Overexpression of *Candida albicans* secretory aspartyl proteinase 2 and its expression in *Saccharomyces cerevisiae* do not augment virulence in mice

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To elucidate the implications of secreted aspartyl proteinase (Sap)2p in the pathogenesis of *Candida* infections, the *SAP2* gene was expressed in *Saccharomyces cerevisiae* and overexpressed in *Candida albicans*. The coding region of *SAP2*, including its signal sequence and propeptide, was amplified by PCR and cloned downstream of the *S. cerevisiae* or *C. albicans* *ADH1* promoter. Plasmid expression of *SAP2* in *S. cerevisiae* showed that the signal peptide was functional. Integrative transformation of *S. cerevisiae* and *C. albicans* was accomplished by homologous recombination within the *URA3* locus for *S. cerevisiae* and the *SAP2* locus for *C. albicans*. Negative control transformants carried plasmids either without the *SAP2* insert or with mutated *sap2*. *S. cerevisiae* and *C. albicans* transformants showed similar growth rates to their parental strains or negative controls, when grown in medium containing amino acids. However, in medium with BSA as sole nitrogen source, constitutive expression of *SAP2* enabled *S. cerevisiae* to grow and increased the growth rate of *C. albicans*. In both media, only *S. cerevisiae* transformants harbouring *SAP2* secreted the enzyme, as confirmed by proteinase activity assays and immunoblotting. When *C. albicans* was grown in amino acids medium, the enzyme was detected exclusively in transformants constitutively expressing *SAP2*. However, in BSA medium these strains secreted enzyme earlier and secreted higher amounts of enzyme and total proteinase activity. In pathogenicity studies in intact mice, expression of Sap2p as a sole putative virulence factor did not cause *S. cerevisiae* to become virulent and constitutive overexpression of *SAP2* did not augment virulence of *C. albicans* in experimental oral or systemic infection.

Keywords: candidiasis, *Candida albicans*, aspartyl proteinase, *Saccharomyces cerevisiae*

INTRODUCTION

Candida albicans is an important opportunistic pathogen causing local or systemic infection in immunocompromised patients (Odds, 1988). Several virulence factors have been proposed in pathogenicity: adhesion, dimorphism, phenotypic switching, molecular mimicry of mammalian integrins and secretion of phospholipases

and aspartyl proteinases (Cutler, 1991; White *et al.*, 1995). The secreted aspartyl proteinases (Saps) have been studied extensively and are encoded by at least eight genes which are expressed and regulated differentially (Hube, 1996). Northern analysis has shown that *SAP1* and *SAP3* are regulated during phenotypic switching between the white and opaque forms of strain WO-1, while *SAP2* was found to be the dominant transcript in budding cells grown in medium containing protein as sole nitrogen source. The expression of *SAP4–6* is detected at neutral pH during serum-induced

Abbreviation: YNB, Yeast Nitrogen Base.

yeast to hyphal transition, but the expression of *SAP7* has not been detected (Hube *et al.*, 1994; White & Agabian, 1995).

In clinical and laboratory strains grown as the yeast form *in vitro*, Sap2p is the predominantly expressed proteinase. Evidence has also been provided that Saps are secreted by *C. albicans* *in vivo* during the course of mucosal and deep-tissue infections (Borg & Ruchel, 1988; De Bernardis *et al.*, 1995). The most commonly used approach to define the role of Saps as virulence attributes has been to compare the lethal effect on mice infected intravenously with parental strains or proteinase-deficient mutants. The mutants, isolated by chemical mutagenesis techniques or UV-irradiation, have been shown to be less virulent than their parental strains. However, definitive proof of the role of Saps in virulence of *C. albicans* has been lacking, because either (i) the mutants had high levels of reversion, (ii) they still produced detectable proteinase activity *in vitro*, (iii) the nature of the mutation was undefined or (iv) they may harbour multiple, independent and unknown lesions (Macdonald & Odds, 1983; Kwon-Chung *et al.*, 1985; Crandall & Edwards, 1987; Ross *et al.*, 1990).

Recently, *C. albicans* strains harbouring targeted disruptions of *SAP* genes have been constructed using the *ura*-blaster protocol (Hube *et al.*, 1997; Sanglard *et al.*, 1997). When tested in a murine model of systemic candidiasis, the virulence of *C. albicans* *sap1*, *sap2* and *sap3* mutants was modestly attenuated (Hube *et al.*, 1997). However, the extent of attenuation of virulence did not correlate directly with the reduction of proteolytic activity *in vitro*. Similarly, mice infected with a *C. albicans* *sap4,5,6* triple homozygous null mutant had survival times significantly longer than that of control animals (Sanglard *et al.*, 1997). In *Candida tropicalis*, disruption of the *SAPT* gene was also achieved by co-transformation with a linear DNA fragment carrying a deletion in *SAPT* (Sanglard *et al.*, 1992). Surprisingly, the virulence of the *sapT*-null mutant and the proteinase-positive strain did not differ significantly after intravenous infection in mice (Togni *et al.*, 1994). These results suggested that the Sap of *C. tropicalis* does not contribute significantly to fungal virulence in systemic infections. Alternatively, potentially decreased virulence may have been masked by the redundancy of members of a putative multigene Sap family in *C. tropicalis* (Monod *et al.*, 1994) and/or the redundancy of other virulence attributes of *Candida*.

