Differential regulation of *SAP8* and *SAP9*, which encode two new members of the secreted aspartic proteinase family in *Candida albicans*

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Secreted aspartic proteinases (Saps) contribute to the virulence of *Candida albicans* in systemic animal models of infection. Seven genes encoding Saps (*SAP1*–*SAP7*) have been identified to date but evidence suggested the existence of additional *SAP* genes. The screening of a *C. albicans* λ EMBL3 genomic library for the presence of other *SAP* genes was undertaken. Two new genes, *SAP8* and *SAP9*, were isolated. The N-terminal amino acid sequence deduced from *SAP8* downstream of a Kex2p-like cleavage site corresponds to the N-terminal amino acid sequence of the 41 kDa Sap isolated and characterized previously. *SAP8* mRNA was expressed preferentially in yeasts at 25 °C after 6 and 9 h growth in BSA-containing medium. *SAP9* encodes an aspartic proteinase with a Kex2p-like cleavage site and contains a putative glycosylphosphatidylinositol-anchor signal at the C-terminus. Although the *SAP9* gene product has not yet been isolated from cultures of *C. albicans*, transcripts of *SAP9* were observed preferentially in later growth phases when *SAP8* expression had decreased.

Keywords: *Candida albicans*, secreted aspartic proteinases, gene family

INTRODUCTION

The opportunistic pathogen *Candida albicans* secretes aspartic proteinases (Saps), which raises a particular interest because of their role as virulence factors (Hube *et al.*, 1997b; Sanglard *et al.*, 1997). Seven gene sequences, *SAP1*–*SAP7*, encoding secreted proteinases are known in *C. albicans* (Monod *et al.*, 1994). Following RNA hybridization experiments, these genes have been shown to be differentially regulated *in vivo* (Hube *et al.*, 1994; White & Agabian, 1995). When BSA is the sole nitrogen source in the medium, *C. albicans* secretes one dominant aspartic proteinase, Sap2p. Two other isoenzymes, Sap1p and Sap3p, have been recovered from a particular *C. albicans* strain, WO-1, which switches from a white to an opaque form at a frequency of 10⁻² to 10⁻⁴ per cell cycle. Opaque cells produce Sap1p, Sap2p and Sap3p, while white cells of the same strain

secrete Sap2p only (Morrow *et al.*, 1992; White *et al.*, 1993). However, a fourth Sap protein with an N-terminal amino acid sequence not identical to any N-terminal sequence of mature forms of Sap1p–Sap7p was isolated by Morrison *et al.* (1993) from the supernatant of *C. albicans* stationary phase cultures in BSA medium at 25 °C. This finding supported the existence in *C. albicans* of *SAP* genes other than those previously reported. The screening of a *C. albicans* λ EMBL3 genomic library for the presence of other *SAP* genes was undertaken and we report here the cloning, sequencing and expression pattern of two additional genes, one of which, *SAP8*, encodes the Sap previously isolated by Morrison *et al.* (1993).

METHODS

Organisms and plasmids. *Escherichia coli* LE392 was used as a host strain for propagation of bacteriophages. The *C. albicans* genomic library was previously constructed in λ EMBL3 (Monod *et al.*, 1994). All plasmid subcloning experiments were performed in *E. coli* strain DH5 α using the plasmid pMTL21 (Chambers *et al.*, 1988). *E. coli* DH5 α was

Abbreviations: GPI, glycosylphosphatidylinositol; Sap, secreted aspartic proteinase.

The GenBank accession numbers for the sequences reported in this paper are AF043330 and AF043331.

transformed using competent cells with standard protocols (Sambrook *et al.*, 1989).

Since the SAP8 gene product was originally detected in *C. albicans* strain DSM 6659 (Morrison *et al.*, 1993), this strain (kindly provided by R. Röchel, University of Göttingen, Germany) was used for gene expression studies. Strain WO-1 (kindly provided by D. Soll, University of Iowa, USA) was used for expression studies during phenotypic switching.

