Polish Journal of Microbiology
2015, Vol. 64, No 3, 203–210

ORIGINAL PAPER

Role of *SAP7-10* and Morphological Regulators (*EFG1*, *CPH1*) in *Candida albicans*' Hypha Formation and Adhesion to Colorectal Carcinoma Caco-2

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Submitted 16 August 2014, revised 25 January 2015, accepted 13 April 2015

Abstract

Secreted aspartic proteases (Saps) are considered as key virulence factors of *Candida albicans*. Hopefully our outlook will widen the knowledge of *SAP7*'s role in *C. albicans* pathogenesis. The goal of our study was to investigate *SAP7* expression during *C. albicans* adhesion to intestinal human cells. Another objective was to study the role of *SAP8-10* and transcriptional regulators: *EFG1* and *CPH1,* using the mutants: *Δsap*, *Δefg1*, *Δcph1* during growth on Caco-2 monolayer. *SAP7* expression was analyzed using real time RT-PCR; relative quantification was normalized against *ACT1* in cells after growth on Caco-2. Adherence assay of *C. albicans* to Caco-2 was performed in a 24-well-plate. The results proved that *SAP7* can play a role during the initial adaptation of *C. albicans* to intestinal tract and decreases over time. Up-regulation of *SAP7* occured in the absence of *SAP8* and *SAP10* (genetic alternations dependence). *SAP7* can be regulated by the morphogensis' regulators during *C. albicans* growth on epithelium. Adhesion of the mutants was indistinguishable from SC5314. The lack of neither *SAP8-10* nor *EFG1*/*CPH1* influences the adhesive behaviour of *C. albicans*. Deletion of *SAP8-10* resulted in no filamentation defects. The results help better understand the role of *SAP7* during adhesion and morphogenesis in *C. albicans*.

Key words: *Candida albicans*, adhesion, *SAP7* gene expression, secreted aspartyl proteinase Sap7-10, true hyphae

Introduction

The incidence and severity of candidiasis in immunocompromised or otherwise debilitated hosts, coupled with diagnostic difficulties and the high cost of treatment, have persuaded several investigators to focus on *Candida albicans* which is by far the most prevalent etiological agent of candidiasis. The most characteristic features of *C. albicans* are its extraordinary range of virulence factors, particularly adherence and spread on and/or through epithelial and endothelial tissues by inducing the filamentous growth and hyphal-associated aspartic proteases (Saps). Adherence to host tissues and morphological versatility are thought to be important in *C. albicans* virulence (Bertini *et al.*, 2013; Braga-Silva and Santos, 2011; Naglik *et al.*, 2011). *C. albicans* contains the hypha-associated genes: *EFG1* and *CPH1* that mediate adhesion of *C. albicans* to mucosal surfaces (Moazeni *et al.*, 2012; Lo *et al.*, 1997). Efg1 and Cph1 play a major role in promoting filamentous growth and regulate the expression of several genes with a crucial function in the invasion of host cells or in biofilm formation (Moazeni *et al.*, 2012; Staniszewska *et al.*, 2013). As adhesion and morphogenesis are crucial for the latter, it is fundamental to study the role of *EFG1* and *CPH1* associated with morphogenesis and adhesion during *C. albicans* epithelial cells colonization. The secreted aspartic protease (Sap) family encompasses at least ten members (Saps1-10) containing a signal peptide, which are secreted, except for Sap9 and Sap10 that remain bound to the cell wall. Individual *SAP* genes are expressed at various stages of the infection process (Correia *et al.*, 2010; Dalle *et al.*, 2010; Jackson *et al.*, 2007; Martin *et al.*, 2011; Pietrella *et al.*, 2013). Saps are characterized by a broad-spectrum proteolytic activity and virulence properties. Sap1-3 are active at acidic pH, whereas Sap4-6 are more active at neutral to slightly alkaline pH and are associated with hyphal growth (Aoki *et al.*, 2011; Cadicamo *et al.*, 2013; Naglik *et al.*, 2011). Saps directly attack and degrade host proteins that are involved in both innate as well as adaptive immunity (for example, complement, E-cadherin, histatins, antibodies),

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and development of inflammations (Aoki *et al.*, 2011; Cassone and Cauda, 2012; Mayer *et al.*, 2013; Pietrella *et al.*, 2013). Although the combined role of Sap1-6 in virulence has now come into question, and the Sap9 and Sap10 isoenzymes' role in the cell surface integrity, cell separation, and adhesion, has been described (Albrecht *et al.*, 2006; Schild *et al.*, 2011), almost nothing is known of Sap7 and Sap8 competence (Taylor *et al.*, 2005). Therefore, we presented our outlook in the hope that it will lead to widening knowledge of the role of *SAP7-10* in *C. albicans* pathogenesis.

