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# *In vitro* Study of Secreted Aspartyl Proteinases Sap1 to Sap3 and Sap4 to Sap6 Expression in *Candida albicans* Pleomorphic Forms

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Abstract

Transition from round budding cells to long hyphal forms and production of secreted aspartic proteases (Saps) are considered virulence-associated factors of *Candida albicans*. Although plenty of data dealing with Saps involvement in the infection process have been published, Saps expression by the different pleomorphic forms as well as the capacity of *C. albicans* filaments to express Sap1-6 under serum influence are poorly investigated. In this study, we used immunofluorescence and immunoelectron microscopy for the detection of Sap1-6 isoenzymes in *C. albicans* pleomorphic cells (blastoconidia, germ tubes, pseudohyphae, true hyphae) grown in Sap-inductive human serum and Sap non-inductive medium – yeast extract-peptone-glucose (YEPD). Isoenzymes were below the detection level in all blastoconidial cells grown in YEPD for 18 h. Sap1-6 expression was hardly detected in *C. albicans* cells cultivated in serum for 20 min. Increasing level of Sap1-6 expression was observed when *C. albicans* was incubated for 2, 6 and 18 h in serum corresponding to the development of germ tubes, pseudohyphae and true hyphae. The expression of Sap1-3 in pseudohyphae and true hyphae was more intensive compared to Sap4-6. Thus, we could show that human serum induced hyphae formation and the expression of Sap1-6 were co-regulated.

Key words: *Candida albicans*, aspartic protease expression, isoenzymes 1-3, isoenzymes 4-6, morphotypes

## Introduction

The opportunistic fungal pathogen *Candida albicans* possesses a repertoire of virulence attributes including adhesion to host tissue, the ability to undergo reversible morphogenetic transition, the secretion of extracellular hydrolases, and rapid switching between different phenotypic forms (Argimón *et al.*, 2007; Naglik *et al.*, 2008; Dalle *et al.*, 2010; Hayek *et al.*, 2010). Among the hydrolytic enzymes, aspartic proteases (Saps) are considered to be key virulence determinants of *C. albicans* which contribute to the adhesive and invasion capabilities of strains from this species (Raška *et al.*, 2007; Tongchusak *et al.*, 2008; Dalle *et al.*, 2010). Saps are the products of a family of 10 *SAP* genes divided into subfamilies based on amino acid sequence homology alignment (*SAP1* to *SAP3*, *SAP4* to *SAP6*, *SAP9*, and *SAP10*). Furthermore, *SAP7* and *SAP8* are divergent and are not represented as subfamily members (Hube *et al.*, 1994; Monod *et al.*, 1994 and 1998; Chen *et al.*, 2002; Correia *et al.*, 2010). Expression of the *SAP* genes varies according to the type and stage of the disease (Schaller *et al.*, 2001; Fradin *et al.*, 2003; Taylor *et al.*, 2005; Jackson *et al.,* 2007; Correia *et al.,* 2010; Naglik *et al.*, 2008; Abegg *et al.*, 2011). Moreover, *SAP* genes expression is also regulated during the morphological transition (Argimón *et al.*, 2007; Décanis *et al.*, 2011). *Candida albicans* is able to grow in different forms (blastoconidia, germ tubes, pseudohyphae and true hyphae), a phenomenon defined as pleomorphism (Whiteway and Bachewich, 2007). Pleomorphic forms enable *C. albicans* to colonize and invade human tissues (Morrison *et al.*, 2003; Kumamoto and Vinces, 2005a; 2005b; Raška *et al.*, 2007; Barnett, 2008). It was found (Gow *et al.*, 2002) that the

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morphogenetic response (transition from budding to hyphal cells) of *C. albicans* as well as expression of *SAP* genes are triggered by factors existing in the environment of the host (pH, temperature, serum).

Previous studies (Schaller *et al.*, 2000; Felk *et al.*, 2002; Naglik *et al.*, 2003; 2008; Lermann and Morschhäuser, 2008; Gropp *et al.*, 2009; Dalle *et al.*, 2010), indicate that the expression of Sap isoenzymes, for the morphogenesis of *C. albicans* varies strongly, depending on the experimental setup having a significant impact on the dependence on protease activity. Felk *et al.* (2002) showed that *in vivo* (in tissue from infected mice) expression of Sap1-3 was detected on the surface of both yeast and hyphal of wild-type cells. In contrast, the Sap4-6 antigens were identified mostly on penetrating hyphal cells (Schaller *et al.*, 2000; 2001; Copping *et al.*, 2005; Hornbach *et al.*, 2009). Those authors suggested that Sap4-6 are the hyphal-associated proteins which is in striking contrast to the results obtained by Lermann and Morschhäuser (2008), who suggested that none of the SAP1-6 genes is required for invasion of vaginal RHE by hyphal morphologies. Moreover, according to Correia *et al.* (2010) Sap1-6 do not play a significant role in *C. albicans* virulence in a murine model of hematogenously disseminated candidiasis and that, in this model, Sap1-3 are not necessary for successful *C. albicans* infection.

