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Saccharomyces cerevisiae Can Secrete Sapp1p Proteinase of *Candida parapsilosis* But Cannot Use It for Efficient Nitrogen Acquisition

Zuzana Vinterová[1], Václava Bauerová[1], Jiří Dostál[1], Hana Sychrová[2], Olga Hrušková-Heidingsfeldová[1*], and Iva Pichová[1*]

[1]*Institute of Organic Chemistry and Biochemistry Academy of Sciences of the Czech Republic, v.v.i., Gilead & IOCB Research Centre, Flemingovo náměstí 2, 166 10 Prague 6, Czech Republic*
[2]*Department of Membrane Transport, Institute of Physiology Academy of Sciences of the Czech Republic, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic*

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Secreted aspartic proteinase Sapp1p of *Candida parapsilosis* represents one of the factors contributing to the pathogenicity of the fungus. The proteinase is synthesized as an inactive pre-pro-enzyme, but only processed Sapp1p is secreted into extracellular space. We constructed a plasmid containing the *SAPP1* coding sequence under control of the *ScGAL1* promoter and used it for proteinase expression in a *Saccharomyces cerevisiae kex2Δ* mutant. Because Sapp1p maturation depends on cleavage by Kex2p proteinase, the *kex2Δ* mutant secreted only the pro-form of Sapp1p. Characterization of this secreted proteinase form revealed that the Sapp1p signal peptide consists of 23 amino acids. Additionally, we prepared a plasmid with the *SAPP1* coding sequence under control of its authentic *CpSAPP1* promoter, which contains two GATAA motifs. While in *C. parapsilosis SAPP1* expression is repressed by good low molecular weight nitrogen sources (e.g., ammonium ions), *S. cerevisiae* cells harboring this plasmid secreted a low concentration of active proteinase regardless of the type of nitrogen source used. Quantitative real-time PCR analysis of a set of genes related to nitrogen metabolism and uptake (*GAT1*, *GLN3*, *STP2*, *GAP1*, *OPT1*, and *PTR2*) obtained from *S. cerevisiae* cells transformed with either plasmid encoding *SAPP1* under control of its own promoter or empty vector and cultivated in media containing various nitrogen sources also suggested that *SAPP1* expression can be connected with the *S. cerevisiae* regulatory network. However, this regulation occurs in a different manner than in *C. parapsilosis*.

Keywords: *Candida parapsilosis*, *Saccharomyces cerevisiae*, secreted aspartic proteinase, *SAPP1*, nitrogen metabolism

*For correspondence. E-mail: iva.pichova@uochb.cas.cz; Tel.: +420-2201 83251; Fax: +420-220183556

Introduction

Pathogenic *Candida* spp. cause a wide spectrum of diseases in immunocompromised patients. *C. parapsilosis* is one of the leading causative agents of nosocomial yeast infections, and is frequently associated with invasive fungal infections in low-birth-weight infants (Trofa *et al*., 2008). In addition to *C. albicans*, *C. parapsilosis* is considered a model organism for investigation of molecular basis of virulence of pathogenic *Candida* spp. (reviewed in Nosek *et al*., 2009). Factors contributing to the virulence of *C. albicans* have been studied extensively, while relatively little is known about regulation of virulent factors in *C. parapsilosis*. However, it has been demonstrated that proteinases and lipases secreted by *C. parapsilosis* contribute to the damage of reconstituted human tissue during experimental infection (Gacser *et al*., 2007).

The well-studied *C. albicans* possesses 10 genes (*SAP1–SAP10*) encoding secreted aspartic proteinases (Sap1–Sap10). The Sap2 isoenzyme is abundantly secreted from the cells. According to the work published to date, the *C. parapsilosis* genome contains three genes (*SAPP1–SAPP3*) encoding secreted aspartic proteinases (Sapp1p–Sapp3p), and the major secreted isoenzyme is Sapp1p (De Viragh *et al*., 1993). The *SAPP2* gene appears to be transcribed under most conditions, but *SAPP1* transcription is induced by the presence of an exogenous protein as the sole source of nitrogen. Sapp2p is secreted in approximately ten-fold lower concentration than Sapp1p (Hrušková-Heidingsfeldová *et al*., 2009). The *SAPP3* gene product has not yet been characterized (the *SAPP3* NCBI accession number is AF339513). The sequence identity between Sapp1p and Sapp2p is 53%, and the sequence identity between all three *C. parapsilosis* isoenzymes is 44%. Sapp1p shares 47% amino acid sequence identity with Sap2 of *C. albicans*. The expression and enzymological properties of Sapp1p and Sapp2p have been described, and in addition, the crystal structure of Sapp1p has been analyzed (Dostál *et al*., 2005, 2009; Majer *et al*., 2006; Merkerová *et al*., 2006; Hrušková-Heidingsfeldová *et al*., 2009).

The *SAPP1* open reading frame is duplicated in *C. parapsilosis*. Deletion of all four *SAPP1* alleles confirmed the importance of Sapp1p in virulence and suggested that Sapp1p contributes to survival of *C. parapsilosis* inside macrophages. Complete deletion of *SAPP1* also revealed the existence of a Sapp protein-production sensing mechanism, which causes upregulation of the remaining *SAPP* genes (Horváth *et al*., 2012). The expression of *SAPs* of *C. albicans* is regulated differentially, depending on ambient pH, temperature, nutrient sources, type and stage of disease, and/or morphological status of the fungus (Naglik *et al*., 2004; Hrušková-Heidingsfeldová, 2008). For example, *SAP2* expression can

Table 1. Plasmids constructed for the expression of *SAPP1* in *S. cerevisiae* wt and/or *kex2Δ* strains

Plasmid	Promoter	Primers	Vector	Strain
pGAL-SAPP^{401}	*pGAL1*	*Cp*SAPP-F/-R	2μ, *URA3*	wt, *kex2Δ*
pSAPP-SAPP	*pSAPP1*	*Cp*PROM-F/-R	YEp352	wt

be induced by micromolar concentrations of amino acids, which may occur in the environment during the degradation of exogenous proteins that serve as a sole source of nitrogen (Martinez and Ljungdahl, 2005). *SAP2* induction likely depends on amino-acid sensing and signaling. Sensing of amino acids is mediated by the SPS sensor, which triggers proteolytic activation of the transcription factors Stp1p and Stp2p. Stp1p activates expression of *SAP2* and two genes encoding oligopeptide transporters, *OPT1* and *OPT3*. Thus, degradation of exogenous proteins and oligopeptide uptake are coregulated (Martinez and Ljungdahl, 2005). Expression of *STP1* is sensitive to nitrogen catabolite repression and is under the control of the general regulators Gln3p and Gat1p (Dabas and Morschhäuser, 2008), which recognize GATAA motifs in the promoter regions. GATAA motifs are conserved in fungi and regulate expression of genes required for utilization of secondary nitrogen sources (Magasanik and Kaiser, 2002).

