Limited Role of Secreted Aspartyl Proteinases Sap1 to Sap6 in *Candida albicans* Virulence and Host Immune Response in Murine Hematogenously Disseminated Candidiasis

Alexandra Correia, Ulrich Lermann, Luzia Teixeira, Filipe Cerca, Sofia Botelho, Rui M. Gil da Costa, Paula Sampaio, Fátima Gärtner, Joachim Morschhäuser, Manuel Vilanova, and Célia Pais

Centre of Molecular and Environmental Biology (CBMA), Department of Biology, University of Minho, 4710-057 Braga, Portugal; Institut für Molekulare Infektionsbioologie, Universität Würzburg, Josef Schneider Str. 2, D-97080 Würzburg, Germany; Unidade Multidisciplinar de Investigação Biomédica (UMIB), Universidade do Porto, Largo Prof. Abel Salazar 2, 4099-003 Porto, Portugal; Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto, Largo Prof. Abel Salazar 2, 4099-003 Porto, Portugal; and Instituto de Biologia Molecular e Celular (IBMC), Rua Campo Alegre, 4099-003 Porto, Portugal

Received 12 March 2010/Returned for modification 23 March 2010/Accepted 25 July 2010

*Candida albicans* secreted aspartyl proteinases (Saps) are considered virulence-associated factors. Several members of the Sap family were claimed to play a significant role in the progression of candidiasis established by the hematogenous route. This assumption was based on the observed attenuated virulence of *sap*-null mutant strains. However, the exclusive contribution of *SAP* genes to their attenuated phenotype was not unequivocally confirmed, as the URA status of these mutant strains could also have contributed to the attenuation. In this study, we have reassessed the importance of *SAP1* to *SAP6* in a murine model of hematogenously disseminated candidiasis using *sap*-null mutant strains not affected in their *URA3* gene expression and compared their virulence phenotypes with those of *ura*blaster mutants. The median survival time of BALB/c mice intravenously infected with a mutant strain lacking *SAP1* to *SAP3* was equivalent to that of mice infected with wild-type strain SC5314, while those infected with mutant strains lacking *SAP5* showed slightly extended survival times. Nevertheless, no differences could be observed between the wild type and a *Δsap456* mutant in their abilities to invade mouse kidneys. Likewise, a deficiency in *SAP4* to *SAP6* had no noticeable impact on the immune response elicited in the spleens and kidneys of *C. albicans*-infected mice. These results contrast with the behavior of equivalent *ura*-blaster mutants, which presented a significant reduction in virulence. Our results suggest that Sap1 to Sap6 do not play a significant role in *C. albicans* virulence in a murine model of hematogenously disseminated candidiasis and that, in this model, Sap1 to Sap3 are not necessary for successful *C. albicans* infection.

The polymorphic yeast *Candida albicans* is an important opportunistic human pathogen causing infections that range from superficial mucosal lesions to life-threatening systemic disease. It is by far the most common cause of fungal invasive infections, which could be attributed to the little immunosuppression required to predispose an individual to invasive *Candida* infections (39). Host physical barriers and immune system integrity are crucial factors in controlling the establishment of infection. However, the high adaptability of *C. albicans* to different host niches, by the expression of appropriate sets of virulence-related genes, is also a determinant (19, 51). Several of these virulence attributes may participate in and influence the infective process, depending on the site and stage of invasion and on the nature of the host response (37). The secretion of hydrolytic enzymes during infection is required as a virulence attribute to aid adhesion, invasion, and the destruction of host immune factors, in addition to nutrient acquisition (21).

Among these enzymes, secreted aspartyl proteinases (Sap), encoded by a 10-member gene family (*SAP1* to *SAP10*) have been the most extensively studied (35). The 10 *SAP* genes that compose this family can be divided into subfamilies based on amino acid sequence homology alignments (*SAP1* to *SAP3*, *SAP4* to *SAP6*, *SAP9*, and *SAP10*). These genes exhibit differential expression profiles at different stages and sites of infection (33, 35, 46, 49) and have been linked with the virulence of the fungus since their discovery (10, 27, 48).