Selective expression/overexpression of genes encoding putative virulence attributes is an attractive strategy to overcome redundancy problems and clarify the contribution of each isoenzyme to virulence. This approach would allow the study of virulence-enhancing genes in already virulent strains (homologous overexpression) and in non-pathogenic but closely related strains (heterologous expression). The rationale for this approach is also based on clinical studies which have shown that the Sap activity of *C. albicans* isolates from patients with vaginitis (Cassone *et al.*, 1987) or HIV infection (Ollert *et al.*, 1995) was significantly higher than that of isolates

from asymptomatic carriers. In this study, we describe the construction of stable *Saccharomyces cerevisiae* and *C. albicans* strains that constitutively secrete Sap2p. Sap2p expression by *S. cerevisiae* allowed it to hydrolyse and use BSA as sole nitrogen source, while Sap2p overexpression in *C. albicans* resulted in early and high-level proteinase secretion. The virulence of these genetically engineered strains was evaluated in murine models of oral or systemic infections.

METHODS

Strains and culture conditions. Yeast strains used in this work are listed in Table 1. *Escherichia coli* DH10B (Gibco-BRL) was routinely used as a plasmid host for cloning procedures. Bacterial and yeast media were prepared as described by Rose *et al.* (1990) and Sambrook *et al.* (1989). Yeast parental strains were grown in YPD medium containing 1% (w/v) yeast extract (Difco), 2% (w/v) Bacto peptone (Difco) and 2% (w/v) dextrose. Uracil/uridine independence selection was performed by using CAT medium [0.67% Yeast Nitrogen Base (YNB) without amino acids (Difco), 0.5 or 1% (w/v) Casamino acids, 0.008% adenine, 0.002% tryptophan] supplemented with 2% dextrose. The medium to regenerate *C. albicans* spheroplasts was supplemented with 1 M sorbitol. Induction of Sap2p was done in YNB-BSA medium containing 0.17% YNB without amino acids and ammonium sulfate (Difco), supplemented with 2% dextrose and 0.2% BSA (fraction V; Sigma). Media were supplemented with 100 µg uracil/uridine ml⁻¹ as required. All yeast cultures were incubated at 30 °C, except for experiments on the kinetics of Sap2p secretion by *S. cerevisiae* and *C. albicans* which were conducted at 37 °C. Growth rates were monitored by measuring OD₆₀₀. Media were solidified with 2% (w/v) agar. Germ tube formation was evaluated by incubating in 5% (v/v) newborn calf serum (Gibco-BRL) for 3 h at 37 °C.

PCR amplification of *SAP2* gene. Genomic DNA from *C. albicans* LAM-1, obtained as described by Magee *et al.* (1987), was used as template for PCR amplification of *SAP2*. The two primers used were 5' CGGGATATCAACAACCCACTAGACATCACC and 5' CTGGAGCTCCACCCCTTCATCTTAGGTCAG. These primers flank the *SAP2* coding sequence with its own signal peptide. The underlined sequences indicate *EcoRV* and *SacI* restriction sites, respectively. The 100 µl PCR reaction contained 1 µg genomic DNA template, 1 µg each of the two primers, 400 µM each of dATP, dGTP, dCTP and dTTP, 10 µl DMSO, 2 U Vent DNA polymerase (New England Biolabs) and 1 × Vent buffer. The PCR program was 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min for 30 cycles. A 1244 bp PCR product was gel-purified and cloned into pBluescript KS II(+) (Stratagene) after digestion with *EcoRV* and *SacI*. The PCR product sequence was verified by double-strand sequencing with universal and internal primers (Sanger *et al.*, 1977).

Plasmid constructions. Recombinant DNA manipulations were done by standard methods (Sambrook *et al.*, 1989). Plasmid pVTU-SAP2 was constructed by inserting the *SAP2* PCR product, digested by *EcoRV* and *Ecl136II*, into the *Ecl136II* site of *S. cerevisiae* shuttle vector pVT102-U (Vernet *et al.*, 1987). A negative control, pVTU-aSAP2, carried *SAP2* in antisense orientation. pVT102-U is a 2 µ-based plasmid bearing the *ADH1* promoter (*ADH1p*), its terminator region and the *URA3* marker gene. From pVTU-SAP2, a 2 kb *Spb1* fragment containing *SAP2* downstream of *ADH1p*, was blunt-ended with T4 DNA polymerase and inserted into the *SmaI* site of pRS306, a *S. cerevisiae* integrative plasmid containing

Table 1. Yeast strains

Strain	Genotype	Source or reference
<i>S. cerevisiae</i>		
cIABYS86	<i>MATα leu2 ura3-52 his3 pra1 prb2 prc1 cps</i>	A. H. Bussey (McGill University, Canada)
PUB754	<i>MATα ura3-52 pra1 prb2 prc1 cps</i>	This study
SEY6210	<i>MATα leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1Δ901 suc2Δ9</i>	Robinson <i>et al.</i> (1988)
Y55	Laboratory isolate, <i>HO</i>	Clemons <i>et al.</i> (1994)
YJM128	Clinical isolate	Clemons <i>et al.</i> (1994)
YJM273	Clinical isolate	Clemons <i>et al.</i> (1994)
<i>C. albicans</i>		
CAI4	<i>Δura3::imm434/Δura3::imm434</i>	Fonzi & Irwin (1993)
LAM-1	Clinical isolate	Lacasse <i>et al.</i> (1990)
SC5314	Clinical isolate	Gillum <i>et al.</i> (1984)

the *URA3* marker gene (Sikorski & Hieter, 1989). This plasmid was named pRS-SAP2. Finally, the *C. albicans* vector containing *C. albicans ADH1p* and *URA3* marker, YPB-ADH1p (kindly provided by A. J. P. Brown, University of Aberdeen, UK), was used to insert the 1244 bp *EcoRV*-*Ecl*136II *SAP2* fragment into *EcoRV* site under the control of *C. albicans ADH1p*. This construct was named pYPB-SAP2. A negative control plasmid, pYPB-mSAP2, was generated by inserting a mutated gene, *sap2*. The mutation was introduced by cleavage of *SAP2* with *Bam*HI, filled-in with Klenow DNA polymerase and religated. This introduced a frameshift mutation (verified by sequencing) after the sequence encoding aa 77 and a premature stop codon 9 bp (3 aa) further downstream.