Culture media and growth conditions. *C. albicans* strains were stored on YPG (1%, w/v, yeast extract; 2%, w/v, peptone; 2%, w/v, glucose) agar plates at 4 °C. For Northern blot analysis, the following liquid media were used: 1.17% (w/v) Yeast Carbon Base (Difco), 1% (w/v) glucose, 0.5% (w/v) BSA (BSA medium; Sigma); modified Lee's medium (Morrow *et al.*, 1992). Each 50 ml culture was inoculated with 1 ml of a *C. albicans* overnight culture grown in the same medium. Cultures were shaken in an orbital incubator at either 25 or 37 °C. Growth was monitored by measuring the OD₆₀₀ (Beckman DV.65 spectrophotometer) and cell samples were harvested at different times for 2 d. White–opaque switching and the stability of both growth forms of strain WO-1 were checked microscopically.

Screening of a *C. albicans* genomic library. Recombinant bacteriophage plaques of the genomic library were immobilized on nylon membranes (Zeta-Probe; Bio-Rad). The membranes were prehybridized for 1 h at 50 °C in a solution containing 5 × SSC, 7% (w/v) SDS, 10 × Denhardt's solution, 20 mM NaH₂PO₄ (pH 7.0) and 100 µg denatured salmon sperm DNA ml⁻¹. Hybridization was performed with γ -³²P-labelled oligonucleotides at 50 °C for 24 h in the same buffer. The membranes were exposed to X-ray films after two washes in 3 × SSC, 5% (w/v) SDS, 25 mM NaH₂PO₄ (pH 7.0) at 40 °C. Oligonucleotides were labelled with polynucleotide kinase as described by Sambrook *et al.* (1989) in the presence of [γ -³²P]ATP (Amersham). Positive plaques were purified and the DNA was isolated as described by Grossberger (1987). Agarose gel electrophoresis of restricted recombinant λ EMBL3 DNA and Southern blotting were performed according to standard protocols (Sambrook *et al.*, 1989).

DNA sequencing. DNA subcloned into plasmid pMTL21 (Chambers *et al.*, 1988) was sequenced using a Licor model automatic sequencer.

RNA isolation and Northern blotting. Total RNA from *C. albicans* was prepared as described by Hube *et al.* (1994). Briefly, cells were extracted with glass beads, extraction buffer (0.1 M Tris/HCl, pH 7.5; 0.1 M LiCl; 0.01 M DTT), 1% (w/v) SDS and phenol/chloroform. The aqueous phase was removed and extracted twice in phenol/chloroform and the RNA was then precipitated with ethanol.

RNA samples were separated on formaldehyde agarose gels as described by Hube *et al.* (1994). Fractionated RNA was blotted (VacuGene; Pharmacia) onto nylon membranes (Hybond-N; Amersham) in 10 × SSC (pH 7.0) for 8 h. After transfer of RNA, the membrane was rinsed with 6 × SSC, air-dried and photographed using a UV transilluminator to determine the position of the 28S and 18S rRNA bands. The RNA was cross-linked to the membrane by baking for 2 h at 80 °C.

PCR was used to prepare hybridization probes for Southern and Northern blotting. Fragments of 1215 bp and 1632 bp in the ORFs of SAP8 and SAP9, respectively, were amplified. Pairs of primers were: 5'-GTTGACACAGGTTCTTCTG-3' and 5'-TCAGCCTTCTATGCCTTTTG-3' for SAP8; 5'-GTGCCACCAATGAGAC-3' and 5'-CCTCTATTGTGTGTAATG-3' for SAP9. DNA fragments were purified by

using the Prep-a-Gene kit (Bio-Rad) and labelled with [α -³²P]dCTP (Amersham) by random priming (Boehringer). To test the specificity of SAP probes, Southern blots of *C. albicans* genomic DNA digested with *Eco*RI were hybridized to each probe under high stringency conditions (Hube *et al.*, 1994). Single bands were detected with both SAP8 and SAP9 probes. Northern blots were then hybridized under the same conditions (Hube *et al.*, 1994).