The contribution of the *SAP1* to *SAP3*, *SAP4* to *SAP6*, *SAP7*, and *SAP9* and *SAP10* genes to virulence in different models of infection has been studied by using *sap*-null mutant strains (1, 13, 16, 20, 23, 25, 26, 29, 34, 43, 54). The subfamily consisting of the *SAP4* to *SAP6* genes, in particular, was shown to contribute significantly to *C. albicans* virulence in models of acute systemic candidiasis, murine peritonitis, and *Candida* gastrointestinal infection (16, 25, 43). These genes are expressed mainly during hypha formation (22, 34), and *SAP5* in particular was found to be upregulated at all time points after either intravenous (i.v.) or intraperitoneal (i.p.) infection of mice (44, 50, 57).

Hube et al. (20) previously reported that *Δsap1*, *Δsap2*, and *Δsap3* null mutants displayed attenuated virulence in models of...
glycerol at study are listed in Table 1. All strains were maintained as frozen stocks in 30%.
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Associates, Birmingham, AL); fluorescein isothiocyanate (FITC) anti-mouse/rat FcXp3 (J5K-16S), PE-C5 anti-mouse CD4 (L3T4) (RM-4-5), and PE anti-mouse F4/80 antigen (BM8) (eBioscience, San Diego, CA); and FITC anti-mouse CD11c (HL3), FITC anti-mouse Ly-6G and Ly-6C (Gr-1) (RB6-8C5), PE anti-mouse CD11c (PC61), FITC anti-mouse CD45R2/B220 (RA3-6B2), PE anti-mouse CD80 (B7-1) (16-10A1), PE anti-mouse CD86 (B7-2) (GL1), PE rat anti-mouse interleukin-4 (IL-4) (BVD4-1D11), FITC anti-mouse gamma interferon (IFN-γ) (XMGl.2), and PE anti-mouse IL-10 (JES5-2A5) (BD Pharminogen, San Diego, CA). Biotin-conjugated markers were revealed with streptavidin-PE-C5 (BD Pharminogen). Cells were preincubated for 15 min with anti-FcγR (a kind gift of Jocelyne Demengeot, Gulbenkian Institute of Science, Oeiras, Portugal) before CD11c and FcXp3 staining. The FcXp3 Staining Buffer set (eBioscience) was used for the fixation and permeabilization of splenocytes previously surface stained with CD4 and CD25 MAbs.

The intracellular expression of the cytokines IFN-γ, IL-4, and IL-10 was detected in splenic CD4+ T lymphocytes. The intracellular expression of the cytokines IFN-γ and IL-4 was also detected in renal CD4+ T lymphocytes. Splenocytes were obtained as described above. Red blood cell lysis was performed by incubation with 0.15 M ammonium chloride. Cells were washed and resuspended in complete RPMI medium (Sigma) (RPMI 1640 medium supplemented with 50 U of penicillin/ml, 50 μg of streptomycin/ml, 1% HEPES buffer [Sigma], 10% fetal calf serum [FCS] [Invitrogen], and 5 μM 2-mercaptoethanol). The kidneys were minced with a razor blade and incubated for 30 min at 37°C in RPMI 1640 medium containing collagenase D (Sigma-Aldrich) at 2 μg/ml. Cells were homogenized to single-cell suspensions, washed, and resuspended in RPMI 1640 complete medium. Mononuclear cells were separated from the above-described suspensions by layering 5 ml onto 2.5 ml of a polysu-}