Posttranslational processing represents an additional level of regulation of extracellular proteolysis. The *SAP* genes encode pre-pro-enzymes consisting of a signal sequence followed by a propeptide and a mature proteinase domain. The signal peptide is removed in the rough endoplasmic reticulum, and the zymogen is, after transport to the Golgi apparatus, activated either by Kex2p proteinases or alternative pathways (Newport and Agabian, 1997; Dostal *et al.*, 2005; Nombela *et al.*, 2006). Propeptides are considered to play a key role in correct maturation of aspartic proteinases (van den Hazel *et al.*, 1993; Fukuda *et al.*, 1994; Koelsch *et al.*, 1994; Beggah *et al.*, 2000). The mature proteinases are transported *via* the secretory pathway to the cell surface and are partially retained in the cell wall during secretion. Recently, we showed that the cell wall-associated Sapp1p is correctly folded and can cleave external substrates (Vinterová *et al.*, 2011).

S. cerevisiae does not produce secreted aspartic proteinases but possesses the yapsin family, which consists of five glycosylphosphatidylinositol-linked aspartic proteinases that are required for maintenance of cell wall integrity. Yapsins are localized at the plasma membrane and are likely involved in the activation and/or shedding of periplasmic proteins implicated in cell wall assembly (Gargnon-Arsenault *et al.*, 2006). Another aspartic proteinase (aspartic proteinase A) is present in vacuoles, and its activity is essential for degradation of proteins targeted to vacuoles during nutritional stress, sporulation, and vegetative growth (Parr *et al.*, 2007).

Here, we show that *S. cerevisiae* represents a good model organism for analysis of the processing of secreted aspartic proteinases of pathogenic *Candida* spp. The *S. cerevisiae* model allowed us to characterize the signal peptide of Sapp1p and to note that, in contrast to *C. parapsilosis*, *S. cerevisiae* cannot fully benefit from extracellular proteolysis.

Materials and Methods

Strains, media, and growth conditions

The following *S. cerevisiae* strains were used in this work: W303-derivative BW31a (MATa *leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 ena1Δ::HIS3::ena4Δ nha1Δ::LEU2*), which has a very high efficiency of transformation and homologous recombination (Kinclová-Zimmermannová *et al.*, 2005), and the *kex2Δ* mutant strain (MATa *his3Δ1 leu2Δ0 met 15Δ0 ura3Δ0 YNL238w::kanMX4*) provided by Euroscarf. The *C. parapsilosis* strain used was clinical isolate CP 69 obtained from the mycological collection of the Faculty of Medicine, Palacky University, Olomouc, Czech Republic.

The compositions of cultivation media were as follows: YNB, 0.67% yeast nitrogen base with ammonium sulfate and without amino acids (pH 5.4) supplemented with either 2% glucose or 2% galactose; YCB-BSA and YCB-Hb, 1.2% yeast carbon base (pH 3.5) supplemented with 0.2% bovine serum albumin or 0.2% hemoglobin, respectively; YCB-BSA-FR and YCB-Hb-FR, 1.2% yeast carbon base (pH 3.5) supplemented with a peptide mixture corresponding to 0.2% bovine serum albumin or hemoglobin cleaved with purified Sapp1p. Cleavage of protein substrates was monitored by SDS-PAGE and mass spectroscopy. The peptide mixture was separated from Sapp1p by centrifugation through a 30 kDa cut-off membrane and was sterilized by filtration (0.20 μm filter) before addition to 1.2% yeast carbon base medium, pH 3.5. If necessary, auxotrophic supplements were added to a final concentration of at least 15 μg/ml.

Escherichia coli XL-1 blue strain was used for DNA manipulation. Selection of transformants was performed on 2% LB medium supplemented with ampicillin (100 μg/ml). All yeast strains were cultivated at 30°C, the bacteria at 37°C.

Construction of plasmids

Plasmids prepared for expression of *SAPP1* in *Saccharomyces*

Table 2. Oligonucleotides used for amplification of the *SAPP1* gene. Underlined sequences are homologous to *SAPP1*.

Primer	Sequence (5'→3')
*Cp*PROM-F	CCATGATTACGAATTCGAGCTCGGTACCGGATCCTCTAGAGTCTACACACACACACACACGCAG
*Cp*PROM-R	CACGACGTTGTAAAACGACGGCCAGTGGCAAGCTTGCATGCATGCATTGCGTAATAGTTAGCG
*Cp*SAPP-F	TACCTCTATACTTTAACGTCAAGGAGAAAAAACTATAATGGACTCCATTTCGTTGTCGTTG
*Cp*SAPP-R	TACTGTTAATTGCTCCAGCACCAGCACCAGCACCTGCTCTTAAACGGCAGAAATGCTCG

cerevisiae are listed in Table 1. Plasmid pGAL-SAPP^{401}, containing the *ScGAL* promoter region and the proteinase gene *SAPP1* encoding the complete pre-pro-Sapp1p, was obtained by homologous recombination in both wild-type (wt) and *kex2Δ S. cerevisiae* cells. Plasmid pSAPP-SAPP, containing the full-length promoter region of *SAPP1* from *C. parapsilosis* and the complete *SAPP1* coding region, was obtained by homologous recombination in wt cells only. Both plasmids were prepared as follows: DNA fragments of the *SAPP1* gene were amplified using the primers listed in Table 2. *C. parapsilosis* genomic DNA was isolated according to Hoffman and Winston (1987). The complete *SAPP1* sequence including its own promoter was cloned into the multi-copy YEp352 vector (Hill *et al.*, 1986), resulting in plasmid pSAPP-SAPP. The DNA fragment SAPP^{401} was cloned in a centromeric *S. cerevisiae* vector containing the *URA3* marker gene and *ScGAL* promoter (a generous gift from Dr. Bruno Andre, Free University of Brussels, Belgium), resulting in plasmid pGAL-SAPP^{401}.

