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Possible role of secreted proteinases in *Candida albicans* infections

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Summary

Extracellular proteolytic activity of the human pathogen *Candida albicans* is due to the activity of at least nine secreted aspartyl proteinase (Sap) isoenzymes. *SAP1-9* genes are differentially regulated both *in vitro* and *in vivo* at the transcriptional level. All *SAP* genes are translated into preproenzymes, which are processed by a signal peptidase and a Kex2-like proteinase. *In vitro* experiments using purified Saps suggested that active Saps may play a role in attachment, colonization, penetration of host tissues and immune evasion. *In vivo* expression of *SAP1-6* during oral candidosis was established. Using *sap* mutants, produced by targeted gene disruption, a prominent role for *SAP1-6* during murine disseminated infections and for *SAP1-3* during rat vaginal infections could be demonstrated. These data underline the general importance of extracellular proteinases for the pathogenesis in *C. albicans* infections.

Key words

Candidosis, Virulence factors, Proteinases, *SAP*, Processing, Expression, Mutants

Posible papel de las proteinasas secretadas en las infecciones por *Candida albicans*

Resumen

La actividad proteolítica extracelular del patógeno humano *Candida albicans* se debe a la actividad de al menos nueve isoenzimas aspartil-proteinasas secretadas (Sap). Los genes *SAP1-9* son regulados de manera diferencial tanto *in vitro* como *in vivo* a nivel de transcripción. Todos los genes *SAP* se traducen en preproenzimas que son procesados por una peptidasa señal y una proteinasa tipo Kex2. Los experimentos *in vitro* con Saps purificadas sugieren que las Saps activas pueden jugar un papel en la adhesión, colonización, penetración de los tejidos del huésped y la evasión del sistema inmune. Se ha demostrado la expresión *in vivo* de *SAP1-6* durante la candidosis oral. Utilizando mutantes *sap* producidos mediante interrupción del gen correspondiente, se pudo demostrar el papel fundamental de *SAP1-6* en la infección diseminada murina y de *SAP1-3* en la infección vaginal en ratas. Estos datos subrayan la importancia general de las proteinasas extracelulares en la patogénesis de las infecciones por *C. albicans*.

Palabras clave

Candidosis, Factores de Virulencia, Proteinasas, *SAP*, Procesamiento, Expresion, Mutantes

Proteolytic activity of the human pathogen *Candida albicans* has been discussed as a virulence factor since 1965 [1-3]. Strains with higher proteolytic activity were shown to be more virulent [5,6,7,10], and proteinase-deficient mutants of *C. albicans* were less pathogenic [4,11,12]. Although these observations associate secreted aspartyl proteinases (Saps) of *C. albicans* with virulence, this early picture was at least simplified. The last eight years have demonstrated that the *SAP* gene family comprises at least nine members [13-19], but the majority of purified proteinases [20-24] were shown to be products of a single gene only (*SAP2*) [30,32]. Consequently, proteolytic activity measured under most *in vitro* conditions, e.g. in medium with protein as the sole source of nitrogen, was due to Sap2 activity. Thus, correlations between *in vitro* proteolytic activity and virulence may only reflect secretion and activity of Sap2, rather than the other Sap isoenzymes. In addition, the proteinase-deficient mutants used in earlier studies were obtained by chemical or UV-treatment and may harbour non-specific mutations at other loci that may impair growth or reduce virulence. Also, virulence studies using proteinase-deficient mutants were focused on mouse models of disseminated candidosis, therefore it was not clear if proteinases would be important for other types of infection such as mucosal candidosis.

The discovery of a *SAP* gene family [15] raised questions about the roles of the nine different *SAP* genes. For example, it has been postulated that the Sap isoenzymes could function at different stages or during different types of infections. With the availability of cloned *SAP* genes and the application of molecular tools, such as targeted gene disruption and RT-PCR technology, significant progress has been made to clarify the regulation and importance of *C. albicans* proteinases *in vitro* and *in vivo*. Furthermore, recent studies using purified proteinases and proteinase-specific antibodies give insights into the possible roles of these enzymes during infection.

