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Secreted aspartic proteases are not required for invasion of reconstituted human epithelia by *Candida albicans*

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A well-known virulence attribute of the human-pathogenic yeast *Candida albicans* is the secretion of aspartic proteases (Saps), which may contribute to colonization and infection of different host niches by degrading tissue barriers, destroying host defence molecules, or digesting proteins for nutrient supply. The role of individual Sap isoenzymes, which are encoded by a large gene family, for the pathogenicity of *C. albicans* has been investigated by assessing the virulence of mutants lacking specific *SAP* genes and by studying the expression pattern of the *SAP* genes in various models of superficial and systemic infections. We used a recombination-based genetic reporter system to detect the induction of the *SAP1–SAP6* genes during infection of reconstituted human vaginal epithelium. Only *SAP5*, but none of the other tested *SAP* genes, was detectably activated in this *in vitro* infection model. To directly address the importance of the *SAP1–SAP6* genes for invasion of reconstituted human epithelia (RHE), we constructed a set of mutants of the wild-type *C. albicans* model strain SC5314 in which either single or multiple *SAP* genes were specifically deleted. Even mutants lacking all of the *SAP1–SAP3* or the *SAP4–SAP6* genes displayed the same capacity to invade and damage both oral and vaginal RHE as their wild-type parental strain, in contrast to a nonfilamentous *efg1*Δ mutant that was avirulent under these conditions. We therefore conclude from these results that the secreted aspartic proteases Sap1p–Sap6p are not required for invasion of RHE by *C. albicans*.

INTRODUCTION

The yeast *Candida albicans* is a harmless commensal on mucosal surfaces of the gastrointestinal and urogenital tract in most healthy people. However, especially in immunocompromised patients, *C. albicans* can also invade into the epithelium and cause superficial as well as life-threatening disseminated infections (Odds, 1988). The secretion of aspartic proteases has long been recognized as a virulence-associated trait of this fungal pathogen (Kwon-Chung *et al.*, 1985; Macdonald & Odds, 1983; Staib, 1969). *C. albicans* possesses a gene family encoding secreted aspartic proteases (Saps), which are thought to have different roles during an infection, e.g. the degradation of tissue barriers during invasion, destruction of host defence molecules, or nutrient supply (Naglik *et al.*, 2003a). The importance of specific Sap isoenzymes for the pathogenicity of *C. albicans* has been investigated by comparing the virulence of mutants deleted for individual or multiple *SAP* genes with that of a wild-type control strain in different infection models. Especially the *SAP1–SAP6* genes have been studied extensively. Initially it was demonstrated that *sap1*Δ, *sap2*Δ and *sap3*Δ single mutants as well as triple mutants lacking the highly homologous *SAP4–SAP6* genes exhibited attenuated virulence after intravenous infection of mice, indicating that all these genes have important roles for the normal progression of a systemic infection (Hube *et al.*, 1997; Sanglard *et al.*, 1997). Mutants deleted for either *SAP1*, *SAP2* or *SAP3* were also found to be less virulent in a rat model of *Candida* vaginitis, whereas mutants lacking *SAP4–SAP6* did not exhibit a detectable virulence defect under these conditions (De Bernardis *et al.*, 1999). Conversely, only the latter mutants showed reduced virulence in a murine model of *Candida* peritonitis, while deletion of *SAP1*, *SAP2* or *SAP3* had no significant effect in this infection model (Kretschmar *et al.*, 1999). These and other studies indicate that the relative importance of specific *SAP* genes for the pathogenicity of *C. albicans* depends on the type of infection.

As the various Sap isoenzymes may have partially redundant functions, the analysis of deletion mutants might not reveal a role of a particular *SAP* gene under all conditions. The individual members of the *SAP* gene family

Abbreviations: IVET, *in vitro* expression technology; LDH, lactate dehydrogenase; MPA, mycophenolic acid; RHE, reconstituted human epithelium/epithelia; Sap, secreted aspartic protease.

are differentially expressed during *in vitro* growth, depending on the cell type and the growth conditions (Hube *et al.*, 1994; White & Agabian, 1995). For example, *SAP2* is specifically induced in media containing proteins as the sole nitrogen source and is required for growth of *C. albicans* under these conditions. *SAP1* and *SAP3* are phase-specific genes and are specifically expressed in opaque cells, the mating-competent form of *C. albicans*, while expression of *SAP4–SAP6* is usually stimulated under conditions that promote hyphal growth. Assuming that the expression of a specific *SAP* gene in a certain tissue reflects its role at that stage of an infection, various investigators have studied the expression patterns of the *SAP* genes in different types of infections. In an early study, expression of *SAP1* and *SAP2* during experimental vaginitis in rats could be demonstrated by Northern hybridization analysis (De Bernardis *et al.*, 1995). Expression of *SAP1*, *SAP2* and *SAP4–SAP6* was detected by RT-PCR during invasion of parenchymal organs after intraperitoneal infection of mice (Felk *et al.*, 2002). In a mouse model of oroesophageal and gastric candidiasis, all *SAP* genes were detectably expressed during gastric candidiasis, while *SAP1* and *SAP3* were only sporadically and weakly expressed at different oral sites of infection (Schofield *et al.*, 2003). Several investigators have also used RT-PCR to analyse the expression of specific *SAP* genes during colonization and infection of humans. Schaller *et al.* (1998) detected *SAP1–SAP3* and *SAP6* transcripts in samples from patients with oral candidiasis, whereas no expression of *SAP4* and *SAP5* was found. In contrast, Naglik *et al.* (2003b) found *SAP2* and *SAP5* to be the most commonly expressed genes in individuals with oral and vaginal *Candida* carriage or infection, while *SAP1* and *SAP3* were preferentially expressed in vaginal, rather than oral, infections.

In vitro models using reconstituted human epithelia (RHE) have also been used to evaluate the importance of specific Sap isoenzymes for tissue invasion and to investigate the expression patterns of individual *SAP* genes. On oral RHE, expression of *SAP1–SAP3* and *SAP6*, but not *SAP4* and *SAP5*, was detected by RT-PCR and each of the *SAP1–SAP3* genes was also found to be required for wild-type levels of tissue damage in this model (Schaller *et al.*, 1998, 1999). *SAP1* and *SAP2*, but not *SAP3–SAP6*, were also required for tissue damage during infection of vaginal RHE, although RT-PCR showed almost all of these *SAP* genes to be expressed under these conditions (Schaller *et al.*, 2003). A role of the Saps during infection of RHE was also demonstrated by the observation that the extent of tissue damage caused by the wild-type strain SC5314 was reduced in the presence of aspartic protease inhibitors (Schaller *et al.*, 1999, 2003).

While all of the above studies point to a differential expression and specific roles of the various *SAP* genes during colonization and infection of different host tissues, there are also discrepancies in the results obtained, which may be related to differences in the sensitivities of the methods used in various laboratories, intrinsic differences even in apparently similar infection models, and variability among different *C. albicans* strains.

Our group has previously employed a genetic reporter system, termed *in vivo* expression technology (IVET), to study the expression of the *SAP1–SAP6* genes in the *C. albicans* model strain SC5314 during experimental infections. We constructed a set of reporter strains which carry a *Candida*-adapted *FLP* gene (*ecaFLP*), encoding the site-specific recombinase FLP, under control of the various *SAP* gene promoters. The induction of the promoter results in expression of the FLP recombinase, which in turn catalyses the excision of a mycophenolic acid resistance marker (MPA^R) from the genome, so that even a transient induction of the target gene during an infection can be detected in individual cells by the MPA-sensitive phenotype of their progeny after reisolation from infected tissue (Staib *et al.*, 1999). Using this reporter system we could also detect a stage- and tissue-specific expression pattern of the *SAP1–SAP6* genes. We found that *SAP5* was induced at an early stage in all infection models examined (intravenous, intraperitoneal, oral and vaginal infections of mice), whereas other *SAP* genes had a more specific expression pattern. Beside its activation after intravenous infection, *SAP6* expression was detected during invasion of the oesophageal mucosa, but not in a mouse model of vaginal candidiasis. Conversely, *SAP4* was significantly expressed during vaginal, but not oesophageal infection. *SAP2* expression was usually found at the late stages of intraperitoneal and systemic infections, although the *SAP2-2* allele was also induced at earlier time points. No significant expression of *SAP2* or the opaque-specific *SAP1* and *SAP3* genes was observed during infection of the oesophageal or vaginal tissue of mice (Staib *et al.*, 2000, 2002a; Taylor *et al.*, 2005). Especially the latter results were in striking contrast to findings of other researchers, who found *SAP1–SAP3* to be expressed during oral and/or vaginal infections of rats and humans and, in contrast to the *SAP4–SAP6* genes, also to be required for invasion of oral and vaginal RHE (see above). Although the results obtained with the *ecaFLP* reporter gene in the mouse model of vaginal candidiasis were fully confirmed using green fluorescent protein (GFP) as an alternative reporter (Taylor *et al.*, 2005), it remained possible that both reporter systems were not sensitive enough to detect a low but biologically relevant expression of the *SAP1–SAP3* genes. Alternatively, it seemed possible that the environment encountered during vaginal infection in mice is different from that to which *C. albicans* is exposed during human and rat vaginitis and also during *in vitro* infection of RHE. To address these issues, we used the reportedly highly reproducible oral and vaginal RHE infection models (Naglik *et al.*, 2003a) to re-examine both the expression pattern and the importance of the *SAP1–SAP6* genes for epithelial invasion and damage by *C. albicans*.

METHODS

Strains and growth conditions. *C. albicans* strains used in this study and, for clarity, the relevant parent strains are listed in Table 1. All strains were stored as frozen stocks with 15 % glycerol at −80 °C.