The goal of the present study was to investigate the aspartic protease *SAP7* gene expression during *C. albicans* adhesion to intestinal human cells. A further objective was to study the role of *SAP8-10* and of the transcriptional regulators: *EFG1* and *CPH1*, using the mutants: *Δsap*, *Δefg1*, *Δcph1* during growth on the Caco-2 monolayer. We tested the ability of the *Δsap8-10* mutants to form hyphae and to adhere to intestinal cells comparing them with the mutants: Δ*cph1* and *efg1* (attenuated in morphogenesis).

Experimental

Materials and Methods

Strains and Media. *C. albicans* strains used in the current study are listed in Table I (Lo *et al.*, 1997; Fonzi and Irwin, 1993; Gillum *et al.*, 1984; Liu *et al.*, 1994; Puri *et al.*, 2012; Schild *et al.*, 2011; Staniszewska, 2009). *C. albicans* (strain no 82) was isolated from blood samples from a patient treated for *Ependymoma anaplasticum* (Staniszewska, 2009; Staniszewska *et al.*, 2014a; 2014b). The clinical isolate had been previously identified according to colony colour on CHROMagar *Candida* medium and evaluated following API 20C AUX carbohydrate assimilation patterns (Staniszewska, 2009; Staniszewska *et al.*, 2012; Staniszewska *et al.*, 2014a; 2014b). Additionally, the identification procedure was confirmed with genetic methods using rDNA sequencing as previously described (Staniszewska, 2009; Staniszewska *et al.*, 2012; Staniszewska *et al.*, 2014b). All the strains used in the present study were stored on ceramic beads in Microbank tube (Prolab Diagnostics, Richmond Hill, ON, Canada) at –70°C. Prior to the respective examinations, routine culturing of strains for growth was conducted at 30°C for 18 h in YEPD (Ness *et al.*, 2010).

Cultivation and Infection of Caco-2 Cell Line (ATCC HTB27, LGC, Poland). Following the supplier's guidelines, monolayers of the colon adenocarcinoma derived cell line were cultured in the Eagle's Minimum Essential Medium (EMEM) containing 10% (v/v) FCS, 1mM pyruvic acid, without antibiotics or antifungal agents and maintained in a humidified incubator at 37°C in 5% (v/v) CO_2. For the experiment 1.2×10^5 of Caco-2 cells (/ml EMEM) were seeded into 24-well-plates (Corning, USA) and cultured up to 18 h. Next, after 18 h post seeding the Caco-2 monolayers

Table I
C. albicans strains used in this study

Strain	Parental strain	Relevant characteristics or genotype	Reference
Reference strains*			
SC5314	none	Prototrophic wild-type strain	(Gillum *et al.*, 1994)
CAI4	SC5314	*ura3Δ::imm434/ura3Δ::imm434*	(Fonzi and Irwin, 1993)
CAF2-1	SC5314	*ura3Δ::imm434/URA3*	(Fonzi and Irwin, 1993)
no 82	none	Prototrophic wild-type strain	(Staniszewska, 2009)
sap8Δ, sap9Δ, sap10 Δ and sap9/10 Δ mutants**			
Δsap8		*Δsap8::hisG/ Δsap8::hisG-URA3-hisG*	(Puri *et al.*, 2012)
Δsap9	CAI4	CAI4, *sap9Δ::hisG/sap9Δ::hisG* + pCIp10 (integration)	(Schild *et al.*, 2011)
Δsap10	CAI4	CAI4, *sap10Δ::hisG/sap10Δ::hisG* + pCIp10 (integration)	(Schild *et al.*, 2011)
Δsap9/10	CAI4	CAI4, *sap10Δ::hisG/sap10Δ::hisG sap9Δ::his/sap9Δ::hisG* + pCIp10 (integration)	(Schild *et al.*, 2011)
efg1Δ and *cph1Δ* mutants**			
Δcph1	CAI4	*ura3::1imm434/ura3::1imm434 cph1::hisG/cph1::hisG-URA3-hisG*	(Liu *et al.*, 1994)
Δcph1 (*CPH1*)	CAI4	*ura3::1imm434/ura3::1imm434 cph1::hisG/cph1::hisG(CPH1)*	(Lo *et al.*, 1997)
Δefg1	CAI4	*ura3::1imm434/ura3::1imm434 efg1::hisG/efg1::hisG-URA3-hisG*	(Lo *et al.*, 1997)
Δefg1 (*EFG1*)	CAI4	*ura3::1 imm434/ura3::1 imm434 efg1::hisG/efg1::hisG (EFG1)*	(Lo *et al.*, 1997)
Δcph1Δefg1 (*EFG1*)	CAI4	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG (EFG1)*	(Lo *et al.*, 1997)

