover, Gpm1 and an elongation factor were responsible for autoaggregation of *Lactobacillus* cells, making them much more resistant to harsh conditions (Pérez Montoro *et al.*, 2018). In our current study, one of the proteins identified on the candidal cell surface was elongation factor 2 – another moonlighting protein that apparently binds Gpm1. These results suggested that in *C. albicans* cells, Gpm1 can be a significant factor that supports adhesion at low pH, a hypothesis especially attractive as the urogenital tract is one of the host niches vulnerable to *C. albicans* infections (Achkar & Fries, 2010).

SPR analysis presented in this study allowed us to compare the Gpm1 isolated from the surface of *C. albicans* with its recombinant version, in terms of kinetic and thermodynamic parameters for interaction with representative host targets – ECM proteins, such as VTR and FN. To our knowledge, this is only a second study after our previous work on enolase (Satala *et al.*, 2020) to compare two forms of a moonlighting protein for differences in the potency of interaction with host ligands. In the conducted experiments, we did not notice any significant differences in these terms; the estimated dissociation constants for both studied Gpm1 forms were in a 10^{-8} M order. Similar analyses, based on SPR measurements, were carried out for *C. albicans* Gpm1 and the proteins of the plasma kinin-generating system (the contact system) – kininogen, prekallikrein and factor XII, and the determined *KD*s for these candidal protein – human protein complexes were in the order of 10^{-7} M (Seweryn *et al.,* 2015). These results suggest that Gpm1 present on the surface of *C. albicans* can bind a variety of host ligands with similar strength, thus undeniably contributing to pathogen adhesion to the host.

VTR and FN occur not only in the ECM structure, but also on the various cell surfaces and in the plasma. VTR can be involved in the hemostasis processes, adhesive cells' function and inhibition of the complement system and plasminogen activation (Schvartz *et al.,* 1998). This 75 kDa monomeric glycoprotein is comparable in size with the Gpm1 tetramer (110 kDa). The interaction of VTR with candidal Gpm1, although known for considerable time as a phenomenon (Gründel *et al.,* 2016; Kozik *et al.,* 2015; Lopez *et al.,* 2015), has not yet been elucidated in more detailed structural aspects. Our cross-linking experiment showed a specific VTR peptide (aa 354-367) that probably touches Gpm1 in the complex of these two proteins. This sequence motif is located in the VTR C-terminal domain with significant homology to hemopexin, and overlaps with the sites of interaction with other important ligands, such as heparin (aa 348-361) and plasminogen activator inhibitor 1 (PAI-1) (aa 361-371) – an important regulator of plasmin formation and, thus, fibrinolysis (Schvartz *et al.,* 1999; Voss *et al.,* 2013). The major high-affinity site for PAI-1 binding is localized in the N-terminal somatomedin B domain (aa 1-39) (Schar *et al.,* 2008; Schvartz *et al.,* 1999) which, however, was not found to interact with Gpm1. Studies on *S. aureus,* in which one of the cell wall components binds to a similar spot on VTR, suggested that VTR can cover the bacterial cell surface and thus inhibit activation of the complement system (Voss *et al.,* 2013). Thus, *C. albicans* cells can get many benefits from being covered with this host protein, not only by defending their colonies but also by hiding from the host immune system.

Another host protein, analyzed hereby for Gpm1 binding, was FN – a 440 kDa dimeric glycoprotein, four times larger than the Gpm1 tetramer. In the FN structure, one can find binding sites for many ligands, such as heparin, fibrin and collagen (Krzyzanowska-Golab *et al.*, 2007). Also, FN possesses cell surface receptor binding domains (Krzyzanowska-Golab *et al.,* 2007). Each chain of FN contains repeated motifs of three types, designated I, II and III, with many glycosylation sites, limiting the protein susceptibility to protease actions (Chen *et al.,* 2009). Hence, it is uncertain whether our cross-linking experiments, followed by trypsin fragmentations, could fully map the sites of FN interactions with Gpm1. Nevertheless, we found putative Gpm1-binding sites (aa 904-922 and aa 1117-1129) in the type III domains 4 and 6, respectively. It should be also mentioned that FN is one of the proteins with a complicated intrinsic disorder nature, especially in fibronectin type III (Peysselon *et al.*, 2011). All of these FN features, as well as the current lack of information on the actual spatial structure of the above selected domains, precluded a molecular modelling of FN-Gpm1 interactions.

The structure of Gpm1 is well known, but molecular analyses, aimed at locating binding sites for various ligands have not been performed so far. One study showed a possible important function of ATP in stabilizing the structure and dimerization of the Gpm1 molecule (Chang *et al.,* 2012; Gardner *et al.,* 2017). Our study on the interactions between Gpm1 and ECM proteins revealed possible interaction sites at motifs aa 116-158 for VTR and aa 138-175 for FN, with aa 138-158 sequence binding to both host proteins. For the Gpm1-VTR complex, a molecular model was successfully developed, with one VTR molecule bound to the Gpm1 tetramer and the sequence motifs identified on Gpm1 (aa 116-136, aa 138-158) and VTR (aa 354-367) facing together, far away from the residues of the catalytic center.

One important area of research on the moonlighting proteins is to consider them for new disease detection methods. Schistosomatosis, one of the most severe parasitic diseases, is caused by *S. japonicum*, *S. mansoni*, *S. bovis* and *S. haematobiumis*. It was shown that in these parasites Gpm1 appears on the cell membrane and is probably responsible for the host-parasite interactions (Zhang *et al.,* 2015). Experiments with buffalos considered the most crucial intermediate hosts for transient schistosomiasis and showed promising results for detecting this parasite using the surface-exposed schistosome Gpm1 (Zhang *et al.,* 2015). In *C. albicans*, Gpm1 was shown to be immunoreactive during human invasive fungal infection and is now considered as a potential diagnostic marker (Pitarch *et al.,* 2011).

In conclusion, in this study, we presented for the first time the tight binding of *C. albicans* Gpm1 to the cell surface of this yeast-like fungus, thus suggesting that the readsorption of exogenous soluble Gpm1 could be likely a mechanism of the appearance of this moonlighting protein on the pathogen surface. Moreover, using SPR measurements, the interactions of Gpm1 with human ECM proteins – VTR and FN – were confirmed but also characterized in terms of kinetic and equilibrium constants, which have not been previously reported. For the first time, using chemical crosslinking and mass spectrometry, structural analysis of the complexes formed was performed, indicating sequence motifs directly interacting with each other in the Gpm1-VTR and Gpm1-FN complexes. Finally, a molecular model of Gpm1-VTR complex was developed. The current detailed description of interaction between Gpm1 and ECM proteins contributes to a better understanding of the role of moonlighting proteins in the host-pathogen interaction during candidal infections.

Competing interests

The authors declare no conflicts of interest.

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