Yeast transformation. *S. cerevisiae* strains grown overnight in YPD medium at 30 °C, were transformed using the lithium acetate/ssDNA/PEG procedure described by Schiestl *et al.* (1993). Strains cIABYS86 (S86), PUB754 (754) and SEY6210 (6210) were transformed with pVTU-SAP2. PUB754, a *His*⁺ *Leu*⁺ derivative of S86, was obtained by two separate 'one-step gene replacement' transformations with *HIS3* and *LEU2* DNA fragments (Rothstein, 1983). Integration of *SAP2* in the *S. cerevisiae URA3* locus was done by transforming strain 754 with pRS-SAP2, linearized by *Nco*I in *URA3*. Negative controls were also constructed by transforming strain 754 with *Nco*I-linearized plasmid pRS306.

Transformation of *C. albicans* CAI4 was done by the spheroplast method as described by Rose *et al.* (1990), with minor modifications. Briefly, CAI4 yeast cells grown overnight in YPD were diluted in YNB-BSA medium (supplemented with uridine) to an OD₆₀₀ of 0.2. Then, exponentially growing cells (OD₆₀₀ of 0.5) were used to prepare the spheroplasts. pYPB-SAP2 and pYPB-mSAP2 plasmids were linearized by cutting at the *Kpn*I site within *SAP2* and used to transform the spheroplasts by integration at the *SAP2* locus. The *S. cerevisiae* and *C. albicans* transformants, selected by complementation of the *ura3* mutation, were grown on CAT plates.

Proper integration was evaluated by Southern blotting analysis (Belhumeur *et al.*, 1993). Genomic DNA from *S. cerevisiae* transformants, prepared as described by Hoffman & Winston (1987), was digested with *Avr*II and probed with a 693 bp *EcoRV*-*Sma*I *URA3* (see Fig. 2a) or a 597 bp *Bam*HI-*Kpn*I *SAP2* restriction fragment. *C. albicans* genomic DNA, prepared according to Magee *et al.* (1987), was digested with *Kpn*I and probed with a 265 bp *Pst*I-*Dra*I *SAP2* DNA fragment (see Fig. 2b).

SDS-PAGE and Western blotting. Intra- and extracellular protein extracts from *S. cerevisiae* plasmid transformants

grown for 18 h in CAT medium at 30 °C were obtained by lysing with glass beads (Harlow & Lane, 1988) and acetone precipitation, respectively. One-tenth of the original extract volume was loaded in a 12% polyacrylamide separating gel to perform SDS-PAGE (Laemmli, 1970). For time course evaluation of Sap2p expression, 60 μ l culture supernatants was analysed by SDS-PAGE. Following electrophoresis, proteins were transferred onto nitrocellulose membranes (Towbin *et al.*, 1979). Membranes were blocked with 2% (w/v) skim milk in Tris-buffered saline (TBS: 10 mM Tris/HCl, pH 7.5, 150 mM NaCl) and washed between steps with TBS containing 0.1% Tween 20 (TBST). Membranes were incubated for 1 h with mouse anti-Sap2p mAb IFG3, kindly provided by T. L. Ray (University of Iowa), diluted 1:500 in TBS/2% skim milk. The bound antibody was detected using alkaline-phosphatase-conjugated goat anti-mouse IgG (Gibco-BRL) diluted 1:5000 in TBS/2% skim milk.

Enzyme assays. Proteolytic activity was quantified by the method of Milewski *et al.* (1994) with minor modifications. Briefly, culture supernatants (150 μ l) were mixed with 1% BSA in 50 mM sodium citrate, pH 3.5 (600 μ l). After 1 h incubation at 37 °C, reactions were stopped by adding 400 μ l 10% (w/v) TCA. Samples were cooled in an ice bath for 10 min and precipitated proteins were removed by centrifugation at 5000 g for 10 min. The A₂₈₀ of the supernatants was measured and corrected for background using a control (for each point) in which TCA solution was added prior to the culture supernatant. One unit of enzyme activity was defined as the amount of enzyme causing a Δ A₂₈₀ of 0.1 in 1 h.

Pathogenicity in vivo. The virulence of *S. cerevisiae* and *C. albicans* transformants as well as their parental strains was evaluated in murine models of oral and systemic candidiasis. Crl:CD-1 (ICR) BR female mice, weighing 22–24 g, were obtained from Charles River Breeding Farms (St Constant, Québec, Canada) and were kept at the University of Montreal Animal Care Unit. For experimental infections, the yeast inoculum was prepared from cells grown overnight in CAT medium at 30 °C.