* apart from indicated features all strains are identical to their parental strain; ** full genotype

were inoculated with 10^5 log phase yeast cells (/ml EMEM) of *C. albicans* wild type and mutants. After 18 h of incubation the Caco-2 was lysed by adding sterile water resulting in recovery of *C. albicans* cells.

RNA Isolation, cDNA preparation and quantification. Total RNA from *C. albicans* cells was extracted as described by Amberg *et al.* (2005). Prior to further examinations *C. albicans* total RNA was stored at –20°C. RNA was reverse transcribed into first-strand cDNA using the Enhanced Avian HS RT-PCR kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. Briefly, total reaction volume (10 µl) contained 1 µl of total RNA, and 1 µl of oligo $(dT)_{23}$ (3.5 µM), and 1 µl of dNTP mix (500 µM each dNTP), and 7 µl of water (nuclease-free) was prepared. Incubation step was carried out at 50°C for 10 min. Then subsequently, the remaining components: 1 µl of Enhanced avian AMV-RT (1 U/µl), 1 µl of 10 × buffer for AMV-RT, 8 µl of water (nuclease-free) were added to obtain 20 µl of final volume. The RT reaction was carried out at 50°C for 50 min. cDNA was quantified using the QuantiTect Taq-Man probe RT-PCR kit (Qiagen, Germany) according to the manufacturer's instructions. Appropriate Taq-Man primer and probe sets for *SAP7* (*SAP7-1* 5'-ATGGACACAGTGTGAAATATGAAGTG-3'; *SAP7-2* 5'-TCAGTGGAGGATGGACCATTAGA-3') and *ACT1* (ACT-1 5'-GACAATTTCTCTTTCAGCACTAGTAGTGA-3'; ACT-2 5'-GCTGGTAGAGACTTGACCAACCA-3') were designed as described previously by Naglik *et al.* (2008). For the real time RT-PCR analysis each reaction mixture contained 1 × RT-PCR buffer, 4 mM $MgCl_2$, 250 nM forward and reverse primer, 200 nM Taq-Man probe, HotStarTaq DNA polymerase, and template RNA (Naglik *et al.*, 2008). To validate our normalization we determined differences in the *SAP7* expression levels between *C. albicans* cells grown for 3 h and 18 h respectively on the Caco-2 cell line. Moreover, for reliable normalization of the *SAP7* gene expression data in *C. albicans* cells grown for 3 h and 18 h respectively we used the housekeeping gene *ACT1* as a reference gene. The real time RT-PCR reactions were performed as described previously by Naglik *et al.* (2008): at 95°C for 15 min, followed by 45 cycles of 15 s at 94°C and 1 min at 60°C with the LightCycler® 96 (Roche Diagnostics GmbH, Germany). The C_T values were provided from real time RT-PCR instrumentation and were imported into a spreadsheet Microsoft Excel 2010. The relative quantification was calculated using Eq. (Livak and Schmittgen, 2001), where

$$\Delta C_T = \text{Avg. } SAP7\ C_T - \text{Avg. } ACT1\ C_T;$$
$$\Delta\Delta C_T = \Delta C_T - \Delta C_{T\ \text{parental strain}} = 2^{-\Delta\Delta CT}.$$