In contrast, previous reports (Naglik *et al.*, 1999; Schaller *et al.*, 2000; Hube, 2004; Hornbach *et al.*, 2009) showed that the Sap4-6 subfamily produced in high level by hyphal cells plays a role in immune evasion and protection from phagocytic killing by murine macrophages. Gropp *et al.* (2009) showed that Sap1-3 degrade and inactivate the central human complement components C3b, C4b as well as C5 and block the damaging effects of the activated complement system.

Although plenty of data dealing with the Saps involved in the infection process have been published the expression of Saps in particular morphotype is not sufficiently described. Given the role of serum in hyphae formation (Lermann and Morschhäuser, 2008; Gropp *et al.*, 2009) and as *SAP1-3* play essential role in the growth of *C. albicans* in medium consisting proteins (Felk *et al.*, 2002; Lermann and Morschhäuser, 2008; Naglik *et al.*, 2008; Gropp *et al.*, 2009) as well as *SAP4-6* are hypha-related genes (Lermann and Morschhäuser, 2008; Naglik *et al.* 2008; Gropp *et al.*, 2009), we asked whether Sap1-3 or Sap4-6 are expressed in each morphotype under human serum influence *in vitro.* In addition, we analyzed whether any differences in expression between these two subfamilies exist. To investigate the expression of the Sap1-3 and Sap4-6 proteins, we studied the media, pH, and temperature shifts. Following to previous conclusion (Hube *et al.*, 1994; Naglik *et al.*, 1999; Wise *et al.*, 2007; Gropp *et al.*, 2009), that different expression profiles of Saps are regulated by pH of the maintenance medium, we studied pH, and temperature shifts during expression of the Sap1-3 and Sap4-6. In contrast to all other members of the Sap family, the proteases Sap9-10 monitored under conditions *in vitro* and *in vivo* are independent of pH and morphotype (Hornbach *et al.*, 2009; Schild *et al.*, 2011). Moreover, the expressions of Sap7 and Sap8 do not correlate with virulence (Hornbach *et al.*, 2009). That is why we did not include Sap7, Sap8, Sap9 and Sap10 respectively in our study.

The aim of this study was to: (i) examine the expression of aspartic proteases (Sap1 to Sap3 and Sap4 to Sap6) *in vitro* in neutral pH during morphogenesis under human serum influence by immunofluorescence and immunoelectron microscopy; (II) establish the relationship between isoenzymes expression and pleomorphism; (iii) determine the localization of Sap1-3 and Sap4-6 in pleomorphic cells of *C. albicans* by immunoelectron microscopy.

## Experimental

### Materials and Methods

**Strains and growth conditions.** The *Candida* strains used in this study are listed in Table I. The clinical isolate of *C. albicans* (strain 82) was recovered from the blood of 3-year-old patient being treated for an anaplastic ependymoma. In the study, we used ATCC SC5314 reference strain to analyse the conceivable differences in Sap1-3 and Sap4-6 expression profile appearing between pleomorphic forms and various *C. albicans* strains. The stock culture of examined strains was stored on ceramic beads (Microbank™, Pro-Lab Diagnostics, Canada) at –70°C. Prior to the respective examinations,

Table I
*Candida* strains used in this study

| Designation | Clone or strain | Relevant characteristics or genotype | Reference |
|---|---|---|---|
| Wild type, clinical isolate | 82 | *URA3*/*URA3* | Staniszewska *et al.* (2011a) |
| Wild type, reference strain | ATCC 5314 | *URA3*/*URA3* | Gillum *et al.* (1984) |
| Wild type, reference strain | ATCC MYA 581 | *URA3*/*URA3* | Sullivan *et al.* (1995) |

routine culturing of strains for growth was conducted at 30°C for 18 h in extract-peptone-glucose broth medium YEPD [10 g yeast extract, 20 g peptone (BBL Trypticase Peptone, Becton Dickinson) and 20 g glucose, pH 5.7] (Ness *et al.*, 2010).

**Phenotypic and biochemical characterization.** The presumptive identification of the clinical strain 82 was conducted using CHROMagar Candida medium (Becton Dickenson, Sparks, MD, USA), as described previously Staniszewska *et al.* (2011b). Colors of the colonies were compared in reference to the *C. albicans* SC5314 strain. The assimilation pattern of the isolate and the reference strains SC5314 was determined using the API 20C AUX identification system (BioMérieux, France). The API 20C AUX test was used according to the manufacturer's instructions. Results of the test were obtained based on the numerical profile read-out (Analytical Profile Index; BioMérieux) (Staniszewska *et al.*, 2011a).

**Molecular examination.** DNA was extracted from blastoconidial cells of the clinical isolate as well as *C. dubliniensis* ATCC MYA 581 and *C. albicans* SC 5314 according to Yeast DNA Miniprep Protocol as described by Amberg and Burke (2005), and DNA quantification was performed using the NanoDrop ND 1000 spectrophotometer at an absorbance of 260 nm. Ribosomal DNA region including a fragment of the 5.8S rDNA gene (GenBank) was amplified by standard PCR. The primers (CALB1, CALB2) were used for species-specific PCR (Luo and Mitchell, 2002). The species-specific PCR products were electrophoresed as previously described Luo and Mitchell (2002). DNA bands were visualized using a transilluminator SYNGENE (Division of Synoptics LTD) under UV 260 nm. Results were documented by using the GenSnap program.

