Proteolytic Cleavage of Covalently Linked Cell Wall Proteins by *Candida albicans* Sap9 and Sap10

Lydia Schild,1 Antje Heyken,1 Piet W. J. de Groot,2,3 Ekkehard Hiller,4 Marlen Mock,1 Chris de Koster,2 Uwe Horn,5 Steffen Rupp,4 and Bernhard Hube1,6†

Department of Microbial Pathogenicity Mechanisms, Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knoell-Institute (HKI), Beutenbergstrasse 11a, 07745 Jena, Germany; University of Amsterdam, Science Park 904, 1098 XH Amsterdam, Netherlands; Regional Centre for Biomedical Research, Alacete Science and Technology Park, University of Castilla—La Mancha, 02006 Albacete, Spain; Fraunhofer Institute for Interfacial Engineering and Biotechnology, Nobelstrasse 12, 70569 Stuttgart, Germany; Bio Pilot Plant, Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knoell-Institute (HKI), Beutenbergstrasse 11a, 07745 Jena, Germany; and Friedrich Schiller University, Jena, Germany

Received 31 August 2010/Accepted 12 November 2010

The cell wall of the human-pathogenic fungus *Candida albicans* is a robust but also dynamic structure which mediates adaptation to changing environmental conditions during infection. Sap9 and Sap10 are cell surface-associated proteases which function in *C. albicans* cell wall integrity and interaction with human epithelial cells and neutrophils. In this study, we have analyzed the enzymatic properties of Sap9 and Sap10 and investigated whether these proteases cleave proteins on the fungal cell surface. We show that Sap9 and Sap10, in contrast to other aspartic proteases, exhibit a near-neutral pH optimum of proteolytic activity and prefer the processing of peptides containing basic or dibasic residues. However, both proteases also cleaved at nonbasic sites, and not all tested peptides with dibasic residues were processed. By digesting isolated cell walls with Sap9 or Sap10, we identified the covalently linked cell wall proteins (CWPs) Cht2, Ywp1, Als2, Rhd3, Rbt5, Ecm33, and Pga4 as *in vitro* protease substrates. Proteolytic cleavage of the chitinase Cht2 and the glucan-cross-linking protein Pir1 by Sap9 was verified using hemagglutinin (HA) epitope-tagged versions of both proteins. Deletion of the *SAP9* and *SAP10* genes resulted in a reduction of cell-associated chitinase activity similar to that upon deletion of *CHT2*, suggesting a direct influence of Sap9 and Sap10 on Cht2 function. In contrast, cell surface changes elicited by *SAP9* and *SAP10* deletion had no major impact on the phagocytosis and killing of *C. albicans* by human macrophages. We propose that Sap9 and Sap10 influence distinct cell wall functions by proteolytic cleavage of covalently linked cell wall proteins.

The polymorphic fungus *Candida albicans* is the most frequent cause of disseminated candidiasis. This opportunistic human pathogen is a frequent colonizer of the gastrointestinal and urogenital tract and skin, where it exists as a member of the normal microbial flora in healthy individuals. However, even a mildly compromised immune system or a minor imbalance of the microbiota can be sufficient for *C. albicans* to cause superficial skin or mucosal infections (50). Furthermore, in cases of impaired immunity or disruption of natural barriers, *C. albicans* can cause fatal systemic disease, disseminating throughout the bloodstream and infecting internal organs (53, 60, 73).

Several virulence attributes contribute to the pathogenic potential of *C. albicans* (7). For example, the ability to switch from yeast to hyphae permits tissue invasion and immune evasion (72). Furthermore, *C. albicans* secretes a multitude of hydrolytic enzymes, namely, lipases, phospholipases, and proteases (62). The Sap protein family of aspartic proteases, consisting of the 10 individual members Sap1 to Sap10, has been described as a key virulence determinant of *C. albicans*. Extensive research on Sap1 to Sap6 has demonstrated the functional association of Saps with *C. albicans* pathogenicity by hydrolyzing host proteins. Sap functions affect a variety of processes, from tissue invasion to immune evasion (for a review see the work by Naglik et al. [46]). Recombinant Sap proteases have been used to determine the biochemical properties of these enzymes, demonstrating substrate cleavage at acidic pH between larger hydrophilic amino acids (4, 31, 59).

Another virulence attribute of *C. albicans* is its metabolic flexibility and the ability to rapidly adapt to changes in the extracellular environment during infection. The fungal cell wall, a robust but also dynamic structure, plays an important role in such environmental adaptation. The *C. albicans* cell wall is a bilayered structure. An inner polysaccharide network consists of tightly packed β-1,3-glucan chains that are covalently linked to β-1,6-glucan molecules and underlying chitin. This inner polysaccharide layer is covered by an outer protein coat of often highly glycosylated mannoproteins (11, 61). Cell wall proteins (CWPs) are either covalently linked via a glycosylphosphatidylinositol (GPI) remnant to the GPI anchor (55) or directly linked to β-1,3-glucan via an alkali-sensitive linkage (non-GPI-CWPs or Pir-CWPs) (61). A third group, the SDS-soluble CWPs, are not covalently linked and represent mainly cell surface-associated cytosolic proteins (67).
The important role of covalently linked CWPs in *C. albicans* fitness and virulence has recently been reviewed by Klis et al. (30). CWPs mediate biofilm formation and promote adherence to host cells and invasion into epithelial cell layers (e.g., the A1s protein family of adhesins, Ywp1 and Ecm33) (24, 27, 37). Other CWPs mask cell wall and cell separation defects and are modified in their interaction with epithelial cells and neutrophils (1, 26). Such phenotypes imply an important role for both proteases in cell wall integrity, which influences *C. albicans*-host cell interaction.

In the present study we aimed to characterize Sap9 and Sap10 enzymatic functions, to identify substrates of these proteases among *C. albicans* cell surface proteins, and to define the impact of cleavage events on substrate function. We used recombinant Sap9 and Sap10 to gain insight into the biochemical properties and cleavage preferences of both proteases. To define protease targets among CWPs, we analyzed soluble CWP profiles of the *sap9Δ sap10Δ* double mutant strain and tested proteolytic cleavage of proteins present in isolated fungal cell walls and of hemagglutinin (HA) epitope-tagged CWPs by Sap9 and Sap10. The activity of selected protease substrates in *sap9Δ* and *sap10Δ* single and double mutants was assayed to reveal a possible connection between proteolytic processing and distinct CWP function. Finally, we determined whether Sap9 and Sap10 are important for the phagocytosis and killing of *C. albicans* by human macrophages.

**MATERIALS AND METHODS**

**Strains and growth conditions.** *Candida albicans* and *Pichia pastoris* strains used in this study are listed in Table 1. Strains were cultured at 30°C in yeast extract-peptone-dextrose (YPD)-rich medium (10 g/liter yeast extract, 20 g/liter Bacto peptone, 20 g/liter d-glucose) or synthetic defined (SD) medium (6.7 g/liter yeast nitrogen base [YNB], YNB without amino acids [Becton Dickinson] supplemented with 75 mM ammonium sulfate, and 20 g/liter d-glucose). For growth on solid media plates, 2% agar was added. To avoid clumping of the *sap9Δ* and *sap10Δ* mutants, strains were precultured by streaking on YPD plates and incubating overnight at 30°C. Routinely, experiments were carried out with the

