Candida albicans proteinases: resolving the mystery of a gene family

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Overview

Fungal infections of mucosal surfaces are extremely common, debilitating and often recurring diseases, which are frequently caused by the yeast Candida albicans. Furthermore, in the severely immunocompromised host, C. albicans may also cause deep-seated or even life-threatening systemic infections. In order to colonize, infect and evade host defence mechanisms, C. albicans possesses a repertoire of virulence attributes. In particular, the secreted aspartic proteinases (Saps), encoded by the SAP gene family with ten members, appear to play a major role in C. albicans virulence. The SAP family is differentially regulated and distinct members are expressed under a variety of laboratory growth conditions and during experimental C. albicans infections in vitro and in vivo. The contribution of the Saps to C. albicans pathogenesis has been clearly demonstrated using SAP-deficient mutants and proteinase inhibitors. These studies demonstrated that different SAP genes appear to be crucial for mucosal and systemic infections, and are involved in C. albicans adherence, tissue damage and evasion of host immune responses. Therefore, the Sap isoenzymes appear to have a variety of functions in vivo, which are probably called upon at different stages and in different types of C. albicans infection. This review aims to summarize the more recent data regarding the contribution of the secreted proteinases to C. albicans virulence and strives to explain why C. albicans possesses such a gene family.

Opportunistic fungal infections: from commensal to pathogen

C. albicans is a ubiquitous mucosal commensal yeast that is commonly isolated from the oral cavity, the gastro-intestinal tract and the vagina, where it resides in equilibrium with the microbial flora and the host immune system. The physiological status of the host is the primary factor governing the aetiology of candidosis. However, the observation that only slight alterations in this physiological state can turn normally harmless commensal yeasts into aggressive pathogens causing mucosal, superficial or even life-threatening systemic infections in the immunocompromised host points to the pathogenic potential of Candida species (Fidel et al., 1999). An increasing number of immunocompromised individuals, such as those with HIV infection, neutropenia, intensive care patients and those treated with immunosuppressive drugs after organ transplantation, experience some form of mucosal Candida infection. In addition, nearly three-quarters of all healthy women experience at least one vaginal yeast infection (Sobel, 1985). Furthermore, while the high mortality rate associated with systemic bacterial infections has declined with the early administration of broad-spectrum antibiotics, systemic fungal infections have become increasingly significant in causing morbidity and mortality in immunocompromised patients (Fidel et al., 1999). As a result, Candida has become the fourth most common nosocomial bloodstream pathogen in the USA (Jarvis, 1995). Yet despite the growing importance of Candida infections in medicine, many of its interactions with the host are still poorly understood.

Virulence attributes of C. albicans

The process, development and course of microbial infections may be regarded as an encounter between the virulence of a micro-organism and the ability of the host to prevent or resist microbial colonization or invasion. Pathogenic bacteria, having a relatively small genome, often develop very specific ways of causing host infections. For example, several bacteria have developed highly specialized virulence factors, which are adapted to intracellular survival within a specific host cell type, and in a few cases single toxins may cause disease. Conversely, C. albicans is not a prokaryote but a
The virulence factors expressed or required by C. selectivity expressed under suitable predisposing conditions, causing pathogen is finely balanced, and is attributable to the nature of the host response. Thus, but non-pathogenic yeast attributes distinct from those of the closely related, Saccharomyces cerevisiae, which indicates that this fungus possesses virulence be highly adapted to an existence on and within the host, and inflict damage directly or in a larger repertoire of virulence attributes in order to adapt to humans as a commensal organism, possesses C. albicans to cause infections may well vary, depending on the nature of the host. Although a number of potential virulence factors have been suggested for C. albicans, cell morphology, adhesion factors, phenotypic switching and extracellular lipolytic or proteolytic activity have long been recognized as the most credible (Odds, 1994). Extracellular proteolytic activity had already been discovered in the mid-sixties (Staib, 1965) but it was not until the early nineties, when molecular methods were introduced into the Candida field, that scientists began to understand the genetic complexity of this fungus. For instance, a gene (SAP1) encoding an extracellular proteinase was cloned in 1991 and was thought to be responsible for the observed secretory aspartic proteinase (Sap) activity of C. albicans (Hube et al., 1991). However, a detailed study of the C. albicans genome in recent years indicated that SAP1 was just the tip of an iceberg and revealed that the fungus possesses an arsenal of ten SAP genes that encode extracellular proteinases. The fact that C. albicans can encounter a large number of different tissues during the infection process in vivo may provide clues as to why it has evolved to possess such a large repertoire of SAP genes.