***In vitro* study of aspartic protease enzyme expression.** The expression of Sap isoenzymes in *C. albicans* strain cultivated in YEPD medium and, subsequently, in filtered undiluted human serum was studied. Expression of Sap1-3 and Sap4-6 was monitored in blastoconidial cells, germ tubes, pseudohyphal and true hyphal forms.

**Induction of pleomorphic cells.** Blastoconidial cells were grown as described Staniszewska *et al.* (2011a). Cells were observed under a phase-contrast microscope (Docuval, Carl Zeiss, Germany). Then, blastoconidia were harvested, washed three times with distilled water, pelleted by centrifugation (300 *g* for 10 min), and stored at –70°C for 96 h.

To induce remaining pleomorphic forms the blastoconidial cells suspensions in YEPD (50 µl) were transferred to 500 µl of filtrated undiluted human serum (pH 7.2–7.4) and incubated separately for 20 min (preincubation), 2 h (to induce germ tubes), 6 h (to induce pseudohyphae), 18 h (to induce true hyphae) at 37°C. Then, pleomorphic forms were harvested, washed, pelleted and stored as described above. Particular morphotypes were observed under a phase-contrast microscope (Docuval, Carl Zeiss, Germany). To examine cell morphology, the pleomorphic cells were fixed in 2.5% glutaraldehyde (Serva, Heidelberg, Germany), dehydrated in graded ethanol, critical point dried in $CO_2$, coated with gold and viewed in FEI Quanta 200 Scanning Electron microscope (Czech Republic) (Staniszewska *et al.*, 2011a).

**Immunofluorescence microscopy (IFM) Leica TCS SP (Leica, Wetz lar, Germany).** The specific anti-Sap2, anti-Sap3 and anti-Sap6 rabbit polyclonal primary antibodies generated by Chen *et al.* (2002) were used in the study. Then, each anti-Sap rabbit antibody was separately mixed with *Candida* cell wall suspension to prevent unspecific labelling. Subsequently, each antibody mixture was centrifuged at 10 000 *g* for 5 min.

For immunofluorescence staining of Saps the cryosection (Frigocut, model 2700, Reichert-Jung) of pleomorphic forms were blocked with donkey serum (1:20; Merck & Kollegen, Ochsenhausen, Germany) and incubated with anti-Sap polyclonal rabbit antibodies (1:100), followed by positive human *Candida* serum (1:60; Merck & Kollegen, Ochsenhausen, Germany). Antibodies were directed against Sap1-3 and Sap4-6. Samples were then incubated with donkey-anti-rabbit Cy5 (1:500; Merck & Kollegen, Ochsenhausen, Germany) and donkey-anti-human serum Cy3 (1:500; Merck & Kollegen, Ochsenhausen, Germany), respectively. This was followed by subsequent nucleus staining with Yopro (1:2000; Invitrogen, Karlsruhe, Germany).

**Immunoelectron microscopy (IEM).** Electron microscopy and postembedding immunogold labelling of sections of pleomorphic forms were performed as described by Schaller *et al.* (1998; 1999). In brief, each pellet was fixed in periodate-lysine-paraformaldehyde (PLP) and after embedding in Lowicryl K4M, the blocks were cut using an ultramicrotome (Ultracut; Reichert, Vienna Austria). Ultrathin sections (30 nm) were mounted on formvar-coated (Serva, Heidelberg, Germany) nickel or cooper grids (Stork Veco, Eerbeek, Netherlands) and incubated with anti-Sap polyclonal rabbit antibodies, directed against Sap1 to Sap3 or against Sap4 to Sap6 followed by 10 nm or 5 nm-gold-conjugated goat-anti-rabbit IgG (Dianova, Hamburg, Germany). In control samples, the primary antibody was omitted. For examination of Sap immunogold labelling, the transmission electron microscope Zeiss Libra 120 (Zeiss, Oberkochen, Germany) operating at 80 kV was used. Evaluation of data obtained from 250 randomly chosen cells was done by determining the intensity of staining with gold particles using a plus scale ranging from 1 (+) to 4 (++++), lack of staining was mark as minus (–).

## Results

**Identification of isolated strain.** Colony color of the clinical strain 82 grown on CHROMagar® *Candida* medium was determined to be light green, which is indicative of *C. albicans* species. Results of this assay indicated that the strain was *beta-N*-acetylhexosaminidase positive. It was capable of assimilating trehalose (TRE) after 48 h and 72 h of incubation. D-Xylose (XYL), DL-lactate (LAC) and *alpha*-methyl-D-glucoside (MDG) assimilation was observed after 72 h of incubation. It utilized sucrose as a carbon source for growth, which is another typical feature of *C. albicans*. The clinical strain 82 and SC5314 reference strain were phenotypically similar in all experimental system described in this study.