| Strain | Description or genotype | Reference or source |
|--------|-------------------------|---------------------|
| *Candida albicans* strains | | |
| SC5314 | Clinical isolate | 23 |
| CAI4 | | 19 |
| CAI4 + pClp10 | | 19, 45 |
| sap9Δ + pClp10 | | 1 |
| sap9Δ + pClp10 | | 1 |
| sup9Δ sup10Δ | | 1 |
| sup9Δ sup10Δ Ura+ | | 1 |
| sup9Δ sup10Δ | | This study |
| sup9Δ sup10Δ + pClp10 | | This study |
| CAI4 Cht2-HA | | This study |
| CAI4 Pir1-HA | | This study |
| CAI4 Rbt5-HA | | This study |
| sup9Δ sup10Δ Rbt5-HA | | This study |
| CAI4 Ecm33-HA | | This study |
| sup9Δ sup10Δ Ecm33-HA | | This study |
| CAI4 Pir1-HA | | This study |
| sup9Δ sup10Δ Pir1-HA | | This study |
| cht2Δ | | 63 |
| rbt5Δ | | 6 |
| *Pichia pastoris* strains | | |
| pCA2 | GS115 + pKJ113 with *C. albicans* SAP2 integration | 4 |
| pCA6 | GS115 + pKJ113 with *C. albicans* SAP6 integration | 4 |
| pCA9 | GS115 + pKJ113 with *C. albicans* SAP9 integration | M. Monod; 1 |
| pCA10 | GS115 + pKJ113 with *C. albicans* SAP10 integration | M. Monod; 1 |
**TABLE 2. Primers used in this study**

| Name | Sequence 5’→3’ |
|------|---------------|
| HA-cassette for | ATCAACAGATCCGTCCTTGTCGGCAGGTTCTCCGGTTCTCTGCTGCTAG |
| HA-cassette rev | ATGACAGATCCCGCCCATCTTGCTGCTAGTGAAGACGCTATAC |
| HA-cassette for 2 | ATCAGACATCTAGTTAGTGAAGTACTTACACTTGTTAGTATTGCTGCTTTTATTAGCACCTCTTTAGCTG |
| ECM33 T1 for | CAATAACAATTAAATCATGAAGTATTCTACACTTGTTAGTATTGCTGCTTTTATTAGCACCTCTTTAGCTG |
| RBT5 T1 rev | TGATGTCACCAACAGCAGTGGCACAAGCAGCAACTTGTTGAACTTGGTTGGAAGCAGTAGCGGTGAAAT |
| YWP1 T1 rev | GAGAAGCAGAACCCGATGGGGAAGCAGAACTTGAACCTGGAACGTAATCAACAACTTCAAGAGTAGAA |
| ACTAG |
| RBT5 T1 for | TAATACTACAATGCTGTTCAATTCTTATGTGCTGCTATTAGAAGAAGATTAATGGGTGAAACTCCAATTGTTAA |
| RBT5 T1 rev | CTGTTCTTTAAATCATTAATGCTGCTACGTGCTTACAAGATCTGGAATCTGGAATCTTCAAGAGTAGAA |
| RBT5 T1 for | AAAACAATTAGAAGCTAATTCTACTGCTGCTATTAGAAGAAGATTAATGGGTGAAACTCCAATTGTTAA |
| ECM33 T1 for | CAAACAGAATAATTGAATCAACAACAGCAATAACAACCAAGTGAATTITATTCAATATACAAAGCTTA |
| YWP1 T1 rev | CAATAACAATATGCTCGCCTTATCCTTATTGTCAATCGTTTCCATTGCTTCAGCTGCTGGTGTCACTGCTA |
| YWP1 T1 for | AAAACAATTAGAAGCTAATTCTACTGCTGCTATTAGAAGAAGATTAATGGGTGAAACTCCAATTGTTAA |
| ACTAGT |

---

*Restriction sites are marked in bold, the (GA)6 linker is in italics, and the 6X/HAs-tag-homologous sequences (in HA cassette primers) and cassette-homologous sequences are underlined.*

pCPl0-containing *sapr9* and *sapr10* mutant strains and the CAH + pCPl0 strain as the wild-type (wt) control.

**Plasmid construction.** Plasmid pH-HA-Ura3-3A was constructed by inserting two direct repeats of the 6X/HAs epitope into the vector pCPl0 (45) (see Fig. 3A); 6X/HAs was amplified from plasmid POM10 (22) (kindly provided by EUROSCARF; http://www.uni-frankfurt.de/fb15/mikro/EUROSCARF), whereby PCR primers introduced terminal restriction sites and the 24-bp linker (GA)n. A SacI/NotI6/H11032 primer fragment was amplified with primers HA-cassette for1 and rev1 and cloned 3/H9004 of the strain as the wild-type (wt) control.

**Strain construction.** For the chromosomal integration of pCPl0 into the sapd/HAs sapd/HAs-Ura* strain, the Ura-positive mutant strain (sapd/HAs sapd/HAs-Ura*) was streaked on 25 μg/mL 5-fluoroorotic acid (FOA)-containing plates to select for loss of the URA3 gene. Next, strains were transformed with Stul-linearized pCPl0, and Ura-positive integrants were selected on SD plates.

**Strains containing insertions of an internal HA epitope tag into open reading frames were obtained as follows (see Fig. 3A).** A TAcl-URA3-tag cassette was amplified from pH-HA-Ura3-3A with HA-cassette for1 and rev1, and cloned 3/H9004 of URA3. Primers are listed in Table 2.

**Cell wall isolation and cell wall digestion.** For cell wall isolation, *C. albicans* sapd/HAs sapd/HAs double mutant cultures were grown in liquid medium supplemented with 0.1 M sodium citrate buffer to pH 4.0 or with 0.1 M potassium phosphate buffer to pH 6.5. Two hundred optical density (OD) units (e.g., 100-mL culture with an OD at 600 nm [OD600] of 2) were harvested after overnight incubation at 37°C, and the OD with the highest fluorescent value was determined. A peptide was selected as processed if its fluorescence reached at least 40% of the highest value. Specific activity of rSap preparations was determined by comparing their activity to the defined activity of 5 U trypsin (0.29 μg; sequencing grade, modified; Promega) in the fluorescence-quantified casein assay. An average of 1.5 μg rSap9, 15 μg rSap10, 3 μg rSap2, and 0.2 μg rSap6 corresponded to the activity of 5 U trypsin.

**Antibody labeling.** To analyze the ability of antibody labeling, cell surface proteins were isolated and freeze-dried, and released peptides were identified by liquid chromatography (LC)-MS/MS analysis. LC-MS/MS analysis was performed as described previously (15). Six milligrams of freeze-dried cell walls were incubated with 2.75 μg rSap9 or 40 μg rSap10 or without protease. After overnight incubation at 37°C, supernatants were collected and freeze-dried, and released peptides were identified by liquid chromatography (LC)-MS/MS analysis. LC-MS/MS analysis was performed as described previously (15). Samples were analyzed with and without digestion with trypsin. Prior to analysis, peptides were desalted and collected on Omix C18 pipette tips (Varian). Protein spectra were processed with MASCOT software (Matrix Science), and MASCOT scores were used to evaluate the identified peptides and proteins. A P value of <0.05 was considered significant for peptide identification. Control samples without addition of rSaps but with trypsin digestion yielded no peptide identifications.

**Western blot analysis of HA epitope-tagged proteins.** Strains expressing HA-tagged CWPs were grown in SD liquid medium for 24 h at 30°C. Cultures of...
Rbt5-HA-expressing strains were supplemented with 500 μM iron chelator bathophenanthrolene disulfonic acid disodium salt (BPS; Sigma-Aldrich) to induce Rbt5 expression by iron starvation (69). Subsequently, cells were removed from the culture medium by centrifugation, and proteins were precipitated out of 30-ml supernatants with methanol-chloroform (70). For comparison of wt and mutant samples, equal amounts (30 to 50 μg) of precipitated proteins were subjected to de-N-glycosylation by incubation with 100 U N-glycosidase F (PNGaseF; New England Biolabs) according to the manufacturer’s instructions. Alternatively, 20 to 40 μg protein was digested with 0.7 μg rSap9 or 8.6 μg rSap10 recombinant proteases or buffer only for 2 h at 37°C. Enzymatically treated supernatant proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% (wt/vol) milk powder and probed with a rat anti-NA-H high-affinity antibody (clone 3F10; Roche Applied Science) and a goat anti-rat IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology) secondary antibody. Signals were visualized with highly sensitive enhanced chemiluminescence (ECL) Western blotting detection reagents (GE Healthcare or Pierce) according to the manufacturer’s instructions. At least three independent experiments were performed.