Assay of adherence to human line Caco-2 epithelial cells. Adherence of *C. albicans* to the Caco-2 cell line (ATCC HTB-37™) was performed as described previously Hashash *et al.* (2011). Briefly, the Caco-2 cell line was cultivated in the EMEM containing 10% (v/v) FCS at 37°C at 5% (v/v) CO_2. After trypsinisation, with the use of 0.25% trypsin (Biomed-Lublin, Lublin, Poland) 1.2×10^5 Caco-2 cells (/ml EMEM) were incubated for at least 16 h on a 24-well-plate (Costar, Corning, NY, USA) to generate a confluent layer. Subsequently, the blastoconidia were grown overnight in the YEPD medium at 30°C. Then, 10^4 blastoconidial cells (/ml saline) were added to each well of the epithelial cells to be afterwards incubated for 90 min (adhesion phase). Next, the non-adherent cells were removed by standard rinsing, and the wells were overlaid with Sabouraud dextrose agar and allowed to solidify. After 18 h growth at 37°C, the number of adherent cells was determined by colony counting and compared with the controls on the Sabouraud dextrose agar plates. Adherence was expressed as a percentage of the total number of cells added (control cells).

Microscopy. The morphological transition from yeast-to-hyphal cells was performed by using LEXT 3D Measuring LASER Microscope OLS4000 (Olympus, USA) and Scanning Electron Tabletop Microscope TM 100 (Hitachi, Japan). Briefly, Caco-2 cells grown on 12 mm glass coverslips were inoculated for 21 days post seeding with 10^4 log phase yeast cells (/ml saline) of strains tested. After 90-min incubation at 37°C, the cells were washed three times with PBS to remove non-adherent yeast and then fixed in 2% glutaraldehyde for 10 min.

Statistical analysis. Each experiment was performed in triplicate on three separate occasions. The percentage of cell adhesion and the *SAP7* expression were formulated as a mean ± standard deviation. Statistical differences were evaluated through comparison with the Wilcoxon test, P values ≤ 0.05 were considered significant. Based on recently published data (Naglik *et al.*, 2008; Staniszewska *et al.*, 2014a), the latter method was used to calculate statistical differences between relative gene expression and cell adhesion.

Results

The *SAP7* gene expression during adhesion to the Caco-2 mMonolayer. The level of *SAP7* transcript normalized to the transcription of *ACT1* is presented in Table II. The expression of the *SAP7* gene in the cells of the parental strain CAF2-1 and Δ*sap8* decreased 1.7- and 1.3-fold respectively after 18-h growth on Caco-2. Conversely, in the cells of Δ*sap8*, *SAP7* mRNA increased 3.6- and 4.8-fold after 3-h and 18-h growth on the intestinal cells respectively compared to the parental strain CAF2-1. Moreover, the expression of *SAP7* decreased 2.3- and 1.1-fold in the cells of Δ*sap9*,

Table II
Analysis of the *SAP7* gene relative expression compared to the *ACT1* reference gene in *C. albicans* cells. The cells were grown on Caco-2 cell line at 37°C

C. albicans	$\Delta C_{(t)}$		$2^{-rrC(t)}$	
	3 h	18 h	3 h	18 h
SC5314	–0.07 ± 0.92	0.05 ± 1.15	[1]	[1]
CAF2-1	–0.53 ± 0.54	–1.23 ± 0.74	1.02 ± 0.63	0.60 ± 0.40
CAI4	–1.61 ± 0.40	–1.60 ± 1.20	1.10 ± 1.30	0.43 ± 0.35
Δ*cph1*	2.04 ± 0.60	0.90 ± 0.15	2.53 ± 1.40	1.10 ± 0.52
Δ*efg1*	–0.43 ± 1.02	–1.24 ± 0.11	0.73 ± 0.24	0.85 ± 0.23
Δ*sap8*	0.90 ± 1.75	0.50 ± 1.22	3.70 ± 3.00	2.90 ± 3.02
Δ*sap9*	–1.44 ± 2.44	–0.36 ± 1.34	1.48 ± 2.35	0.63 ± 0.48
Δ*sap10*	–3.03 ± 7.43	–0.29 ± 2.03	1.70 ± 1.60	1.68 ± 1.10
Δ*sap9/10*	–0.85 ± 2.40	–0.21 ± 1.45	1.45 ± 1.23	1.34 ± 1.50