Application of *C. albicans* specific primers (CALB1, CALB2) allowed detecting the expected product size (273 bp) of *C. albicans* isolate. Subsequently, DNA of strain 82, ATCC SC5314 strain, excluding *C. dubliniensis* MYA 581, gave readily a PCR product with CALB primers. Primers used in this study were determined to discriminate between *C. albicans* and *C. dubliniensis* (Fig. 1).

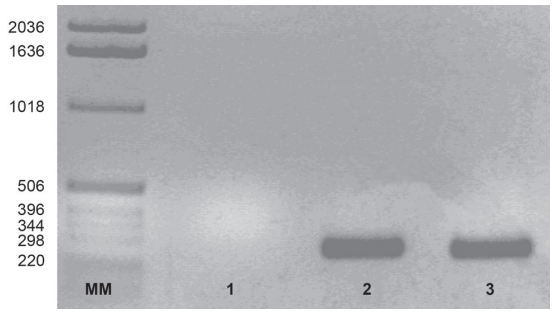


Fig. 1. *Candida albicans* identification based on PCR amplification of the 5.8S rDNA gene fragment using the CALB1 and CALB2 primers.

(MM) Molecular marker mass; (lane 1) analysis of *C. dubliniensis* ATCC MYA 581 showed absence of PCR product size (273 bp); (lane 2) PCR product size (273 bp) of *C. albicans* ATCC 5314; (lane 3) PCR product size (273 bp) of *C. albicans* clinical strain 82. PCR products were separated on an agarose gel (0.8%) and stained with ethidium bromide.

**Detection of Sap1-3 and Sap4-6 antigens by immunofluorescence and immunoelectron staining techniques.** Fractions of pleomorphic forms that were examined for their Saps expression are shown in Fig. 2. For strain 82, 100% of cells were found to form blastoconidia after 18 h of incubation in YEPD medium. In undiluted human serum, germ tube formation was observed at 2 h induction and approached 100%. Hyphae were formed as pseudohyphae (chains of elongated blastoconidial cells) at 6 h induction, with addition of budding blastoconidial cells and uniformly elongated the hyphal cells. True hyphal forms appeared as homogeneous fraction after incubation for 18 h in serum. No differences were observed between the clinical strain 82 and SC 5314 reference strain (data not shown) in the ability to form pleomorphic cells.

IFM and IEM were carried out for intracellular detection of Sap1-3 and Sap4-6 in *C. albicans* pleomorphic cells grown in Sap-inductive human serum and Sap non-inductive media – YEPD. Yeast cells cultivated in YEPD medium for 18 h as well as cells transferred to undiluted human serum for 20 min showed a lack of blue fluorescence protein of Sap1-3 and Sap4-6 expressing signal. After 2 h of incubation in Sap-inductive undiluted human serum, Sap labelling became more distinct. Analysis of the different pleomorphic forms cultivated *in vitro* demonstrated almost similar results for all tested Sap antigens (Table II).

Table II
Expression of aspartic protease (Sap) isoenzymes by pleomorphic forms of *Candida albicans*

| Pleomorphic cells | Immunogold labelling intensity (immunoelectron microscopy) | | | | Immunofluorescence labelling intensity (fluorescence microscopy) | | | |
|---|---|---|---|---|---|---|---|---|
| | Sap1-3 | | Sap4-6 | | Sap1-3 | | Sap4-6 | |
| Blastoconidia[1] | – | $22^{N}$ | – | $10^{N}$ | – | $4^{N}{}_{1}$ | – | $4^{N}{}_{1}$ |
| Blastoconidia[2] | + | 12 | + | 13 | + | 5 | + | 2 |
| Germ tube[3] | ++ | 29 | +++ | 13 | ++ | 5 | +++ | 5 |
| Pseudohyphae[4] | ++++ | 30 | +++ | 15 | ++++ | 7 | +++ | 2 |
| True hyphae[5] | ++++ | 24 | +++ | 36 | ++++ | 8 | +++ | 4 |

[1] blastoconidia cultivated in YEPD medium (pH 5.7) for 18 h at 30°C
[2] blastoconidia transferred to undiluted human serum (pH 7.2–7.4) for 20 min at 37°C
[3] germ tubes grown in human serum for 2 h at 37°C
[4] pseudohyphae grown in human serum for 6 h at 37°C
[5] true hyphae grown in human serum for 18 h at 37°C
$^{N}$ number of analyzed cells
$^{N}{}_{1}$ number of analyzed images
– lack of Sap immunogold or immunofluorescence staining; from + to ++++ the intensity of Sap staining with gold particles or immunofluorescence. Cell and images were observed by three persons independently. The experiment repeated three times gave similar results.