The oral infection model described by Chakir *et al.* (1994) was used with minor modifications. Briefly, both *S. cerevisiae* and *C. albicans* yeast cells were washed twice in sterile 0.01 M PBS (pH 7.4) and counted in a haemocytometer. Cells (1×10^8) were distributed in sterile plastic microfuge tubes and pelleted by centrifugation at 13000 g. Six mice per strain were anaesthetized intraperitoneally with 0.35 ml ketamine (10 mg ml⁻¹). They were then inoculated by topical application into the oral cavity using a sterile calcium alginate swab

(Fisher Scientific). A longitudinal quantification of the microorganisms in the oral cavities of individual mice was done by daily oral swabbing until 8 d post-inoculation. Swabs used for sampling were dissolved in 2 ml volumes of Ringer's citrate buffer (Rodrigue *et al.*, 1989) and serial dilutions in PBS were plated on CAT medium supplemented with 50 mg chloramphenicol l⁻¹ (CAT-Chl). Plates were incubated at 30 °C and c.f.u. were counted.

Experimental murine systemic infection (Clemons *et al.*, 1994) was used to study *S. cerevisiae* virulence. Mice were inoculated intravenously with 2×10^7 or 6×10^7 cells, previously washed twice in sterile saline. Six mice per yeast strain were killed at 1, 2 or 4 weeks after infection. To determine organ burden, brains and kidneys were removed aseptically, homogenized in 5 ml sterile saline and plated on CAT-Chl and Sabouraud supplemented with chloramphenicol (SDA-CHL) for determination of c.f.u.

The virulence of *C. albicans* strains was also evaluated in a model of systemic candidiasis. Mice were inoculated intravenously with 5×10^5 cells, previously washed twice in PBS. Mice were observed daily for signs of morbidity over 15 d. Moribund animals were scored as non-survivors and euthanized by CO₂ inhalation. To evaluate tissue invasion, additional groups of infected mice were sacrificed at 1, 3 or 6 d post-inoculation. One kidney from each animal was removed and prepared for histological analysis. They were fixed in 10% formalin, embedded in paraffin, sectioned and stained by the Grocott procedure (Luna, 1992). The remaining kidney and the liver were also removed aseptically, weighed and homogenized in 5 ml PBS. Homogenates were plated on CAT-Chl and SDA-Chl and incubated at 30 °C for colony counting. Data were expressed as c.f.u. (g kidney or liver)⁻¹. Several colonies of *C. albicans* were isolated from both media to verify the *in vivo* stability of SAP2 integration by Southern blotting as described above.

Zymolyase and NaCl resistance. A zymolyase test was used to compare the sensitivity of the cell wall of transformants and parental strains (Lussier *et al.*, 1997). *S. cerevisiae* and *C. albicans* strains were grown in CAT medium to exponential phase (OD₆₀₀ of 0.6), washed twice in sterile saline and resuspended in sterile zymolyase buffer (50 mM Tris/HCl, pH 7.4) at an OD₆₀₀ of 0.635. Aliquots of 400 µl were mixed with 100 µl zymolyase 100T (1 mg ml⁻¹) or 100 µl zymolyase buffer (controls). After 1 h incubation at 37 °C with gentle shaking, 5 µl serial dilutions were spotted on CAT agar medium. *C. albicans* and *S. cerevisiae* strains were also tested for NaCl resistance. Briefly, cultures grown overnight in CAT medium were patched onto CAT plates with 0, 0.2, 0.6, 1.0, 1.4, 1.8, 2.2 or 2.6% NaCl. Sensitivity to zymolyase and NaCl was determined by comparison of the level of growth after incubation at 30 °C.

Statistical analysis. The data were fed to the SAS system (SAS Institute, Cary, NC, USA). The Kaplan & Meier (1958) product limit estimate was used to analyse survival data and plot the survival function. The Wilcoxon method (Dawson-Saunders & Trapp, 1990) was applied to compare the survival functions of experimentally infected mouse populations and differences were considered significant at the 0.01 level.

Differences in weights and c.f.u. (g kidney)⁻¹ were compared among mouse populations by using the general linear models procedure two-way analysis of variance, conducted with two factors, one between (strain) and one within (time). Significant interactions ($P < 0.01$) were further analysed using the Bonferroni (Dunn) *t*-test (Dawson-Saunders & Trapp, 1990) and the resulting comparisons were considered significant at the 0.01 level.

RESULTS

Expression and secretion of Sap2p by *S. cerevisiae*

As an initial approach toward heterologous expression of *C. albicans* SAP2 in *S. cerevisiae*, we performed a PCR amplification of this gene from *C. albicans* LAM-1. The 1244 bp PCR product containing the native prepro and mature coding regions was cloned into the multicopy expression vector pVT102-U downstream of the constitutive *ADH1* promoter, to create the plasmid pVTU-SAP2 (sense orientation). A negative control, pVTU-aSAP2 (antisense orientation), bearing the PCR product in opposite orientation was also constructed. These recombinant plasmids were transformed into *S. cerevisiae* 6210 and S86, the latter defective in intracellular proteinases (Table 1).

The expression of Sap2p was evaluated by Western blotting in intra- and extracellular extracts. As shown in Fig. 1 (lanes 3, 4 and 6), a single positive signal of approximately 42 kDa was detected in the extracts of strains transformed with pVTU-SAP2 (sense orientation). A decreased loading revealed additional forms of 39 and 45 kDa (data not shown). The largest amount of Sap2p was detected in the extracellular fraction (secreted), indicating that the secretion signal was fully functional in *S. cerevisiae*. In addition, there was more abundant production of Sap2p by strain S86, the intracellular proteinases mutant, compared to 6210 (Fig. 1, lanes 4 and 6). We could not determine whether this difference was due to the lack of these proteinases, since these strains are not isogenic. In addition, degradation of casein was demonstrated in a Petri dish assay (Colina *et al.*, 1996a) using unconcentrated culture supernatant

Fig. 1. Western blot analysis of intra- (I) and extracellular (E) proteins of *S. cerevisiae* S86 and 6210 plasmid transformants (with sense or antisense SAP2 constructs) grown in CAT medium for 18 h at 30 °C and probed with an anti-Sap2p mAb. Loaded sample volumes were adjusted to equivalent mass cultures.