Table 1. *C. albicans* strains

Strain	Parent	Relevant characteristics or genotype*	Reference
SC5314		Prototrophic wild-type strain	Gillum *et al.* (1984)
CAI4	SC5314	*ura3*Δ::*imm434/ura3*Δ::*imm434*	Fonzi & Irwin (1993)
CFI1	CAI4	*ACT1/act1*::*FRT-MPA^R-FRT*	Staib *et al.* (1999)
Can33	CAI4	*efg1*::*hisG/efg1*::*hisG-URA3-hisG*	Dieterich *et al.* (2002)
Reporter strains			
S1FI2A	CFI1	*SAP1-1/sap1-2*::P$_{SAP1}$-*ecaFLP-URA3*	Staib *et al.* (2000)
S1FI2B	CFI1	*sap1-1*::P$_{SAP1}$-*ecaFLP-URA3/SAP1-2*	Staib *et al.* (2000)
S2FI5B	CFI1	*sap2-1*::P$_{SAP2-1}$-*ecaFLP-URA3/SAP2-2*	Staib *et al.* (2000)
S2FI7A	CFI1	*SAP2-1/sap2-2*::P$_{SAP2-2}$-*ecaFLP-URA3*	Staib *et al.* (2002a)
S3FI2B	CFI1	*SAP3/sap3*::P$_{SAP3}$-*ecaFLP-URA3*	Staib *et al.* (2000)
S3FI2C	CFI1	*SAP3/sap3*::P$_{SAP3}$-*ecaFLP-URA3*	Staib *et al.* (2000)
S4FI2A	CFI1	*SAP4-1/sap4-2*::P$_{SAP4}$-*ecaFLP-URA3*	Staib *et al.* (2000)
S4FI2B	CFI1	*sap4-1*::P$_{SAP4}$-*ecaFLP-URA3/SAP4-2*	Staib *et al.* (2000)
S5FI2A	CFI1	*sap5-1*::P$_{SAP5}$-*ecaFLP-URA3/SAP5-2*	Staib *et al.* (2000)
S5FI2B	CFI1	*SAP5-1/sap5-2*::P$_{SAP5}$-*ecaFLP-URA3*	Staib *et al.* (2000)
S6FI2A	CFI1	*sap6-1*::P$_{SAP6-1}$-*ecaFLP-URA3/SAP6-2*	Staib *et al.* (2000)
S6FI3A	CFI1	*SAP6-1/sap6-2*::P$_{SAP6-2}$-*ecaFLP-URA3*	Taylor *et al.* (2005)
S2UI1A	CFI1	*sap2-1*::*URA3/SAP2-2*	Staib *et al.* (1999)
***sap1*Δ single mutants**			
SAP1MS1A	SC5314	*sap1-1*Δ::*SAT1-FLIP*†*/SAP1-2*	This study
SAP1MS1B	SC5314	*SAP1-1/sap1-2*Δ::*SAT1-FLIP*	This study
SAP1MS2A	SAP1MS1A	*sap1-1*Δ::*FRT/SAP1-2*	This study
SAP1MS2B	SAP1MS1B	*SAP1-1/sap1-2*Δ::*FRT*	This study
SAP1MS3A	SAP1MS2A	*sap1-1*Δ::*FRT/sap1-2*Δ::*SAT1-FLIP*	This study
SAP1MS3B	SAP1MS2B	*sap1-1*Δ::*SAT1-FLIP/sap1-2*Δ::*FRT*	This study
SAP1MS4A	SAP1MS3A	*sap1-1*Δ::*FRT/sap1-2*Δ::*FRT*	This study
SAP1MS4B	SAP1MS3B	*sap1-1*Δ::*FRT/sap1-2*Δ::*FRT*	This study
***sap2*Δ single mutants**			
SAP2MS4A and -B	SC5314	*sap2-1*Δ::*FRT/sap2-2*Δ::*FRT*	Staib *et al.* (2008)
***sap3*Δ single mutants**			
SAP3MS1A and -B	SC5314	*SAP3/sap3*Δ::*SAT1-FLIP*	This study
SAP3MS2A	SAP3MS1A	*SAP3/sap3*Δ::*FRT*	This study
SAP3MS2B	SAP3MS1B	*SAP3/sap3*Δ::*FRT*	This study
SAP3MS3A	SAP3MS2A	*sap3*Δ::*SAT1-FLIP/sap3*Δ::*FRT*	This study
SAP3MS3B	SAP3MS2B	*sap3*Δ::*SAT1-FLIP/sap3*Δ::*FRT*	This study
SAP3MS4A	SAP3MS3A	*sap3*Δ::*FRT/sap3*Δ::*FRT*	This study
SAP3MS4B	SAP3MS3B	*sap3*Δ::*FRT/sap3*Δ::*FRT*	This study
***sap4*Δ single mutants**			
SAP4MS1A	SC5314	*sap4-1*Δ::*SAT1-FLIP/SAP4-2*	This study
SAP4MS1B	SC5314	*SAP4-1/sap4-2*Δ::*SAT1-FLIP*	This study
SAP4MS2A	SAP4MS1A	*sap4-1*Δ::*FRT/SAP4-2*	This study
SAP4MS2B	SAP4MS1B	*SAP4-1/sap4-2*Δ::*FRT*	This study
SAP4MS3A	SAP4MS2A	*sap4-1*Δ::*FRT/sap4-2*Δ::*SAT1-FLIP*	This study
SAP4MS3B	SAP4MS2B	*sap4-1*Δ::*SAT1-FLIP/sap4-2*Δ::*FRT*	This study
SAP4MS4A	SAP4MS3A	*sap4-1*Δ::*FRT/sap4-2*Δ::*FRT*	This study
SAP4MS4B	SAP4MS3B	*sap4-1*Δ::*FRT/sap4-2*Δ::*FRT*	This study
***sap5*Δ single mutants**			
SAP5MS1A	SC5314	*sap5-1*Δ::*SAT1-FLIP/SAP5-2*	This study
SAP5MS1B	SC5314	*SAP5-1/sap5-2*Δ::*SAT1-FLIP*	This study
SAP5MS2A	SAP5MS1A	*sap5-1*Δ::*FRT/SAP5-2*	This study
SAP5MS2B	SAP5MS1B	*SAP5-1/sap5-2*Δ::*FRT*	This study
SAP5MS3A	SAP5MS2A	*sap5-1*Δ::*FRT/sap5-2*Δ::*SAT1-FLIP*	This study
SAP5MS3B	SAP5MS2B	*sap5-1*Δ::*SAT1-FLIP/sap5-2*Δ::*FRT*	This study
SAP5MS4A	SAP5MS3A	*sap5-1*Δ::*FRT/sap5-2*Δ::*FRT*	This study
SAP5MS4B	SAP5MS3B	*sap5-1*Δ::*FRT/sap5-2*Δ::*FRT*	This study
***sap6*Δ single mutants**			
SAP6MS1A	SC5314	*sap6-1*Δ::*SAT1-FLIP/SAP6-2*	This study

Table 1. cont.