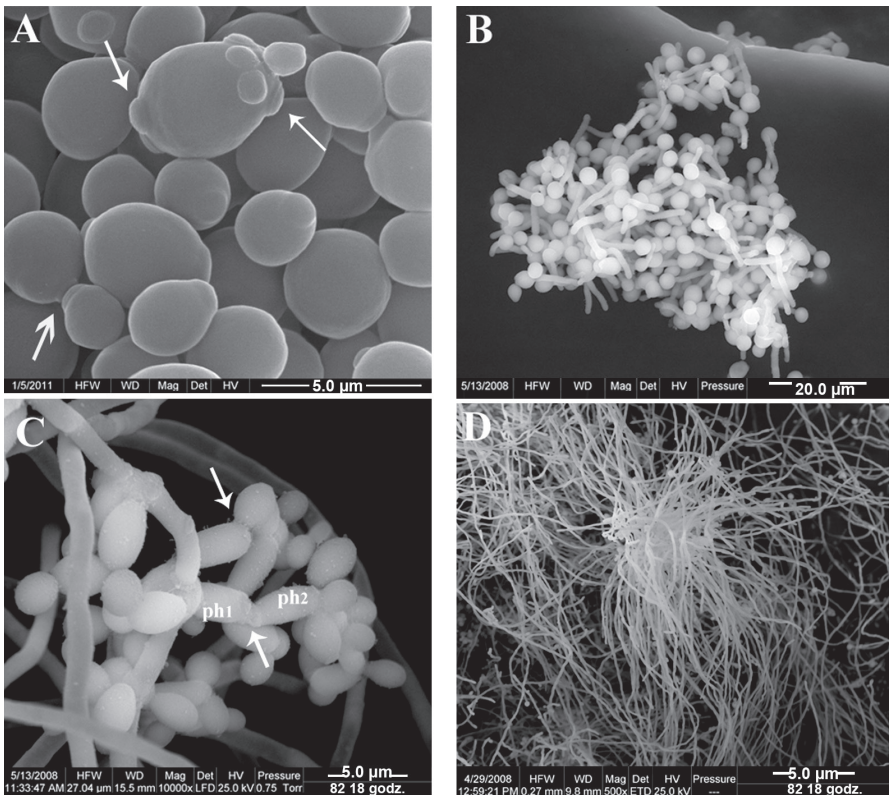


Fig. 2. *Candida albicans* pleomorphic forms. (A) Blastoconidial cells incubated at 30°C for 18 h in YEPD medium.

Oval-shaped blastoconidial cells exhibiting bipolarly-located bud scars (arrows). Fragile blastoconidial septa are seen (open arrow). Blastoconidial poles are the privileged budding areas. (B) In undiluted human serum blastoconidial cells undergo the process of unipolar germination. After 2 h of incubation germ tube forms were abundantly observed. (C) 6 h of cultivation in undiluted human serum at 37°C. A mixture of pseudohyphae and true hyphae, pseudohyphae forms are visible as chains of elongated blastoconidial cells ($ph_1$, $ph_2$). Fragile septa junctions are typical for pseudohyphal forms (arrows). (D) True hyphae grown for 18 h. Uniformly elongated true hyphal forms exhibit lack of constrictions at the solid septum. Scanning electron micrographs

The results obtained for Sap1-3 (Fig. 3) express nearly similar data obtained for remaining proteins *i.e.*, Sap4-6. The increasing immunoreactivity of Sap1-3 and Sap4-6 was seen in germ tubes, pseudohyphae and true hyphae. On the other hand, there was more intensive Sap1-3 labelling than Sap4-6 in germ tubes, pseudohyphae and true hyphae. Lack of blue fluorescence protein expressing signal was seen in the control without anti-Sap rabbit polyclonal antibodies.

Sap1-3 and Sap4-6 immunoreactivity results obtained for IEM were similar as described for IFM studies (Table II). Sap immunogold labelling was positive for all pleomorphic cells incubated in human serum for 2, 6 and 18 h at 30°C. In contrast, isoenzymes were below the detection level (estimated as minus –) in all blastoconidial cells grown in YEPD medium for 18 h. Sap1-3 and Sap4-6 were found to be active at a neutral pH in Sap-inductive medium.

Immunogold labelling showed that in pleomorphic forms Sap localizes mainly in the cell wall and in the cytoplasm. Labelling with Sap antibodies directed against Sap1-3 or Sap4-6 demonstrated lack of reactivity in blastoconidial cells cultivated in YEPD medium and intensive immunoreactivity in cells grown in Sap-inductive medium (human serum). In pleomorphic forms grown in Sap-inductive medium, intensive labelling was observed, often seen as vesicles-associated gold particles. A correlation between germination and Sap1-3 and Sap4-6 expression was demonstrated. Control experiments without polyclonal antibodies showed a complete absence of immunogold labelling with Sap1-3 or Sap4-6. The intensity of Sap1-3 gold particles immunoreactivity (Fig. 4.) bears higher immunogold labelling in germ tubes, pseudohyphae and true hyphae compared with Sap4-6 (Table II). No evidence of gold particles in control cells (without polyclonal rabbit antibodies) was observed. Strain 82 and SC5314 reference strain (data not shown) showed similar Sap1-6 expression in experimental system described in this study.