$C_{(t)}$ – mean for three independent experiment ± SD; [1] strain SC5314 – calibrator in $2^{-\Delta\Delta C(t)}$

and Δ*sap9-10* respectively after 18-h growth on Caco-2. On the other hand, the *SAP7* mRNA slightly increased 1.1-fold in the null Δ*sap10* mutant. In the cells of the parental strain CAI4, *SAP7* decreased 2.5-fold after 18-h growth on Caco-2. After 3-h growth on Caco-2, *SAP7* was up-regulated in Δ*sap9*, Δ*sap10*, and Δ*sap9-10* (1.3-, 1.5- and 1.3-fold as compared respectively to the parental strain CAI4). After 18-h growth, *SAP7* was up-regulated in Δ*sap9*, Δ*sap10*, and Δ*sap9-10* (1.4-, 3.9-, and 3.1-fold as compared respectively to the parental strain CAI4). We showed that after 18-h growth, Δ*cph1* cells showed *SAP7* decreased 2.3-fold, while Δ*efg1* displayed a slight up-regulation of this gene (1.2-fold). The expression of *SAP7* on the line Caco-2 at 3 h post-inoculation was higher than in 18-h grown cells. The obtained results proved that *SAP7* can play a role during the initial adaptation of *C. albicans* to the intestinal tract and that it decreases over time.

Determination of *C. albicans* adhesion to Caco-2 monolayer. To study whether the genetic alternations found in *SAP* and *EFG1* and *CPH1* genes could have an impact on *in vitro* virulence, we investigated the adherence of the *C. albicans* null mutants in a model of epithelial cells, and compared them with that of the wild type strains: SC5314 and no 82 as well as the rescued strains. As depicted in Table III, the adherence ability varied within each species with the values from 0.533 ± 0.321 to 11.3 ± 10.324 for the wild type strains: SC5314 and no 82, and from 0.68 ± 0.593 to 11.8 ± 7.532 for the mutants. A significant trend toward an affected adhesion of morphogenesis mutants was noted by comparison with the wild type isolate no 82 ($P \leq 0.05$). Our results demonstrated that lack of Cph1 protein influence significant reduction in their adhesive ability ($P \leq 0.05$) compared to the wild type strain no 82. With *Δcph1* adhesion was significantly lower (16.6-fold) than with *Δefg1* (2.1-fold) compared to the wild type strain no 82. In opposition, the SC5314 and *Δcph1* strains showed almost similar adhesion, whereas *Δefg1* revealed 9.85-fold increase in adhesive mode compared to the parental strain (SC5314). In this particular case of *Δcph1*, reintroduction of the one copy of *CPH1* restored adhesion. Thus the presence or absence of *CPH1* affected the adhesiveness behaviour of *C. albicans* cells.

The adhesion properties depended on the morphogenesis mutants (compared to the wild type strain 82) while for the mutants *Δsap9* and *Δsap10* almost the same level of adhesion was observed ($P \geq 0.05$). Moreover, the mutant *Δsap8* adhered less efficiently to epithelial cells (6.2-fold reduction) compared to strain no 82. In the case of the *Δsap8* mutant, adhesion of the latter strain was reduced to statistically significant levels ($P \leq 0.05$). Moreover, when strain SC5314 was analyzed, there were no detectable differences in its percentage of adhesion in comparison to the mutants ($P \geq 0.05$,

Table III
Adherence of *C. albicans* morphologies in an *in vitro* model of intestinal candidiasis (monolayer of Caco-2 cell line ATCC). Data are expressed as the mean ± SD of three independent experiments

Strains 10^6 cells (ml saline)$^{-1}$	Percentage of cells adhesion
SC5314	0.533 ± 0.321
no 82	11.30 ± 10.32
Δsap8	**1.83 ± 1.55**
Δsap9	9.92 ± 8.10
Δsap10	11.80 ± 7.53
Δcph1	**0.68 ± 0.59**
Δcph1 (*CPH1*)	**2.60 ± 1.84**
Δefg1	**5.25 ± 6.72**
Δefg1 (*EFG1*)	**1.01 ± 1.11**
Δcph1Δefg1 (*EFG1*)	**1.45 ± 0.35**

Significant reduction of adhesive properties ($P \leq 0.05$) in bold (compared to the wild type strain no 82)