TABLE 2. *SAP* and *ACT1* primers and expected fragment lengths

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’–3’)</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ACT1</em></td>
<td>TGGCAGGTTGATGCTATG</td>
<td>186</td>
</tr>
<tr>
<td><em>SAP1</em></td>
<td>TGGACTGCTTGGTTGGATG</td>
<td>224</td>
</tr>
<tr>
<td><em>SAP2</em></td>
<td>ATCGACTGTTTGGTGGTC</td>
<td>105</td>
</tr>
<tr>
<td><em>SAP3</em></td>
<td>TTGTTACTGGTCCCTTCTCTC</td>
<td>209</td>
</tr>
<tr>
<td><em>SAP4</em></td>
<td>TGGACATAGGCAATGGGACAGG</td>
<td>155</td>
</tr>
<tr>
<td><em>SAP5</em></td>
<td>ATCTCCCTCGTCGAGACTGC</td>
<td>205</td>
</tr>
<tr>
<td><em>SAP6</em></td>
<td>GTCAGCTCGTGTAAGTCCTC</td>
<td>197</td>
</tr>
<tr>
<td><em>SAP7</em></td>
<td>TCTCTGATGCTGTCGCAAG</td>
<td>183</td>
</tr>
<tr>
<td><em>SAP8</em></td>
<td>TTCTGCTGAGGTTTGAATTAC</td>
<td>198</td>
</tr>
<tr>
<td><em>SAP9</em></td>
<td>ACGGGCTTCCAGATTGTTG</td>
<td>180</td>
</tr>
<tr>
<td><em>SAP10</em></td>
<td>ACGGAAATGTTGCTTCTGG</td>
<td>192</td>
</tr>
</tbody>
</table>

nificance of results was determined by the unpaired Student t test, and survival data were analyzed with the log-rank test by using GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA). Results were considered statistically significant with P values of less than 0.05.

RESULTS

Virulence of *C. albicans* in a murine model of hematogenously disseminated infection. Prior to the virulence studies, we determined the generation time for each strain in synthetic defined and complex media at 30° and 37°C and the ability to form hyphae in serum- or N-acetylglucosamine-containing media. No yeast growth defects were observed under the conditions tested (similar generation times), and filamentous growth was similar to that of wild-type (WT) strain SC5314 under the hypha-inducing conditions analyzed (data not shown).

To explore the role of the aspartyl proteinases Sapl to Sap6 as virulence factors in the course of hematogenously disseminated candidiasis, BALB/c mice were initially infected i.v. with 5 × 10^5 and 1 × 10^5 cells of *C. albicans* WT strain SC5314 and two independent series of homoygous-deletion triple-mutant strains lacking the *SAPI* to *SAP5* genes (SAPI2MS4C and SAPI2MS4D) or lacking the *SAP4* to *SAP6* genes (SAPI456MS4A and SAPI456MS4B) (29). The independent mutant strains behaved indistinguishably (data not shown), and therefore, the results presented below correspond to those obtained with strains SAPI2MS4C and SAPI456MS4A.

Mice infected with the highest inoculum showed 100% mortality for every group by day 6 after infection, and no differences could be observed (data not shown).

No differences in survival time were observed after i.v. infection of BALB/c mice with 1 × 10^5 CFU of the WT or Δsap123 mutant strain SAP123MS4C (P = 0.5698 by log-rank test) (Fig. 1A). Mice infected with Δsap456 mutant strain SAP456MS4A had an extended overall survival time compared to that of their WT-infected counterparts (Fig. 1). The median survival times of SAP456MS4A-infected mice were 13 and 16 days, and Δsap456 mutant survived infection. Nevertheless, the kidneys of the surviving mice had Candida microabscesses and gran-
were performed by using the same batch of BALB/c mice and the same experimental conditions. Strain CAF2-1 (17) was also included. As shown in Fig. 1C, significant differences in survival times were observed between mice infected with Δsap123 strain M119 and Δsap456 strain DSY459 and control strain CAF2-1 (P = 0.0089 and P = 0.0065, respectively). Mice infected with the Ura-blaster mutants survived significantly longer than mice infected with the equivalent SAT1-flipping mutants. The median survival times of mice infected with mutants lacking SAP1 to SAP3 were 21 days for the Ura-blaster-constructed Δsap123 mutant strain M119 and 10 days for Δsap123 mutant strain SAP123MS4C (P = 0.0127). Similar results were found when comparing the triple mutants lacking the SAP4 to SAP6 genes. The median survival times were 51 days for mice infected with the Ura-blaster mutant and 13 days for mice infected with the SAT1-flipping mutant (P = 0.0096). The survivals of mice infected with WT strain SC5314 and with URA3 heterozygous strain CAF2-1 were similar (P = 0.7005).