RESULTS

Cloning of two new members of the *C. albicans* SAP gene family

The overall number of SAP genes in *C. albicans* was tentatively estimated by Southern blot analysis of *Eco*RI-digested genomic DNA of different *C. albicans* strains and hybridization with SAP1–SAP7 probes encoding the N-terminal aspartyl domain of the Sap active site (Monod *et al.*, 1994). Eight distinct bands, designated A1–A8, were identified. Five of these bands, A1, A3, A5, A6 and A8, corresponded to each of the *Eco*RI fragments characteristic of SAP2, SAP7, SAP1, SAP5 and SAP3, respectively. The band A7 corresponded to unresolved 1.2 kb fragments characteristic of SAP4 and SAP6. Two bands, A2 and A4, for two fragments of 6.0 and 3.7 kb, respectively, corresponded to none of the previously isolated SAP1–SAP7. Furthermore, both fragments hybridized with the oligonucleotide probe 5'-GTT ATT GTT GA(T/C) ACT GG(A/G) TCT TCT GAT TT(A/G) TGG-3' designed as a consensus of the nucleotide sequence encoding the N-terminal aspartyl domain V(V/I/M)(I/L/V)DTGSSD(L/F)W of the catalytic site of the Sap isoenzymes. Therefore, the existence of two other genes encoding aspartic proteinases in *C. albicans* was inferred in addition to SAP1–SAP7. A further screening of a *C. albicans* λ EMBL3 genomic library, from which SAP1–SAP7 were isolated (Monod *et al.*, 1994), was performed using the oligonucleotide probe and low stringency conditions of hybridization. Among 10⁴ individual recombinant bacteriophage plaques, corresponding to 10 *C. albicans* genome equivalents, all hybridizing clones (100 in total) were purified. The DNA obtained from these clones was restricted with *Eco*RI, blotted onto membranes and hybridized with the selecting oligonucleotide probe. The clones containing a hybridizing *Eco*RI fragment corresponding

Fig. 1. Restriction maps of inserts of the plasmids containing the *C. albicans* SAP8 and SAP9 genes. The SAP ORFs are represented by solid arrows. The N-terminal domains of the catalytic site of the enzymes are indicated by solid triangles. The GenBank accession numbers of the sequenced regions of pCA8 and pCA9 represented by a dashed line are AF043330 and AF043331, respectively. A, Asp718; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; S, *Sal*I; X, *Xho*I; Xb, *Xba*I.

Fig. 2. Comparison of deduced amino acid sequences of aspartic proteinases Sap1p–Sap9p from *C. albicans*. ▼ Aspartic acid residues corresponding to those found in the active site of proteinases of the pepsin family; ●, conserved cysteine residues possibly involved in disulphide bridge formation in the three-dimensional structure of aspartic proteinases. Asterisks and dots show identical and similar amino acids between aligned sequences, respectively. An arrow delimits the N-terminal positions of the mature secreted proteinases documented for Sap1p, Sap2p, Sap3p and Sap8p. Tandem Lys–Arg and Lys–Lys sequences known to be proteolytic processing sites precede these positions. The alignment was performed with the PILEUP algorithm implemented in the GCG package of the University of Wisconsin. The nucleotide sequences of the genes encoding the aspartic proteinases are available in the GenBank database with the accession numbers X56867 (SAP1), M83663 (SAP3), L22358 (SAP2), L25388 (SAP4), Z30191 (SAP5), Z30192 (SAP6), Z30193 (SAP7), AF043330 (SAP8) and AF043331 (SAP9).

Table 1. Pairwise comparisons of Sap proteins from *C. albicans*

The percentage similarity (top right-hand corner) and percentage divergence (bottom left-hand corner) values were obtained using the program CLUSTAL (Higgins & Sharp, 1989) with a PAM 250-residue weight table.

	Sap1p	Sap2p	Sap3p	Sap4p	Sap5p	Sap6p	Sap7p	Sap8p	Sap9p
Sap1p	***	67.1	67.6	57.4	55.4	56.6	20.4	50.0	28.1
Sap2p	26.7	***	69.8	55.0	52.5	55.0	27.4	50.0	28.6
Sap3p	29.0	27.5	***	53.0	49.2	52.3	26.9	50.5	22.1
Sap4p	40.8	42.4	43.7	***	77.2	89.9	26.4	43.4	29.5
Sap5p	42.6	45.2	47.5	22.1	***	80.4	26.1	41.2	26.8
Sap6p	41.0	42.1	44.2	9.4	19.6	***	27.3	42.4	26.3
Sap7p	74.8	75.9	75.7	76.6	75.9	76.4	***	25.3	17.1
Sap8p	48.5	46.6	45.8	54.6	56.2	55.0	74.8	***	26.3
Sap9p	66.4	67.2	67.8	65.8	68.2	66.7	76.8	68.6	***