Measurement of chitinase activity. Endochitinase activity was determined with a fluorescence-based assay as previously described (63), with minor modifications. Briefly, the cell-bound activity of 2 × 10^7 phosphate-buffered saline (PBS)-washed C. albicans cells precultured on YPD agar plates was assayed with 50 μM substrate 4-methylumbelliferyl-β-D-ν-triactetylchitotrioside (4-MU-[GlcNAc]3; Sigma Aldrich). Fluorescence at 340-nm excitation/465-nm emission and an OD 600 were monitored in a microplate reader at the reaction start and endpoint (30 min). In parallel, the OD 600 was monitored. The relative chitinase activity of C. albicans mutants was calculated as a percentage of wt values, normalized against the OD 600.

**RESULTS**

**Recombinant Sap9 and Sap10 exhibit an unusually broad pH spectrum, with activities above neutral pH and an incomplete inhibition by pepstatin A.** Secreted Sap proteases display typical features of aspartic proteases, including acidic pH optima ("acid proteases"), a bell-shaped (Gaussian) pH dependence of activity (55), and sensitivity to the inhibitor pepstatin A (43). To test whether Sap9 and Sap10 follow this prototypic scheme, we measured the proteolytic activities of recombinant Sap9 and Sap10 (rSap9 and rSap10) against the fluorescence-quenched substrate casein and compared these to Sap2 and Sap6 (representing the two main subfamilies of Sap1 to 3 and Sap4 to 6). The activity of recombinant Sap2 (rSap2) was highest at pH 3 or 4, with significant reduction at pH 6 to 8, as expected from previous studies (4, 31, 59) (Fig. 1A). Recombinant Sap6 (rSap6) showed a narrow pH optimum at pH 6, with activity sharply declining at pH values higher than pH 6 or lower than pH 5 (Fig. 1B), confirming and extending previous data (4). Strikingly, rSap9 and rSap10 exhibited high activity over a broad pH range, even above neutral pH (pH 5 to 8). Both proteases displayed a pH optimum between pH 6 and pH 7 and showed a significantly reduced activity at lower acidic pH values (pH 3 or 4) (Fig. 1C and D).

As predicted, activity of rSap2 and rSap6 was almost completely inhibited by the addition of the potent aspartic protease inhibitor pepstatin A (20 μM) at all tested pH values (Fig. 1A and B). In contrast, pepstatin A only partially inhibited rSap9 and rSap10 (Fig. 1C and D), and high residual activity was observed. The residual rSap9, but not rSap10, activity could be further reduced at higher pepstatin A concentrations, with 40% and 27% residual activity at pH 6 when applying 50 and 100 μM pepstatin A, respectively. These data demonstrate that although Sap9 and Sap10 do possess characteristics of aspartic proteases, they also exhibit distinct features, such as decreased sensitivity to pepstatin A and near-neutral pH optima.

**Sap9 and Sap10 cleavage preference for basic or dibasic residues is not exclusive.** In a previous study on a limited set of synthetic peptides we showed that rSap9 and rSap10 cleave at basic lysine or arginine (K or R) or dibasic residues, similar to the S. cerevisiae yapsins, and that rSap10 cleavage can also
occur at yapsin-atypical amino acids F and H (1, 32, 51). In order to gain more detailed knowledge of Sap9 and Sap10 substrate cleavage sites, we applied a systematic screen, digesting a set of 360 fluorescence-quenched peptides with rSap9 or rSap10 (protease substrate set; JPT Peptide Technologies). The peptides represented the cleavage sequences of a large set of known protease substrates. Sequences of peptides cleaved by rSap9 and rSap10 are listed in Table 3.

Out of the 360 peptides tested, almost 25% were processed exclusively by rSap9, only a minor fraction of peptides was cleaved exclusively by rSap10, and an overlapping fraction of 31 peptides was processed by both proteases (Fig. 2A). Peptides with dibasic residues were clearly enriched among cleaved peptides, with 45% among rSap9-cleaved peptides (51 out of 113) and 41% among rSap10-cleaved peptides (16 out of 39) compared to 23% in the whole protease substrate set (82 out of 360). However, 29 dibasic residue-containing peptides were not processed by rSap9 or rSap10 (Fig. 2A). This could be due to the influence of residues surrounding basic amino acids on cleavage preferences, as certain amino acids were enriched at these positions among cleaved peptides (Fig. 2B; summarized in Fig. 2C). As expected, basic residues were in the enriched fraction, but other residues were also present. Hydrophobic amino acids (A, I, L, Y, and V) were enriched N-terminal to K and R residues among rSap10 cleaved peptides and were also present in the rSap9-cleaved fraction. C-terminal to basic amino acids, acidic amino acids seem to be tolerated, as D and E were enriched in rSap9-cleaved peptides and were also present in the rSap10-cleaved fraction. Thus, basic or dibasic residues are common but not unique among cleaved peptides, and residues neighboring basic amino acids have an impact on cleavage. Eighteen rSap9-cleaved peptides and four rSap10-cleaved peptides contained no basic residues. Consequently, cleavage at amino acids other than lysine or arginine must have occurred not only by rSap10 (1) but also by rSap9.

A pattern-matching search of the cleaved peptides against C. albicans-translated ORF sequences using the Candida Genome Database (http://www.candidagenome.org) generated no hits. However, one of the peptides cleaved by rSap9 and rSap10, AKRAASQI, represents a sequence present in the S. cerevisiae covalently linked cell wall protein (CWP) ScPir2, which is important for ScPir2 propeptide cleavage by Kex2 (44). Interestingly, the C. albicans homolog CaPir1, possesses dibasic residues at a similar position in the protein sequence (N25K25) (see Fig. 5), suggesting that this protein is a potential Sap9 and Sap10 substrate in C. albicans.

**Minor influence of Sap9 and Sap10 on the soluble cell wall proteome.** Localization of Sap9 and Sap10 at the cell surface suggested that both proteases cleave fungal CWPs. We therefore examined the influence of Sap9 and Sap10 cleavage events on noncovalently linked SDS-soluble CWPs by comparing soluble CWP profiles of the wild type (wt) with those of a sap9Δ sap10Δ double mutant. Cell surface proteins that have been labeled with a membrane-impermeable biotin derivative were solubilized using detergents, affinity purified, and separated via 2D-PAGE (for gel images and a list of proteins identified by mass spectrometry see Fig. S1 and Table S1 in the supplemental material).