The evaluation of the fungal ability to invade the kidneys has been frequently used to measure the virulence of C. albicans strains (30, 38). The numbers of C. albicans CFU in the kidneys of mice infected with either the SAT1-flipping or the Ura-blaster mutants as well as with the respective control strains were similar, except for mice infected with Δsap456 strain DSY459 (Fig. 2). Seven days after infection, CFU numbers were significantly reduced in mice infected with the latter mutant (P = 0.0149 for CAF2-1 versus Δsap456 strain DSY459).

The two sets of mutants tested behaved distinctly in the same experimental model, suggesting that the observed differences could be due to the effect of the ectopic URA3 insertion and not caused by the disruption of SAP genes. However, the limited impact of the SAP gene deletion in C. albicans viru-
lence could be due to a compensatory expression of the non-deleted SAP genes, as reported previously for Ura-blaster mutants (34, 45). The expression of SAP1 to SAP10 in SC5314 and in SAT1-flipping triple mutants was evaluated by qRT-PCR in kidney samples 3 and 7 days after infection. Only the results for the latter time point analyzed are presented, since after 3 days, the fungal burden was often insufficient to obtain reproducible results. The expression levels were always inferior or similar to the level of ACT1 expression in the WT strain, except for SAP7.

No significant differences in SAP1 to SAP10 expression were observed for either mutant compared with SC5314. However, mRNA levels of SAP4 were higher in the Δsap123 mutant, and the levels of expression of SAP1, SAP2, and SAP3 trended higher in the Δsap456 mutant (Fig. 3). Thus, the virulence phenotypes observed do not seem to be due to significant compensatory upregulation.

Although not significant, a reduction in virulence was consistently seen for the Δsap456 mutant. Therefore, the impact of a deficiency of SAP4 to SAP6 on C. albicans virulence was further evaluated.

The abilities of WT and Δsap456 triple-mutant strains to infect and injure several organs were assessed by histopathological analysis of the kidneys, liver, lungs, and brain 3 and 7 days after infection. As shown in Fig. 4, similar C. albicans cell morphologies and invasive abilities of the WT and mutant strains were observed. The kidneys of both mouse groups showed moderate multifocal renal medullary interstitial neutrophilic infiltration, with small areas of ductular necrosis. Intratrasional PAS-positive organisms both in yeast and with septated, branched hyphal morphology were detected 3 days after C. albicans i.v. infection with strains SC5314 (Fig. 4A) and SAP456MS4A (Fig. 4B). At the later time point tested, 7 days after infection, analysis of the kidneys of WT-infected (Fig. 4C) and mutant-infected (Fig. 4D) mice showed moderate to severe, focally extensive to coalescing, renal medullary interstitial neutrophilic infiltration surrounding numerous PAS-positive organisms. These organisms were present mainly as septated, branched hyphal structures, which largely effaced the medulla and invaded the urothelium. Invasion of liver, lungs, and brain was not consistently seen (data not shown). Altogether, these results suggest that the SAP4 to SAP6 genes are not essential for the invasion of the kidneys during hematogenously disseminated candidiasis.

