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ROLE OF ASPARTIC PROTEINASES IN *CANDIDA ALBICANS* VIRULENCE. PART I. SUBSTRATE SPECIFICITY OF ASPARTIC PROTEINASES AND *CANDIDA ALBICANS* PATHOGENESIS

ROLA PROTEAZY ASPARTYLOWEJ W WIRULENCJI *CANDIDA ALBICANS* CZĘŚĆ I. SPECYFICZNOŚĆ SUBSTRATOWA PROTEAZY ASPARTYLOWEJ A PATOGENEZA ZAKAŻEŃ *CANDIDA ALBICANS*

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Abstract: *Candida albicans* resides mainly as a harmless commensal in the gastrointestinal tract, vagina and some cutaneous areas of humans. However, in individuals who are immunocompromised or debilitated in some other way, *C. albicans* is responsible for superficialy-localized or systemic infections. *Candida albicans* produces a large family of secreted aspartyl proteinases (Saps) which are key virulence factors in *C. albicans* pathogenesis. Saps contribute to infection by degrading tissue barriers and destroying host defense molecules. The secretion of Saps varies depending on the *C. albicans*' morphologies, the site and stage of infection, and the nature of the host response. This review the focuses on characteristics and function of the members of aspartyl proteinases, which have been studied in more detail. It should be noted that the discrepancies in individual Sap's role in the virulence of *C. albicans* may result from differences in the sensitivity of methods used, or differences in infection models and stage of the epithelial cells, or variability among *C. albicans* strains.

Słowa kluczowe: *Candida albicans*, kandydoza, proteaza aspartylowa, wirulencja
Key words: *Candida albicans*, aspartic proteinases, candidiasis, virulence

1. Introduction

1.1. *Candida* as a commensal and as a pathogen

Candida albicans is a diploid microorganism belonging to genus *Candida* of ascomycetous-like fungal species which has no known teleomorph [1]. *Candida albicans* is an ubiquitous human commensal yeast which resides mainly on mucosal surfaces of the oral cavity as well as in the urogenital, gastrointestinal and vaginal tracts and some cutaneous areas of healthy individuals without symptoms of disease [5, 53]. The latter strain in commensal form can be detected in approximately 50% of the human population [75]. Furthermore, in all body locations, *C. albicans* represented the predominant *Candida* species (70%). On the other hand, results of previous studies established association between fungal colonisation and candidaemia, and furthermore the rate of progression from colonisation to invasive infection ranges from 15 to 40% [79]. *Candida albicans* is considered to be a major causative factor of opportunistic human infections (invasive candidiasis) with high morbidity and mortality rate of 30 to 70% [5, 9–11, 13–15, 53, 96].

The status of the host immune system is the major factor balancing the transition of *C. albicans* from a commensal to a pathogen [84]. Much has been done to elucidate the host defence mechanisms against systemic candidiasis [30, 37, 68]. Moreover, many of the previous studies showed that *C. albicans* aspartic proteinases are immunogenic and elicit mucosal and systemic antibody responses [7, 44, 53, 55, 92, 99]. Recently, it was reviewed [37] that transition from healthy to pathogenic

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state occurs at the mucosal-fungal interface. Mucosal immune mechanisms are vital to prevent the systemic spread of pathogens from localized infection. The innate and adaptive arms of the immune response play key role in immunity to *Candida*. Several studies have demonstrated that innate immunity in mucosal infection involves many cell types: neutrophils, monocytes/macrophages, Natural Killer (NK) cells, dendritic cells (DC), $CD4^+$ and $CD8^+$ T cells, non-MHC restricted T cells such as γδ-T cells, mucosal epithelial cells, stromal cells and keratinocytes [30, 37, 68]. In the case of systemic infection, the release of interferon-γ (INF-γ) and lymphotoxin-α (LTA) from Th1 cells is responsible for activation of the antifungal properties of neutrophils and macrophages in the deep tissues. Investigation in mice and humans has provided evidence for a protective role of the Th17 pathway in anti-fungal immunity at mucosal and epithelial surfaces, particularly the oral cavity and skin. During infection of mucosa, the release of IL-17 and IL-22 from specific Th17 lymphocytes recruits and activates neutrophiles for the elimination of infection [37, 49, 50, 52]. Moreover, commensal flora in the oral, gastrointestinal and vaginal tracts plays a vital role in limiting infections [46]. *Candida albicans* has three major mechanisms of its own that mediate pathogenicity and invasiveness: (I) escape from host immune responses, (II) morphogenic change from the yeast to the hyphal form, which increases adherence and ability to invade host cells, and (III) host cell invasion, which is supported by factors associated with hyphae including adhesion molecules, invasion-like molecules, and secreted hydrolytic enzymes [53].