to SAP1–SAP7 were eliminated. Four groups of clones containing 6.0, 3.7, 3.2 and 2.4 kb *Eco*RI fragments which did not correspond to any of SAP1–SAP7 were retained and further analysed. The 6.0 and 3.7 kb *Eco*RI fragments corresponded to the bands A2 and A4. The four *Eco*RI fragments were subcloned into pMTL21 (Chambers *et al.*, 1988) and partial sequencing of their extremities revealed that the 6.0 and 3.7 kb fragments contained parts of new aspartic proteinase genes encoding the N-terminal aspartyl domain of the protein. Sequencing of the 3.2 and 2.4 kb *Eco*RI fragments, which show only a weak signal with the oligonucleotide probe, revealed no sequence similarities with any of the SAP genes and were not studied further. The genes corresponding to the 3.7 and 6.0 kb *Eco*RI fragments were called SAP8 and SAP9, respectively. Further restriction analysis of bacteriophage DNA permitted the identification of fragments of about 6 kb that contained the entire new SAP genes. These fragments were subcloned into pMTL21, generating the plasmids pCA8 and pCA9 for which the map of the inserts is shown in Fig. 1. SAP8 and SAP9 are localized on chromosome 3 (S. Scherer, personal communication; data available at <http://alces.med.umn.edu/bin/genelist?genes>).

Amino acid sequences deduced from SAP8 and SAP9

SAP8 and SAP9 contain ORFs of 1215 and 1632 bp encoding proteins of 405 and 544 amino acids, respectively. The Sap8p and Sap9p proteins showed similarity with other aspartic proteinases, in particular within the regions which contained the two reactive aspartic acid residues of the active site for these enzymes. The amino acid sequences of Sap8p and Sap9p, which were aligned with those of Sap1p–Sap7p, suggested the existence of a signal peptide with putative signal peptidase cleavage sites (von Heijne, 1986) and the existence of a prosequence with pairs of Lys–Arg residues which are known to be proteolytic processing sites for the proconvertase Kex2p in *Saccharomyces cerevisiae* (Fig. 2). The Sap8p and Sap9p protein molecules generated by cleavage at the Kex2p-like cleavage site

Fig. 3. Dendrogram of the currently known *Candida* Saps and the GPI-anchored proteinase Yap3p of *S. cerevisiae*. Sapp and Sapt denote the proteinases secreted by *Candida parapsilosis* and *C. tropicalis*, respectively. The branch lengths are proportional to the similarity between amino acid sequences. The dendrogram was created by the PILEUP program by the unweighted pair-group method using arithmetic means (GCG package, University of Wisconsin).

have a calculated molecular mass of 35 and 52 kDa, respectively. Pairwise comparisons for all members of the Sap family in *C. albicans* are shown in Table 1. The dendrogram deduced from the alignments of different *Candida* Saps and the aspartic proteinase Yap3p of *S. cerevisiae* shows that Sap8p and Sap9p belong to branches distinct from the Sap1p, Sap2p and Sap3p group, and from the Sap4p, Sap5p and Sap6p group (Fig. 3). Sap8p is clustered with Sapt1p, the aspartic pro-

Fig. 4. Northern blot analysis of *SAP8* and *SAP9* expression. *C. albicans* DSM 6659 was grown in BSA medium at 37 and 25 °C, where the stationary phase starts from 24 and 36 h, respectively. Six and eight samples, respectively, were taken over 3 d during batch growth at the times indicated, and Northern blots of the prepared RNA were hybridized to radiolabelled PCR fragments of *SAP8* and *SAP9* as described in Methods. Approximately equal amounts of total RNA were loaded in each lane of the Northern blots and allowed assessment of the mRNA levels of *SAP8* and *SAP9*. In addition, the *TEF3* mRNA (Colthurst *et al.*, 1992) was also probed as a positive but non-quantitative control. *TEF3* expression is down-regulated in the stationary phase during batch growth (Swoboda *et al.*, 1994). Probed *TEF3* mRNA and ethidium-bromide-stained rRNAs are shown for all samples.

teinase secreted by *Candida tropicalis* in BSA medium (Togni *et al.*, 1991), and Sap9p is clustered with Yap3p.