Isolation and purification of authentic Sapp1p

Isolation and purification of Sapp1p was performed as described by Dostál *et al.* (2009). Briefly, Sapp1p was isolated from the cell-free culture supernatants obtained from *C. parapsilosis* incubation in YCB-BSA medium, pH 3.5, for 72 h at 30°C. The cells were harvested by centrifugation (5,000×g for 15 min). The supernatant was filtered using Stericup (Millipore), and the cell-free supernatant containing secreted proteases was precipitated by addition of ammonium sulfate [final concentration 80% (w/v)] and centrifuged at 20,000×g for 10 min. The sediment was diluted in 15 mM sodium citrate, pH 3.75, and dialyzed against the same buffer in order to remove ammonium sulfate. Sapp1p was purified by anion-exchange chromatography using an FPLC equipped with a MonoS column (Amersham) equilibrated in 15 mM sodium citrate, pH 3.75. Elution was carried out using a NaCl gradient (0–300 mM). Samples containing active Sapp1p were dialyzed into 25 mM BIS-Tris, pH 6.3, and further purified using a MonoP HR 5/20 column (Amersham). Sapp1p elution was performed using a gradient of 10% (v/v) Polybuffer 74, pH 4.0 (Amersham). The efficiency of the purification steps was analyzed using SDS-PAGE and the proteinase activity assay described below.

Preparation of total cell protein extracts

The technique of total cell protein extraction (TCP) was based on a method described by Beggah *et al.* (2000). Briefly, cells from 10 ml of culture (OD_{550}~2) were harvested and resuspended in 1 ml of a solution consisting of 1.85 M sodium hydroxide and 1.2 M 2-mercaptoethanol. The suspension was incubated for 5 min on ice. Then, the same volume of 25% trichloroacetic acid (v/v) was added. The mixture was incubated on ice for an additional 5 min and then centrifuged (15,000×g, 5 min, 4°C). The resulting pellet was resuspended in a loading buffer for SDS electrophoresis and boiled for at least 10 min prior to SDS-PAGE.

Analysis of secreted proteins

For detection of proteins secreted from *S. cerevisiae* cells harboring plasmids with *C. parapsilosis* proteinase coding regions, culture supernatants were dialyzed against distilled water, frozen at -70°C overnight, and concentrated by lyophilization in a Beta 2-8 LD Plus freeze dryer. The lyophilized samples were dissolved in water and subjected to SDS-PAGE and Western blot analysis.

Western blotting

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane. For Sapp1p detection, the membrane was incubated with polyclonal rabbit antibodies raised against Sapp1p and then with peroxidase-labeled swine anti-rabbit immunoglobulins (Dostál *et al.*, 2005). The protein bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Proteinase activity assay

The Sapp1p activity in culture supernatants was examined using the fluorogenic substrate Dabcyl-Glu-His-Val-Lys-Leu-Val-Glu-EDANS in 0.1 M sodium citrate buffer, pH 3.5, as previously described (Merkerová *et al.*, 2006; Hrušková-Heidingsfeldová *et al.*, 2009). Analysis of the cleavage products was performed using an HPLC equipped with an Agilent C-18 column and a fluorescence detector set at an excitation wavelength of 350 nm and an emission wavelength of 480 nm. A linear methanol gradient was used for elution.

Controls containing the specific aspartic proteinase inhibitor pepstatin A were prepared similarly. Pepstatin was dissolved in dimethylsulfoxide and added to reaction mixtures to a final concentration of up to 1 μM.

N-terminal sequencing of proteins

Proteins separated by SDS-PAGE were transferred onto a poly (vinylidene difluoride) membrane (PVDF) by semi-dry blotting method. The membrane was stained with BioSafe Coomassie Blue (Bio-Rad). Bands of interest were excised, and the N-terminal protein sequence was determined by

Table 3. Primers of selected genes for expression study

Primer	Sequence (5′→3′)
ACT1-F	CAATGGATTCTGAGGTTGCTG
ACT1-R	GCTTCATCACCAACGTAGGAGTCT
GAT1-F	CCTAGCTTGCCCAATACTTCTGAG
GAT1-R	GGTTGCTGGTTGAAGTTGGATTAC
GAP1-F	GATGGTGTCTTGCCCCTGCTTAC
GAP1-R	TGCCCCTATTCTCCTTTGTGTCTG
STP2-F	TGCGAAACAGAACCATCCACAG
STP2-R	AAATAATTCGCTCACGTCAACCAG
OPT1-F	GCAACGAATCAAACAAGGACACTG
OPT1-R	GCCCAGGGAAGCTTTTACAACAT
PTR2-F	GCCCGCCCTACTGACATCC
PTR2-R	AAGGCCAATCAAAGAGCAAGTCAC
GLN3-F	CGAAGTAATGAAGAGCCGAGACA
GLN3-R	TGCCGCCGTTTAATCCACTG
SAPP1-F	GGTTTGTTGCCTTGGACTTTGATG
SAPP1-R	ACGGTTTGCTGCTGTTTGTTTGAA

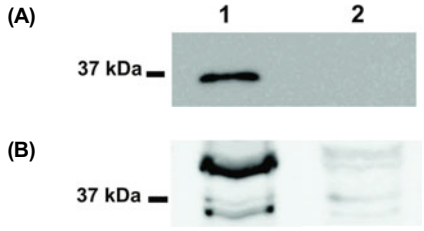

Fig. 1. Western blot analysis of Sapp1p secretion and processing in *S. cerevisiae* transformed with pGAL-SAPP^{401} (Lane 1) or empty plasmid (Lane 2). (A) culture supernatants after overnight incubation at 30°C in YNB supplemented with galactose; (B) total cell protein extracts (TCP) of cells grown in the same medium as above. Detection was performed using polyclonal antibodies against Sapp1p.

Edman degradation performed on an ABI Procise Sequencer (Applied Biosystems).

RNA isolation and cDNA synthesis

RNA from *S. cerevisiae* was isolated using the YeaStar RNA kit from Zymo Research. RNA was treated with DNAase I to remove contaminating DNA. cDNA was prepared from 1 µg of isolated RNA by reverse transcription using SuperScript II reverse transcriptase and oligo(dT)$_{12\text{-}18}$ primer (both from Invitrogen), according to the manufacturer's protocol.