Host immune response to hematogenously disseminated candidiasis. To determine the effect of the disruption of SAP4 to SAP6 on the immune response elicited by C. albicans systemic infection, BALB/c mice were infected i.v. with $5 \times 10^4$ C. albicans yeast cells of the WT and Δsap456 mutant strains. At days 3 and 7 upon infection, absolute numbers and phenotypes of different splenic leukocyte populations were determined by flow cytometric analysis. Macrophages and neutrophils represent the first line of host immune defense when C. albicans cells infect the bloodstream or the endothelia (47, 58). Macrophages typically express the F4/80 cell surface marker, whereas neutrophils have a Gr-1^{high} surface phenotype. Murine splenic cells expressing both antigens with either inflamm-
matory or immunosuppressive function have also been described in the context of *C. albicans* infections (31, 56). According to the expression of these two surface markers, three cell populations were analyzed in this study: F4/80\textsuperscript{high} Gr-1\textsuperscript{neg}, F4/80\textsuperscript{high} Gr-1\textsuperscript{high}, and F4/80\textsuperscript{neg/low} Gr-1\textsuperscript{high}. These cell populations were designated macrophages, inflammatory monocytes, and neutrophils, respectively (55). An extensive recruitment of neutrophils and inflammatory monocytes into the spleen could be observed for infected mice compared to noninfected controls at 3 and, more markedly, 7 days after infection. Higher numbers of spleen macrophages were also detected in the infected mice at 7 days after infection. The total numbers of these myeloid cell populations in WT-infected mice were equivalent to the ones in mice infected with the Δsap456 triple mutant (Fig. 5A).

Dendritic cells were previously shown to play a major role in the induction of the cell-mediated immune response to *C. albicans* infection (9, 14) and to directly influence the infection outcome (6). Therefore, the numbers and surface maturation markers of splenic conventional dendritic cells (cDC), defined as CD11c\textsuperscript{high} cells, were assessed upon *C. albicans* infection. High numbers of splenic cDC, compared to noninfected controls, were observed at the earlier time point analyzed. The cDC surface expression of the costimulatory molecules CD40 and CD80 remained practically unchanged after *C. albicans* i.v. infection, as evaluated by flow cytometry and recorded as the mean fluorescence intensities (MFIs). In contrast, the costimulatory molecule CD86 was upregulated on the surface of splenic cDC from *C. albicans*-infected mice 3 and 7 days after infection. The cDC surface expression of MHC class II molecules was slightly downregulated in infected mice 3 days after infection and upregulated at day 7 postinfection compared to that of noninfected controls. No significant differences of cDC numbers (Fig. 5A) and surface expression of any of the assessed costimulatory and antigen-presenting molecules were detected between WT- and SAP456MS4A-infected mice at the two tested time points (Fig. 6).

**FIG. 5.** (A) Scatter plots of the total numbers of neutrophils (F4/80\textsuperscript{neg/low} Gr-1\textsuperscript{high}), inflammatory monocytes (F4/80\textsuperscript{high} Gr-1\textsuperscript{high}), macrophages (F4/80\textsuperscript{high} Gr-1\textsuperscript{neg}), cDC (CD11c\textsuperscript{high}), B cells (B220\textsuperscript{+}), Treg cells (CD4\textsuperscript{+} CD25\textsuperscript{+} Foxp3\textsuperscript{+}), and T effector cells (CD4\textsuperscript{+} CD25\textsuperscript{−} Foxp3\textsuperscript{−}), as indicated, observed 3 and 7 days after infection in the spleens of noninfected control mice (open triangles) and mice challenged i.v. with 5 × 10\textsuperscript{8} WT (open squares) and SAP456MS4A (filled circles) *C. albicans* cells. Data are representative of three independent experiments. Each symbol represents an individual mouse, and horizontal bars are means of cell numbers for each group (n = 3 for control and n = 4 to 5 for infected mouse groups). Statistically significant differences between controls and *C. albicans*-infected mice are indicated: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