Expression of *SAP8*

The N-terminal amino acid sequence deduced from *SAP8* downstream of the Kex2p-like cleavage site corresponds to the N-terminal sequence of the mature 41 kDa Sap isolated and characterized by Morrison *et al.* (1993). This Sap was detected during growth of *C. albicans* DSM 6659 in BSA medium at 25 °C. To investigate the expression pattern of *SAP8*, we isolated total RNA from strain DSM 6659 grown under these conditions and probed for *SAP8* mRNA. *SAP8* was expressed preferentially during the early exponential phase of growth of the yeast cells, i.e. after 6 and 9 h growth at 25 °C and after 3 h at 37 °C in BSA medium (Hube *et al.*, 1994). However, *SAP8* was expressed at lower levels at 37 °C than at 25 °C (Fig. 4). Interestingly, *SAP8* mRNA levels detected in the early exponential phase at 37 °C disappeared within 3 h (Fig. 4). Such a sharp regulation was also found for the regulation of *SAP2* expression (Hube *et al.*, 1994). Since we observed *SAP2* and *SAP3* expression under the same conditions (Hube *et al.*, 1994), it can be concluded that these three proteinase genes may be expressed by *C. albicans* DSM 6659 under similar physiological conditions. The higher level of *SAP8* mRNA during growth at 25 °C may explain the earlier observed temperature-dependent sensitivity effect of proteinase production during growth of *C. albicans* DSM 6659 in BSA medium at 25 °C (Crandall & Edwards, 1987).

The temperature dependence of *SAP8* expression was also tested in the switching strain WO-1 (Soll, 1992). Several genes, including *SAP* genes, are regulated during the white-opaque switching of WO-1 (Morrow *et al.*, 1992, 1994). RNA isolated from both white and opaque forms of WO-1 grown in BSA medium at 25 and 37 °C was tested for *SAP8* transcripts. The *SAP8* mRNA was

found to be abundant in opaque cells at 25 °C, but poorly expressed in the white form at 37 °C (not shown). Thus the opaque form was able to express at least four different proteinase genes (*SAP1*, *SAP2*, *SAP3* and *SAP8*) under the same conditions, all of which together may be responsible for the higher extracellular proteolytic activity of this switching form (Hube *et al.*, 1997a).

Expression of *SAP9*

To investigate the expression of *SAP9*, Northern blots which showed *SAP1*–*SAP3* and *SAP8* signals were stripped and reprobed with *SAP9*. A low level of *SAP9* transcripts was found in most RNA samples (not shown). However, a strong signal was observed at 25 °C after 45 h corresponding to stationary phase of growth in BSA medium (Fig. 4) (Hube *et al.*, 1994). This expression is different from most identified *SAP* genes and may indicate a divergent role for this gene.

DISCUSSION

Several points support the conclusion that *SAP8* encodes the 41 kDa protein detected by Morrison *et al.* (1993). (i) The N-terminal amino acid sequence deduced from *SAP8* downstream of the Kex2p-like cleavage site corresponds to the N-terminal amino acid sequence of the mature 41 kDa form of this protein. (ii) The *SAP8* mRNA and the 41 kDa protein are both expressed in BSA-containing media at 25 °C. These are growth conditions corresponding to those in which the 41 kDa protein was produced. One possible reason for the difference between the observed (41 kDa) and the predicted (36 kDa) molecular mass of Sap8p is that the value of 41 kDa reported by Morrison *et al.* (1993) was an overestimate. Indeed, Sap2p was described at the same time as a 48–49 kDa protein whereas its apparent molecular mass on SDS-PAGE is 40 kDa (de Viragh *et al.*, 1993). Since *SAP8* is expressed preferentially at