The incidence of *Candida* endogenous infections appeared to be due to several predisposing factors such as immunosuppressant or steroids treatments, long-term catheterization, abdominal surgery, treatment with broad-spectrum antibiotics, perforation of the gastrointestinal tract, destruction of the skin by deep burns, hyperalimentation, mechanical ventilation, renal failure, bone marrow transplant, premature very low birth weight infants, critically ill neonates, diabetes mellitus, Crohn's disease, immunologically comprised individuals, spread of HIV infections [16–19, 21, 23, 45, 79, 83]. However, rare cases of exogenous acquisition have also been reported, they occurred due to contaminated solutions and material (catheter-related), also via healthcare-associated cross-transmission of *C. albican*s [6, 71].

1.2. Candidiasis

Candidiasis may be classified as superficially-localized or a systemic [5, 20, 22, 24, 25, 27]. The surface-localized, mucocutaneous candidiasis can affect epidermal and mucosal surface including oral cavity, pharynx, esophagus, intestines, urinary bladder and vagina. It occurs in the form of oropharyngeal candidiasis (OPC), cutaneous candidiasis (CC), esophageal candidiasis and vulvovaginal candidiasis (VVC) [26]. Increased risk of OPC is seen either in smokers of tobacco products or in patients with the following disorders: xerostomia, Sjögren's syndrome (SjS), cancer therapy followed by local mucosal injury, hyposalivation as well as in patients with local or systemic steroid and antibiotic treatments [8]. Furthermore, OPC is one of the first clinical signs of HIV infection, and is diagnosed in up to 95% of HIV+ patients [37]. The main described clinical forms of OPC are pseudomembranous (so-called thrush), erythematous, and angular cheilitis [8]. A chronic mucocutaneous candidiasis (CMC) is characterized by chronic or recurring infection of the skin, nails, oropharyngeal and esophageal involvement without the tendency for systemic dissemination and with increased frequency of endocrinopathy [5]. The CC has a number of predispositions, such as warmth, moisture and immunosuppression [26]. *Candida albicans* is responsible in over 85% for vulvovaginal candidiasis cases [28]. Fungal infection are very frequent in women, in fact, vulvovaginal candidiasis (VVC) affected up to 75% of all women [52]. Moreover, about 5–10% of women developed recurrent form – RVVC [5, 31–34]. Unlike systemic candidiasis, characterized by the presence of *Candida* in normally sterile sites locations in the body, VVC and RVVC affect vaginal tissue, where *C. albicans* is a natural commensal [5]. The systemic, invasive *C. albicans* infection (ICI) can affects organs causing pneumonia, endocarditis, myocarditis, pericarditis, meningitis, endophthalmitis, arthritis, osteomyelitis [35, 36]. *Candida albicans* is the most frequent causative factor of 50–70% of all invasive infections [38].