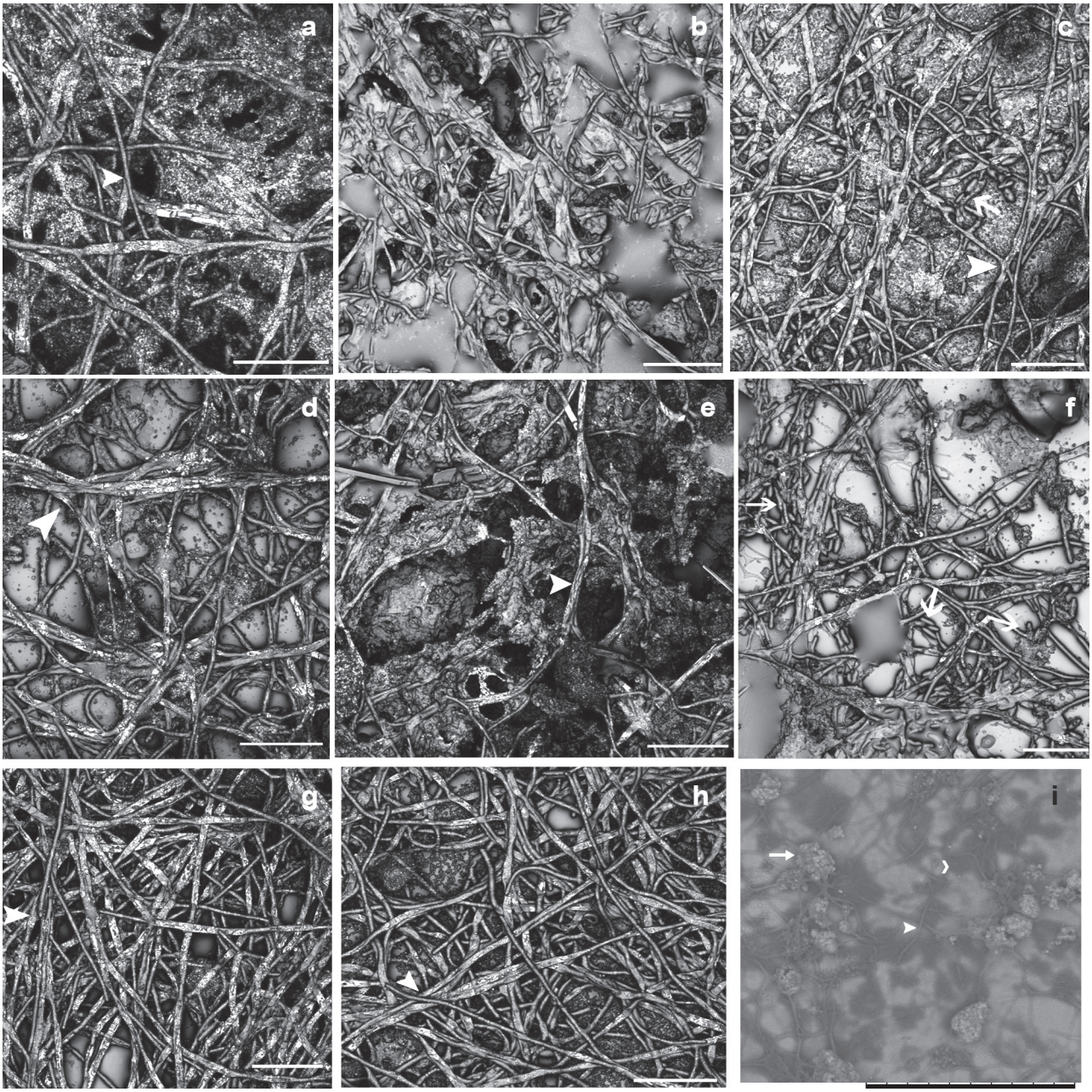

Fig. 1. Microscopic view of *C. albicans* strains hyphae production after 90-min incubation on Caco-2 monolayer at 35°C (5% CO_2).

(a) SC5314 presents true hyphal forms on the cell line surface (arrowhead). (b) The double Δ*cph1*/Δ*efg1* mutant containing an integrated copy of *EFG1* and (c) the Δ*efg1* mutant with one copy of *EFG1* display abundant true hyphae formation (arrowhead). In the case of the latter elongated blastoconidial cells can be seen (open arrow). (d) The Δ*cph1* mutant and (e) The Δ*cph1* strain reintroduced with one copy of *CPH1* show true hypha formation (arrowheads). (f) The Δ*efg1* mutant displays true hyphal forms and abnormally elongated blastoconidia (open arrows). (g) The strains: Δ*sap9* and (h) Δ*sap10* as well as (i) Δ*sap8*, display abundant hyphae formation (arrowheads) as well as conglomerate of morphologies (arrow) on enterocyte monolayer (open arrowhead). (a-h) Cells were imaged using LEXT 3D Measuring LASER Microscope OLS4000, bars 20 μm. (i) Cells were imaged using Scanning Electron Microscope TM100, bar 100 μm

Table III). In this regard, all the tested *C. albicans* strains were able to adhere to the epithelial cell line to a different degree.