**The SAP gene family**

Shortly after SAP1 was discovered, more genes encoding extracellular aspartic proteinases were identified, by PCR-based cloning strategies (Wright et al., 1992), by using SAP1 as a probe (Monod et al., 1994, 1998; White et al., 1993), by sequencing the promoter region of SAP1 (Miyasaki et al., 1994) or by BLAST searches and sequence alignments in the Candida genome databases (e.g. SAP10, accession number AF146440). The deduced proteins are all aspartic proteinases and share a number of Sap-specific characteristics (Fig. 1). All ten SAP genes encode preproenzymes approximately 60–200 amino acids longer than the mature enzyme. The N-terminal secretion signal is cleaved by a signal peptidase in the endoplasmic reticulum (ER) (Fig. 2). The propeptide is removed to activate the proteinases by the subtilisin-like Kex2 proteinase in the Golgi before being transported via vesicles to the cell surface for secretion or glycosylphosphatidylinositol (GPI)-anchoring. Although Kex2 may be a key regulatory proteinase of Saps (Newport & Agabian, 1997), other alternative processing pathways are thought to exist (Togni et al., 1996), and autoactivation was shown to occur extracellularly for Sap1–3 and Sap6 at certain pH values (Koelsch et al., 2000). The mature enzyme contains sequence motifs typical for all aspartic proteinases, including the two conserved aspartate residues of the active site. Conserved cysteine residues are probably implicated in maintaining the three-dimensional structure of the enzyme (Hube, 1996). Unlike Sap1–8, Sap9 and Sap10 both have C-terminal consensus sequences typical for GPI proteins (Fig. 2).

While in the early nineties efforts concentrated on the collection of sequence data, the increasing numbers of cloned SAP genes soon shifted the interest towards the role and function of the SAP gene family during the infection process. The fact that the presence of a SAP gene family was unique to only the most pathogenic Candida species, such as C. albicans (Mage et al., 1993, 1994), C. dubliniensis (Gilfillan et al., 1998), C. tropicalis (Zaugg et al., 2001) and C. parapsilosis (Monod et al.,

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**Fig. 1.** Dendrogram of the C. albicans Sap isoenzyme family based on amino acid sequences. Three distinct groups are clustered within the family. Sap1–3 are up to 67% and Sap4–6 up to 89% identical, while Sap7 is only 20–27% identical to other Saps. Sap9 and Sap10 both have C-terminal consensus sequences typical for GPI proteins. Reprinted from Stehr et al. (2000) with permission of the publisher.
**Candida SAP gene family**

Fig. 2. Processing and secretion of Saps via the secretory pathway and their optimal pH activity ranges. **SAP** genes are translated as preproprotein into the ER. The signal peptide is processed by the signal peptidase (arrow) in the ER. The propeptide contains two Lys-Arg processing sites, which are targeted by the proteinase Kex2 (arrow) in the Golgi apparatus or by alternative activation processes (see text). The propeptide was found to be essential for proper folding of Saps (Beggah et al., 2000). The mature Sap proteins are transported by vesicles via the cell membrane into the cell wall and secreted into the extracellular space. Sap1–3 are mostly active between pH 2 and 5 while Sap4–6 are active at a higher pH range (3–7) and thus may act in different host environments. Sequence analysis of Sap9 and Sap10 suggested that both proteinases are GPI-proteins anchored in the cell membrane or cell wall (Caro et al., 1997).

1994), but was absent in the non-pathogenic yeast *S. cerevisiae*, supported the view that these proteinases may be involved in virulence. However, in the light of the discovery that only a single isoenzyme, Sap2, was necessary and sufficient to allow rapid growth of *C. albicans* in media containing protein as the sole source of nitrogen (Hube et al., 1994; White & Agabian, 1995), it remained a mystery as to why this fungus possessed a whole family of **SAP** genes. As one possible explanation may be that different proteinases are required to act upon different host proteins and tissues in vivo, a number of studies dealt with the possible targets of Saps.