Strain	Parent	Relevant characteristics or genotype*	Reference
SAP6MS1B	SC5314	*SAP6-1*/*sap6-2*Δ::*SAT1-FLIP*	This study
SAP6MS2A	SAP6MS1A	*sap6-1*Δ::*FRT*/*SAP6-2*	This study
SAP6MS2B	SAP6MS1B	*SAP6-1*/*sap6-2*Δ::*FRT*	This study
SAP6MS3A	SAP6MS2A	*sap6-1*Δ::*FRT*/*sap6-2*Δ::*SAT1-FLIP*	This study
SAP6MS3B	SAP6MS2B	*sap6-1*Δ::*SAT1-FLIP*/*sap6-2*Δ::*FRT*	This study
SAP6MS4A	SAP6MS3A	*sap6-1*Δ::*FRT*/*sap6-2*Δ::*FRT*	This study
SAP6MS4B	SAP6MS3B	*sap6-1*Δ::*FRT*/*sap6-2*Δ::*FRT*	This study
***sap1*Δ *sap2*Δ double mutants**			
SAP12MS1A	SAP2MS4A	*SAP1*/*sap1*Δ::*SAT1-FLIP*; *sap2-1*Δ::*FRT*/*sap2-2*Δ::*FRT*	This study
SAP12MS1B	SAP2MS4B	*SAP1*/*sap1*Δ::*SAT1-FLIP*; *sap2-1*Δ::*FRT*/*sap2-2*Δ::*FRT*	This study
SAP12MS2A	SAP12MS1A	*SAP1*/*sap1*Δ::*FRT*; *sap2-1*Δ::*FRT*/*sap2-2*Δ::*FRT*	This study
SAP12MS2B	SAP12MS1B	*SAP1*/*sap1*Δ::*FRT*; *sap2-1*Δ::*FRT*/*sap2-2*Δ::*FRT*	This study
SAP12MS3A	SAP12MS2A	*sap1*Δ::*SAT1-FLIP*/*sap1*Δ::*FRT*; *sap2-1*Δ::*FRT*/*sap2-2*Δ::*FRT*	This study
SAP12MS3B	SAP12MS2B	*sap1*Δ::*SAT1-FLIP*/*sap1*Δ::*FRT*; *sap2-1*Δ::*FRT*/*sap2-2*Δ::*FRT*	This study
SAP12MS4A	SAP12MS3A	*sap1*Δ::*FRT*/*sap1*Δ::*FRT*; *sap2-1*Δ::*FRT*/*sap2-2*Δ::*FRT*	This study
SAP12MS4B	SAP12MS3B	*sap1*Δ::*FRT*/*sap1*Δ::*FRT*; *sap2-1*Δ::*FRT*/*sap2-2*Δ::*FRT*	This study
***sap1*Δ *sap3*Δ double mutants**			
SAP13MS1A	SAP1MS4A	*sap1-1*Δ::*FRT*/*sap1-2*Δ::*FRT*; *SAP3*/*sap3*Δ::*SAT1-FLIP*	This study
SAP13MS1B	SAP1MS4B	*sap1-1*Δ::*FRT*/*sap1-2*Δ::*FRT*; *SAP3*/*sap3*Δ::*SAT1-FLIP*	This study
SAP13MS2A	SAP13MS1A	*sap1-1*Δ::*FRT*/*sap1-2*Δ::*FRT*; *SAP3*/*sap3*Δ::*FRT*	This study
SAP13MS2B	SAP13MS1B	*sap1-1*Δ::*FRT*/*sap1-2*Δ::*FRT*; *SAP3*/*sap3*Δ::*FRT*	This study
SAP13MS3A	SAP13MS2A	*sap1-1*Δ::*FRT*/*sap1-2*Δ::*FRT*; *sap3*Δ::*SAT1-FLIP*/*sap3*Δ::*FRT*	This study
SAP13MS3B	SAP13MS2B	*sap1-1*Δ::*FRT*/*sap1-2*Δ::*FRT*; *sap3*Δ::*SAT1-FLIP*/*sap3*Δ::*FRT*	This study
SAP13MS4A	SAP13MS3A	*sap1-1*Δ::*FRT*/*sap1-2*Δ::*FRT*; *sap3*Δ::*FRT*/*sap3*Δ::*FRT*	This study
SAP13MS4B	SAP13MS3B	*sap1-1*Δ::*FRT*/*sap1-2*Δ::*FRT*; *sap3*Δ::*FRT*/*sap3*Δ::*FRT*	This study
***sap2*Δ *sap3*Δ double mutants**			
SAP23MS1C	SAP3MS4A	*sap2-1*Δ::*SAT1-FLIP*/*SAP2-2*; *sap3*Δ::*FRT*/*sap3*Δ::*FRT*	This study
SAP23MS1D	SAP3MS4B	*sap2-1*Δ::*SAT1-FLIP*/*SAP2-2*; *sap3*Δ::*FRT*/*sap3*Δ::*FRT*	This study
SAP23MS2C	SAP23MS1C	*sap2-1*Δ::*FRT*/*SAP2-2*; *sap3*Δ::*FRT*/*sap3*Δ::*FRT*	This study
SAP23MS2D	SAP23MS1D	*sap2-1*Δ::*FRT*/*SAP2-2*; *sap3*Δ::*FRT*/*sap3*Δ::*FRT*	This study
SAP23MS3C	SAP23MS2C	*sap2-1*Δ::*FRT*/*sap2-2*Δ::*SAT1-FLIP*; *sap3*Δ::*FRT*/*sap3*Δ::*FRT*	This study
SAP23MS3D	SAP23MS2D	*sap2-1*Δ::*FRT*/*sap2-2*Δ::*SAT1-FLIP*; *sap3*Δ::*FRT*/*sap3*Δ::*FRT*	This study
SAP23MS4C	SAP23MS3C	*sap2-1*Δ::*FRT*/*sap2-2*Δ::*FRT*; *sap3*Δ::*FRT*/*sap3*Δ::*FRT*	This study
SAP23MS4D	SAP23MS3D	*sap2-1*Δ::*FRT*/*sap2-2*Δ::*FRT*; *sap3*Δ::*FRT*/*sap3*Δ::*FRT*	This study
***sap4*Δ *sap5*Δ double mutants**			
SAP45MS1A	SAP4MS4A	*sap4-1*Δ::*FRT*/*sap4-2*Δ::*FRT*; *sap5-1*Δ::*SAT1-FLIP*/*SAP5-2*	This study
SAP45MS1B	SAP4MS4B	*sap4-1*Δ::*FRT*/*sap4-2*Δ::*FRT*; *sap5-1*Δ::*SAT1-FLIP*/*SAP5-2*	This study
SAP45MS2A	SAP45MS1A	*sap4-1*Δ::*FRT*/*sap4-2*Δ::*FRT*; *sap5-1*Δ::*FRT*/*SAP5-2*	This study
SAP45MS2B	SAP45MS1B	*sap4-1*Δ::*FRT*/*sap4-2*Δ::*FRT*; *sap5-1*Δ::*FRT*/*SAP5-2*	This study
SAP45MS3A	SAP45MS2A	*sap4-1*Δ::*FRT*/*sap4-2*Δ::*FRT*; *sap5-1*Δ::*FRT*/ *sap5-2*Δ::*SAT1-FLIP*	This study
SAP45MS3B	SAP45MS2B	*sap4-1*Δ::*FRT*/*sap4-2*Δ::*FRT*; *sap5-1*Δ::*FRT*/ *sap5-2*Δ::*SAT1-FLIP*	This study
SAP45MS4A	SAP45MS3A	*sap4-1*Δ::*FRT*/*sap4-2*Δ::*FRT*; *sap5-1*Δ::*FRT*/ *sap5-2*Δ::*FRT*	This study
SAP45MS4B	SAP45MS3B	*sap4-1*Δ::*FRT*/*sap4-2*Δ::*FRT*; *sap5-1*Δ::*FRT*/ *sap5-2*Δ::*FRT*	This study

Table 1. cont.

Strain	Parent	Relevant characteristics or genotype*	Reference
***sap4Δ sap6Δ* double mutants**			
SAP46MS1A	SAP6MS4A	*sap4-1Δ::SAT1-FLIP/SAP4-2*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP46MS1B	SAP6MS4B	*SAP4-1/sap4-2Δ::SAT1-FLIP*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP46MS2A	SAP46MS1A	*sap4-1Δ::FRT/SAP4-2*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP46MS2B	SAP46MS1B	*SAP4-1/sap4-2Δ::FRT*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP46MS3A	SAP46MS2A	*sap4-1Δ::FRT/sap4-2Δ::SAT1-FLIP*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP46MS3B	SAP46MS2B	*sap4-1Δ::SAT1-FLIP/sap4-2Δ::FRT*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP46MS4A	SAP46MS3A	*sap4-1Δ::FRT/sap4-2Δ::FRT*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP46MS4B	SAP46MS3B	*sap4-1Δ::FRT/sap4-2Δ::FRT*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
***sap5Δ sap6Δ* double mutants**			
SAP56MS1A	SAP6MS4A	*sap5-1Δ::SAT1-FLIP/SAP5-2*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP56MS1B	SAP6MS4B	*sap5-1Δ::SAT1-FLIP/SAP5-2*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP56MS2A	SAP56MS1A	*sap5-1Δ::FRT/SAP5-2*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP56MS2B	SAP56MS1B	*sap5-1Δ::FRT/SAP5-2*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP56MS3A	SAP56MS2A	*sap5-1Δ::FRT/sap5-2Δ::SAT1-FLIP*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP56MS3B	SAP56MS2B	*sap5-1Δ::FRT/sap5-2Δ::SAT1-FLIP*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP56MS4A	SAP56MS3A	*sap5-1Δ::FRT/sap5-2Δ::FRT*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP56MS4B	SAP56MS3B	*sap5-1Δ::FRT/sap5-2Δ::FRT*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
***sap1Δ sap2Δ sap3Δ* triple mutants**			
SAP123MS1C	SAP12MS4A	*sap1Δ::FRT/sap1Δ::FRT*; *sap2-1Δ::FRT/sap2-2Δ::FRT*; *SAP3/sap3Δ::SAT1-FLIP*	This study
SAP123MS1D	SAP12MS4B	*sap1Δ::FRT/sap1Δ::FRT*; *sap2-1Δ::FRT/sap2-2Δ::FRT*; *SAP3/sap3Δ::SAT1-FLIP*	This study
SAP123MS2C	SAP123MS1C	*sap1Δ::FRT/sap1Δ::FRT*; *sap2-1Δ::FRT/sap2-2Δ::FRT*; *SAP3/sap3Δ::FRT*	This study
SAP123MS2D	SAP123MS1D	*sap1Δ::FRT/sap1Δ::FRT*; *sap2-1Δ::FRT/sap2-2Δ::FRT*; *SAP3/sap3Δ::FRT*	This study
SAP123MS3C	SAP123MS2C	*sap1Δ::FRT/sap1Δ::FRT*; *sap2-1Δ::FRT/sap2-2Δ::FRT*; *sap3Δ::SAT1-FLIP/sap3Δ::FRT*	This study
SAP123MS3D	SAP123MS2D	*sap1Δ::FRT/sap1Δ::FRT*; *sap2-1Δ::FRT/sap2-2Δ::FRT*; *sap3Δ::SAT1-FLIP/sap3Δ::FRT*	This study
SAP123MS4C	SAP123MS3C	*sap1Δ::FRT/sap1Δ::FRT*; *sap2-1Δ::FRT/sap2-2Δ::FRT*; *sap3Δ::FRT/sap3Δ::FRT*	This study
SAP123MS4D	SAP123MS3D	*sap1Δ::FRT/sap1Δ::FRT*; *sap2-1Δ::FRT/sap2-2Δ::FRT*; *sap3Δ::FRT/sap3Δ::FRT*	This study
***sap4Δ sap5Δ sap6Δ* triple mutants**			
SAP456MS1A	SAP56MS4A	*sap4-1Δ::SAT1-FLIP/SAP4-2*; *sap5-1Δ::FRT/sap5-2Δ::FRT*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP456MS1B	SAP56MS4B	*sap4-1Δ::SAT1-FLIP/SAP4-2*; *sap5-1Δ::FRT/sap5-2Δ::FRT*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP456MS2A	SAP456MS1A	*sap4-1Δ::FRT/SAP4-2*; *sap5-1Δ::FRT/sap5-2Δ::FRT*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study
SAP456MS2B	SAP456MS1B	*sap4-1Δ::FRT/SAP4-2*; *sap5-1Δ::FRT/sap5-2Δ::FRT*; *sap6-1Δ::FRT/sap6-2Δ::FRT*	This study

Table 1. cont.