Fig. 2. Southern blot analysis of wild-type and selected transformants of (a) *S. cerevisiae* and (b) *C. albicans*. Top, integration sites with probes indicated by thick lines. Bottom, *S. cerevisiae* and *C. albicans* genomic DNAs digested with *AvrII* and *KpnI*, respectively, and probed with 32 P-labelled *EcoRV*-*SmaI* (*S. cerevisiae*) or *PstI*-*DraI* *SAP2* (*C. albicans*) restriction fragments. Shaded areas represent the chromosomal copy of *URA3* and *SAP2*.

of S86 (sense orientation), but was absent in the antisense orientation (data not shown). Based on these results, we decided to select strain S86 harbouring pVTU-SAP2 for further experiments. No anti-Sap2p-reacting material was detected in the strain transformed with pVTU-aSAP2 (antisense orientation; Fig. 1, lanes 1 and 2).

Overall, these findings indicated that the recombinant enzyme was efficiently expressed and secreted into the culture medium and that the signal sequence of *C. albicans* SAP2 was functional in *S. cerevisiae*.

Integrative transformation of *S. cerevisiae* and *C. albicans*

To produce stable *S. cerevisiae* and *C. albicans* transformants that could be used in animal models and for further genetic analysis, plasmids carrying *SAP2* were integrated into the genome by homologous recombination.

(i) *S. cerevisiae*. The integrative plasmid, pRS-SAP2 (see Methods), was linearized at the *NcoI* site in *URA3* and transformed into strain 754. A negative control was obtained by integrating pRS306 without insert. Proper integration was evaluated by Southern blotting after digestion of parental and transformant genomic DNAs with *AvrII*. As shown in Fig. 2(a), a single 2.7 kb fragment hybridized to the *URA3* probe in the parental strain (754) as predicted. In positive transformants (884,

885), we expected a 9.1 kb fragment, resulting from the integration of pRS-SAP2. Unexpectedly, both strains gave a positive signal larger than 15 kb which can be interpreted as a double integration. Because of satisfactory expression of Sap2p, these strains were selected for further studies (see below). A 7.1 kb band was detected in negative control 894, corresponding to the integrated vector without *SAP2*. These findings were confirmed by probing the same blot with a radiolabelled *SAP2* DNA fragment (data not shown).

(ii) *C. albicans*. Plasmids pYPB-SAP2 and pYPB-mSAP2 carrying the wild-type and mutated *SAP2*, respectively, under the control of *ADH1p* were linearized at the *KpnI* site to target the integration into the *SAP2* locus. Genomic DNAs were digested with *KpnI* and probed with the *SAP2* fragment (see Methods). In the parental strain (CAI4), the *SAP2* probe hybridization resulted in a single band larger than 13 kb derived from the wild-type alleles of *SAP2*. The transformants S1, S2, N1 and N2 had an additional 13 kb fragment, which corresponds in size to the integrated construct (Fig. 2b).

Kinetics of Sap2p secretion by *S. cerevisiae*

The time course for proteinase secretion was investigated in media containing free amino acids from acid hydrolysis of casein (CAT), or BSA as nitrogen source (YNB-BSA). In CAT medium, positive transformants 884 and 885 and negative control 894 showed growth

Fig. 3. Kinetics of Sap2p secretion by *S. cerevisiae* positive transformants 884 (●) and 885 (○), negative control 894 (□) and parental strain 754 (◆) grown in 0.5% CAT (left) or YNB-BSA (right) media at 37 °C. At the indicated times, culture samples were evaluated for growth (a), extracellular proteolytic activity (b) and Sap2p expression in 885 (Western blot) (c). Data are mean values of duplicate experiments.

rates similar to parental strain 754 (Fig. 3a, left). Growth in YNB-BSA medium was restricted to strains harbouring SAP2, suggesting their ability to use BSA as sole nitrogen source (Fig. 3a, right). However, it is noteworthy to mention that the growth rates in this medium were lower than in CAT.

In both media, secreted proteolytic activity was detected exclusively in those transformants carrying SAP2 (Fig. 3b). However, the level of activity was 1.7-fold higher in CAT than in YNB-BSA for the same cell density. This

could reflect the greater physiological activity of the cells grown in the former medium. In CAT medium, the maximum level of proteinase activity was attained at the beginning of the stationary phase (Fig. 3b, left).

Additional information on Sap2p secretion was obtained from Western blotting analysis of 885 transformant supernatants. Sap2p was detectable at the beginning of the exponential phase, after 6 and 24 h of incubation in CAT and YNB-BSA media, respectively (Fig. 3c). In both media, the protein was processed to three forms

Fig. 4. Kinetics of Sap2p secretion by *C. albicans* positive transformants S1 (●) and S2 (○), negative controls N1 (■) and N2 (□), and parental strain CAI4 (◆) grown in 1% CAT (left) or YNB-BSA (right) media at 37 °C. At the indicated times, culture samples were evaluated for growth (a), extracellular proteolytic activity (b) and Sap2p expression in S2 and N1 (Western blot) (c). Data are mean values of duplicate experiments.

with approximate molecular masses of 45, 42 and 39 kDa. The middle 42 kDa form co-migrated with Sap2p purified from concentrated culture filtrate of *C. albicans* LAM-1, consistent with our previous observations (Colina *et al.*, 1996b). The larger 45 kDa form may result from altered post-translational modification or incomplete processing of the protein by *S. cerevisiae* and the smaller 39 kDa form may represent a degradation product. Using a zymogram procedure described previously (Colina *et al.*, 1996b), it was shown that each of the three bands had enzymic activity using mucin as substrate (data not shown). No Sap2p was detected in negative control 894 (data not shown).