(B) Relative proportions of splenic T effector (black bars) and Treg (white bars) cells in the spleens of noninfected controls (PBS) and SC5314 or SAP456MS4A i.v. infected mice 3 and 7 days after challenge.
established *C. albicans* systemic infection (61). B-cell numbers were significantly increased 7 days after infection in either WT- or SAP456MS4A-challenged mice (Fig. 5A). As observed for splenic cDC, an upregulation of the costimulatory molecule CD86 was observed 3 and 7 days postinfection on the surface of B cells of the infected mice compared to noninfected controls. The expression of CD80 and MHC class II molecules on the surface of B cells was observed to be upregulated only at day 7 after challenge. Challenge with the mutant deficient in SAP4 to SAP6 did not result in any differences compared to the WT strain, regarding either B-cell numbers or costimulatory molecule expression levels (Fig. 5A and 6).
Although CD4+ T cells have been reported to have little influence on survival and on fungal burden during acute systemic candidiasis (3, 24), the CD4+ T-cell subset of naturally occurring regulatory T (Treg) cells was shown to promote host susceptibility to *C. albicans* (36, 53) and to limit tissue damage and/or enhance healing but not to directly augment the clearance of the organism from infected tissues (32). To ascertain the impact of a deficiency of SAP4 to SAP6 on the immune response mediated by CD4+ T-lymphocyte cell populations, the numbers of CD4+ and CD4+ CD25+ T cells, and CD4+ CD25+ Foxp3+ (Treg) cells and also of CD4+ CD25+ Foxp3− (Teffector) cells in the spleens of the infected BALB/c mice were assessed. The numbers of splenic CD4+ and CD4+ CD25+ T cells of infected mice were not significantly different from those of noninfected controls (data not shown). However, as assessed by Foxp3 expression within the CD4+ CD25+ T-cell population, reduced percentages and numbers of Treg cells were observed 7 days after *C. albicans* infection. This correlated with higher splenic percentages and numbers of Teffector cells in these mice (Fig. 5A), resulting in higher Teffector/Treg ratios than those for noninfected controls (Fig. 5B). The percentages and numbers of both Teffector and Treg cells were not significantly different between the two *C. albicans*-infected groups.

To better elucidate the effector function of the CD4+ T cells from WT- and SAP456MS4A-infected mice, the proportion of splenic CD4+ T cells producing IFN-γ, IL-4, and IL-10 was determined by intracytoplasmic cytokine staining analysis. An increased frequency of CD4+ T cells expressing IFN-γ or IL-4 was observed for *C. albicans*-infected mice compared to the noninfected controls. Although the frequency of cells producing either cytokine increased upon infection in the spleens of *C. albicans*-challenged mice, a bias toward a Th1-type response was observed (high IFN-γ/IL-4 ratio). The frequency of CD4+ T cells expressing IL-10 was also increased in the spleens of infected mice (Fig. 7). The intracellular expression of IFN-γ and IL-4 was also evaluated in kidney CD4+ T cells, as it is associated with the outcome of infection (48). As observed for
the spleen, the frequency of CD4+ T cells expressing the cytokines IFN-γ and IL-4 increased in infected mice. Although a trend toward extended survival was observed for mice infected with mutant strain SAP456MS4A, the percentages of CD4+ T cells producing the cytokine IFN-γ or IL-4 in the kidneys of either group of infected mice were similar, resulting in equivalent Th1/Th2 cell ratios (Fig. 7). No serum IFN-γ, IL-4, and IL-10 was detected by ELISA and 7 days after infection of either infected or uninfected mice (data not shown).

Overall, these results indicate that a deficiency of SAP4 to SAP6 does not have a significant impact on the immune response elicited in the spleen and kidneys of BALB/c mice hematogenously challenged with C. albicans.