Possible target proteins of *C. albicans* proteinases in vivo

At the most basic level, one role of the *C. albicans* Saps may be to simply digest host proteins to provide nitrogen for the cells. Since Sap2 activity enables the fungus to grow efficiently in media containing serum albumin or other proteins as a sole source of nitrogen, nutrient acquisition is a likely function of the proteinases. However, the Saps may have also adapted and evolved to have more direct virulence functions. For example, Saps could contribute to host tissue adhesion and invasion by degrading or distorting host cell surface structures and intercellular substances, or by destroying cells and molecules of the host immune system to avoid or resist antimicrobial attack. Such activities for Sap2 have been shown in vitro (reviewed by Ruechel, 1992; and Hube, 1996, 1998). Extracellular matrix and host surface proteins such as keratin, collagen, laminin, fibronectin and mucin are efficiently degraded by Sap2. Several host defence proteins, such as salivary lactoferrin, the proteinase inhibitor α-macroglobulin, enzymes of the respiratory burst of macrophages and almost all immunoglobulins, including secretory IgA, which is normally resistant to most bacterial proteinases, can also be hydrolysed by Sap2. *C. albicans* Saps may also act on host proteolytic cascades with numerous effects, which have no obvious advantage for the fungus. For example, Sap2 can activate host protein precursors of the blood clotting cascade (Kaminishi et al., 1994), inactivate the epidermal cysteine proteinase inhibitor cystatin A (Tsushima et al., 1994), and cleave human endothelin-1 precursor (a vasoconstrictive peptide) to alter vascular homeostasis (Tsushima & Mine, 1995). Such activities may be responsible for the enhanced overall circulating proteolytic activity observed in traumatized mice challenged with *C. albicans* (Neely et al., 1994). Furthermore, Candida proteinases have been shown to activate the.
proinflammatory cytokine interleukin-1β from its precursor, suggesting a role for Sap5s in the activation and maintenance of the inflammatory response at epithelial surfaces in vivo (Beausejour et al., 1998).

These studies suggested that Sap2, in contrast to the highly substrate-specific enzymes produced by certain bacteria, has very broad substrate specificity and may have multiple targets in vivo. However, this raises the question of why, if a single proteinase has such a range of activities and functions, does C. albicans need a family of ten SAP genes? Magee et al. (1993) postulated that the Sap isoenzymes might have a variety of functions in vivo, which may be called upon at different sites, and during different stages and types of C. albicans infection.

**SAP gene expression in vitro and in vivo**

The presence of ten SAP genes indicated that different proteinases might target a variety of host cells and tissues during the infection process. If this were the case, one would expect the various members of the SAP family to be differentially regulated and expressed under a variety of laboratory growth conditions and during C. albicans infections in vivo. Accordingly, a number of studies have addressed this issue (Hube, 2000).

Under most proteinase-inducing conditions in the laboratory, the major proteinase gene expressed in C. albicans yeast forms is SAP2, which was found to be regulated by a positive feedback mechanism: peptides resulting from proteolysis of high-molecular-mass proteins were proposed to lead to the induction of SAP2 gene expression (Hube et al., 1994). In contrast, SAP1 and SAP3 were discovered to be differentially expressed during phenotypic switching in certain strains (Morrow et al., 1992; White et al., 1993). However, later studies indicated the regulation of SAP3 during switching was not absolute (Hube et al., 1994; White & Agabian, 1995; Smolenski et al., 1997). Expression of SAP8 is temperature-regulated (Monod et al., 1998) and SAP9 and SAP10 are constitutively expressed under most environmental conditions in both yeast and hyphal forms (A. Felk, W. Schäfer & B. Hube, unpublished results). Since most aspartic proteinases are only active under acidic conditions, it was perhaps a surprising discovery that the SAP4–6 genes were almost exclusively expressed during hyphal formation at neutral pH, even in defined protein-free media (Hube et al., 1994; White & Agabian, 1995). These studies demonstrated that the SAP gene family was differentially expressed in vitro and further suggested that, in contrast to the induction of SAP2, expression of other SAP genes was not dependent on the presence of exogenous protein or peptides.

The in vitro demonstration of distinct SAP expression patterns in yeast, hyphal and phenotypically switched cells indicated that proteinase expression was a highly regulated and tightly controlled process. However, this did not address the issue of whether the Sap family contributed to the pathogenicity of C. albicans in vivo. For this reason it was crucial to first ascertain whether the proteinases were also differentially expressed during C. albicans infections; this was demonstrated using in vitro and animal infection experimental models.