Sap9p	1	..MRINSVALLSLVATAA..APFFIDFEVRR.....G	31
Yap3p	1	MKLKTVRSVAVLSSLFASQVLGKIIPAANKRDDSDNSKFVKLPFHKFTLYG	50
Sap9p	32	ESKDDLSPEDDSNPRFVKR..DGLDMTLTNKQTFYMATLKIGSNEDENRV	80
Yap3p	51	DSLENVGSDKKPEVRLKLRADGYEEIIITNQSFYSVDLEVGTTPQNVTV	100
Sap9p	81	LEDTGS..SDLVMSHDLKCVSAPISKRNERSFGHGTGVKLNRELMQKR	128
Yap3p	101	LVDTSGSTDLWIMSGSNPYCSS.....NSMGSS.....	129
Sap9p	129	KNLYQPSRTIETDEEKEALEKIHNLKLFSGFSYSTVYITEGPGAYST..FS	177
Yap3p	130	RRRVIDKRDDSSSGSLINDINPFWLTGTG..SAIGPTAFTTGLG	173
Sap9p	178	PLVGTGGSS..GGSGSNITCRSYGSFNTENDTFKKNTNDFEIQVADTTS	226
Yap3p	174	GGSGTATQSVFASATMDQQYGTFTSGSSTFRSNNTY..FSISYGDGT	222
Sap9p	227	AIGWGY..DDVTISNVTYKDLSPAANETSSDVGVGLGIGLPLEVT..	271
Yap3p	223	ASGTFGFTDVLDSLNVGTLSFAVANETNSTMGVLGIGLPLEVITYSG	272
Sap9p	272	TQLRYTYQNPL..KLKADGIIAKSLYSLYLNTADAKAGSILFGA	314
Yap3p	273	STASHSGKAYKYDNFPFIPTVLKNSGAIKSNNTYSLYLNDSAMHGTLFGA	322
Sap9p	315	IDHAKYQCDLVTVKMMRTYSQISYVPII...QPVFKIDVESSSGSTTN	360
Yap3p	323	VDHSKYTGTLTYTIPIVNTLSASGFSSPIPTQFDVTINGIGISDSSGNKT	372
Sap9p	361	ILSGTGVVLDGSGTSLSYVSDTLQLLKGALNGQYSNSV..GAYVNCNL	408
Yap3p	373	LTITKIPALSDSGTTLTYLPQTVSMIATLGAQYSSRIFTGYVLDPC.	421
Sap9p	409	ADSSRTVDIEFGNKTIKVPIIDLVLQASKSTCILGVMMQSSSSSYMLFG	458
Yap3p	422	SDDSMELVDFDGGFH..INAFLSFSLITSG..TCLLGIPTSDDTG..TILG	468
Sap9p	459	DNIL..RSAIVYDLDYEVSLAQVSY..TNKESIEVIGA.....	494
Yap3p	469	DSFLFTNAYVYDLENLBISMAQARYNTSENIEITSSVPSAVKAPGY	518
Sap9p	495	SGITNSGSGTSSSGTSTSTSTRHSAG	522
Yap3p	519	TNTWSTSASIVTGGNFTIPTVMSQTASPSGNLTSTASATSTSSKRNVG	568
Sap9p	523	SIISNPVYGLLLSLLSYYVLV	544
Yap3p	569	DHIVPSLPLTLISLFAFI...	587

Fig. 5. Comparison of the *S. cerevisiae* YAP3 and *C. albicans* SAP9 gene products. The identity and similarity between Yap3p and Sap9p are 37.7 and 58.9%, respectively. The comparison was performed with the program GAP implemented in the GCG package of the University of Wisconsin. Bars indicate identity between amino acids, whereas colons and stops represent high and low conservation between amino acids of each sequence.

25 °C, it seems unlikely that this gene plays an important role during infection at the body temperature of the host. However, transcripts of *SAP8* have recently been found in models of oral candidosis (M. Schaller, W. Schaefer, H. C. Korting & B. Hube, unpublished results). It may therefore be possible that other inducing factors are important for *SAP8* expression during host infection.