Comparison of protein patterns of the protease double mutant and wt demonstrated high similarity. After the matching of 318 spots and quantification of spot volume, only seven proteins with low abundance were shown to be differentially regulated, with one protein upregulated and six proteins downregulated in the sap9Δ sap10Δ mutant. One protein spot with less abundance was identified as the aldehyde dehydrogenase Ald5, a protein previously detected in cytosolic protein fractions (5) but also among soluble CWPs (8). Other proteins

### TABLE 3. Sequences of peptides of the protease substrate set cleaved by Sap9 or Sap10

| Protease used for cleavage | Peptide sequence⁴ |
|---------------------------|-------------------|
| **Sap9**                  |                   |
| 1⁵                        |                   |
| 14                        |                   |
| 27                        |                   |
| 40                        |                   |
| 66                        |                   |
| 79                        |                   |
| 105                       |                   |
| GKKRIMSS                  | RKRRVDRVEQ        |
| GKRIMSS                   | RKRELAP           |
| AEPPYGAL                  | GKRESQDA          |
| PANFAEG                   | PQRFRGNT          |
| RAKRSHVF                  | ERKR11GG          |
| KTERAEED                  | AATRQAV           |
| ERERRLDP                  | ALADSLGRK        |
| MSKRALQQ                  | QSPRSFKQ          |
| VERRVIVO                  | RARALRKGKD        |
| ERERRLDP                  | RRKREKTR          |
| SEVMADAE                  | CVKVKKEREQ        |
| VEMDSLSE                  | RKRRAVLT          |
| DGVDLKTQ                  | DGVDLKTQ          |
| VARRKLPT                  | VARRKLPT          |
| VMRDPASK                  | VMRDPASK          |
| VEMDSLSE                  | VEMDSLSE          |
| VERRVDEQ                  | VERRVDEQ          |
| RRRRELDP                  | RRRRELDP          |
| VEMDSLSE                  | VEMDSLSE          |
| VERRVDEQ                  | VERRVDEQ          |
| RRRRELDP                  | RRRRELDP          |
| VEMDSLSE                  | VEMDSLSE          |
| RRRRELDP                  | RRRRELDP          |
| VEMDSLSE                  | VEMDSLSE          |
| RRRRELDP                  | RRRRELDP          |

### Notes

⁴ Peptides cleaved by both Sap9 and Sap10 are underlined.

⁵ Lists start with peptides with the highest cleavage efficiency.
characteristic of the soluble cell surface proteome (8, 36, 54) were found in similar patterns in the wt and sap9/H9004 sap10/H9004 mutant: Atp2, Ssb1, Pdc11, Rpp0, Eno1, Eft2, Cdc19, Atp1, Tdh3, Fba1, and Gpm1. Consequently, the lack of Sap9 and Sap10 had only a minor influence on soluble CWPs.

Similarly, deletion of SAP9 and SAP10 appears to have a minor influence on the presence and migration of major secreted proteins, as suggested from 2D-PAGE experiments and determination of protein concentration in culture supernatants of the protease double mutant (data not shown).

Recombinant Sap9 and Sap10 proteases cleave GPI-CWPs on isolated C. albicans cell walls. We next applied a more specific approach, monitoring cleavage events by rSap9 and rSap10 targeting covalently linked CWPs. Isolated cell walls of the sap9Δ sap10Δ mutant were incubated with rSap9 and rSap10 proteases. Peptides released from cell walls after digestion were analyzed by LC-MS/MS.

Incubation with rSap9 and rSap10 specifically liberated protein species from cell walls, while no peptides were detected in samples without protease treatment. Identified proteins belonged to the class of covalently linked GPI-modified cell wall proteins (GPI-CWPs), with functions from cell wall remodeling (Cht2, Ecm33, Pga4 and Rhd3) (14, 37, 39, 56) to host cell interaction and virulence (Ywp1, Als2, Ecm33, and Rhd3) (14, 24, 37, 64) and iron metabolism (Rbt5) (69) (Table 4). Cell walls were isolated from logarithmic- or stationary-phase cultures grown at pH 4 or pH 6.5. Release of peptides after protease digest varied mainly with the growth phase rather than with the culture pH. These data provide the first in vitro evidence of proteolytic targets of Sap9 and Sap10 among covalently linked CWPs. This reinforces the view that Sap9 and Sap10 activity may regulate CWP functions and supports our earlier observations that deletion of SAP9 and SAP10 influences cell wall integrity and adhesion to host cells (1).

Processing of epitope-tagged Cht2 and Pir1 by Sap9. To further characterize Sap9 and Sap10 cleavage events on CWPs, we integrated the small internal hemagglutinin epitope tag (HA) into the protein sequences of the previously identified proteolytic targets Cht2, Ywp1, Rbt5, and Ecm33 (Fig. 3A). Pir1, a non-GPI-CWP with a proposed role in β-1,3-glucan cross-linking (28) was also included for analysis because peptide cleavage data had suggested processing of this CWP by
Sap9 and Sap10 (see above). The HA tag was inserted close to the protein N terminus, leaving protein N- and C-terminal regions unmodified to ensure proper secretion and cell surface localization. This approach allows the detection of HA-tagged mature proteins or N-terminal cleavage fragments with an epitope-specific antibody (Fig. 3B) and was used for comparison of epitope-tagged protein characteristics in wt and SAP9/SAP10 double mutant strain backgrounds.

Successful epitope tagging and surface localization of tagged proteins was confirmed by immunofluorescence staining with an HA-specific antibody (Fig. 3C). The presence of HA-tagged fragments in C. albicans culture supernatants was then examined by Western blot analysis. All tested proteins were not only present on the cell surface (Fig. 3C) but were also partly released into the extracellular space (Fig. 4A). In contrast, the Cht2-HA protein was present in protease mutant samples (Fig. 4B). The observed differences in patterns of Cht2-HA and Pir1-HA further suggested that these two CWPs are in vitro proteolytic targets of Sap9 and that Cht2 may also constitute a Sap10 target.

Proteolytic digestion with recombinant rSaps did not release defined cleavage fragments from Ywp1-HA, Rbt5-HA, or Ecm33-HA (Fig. 4A). In contrast, the Cht2-HA protein pattern changed after protease digestion: a ~30-kDa fragment was enriched in the rSap9-digested sample and also slightly in the rSap10-digested sample. In addition, several distinct bands in the molecular weight range of 25 to 45 kDa were detected (Fig. 4B). A large portion of Pir1-HA migrated with a high molecular mass (180 kDa) (35). Proteolytic digestion with rSap9 reduced the amount of a distinct ~100-kDa band and generated a slightly smaller fragment (Fig. 4B). Interestingly, wt samples showed an ~90-kDa band, while an ~100-kDa band was present in protease mutant samples (Fig. 4B). The observed differences in patterns of Cht2-HA and Pir1-HA further suggested that these two CWPs are in vitro proteolytic targets of Sap9 and that Cht2 may also constitute a Sap10 target.

**In silico prediction of Sap9 and Sap10 cleavage sites in Cht2 and Pir1.** In order to assign cleavage fragments detected in anti-HA-Western blot analysis to potential cleavage sites, we analyzed the Cht2 and Pir1 protein sequences for the presence of the following sequence motifs: (i) a basic residue flanked by two amino acids that were found enriched when comparing rSap9 and rSap10 compared with the wt (data not shown). We therefore directly subjected sap9Δ sap10Δ supernatant proteins to digestion with rSap9 or rSap10.