**In vitro** experimental models of oral (Schaller et al., 1998) and cutaneous (Schaller et al., 2000) C. albicans infections suggested that SAP1–3 were the main proteinases expressed during superficial infections. This supposition was supported by the detection of SAP1 and SAP2 transcripts in a rat vaginitis model (De Bernardis et al., 1995). In contrast to mucosal infection models, experimental models of systemic C. albicans infections correlated SAP4–6 expression with systemic disease (A. Felk and others, unpublished results; Staib et al., 2000). However, using in vivo expression technology, Staib et al. (1999) also demonstrated SAP2 expression in late stages of systemic infections. Taken together, these in vitro experimental and animal model data correlated SAP gene expression with C. albicans virulence and demonstrated differential SAP gene expression during different types of C. albicans infection.

Whether these models were representative of proteinase expression during human mucosal and systemic infections was not known. However, high titres of anti-Sap antibodies have been observed in sera of candidosis patients, indicating the presence of Sap antigen during infection (Rüchel, 1992). In addition, Sap antigens have been detected in biopsies of oral epithelial lesions collected from HIV-infected patients (Schaller et al., 1999a) and in almost all organs of immunocompromised patients who had died of systemic C. albicans infections (Rüchel et al., 1991). Nevertheless, only two studies have investigated the expression of the SAP gene family during human C. albicans infections. Schaller et al. (1998) showed that the SAP genes were indeed expressed during oral candidosis. Moreover, Naglik et al. (1999) and J. R. Naglik and others (unpublished results) were able to demonstrate differential expression of the SAP gene family, with SAP1, SAP3 and SAP7 transcripts predominantly being expressed in patients with oral C. albicans infection as opposed to oral C. albicans carriers.

Therefore, differential expression of the proteinase family has been demonstrated in culture media, during in vitro and animal experimental infection models and during human C. albicans infections. Such differential expression suggested a more specific role or function for the different SAP genes. However, although these studies correlated SAP gene expression with the ability of C. albicans to cause infection, they did not directly demonstrate that Sap proteinases actually contributed to the pathology or virulence of C. albicans infections.

**Contribution of Saps to the pathogenesis of C. albicans infections**

Strong evidence existed to indicate that Candida proteinases were differentially expressed in vivo and had the potential to degrade a large number of host proteins, but their direct contribution to pathogenesis was still unknown. However, recent studies using proteinase
inhibitors and SAP-disrupted or SAP-overexpressing mutants have compellingly demonstrated that Saps indeed contribute to the virulence of *C. albicans*. Inhibition of Saps with the aspartic protease inhibitor pepstatin A prevented the initial penetration of *C. albicans* through mucosal surfaces, but not the dissemination of the fungus once the cells had already reached the blood vessels (Fallon et al., 1997). Moreover, pepstatin A prevented *C. albicans* invading and causing tissue damage in oral, vaginal and skin experimental infection models (Schaller et al., 1999b; De Bernardis et al., 1997). In addition, HIV aspartic protease inhibitors, which also act on the *C. albicans* Saps (Gruber et al., 1999; Borg-von Zepelin et al., 1999; Cassone et al., 1999; Korting et al., 1999), can inhibit *C. albicans* adherence to epithelial cells (Borg-von Zepelin et al., 1999). Taken together, these data indicate that Sap activity may be crucial during the early stages of *C. albicans* infections.

Although Sap2 was the dominant *C. albicans* extracellular proteinase in *vitro*, the *in vivo* expression pattern of SAP genes suggested that Sap2 was not the only proteinase and certainly not the most dominant proteinase acting *in vivo*. Therefore, to determine to what extent the different Saps contributed to the different types of *Candida* infection, mutants lacking Table 1 or overexpressing (Dubois et al., 1998; Kvaal et al., 1999) distinct SAP genes were created and used in experimental infections. These studies showed that not only were distinct Sap isoenzymes required during different types of infection, but also that not all *Candida* proteinases were important for virulence *per se* (Dubois et al., 1998). Using SAP-deficient mutants, Schaller et al. (1999b) demonstrated that SAP1–3, but not SAP4–6, contributed significantly to experimental *C. albicans* infections of artificial oral mucosa. A major role for SAP1–3, but not SAP4–6, was also demonstrated in experimental vaginal infections using SAP-disrupted mutants (DeBernardis et al., 1999). Using a gene misexpression strategy in the switching strain WO-1 in which white-phase cells misexpressed the opaque-specific gene SAP1, Kvaal et al. (1999) demonstrated in a cutaneous mouse model that SAP1 caused a dramatic increase in cutaneous infection, probably as a result of increased adherence to, and ‘cavitation’ of, the skin.