Quantitative real-time PCR analysis and data processing

Primers for PCR analysis were designed with the DNASTAR program (DNASTAR, Inc.) and were checked with BLAST (http://blast.ncbi.nlm.nih.gov). Selected primers are listed in Table 3. Real-time PCR was performed using the DyNAmo™ SYBR® Green 2-Step qRT-PCR Kit (Finnzymes). Each reaction was set up in duplicate with 1 µl of cDNA in a total reaction volume of 20 µl. Both the composition of the reaction and the cycling parameters were set up as recommended by the manufacturer. Levels of mRNA expression were measured on a LightCycler 480 (Roche Applied Science), and acquired expression profiles were analyzed with GenEx software (MultiD Analyses). Data were normalized to the reference gene actin (*ACT1*), which was selected using NormFinder (MultiD Analyses), and expressed as a fold change ($2^{-\Delta\Delta Ct}$) relative to lowest expression. Statistical analysis of the data was performed using the GenEx program (MultiD Analyses), and P values were determined by 2-tailed t-test. Changes in expression with P values lower than 0.05 were considered significant.

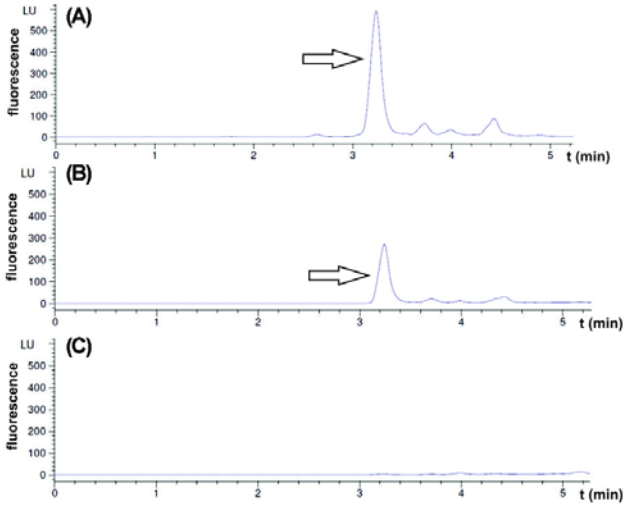

Fig. 2. HPLC chromatograms of samples from cleavage of the Dabcyl-Glu-His-Val-Lys-Leu-Val-Glu-EDANS substrate by Sapp1p secreted from (A) *C. parapsilosis*, (B) *S. cerevisiae*, (C) *S. cerevisiae* supplemented with pepstatin A to a final concentration of 1 µM. Both *C. parapsilosis* and *S. cerevisiae* cells were cultivated in YCB-BSA for 48 h at 30°C. The cell culture was centrifuged, and only the cell-free culture supernatant was used for the proteinase activity assay. The arrows indicate the peak characteristic of the Sapp1p substrate cleavage product.

Results

Mature Sapp1p is secreted from *S. cerevisiae* cells transformed with a plasmid containing the *SAPP1* sequence and *GAL1* promoter

To examine whether Sapp1p is readily secreted from *S. cerevisiae* cells and verify that this model organism could be used for analysis of Sapp1p processing and trafficking, we placed the *SAPP1* gene encoding the pre-pro-proteinase form of Sapp1p under the control of the *ScGAL1* promoter and prepared the plasmid pGAL-SAPP^{401}. Proteinase expression was induced in YNB medium containing 2% galactose (the inoculum was cultivated in YNB with glucose overnight). As controls, we analyzed cells harboring pGAL-SAPP^{401} cultivated only in glucose and cells transformed with the control vector lacking the proteinase coding sequence and cultivated in galactose. We monitored the production of proteinase by Western blot analysis of the culture supernatant and of the total cell protein extracts using polyclonal antibodies against Sapp1p. As illustrated in Fig. 1A, the cells harboring pGAL-SAPP^{401} secreted proteinase with a molecular weight of approximately 36 kDa, which is similar to that of mature Sapp1p. The N-terminal sequence of the released protein (DSISL-) corresponded to that of correctly processed mature Sapp1p. Using a Sapp1p-specific fluorescent substrate, we detected Sapp1p activity in the culture supernatant. Moreover, the activity was blocked by the specific inhibitor pepstatin A (Fig. 2), which confirmed that *S. cerevisiae* secreted correctly folded Sapp1p. A rela-

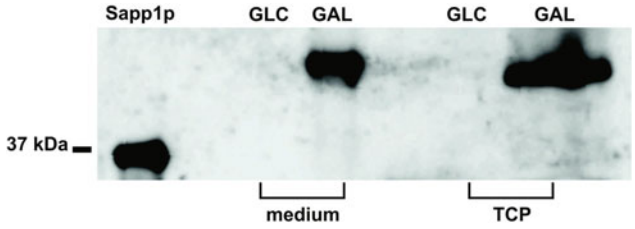

Fig. 3. Western blot analysis of Sapp1p secretion and processing in the *kex2Δ S. cerevisiae* mutant strain harboring plasmid pGAL-SAPP^{401} grown in YNB supplemented with glucose (GLC) or galactose (GAL). Detection of Sapp1p in culture supernatant and in TCP was performed using polyclonal antibodies against Sapp1p.

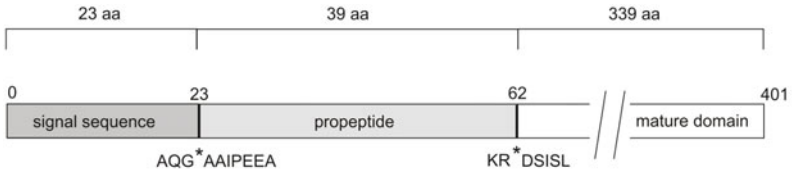

Fig. 4. Schematic representation of pre-pro-Sapp1p. The lengths of the pre- and propeptide as well as the mature domain are marked, and the cleavage sites are indicated by asterisks.

tively high concentration of unprocessed and mature proteinase was, however, retained in the cells (Fig. 1B), which could explain the low concentration of Sapp1p observed in the culture supernatant (about 0.02 mg/L in contrast to approximately 3 mg/L, the concentration of Sapp1p secreted by *C. parapsilosis* cells).