Kinetics of Sap2p secretion by *C. albicans*

As for *S. cerevisiae*, *C. albicans* was grown in CAT or YNB-BSA medium, not inducing or inducing SAP2 expression, respectively. In CAT medium, the growth rates of the secretor-transformant S2, as well as the negative controls N1 and N2 (with mutated *sap2*), were indistinguishable from that of the parental strain, CAI4. However, the secretor-transformant S1 grew far more slowly than CAI4 (Fig. 4a, left). In YNB-BSA medium, transformants S1 and S2 displayed similar growth rates, which were greater than those of CAI4 and negative controls (Fig. 4a, right). Overall, cultures of S2, N1, N2 and CAI4 reached a higher cell density when media were supplemented with amino acids rather than BSA as nitrogen source.

Under non-inducing conditions (CAT medium), secreted proteolytic activity was detectable only in the supernatants of transformants S1 and S2 (Fig. 4b, left). These results indicated that SAP2, placed under ADH1p, was constitutively expressed in transformants S1 and S2. In YNB-BSA medium, conditions in which wild-type SAP2 is induced, proteolytic activity was detected in CAI4 and negative controls as well as in secretor-transformants. However, in the latter strains, activity was 3.3-fold (S1) and 1.75-fold (S2) higher than in CAI4 or negative controls (Fig. 4b, right). Although higher proteolytic activity in S1 could have possibly resulted from the integration of multiple copies of the ADH1 fusion, this was unlikely because the intensities of the bands for wild-type SAP2 and integrated vector were individually comparable in the Southern analysis of S1 and S2 (Fig. 2b). Enhanced proteolytic activity in YNB-BSA compared to CAT medium (Fig. 4) may have resulted from the presence of other Saps produced in YNB-BSA (Hube *et al.*, 1994).

Finally, the supernatants of S2 and N1 strains were selected to evaluate the presence of Sap2p. In CAT medium, Sap2p of S2 (apparent molecular mass 42 kDa) was detectable after 9 h incubation, corresponding to the exponential phase (Fig. 4c, left). No protein was detected in N1 supernatants (data not shown). Interestingly, when BSA was included in the culture medium (Fig. 4c, right), S2 produced detectable Sap2p 3 h earlier than N1. The slower growth rate of N1

compared to S2 correlated with the apparent lower level of secretion of Sap2p.

Germination rates of the transformants and parental strain were indistinguishable. After 3 h of incubation at 37 °C, more than 70 % of blastoconidia had germinated.

Pathogenicity *in vivo*

The virulence of *S. cerevisiae* negative (894) and positive (884, 885) transformants was evaluated in experimental oral or systemic infection in mice. In the former, all three strains were completely cleared from the oral cavity 5 h after inoculation (data not shown). A similar result was obtained after systemic infection. No c.f.u. were recovered from kidneys or brains of mice over a period of 28 d after infection with each of the *S. cerevisiae* strains. The inoculum of 2×10^7 cells was selected in accordance with a study by Clemons *et al.* (1994) which evaluated the pathogenic potential of various clinical and laboratory isolates of *S. cerevisiae*. In addition, when the experiment was repeated using 6×10^7 cells to exclude the possibility of an insufficient inoculum, cultures of homogenized organs remained completely negative for all strains. A control experiment was conducted using a 2×10^7 cells inoculum of *S. cerevisiae* Y55 (avirulent laboratory strain) and virulent clinical isolates YJM128 and YJM273, as described by Clemons *et al.* (1994). Quantification of c.f.u. in kidneys and brains of mice produced results very close to those reported by Clemons *et al.* (1994) (data not shown). This confirmed that lack of virulence with strains 884, 885 and 894 was not due to a technical problem.

C. albicans parental strain SC5314 and positive (S1, S2) and negative (N1, N2) transformants were compared for their virulence in two models of candidiasis. In the murine oral model, all four transformants were completely cleared 24 h post-inoculation. Positive control *C. albicans* LAM-1 produced an initial decrease in c.f.u. at 24 h followed by a mean peak of 5.6×10^3 c.f.u. 48 h after infection, as reported by Lacasse *et al.* (1990). *C. albicans* parental strain SC5314 was less virulent than LAM-1 in the oral model, producing a peak of 3.0×10^3 c.f.u. Virulence of *C. albicans* transformants was also determined in a murine model of systemic candidiasis. In a first experiment, in which survival of mice was used as end point (Fig. 5), no mice died after intravenous infection with strain S1 which was thus completely avirulent. However, strain SC5314 was significantly ($P < 0.007$) more virulent than S2, N1 and N2, which were not significantly different between themselves ($P > 0.15$). A second experiment evaluated kidney and liver burdens of the same strains after intravenous infection. Comparison of organ weights revealed no reproducibly significant differences 1, 3 and 6 d after infection with strains SC5314, S1, S2, N1 and N2 (data not shown). However, comparison of mean log(c.f.u.) recovered from kidneys on these same days showed a strikingly decreased c.f.u. g^{-1} for strain S1 compared to the other four strains, which between themselves were

Fig. 5. Survival of CD-1 mice infected intravenously with 5×10^5 cells of *C. albicans* transformants S1 (●, $n = 12$), S2 (○, $n = 15$), N1 (■, $n = 14$), N2 (□, $n = 12$) and parental strain SC5314 (◆, $n = 14$).