Strain	Parent	Relevant characteristics or genotype*	Reference
SAP456MS3A	SAP456MS2A	*sap4-1*Δ::*FRT*/*sap4-2*Δ::*SAT1-FLIP*; *sap5-1*Δ::*FRT*/*sap5-2*Δ::*FRT*; *sap6-1*Δ::*FRT*/*sap6-2*Δ::*FRT*	This study
SAP456MS3B	SAP456MS2B	*sap4-1*Δ::*FRT*/*sap4-2*Δ::*SAT1-FLIP*; *sap5-1*Δ::*FRT*/*sap5-2*Δ::*FRT*; *sap6-1*Δ::*FRT*/*sap6-2*Δ::*FRT*	This study
SAP456MS4A	SAP456MS3A	*sap4-1*Δ::*FRT*/*sap4-2*Δ::*FRT*; *sap5-1*Δ::*FRT*/*sap5-2*Δ::*FRT*; *sap6-1*Δ::*FRT*/*sap6-2*Δ::*FRT*	This study
SAP456MS3B	SAP456MS3B	*sap4-1*Δ::*FRT*/*sap4-2*Δ::*FRT*; *sap5-1*Δ::*FRT*/*sap5-2*Δ::*FRT*; *sap6-1*Δ::*FRT*/*sap6-2*Δ::*FRT*	This study

*Apart from the indicated features all strains are identical to their parental strains.
†*SAT1-FLIP* denotes the *SAT1* flipper cassette. In strain SC5314, the two *SAP1* alleles can be distinguished by a *Kpn*I restriction fragment length polymorphism (RFLP), the two *SAP2* alleles by a *Cla*I RFLP, the two *SAP4* and the two *SAP5* alleles by a *Bgl*II RFLP, and the two *SAP6* alleles by an *Eco*RI RFLP. In those cases in which we determined which of the two alleles was inactivated first, the alleles are distinguished by a suffix.

The strains were routinely grown in YPD medium [10 g yeast extract, 20 g peptone (BBL Trypticase Peptone, Becton Dickinson) and 20 g glucose per litre] at 30 °C. Reporter strains were grown in SD medium [6.7 g yeast nitrogen base without amino acids (YNB; Bio 101) and 20 g glucose per litre]. To prepare solid media, 1.5 % agar was added before autoclaving. For excision of the *SAT1* flipper from nourseothricin-resistant transformants by FLP-mediated recombination, the strains were cultivated overnight in YPM medium (10 g yeast extract, 20 g peptone and 20 g maltose per litre) without selective pressure to induce the *MAL2* promoter. One hundred to two hundred cells were spread on YPD plates containing 20 μg nourseothricin ml^{-1} (Werner Bioagents) and grown for 2 days at 30 °C. Nou^S clones were identified by their small colony size and confirmed by restreaking on YPD plates containing 100 μg nourseothricin ml^{-1} as described previously (Reuß *et al.*, 2004). To test for growth on BSA as the sole nitrogen source, strains were grown at 30 °C or 37 °C in YCB-BSA medium [23.4 g yeast carbon base, 4 g bovine serum albumin (Fraction V; Gerbu) per litre, pH 4.0].

Plasmid constructions. Plasmid pSAP2MS2, which was used to delete the *SAP2* gene, has been described before (Staib *et al.*, 2008). Analogous constructs were generated for the deletion of *SAP1* and *SAP3*–*SAP6*. *Apa*I–*Sal*I fragments containing the upstream sequences of these genes were obtained from plasmids pSFL13, pSFL33, pSFL43, pSFL53 and pSFL63 (Staib *et al.*, 2000) and ligated into *Apa*I/*Xho*I-digested pOPT1M3 (Reuß *et al.*, 2004), resulting in plasmids pSAP1MS1, pSAP3MS1, pSAP4MS1, pSAP5MS1 and pSAP6MS1, respectively. The downstream regions of the *SAP* genes were then amplified using the primer pairs SAP1K/SAP1L, SAP3K/SAP3L, SAP4K/SAP4L, SAP5N/SAP5Q and SAP6N/SAP6Q (primer sequences are given in Table 2). The PCR products were digested at the introduced *Sac*II and *Sac*I sites and ligated between the same sites of the plasmids described above, resulting in pSAP1MS2, pSAP3MS2, pSAP4MS2, pSAP5MS2 and pSAP6MS2 (see Fig. 2a and c, Fig. 3a–c).

***C. albicans* transformation.** *C. albicans* strain SC5314 was transformed by electroporation (Köhler *et al.*, 1997) with the gel-purified *Apa*I–*Sac*I fragments from plasmids pSAP1MS2, pSAP2MS2, pSAP3MS2, pSAP4MS2, pSAP5MS2 and pSAP6MS2. Nourseothricin-resistant transformants were selected on YPD agar plates containing 200 μg nourseothricin ml^{-1} as described previously (Reuß *et al.*, 2004). Single-copy integration of all constructs was confirmed by Southern hybridization.

Isolation of genomic DNA and Southern hybridization. Genomic DNA from *C. albicans* strains was isolated as described previously (Millon *et al.*, 1994). A 10 μg sample of DNA was digested with appropriate restriction enzymes, separated on a 1 % agarose gel and, after ethidium bromide staining, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. Southern hybridization with enhanced chemiluminescence-labelled probes was performed with the Amersham ECL Direct Nucleic Acid Labelling and Detection System (GE Healthcare) according to the instructions of the manufacturer.

Cultivation of reconstituted human epithelia. The human epithelia for the *in vitro* model of oral and vaginal candidiasis were supplied by SkinEthic Laboratories. Oral and vaginal RHE were obtained by culturing the human cell lines TR146 and A431, respectively, on an inert supporting membrane. According to the guidelines of the supplier, uninfected and infected RHE were incubated in 1 ml SkinEthic maintenance medium at 37 °C with 5 % CO_2 at 100 % humidity. After 24 h, the culture medium was removed and fresh medium was added.

Detection of *SAP* induction during infection of vaginal RHE. Reporter strains were grown overnight at 30 °C in SD medium, washed, and resuspended in PBS at a density of 10^7 cells ml^{-1}. Vaginal RHE was infected with 5×10^5 *C. albicans* cells. After 48 h of incubation, the RHE was lysed by the addition of sterile distilled water and the *C. albicans* cells were recovered and plated at an appropriate density on indicator plates (SD agar containing 1.8 μg MPA ml^{-1}).

Table 2. Primers used in this study

Restriction sites introduced into the primers are underlined.

Primer	Sequence (3′–5′)
SAP1K	GAGATACCTACTTCCGCGGTTATAAGTGTC
SAP1L	TCCAAGAGCTCAAGTAGCTTTCCAAC
SAP3K	GCTGCTCTTACCGCGGGGGAAGTACAC
SAP3L	CCAATTGAATTAGAGCTCTTTTTACCAGCC
SAP4K	GACGACCGCGGTTTTAGATTAATTGTCGG
SAP4L	GGTGTAATTGCTGAGCTCTTTTTGATGG
SAP5N	ATTAACCGCGGAACTTTGACTTTAGATTAATTATC
SAP5Q	TAAGTGAGCTCATGATTTTGTTGTATCTATGG
SAP6N	GAAAACCGCGGTTTTAGATTAATTATCGATTTGC
SAP6Q	GAAACTGCGTGAGCTCTCACATTGCACG

The percentage of small colonies was determined after 2 days of growth at 30 °C. *C. albicans* cells from the precultures were also plated on the MPA indicator plates to verify that no promoter induction had occurred before the infection.

Light microscopy. Oral or vaginal RHE was infected as described above with *C. albicans* strains grown overnight at 30 °C in YPD medium. In some experiments, pepstatin A (Sigma) was added at a final concentration of 15 μM. After 48 h of incubation, the RHE was fixed with 2.5 % glutaraldehyde and 2 % formaldehyde in a 0.05 M cacodylate-buffered solution (pH 7.2) at room temperature. After several washing and dehydration steps with chilled solutions, the tissues were embedded in glycide ether. Semi-thin sections (300 nm) of the embedded tissues were obtained by using an RMC MT 7000 ultramicrotome, stained with 0.5 % methylene blue and 0.5 % azur II, and observed by light microscopy.

Determination of LDH activity. The release of lactate dehydrogenase (LDH) from infected epithelial cells into the surrounding medium was monitored as a measure of tissue damage. LDH activity was determined with the CytoTox 96 non-radioactive cytotoxicity assay (Promega) according to the instructions of the manufacturer. Controls consisted of uninfected RHE (target spontaneous) and *C. albicans* cells grown in culture medium without RHE (effector spontaneous). Total LDH activity in the epithelial cells was determined after complete lysis of an uninfected sample with the lysis buffer provided in the kit (target maximum). After subtracting the absorbance values of the culture medium, tissue damage caused by *C. albicans* was calculated according to the following formula: % tissue damage=(experimental−effector spontaneous−target spontaneous)/(target maximum−target spontaneous).