The Sapp1p signal peptide consists of 23 amino acids

The exact sequence of the propeptide of Sapp1p has not yet been determined. To investigate the signal peptide cleavage site, we transformed *kex2Δ S. cerevisiae* cells with the pGAL-SAPP^{401} plasmid and cultivated the yeast cells under conditions similar to those used for the wt strain. In the presence of galactose, we detected production of a roughly 45 kDa protein both in TCP and in the culture supernatant (Fig. 3). N-terminal sequence analysis of the secreted protein revealed the sequence AAIPEEAAKRDD-, and we identified this protein as Sapp1p flanked by 39 amino acids at the N-terminus (Fig. 4). We labeled this species Sapp1p^{+39}. Because the Kex2p proteinase, which cleaves the bond between propeptides and mature proteinases in *C. albicans* (Naglik *et al.*, 2004), was absent, Sapp1p^{+39} most likely resulted from cleavage of pre-pro-Sapp1p by a signal peptidase in endoplasmic reticulum. We used the SignalP 4.0 program to predict the sequence of the signal peptide (Petersen *et al.*, 2011; www.cbs.dtu.dk/services/SignalP/) and compared the output with the Sapp1p^{+39} N-terminal sequence. One of the two most probable sequences agreed with the experimentally detected N-terminus of Sapp1p^{+39}. These results indicate that the Sapp1p signal peptide consists of 23 residues and that the cleavage site between pre- and propeptide is located between Gly^{23} and Ala^{24} (Fig. 4). The N-terminal extension did not preclude the release of Sapp1p^{+39} (i.e., pro-Sapp1p) into the extracellular space. Importantly, we confirmed that pro-peptide cleavage and complete proteinase maturation inside the cells are not essential for successful secretion of Sapp1p.

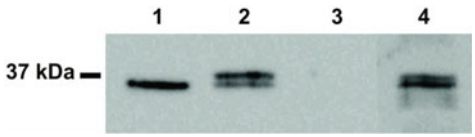

Fig. 5. Western blot analysis of Sapp1p forms present in culture supernatants from *S. cerevisiae* cells transformed with a vector bearing the full-length *SAPP1* with its own promoter (pSAPP-SAPP). Lanes: 1, cultivation in YNB; 2, cultivation in YCB-BSA; 3, cultivation in YCB; 4, cultivation in YCB-Hb. All cultivations were performed at 30°C. Cells in YNB were cultivated overnight, and cells in YCB and supplemented YCB were cultivated for 48 h. Detection was performed using antibodies against Sapp1p.

SAPP1 expression in *S. cerevisiae* can be driven by its own promoter

The type of nitrogen source plays an important role in regulation of *SAP* gene expression not only in *C. albicans* but also in other *Candida* species, including *C. parapsilosis* and *C. dubliniensis* (Hrušková-Heidingsfeldová *et al.*, 2009; Loaiza-Loeza *et al.*, 2009). We hypothesized that GATA transcription factors, which regulate transcription of *SAP2* in *C. albicans*, are also involved in regulation of Sapp1p secretion in *C. parapsilosis*. The *SAPP1* promoter region contains two GATAA motifs at positions -161 to -165 and -236 to -240 relative to the *SAPP1* start codon. *C. parapsilosis* does not produce Sapp1p in the presence of low molecular weight nitrogen sources (e.g., ammonium ions). The *SAPP1* gene is transcribed and the proteinase Sapp1p is secreted only in the presence of protein as a sole source of nitrogen in the

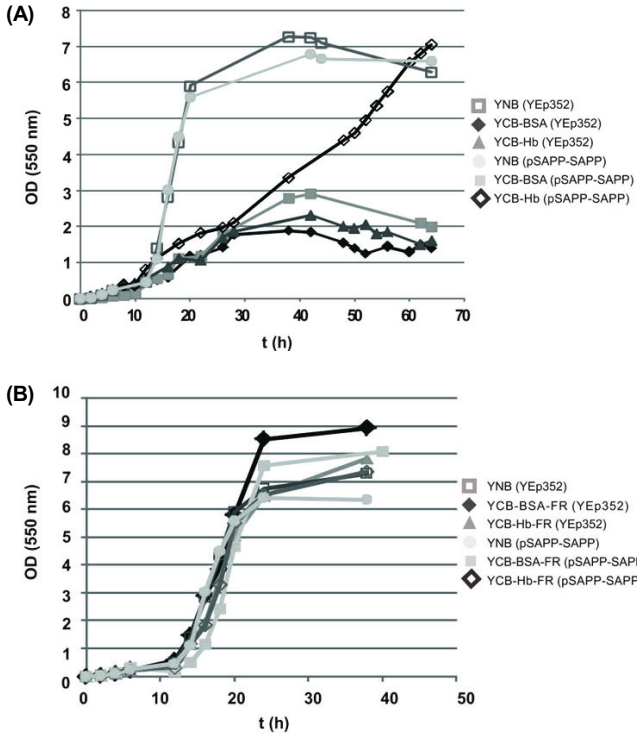

Fig. 6. Growth curves of *S. cerevisiae* cells transformed with pSAPP-SAPP and empty plasmid YEp352 cultivated under various nutrient conditions at 30°C. (A) YNB, 0.67% yeast nitrogen base supplemented with 2% glucose; YCB-BSA, 1.2% yeast carbon base supplemented with 0.2% BSA; YCB-Hb, 1.2% yeast carbon base supplemented with 0.2% hemoglobin. (B) YCB-BSA-FR, 1.2% yeast carbon base supplemented with peptide mixture in a concentration equivalent to 0.2% BSA pre-cleaved with Sapp1p; YCB-Hb-FR, 1.2% yeast carbon base supplemented with peptide mixture in a concentration equivalent to 0.2% Hb pre-cleaved with Sapp1p (see Materials and Methods for preparation of peptide mixtures).

medium. In order to investigate whether *S. cerevisiae* cells could express the *SAPP1* gene driven by its inducible promoter, we replaced the *ScGAL* promoter region with the full-length promoter of *SAPP1* from *C. parapsilosis* in a YEp352 multi-copy vector containing *SAPP1* encoding pre-pro-Sapp1p. This new plasmid, pSAPP-SAPP, was transformed into *S. cerevisiae* cells, and *SAPP1* expression was tested in the presence of BSA as the only source of nitrogen and in the presence of ammonium ions (YNB), which repress *SAPP1* expression in *C. parapsilosis*. *S. cerevisiae* cells transformed with pSAPP-SAPP were cultivated in YCB containing 0.2% BSA, YNB with 0.5% ammonium sulfate, and YCB without any additional nitrogen source. As a control of proteinase production, we cultivated *S. cerevisiae* cells transformed with empty YEp352 vector under the same conditions. While cultivations in YCB-BSA yielded a mixture of mature and partially processed Sapp1p, cultivations in YNB with ammonium ions yielded only fully processed active Sapp1p (Fig. 5). In YCB without an additional nitrogen source, we did not detect any Sapp1p secretion. These results show that the authentic promoter of Sapp1p functions in *S. cerevisiae*, albeit in a different manner than in *C. parapsilosis*. In addition to responding to a proteinaceous nitrogen source, the promoter also responded to ammonium ions in supplemented YNB media.