***C. albicans* morphology on the intestinal Caco-2 monolayer.** Electron microscope studies revealed a, clearly reduced capacity of hyphal growth of the mutant *Δefg1* in comparison with the wild type strain SC5314 on enterocyte monolayer (Fig. 1). As shown microscope micrographs, following an 90-min attachment phase of yeast cells, we observed that all fungal strains had switched to hyphal growth form. The Δ*efg1* mutant latter strain showed morphologies tended to be slightly distorted compared to those of the wild type strain. Microscopically, no differences in the Δ*sap8-10* mutants' morphogenesis potential during adhesion to Caco-2 monolayer were observed after 90 min, indicating epithelial adherence and possibly cell proliferation.

Discussion

As far it is known (Cadicamo *et al.*, 2013; Correia *et al.*, 2010; Dalle *et al.*, 2010; Jackson *et al.*, 2007; Martin *et al.*, 2011; Naglik *et al.*, 2003; 2008; Taylor *et al.*, 2005), the increased expression of specific Saps at various stages of the infection plays a special role in tissue invasion. As the role of *SAP7* in *C. albicans* virulence had remained unknown, we showed in our study, that the level of the *SAP7* expression correlates with the importance of this gene for the early stage of the Caco-2 intestinal tissues invasion. It is worth noting, that differences in the *SAP7* expression between *C. albicans* cells colonizing Caco-2 depend on genetic alternations. These results showed clearly that the up-regulation of *SAP7* occurs in the absence of *SAP8* and *SAP10*. The compensation for the absence of Sap10 activity by Sap7 demonstrated their similar function. As showed Bocheńska *et al.* (2013) these two Saps out of 10 isoenzymes are unable to release bactericidal peptides from human Hb. As Sap10 participated in the processing of cell wall proteins (Schild *et al.*, 2011), the function of Sap7 was speculated and needed experimental verification. Our results perfectly agree with the previous study concerning Sap7 activity at neutral pH. We demonstrated (Staniszewska *et al.*, 2014a) that *SAP7* may help the fungus to cause systemic infections. We also showed that morphogenesis factors can be regulators of *SAP7* at early stage of epithelial infection. As desribed Pierce and Kumamoto (2012), Efg1 is a transcritional regulator of a large number of genes and many differences in the gene expression are suspected to occur in response to changes in the *EFG1* expression. Moreover, according to the latter authors, in the human gastrointestinal (GI) tract, wild type cells and cells with low Efg1 activity are expected to express factors that allow them to interact with host epithelial cells. We proposed that the *SAP7* activity provides a mechanism which allows the colonizing of human cells. As showed Nobile *et al.* (2012) *C. albicans* is one of the very few fungal species that can efficiently form biofilms in healthy mammalian cells. Following Nobile *et al.* (2012), we suggested that *SAP7* is involved in the biofilm network in *C. albicans* as a 'young' gene engaged in an early organisation of biofilm (adhesion to the intestinal monolayer). On the other hand, *C. albicans* hydrolytic enzymes might be regulated differently during various experiments and what is more, these genes' expression is strain-specific. Lermann and Morschhäuser (2008) showed that Saps are not required for the invasion of reconstituted human epithelia (RHE) by *C. albicans*. Therefore, environmental conditions have an important impact on the *SAP* expression pattern in *C. albicans*. Moreover, the proteinase encoded by the *SAP7* gene, if translated, may be associated with *C. albicans* early infection and is quite different from laboratory culture conditions (Cadicamo *et al.*, 2013; Taylor *et al.*, 2005).