In contrast, SAP4–6, but not SAP1–3, appeared to be critical for systemic infections, as mutants lacking SAP4–6 were strongly attenuated in two rodent models of intravenous infection (Hube et al., 1997; Sanglard et al., 1997) and during intraperitoneal infection (Kretschmar et al., 1999) (Table 1). In this model, SAP4–6 may contribute to virulence by resisting phagocytic attack, since the corresponding proteinases were mostly expressed by *C. albicans* in phagolysosomes of murine peritoneal macrophages and neutrophils (Borg-von Zepelin et al., 1998; A. Felk and others, unpublished)

### Table 1. Use of SAP-deficient mutants to determine a role for the proteinases during *C. albicans* infections

<table>
<thead>
<tr>
<th>Study</th>
<th>Main findings</th>
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<tr>
<td><strong>Mucosal infections</strong></td>
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<tr>
<td>Watts et al. (1998)</td>
<td><em>sap1</em>, <em>sap2</em> and <em>sap3</em> null mutants were less adherent to buccal epithelial cells than the parental strain. The <em>sap4–6</em> triple mutant showed significantly increased adherence.</td>
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<td>Schaller et al. (1999b)</td>
<td><em>sap1</em>, <em>sap2</em> and <em>sap3</em> mutants caused less tissue damage than the parental strain. A <em>sap1</em>/<em>3</em> double mutant caused fewer lesions than the two <em>sap1</em> or <em>sap3</em> mutants individually. <em>sap4–6</em> mutant showed equal or enhanced tissue damage.</td>
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<td>De Bernardis et al. (1999)</td>
<td><em>sap1</em>, <em>sap2</em> and <em>sap3</em> mutants, but not the <em>sap4–6</em> mutant, were less virulent in a rat vaginitis model compared with the parent strain. The <em>sap2</em> mutant was almost avirulent.</td>
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<td><strong>Systemic infections</strong></td>
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<td>Ibrahim et al. (1998)</td>
<td>No difference in adherence to endothelial cells between the <em>sap1</em>, <em>sap2</em> and <em>sap3</em> null mutants and the parental strain. The <em>sap2</em> mutant caused less damage to endothelial cells.</td>
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<td>Kretschmar et al. (1999)</td>
<td>The <em>sap4–6</em> mutant, but not <em>sap1</em>, <em>sap2</em> or <em>sap3</em> mutants, caused less tissue damage and invasion in peritoneal infections.</td>
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<td>Hube et al. (1997);</td>
<td><em>sap1</em>, <em>sap2</em>, <em>sap3</em> and, in particular, <em>sap4–6</em> mutants were less lethal in two animal models compared with the parent strain. The <em>sap4–6</em> mutant displayed the greatest attenuation.</td>
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<td>Sanglard et al. (1997)</td>
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<td><strong>Interactions with immune system</strong></td>
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<tr>
<td>Borg-von Zepelin et al. (1998)</td>
<td>The <em>sap4–6</em> triple mutant was eliminated 53% more effectively after phagocytosis by macrophages than the parent strain.</td>
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results). Furthermore, SAP4–6-deficient mutants were shown to be hypersensitive to phagocytic killing by macrophages (Borg-von Zepelin et al., 1998).

These data clearly demonstrate that Saps contribute to the pathogenesis of Candida infections. However, it should be noted that mutants lacking individual SAP genes rarely exhibit a full avirulent phenotype in a particular infection model. This may indicate a synergistic effect of a number of proteinases during certain infections, or the involvement of other virulence factors. Nonetheless, these studies illustrate that not all of the proteinases are required at the same time or stage of the infection process and not all contribute to the same types of infection. Determining the precise roles and functions of the C. albicans proteinases in vivo should provide a big step forward in identifying which proteinases, or set of proteinases, may contribute to, or even be responsible for, specific types of C. albicans infection.