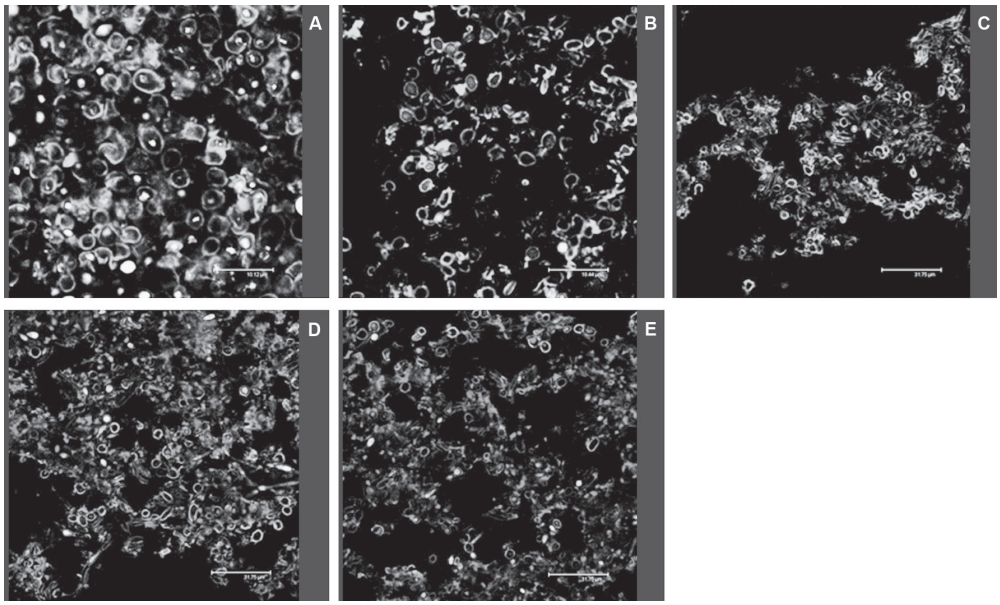


Fig. 3. Visual estimation of Sap1-3 immunolabelling in pleomorphic forms of *Candida albicans*.

(A) Blastoconidial cells incubated in YEPD (yeast extract-peptone-glucose, YEPD-grown blastoconidial cells) at 30°C for 18 h, (–) lack of blue fluorescence of Sap1-3 expressing signal. (B) YEPD-grown blastoconidial cells incubated in undiluted human serum for 20 min, (+) weak Sap1-3 immunoreactivity. (C) Germ tube forms developed from YEPD-grown blastoconidial cells by incubation in undiluted human serum for 2 h at 37°C, (++) increased content of Sap1-3. (D) Pseudohyphae developed from YEPD-grown blastoconidial cells by incubation in undiluted human serum for 6 h at 37°C, (++++) very high content of Sap1-3. (E) True hyphae developed from YEPD-blastoconidial cells by incubation in undiluted human serum for 18 h at 37°C, (++++) very high immunoreactivity of Sap1-3. The immunoreactivity of Sap4-6 was of low intensity at (+++) compared with Sap1-3 in germ tubes, pseudohyphae or true hyphae (Table II)

## Discussion

In this study, the expression of Sap1-6 during *C. albicans* morphogenesis in undiluted human serum was evaluated. Our data indicate that (1) Sap1-3 and Sap4-6 are the isoenzymes whose expression was observed in germ tubes, pseudohyphae and true hyphae of *C. albicans* (2) Sap1-3 antigens expression was significantly raised during hyphae formation compared with Sap4-6.

Recent studies (Taylor *et al.*, 2000; Leinberger *et al.*, 2005; Okawa *et al.*, 2007) demonstrated that conventional biochemical tests can misidentify clinical isolates. That is why, in this study, sequence analysis of the 5.8 rDNA region amplified by using the species-specific primer pair (CALB1 CALB2) (Luo and Mitchell, 2002) confirmed that the examined isolate belongs to the *C. albicans*. In our study, identification of the clinical isolate (strain 82) based on genotypic differences confirmed results obtained through phenotypic studies.

Many authors (Lermann and Morschhäuser, 2008; Naglik *et al.*, 2008; Gropp *et al.*, 2009; Dalle *et al.*, 2010), included the reference strain SC5314 and its mutants in studying the roles of secreted Sap hydrolases in the pathogenesis process in humans. We selected the clinical isolate in purpose in view of inconciliable results referring to strain SC5314, which are presented below. In this study, we compared Saps expression profile of pleomorphic forms of the *C. albicans* clinical isolate recovered from blood samples as well as SC5314, which was similar (data not shown).

Taylor *et al.* (2000) showed that *C. albicans* strain SC5314 well known from animal experiments is a poor colonizer and invader of mammalian epithelia. On the contrary, it was established (Schaller *et al.*, 2000; Felk *et al.*, 2002; Dalle *et al.*, 2010), that this strain was able to invade the host tissues, which was followed by systemic dissemination, as well as it caused damage in an *in vitro* model. It may be said that the virulence (in view of the place of recovering) of the clinical isolate and the reference strain is comparable; both strains are virulent by intravenous challenge. The clinical strain was chosen because it caused candidaemia in the patient, proving that it develops virulence factors.

Previously, we demonstrated (Staniszewska *et al.*, 2011a) that the clinical isolate showed virulence determinants, it produced germ tubes, pseudohyphae, and true hyphae in undiluted human serum. Furthermore, strain 82 showed distinct differences in activity profiles of hydrolytic enzymes between hyphae and blastoconidia by using the api®ZYM test (Staniszewska *et al.*, 2011b).