Although the incidence of candidaemia in Europe (0.5 episodes per 10,000 patient days) is lower than in the United States (2 episodes per 10,000 patient days), the incidence in Europe has recently increased [36]. The recent study by Schelenz [93] on surveillance conducted in hospitals in the United Kingdom showed that *C. albicans* remains the most common species causing candidaemia in Intensive Care Unit patients. Furthermore, study of Zaoutis [100] and Nguyen et al. [79] conducted in neonatal intensive care unit in the United States and France respectively showed that *C. albicans* was the most frequent species responsible for invasive candidiasis infection in neonatal patient group. According to Schofield et al. [95], Morrison et al. [73], Bialková and Šubík [9], Schelenz [93], *C. albicans* was responsible for 59% of nosocomial candidaemia and for 55% of bloodstream infections. Enoch et al. [47] noted that *C. albicans* was responsible for 79.4% of candidaemias in intensive-care patients, but only for 37.5% in haematology patients. The contribution of *C. albicans* in invasive and disseminated

candidiasis appears highly similar in United States and Europe and accounts from 49 to 55% [36]. According to Kullberg and Filler [65] among the risk factors that predispose to candidaemia and disseminated candidiasis are: cancer (26%), abdominal surgery (14%), diabetes mellitus (13%) or human immunodeficiency virus (10%).

In the light of the above-mentioned literature data systemic mycoses of *C. albicans* etiology constitute a serious clinical problem world-wide. The high frequency of occurrence of these infections as well as the high mortality of patients with immunosuppression cause a tendency toward better understanding of *C. albicans* virulence factors and developing sensitive and specific diagnostic methods and appropriate strategies for candidiasis treatment.

Candida albicans strains represent an important clinical problem as they from this species possess more virulence factors than non-*Candida albicans* strains [5, 28, 39, 41]. Virulence factors identified so far include such phenomena as morphogenetic transition from yeast to pseudo- and true hyphae, adhesion to inert and biological substrates, production and secretion of hydrolytic enzymes, biofilm formation, antigenic variability and phenotype switching [1, 5, 13, 27, 43, 47, 48]. This review focuses on characteristic and function of the members of aspartyl proteinase, which have been studied in more detail, and are key virulence factors in *C. albicans* pathogenesis.

2. Extracellular enzymes and *C. albicans* virulence

Candida albicans is a producer of extracellular hydrolytic enzymes [9]. The hydrolytic enzymes produced by *C. albicans* in addition to the simple role of digesting molecules for nutrient acquisition, fulfil a number of functions [2]. It is generally considered that some of hydrolytic enzymes, such as *beta*-N-acetylhexosaminidase (HexNAcase), formerly known as N-acetyl-*beta*-D-galactosaminidase (NAGase), acid phosphatase and *beta*-D-glucosidase contribute specifically to the differentiation of *C. albicans* yeast strains [59, 60]. Niimi *et al.* [80] reported the HexNAcase enzyme to be a virulence factor for *C. albicans* since the HexNAcase-deficient mutant (EOB4) of the ATCC 10261 strain was less pathogenic than the parental wild type strain in a mouse infection model. Hube and Naglik [58] reported that production of hydrolases contributes to colonization of host surfaces, enhances adhesion by degrading host surface molecules, and allows penetration into deeper host tissues by digesting host cell membranes or evasion of host defence mechanism by digesting cells and molecules of the host immune system. The three most significant extracellular hydrolytic enzymes produced by *C. albicans* i.e., the phospholipases, lipases and secreted aspartyl proteinases (Saps) are linked to virulence [12, 58]. Phospholipases are important pathogenicity determinants in *C. albicans*. They play a significant role in damaging cell membranes by destroying phospholipids in host cells, therefore inducing cell lysis and facilitating tissue invasion [3, 12]. There are four types of secreted phospholipases: A, B, C and D [27]. Among them phospholipase B contributes to the pathogenicity of *C. albicans* by abetting the fungus in damaging and traversing host cell membranes [12]. Lipases are enzymes that catalyze both the hydrolysis and synthesis of triacylglycerols [51]. *Candida albicans* can produce at least nine lipases which can hydrolyze ester bonds of mono-, di-, and triacylglycerols [92]. Moreover, secreted lipases may play role in the adhesion and penetration steps of infection process in murine model of haematogenously disseminated candidiasis [29, 51]. Based on observations made by Kitanovic et al. [62], esterase activity (hydrolysis of ester bounds of triacylglycerols) is a common feature of *C. albicans* strains isolated from clinical specimens. The ability to secrete hydrolytic enzymes that destroy barriers to enable growth and break polymers to provide nutrients as well as inactivate the host defense molecules is considered as one of the virulence factors of *C. albicans* [54]. Among hydrolytic enzymes of *Candida* spp., aspartyl proteinases are by far the most commonly associated with virulence [75].