Why does C. albicans possess a SAP gene family?

There are several possible reasons or circumstances that would make the assembly of a family of proteinases necessary for C. albicans.

Firstly, the different Sap isoenzymes may have adapted and evolved to function in different tissues and environments. Such a view may also explain how the gene family evolved: C. albicans may have duplicated a successful factor (an ancestral proteinase), which in turn adapted to different host environments and thus evolved into homologous but functionally distinct Sap isoenzymes. This hypothesis may be supported by the fact that all the SAP genes encode similar amino acid sequences (Fig. 1). However, since only SAP1 and SAP4 are clustered in tandem and all other SAP genes are located on five different chromosomes, this gene duplication event probably occurred sometime in the distant past. Alternatively, it is possible that the high genetic flexibility of C. albicans may have accelerated this widespread distribution of the SAP genes.

Although the overall similarity of the SAP genes is high, differences in their promoter sequences indicate that expression of the various SAP genes is controlled by different SAP-specific transcriptional regulators. This would suggest that the SAP genes might have evolved to possess distinct properties and functions, which indeed appears to be the case. For example, different Saps have been shown to have different pH optima for activity (Borg-von Zepelin et al., 1998). Sap2 acts mainly at acidic pH values around pH 4.0, Sap4–6 are significantly active at physiological pH and Sap3 still has activity at pH 2.0. This provides C. albicans with a range of proteolytic activity from pH 2.0 to 7.0, a property that may prove essential for the specific adaptation of individual Saps to different host environments.

It seems surprising that all secreted proteinases of C. albicans are aspartic proteinases and that neither extra-}

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fails (i.e. the enzyme may be functionally redundant), is removed or is otherwise lost. There is evidence for such compensatory mechanisms in C. albicans. For instance, disruption of SAP1 and SAP3 genes, which may be required for oral infection, lead to increased expression of SAP5 and SAP8, suggesting that C. albicans may be attempting to compensate for the loss of key genes by upregulating alternative genes (Schaller et al., 1999b). Such balanced regulation is likely to be a general phenomenon in C. albicans.

Conclusion and future directions

The presence of a SAP gene family in C. albicans clearly provides the fungus with an efficient and flexible proteolytic system that may prove vital to its success as an opportunistic pathogen. Furthermore, Sap production is a highly regulated and tightly controlled process, which appears to be a central factor in many aspects of C. albicans virulence and is indicative of the multiple functions this gene family possesses. These include the simple role of digesting molecules for nutrient acquisition, the contribution to host tissue invasion by digesting or distorting host cell membranes, the degradation of host surface molecules to enhance adhesion, and the digestion of cells and molecules of the host immune system to avoid or to resist antimicrobial attack.

However, there are still many unanswered questions that need to be addressed. (1) How is proteinase expression regulated? The signal transduction pathways that control the differential expression of SAP genes are currently unknown and no receptor has yet been implicated in the regulation of C. albicans proteinase secretion. (2) What are the actual targets of Saps during human infections? Although the possible targets of the proteinases have been deduced from in vitro studies, these need to be confirmed in the in vivo environment. Furthermore, as the substrate specificity of the Saps is so broad and most Sap antigen is detected within the C. albicans cell wall (Rüchel et al., 1991; Schaller et al., 1999a), how does the fungus manage to protect itself from proteolytic digestion? (3) The precise roles and functions of the SAP genes, especially SAP7, SAP9 and SAP10, during human infections are currently unknown, as is the reason why Sap9 and Sap10 have C-terminal GPI-anchoring sequences. Do these two proteinases have functions similar to the GPI-anchored yapsins of S. cerevisiae (Komano et al., 1999) or do they contribute to C. albicans infection? (4) The immunological consequences of Sap secretion and other interactions of the proteinases with host factors in vivo are largely unknown and need to be addressed.

In summary, this review has endeavoured to elucidate the possible reasons why C. albicans is equipped with a family of proteinase genes and suggests a number of possible explanations for their evolution in light of the information available. However, there are several shortfalls that need to be tackled before the biological significance of this gene family is fully understood. With access to the complete genome sequence of C. albicans and with the development of new and exciting tools such as DNA microarray analysis, our understanding of the SAP gene family and its interaction with the human host should rapidly increase.

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