---

**TABLE 4. Mass spectrometric analysis of GPI-CWPs released from isolated cell walls after digestion with Sap9 or Sap10**

| Protein          | Description                                      | Identified peptide (1*sequence*+1) (position) | pH at which protein released* |
|------------------|--------------------------------------------------|---------------------------------------------|------------------------------|
|                  |                                                  |                                             | Sap9 | Sap10 |
| **Logarithmic-phase cell walls** |
| Cht2             | Chitinase; required for normal filamentous growth | L*LSLGGGVDGFDVASATK*F (102–121)              | 4    |
| Cht2             |                                                  | L*LSLGGGVDGFDVASATK*F (102–121)              | 6.5  |
| Cht2             |                                                  | L*SAAAAPCPYPDLGGLLSK*V (182–200)             | 4    |
| Cht2             |                                                  | S*AAPQCPYPDLGGLLSK*V (183–200)              | 6.5  |
| Ywp1             | Cell wall and secreted protein with suggested role in dispersal in host | R*DQIDDFIASIENTEGTALGSTL*E (134–156)       | 4    |
| Als2 (or Als9)*  | ALS family protein; role in adhesion, biofilm formation, germ tube induction | I*TQVFN6FDSLTWTR*S (21–34)                 | 4    |
| Als10d           | ALS family protein                               | F*NVGGTGLVDLESSK*C (135–149)               | 6.5  |
| **Stationary-phase cell walls** |
| Rhd3/Pga29       | Unknown function; contributes to virulence       | TISSIOLFAK*S (16–25)*                      | 4    |
| Rbt5 (or Pga10, Csa1) | Hemoglobin acquisition                           | R*TYDOLPECAK*E (47–56)                    | 4    |
| Rbt5 (or Csa2)   |                                                  | K*QSTSSTPACPYWDGTC*L*C (61–76)              | 4    |
| Rbt5             |                                                  | L*CVMPQFAGAVGNCVAK*N (77–92)               | 6.5 |
| Rbt5             |                                                  | Q*FAGAVGNCVAK*N (82–92)                    | 4    |
| Ecm33            | Important for cell wall integrity                | K*TIGGALQISDNSELR*S (276–290)             | 4    |
| Fga4             | Transglucosidase                                 | K*AGIVYVLDVTNPSSITER*S (99–116)           | 6.5  |

* According to Candida Genome Database (http://www.candidagenome.org/).

**Proteolytic digestion of recombinant rSaps did not release defined cleavage fragments from Ywp1-HA, Rbt5-HA, or Ecm33-HA (Fig. 4A). In contrast, the Cht2-HA protein pattern changed after protease digestion: a ~30-kDa fragment was enriched in the rSap9-digested sample and also slightly in the rSap10-digested sample. In addition, several distinct bands in the molecular weight range of 25 to 45 kDa were detected (Fig. 4B). A large portion of Pir1-HA migrated with a high molecular mass (180 kDa) (35). Proteolytic digestion with rSap9 reduced the amount of a distinct ~100-kDa band and generated a slightly smaller fragment (Fig. 4B).**
but significant reduction in activity, as described previously to that of a mutant, but not the single mutants, exhibited significantly decreased chitinase activity, with 13.5% reduction compared to that of the wt (Fig. 6). This reduction was similar to that of a cht2Δ deletion mutant, which also showed a small but significant reduction in activity, as described previously.

FIG. 3. Internal hemagglutinin (HA) epitope tagging of potential protease substrates. (A) Two-step PCR-based epitope-tagging procedure as used in this study. Tagging constructs with URA3 marker flanked by two direct repeats of the HA epitope were introduced into the target gene open reading frames. Growth on FOA (5-fluoroorotic acid) selected for clones in which recombination between the HA epitope repeats had generated one intact HA epitope tag. (B) GPI-CWP with an internal HA epitope tag close to the N terminus and a possible cleavage product. SP, N-terminal signal peptide for secretion; GPI, C-terminal glycosylphosphatidylinositol addition signal; asterisks, serine- and threonine-enriched region, often highly O glycosylated. (C) HA-epitope-tagged CWPs localize to the cell surface as shown by immunofluorescence staining with an anti-HA antibody for wt and sap9Δ sap10Δ (ΔΔ) HA clones. Bars, 10 μm.

Chitinase activity is impaired in a sap9Δ sap10Δ mutant. Together, our data demonstrated in vitro cleavage of CWPs by Sap9 and Sap10. To evaluate if such cleavage may affect functions of CWPs in living C. albicans cells, we applied activity assays testing the function of two potential protease substrates in sap9Δ and sap10Δ single and double mutant strains. First, we examined cell-associated chitinase activity with a fluorescence-based enzymatic assay (38). The sap9Δ sap10Δ double mutant, but not the sap9Δ or sap10Δ single mutants, exhibited significantly decreased chitinase activity, with 13.5% reduction compared to that of the wt (Fig. 6). This reduction was similar to that of a cht2Δ deletion mutant, which also showed a small but significant reduction in activity, as described previously (63).

Second, we tested the function of Rbt5. This protein is necessary for utilization of iron from hemoglobin. While an rbt5Δ mutant exhibited reduced growth with hemoglobin as the sole iron source (69), single and double protease mutants grew as well as the wt under these conditions (data not shown), indicating that Rbt5 function is not impaired in the absence of SAP9 and SAP10. Consequently, Sap9 and Sap10 in combination are necessary for full chitinase activity but not for Rbt5 function.

Sap9 and Sap10 functions are dispensable for survival during interaction with macrophages. The importance of Sap9 and Sap10 for cell wall integrity and an intact cell wall structure (1) suggested that these proteases might influence the recognition of C. albicans by macrophages or fungal survival after phagocytosis. To test this hypothesis, we analyzed the interaction of C. albicans wt and protease mutants with human macrophages, using the monocytic cell line THP-1. Uptake of fungal cells into macrophages as well as survival after coincubation with macrophages were quantified. Neither uptake nor survival rates were altered for sap9Δ and sap10Δ single and double mutants compared with the wt (phagocytosis: wt, 52.9% [±12.9], sap9Δ sap10Δ mutant, 51.2% [±17.4]; killing: wt, 35.4% [±11.6], sap9Δ sap10Δ mutant, 35.7% [±5.1]). Protease mutants were able to form wt-like filaments inside macrophages and to escape from macrophages like the wt. Similar results were obtained with primary human blood monocyte-derived macrophages (data not shown).

Thus, cell surface changes in the absence of SAP9 and SAP10 do not seem to cause major alterations in cell surface immunogenicity. This observation is further supported by the finding that accessibility of β-1,3-glucan, a molecule important for immune recognition of C. albicans by macrophages (48), was similar in wt and sap9Δ sap10Δ single or double mutants,
as determined by flow cytometry analysis using an anti-β-1,3-glucan antibody (data not shown).

**DISCUSSION**

The current study we have characterized the enzymatic properties of Sap9 and Sap10 and identified cell wall protein substrates for these cell surface-associated aspartic proteases.

**Sap9 and Sap10 activity and specificity.** The near neutral pH optima and activity over a broad pH range distinguishes Sap9 and Sap10 from the other secreted Saps, which exhibit activity at more acidic pH optima and a more narrow pH range. The *S. cerevisiae* yapsins show similar activity over a broad pH range, depending on the substrate cleaved (20, 52), but the significant activity at neutral to basic pH seems to be specific for Sap9 and Sap10. This may reflect the host-associated lifestyle of *C. albicans* requiring regulatory proteolytic digests at several different host niches with different pH values.

Peptatin A was previously shown to be a potent inhibitor of secreted Sap activity (31, 59) and of Sap9 activity at acidic pH (10). In our experiments, however, peptatin A concentrations that inhibited Sap2 and Sap6 activity efficiently, were too low to elicit full Sap9 and Sap10 inhibition. This effect was also observed in a different buffer system (25). A low inhibition potential by peptatin A may be due to a lower inhibitor binding capacity at pH values higher than pH 5 (2) and has also been described for *S. cerevisiae* yapsins (32). Cutfield et al. (13) have determined residues in the Sap2 sequence which are involved in peptatin A binding. Strikingly, two of these amino acids differ in both Sap9 and Sap10 compared to Sap2 (I86 in Sap2 to L81 in Sap9 and S68 in Sap10; T221 in Sap2 to S374 in Sap9 and S269 in Sap10), possibly leading to reduced inhibitor binding.