DISCUSSION

The secretion of aspartyl proteinases has long been recognized as a virulence-associated trait of Candida albicans (10, 27, 49). The importance of specific Sap isoenzymes for the pathogenicity of this fungus has been investigated with different infection models by comparing the virulence of mutants deficient in individual or multiple SAP genes with that of a WT control strain. In this study, the importance of SAP1 to SAP6 gene expression for C. albicans virulence was evaluated by using sap-null mutants derived from WT strain SC5314. The virulence of mutant strains lacking SAP1 to SAP3 was indistinguishable from that of WT strain SC5314, while the deletion of SAP4 to SAP6 caused a slight attenuation in virulence. Previous reports have shown that a deficiency in SAP4 to SAP6 attenuated virulence to a higher extent than did a deficiency in SAP1, SAP2, or SAP3 (20, 43). Here, an increased median survival time was consistently observed for mice infected with mutants lacking the SAP5 gene, such as the single-deletion mutant strain SAP5MS4A or the triple-deletion mutant strain SAP456MS4A, compared to that of animals infected with WT strain SC5314. However, the differences found were small and not always significant. Moreover, histopathology analysis did not indicate a reduced ability of the SAP456MS4A mutant to invade the kidneys, although sap-null mutant strains lacking the SAP6 gene were previously shown to have reduced invasiveness in a model of experimental peritonitis (16). The deletion of SAP4 to SAP6 did not result in clear differences in hypha formation, and similar morphotypes were observed, both in vitro and in vivo, for WT and mutant strains. This is not unexpected, as SAP4 to SAP6 expression is associated with, but not required for, hyphal morphology (16). Additionally, expression levels of SAP4 to SAP6 may not be directly linked to organ invasion, since a previously reported C. albicans strain expressing high levels of SAP4 to SAP6 was noninvasive (57).

The results obtained with SAT1-flipping mutants contrast with those obtained when using the Ura-blaster sap-null mutants, which survived much longer. When another parameter associated with C. albicans virulence was analyzed, such as kidneys CFU, no differences were observed among mouse groups, except for the ones infected with strain DSY459, which presented a lower fungal burden. Discrepancies between different methods of evaluating virulence have already been reported for mice intravenously infected with C. albicans mutant strains, including sap-null mutants, where differences in mouse survival were not associated with differences in organ fungal burden (20, 54, 62). The differences found between the two sets of mutants are most likely due to the ectopic insertion of URA3, which must have contributed to the reduced virulence of the Ura-blaster-constructed mutant strains. It is widely known that the Ura status of C. albicans strains influences adherence (5) and virulence (28, 52). Although this can be overcome by the integration of URA3 at the ENO1 (52) or RPS10 (8) locus, the strains used in this study and in previously reported studies (20, 43) did not share a site of URA3 integration.

The disruption of SAP1 to SAP3 and SAP4 to SAP6 led to an increased level of expression of SAP4 and SAP1 to SAP3, respectively, suggesting that C. albicans attempts to compensate the functional loss of these subfamilies by upregulating alternative SAP genes during hematogenously disseminated candidiasis. Therefore, the compensatory upregulation observed could be, to some extent, contributing to the lack of a phenotype seen for these mutants. However, the equivalent Ura-blaster mutants, despite the compensatory upregulation reported previously (34, 45), showed markedly reduced virulence in this model.

Recently, Lermann and Morschhäuser (29) and Naglik et al. (34) reevaluated the role of SAP1 to SAP6 in a model of reconstituted human epithelia (RHE) and reported that SAP1 to SAP6 were not essential for successful C. albicans RHE infection, in contrast to previous reports (45, 46). The present study thus reports an additional model in which the SAP gene subfamilies SAP1 to SAP3 and SAP4 to SAP6 seem to have little influence on the outcome of infection.

As mice infected with the Δsap456 triple mutant displayed a slightly extended survival time, it could be expected that it might result from a more effective host immune response. This would be in agreement with a previous report suggesting an immunomodulatory role of Sap4 to Sap6 upon macrophage phagocytosis (7). However, the analysis of diverse features of the innate and acquired immune response elicited in BALB/c mice upon infection with either the WT or the Δsap456 triple mutant did not show any significant differences between these two yeast strains. The similar abilities of both strains to recruit inflammatory cells are in accordance with their similar observed virulences, taking into account the prominent role of innate immunity and of neutrophils in particular, in host protection against disseminated candidiasis (2, 60).