3. *Candida albicans* aspartic proteinases

Candida albicans secreted aspartic proteinases (Saps) represent a family of 10 related proteinases [57, 76] which catalyze the hydrolysis of peptide bonds (CO-NH) in proteins [35, 62, 69, 75, 99]. Ten *SAP* genes are located on five different chromosomes [43]. Naglik et al. [114] noted that within Sap isoenzyme family distinct groups based of sequence homology are clustered: Sap1 to Sap3 are up to 67% identical, and Sap4 to Sap6 are up to 89% identical, while Sap7 is only 20 to 27% identical to other Sap proteins, which makes it the most diverged member of family. It seems apparent from the previous studies [58, 75, 92] that Sap1-Sap8 proteins are secreted extracellularly, whereas Sap9 and Sap10 remain anchored in the fungal membrane. The proteases Sap9 and Sap10 are bound to the fungal cell surface by a glycosylphosphatidylinositol (GPI) anchor motif. Of these, Sap9 seems to be predominantly located in the cell membrane, and Sap10 is located in the cell wall and membrane [4]. Many of the early proteinase studies focused on the detection of Sap antigens inside the morphologies of *C. albicans* using polyclonal antibodies [35, 73, 97]. Accordingly, reports using

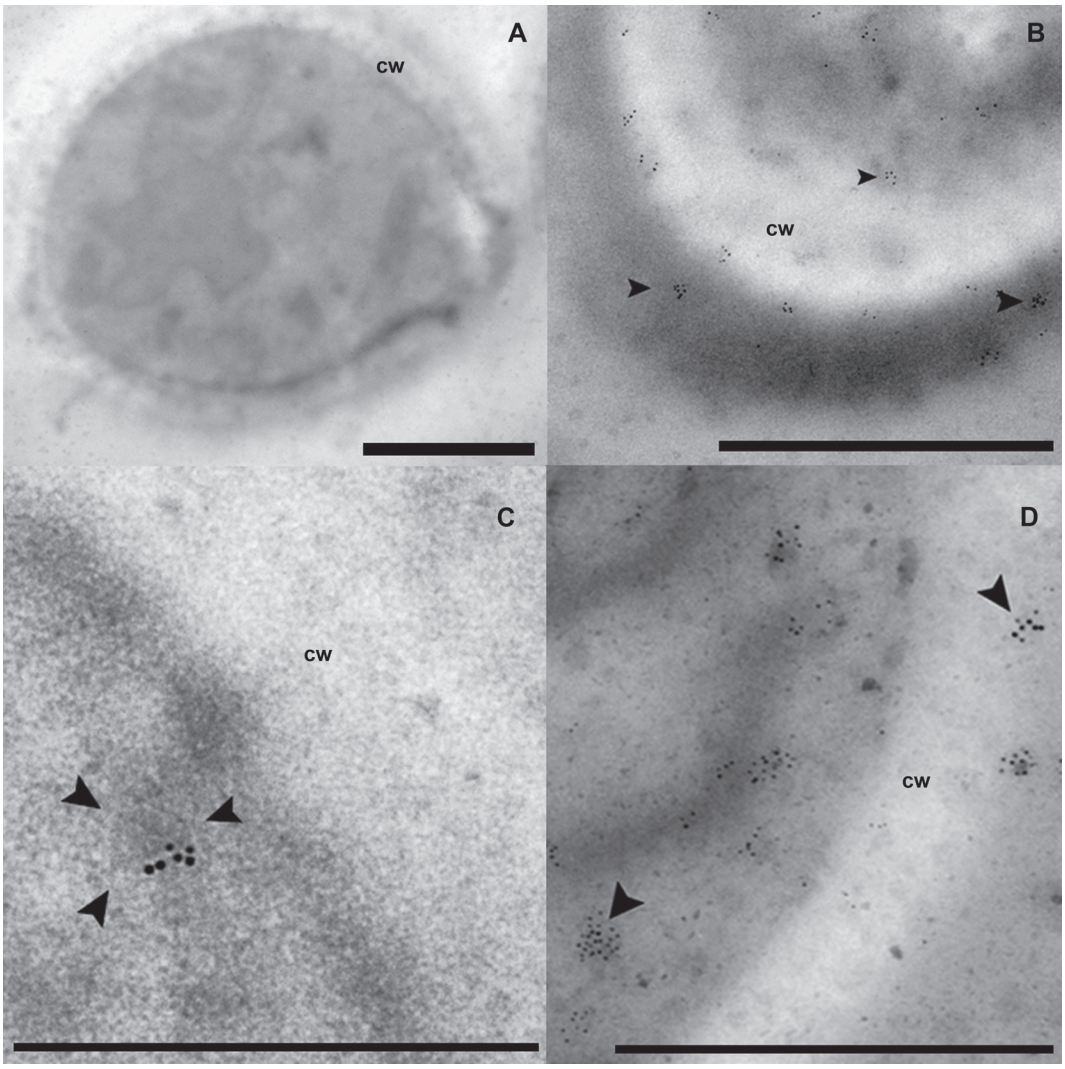

Fig. 1. Immunoelectron microscopy (IEM). Detection of Sap3 in pleomorphic cells of *Candida albicans* clinical strain using polyclonal rabbit anti-Sap3 serum and goat-anti-rabbit IgG conjugated to 5 nm gold particles. (**A**-**D**) Cells cultivated in Sap-inductive undiluted human serum for 18 h. **(A)** Control without anti-Sap3 antibody. No evidence of gold particles in control cells is seen. (**B**) Separate gold particles and clusters of them are visible in the cytoplasm (arrows) and in the cell wall (cw). (**C**) Note the vesicle packed with gold clusters in the cytoplasm (arrows). (**D**) Note the cytoplasm- and cell wall-located clusters of the enzyme marker (arrows). Bars = 1 μm