As illustrated in Fig. 6A, the culture grew well only in YNB with ammonium ions. The growth curve in YCB-BSA medium did not differ between control cells and cells secreting Sapp1p, and the cell growth was substantially lower than in YNB. Monitoring of pH in all cultivations, including cultivations of *S. cerevisiae* cells transformed with empty plasmid, confirmed that secretion of Sapp1p did not alter the pH of cultivation media (the pH of each culture medium did not exceed 5 during cultivation; Sapp1p is able to cleave a substrate up to pH 6). We further tested different proteinaceous sources of nitrogen for induction of Sapp1p secretion in *S. cerevisiae* cells. We performed cultivation of *S. cerevisiae* cells transformed with pSAPP-SAPP in YCB supplemented with 0.2% hemoglobin (YCB-Hb). Although cells harboring pSAPP-SAPP grew better in YCB-Hb than in YCB-BSA, the cell growth was still significantly slower than in YNB with ammonium ions (Fig. 6A), and both proteinase precursor and mature Sapp1p were found in the culture supernatant (Fig. 5).

To elucidate whether the lack of protein utilization was caused by insufficient expression of Sapp1p or by inefficient uptake of protein cleavage products by *S. cerevisiae*, we cultivated cells harboring pSAPP-SAPP in YCB medium supplemented with a peptide mixture resulting from fractionation of BSA and hemoglobin by purified Sapp1p (YCB-BSA-FR and YCB-Hb-FR, respectively). Addition of pre-cleaved proteins to the medium improved the growth of *S. cerevisiae* cells harboring pSAPP-SAPP; OD_{550} values were comparable to those obtained in YNB medium containing ammonium ions (Fig. 6B). Western blot analysis of YCB-BSA-FR and YCB-Hb-FR culture supernatants confirmed that only fully processed Sapp1p was secreted into the medium (data not shown). Together, these data show that the lack of protein utilization by *S. cerevisiae* cells producing Sapp1p is likely a result of inefficient proteinase secretion.

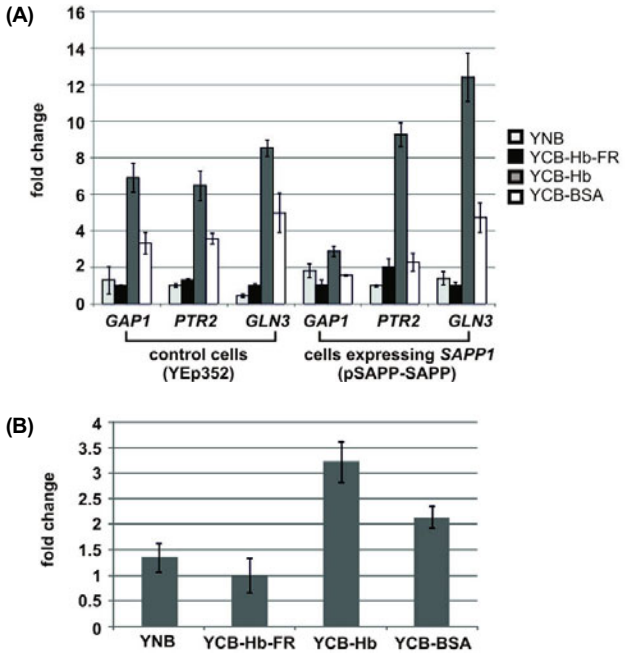

Fig. 7. Real-time PCR analyses. (A) gene expression fold change of *GAP1*, *PTR2*, and *GLN3* in *S. cerevisiae* cells transformed with empty YEp352 vector (control cells) and cells transformed with pSAPP-SAPP and expressing *SAPP1*. The cells were grown in YNB, YCB-Hb-FR, YCB-Hb, or YCB-BSA for 16 h at 30°C. (B) *SAPP1* expression fold change in *S. cerevisiae* transformed with pSAPP-SAPP and grown in YNB, YCB-Hb-FR, YCB-HB, or YCB-BSA for 16 h at 30°C.

Transcription analysis of genes associated with nitrogen utilization in *S. cerevisiae* expressing *SAPP1*

As described above, we observed different patterns of Sapp1p bands on Western blots when cells harboring pSAPP-SAPP were cultivated in media with different nitrogen sources (Fig. 5) and noted distinct culture growth of cells expressing *SAPP1* in various media (Fig. 6). Therefore, we decided to investigate the potential effect of nitrogen sources on the expression of genes related to nitrogen metabolism. We set out to examine whether the secretion of Sapp1p from *S. cerevisiae* cells harboring pSAPP-SAPP cultivated under various conditions (BSA, Hb, a peptide mixture originating from fractionated Hb, or ammonium sulfate as nitrogen sources) affects the expression profiles of genes encoding amino acid permease (*GAP1*), transcription factors (*GAT1*, *GLN3*, and *STP2*), or transporters (*OPT1*, an oligopeptide transporter, and *PTR2*, a di/tripeptide transporter). We selected *ACT1* as a reference gene because its expression profile was the most stable of five genes tested (*RPP2*, *PGK1*, *18S*, *IPP1*, and *ACT1*). We analyzed RNA from cells that were cultivated for 16 h in YCB-BSA, YNB with ammonium sulfate, YCB-Hb, or YCB-Hb-FR. As a control, we used RNA isolated from BW31a cells transformed with the empty YEp352 vector and cultivated under the same conditions as cells harboring the pSAPP-SAPP plasmid.