**FIG. 4.** Cht2-HA and Pir1-HA are cleaved by Sap9. Proteins concentrated from culture supernatants of HA-protein-expressing clones were separated by SDS-PAGE and subjected to Western blot analysis with an anti-HA antibody. (A) Equal amounts of proteins from *sap9Δ* sap10Δ Ywp1-HA, Rbt5-HA, and Ecm33-HA clones were subjected to a 2-h protease digest with rSap9 (9) or rSap10 (10), in parallel to a buffer control without protease (−). (B) Protease digests of Cht2-HA and Pir1-HA clones and comparison of Pir1-HA patterns in wt and *sap9Δ* sap10Δ (ΔΔ) strain backgrounds. Asterisks, cross-reacting bands; arrowhead, rSap9/rSap10 protein bands. Arrows indicate differences in protein patterns. Molecular masses are given in kDa.

**FIG. 5.** Schematic depiction of Cht2 and Pir1 major domains and predicted Sap9 and Sap10 cleavage sites. SP, N-terminal signal peptide; GPL, C-terminal glycosylphosphatidylinositol addition signal; GH18, glycoside hydrolase family. Amino acid positions are given for domain borders and potential processing sites. Letters in bold, potential Sap9 cleavage; italics, potential Sap10 cleavage; bold italics, Sap9 and Sap10 cleavage. The proposed cleavage site labeled with an asterisk was predicted from data published by Albrecht et al. (1).

**FIG. 6.** *SAP9* and *SAP10* are necessary for full cell-associated chitinase activity. Shown are chitinolytic activities of *sap9Δ* and *sap10Δ* and *cht2Δ* mutant strains against 4-MU-[GlcNAc]₃, in percentage of wt activity (100%). Statistically significant differences to wt values are marked with asterisks. *, *P* < 0.05; **, *P* < 0.01.
independent of the pH. Our data imply that experiments using pepstatin A to address the importance of Saps for C. albicans pathogenicity should be interpreted with caution. For example, near-neutral pH values used in most cell culture systems could allow substantial residual Sap9 and Sap10 activity despite pepstatin A treatment.

A systematic screen of synthetic peptides for Sap9 and Sap10 cleavage confirmed our previous observation that both proteases prefer cleavage at basic lysine or arginine or dibasic residues, similar to S. cerevisiae yapsins (1, 21). However, both Sap9 and Sap10 cleavage events also occurred independent of basic residues, an ability that seems to be specific for the C. albicans yapsin-like proteases. Furthermore, several peptides containing dibasic residues were not digested, suggesting that the presence of such sites alone is not a definite indication for cleavage. Thus, the generation of a cleavage consensus sequence for systematic prediction of proteolytic substrates by searching the C. albicans genome is not possible on the basis of our data. Comparing Sap9 and Sap10 behavior, our cleavage assays suggest common as well as specific processing sites for the two proteases. This confirms our previous observation that Sap9 can only partially substitute Sap10 and vice versa (1). Of note was the absence of certain amino acid combinations among Sap10-cleaved peptides (e.g., DK, DR, EK, TK, and TR) and the lower number of Sap10-cleaved peptides compared to Sap9-cleaved peptides. This may indicate a more specific action of Sap10 compared to Sap9 but may also be due to a lower specific activity of our Sap10 preparation (see Materials and Methods). However, increasing the amount of Sap10 protein in the protease substrate set assay did not significantly enhance the number of processed peptides (data not shown), suggesting rather that Sap10 may indeed have a more limited number of potential substrates.

**Sap9 and Sap10 in vitro substrates and suggested role of processing.** Cleavage experiments with isolated C. albicans cell walls and synthetic peptides facilitated the identification of potential Sap9 and Sap10 substrates among previously described covalently linked C. albicans CWPs: Cht2, Ywp1, Als2, Rhd3, Rbt5, Ecm33, Pga4, and Pirl (14, 15). Interestingly, mutants lacking these proteins showed sap9Δ sap10Δ-similar phenotypes, such as altered adhesion (ywp1Δ and als2Δ mutants), cell wall structural or integrity defects (rhd3Δ, ecm33Δ, and pirlΔ mutants), and cell separation defects (ecm33Δ and pirlΔ mutants) (14, 24, 35, 37, 64), suggesting a functional connection between these cell wall proteins and the two proteases. Surprisingly, of the eight potential substrates, it appeared that only Cht2-HA and Pirl1-HA were cleaved by Sap9 and Sap10 following epitope tagging. One reason for this unexpected result may be that cleavage sites, which were accessible in isolated cell walls or synthetic peptides, were masked in living fungal cells, e.g., due to glycosylation or protein folding. Most of the tagged proteins were detected in Western blots as highly diffuse bands, as expected for heavily N- and O-glycosylated CWPs (30). Thus, slight molecular weight shifts of protein bands may not have been detectable. Notable, epitope-tagged CWPs were detected not only on the cell surface, but also in the culture medium. GPI-anchored proteins can be either membrane localized or covalently linked to the cell wall β-1,6-glucan after cleavage of the GPI anchor (28). As discussed by Granger et al. (24), an intermediate form, lacking the GPI anchor may exist that could possibly diffuse into the medium. Noncovalently linked, soluble surface proteins also represent possible proteolytic targets for Sap9 and Sap10. However, our experiments did not detect a major influence of Sap9 and Sap10 on this class of proteins. The reduced abundance of the aldehyde dehydrogenase Ald5 in the sap9Δ sap10Δ mutant compared with the wt may rather be due to secondary effects (5).

Cell wall integrity and cell separation defects of mutants lacking SAP9 and SAP10 (1), as well as upregulation of SAP9 expression during cell wall stress (12) and cell wall regeneration (9), suggest that proteolytic cleavage has a direct impact on CWP functions. This is similar to S. cerevisiae, as yapsin deletion mutants develop cell wall integrity defects (33) and ScYps1 was shown to cleave the cell wall remodeling CWPs ScPir4 and ScGas1 (20). However, the consequences of proteolytic processing of distinct CWPs for their function remains unclear. We propose three possible consequences of cleavage: activation, structural rearrangements, and shedding, based on the following findings. Our assays revealed that C. albicans cell-bound chitinase activity is reduced in the absence of SAP9 and SAP10. C. albicans possesses at least four chitinase-encoding genes, CHT1 to -4. Besides Cht2, Cht3 has been shown previously to contribute to yeast chitinase activity, while Cht1 and Cht4 likely play a minor role (17, 63). Constitutive CHT3 expression did not reconstitute wt-like chitinase activity in a sap9Δ sap10Δ mutant (data not shown). Thus, a Cht2 defect rather than a dysfunction of Cht3 is likely to be the main cause of reduced chitinase activity in the sap9Δsap10Δ mutant, although the influence of further factors cannot be ruled out. Therefore, a direct activating effect of Sap9 and Sap10 proteolytic cleavage on Cht2 function is proposed. A connection between chitinase activity and proteolytic cleavage has already been described for a C. albicans microsomal zymogenic chitinase (16).