The putative N-terminal amino acid sequence of the mature Sap9p does not correspond to any previously isolated *C. albicans* Sap proteins. Interestingly, the deduced translation product of *SAP9* has a C-terminal extremity that is 51 amino acids longer than that of all other Saps (Fig. 2). This C-terminal extremity is serine/threonine-rich and can be aligned to the C-terminal extremity of the aspartic proteinase Yap3p of *S. cerevisiae* (Fig. 5), which was shown to be a glycosylphosphatidylinositol (GPI)-anchored protein (Ash *et al.*, 1995). The Sap9p amino acid sequence GASG at positions 493–496 is also identical to the consensus sequence of the site of cleavage and attachment of GPI-anchored proteins (Gerber *et al.*, 1992). Therefore, it is

probable that Sap9p is a GPI-anchored aspartic proteinase. Moreover, Sap9p is more related to Yap3p than to any other *Candida* Saps (Fig. 3). Because GPI-anchored proteins follow the usual secretory pathway, we propose here to leave the gene encoding this protein in the *SAP* gene family.

The Sap isoenzymes have a propeptide containing one Lys-Arg Kex2p processing sequence (Julius *et al.*, 1984) immediately preceding the N-terminus of the mature form of the enzyme. *YAP3* was cloned based upon its ability to suppress the processing defect of an *S. cerevisiae* *kex2* null mutant (Egel-Mitani *et al.*, 1990). It has been shown recently that *C. albicans* *kex2* null mutants continue to secrete active Sap2p (Newport & Agabian, 1997) but the enzyme was abnormally processed with an additional amino acid at its N-terminus, and was secreted at reduced levels. Although autocatalysis of the enzyme cannot be excluded, processing of Sap2p in *C. albicans* *kex2* null mutants may involve activation by a Yap3p homologue which could be Sap9p. However, the exact role of Sap9p remains to be investigated.

In total, nine *SAP* genes have now been cloned by low stringency hybridization with *SAP* gene probes and a consensus oligonucleotide probe designed on the basis of the sequence encoding the N-terminal aspartyl domain of the catalytic site of Sap isoenzymes. All bands A1–A8 identified by Southern blotting of *EcoRI*-digested genomic DNA of *C. albicans* and hybridization with both types of probes (Monod *et al.*, 1994) can now be assigned to a specific *SAP* gene. No other *SAP* genes were found on cloned *EcoRI* fragments showing only a slight signal with the oligonucleotide probe. Sequencing of the entire *C. albicans* genome, which is under way, may reveal other *SAP*-like genes in addition to those described. However, with the cloning methods used previously (Monod *et al.*, 1994) and in this study, the likelihood of finding additional *SAP* genes in the *C. albicans* genome is rather low.

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REFERENCES

- Ash, J., Dominguez, M., Bergeron, J. J. M., Thomas, D. Y. & Bourbonnais, Y. (1995). The yeast proprotein convertase encoded by *YAP3* is a glycosylphosphatidylinositol-anchored protein that localizes to the plasma membrane. *J Biol Chem* **270**, 20847–20854.
- Chambers, S. P., Prior, S. E., Barstow, D. A. & Milton, N. P. (1988). The pMTL *nic*⁺-cloning vectors. 1. Improved polylinker region to facilitate the use of sonicated DNA for nucleotide sequencing. *Gene* **68**, 139–149.
- Colthurst, D. R., Schauer, B. S., Hates, M. V. & Tuite, M. F. (1992). Elongation factor 3 (*EF-3*) from *Candida albicans* shows both structural and functional similarity to *EF-3* from *Saccharomyces cerevisiae*. *Mol Microbiol* **6**, 1025–1033.
- Crandall, M. & Edwards, J. E. (1987). Segregation of proteinase-negative mutants from heterozygous *Candida albicans*. *J Gen Microbiol* **133**, 2817–2824.