immunogold-labelling techniques revealed that Sap proteins are localized inside the cell wall of yeast and hyphal cells of *C. albicans* [86–88, 97]. Moreover, S t r i n g a r o et al. [97] demonstrated that in rats with developed vaginitis the ultrastructural localization of Sap is observed only within the cell wall of hyphal cells. Similar results were observed in the *in vitro* model of experimental oral candidiasis [86] and reconstituted human epidermis (RHE model) [88]. In our study (unpublished data), immunogold labeling showed that in pleomorphic forms Sap1-6 localize mainly in the cell wall and in the cytoplasm. The cytoplasm-located clusters of the enzyme marker surrounded by a membrane-like structure were observed (Fig. 1). For more information on processing cultivation and regulation of Sap, the reader is guided to reference by N a g l i k et al. [75].

Many authors [35, 42, 58, 62, 69, 72, 73, 75, 76, 82, 88, 91, 92, 99] pointed out that Sap production is associated with a number of *C. albicans* virulence features, including hyphae formation, adhesion and phenotypic switching. These findings are supported by the fact that *C. albicans SAP* genes appear to have no equivalents in less pathogenic yeast specie – *Saccharomyces cerevisiae* [69]. Furthermore, the proteinases appear to have adapted their biochemical properties to fulfil a number of specialized functions during the of infection process, e.g., digestion of host cell membranes and molecules of the host immune system to avoid antimicrobial attack by the host [56, 75, 76, 98]. For example, a more recent study by G r o p p et al. [53] found that the Sap proteinases degrade and inactivate the central human complement components C3b, C4b as well as