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SAP EXPRESSION *IN VITRO*

All members of the *SAP* gene family encode preproenzymes, therefore, there are at least two possible ways to regulate the expression of these proteins: regulation at the mRNA- or protein- level. Sap preproenzymes are processed and activated by a signal peptidase in the endoplasmatic reticulum and a Kex2-like proteinase in the late golgi compartment [25,26]. The *KEX2* gene of *C. albicans* has been recently cloned and disrupted, and *kex2* null mutants were shown to secrete active Sap2; however, the enzyme was abnormally processed and secreted at a reduced level [26]. This indicated that Kex2 is partially involved in processing Sap2, but alternative Sap processing routes exist in *C. albicans*. However, kinetic studies of Sap2 secretion by protein-labelling and immunoprecipitation (pulse chase experiments) suggested that proteinase synthesis and secretion were coupled and no intracellular Sap forms were accumulated [27,28]. Therefore, regulation of Sap2 activity must occur predominantly at the mRNA level.

SAP genes are regulated differentially *in vitro* at the transcriptional level [3]. *SAP*1 and *SAP*3 are thought to be regulated during phenotypic switching [29-32], however, *SAP*3 mRNA was also detected in yeast cells of some strains under conditions where *SAP*2 was highly expressed [30,33]. *SAP*2 mRNA was the main transcript in log-phase yeast cells and was induced by peptides [30,32]. *SAP*4 - *SAP*6 were expressed in cells undergoing yeast to hyphal transition at neutral pH [30,32], while *SAP*7 was transcriptionally silent under all conditions tested *in vitro* [30]. Expression of *SAP*8 was increased at 25°C physiological temperatures [34], and transcripts of *SAP*9 were observed preferentially in later growth phases, when expression of other *SAP* genes had decreased [19]. This *in vitro* expression pattern suggested that various members of the *SAP* gene family may have distinct roles during *Candida* infections.

POSSIBLE TARGET PROTEINS OF SAPS

The most manifest property of Sap2 is its broad substrate specificity, and this is likely the case for the other Sap isoenzymes [35]. Only a few proteins, such as lysozyme and ferritin, resist Sap2 activity [2], but the majority of proteins such as keratin, collagen and vimentin [36,37] can be degraded. Not only could this provide essential nitrogen for growth, but could also enhance attachment, colonization and penetration of host barriers. For example, hydrolysis of mucin by Sap2 may help *C. albicans* to penetrate the gastrointestinal mucosa [38], and degradation of subendothelial extracellular matrix proteins may aid invasion of endothelial cell layers [39].

A role for Sap2 during invasion of *C. albicans* through endothelial cell layers was also demonstrated using *sap* null mutants, obtained by targeted gene disruption. Only *sap2* mutants caused significantly less endothelial cell injury than the parental strain and *sap1, sap3* or *sap4-6* mutants [40].

Adherence of *C. albicans* to human mucosa [41], epidermal corneocytes [42] and epidermal keratinocytes [43] has been shown to be inhibited by pepstatin A, a potent inhibitor of aspartyl proteinases. Therefore, surface proteins may be targets of the Saps. Each of the single *sap1, sap2,* and *sap3* null mutants were moderately less adherent to buccal epithelial cells, suggesting that these genes may act synergistically to enhance adherence [44[. Surprisingly, a *sap4-6* triple mutant had significantly increased adherence. This may indicate that Sap4-6 normally remove or degrade cell surface components on the yeast or epithelial cells that are inhibitory to the host-fungus recognition and adhesion mechanism.

Adherence of the *sap* mutants to endothelial cells was unaffected, indicating that Sap1-6 activity is not essential [40]. However, it can not be excluded that in single *sap* mutants a compensatory up-regulation of other *SAP* genes occurs.

Many proteins of the immune system such as enzymes of leucocytes (e.g. cathepsin D), immunoglobulins (including secretory IgA), complement factors and alpha macroglobulin are degraded by Sap2 [2,37,45]. Therefore, Saps may help the fungus to evade the host immune system. Recently this has been confirmed by Borg-von Zepelin *et al.* [35], who showed that *Sap4-6* are expressed in murine macrophages. In addition, a sap4-6 triple mutant was killed 53% more effectively after contact with macrophages than the wild type strain, indicating a role for Sap4-6 in this host-fungus interaction.

Finally, proteolytic activity of Sap2 could activate host proteolytic cascades such as the blood clotting system [8,9], which may not be advantageous to the fungus but may have deleterious effects on the host.

These *in vitro* experiments illustrate the potential of Sap activity. However, to elucidate the precise role of Saps in pathogenicity, the expression, function and importance of each *SAP* gene during the *Candida* infection must be investigated.