**In silico analysis of the Pirl1 sequence predicted cleavage sites between repeats that are likely involved in β-1,3-glucan-binding and cross-linking of glucan chains (18, 30). It is possible that cleavage in this region alters the degree of glucan cross-linking by Pirl1, thus assigning Sap9 and Sap10 a function in rearranging cell wall components. A third function of cleavage could be the shedding of CWPs or CWP fragments into the surrounding medium. Shedding of a substrate by Sap9 has been suggested to induce chemotaxis of polymorphonuclear neutrophils toward C. albicans (26).**

Depending on environmental conditions and fungal morphology, the cell wall proteome, and thus the proteins potentially exposed to Sap9 and Sap10, can change dramatically. In addition, target protein localization might be restricted to certain cell surface compartments, such as bud sites (30). The constitutive expression of SAP9 and SAP10 under both yeast- and hypha-inducing growth conditions (47) and the apparently even distribution of Sap9 and Sap10 over the C. albicans cell surface (1) suggest that proteolytic activities are important during both yeast and hyphal growth and are not restricted to certain cell surface components. The experiments in this study focused on yeast cells since the most prominent phenotype of deletion mutants, a cell separation defect, was observed for yeast cells, while no alteration in hyphal induction, growth, or appearance was evident.
Many of our experiments comparing the wt with the sap9Δ and/or sap10a mutants showed no differences in protein patterns or processing. It may be possible that lack of Sap9 and/or Sap10 activity can be complemented by other proteases such as the proprotein-converting serum protease Kex2 (3, 49). As discussed by Albrecht et al. (1), Kex2, which uses processing sites similar to those of Sap9 and Sap10, is active in the trans-Golgi network and may process target proteins of Sap9 and Sap10 that are transported to the surface via the secretory pathway. Two observations from the present study indicate overlapping functions between Kex2 and Sap9 and Sap10. First, the Ywp1-fragment KR133*DQIDDFIASIENTEGTAL overlapping functions between Kex2 and Sap9 and Sap10.

First, the Ywp1-fragment KR133*DQIDDFIASIENTEGTAL overlapping functions between Kex2 and Sap9 and Sap10. First, the Ywp1-fragment KR133*DQIDDFIASIENTEGTAL overlapping functions between Kex2 and Sap9 and Sap10. First, the Ywp1-fragment KR133*DQIDDFIASIENTEGTAL overlapping functions between Kex2 and Sap9 and Sap10. First, the Ywp1-fragment KR133*DQIDDFIASIENTEGTAL

**Interaction with macrophages.** Despite their importance for cell wall structure and integrity. Sap9 and Sap10 were shown to be dispensable during interaction of *C. albicans* with human macrophages in this study. McKenzie et al. (40) suggest that CWP defects alone have a minor impact on macrophage phagocytosis and killing, as seen for the CWP hwp1Δ and als3Δ mutants. Fungal yapsins, however, have the potential to influence fungus-macrophage interactions, as shown for *C. glabrata*, in which mutants lacking yapsins exhibit reduced survival in murine macrophages (29). Sap9 has recently been shown to degrade and inactivate the oral salivary antimicrobial peptide histatin-5 (41), and further, yet undefined, protease targets on the host side may be possible.

**Conclusions.** In summary, our data demonstrate that Sap9 and Sap10 are distinct *C. albicans* aspartic proteases: they differ from other Sap members (i) in exhibiting enzymatic activity over a broad range of pH, even at neutral (physiological) pH, (ii) in a limited inhibition potential by pepstatin A, and (iii) in distinct cleavage preferences. We also, for the first time, provide evidence for Sap9/Sap10 processing of a set of *C. albicans* cell wall proteins in vitro. Finally, the deletion of SAP9 and SAP10 genes in *C. albicans* resulted in a reduction in chitinase activity similar to that upon deletion of the chitinase-encoding gene CHT2. This result indicates that Sap9/Sap10 cleavage has the potential to directly influence cell wall protein functions. It opens up perspectives for future experiments focusing on the characterization of the particular consequence of proteolytic cleavage for the functions of Cht2 and other cell wall protein targets.

**ACKNOWLEDGMENTS**

We acknowledge the financial support by the Deutsche Forschungsgemeinschaft DFG project Hu 528/14-1. We are grateful to Olaf Kniemeyer and Maria Poetsch (HKI) for supporting 2D-PAGE analyses. We thank Frans Kils and Henk Dekker (Swammerdam Institute for Life Sciences, Netherlands) for helpful discussions regarding potential Sap targets and support in LC-MS/MS analyses, respectively, and Duncan Wilson (HKI) for critical reading of the manuscript. *C. albicans* cht2Δ and rbg5a strains were kindly provided by Carol Munro (University of Aberdeen, United Kingdom) and Daniel Kornitzer (Rappaport Institute for Research in the Medical Sciences, Haifa, Israel), and Sap-producing *Pichia pastoris* strains were kindly provided by Michel Monod (CHUV Lausanne, Switzerland). Furthermore, we thank Betty Wächter, Katja Seider, and Sascha Brunke (HKI) for fruitful discussions.

**REFERENCES**

1. Albrecht, A., et al. 2006. Glycosylphosphatidylinositol-anchored proteases of *Candida albicans* target proteins necessary for both cellular processes and host-pathogen interactions. J. Biol. Chem. 281:688–694.

2. Backman, D., and U. H. Danielson. 2003. Kinetic and mechanistic analysis of the association and dissociation of inhibitors interacting with secreted aspartic acid proteases 1 and 2 from *Candida albicans*. Biochim. Biophys. Acta. 1646:134–139.

3. Bader, O., Y. Krause, and B. Hube. 2003. Processing of predicted substrates of fungal Kex2 proteases from *Candida albicans*, *C. glabrata*, Saccharomycetes cerevisiae and *Pichia pastoris*. BMC Microbiol. 3:116.

4. Borg-von Zepelin, M., S. Beggah, K. Boggian, D. Sanglard, and M. Monod. 1998. The expression of the secreted aspartyl proteinases Sap to Sap6 from *Candida albicans* in murine macrophages. Mol. Microbiol. 28:543–554.

5. Brand, A., D. M. MacCallum, A. J. Brown, N. A. Gow, and F. C. Odds. 2004. Ectopic expression of *URA3* can influence the virulence phenotypes and proteome of *Candida albicans* but can be overcome by targeted reintroduction of *URA3 at the RPS10 locus*. Eukaryot. Cell. 3:909–909.

6. Braun, B. R., W. S. Head, M. X. Wang, and A. D. Johnson. 2000. Identification and characterization of *TUP1*-regulated genes in *Candida albicans*. Genetics 156:31–44.

7. Calderone, R. A., and W. A. Fonzi. 2001. Virulence factors of *Candida albicans*. Trends Microbiol. 9:327–335.

8. Castillo, L., et al. 2008. A study of the *Candida albicans* cell wall proteome. Proteomics 8:3871–3881.

9. Castillo, L., et al. 2006. Genomic response programs of *Candida albicans* following proteolysis and regeneration. Fungal Genet. Biol. 43:124–134.

10. Cawley, N. X., et al. 2003. Synthesis and characterization of the first potent inhibitor of yapsin 1. Implications for the study of yapsin-like enzymes. J. Biol. Chem. 278:5523–5530.

11. Chaffin, W. L. 2008. *Candida albicans* cell wall proteins. Microbiol. Mol. Biol. Rev. 72:495–544.

12. Copping, V. M., et al. 2005. Exposure of *Candida albicans* to antifungal agents affects expression of *SAP2* and *SAP9* secreted protease genes. J. Antimicrob. Chemother. 55:645–654.

13. Cutfield, S. M., et al. 1995. The crystal structure of a major secreted aspartic proteinase from *Candida albicans* in complexes with two inhibitors. Structure 3:1261–1271.

14. de Boer, A. D., et al. 2010. The *Candida albicans* cell wall protein Rhd/ Pg29 is abundant in the yeast form and contributes to virulence. Yeast 27:611–624.

15. de Groot, P. W. J., et al. 2006. Proteinolytic activity of *Candida albicans* cell wall cells reveals covalently bound carbohydrate-active enzymes and adhesins. Eukaryot. Cell 5:955–965.

16. Dickinson, K., V. Keer, C. A. Hitchcock, and D. J. Adams. 1991. Micromosaic chitinase activity from *Candida albicans*. Biochem. Biophys. Acta. 1073:177–182.

17. Dinkler, A., A. Walther, C. A. Specht, and J. Wendland. 2005. *Candida albicans* CHT3 encodes the functional homolog of the Cts1 chitinase of Saccharomyces cerevisiae. Fungal Genet. Biol. 42:935–947.