Expression of genes related to nitrogen metabolism revealed that cells responded to fractionated hemoglobin (Hb-FR) and ammonium ions as good sources of nitrogen, whereas BSA

and hemoglobin were poor nitrogen sources. Figure 7A shows the qRT PCR analysis of those genes whose expression levels were differently regulated in cells harboring pSAPP-SAPP and cells harboring empty plasmid (i.e., genes *GAP1*, *OPT1*, and *PTR2*). Interestingly, relative expression of *GAP1* was upregulated in the control cells grown in proteinaceous sources of nitrogen, whereas cells expressing *SAPP1* showed similar levels of *GAP1* fold change when grown in either poor or good nitrogen sources (Fig. 7A). *PTR2* and *GLN3* transcription displayed similar patterns both in control cells and in cells harboring *SAPP1*. This effect was more pronounced in the cells harboring *SAPP1*. Differences in transcription of *GAT1*, *STP1*, and *OPT1* in the control cells vs cells harboring *SAPP1* were less significant (data not shown). While *GAT1* regulation did not change at all, *STP2* and *OPT1* were upregulated in both protein-containing media. *STP2* fold change in the presence of proteinaceous nitrogen sources was similar for both control cells and cells expressing *SAPP1*, and *OPT1* levels were upregulated in control cells.

Relative expression levels of *SAPP1* showed a similar pattern as those of the other genes tested (Fig. 7B). The lowest expression levels were observed when cells were grown in YNB and YCB-Hb-FR. These levels were comparable to each other and at least two-fold lower than those observed for cells grown in YCB-BSA and YCB-Hb. The expression level in YCB-Hb was about 1.5-fold higher than that in YCB-BSA and about three-fold higher than those in YNB and YCB-Hb-FR. These data correlate well with the results of our other experiments, suggesting that there might be a difference in regulation of *SAPP1* expression depending on whether the gene is under control of the galactose promoter or the authentic inducible *SAPP1* promoter.

Discussion

Several medically important *Candida* species, including *C. parapsilosis*, produce extracellular aspartic proteinases with broad substrate specificity. Although *S. cerevisiae* expresses aspartic proteinases, these proteinases are not secreted from the cells, they are either localized in vacuoles or attached to the cell wall or plasma membrane by a GPI-anchor, and their mode of action is mostly processive (Gagnon-Arsenault *et al.*, 2006). Thus, *S. cerevisiae* does not produce any aspartic proteinase that might interfere with the analysis of Sapp1p secretion and seems to be an ideal model organism for analysis of trafficking and processing of individual, heterologously expressed *C. parapsilosis* proteinases. Indeed, we detected mature Sapp1p secreted from *S. cerevisiae* cells transformed with a yeast expression vector containing the *SAPP1* gene sequence under control of the *ScGAL1* promoter. However, the concentration of Sapp1p secreted by *S. cerevisiae* was significantly lower than that secreted by *C. parapsilosis.* The decreased secretion might be caused by inefficient processing of Sapp1p precursor and transport of proteinase within the cells and into the extracellular space. This speculation was supported by the observation of both unprocessed (approximately 45 kDa) and mature Sapp1p in the total cell protein extracts.

Expression of the pre-pro form of Sapp1p driven by the *ScGAL1* promoter in a *kex2Δ S. cerevisiae* strain enabled us to identify the cleavage site between the signal peptide and Sapp1p zymogen (Gly^{23}-Ala^{24}) and confirmed that Kex2p proteinase is necessary for correct propeptide processing. These experiments further showed that the N-terminal extension does not influence proteinase secretion into the extracellular space. In our previous work, we found that part of the N-terminal domain of Sapp1p likely remains buried in the cell wall, while the C-terminal domain and the active site are most likely exposed on the cell surface during temporary retention of Sapp1p in the cell wall before final proteinase release into the extracellular space (Vinterová *et al.*, 2011).

Transcription of *SAPP1* in *C. parapsilosis* can be induced merely by the presence of an exogenous protein (e.g., BSA or hemoglobin) that serves as the sole source of nitrogen (Hrušková-Heidingsfeldová *et al.*, 2009). A similar type of regulation has been thoroughly characterized for *C. albicans SAP2*, which can also be induced by micromolar concentrations of amino acids likely formed from degradation of exogenous proteins (Martinez and Ljungdahl, 2005). In this work, we showed that *SAPP1* can be transcribed in *S. cerevisiae* cells in the presence of good low molecular weight nitrogen sources such as ammonium ions or peptide fragments. We also found that expression of the *C. parapsilosis* proteinase Sapp1p under control of its own promoter did not render *S. cerevisiae* able to utilize exogenous BSA. The lower concentration of secreted proteinase obviously was not caused by a low level of *SAPP1* transcription but rather by inefficient processing and secretion of Sapp1p. In addition, coordination of proteinase secretion and peptide uptake was probably insufficient when BSA served as a sole source of nitrogen. Using hemoglobin instead of BSA resulted in a better culture growth of the cells harboring *SAPP1*; however, both YCB-BSA and YCB-Hb cultivations yielded a mixture of partially unprocessed and mature Sapp1p in the culture supernatant. Unfortunately, we were unable to confirm the identity of the higher molecular weight band using N-terminal sequencing. The proteinase concentration in these samples was rather low (approximately 0.02 mg/L), and the analysis was complicated by the presence of impurities. However, this two-band pattern strongly resembles mature recombinant Sapp1p accompanied by the intermediate of pro-Sapp1p (flanked by 29 amino acids at the N-terminus, $Sapp1p^{+29}$) cleavage, which we observed in our previous work (Dostál *et al.*, 2005).

The distinct behaviors of cultures incubated in the presence of BSA and Hb were supported by qRT PCR analysis of genes related to utilization of alternative nitrogen sources. Martinez and Ljungdahl (2005) showed that the *SAP2* gene in *C. albicans* is co-regulated with genes encoding the oligopeptide transporters *OPT1* and *PTR2*, but not the gene encoding general amino acid permease, *GAP1*. Our results correspond to this observation in that *GAP1* transcription is decreased in cells expressing *SAPP1*. In our experimental setting, however, *OPT1* was not upregulated in *SAPP1*-expressing cells. This is very likely due to the lack of *STP1* upregulation, which, in turn, might be caused by an insufficient concentration of amino acids in culture media. Despite the *SAPP1* upregulation in YCB-Hb, the Sapp1p secretion and subsequent hemoglobin cleavage may not yield a sufficient