In the current work, the antibodies generated by Chen *et al.* (2002) were used. The authors highlighted

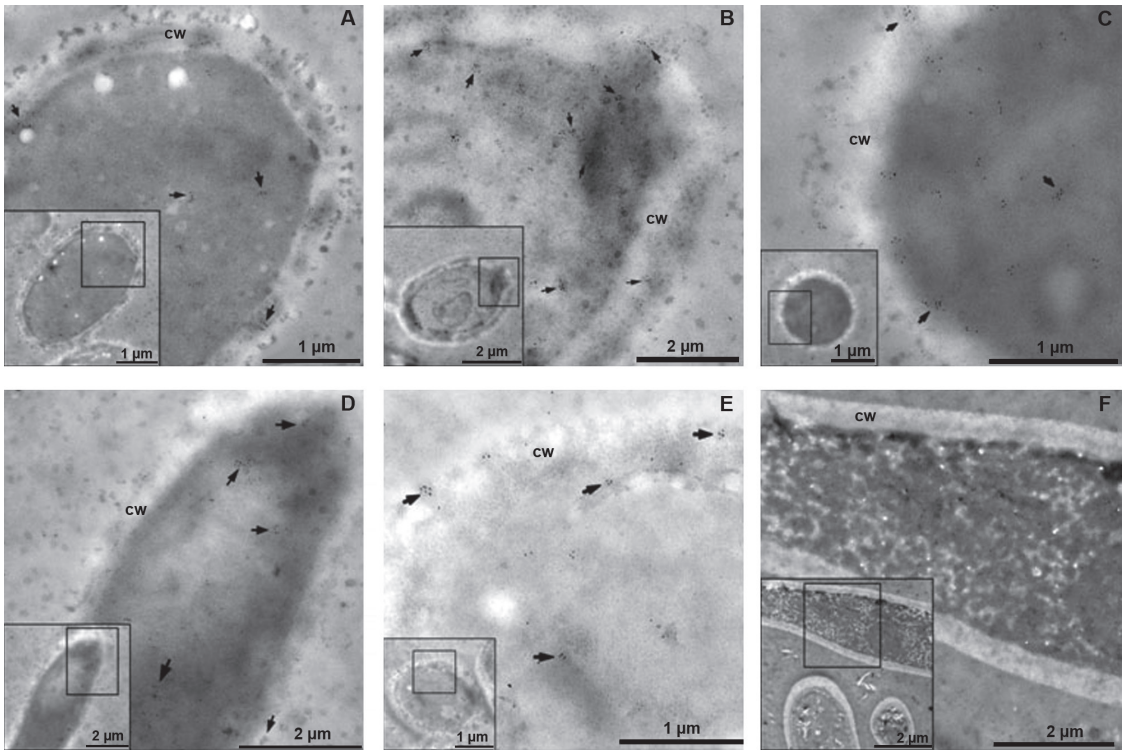


Fig. 4. Immunoelectron microscopy (IEM).

Detection of Sap1-3 in pleomorphic cells of *Candida albicans* using polyclonal rabbit anti-Sap2 serum and goat-anti-rabbit IgG conjugated to 5 nm gold particles. (A) Cells cultivated in Sap non-inductive medium YEPD and (B-E) in Sap-inductive undiluted human serum for (B) 20 min, (C) 2 h, (D) 6 h and (E) 18 h. (A) For blastoconidial cells, the gold particle labelling intensity is evaluated as (–). The gold labelling was not visible in the cytoplasm neither cell wall. (B) Blastoconidial cells cultivated for 20 min in human serum. The gold particle labelling density is estimated as (+). In comparison to the cell wall (cw), labelling is seen mainly in the cytoplasm (arrows). (C) Germ tube forms. The gold particle labelling density is estimated as (++). Labelling is seen mainly in the cell wall (cw) (arrow). (D) Pseudohyphae. Note the cytoplasm-located clusters of the enzyme marker surrounded by a membrane-like structure (arrows). (E) True hyphae. In comparison to the cytoplasm, labelling is seen mainly in the cell wall (arrow). Enhanced clusters of gold particles in the cell wall are seen . The gold particle labelling density is estimated as (++++). (F) The immunoreactivity of Sap4-6 was less intensive compared with that of Sap1-3 (Table II)

the difficulty with generating specific and sensitive antibodies against each Sap. It was mentioned (Chen *et al*., 2002) that antibodies against Sap3 showed cross reactivity with Sap2 or Sap4. The cross reactivity of anti-Sap3 might have affected results obtained in the present study. That is why in our study, additionally anti-Sap2 and anti-Sap6 were used which reacted specifically with Sap1-3 or Sap4-6, respectively (Chen *et al*., 2002).