Table 2. Comparative c.f.u. of *C. albicans* parental strain and transformants recovered from kidneys at various times during disseminated infection of mice

Day	Strain	No. of animals	log[c.f.u. (g kidney) ⁻¹] (mean $\pm$ SD)
1	SC5314	7	4.45 $\pm$ 0.75
	S1	7	0.88 $\pm$ 1.39
	S2	7	4.57 $\pm$ 0.27
	N1	4	5.93 $\pm$ 0.07
	N2	5	5.53 $\pm$ 0.52
3	SC5314	7	5.04 $\pm$ 0.51
	S1	6	0.88 $\pm$ 0.88
	S2	6	5.99 $\pm$ 0.74
	N1	6	5.15 $\pm$ 0.60
	N2	6	5.10 $\pm$ 0.73
6	SC5314	7	4.98 $\pm$ 0.59
	S1	6	0
	S2	4	5.58 $\pm$ 0.15
	N1	5	5.78 $\pm$ 0.28
	N2	6	5.55 $\pm$ 0.31

not significantly different (Table 2). Counts in liver were 2 (day 1) and 4 log(c.f.u.) (days 3 and 6) lower than those obtained from the kidneys. However, the relationship between strains remained the same. All strains produced equivalent liver c.f.u. on the three observation days, except for strain S1 which yielded negative cultures (data not shown). Finally, randomly selected colonies of S1, S2, N1 and N2 isolated from the kidneys of mice showed no modification at the *SAP2* locus by Southern blotting.

In the previous experiment, one kidney was removed for c.f.u. quantification and the other for histological analysis. Macroscopic examination of the kidneys showed several surface microabscesses 3 and 6 d after infection for all strains except S1. Interestingly, larger microabscesses were noted for strain S2 after 3 d, compared to the other strains. Histopathological exam-

ination demonstrated a mixture of blastoconidia, hyphae and pseudohyphae in the cortex on days 1 and 3 with strains SC5314, N1, N2 and S2. However, no fungi were observed on these same days in kidneys of mice infected with strain S1. Moreover, strain SC5314 produced several cortical abscesses on day 1, while strains N1, N2 and S2 produced very rare blastoconidia and hyphae. On day 3, more widespread fungi were seen with strain SC5314 compared to day 1, with extension into the renal medulla; N1, N2 and S2 were very sparse or absent.

Zymolyase and NaCl resistance

We observed decreasing viability of *S. cerevisiae* and *C. albicans* transformants expressing or overexpressing Sap2p on CAT medium held for 5 d at 4 °C, after initial growth at 30 °C for 48 h. This could have potentially resulted from cell wall damage produced by massive expression and/or secretion of the protease. The same hypothesis could also account for the absence of increased virulence of positive transformant S2 and the decreased virulence of S1 *in vivo*, compared to the negative controls. To assess this possibility, two different cell wall resistance assays were done. In both the zymolyase and NaCl resistance assays, all *S. cerevisiae* negative control and positive transformants demonstrated the same level of sensitivity. However, treatment with zymolyase resulted in a 2 log greater decrease in viability of *C. albicans* strain S1, compared to SC5314, S2, N1 and N2. In addition, growth of strain S1 was inhibited by 1.4% NaCl, compared to 2.2% for the other four *C. albicans* strains.

DISCUSSION

This is the first report describing the construction of stable *S. cerevisiae* and *C. albicans* integrative transformants constitutively secreting Sap2p. We used the *S. cerevisiae* or *C. albicans* *ADH1* promoter (Bertram *et al.*, 1996) on expression plasmids to overexpress *C. albicans* *SAP2* in both yeast species. The *ADH1* promoter has been used in studies of gene expression in *C. albicans* (Cormack *et al.*, 1997).

On the basis of *SAP2* DNA and Sap2p N-terminal sequences, the mRNA is translated as a prepro form, 56 aa larger than the mature protein. The prepropeptide has a signal peptide sequence of 14–21 aa with 1–4 putative signal peptidase cleavage sites and two Lys-Arg sequences, one of which is immediately before the N terminus of the mature form (Wright *et al.*, 1992; Hube, 1996). Thus, Sap2p was expected to be processed in *S. cerevisiae* because it has been shown that the cleavage of *C. tropicalis* Sap heterologously expressed in *S. cerevisiae* is mediated by KEX2-dependent proteolysis (Togni *et al.*, 1996).

The Sap2p signal peptide was functional in *S. cerevisiae*, allowing the secretion of three active forms of Sap2p. While we cannot rule out the possibility of post-translational modification, the higher molecular mass

form (45 kDa) may result from incomplete processing of the proteinase by *S. cerevisiae*. The secretion of a premature form may be due to saturation of the processing system as a consequence of overexpression of the protein. Previous reports have shown that heterologous expression of *C. tropicalis* SAPT1 (Togni *et al.*, 1996) or *C. albicans* SAP1 (Smolenski *et al.*, 1997) in *S. cerevisiae* resulted in secretion of extracellular active enzyme identical to the native form, indicating that no oligosaccharide chains were added. In both cases, expression was under the control of inducible promoter of *GAL10* and the hosts were not deficient in intracellular proteinases. Although no extensive studies have been done on the intracellular processing of *C. albicans* Sap2p, Banerjee *et al.* (1991) and Homma *et al.* (1992) reported the detection of a 45 kDa intracellular precursor of Sap2p. More recently, it was shown that heterologous expression of SAPT1 of *C. tropicalis* by *S. cerevisiae* resulted in intracellular forms 4–6 kDa larger than the mature protein (Togni *et al.*, 1996). Further experiments will be required to clarify these issues.