RESULTS

Expression pattern of the *SAP1–SAP6* genes on reconstituted human vaginal epithelium

To investigate the expression of the *SAP1–SAP6* genes during infection of vaginal RHE, we used a set of reporter strains expressing *ecaFLP* under control of the respective *SAP* gene promoters (see Table 1). Vaginal RHE was infected with each of the reporter strains as well as with a similarly constructed control strain that does not contain the *ecaFLP* gene and, therefore, stably retains the MPA^R marker in the genome. *C. albicans* cells were recovered after 48 h of infection, appropriately diluted, and spread on MPA-containing indicator plates to determine the percentage of small colonies, which are generated from cells that have lost the MPA^R marker by FLP-mediated recombination. As can be seen in Fig. 1, only the *SAP5* promoter was significantly activated under these conditions, whereas the other reporter strains generated only a few small colonies, similar to the control strain, which produced a background of about 3 % small colonies due to accidental slower growth, which is in agreement with previously reported values in other infection models (Staib *et al.*, 1999). The *SAP* gene expression pattern observed in this *in vitro* model of vaginal infection partially corresponds to previous results obtained with the same set of reporter strains in a mouse model of vaginal candidiasis, in that no significant expression of *SAP1–SAP3* and *SAP6* was detected and *SAP5* was induced in both models. However, in the *in vivo* model *SAP4* was also expressed in addition to *SAP5*, albeit at lower levels (Taylor *et al.*, 2005), while this was not the case in the *in vitro* model. In addition, while *SAP5* expression was detected in virtually all infecting cells in the mouse model, only about 20 % of the cells had detectably activated the *SAP5* promoter in the RHE model (see Fig. 1). These results indicated that the signals inducing *SAP5* (and *SAP4*) expression during vaginal infection in mice are stronger than those that result in *SAP5* activation during *in vitro* infection of the RHE.

Construction of mutants of the *C. albicans* wild-type strain SC5314 lacking individual or multiple *SAP* genes

The *SAP* gene expression pattern observed during infection of the vaginal RHE with our reporter strains is in striking contrast to the results obtained by other researchers, who detected expression of *SAP1*, *SAP2* and *SAP4–6* in the same model by RT-PCR. These researchers also found that tissue damage was drastically reduced in mutants lacking either *SAP1* or *SAP2*, implying an important role of these genes for invasion of the vaginal epithelium (Schaller *et al.*, 2003). Although we had previously shown that the activation of the *SAP2* promoter under known inducing

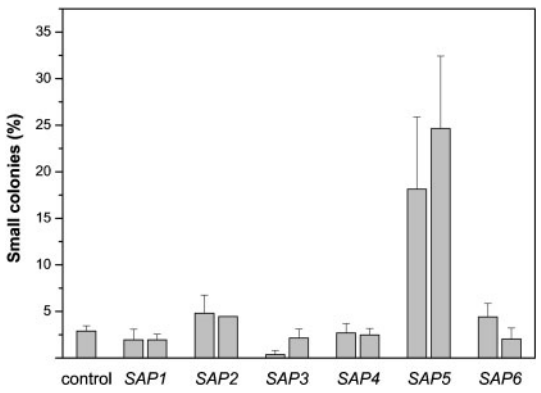
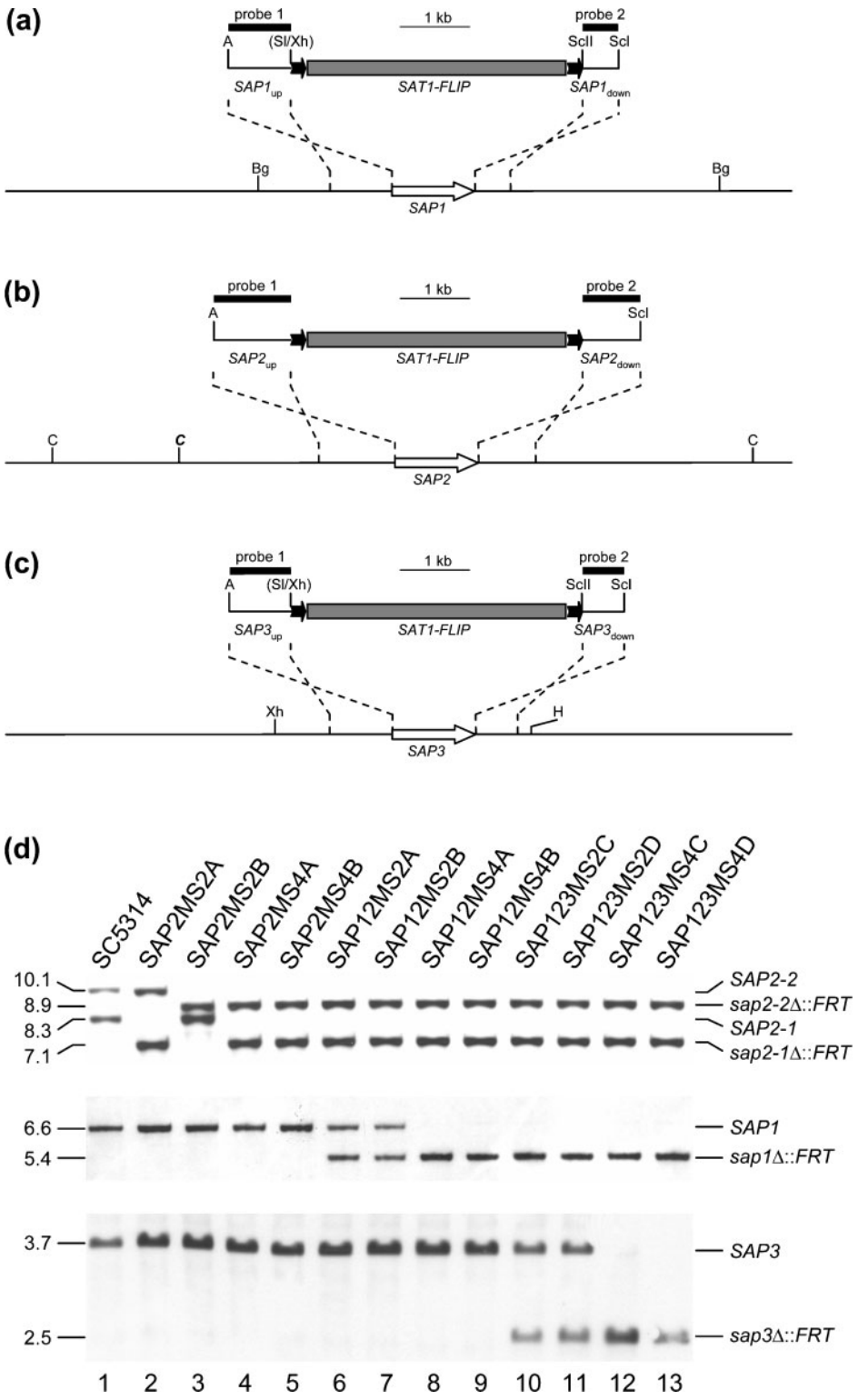


Fig. 1. Induction of the *SAP1–SAP6* promoters during invasion of vaginal RHE. Reporter strains expressing the *ecaFLP* gene under control of the indicated *SAP* gene promoters were used to infect vaginal RHE. *C. albicans* cells were recovered after 48 h of incubation and plated on MPA indicator plates. The percentage of small colonies was determined after 2 days of growth at 30 °C. For each *SAP* gene, two independently constructed reporter strains were used (see Table 1). Results are the means ± SD from two to four experiments, except for the *SAP2* reporter strain S2FI7A (second *SAP2* column), where the value is from a single experiment because in the other experiments too many cells had lost the MPA^R marker already in the preculture due to basal activity of the *SAP2-2* promoter. Strain S2UI1A, which does not contain the *ecaFLP* gene, was used as a control to estimate the background of small colonies appearing as a result of accidental slow growth instead of excision of the MPA^R marker.



conditions *in vitro* could easily be detected with the *ecaFLP* reporter gene in all cells of the population (Staib *et al.*, 2000, 2002a), we could not exclude the possibility that *SAP2* and other *SAP* genes are induced during RHE infection at low, but biologically relevant levels that are below the sensitivity limit of the reporter system. We therefore decided to readdress the importance of each of the *SAP1–SAP6* genes for infection of RHE by assessing the virulence of mutants lacking specific *SAP* genes in these models. The *sap* mutants used in earlier studies were generated from the auxotrophic laboratory strain CAI4 using the Ura-blaster protocol (Hube *et al.*, 1997; Sanglard

Fig. 2. Construction of *sap1*Δ *sap2*Δ *sap3*Δ triple mutants. (a–c) Structure of the deletion cassettes from plasmids pSAP1MS2 (a), pSAP2MS2 (b) and pSAP3MS2 (c), which were used to delete the *SAP1*, *SAP2* and *SAP3* alleles, respectively, and genomic structure of the wild-type loci in strain SC5314. The *SAP1–SAP3* coding regions are represented by white arrows and the upstream and downstream regions by the solid lines. Details of the *SAT1* flipper cassette [grey rectangle bordered by FRT sites (black arrows)] have been presented elsewhere (Reuß *et al.*, 2004). The 34 bp FRT sites are not drawn to scale. The probes used for Southern hybridization analysis of the mutants are indicated by the black bars. Only relevant restriction sites are given: A, *Apa*I; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; ScI, *Sac*I; ScII, *Sac*II; SI, *Sal*I; Xh, *Xho*I. Sites shown in parentheses were destroyed by the cloning procedure. The *Cla*I site in bold italic is present only in the *SAP2-1* allele. (d) Southern hybridizations of *Cla*I- (for *SAP2*), *Bgl*II- (for *SAP1*) and *Xho*I/*Hin*dIII- (for *SAP3*) digested genomic DNA of the parental strain SC5314 and the indicated mutants with the *SAP2*-specific probe 1 (top), the *SAP1*-specific probe 1 (middle) and the *SAP3*-specific probe 1 (bottom). Lane 1, wild-type; lanes 2 and 3, *SAP2*/*sap2*Δ; lanes 4 and 5, *sap2*Δ/*sap2*Δ; lanes 6 and 7, *SAP1*/*sap1*Δ *sap2*Δ/*sap2*Δ; lanes 8 and 9, *sap1*Δ/*sap1*Δ *sap2*Δ/*sap2*Δ; lanes 10 and 11, *sap1*Δ/*sap1*Δ *sap2*Δ/*sap2*Δ *SAP3*/*sap3*Δ; lanes 12 and 13, *sap1*Δ/*sap1*Δ *sap2*Δ/*sap2*Δ *sap3*Δ/*sap3*Δ. The sizes of the hybridizing fragments (in kb) are given on the left side of the blots and their identities are indicated on the right.