EXPRESSION AND RELEVANCE OF SAPS DURING MUCOSAL INFECTIONS

The most common type of candidosis are mucosal infections, such as infections of the oral cavity, the gastrointestinal tract and the vagina. Activity of Sap2, like most aspartyl proteinases, was found to be optimal at acidic pH values [3]. Since saliva has an approximately neutral pH, it was thought that Saps may not be important in oral candidosis. However, Sap4 - Sap6 are expressed and active at neutral pH [30, 35], and Sap2 was shown to be active at pH values near neutral [46]. Also, other Sap isoenzymes may act in acidic microniches [3]. A positive correlation between *C. albicans* isolates from the oral cavity of HIV positive patients and proteinase production has been demonstrated by Ollert *et al.* [47] and De Bernardis *et al.* [10]. In a skin model of human oral candidosis Schaller *et al.* [48] showed a temporal regulation of *SAP* expression by RT-PCR. The expression of *SAP*1, *SAP*3 and *SAP*6 correlated temporally with severe lesions of the epithelial tissue, suggesting a role of these *SAP* genes in the first steps of an oral infection. It should be noted that no *SAP*2 transcript was detected in the early stages of infection, although *SAP*2 mRNA is the dominant transcript under several *in vitro* conditions. Expression of *SAP*1, *SAP*3 and *SAP*6 was also detected by RT-PCR in a sample from a young female patient suffering from acute oral candidosis at an early stage [48].

Sap production is thought to play a role in the mechanisms by which *C. albicans* infects and persists in the vagina. Expression of *SAP*1 and *SAP*2 has been demontrated by Northern blotting in experimental vaginitis in rats [49], and polyclonal antibodies against Sap2 conferred partial protection in the same model [50]. Furthermore, *sap2* null mutants were almost avirulent in the rat vaginitis model [51]. In addition, the virulence of *sap1* and *sap3*, but not *sap4 - sap6* null mutants, was attenuated in comparison to the parental strain, indicating that

SAP4-6 may not be important for this type of candidosis. These data clearly demonstrate that distinct Sap isoenzymes are essential for initiation and persistence of experimental vaginitis.

ROLE OF SAPS IN DISSEMINATED INFECTIONS

Antigen-antibody studies provided the strongest evidence that Saps are expressed during systemic candidosis [52]. In a more recent study, when *sap1, sap2 , sap3* and *sap4-6* null mutants were injected intravenously in guinea pigs and mice, the animals had increased survival-rate compared to control animals infected with the parental strain [53,54]. However, reduction of proteolytic activity *in vitro* did not correlate directly with the extent of attenuation of virulence observed for all Sap-deficient mutants. These data suggest that *SAP*1, *SAP*2, *SAP*3 and *SAP*4-6 all contribute to the overall virulence of *C. albicans,* and presumably all play important roles during disseminated infections. For example, Sap2 may be needed for invasion through the endothelial cell barrier [40] and Sap4-6 for resistance to phagocytosis by macrophages [35] (see above).

If Saps are important for disseminated candidosis, the inhibition of these aspartyl proteinases *in vivo* will protect the host against infection. Only slight or no protection by pepstatin A was observed when mice were challenged intravenously [55,56]. In contrast, pretreatment of neutropenic mice with pepstatin A by intraperitoneal injection afforded strong dose-dependent protection against a subsequent lethal intranasal dose of *C. albicans* [56]. These data show that Saps may play an essential role during early dissemination and suggest that aspartyl proteinase inhibitors may prove useful as a prophylactic drug to prevent systemic *C. albicans* infections.

CONCLUSIONS

Extracellular proteolytic activity of *C. albicans* is due to the activity of at least nine *SAP* genes. Although the substrate specificity of the Sap isoenzymes seem to be similar, they differ in pH optima, thermal stability and, more importantly, in expression pattern. [29-35,49]. It seems that *C. albicans* provides a set of genes, whose regulated expression allows the fungus to persist on different host surfaces, to resist host defence mechanisms and to penetrate into deeper tissue [57,58]. Expression of distinct *SAP* genes during infection has been demonstrated [48,49], and in one study inhibition of Saps efficiently prevents disseminated infection [56]. In addition, the disruption of a single *SAP* gene (*SAP*2) produced a strain avirulent in experimental vaginal infection. However, it remains to be investigated which Saps are essential for other types of infection. Further purification and characterization of those essential Sap isoenzymes will allow the future design of new Sap inhibitors with potential anticandidal activity [33,37].

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