Considering that *S. cerevisiae* is non-pathogenic, the study of the virulence of strains secreting Sap2p is an attractive system to address the role of Sap2p in pathogenicity in the absence of other putative factors. Although episomal expression of Sap1p has been achieved in *S. cerevisiae* (Smolenski *et al.*, 1997), we have now obtained expression of the major transcription product, Sap2p, from an integrated construct, suitable for studies of virulence in animal models. The constitutive expression of Sap2p by *S. cerevisiae* clearly allowed it to hydrolyse and use BSA. This may be important for survival in animal models because it would enable the yeast to use host proteins as nitrogen source.

A model of the regulation of SAP2 expression suggests that *C. albicans* has a basal level of proteinase expression, which yields peptides that act as inducers of SAP2 expression, via a positive feedback mechanism (Hube *et al.*, 1994). Our data indicate that SAP2 expression was deregulated in those *C. albicans* strains constitutively producing Sap2p. In the presence of high-molecular-mass protein, the enzyme was secreted precociously, which resulted in earlier induction of the wild-type SAP2 gene and higher amounts of total proteinase activity. These strains constitute a novel approach to explore the role of early and high-level expression of Sap2p during experimental infections. In fact, De Bernardis *et al.* (1995) have shown that highly vaginopathic strains expressed both SAP1 and SAP2 earlier in experimental vaginitis than moderately or non-vaginopathic strains. Furthermore, it has been hypothesized that Saps may play a role early in systemic dissemination of *C. albicans* to deep organs from mucosal sites (Kimberly *et al.*, 1997). Finally, *C. albicans* isolates from oral cavities of symptomatic HIV-infected patients secreted significantly higher levels of proteinase than those from asymptomatic or non-infected patients (Ollert *et al.*, 1995; De Bernardis *et al.*, 1996). When tested in experimentally infected animals, the high-level

producers had enhanced virulence (De Bernardis *et al.*, 1996). However, whether the increased proteinase activity was due to a particular Sap(s) was not determined.

In pathogenicity studies *in vivo*, expression of SAP2 as a sole putative virulence factor did not cause an avirulent strain of *S. cerevisiae* to become virulent and constitutive expression of SAP2 did not augment virulence of *C. albicans* in experimental oral or systemic infection in intact mice. The absence of enhanced virulence of *S. cerevisiae* SAP2 transformants is consistent with the long-held view that *C. albicans* possesses a multiplicity of properties, each with a low propensity for enhancing fungal infectivity, none necessarily dominant and all, even in combination, unable to overcome fully intact host defences (Odds, 1988).

Unknown mutations occurring concurrently in all strains would be unlikely to explain the significantly enhanced survival of mice infected with the *C. albicans* negative control (N1, N2) or positive SAP2 transformant (S2), compared to parental strain SC5314. In addition, although N1, N2 and S2 only contain one copy of *URA3*, while the wild-type SC5314 has two copies, a gene dosage effect is also unlikely to be responsible for the attenuated virulence of N1, N2 and S2 since heterozygous *URA3* mutants were as virulent as parental strains in experimentally infected mice (Leberer *et al.*, 1997). Because the negative controls (N1, N2) would have been expected to be as virulent as the wild-type SC5314, mutated SAP2 may have been translated into a truncated protein, resulting in saturation of the secretory pathway.

Although positive *C. albicans* transformants S1 and S2 were expected to be isogenic, they had different phenotypes. S1 grew much slower in CAT medium compared to all other strains, including the wild-type SC5314. In addition, this strain had higher proteolytic activity in YNB-BSA medium and was avirulent after systemic infection in intact mice. The strikingly decreased virulence of strain S1 and its enhanced cell wall sensitivity compared to the other *C. albicans* transformants suggest that SAP2 overexpression may have damaged the cell wall, resulting in enhanced killing by polymorphonuclear leukocytes and other intact host defences. Alternatively, an unknown mutation in S1 causing slower growth in CAT medium may also have attenuated virulence. With regard to strain S2, a mutation may have caused a reduced secretion of Sap2p, which in turn caused less damage to the cell wall of *C. albicans*. Lack of enhancement of virulence of positive *C. albicans* transformant S2 compared to negative controls N1 and N2 may have resulted from unlikely spurious mutations during transformation, the route of infection, the absence of immunosuppression, or defects in other virulence factors due to saturation of secretory pathways. In this regard, all *C. albicans* transformants maintained the ability to form pseudohyphae and hyphae *in vivo*. Although Sap2p is the most abundant transcript of *C. albicans* yeast cells, increased virulence *in vivo* may require not only its enhanced expression, but also the early participation of other putative

virulence factors, including members of the Sap isoenzyme family such as Sap4, 5 and 6 which are produced by the hyphal form of *C. albicans*. Finally, an absence of SAP2 constitutive expression in the animal models was unlikely because *ADH1* is a strong constitutive promoter. Furthermore, *C. albicans* transformants isolated from the kidneys of mice showed no modification at the SAP2 locus. Further studies will be required to determine whether differences in virulence would be observed in a rat vaginal model between the wild-type and strains overexpressing SAP2. Although SAP2 constitutive expression did not augment virulence, this new approach may be a useful paradigm in the study of virulence attributes in *Candida* and other fungal pathogens.

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