18. Ecker, M., R. Deutzmann, L. Lehle, V. Mrsa, and W. Tanner. 2006. Piproteins of *Saccharomyces cerevisiae* are attached to beta-1,3-glucan by a new protein-carbohydrate linkage. Yeast 23:1152–11529.

19. Fonzi, W. A., and M. Y. Irwin. 1993. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics 134:717–728.

20. Gagnon-Arsenault, I., L. Parise, J. Tremblay, and Y. Bourbonnais. 2008. Activation mechanism, functional role and shedding of glycosylphosphatidylinositol-anchored Yps1p at the *Saccharomyces cerevisiae* cell surface. Mol. Microbiol. 69:982–993.

21. Gagnon-Arsenault, I., J. Tremblay, and Y. Bourbonnais. 2006. Fungal yapsins and cell wall: a unique family of aspartic peptides for a distinctive cell wall function. FEMS Yeast Res. 6:966–978.

22. Gauss, R., M. Trautwein, T. Sommer, and A. Spang. 2005. New modules for the repeated internal and N-terminal epitope tagging of genes in *Saccharomyces cerevisiae*. Yeast 22:1–12.

23. Gillum, A. M., E. Y. Tsay, and D. R. Kirsch. 1984. Isolation of the *Candida albicans* allele for orotidine-5’-phosphate decarboxylase by complementation of *S. cerevisiae ura3* and *E. coli pyrF* mutations. Mol. Gen. Genet. 198:179–182.

24. Granger, B. L., M. L. Flemmiken, D. A. Davis, A. P. Mitchell, and J. E. Cutler. 1999. Yeast wall protein 1 of *Candida albicans*. Microbiology 151:1631–1641.

25. Gropp, K., et al. 2009. The yeast *Candida albicans* evades human complement attack by secretion of aspartic proteases. Mol. Immunol. 47:465–475.

26. Hornbach, A., et al. 2009. The glycosylphosphatidylinositol-anchored pro-
tease Sap9 modulates the interaction of Candida albicans with human neutrophils. Infect. Immun. 77:5216–5224.

27. Hoyer, L. L., C. B. Green, S. H. Oh, and X. Zhao. 2008. Discovering the secrets of the Candida albicans agglutinin-like sequence (ALS) gene family—a sticky pursuit. Med. Mycol. 46:1–15.

28. Kaptyn, J. C., et al. 2000. The cell wall architecture of Candida albicans wild-type cells and cell wall-defective mutants. Mol. Microbiol. 35:601–611.

29. Kaur, R., B. Ma, and B. P. Cormack. 2007. A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of Candida glabrata. Prot. Natl. Acad. Sci. U. S. A. 104:7628–7633.

30. Kilis, F. M., G. J. Sosinska, P. W. J. de Groot, and S. Bruil. 2009. Covalently linked cell wall proteins of Candida albicans and their role in fitness and virulence. FEBS Yeast Res. 9:1013–1028.

31. Koelsch, G., et al. 2006. Enzymic characteristics of secreted aspartic proteases of Candida albicans. Biochim. Biophys. Acta 1780:117–131.

32. Komano, H., N. Rockwell, G. T. Wang, G. A. Krafft, and R. S. Fuller. 1999. Purification and characterization of the yeast glycosylphosphatidylinositol-anchored, monobasic-specific aspartyl protease yapasin 2 (Mkp7p). J. Biol. Chem. 274:24431–24437.

33. Krysan, D. J., E. L. Ting, C. Abeijon, L. Kroos, and R. S. Fuller. 2003. CIp10, an efficient and convenient integrating vector for O-mannosylated proteins of Candida albicans. Cell 113:644–654.

34. Krysan, D. J., E. L. Ting, C. Abeijon, L. Kroos, and R. S. Fuller. 2000. CIp10, an efficient and convenient integrating vector for O-mannosylated proteins of Candida albicans. Cell 113:644–654.

35. Martinez, A. L., et al. 2004. Role of PirI in the construction of the Candida albicans cell wall. Microbiology 150:3151–3161.

36. Martinez-Lopez, R., C. Nombela, R. Díez-Orejas, L. Monteoliva, and C. Gil. 2008. Immunoprotoomic analysis of the protective response obtained from vaccination with Candida albicans Ecm33 cell wall mutants in mice. Proteomics 8:2651–2664.

37. Martinez-Lopez, R., H. Park, C. L. Myers, C. Gil, and S. G. Filler. 2006. Candida albicans Ecm33p is important for normal cell wall architecture and interactions with host cells. Eukaryot. Cell 5:140–147.

38. McCleath, K. J., and G. W. Gooday. 1992. A rapid and sensitive microsieve assay for determination of chitinolytic activity. J. Microbiol. Methods 14:229–237.

39. McCleath, K. J., C. A. Specht, and P. W. Robbins. 1995. Molecular cloning and characterization of chitinase genes from Candida albicans. Proc. Natl. Acad. Sci. U. S. A. 92:5244–5248.

40. McKenzie, C. G., et al. 2010. Contribution of Candida albicans cell wall components to recognition by and escape from murine macrophages. Infect. Immun. 78:1630–1637.

41. Meiller, T. F., et al. 2009. A novel immune evasion strategy of Candida albicans: proteolytic cleavage of a salivary antimicrobial peptide. PLoS One 4e10539.

42. Monge, R. A., E. Roman, C. Nombela, and J. Pla. 2006. The MAP kinase signal transduction network in Candida albicans. Microbiology 152:905–912.

43. Morishima, H., T. Takita, T. Aoyagi, T. Takeuchi, and H. Umezawa. 1970. The structure of peptatin. J. Antibiot. (Tokyo) 23:621–623.

44. Mrsa, V., T. Seidl, M. Gentzsch, and W. Tanner. 2007. Yeast proteolytic cleavage of a salivary antimicrobial peptide. PLoS One 2:14102.

45. Murad, A. M., P. R. Lee, I. D. Broadhurst, C. J. Barell, and A. J. Brown. 2000. Clp10, an efficient and convenient integrating vector for Candida albicans. Yeast 16:325–337.

46. Naglik, J. R., S. J. Challacombe, and B. Hube. 2003. Candida albicans secreted aspartyl proteasines in virulence and pathogenesis. Microbiol. Mol. Biol. Rev. 67:400–428.

47. Naglik, J. R., et al. 2008. Quantitative expression of the Candida albicans secreted aspartyl protease gene family in human oral and vaginal candidiasis. Microbiology 154:236–3280.

48. Netea, M. G., G. D. Brown, B. J. Kulberg, and N. A. Gow. 2008. An integrated model of the recognition of Candida albicans by the innate immune system. Nat. Rev. Microbiol. 6:571–78.

49. Newport, G., and N. Agabian. 1997. KEX2 influences Candida albicans protease secretion and hyphal formation. J. Biol. Chem. 272:28954–28961.

50. Odds, F. C. 1988. Candida and candidosis. Baillière Tindall, London, United Kingdom.

51. Olsen, V., N. X. Cawley, J. Brandt, M. Egel-Mitani, and Y. P. Loh, 1999. Identification and characterization of Saccharomyces cerevisiae yps3, a new member of the ypsin family of aspartic proteases encoded by the YPS3 gene. Biochem. J. 339:2407–2411.

52. Olsen, V., et al. 1998. Cleavage efficiency of the novel aspartic protease ypsin 1 (Yap3p) enhanced for substrates with arginine residues flanking the P1 site: correlation with electoreactive active-site pockets predicted by molecular modeling. Biochemistry 37:2768–2777.

53. Olsen, V., et al. 2000. New member of the yapsin family of aspartic proteases encoded by the YPS3 gene. Biochem. J. 339:2407–2411.