- Egel-Mitani, M., Flygenring, H. P. & Hansen, M. T. (1990). A novel aspartyl protease allowing KEX2-independent MF α propheromone processing in yeast. *Yeast* 6, 127–137.
- Gerber, L. D., Kodukula, K. & Udenfriend, S. (1992). Phosphatidylinositol glycan (PI-G) anchored membrane proteins. *J Biol Chem* 267, 12168–12173.
- Grossberger, D. (1987). Minipreps of DNA from bacteriophage lambda. *Nucleic Acids Res* 15, 6737.
- von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res* 14, 4683–4690.
- Higgins, D. G. & Sharp, P. M. (1989). Fast and sensitive multiple sequence alignments on a microcomputer. *CABIOS* 5, 151–153.
- Hube, B., Monod, M., Schofield, A., Brown, A. J. P. & Gow, N. A. R. (1994). Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. *Mol Microbiol* 14, 87–99.
- Hube, B., Sanglard, D., Monod, M., Brown, A. J. P. & Gow, N. A. R. (1997a). Extracellular proteolytic activity of *Candida* species. In *Host–Fungus Interplay. Proceedings of the 5th Symposium on Topics in Mycology 1995*, pp. 109–122. Edited by H. Vanden Bossche, D. A. Stevens & F. C. Odds. Bethesda, MD: National Foundation for Infectious Diseases.
- Hube, B., Sanglard, D., Odds, F. C., Hess, D., Monod, M., Schäfer, W., Brown, A. J. P. & Gow, N. A. R. (1997b). Disruption of each of the aspartyl proteinase genes SAP1, SAP2, and SAP3 of *Candida albicans* attenuates virulence. *Infect Immun* 65, 3529–3538.
- Julius, D., Brake, A., Blair, L., Kunisawa, R. & Thorner, J. (1984). Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro- α -factor. *Cell* 37, 1075–1089.
- Monod, M., Togni, G., Hube, B. & Sanglard, D. (1994). Multiplicity of genes encoding secreted aspartic proteases in *Candida* species. *Mol Microbiol* 13, 357–368.
- Morrison, C. J., Hurst, S. F., Bragg, S. L., Kuykendall, R. J., Diaz, H., Pohl, J. & Reiss, E. (1993). Heterogeneity of the purified extracellular aspartyl proteinase from *Candida albicans*: characterization with monoclonal antibodies and N-terminal amino acid sequence analysis. *Infect Immun* 61, 2030–2036.
- Morrow, B., Srikantha, T. & Soll, D. R. (1992). Transcription of the gene for a pepsinogen, PEP1, is regulated by white–opaque switching in *Candida albicans*. *Mol Cell Biol* 12, 2997–3005.
- Morrow, B., Ramsey, H. & Soll, D. R. (1994). Regulation of phase-specific genes in the more general switching system of *Candida albicans* strain 3153A. *J Med Vet Mycol* 32, 287–294.
- Newport, G. & Agabian, N. (1997). KEX2 influences *Candida albicans* proteinase secretion and hyphal formation. *J Biol Chem* 272, 28954–28961.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanglard, D., Hube, B., Monod, M., Odds, F. C. & Gow, N. A. R. (1997). A triple deletion of the aspartyl proteinase genes SAP4, SAP5, and SAP6 of *Candida albicans* causes attenuated virulence. *Infect Immun* 65, 3539–3546.
- Soll, D. R. (1992). High-frequency switching in *Candida albicans*. *Clin Microbiol Rev* 5, 183–185.
- Swoboda, R. K., Bertram, G., Colthurst, D. R., Tuite, M. F., Gow, N. A. R., Gooday, G. W. & Brown, A. J. P. (1994). Regulation of the gene encoding translation elongation factor 3 during growth and morphogenesis in *Candida albicans*. *Microbiology* 140, 2611–2616.
- Togni, G., Sanglard, D., Falchetto, R. & Monod, M. (1991). Isolation and nucleotide sequence of the extracellular acid protease gene (ACP) from the yeast *Candida tropicalis*. *FEBS Lett* 286, 181–185.
- de Viragh, P. A., Sanglard, D., Togni, G., Falchetto, R. & Monod, M. (1993). Cloning and sequencing of two *Candida parapsilosis* genes encoding acid proteases. *J Gen Microbiol* 139, 335–342.
- White, T. C. & Agabian, N. (1995). *Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. *J Bacteriol* 177, 5215–5221.
- White, T. C., Miyasaki, S. H. & Agabian, N. (1993). Three distinct secreted aspartyl proteinases in *Candida albicans*. *J Bacteriol* 175, 6126–6133.

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