Table I

Expression of secreted aspartyl proteinases in human and in animal models

Model	Infection	Secreted aspartyl proteinases	Assay	Main findings	References
Murine and guinea pig	Systemic infection	*SAP*4-6	Southern blotting, survival curves	*SAP*4, *SAP*5 and *SAP*6 expression was observed during progression of systemic infection by *C. albicans* in animals	[43]
Murine and guinea pig	Disseminated infection	*SAP*1-3	Southern blotting, hybridization autoradiography	*SAP*1, *SAP*2 and *SAP*3 were detected during *C. albicans* disseminated infections	[20]
Rat	Infected rat vagina	Sap	Immunoelectron microscopy, SDS-PAGE with antirabbit IgG or antimouse IgG-peroxidase conjugated	Sap secreted by *C. albicans* during rat vaginitis; Sap localized in the cell wall of hyphal forms during infection	[53]
Human	Sera	Sap	Western blot with monoclonal antibody (MAb)	Detection of Sap antigen in the sera of patients with invasive candidias	[7]
Human	Oral candidiasis	*SAP*1-7	RT-PCR	*SAP*1-3 transcripts were observed in patients with oral candidiasis	[34]
Human	Oropharyngeal candidiasis	Sap1-3	Immunoelectron microscopy with murine monoclonal antibody	The expression of Sap1-3 does not confirm a pathogenetic role of the Sap1-3 in host-fungal interaction	[46]
Human	Oral and cutaneous candidiasis	Sap1-3 or Sap4-6	Immunoelectron microscopy	Sap1-3 crucial for mucosal and cutaneous candidiasis	[47]
Mice	Gastrointestinal infection	*SAP*1-6	RT-PCR, IVET	Expression of *SAP*4-6 was detected in higher percentage than *SAP*1-3; individual Saps are not indispensable factors for virulence	[25]
Mice	Disseminated candidiasis	*SAP*4-6	RT-PCR; immunoelectron microscopy	Hyphal morphologies without expression of *SAP*6 are less virulent; Sap1-3 antigens were found on yeast and hyphal cells, Sap4-6 were predominantly found on hyphal cells in close contact with host cells	[15]
Human	Vaginal candidiasis	*SAP*1-10	RT-PCR	*SAP*5 and *SAP*2 transcript were expressed in *C. albicans* cells infecting human epithelia *in vivo*	[48]
Mice	Keratitis infection model	*SAP*4-6	Southern blot analysis	*SAP*6 appears to be associated with morphogenetic transformation of yeast to invasive filamentous forms, *SAP*6 contributes to corneal pathogenicity,	[23]
Human	Oral candidiasis	*SAP*1-6	qRT-PCR	*SAP*5 and *SAP*9 are the most highly expressed proteinase genes *in vivo*	[37]
Mice	Disseminated infections	*SAP*1-6	qRT-PCR	Expression of *SAP*1-6 was low in murine model of haematogenously disseminated candidiasis	[11]

C5 and block the damaging effects of the activated complement system.

Numerous studies (see the following section) have correlated the *SAP* expression and morphogenesis process under hyphae inducing conditions. *SAP* genes were shown to be expressed differentially according to the morphological form of the fungus and the surrounding environment supporting transition [74]. Nantel et al. [78] showed that the serum favoured hyphae formation and expression of Sap4-6. In contrast, Hube and Naglik [58] reported that hyphae induction alone is sufficient for Sap expression and that the protein components of the serum are not necessary. On the contrary, early study of Brown and Gow [21] demonstrated that Sap6 is expressed when hyphae growth is stimulated using polypeptide-containing culture media. To address this, two reports [35, 38] showed that hyphae formation was induced in modified Lee's medium after 18 h and under the same growth condition expression of Sap4-6 was detected. Finally, it was clearly established [35, 66, 75, 76, 91, 92] that yeast cells predominantly produce Sap1-3, while hyphae produce mainly Sap4-6. Supporting these data, more recent report by dos Santos [43] noted that the expression of *SAP*1-*SAP*3 and *SAP*8-*SAP*10 is detected in yeast cells, while the expression of *SAP*4-*SAP*6 is associated with the filamentous forms. However, Naglik et al. [77] concluded that although Sap5 may play an indirect role in facilitating

hyphal invasion, but *SAP*5 expression can be hypha-independent during oral and vaginal (RHE) infection. Yet, detailed information on Sap expression in different pleomorphic forms of *C. albicans* is still lacking.