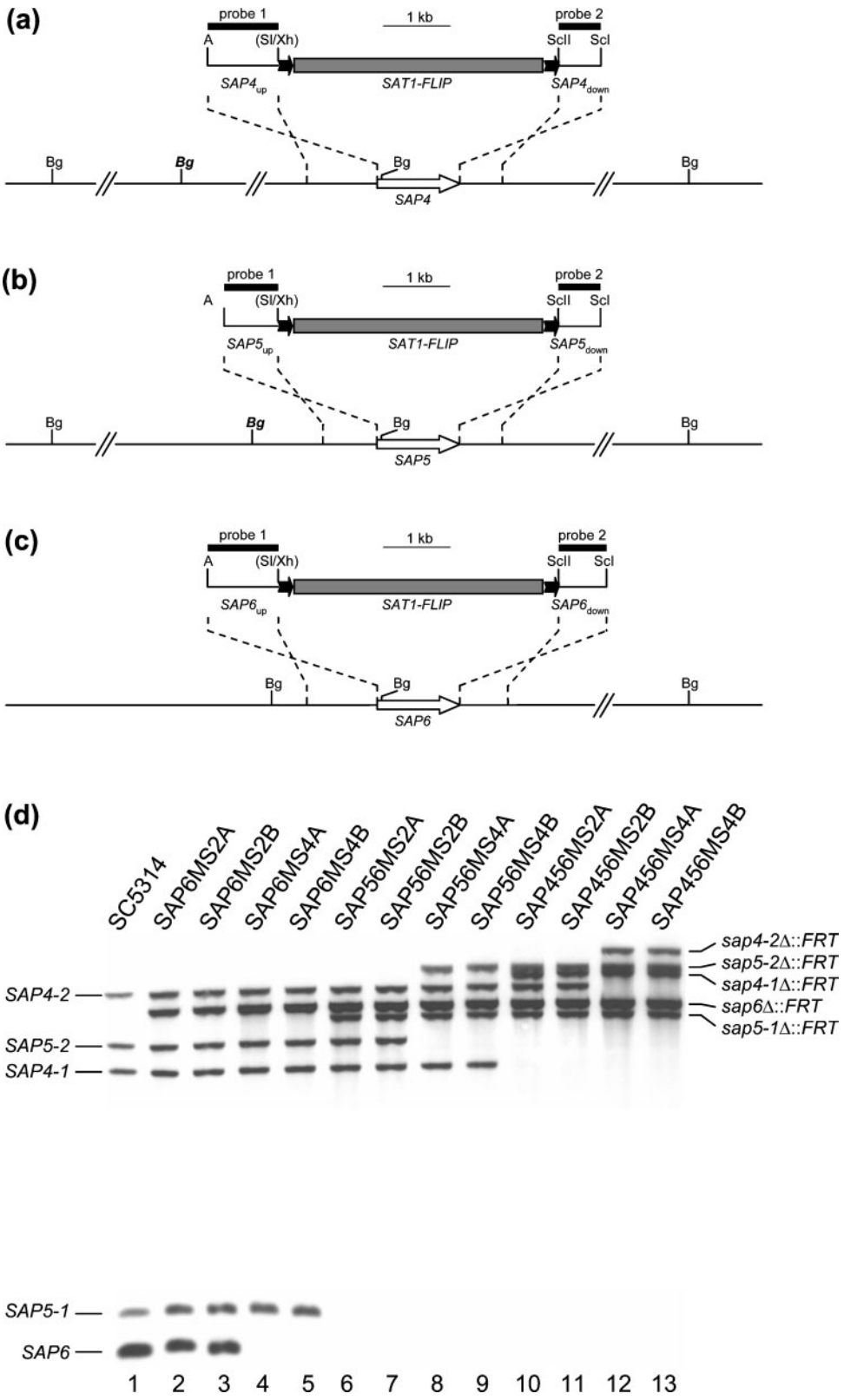
et al., 1997). As the use of the *URA3* marker for mutant construction in *C. albicans* can sometimes cause problems in the interpretation of mutant phenotypes (Bain *et al.*, 2001; Brand *et al.*, 2004; Cheng *et al.*, 2003; Lay *et al.*, 1998; Sharkey *et al.*, 2005), we constructed a new set of *sap* mutants from the prototrophic wild-type model strain SC5314 using the *SAT1*-flipping strategy (Reuß *et al.*, 2004). For each of the *SAP1–SAP6* genes, two independent series of homozygous deletion mutants were generated. In addition, within the *SAP1–SAP3* and *SAP4–SAP6* subgroups, we constructed two series of all possible double and triple mutants, starting from two independent single mutants. The generation of each mutant from its progenitors can be followed in Table 1, in which all strains are described, and the construction of the triple mutants is illustrated and documented in Figs 2 and 3. After each round of insertion and FLP-mediated excision of the *SAT1* flipper cassette the resulting strains were analysed by Southern hybridization with upstream and downstream probes of the target genes to confirm their specific inactivation and to exclude, as far as possible, undesired recombination events involving the previously inactivated loci. The absence of the target genes from the genome of the mutants was also confirmed by hybridization with the corresponding ORFs (data not shown). Apart from the deletion of the *SAP* genes, the final mutants should therefore be identical with the wild-type parental strain SC5314.

SAP2, but not *SAP4–SAP6*, is required for utilization of protein as a nitrogen source

A well-known and easily testable function of the Saps is the degradation of proteins for use as a nitrogen source (Staib, 1965). In YCB-BSA medium, which contains BSA as the sole nitrogen source, expression of the *SAP2* gene is specifically induced and allows growth of *C. albicans*. No significant expression of other *SAP* genes occurs under these conditions and *sap2*Δ mutants are unable to grow in this medium, but forced expression of most other *SAP* genes from an inducible promoter can fully or partially rescue the growth defect of *sap2*Δ mutants (Hube *et al.*, 1994, 1997; Staib *et al.*, 2002b, 2008). Interestingly, the *sap4*Δ *sap5*Δ *sap6*Δ triple mutants used in previous studies have been reported to have a similar growth defect as *sap2*Δ mutants in this medium, and it was concluded that Sap4p, Sap5p or Sap6p is required for the induction of *SAP2* expression (Sanglard *et al.*, 1997). We therefore tested the capacity of our *sap4*Δ *sap5*Δ *sap6*Δ triple mutants to grow in YCB-BSA. As can be seen in Fig. 4, mutants lacking the *SAP4–SAP6* genes grew as well as the wild-type parental strain SC5314, both at 30 °C, which is used as the standard condition in our laboratory, and at 37 °C, which was used in the experiments in which a growth defect of *sap4*Δ *sap5*Δ *sap6*Δ triple mutants was observed by other researchers (Sanglard *et al.*, 1997). In contrast, the *sap2*Δ mutants failed to grow in YCB-BSA at both temperatures, in line with previous observations (Hube *et al.*, 1997; Staib *et al.*, 2002a, 2008). When the supernatants of the cultures were analysed by SDS-PAGE, we found that the BSA in the medium was degraded by the *sap4*Δ *sap5*Δ *sap6*Δ triple mutants and comparable ampounts of Sap2p were produced by these mutants and the wild-type strain both at 30 °C and at 37 °C (Fig. 5). These results demonstrate that *SAP2*, but not any of the *SAP4–SAP6* genes, is required for growth of *C. albicans* on BSA as the sole nitrogen source and Sap2p is normally expressed in the absence of *SAP4–SAP6*. Apparently, there are differences in the phenotypes of *sap4*Δ *sap5*Δ *sap6*Δ mutants constructed previously from strain CAI4 and those generated in the present study from the wild-type strain SC5314.

SAP1–SAP6 are not required for invasion and damage of reconstituted human epithelia

We then tested the capacity of our mutants to invade and damage epithelial tissue during *in vitro* infection. In an initial set of experiments, all single mutants lacking one of the *SAP1–SAP6* genes were used for infection of RHE; however, we did not observe a virulence defect of any of these mutants (data not shown). As other *SAP* genes might be upregulated and compensate for the loss of individual Sap isoenzymes, we then used the triple mutants lacking all of *SAP1–SAP3* or *SAP4–SAP6* in further infection experiments. In addition to the wild-type control strain SC5314, a nonfilamentous *efg1*Δ mutant, which has been reported



to be noninvasive in a similar epithelial infection model (Dieterich *et al.*, 2002), was included for comparison. As shown in Fig. 6, no differences between the wild-type and either of the triple mutants in their capacity to invade and damage vaginal RHE could be observed. In each case, yeast and hyphal cells could be seen penetrating throughout the epithelium to the supporting filter. In contrast, the *efg1*Δ mutant was unable to invade and damage the epithelium