The immunofluorescence microscopy studies revealed that the pattern of enzyme expression in blastoconidia grown in YEPD medium differs significantly from other *C. albicans* forms cultivated in the human serum, possibly due to the fact that YEPD medium contains no suitable substrate for Sap. Higher level of Sap1-6 expression was correlated with the course of germination process and germ tubes, pseudohyphae and true hyphae appearance during incubation blastoconidial cells respectively for 2, 6 and 18 hours in human serum. Analysis using microscopy techniques determine that Sap expression profiles demonstrate significant differences between particular pleomorphic forms grown in human serum. Sap1-3 expression gradually increased in cells during germination and was much more intensive than Sap4-6. A previous report showed (Hube *et al.*, 1994) that deletion of *SAP4-6* did not result in differences in hyphae formation both *in vitro* and *in vivo*.

Moreover, Felk *et al.* (2002) showed that *SAP4-6* expression is associated with, but not required for hyphal morphology. Our findings are in line with previous data (Felk *et al.*, 2002), showing that Sap4-6 expression is related with germination process. Additionally, we showed that Sap4-6 are expressed in each morphotype (including blastoconidial cells) under human serum influence.

These results were consistent with the *in vivo* expression pattern (Staib *et al.*, 2001) and suggested that expression of Sap1-6 proteins is regulated by factors that also regulate *C. albicans* morphology in human serum. Yet, information on secretion of particular Sap

isoenzymes by *C. albicans* germ tubes and pseudohyphae is still missing in the literature. In our studySap1-6 expression was observed in both morphotypes (germ tubes; pseudohyphae).

Our data allow postulating that there is a correlation between human serum induced hyphae growth *in vitro* and expression of Sap1-6. Here, we showed that the presence of high level of Sap1-3 expression throughout 18-h-incubation in human serum supports the view that Sap1-3 are probably key proteases that promote cell growth and may be dependent on morphology. Although Sap1-3 expression level was higher than Sap4-6, the latter were consistently detected in germ tubes, pseudohyphae and true hyphae at steady levels, which may also support a contributory role of Sap4-6 in *C. albicans* cell growth and fitness. Our observation is not in line with results published previously (Correia *et al.*, 2010), which suggested that yeast growth may be protease independent when *C. albicans* cells were delivered directly into the bloodstream. On the contrary, Gropp *et al*. (2009) and Staib *et al*. (2001) showed that Sap2 is essential for growth when the protein is the only nitrogen source. Another support for our findings may be the previous report (Gropp *et al*., 2009) showing the strong complement inhibitory activity of Sap1-3 in human plasma. In the present study, it was shown that the Sap1-3 expression was highly induced in *C. albicans* hyphal cells upon host body fluid (serum) influence suggesting that also *in vivo* each of the three Sap proteins may contribute to blood infection, complement inactivation and immune evasion (Gropp *et al*., 2009). Here, we have provided evidence that the human serum plays a important role in hyphae formation and Sap1-3 expression. However, more investigations about the expression of *SAP1-3* as well as *SAP4-6* under human serum influence by using RT-PCR are going to be done.

In the current study, increasing Sap1-3 and Sap4-6 expression was detected during hyphae formation induced by shift of temperature (from 30°C to 37°C) and pH (from 5.7 to 7.2) in human serum. The same results were obtained for another clinical isolate (Taylor *et al.*, 2000) as well as SC5314 reference strain (data not shown). We did not observe differences between *C. albicans* strains due to pH changes. There was no Sap1-3 or Sap4-6 activity in yeast cells grown in medium at pH 5.7, while germ tubes, pseudohyphae and true hyphae expressed these isoenzymes at a neutral pH (7.2–7.4). These characteristics may indicate the ability of *C. albicans* to survive and cause infection in a variety of host tissues.

The results obtained for the expression pattern of the immunofluorescence study were confirmed by immunoelectron microscopy studies (IEM). The use of antibodies raised against Sap1-3 or Sap4-6 made it possible to determine the precise location of these proteins in the pleomorphic forms that have been precultured in human serum. Sap proteins were detected inside the cytoplasm and within the cell wall of blastoconidia, germ tubes, pseudo- and true hyphae forms, but not in the surrounding of the cell wall, which was confirmed by the negative control (non-antigens in the surrounding of the cell wall). The Sap1-3 and Sap4-6 were observed to be organized in groups and packed with the vesicles localized in the cytoplasm. The results of our study are in line with those of Stringaro *et al*. (1997) demonstrating that during murine vaginitis Sap antigen is located within the cell wall of hyphal cells. Similar results were observed in the *in vitro* model of experimental oral candidiasis (Stringaro *et al*., 1997) and reconstituted human epidermis (Schaller *et al.*, 2000).

Our studies may support the data of Brown (2002) that both blastoconidia and filamentous forms of *C. albicans* are pathogenic and both contribute to different stages in the establishment and progress of the infection. Sap1-6 proteins tested in the above-mentioned study were determined to be expressed in pleomorphic forms during incubation in human serum which mimics *in vivo* conditions encountered during systemic blood infections. These data may indicate an important role of Sap1-6 proteins during blood infections and immune evasion (Hornbach *et al.*, 2009). We therefore conclude that there is a correlation between the increased expression of Sap1-6 proteins and germ tubes, pseudohyphae and true hyphae formation.

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