Research efforts by many investigators in different laboratory have concentrated on the correlation study between Sap production *in vitro* and the virulence of *C. albicans* [4, 13, 40, 56, 58, 61, 63, 64, 67, 76, 81, 83, 85–91]. The first observation of extracellular proteinase involvement in *C. albicans* attachment to oral mucosa was presented by Borg and Rüchel [13]. To address this, Sap production by *C. albicans* was demonstrated by Schaller et al. [85, 86, 88, 91]. Although the gene expression pattern of the vaginal RHE model was different from that in the oral RHE model, it was clearly suggested that Sap1 to Sap3 are the main proteinases contributing to the early stages of mucocutaneous infections. Moreover, Schaller et al. [87] also analyzed *SAP* expression in the *in vitro* model of cutaneous candidiasis based on reconstituted human epidermis. This analysis was undertaken to confirm the *SAP*1-3 predominant expression in this type of infection. Two recent reports have correlated proteolytic activity of Saps *in vitro* with the virulence of *C. albicans* species [67, 77]. The latter authors studied the role of Sap isoenzymes in *C. albicans* pathogenesis by comparising virulence of mutants with one or multiple *SAP* genes disturbed to wild-type control strain. As a result, using RHE models of oral and mucosal infections in human, it was founded that only Sap5 is potentially contributed to *C. albicans* virulence. Moreover, Naglik et al. [77] indicated that *SAP*9 is consistently the most highly expressed proteinase gene in monolayers and RHE models. According to the same group [77] *SAP*5 is the only gene significantly upregulated as infection progressed in oral and vaginal RHE models. This study [77], therefore allows to conclude that both genes (*SAP*5 and *SAP*9) do not influence fungus morphology. Finally, the latter studies abolished earlier findings that Sap1-3 subfamily is required for invasion of human epithelia as previously concluded [64, 74–76, 85–89, 91]. For example, Naglik et al. [77] indicated that the overall individual contribution of Sap1-3 and Sap4-6 subfamilies in inducing epithelial damage in the RHE model appears to be low. On the other hand, studies [40] using fungal mutants as well as set of fungal and host cells inhibitors demonstrated that proteases Sap1-6 support invasion into oral or intestinal epithelial cells. Finally, one should be noted that all of the above discrepancies in the results presented by many authors may be related either to differences in the sensitivity of methods used, or differences in infection models and stage of the epithelial cells, as well as variability among *C. albicans* strains [40, 67]. In fact, there are no directly comparable studies by different authors using the same technique, model, or site of infection (Table I).

4. Summary

In this review the of involvement secreted aspartic proteinase (Sap) members' in the pathogenesis of *C. albicans* infections, risk factors for candidiasis, as well as the incidences of candidiasis. The proteolytic activity of secreted aspartyl proteinases has been extensively studied in the last years [13, 24, 35, 57, 63, 75, 86–88, 90]. Aspartic proteinases are considered most significant extracellular hydrolytic enzymes as they are the key virulence factor in *C. albicans* pathogenesis. Saps have a number of specialized functions during infection e.g.: their proteolytic activity has been associated with tissue invasion as they degrade host proteins at mucosal sites; also Sap isoenzymes degrade and inactivate the central human complement components [3, 4, 53, 75, 76]. The Sap isoenzymes' role in *C. albicans* virulence has been supported by the fact that Sap production is associated with a number of other virulence factors such as adhesion, hyphal formation, pleomorphism, phenotypic switching [70]. At present, the roles and functions of the Saps during the infective process in humans are not clear and thus future functional studies have to be conducted.

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