Fig. 3. Construction of *sap4*Δ *sap5*Δ *sap6*Δ triple mutants. (a–c) Structure of the deletion cassettes from plasmids pSAP4MS2 (a), pSAP5MS2 (b) and pSAP6MS2 (c), which were used to delete the *SAP4*, *SAP5* and *SAP6* alleles, respectively, and genomic structure of the wild-type loci in strain SC5314. The *SAP4–SAP6* coding regions are represented by white arrows and the upstream and downstream regions by the solid lines. Other details are as described in the legend to Fig. 2. The polymorphic *Bgl*II sites are in bold italic. (d) Southern hybridization of *Bgl*II-digested genomic DNA of the parental strain SC5314 and the indicated mutants with the *SAP6*-derived probe 1, which also cross-hybridizes with *SAP4* and *SAP5*. Lane 1, wild-type; lanes 2 and 3, *SAP6*/*sap6*Δ; lanes 4 and 5, *sap6*Δ/*sap6*Δ; lanes 6 and 7, *SAP5*/*sap5*Δ *sap6*Δ/*sap6*Δ; lanes 8 and 9, *sap5*Δ/*sap5*Δ *sap6*Δ/*sap6*Δ; lanes 10 and 11, *SAP4*/*sap4*Δ; *sap5*Δ/*sap5*Δ *sap6*Δ/*sap6*Δ; lanes 12 and 13, *sap4*Δ/*sap4*Δ *sap5*Δ/*sap5*Δ *sap6*Δ/*sap6*Δ. Wild-type alleles are indicated on the left side of the blot and mutated alleles on the right.

and only yeast cells were found at the surface of the epithelium. These results suggested that none of the *SAP1–SAP6* genes is required for invasion of vaginal RHE, a conclusion that was further corroborated by the fact that, in our hands, the addition of the aspartic protease inhibitor pepstatin A did not affect the ability of the wild-type strain SC5314 to invade the vaginal RHE (Fig. 6). Similar results were obtained in experiments in which oral RHE was infected (data not shown).

Fungal invasion and tissue damage did not occur evenly within the whole RHE samples. To better compare and quantify the tissue damage caused by the various strains, we determined the LDH released from the epithelial cells after infection with *C. albicans*, as previously described (Schaller *et al.*, 2003). Fig. 7 shows that there were no appreciable differences in the tissue damage caused by the wild-type and the *sap* mutants, and pepstatin A also did not reduce the tissue damage caused by the wild-type. Only the *efg1*Δ mutant was unable to cause significant tissue

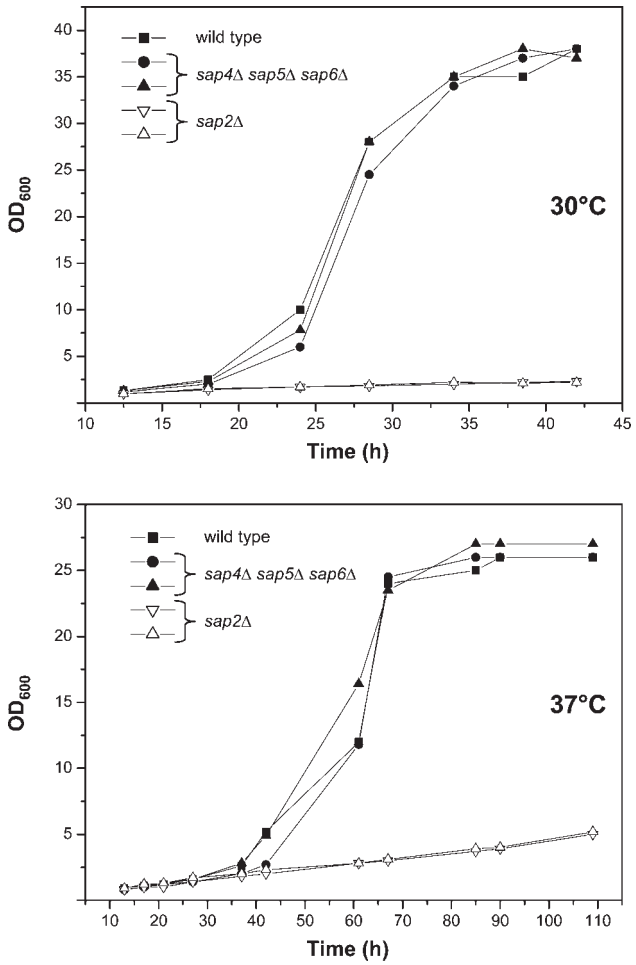


Fig. 4. Only *SAP2* is required for growth of *C. albicans* on proteins as the sole nitrogen source. YPD precultures of the wild-type strain SC5314, *sap2*Δ single, and *sap4*Δ *sap5*Δ *sap6*Δ triple mutants were diluted 10^{-2} into YCB-BSA medium and incubated at 30 °C (top) or 37 °C (bottom). Growth was monitored by measuring the optical density of the cultures. Two independently constructed mutants were used in each case.

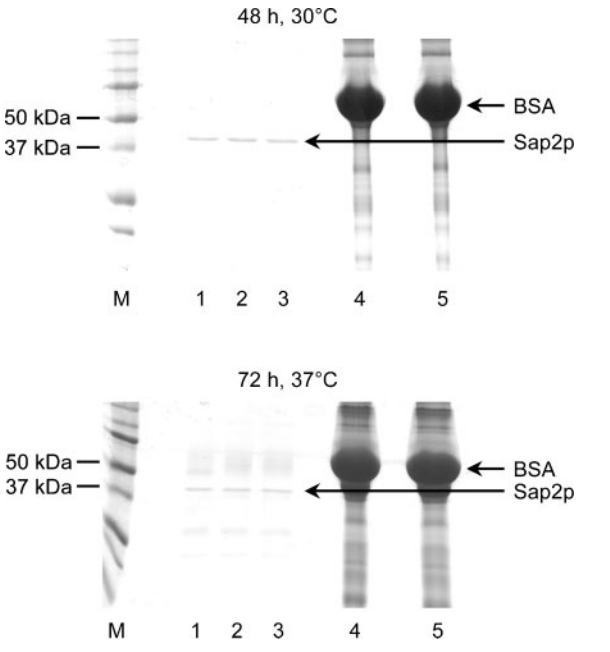


Fig. 5. Secretion of Sap2p by the wild-type strain SC5314 (lane 1) and the *sap4*Δ *sap5*Δ *sap6*Δ triple mutants SAP456MS4A (lane 2) and SAP456MS4B (lane 3) grown for 48 h at 30 °C (top) or 72 h at 37 °C (bottom) in YCB-BSA. The culture supernatants of the strains were analysed by SDS-PAGE. Supernatants of the *sap2*Δ mutants SAP2MS4A (lane 4) and SAP2MS4B (lane 5) were included for comparison. The bands corresponding to BSA and Sap2p are labelled by arrows. M, molecular size markers.

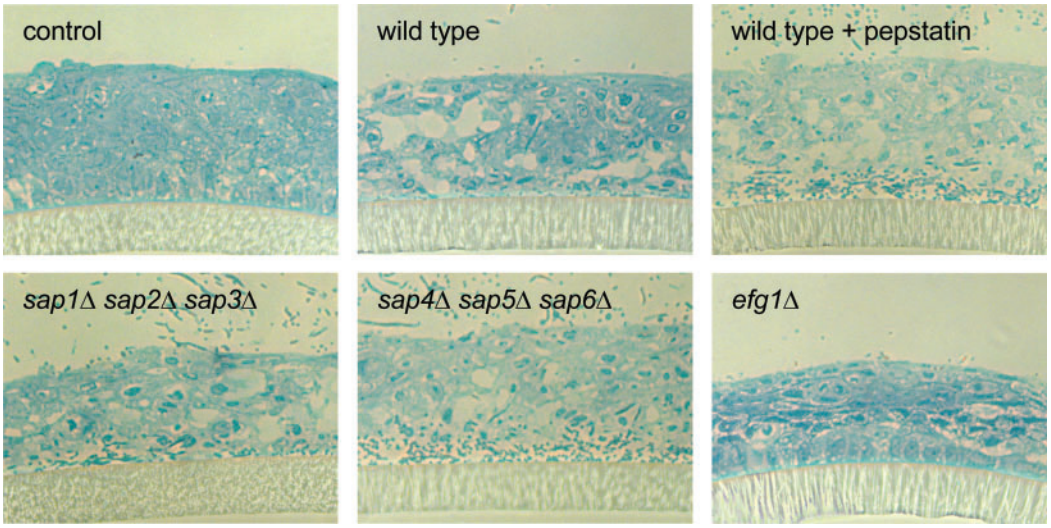


Fig. 6. Invasion of vaginal RHE by the *C. albicans* wild-type strain SC5314, the *sap1*Δ *sap2*Δ *sap3*Δ triple mutants SAP123MS4C and SAP123MS4D, the *sap4*Δ *sap5*Δ *sap6*Δ triple mutants SAP456MS4A and SAP456MS4B, and the *efg1*Δ mutant Can33. The independently constructed series of *sap* mutants behaved identically and only one of them is shown in each case. Infection by the wild-type strain was also performed in the presence of the aspartic protease inhibitor pepstatin A. An uninfected control sample is shown for comparison. After 48 h of infection, the tissue was processed for microscopy as described in Methods.

damage. Similar results were obtained in both vaginal (Fig. 7a) and oral (Fig. 7b) RHE infection models. We conclude from these experiments that Saps are not required for invasion of RHE by *C. albicans*.