C. albicans possesses a remarkable capacity to adhere to tissues (Dalle *et al.*, 2010; Yan *et al.*, 2013). Moreover, adhesion is influenced by enhanced expression and production of Saps (Albrecht *et al.*, 2006; Braga-Silva and Santos, 2011; Dalle *et al.*, 2010; Martin *et al.*, 2011; Seabra *et al.*, 2013). In our *in vitro* model of epithelial infection, Δ*sap8-10* and morphogenesis mutants displayed adherence to Caco-2 monolayer, where hyphae germinated. We characterized the morphology of the two morphogenesis mutants and four Δ*sap* compared to the wild type by CLSM, using the Caco-2 cell line. The strains proliferated, underwent morphogenesis and caused epithelial cells lysis. Although deletion of *CPH1* reduces hyphal growth on solid medium (Tsai *et al.*, 2013), we showed that it still forms hyphae during adhesion to a polarized monolayer of Caco-2 epithelial cells (Fig. 1). In stark contrast, *Δefg1* was slightly attenuated in developing filaments (Fig. 1). While, the extent of adhesion was strain-depended, a general trend was observed among all the strains tested, confirming a slightly reduced adhesion ability of the morphogenesis mutants, with *Δsap9* and *Δsap10* strongly adhering to the epithelial cells, followed by the wild type strain no 82 (Table III). Although, Cph1 appears to contribute to adhesion (*Δcph1* was reduced in this capacity, Table III), other factors, particularly agglutinin-like sequence (*ALS*) are the major contributor to epithelial cells adhesion (Tsai *et al.*, 2013). As discussed Brand (2012), deletion of one member of *SAP*s effected on the compensation of genes encoding surface proteins that are involved in adhesion i.e., *ALS3*, *HWP1*.

In our study, deletion of *SAP9* resulted in only slightly altered adhesion to Caco-2 compared to the wild type strains (Table III). To be more precise, *Δsap9* was more effective in adhesion than its parental strain SC5314. Strikingly, our data showed that adhesion activity of *Δsap10* was reduced in higher level compared to SC5314. We also found that strains were not adhesion activity correlated, pointing to a strain-dependent phenomenon rather than a significant association between Saps and adhesion. It was demonstrated that the ability of the mutants: Δ*sap,* Δ*efg1,* and Δ*cph1* to adhere to epithelial cells is not altered significantly compared with the wild types. Thus we suggested that a lack of morphogenesis factors as well as Δ*sap8-10* cannot be important for adhesion to the intestinal layer. Furthermore, deletion of the *SAP8-10* genes resulted in no filamentation defects; however these have not been reported to date. Overall, this analysis indicates that unlike SC5314, the significant differences in adhesion of Δ*sap*, Δ*efg1* and Δ*cph1* during the growth on Caco-2 relative to the clinical strain no 82 are not meaningful for this process if the strains genetic backgrounds are

too different. On the other hand, as reviewed by Naglik *et al.* (2011), Sap9 and Sap10 may indirectly contribute to adhesion by targeting covalently linked fungal cell wall proteins such as Cht2, Ywp1, Als2, Rhd3, Rbt5, Ecm33 and Pga4 and glucan cross-linking protein Pir1. Furthermore, the findings of Schild *et al.* (2011) on the *in vivo* expression profile of Saps1-6 supports a role of Sap1-3 in the adherence process to epithelial cells and Sap4 to Sap6 in assisting *C. albicans* cells to evade phagocytosis (Bertini *et al.*, 2013; Braga-Silva and Santos, 2011; Han *et al.*, 2011; Naglik *et al.*, 2011).

Conclusions. To our knowledge, ours is the first study of the role of *SAP7* in the early stage of the colorectal carcinoma Caco-2 invasion In order to benefit from these results in therapy (development of proper anti-fungal compounds or potentially effective combination vaccines including Sap7) additional *in vivo* experiments should be conducted.

Acknowledgements

The work was supported by the grant No DEC-2011/03/D/NZ7/06198 from National Science Center, Kraków, Poland. The authors would like to thank sincerely Prof. B. Hube for the strains CAF2-1, CAI4, *Δsap9*, *Δsap10*, *Δsap9-10*, *Δsap8*. We are extremely grateful to Professor Hsiu-Jung Lo from the National Health Research Institute in Zhunan (Taiwan) for providing us with the following strains: Can16, YLO323, HLC52, HLC74 and HLC84. We are very grateful to the Laboratory of Miniaturized Chemical System (Warsaw University of Technology) for access to confocal and electron microscopes.

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