The proportions of splenic T effector and Treg cells in the spleens of mice infected with either the WT or the Δsap456 mutant were highly similar. Likewise, the frequencies of CD4+ T cells expressing IFN-γ, IL-4, and IL-10, cytokines previously shown to be relevant for resistance or susceptibility to systemic candidiasis (48), were similar in the two infected mouse groups. In vivo models indicate that regulatory T cells attenuate Th1-type antifungal responses and induce tolerance to the fungus (32, 36). As higher IFN-γ/IL-4 ratios were observed for splenic and renal CD4+ T cell of infected mice than those of noninfected controls, it can be assumed that even though the kidneys of infected mice presented a high fungal burden 7 days after challenge, a protective Th1-type response of an equivalent magnitude might be occurring in both WT- and Δsap456 mutant-infected mice.

Although our results suggest that B cells may have a role in
the activation of T cells during experimental disseminated candidiasis, in accordance with the increased susceptibility observed for B-cell-deficient mice (61), they also indicate that the deficiency of Sap4 to Sap6 does not affect such a role of B cells.

Differences in C. albicans morphology have been frequently shown to influence both the type and magnitude of the host immune response in the course of candidiasis. Dendritic cells, and also neutrophils, modulate adaptive responses to the fungus, depending on the Candida morphotype encountered (14, 42, 47). As indistinguishable morphotypes were found for the WT and the sαp456 mutant strain both in vitro and in vivo, this is also in agreement with the lack of significant differences observed in the immune responses elicited by these strains.

A relative independence on aspartyl protease activity for the establishment of hematogenously disseminated candidiasis was previously reported (15), as treatment with pepstatin A, a potent protease inhibitor, did not protect mice against intravenous infection with C. albicans. As previously suggested, an explanation for these observations may be the requirement for Sap only where anatomical barriers had to be crossed prior to dissemination (15, 26). When C. albicans cells are delivered directly into the bloodstream, low-molecular-weight peptides are available, and yeast growth may be protease independent.

The relative importance of specific SAP genes for C. albicans pathogenicity is greatly determined by the type of infection and its dependence on protease activity for the successful invasion and colonization of various host niches. Treatment with pepstatin A resulted in reduced virulence in intranasal (15) and intraperitoneal (26) models but had no protective effect in the intravenous model. The subfamily of the SAP genes SAP1 to SAP3, and SAP2 in particular, was proven to be important in a model of rat vaginal infection, while SAP4 to SAP6 had little impact on this infection model (12). In contrast, only Δsαp456 mutants, and Δsαp6 in particular, showed reduced virulence in a murine model of Candida peritonitis and keratitis, while the deletion of the SAP1, SAP2, or SAP3 gene had no significant effect on these infection models (16, 23). Moreover, immunological neutralization of Sap2 was shown to have a protective effect on C. albicans-infected hosts during vaginal and oral infection (12, 40) and also in experimental peritonitis (59).

Although individual processes resulting from the action of a single gene or a small group of genes may be important in specific stages of infection, cooperative gene functions are essential for the multiple processes of C. albicans infection (38). Thus, although the protease family as a whole may contribute to C. albicans virulence in the course of acute systemic candidiasis, other factors must be the major contributors to invasion and cell damage in this model.

ACKNOWLEDGMENTS

We are indebted to Bernhard Hube from Hans Knoll Institute, Jena, Germany, for providing C. albicans CAF2-1 and the Ura-blaster sap mutant strains.

This work was supported by Fundação para a Ciência e Tecnologia grant POCI/SAU-IMI/58014/2004 and FEDER. Alexandra Correia and Filipe Cerca were supported by FCT fellowships SFRH/BD/31354/2006 and SFRH/BD/27638/2006, respectively. Luzia Teixeira was supported by FSE and MCTES through POPH-QREN-Tipologia 4.2.

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