DISCUSSION

The role of the Saps of *C. albicans* in the pathogenicity of the fungus has been investigated in numerous studies (for a comprehensive review, see Naglik *et al.*, 2003a). Various laboratories have analysed the gene expression pattern of the *SAP* gene family during infection, as it might provide clues about the role of individual Sap isoenzymes in the host–pathogen interaction. The recombination-based IVET is highly useful for the detection of even a transient *in vivo* induction of genes that, like *SAP1–SAP6*, are not significantly expressed under standard growth conditions *in vitro*. An additional advantage of this reporter system is that the activation of a target gene promoter can be observed at the level of single cells. However, a limitation of the system is that only a yes or no answer is obtained for each cell recovered from infected tissue and, as previously noted, it may not be sensitive enough for genes that are expressed only at low levels (Staib *et al.*, 1999, 2000, 2002b; Taylor *et al.*, 2005). The sensitivity of the *ecaFLP* reporter gene was similar to that of the more frequently used *GFP* reporter gene when corresponding reporter strains were compared under identical conditions. Both reporter systems detected the induction of the *SAP2* promoter in YCB-BSA medium in all cells of the population and also the activation of the *SAP4* and *SAP5* promoters during vaginal infection of mice, whereas no expression of the *SAP1–SAP3* genes was observed in the latter model with either of the two reporter genes (Morschhäuser *et al.*, 1998; Staib *et al.*, 2000; Taylor *et al.*, 2005). The expression of the phase-specific *SAP1* gene was also easily detected in opaque cells of strain WO-1 using *GFP* as a reporter (Strauß *et al.*, 2001). While our failure to detect expression of the *SAP1* and *SAP2* genes during infection of vaginal RHE may therefore be due to a limited sensitivity of the FLP-based IVET, the expression levels of these genes in this infection model seem to be considerably below their fully induced state. Nevertheless, the importance of *SAP1* and *SAP2* for invasion and damage of vaginal RHE and of *SAP1–SAP3* for infection of oral RHE has been demonstrated by the reduced virulence of mutants lacking the corresponding genes in these *in vitro* infection models (Schaller *et al.*, 1998, 1999, 2003). In contrast, we were unable to confirm a role of any of the *SAP1–SAP6* genes in the same infection models. Apart from a possible compensatory upregulation of other *SAP* genes in our *sap1*Δ *sap2*Δ *sap3*Δ triple mutants, there are several possible explanations for the discrepancies in the results obtained in our present work and those of previous studies by other researchers. Although the RHE models have been reported to be highly reproducible (Naglik *et al.*, 2003a), it seems that variabilities may nevertheless arise when they are used in different laboratories. Schaller *et al.* (1999) reported that the damage to oral RHE caused by infection with the wild-type strain SC5314 was reduced in the presence of the aspartic protease inhibitor pepstatin A, but we did not see such an effect in our present study. Pepstatin A completely

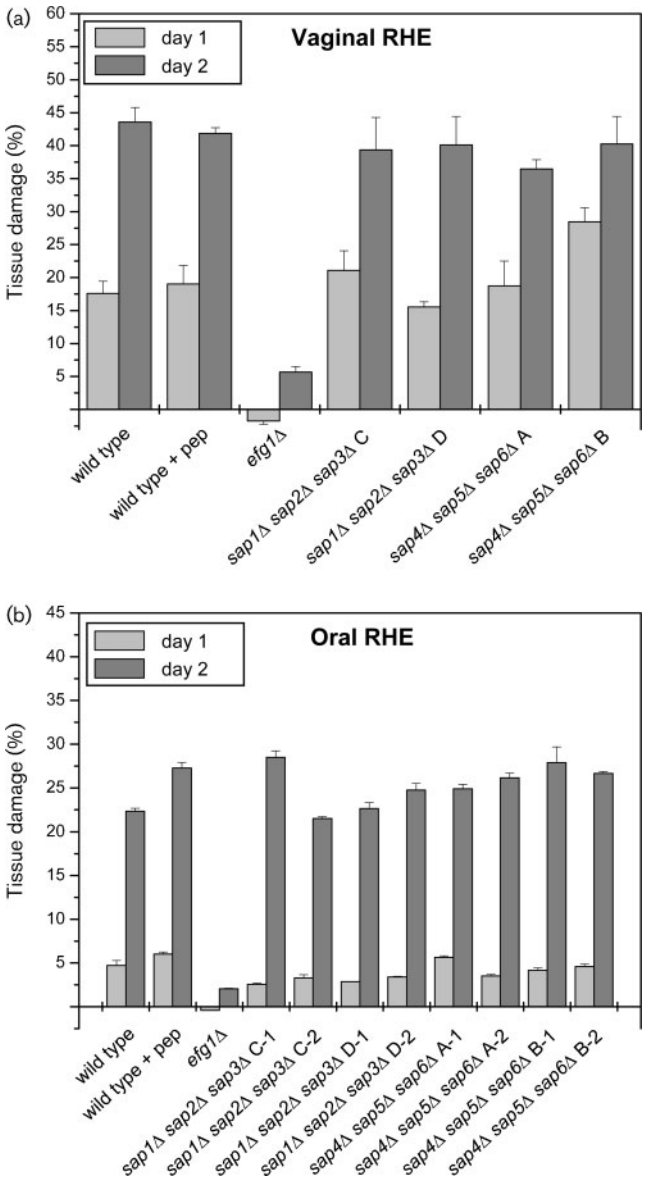


Fig. 7. Epithelial damage caused by the *C. albicans* wild-type strain SC5314 and *sap1*Δ *sap2*Δ *sap3*Δ and *sap4*Δ *sap5*Δ *sap6*Δ triple mutants. Vaginal (a) or oral (b) RHE was infected with the indicated *C. albicans* strains. Infections with the wild-type strain SC5314 were also performed in the presence of pepstatin A (+pep). Tissue damage was determined by measuring the LDH activity in the supernatant after 24 h and 48 h as described in Methods. Values are the means ± SD from three replicate measurements. Two independently constructed *sap1*Δ *sap2*Δ *sap3*Δ triple mutants (strains SAP123MS4C and SAP123MS4D) and *sap4*Δ *sap5*Δ *sap6*Δ triple mutants (strains SAP456MS4A and SAP456MS4B) were used in these experiments. Infection of the oral RHE (b) was performed in duplicate for all *sap* mutants, as indicated by the suffix.

inhibits Sap-dependent growth in YCB-BSA medium at even lower concentrations than those used in the infection assays (Staib *et al.*, 2008); therefore, a contribution of the Saps to tissue damage should have been detected, although we can not exclude the possibility that pepstatin A did not fully inhibit Sap activity under the conditions used in the RHE infection experiments. These results indicate that the differences observed in this and previous studies with respect to the role of the Saps for invasion of RHE can not solely be attributed to phenotypic differences of the constructed deletion mutants. Differences in the experimental setup may therefore cause invasion of RHE to be protease-dependent or not. In contrast to the *sap* mutants, an *efg1*Δ mutant that had previously been reported to be noninvasive in a similar epithelial invasion model (Dieterich *et al.*, 2002) was also unable to invade and damage vaginal and oral RHE in our hands. An alternative possible explanation for the differences in the effect of pepstatin A on the ability of the wild-type strain SC5314 to damage RHE is that the stocks of this strain maintained in various laboratories are not identical and may vary in their dependence on Sap activity for epithelial invasion.

Apart from these considerations, there also seem to be differences between the *sap* mutants constructed previously from the auxotrophic laboratory strain CAI4 and those generated in the present study from its prototrophic parental strain SC5314. In agreement with the gene expression pattern, we found that only *SAP2*, but none of the other *SAP* genes tested, was required for growth of *C. albicans* in YCB-BSA medium, i.e. when proteins are the only available nitrogen source. In contrast, Sanglard *et al.* (1997) found that a mutant lacking the *SAP4–SAP6* genes also failed to grow in this medium and they concluded that one of the corresponding proteases is required for *SAP2* expression. They did not report whether mutants lacking only one or two of the *SAP4–SAP6* genes exhibited the same growth defect and if reintroduction of any of the genes into the triple mutant rescued the growth defect. The *sap4*Δ *sap5*Δ *sap6*Δ triple mutants constructed in our present study secreted wild-type levels of Sap2p (see Fig. 5), demonstrating that *SAP2* expression does not depend on any of those other proteases.

The importance of Saps for tissue invasion and damage seems to depend on the infection model used. For example, when the interaction of mutants lacking one of the *SAP1–SAP3* genes with endothelial cells was investigated, only Sap2p, but not Sap1p or Sap3p, was found to contribute to the ability of *C. albicans* to damage endothelial cells (Ibrahim *et al.*, 1998). Other researchers found that proteases mediate invasion of *C. albicans* into human oral mucosa by degrading E-cadherin, as E-cadherin degradation was completely inhibited in the presence of protease inhibitors (Villar *et al.*, 2007). In this case, Sap5p was implicated in tissue invasion, as forced overexpression of *SAP5* rescued the invasion defect of a *rim101* mutant, in which expression of the *SAP4–SAP6* genes was severely reduced. On the other hand, *C. albicans* can also invade

epithelial and endothelial cells by inducing its own endocytosis (Phan *et al.*, 2007). These studies and our present work indicate that the importance of the Saps in general, and of individual Sap isoenzymes, for the virulence of *C. albicans* varies strongly, depending on the infection model, with even minor differences in the experimental setup having a significant impact on the dependence on protease activity for successful invasion and establishment in various host niches. New and more sophisticated animal models of superficial and disseminated *Candida* infections have been established in the past years and continue to be developed (de Repentigny, 2004). The set of mutants generated in this study from the wild-type model strain SC5314, which lack single or multiple *SAP* genes, will be a valuable tool to study the role of these enzymes in the host–pathogen interaction in more detail.

ADDENDUM

In a related paper in this issue of *Microbiology*, Naglik *et al.* (2008), using quantitative real-time RT-PCR, now also report that only *SAP5*, but not the other *SAP* genes, is significantly upregulated during infection of RHE and that the *SAP1–SAP6* genes are not required for invasion of RHE, which is in contrast to their previous results and completely supports our findings. However, they observe a partial inhibition of RHE invasion and damage by *C. albicans* in the presence of the aspartic protease inhibitor pepstatin A.

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