amount of amino acids to increase *STP1* transcription in comparison with the control. On the other hand, *PTR2* was upregulated in *SAPP1*-expressing cells, although its induction also depends on amino acids present in the cultivation medium (Xia *et al*., 2008). However, *PTR2* is regulated *via* the transcription repressor Cup9p, and not *via* an SPS sensor such as *OPT1*. Transcription analysis of the genes encoding two general regulators, *GAT1* and *GLN3*, indicates that *SAPP1* regulation in *S. cerevisiae* may be connected with *GLN3* rather than *GAT1*. However, it should be stressed that the real-time PCR analysis was performed only for one time point and does not indicate effects that might have occurred in a time-dependent manner. Nevertheless, analysis of the *SAPP1*-expressing *S. cerevisiae* cells showed that the *S. cerevisiae* regulatory network is not tuned for the presence of *SAPP1*. Sapp1p expression in *S. cerevisiae* can be driven by the authentic *C. parapsilosis* promoter and can be induced by hemoglobin or BSA as a sole source of nitrogen, but this process is not as efficient and clear-cut as in *C. parapsilosis*. The *SAPP1* authentic promoter also positively responded to low molecular weight nitrogen sources, despite the fact that expression of *SAPP1* is repressed under these conditions in *C. parapsilosis*. Our preliminary experiments with the *SAPP3* gene, which also possesses a GATA-motif in its promoter region, showed that this gene is expressed both in the presence of protein in the medium as well as in the presence of a good low molecular weight nitrogen source, conditions under which a promoter inducible by GATA-transcription factors should be repressed (unpublished data). This suggests specific roles for *SAPP* promoters and growth conditions in the function of *C. parapsilosis* cells.

S. cerevisiae is widely used as a model organism, and many hypotheses and paradigms can be inferred from studies of this species. Here, we demonstrated that *S. cerevisiae* could transcribe, translate, and secrete an extracellular proteinase of *C. parapsilosis* even when it was placed under the control of its own promoter. Nevertheless, for several reasons, *S. cerevisiae* was not able to fully benefit from extracellular proteolysis.

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References

Beggah, S., Lechenne, B., Reichard, U., Foundling, S., and Monod, M. 2000. Intra- and intermolecular events direct the propeptide-mediated maturation of the *Candida albicans* secreted aspartic proteinase Sap1p. *Microbiology* **146**, 2765–2773.

Dabas, N. and Morschauser, J. 2008 A transcription factor regulatory cascade controls secreted aspartic protease expression in *Candida albicans*. *Mol. Microbiol*. **69**, 586–602.

de Viragh, P.A., Sanglard, D., Togni, G., Falchetto, R., and Monod, M. 1993. Cloning and sequencing of two *Candida parapsilosis* genes encoding acid proteases. *J. Gen. Microbiol.* **139**, 335–342.

Dostál, J., Brynda, J., Hrušková-Heidingsfeldová, O., Sieglová, I., Pichová, I., and Řezáčová, P. 2009. The crystal structure of the secreted aspartic protease 1 from *Candida parapsilosis* in complex with pepstatin A. *J. Struct. Biol.* **167**, 145–152.

Dostál, J., Dlouhá, H., Maloň, P., Pichová, I., and Hrušková-Heidingsfeldová, O. 2005. The precursor of secreted aspartic proteinase Sapp1p from *Candida parapsilosis* can be activated both autocatalytically and by a membrane-bound processing proteinase. *Biol. Chem.* **386**, 791–799.

Fukuda, R., Horiuchi, H., Ohta, A., and Takagi, M. 1994. The prosequence of *Rhisopus niveus* aspartic proteinase-I supports correct folding and secretion of its mature part in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **269**, 9556–9561.

Gacser, A., Schafer, W., Nosanchuk, J.S., Salomon, S., and Nosanchuk, J.D. 2007. Virulence of *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis* in reconstituted human tissue models. *Fungal Genet. Biol.* **44**, 1336–1341.

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

Hill, J.E., Myers, A.M., Koerner, T.J., and Tzagoloff, A. 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* **2**, 163–167.

Hoffman, C.S. and Winston, F. 1987 A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**, 267–272.

Horváth, P., Nosanchuk, J.D., Hamari, Z., Vágvölgyi, C., and Gácser, A. 2012. The identificationof gene duplication and the role of secreted aspartyl proteinase 1 in *Candida parapsilosis* virulence. *J. Infect. Dis*. **205**, 923–933.

Hrušková-Heidingsfeldová, O. 2008. Secreted proteins of *Candida albicans*. *Front. Biosci.* **13**, 7227–7242.

Hrušková-Heidingsfeldová, O., Dostál, J., Havlíková, J., Hradilek, M., and Pichová, I. 2009. Two aspartic proteinases secreted by the pathogenic yeast *Candida parapsilosis* differ in the expression pattern and catalytic properties. *Biol. Chem.* **390**, 259–268.

Kinclová-Zimmermannová, O., Zavřel, M., and Sychrová, H. 2005. Identification of conserved prolyl residue important for transport activity and the substrate specificity range of yeast plasma membrane Na^+/H^+ antiporters. *J. Biol. Chem.* **280**, 30638–30647.

Koelsch, G., Mareš, M., Metcalf, P., and Fusek, M. 1994. Multiple functions of pro-parts of aspartic proteinase zymogens. *FEMS Lett*. **343**, 6–10.

Loaiza-Loeza, S., Parra-Ortega, B., Cancino-Diaz, J.C., Illades-Aguiar, B., Hernandez-Rodriguez, C.H., and Villa-Tanaca, L. 2009. Differential expression of *Candida dubliniensis*-secreted aspartyl proteinase genes (*CdSAP1-4*) under different physiological conditions and during infection of a keratinocyte culture. *FEMS Immunol. Med. Microbiol*. **56**, 212–222.

Magasanik, B. and Kaiser, C.A. 2002. Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene* **290**, 1–18.

Majer, F., Pavlíčková, L., Majer, P., Hradilek, M., Dolejší, E., Hrušková-Heidingsfeldová, O., and Pichová, I. 2006. Structure-based specificity mapping of secreted aspartic proteases of *Candida parapsilosis*, *Candida albicans*, and *Candida tropicalis* using peptidomimetic inhibitors and homology modeling. *Biol. Chem.* **387**, 1247–1254.

Martinez, P. and Ljungdahl, P.O. 2005. Divergence of Stp1 and Stp2 transcription factors in *Candida albicans* places virulence factors required for proper nutrient acquisition under amino acid control. *Mol. Cell. Biol.* **25**, 9435–9446.

Merkerová, M., Dostál, J., Hradilek, M., Pichová, I., and Hrušková-Heidingsfeldová, O. 2006. Cloning and characterization of Sapp2p,

the second aspartic proteinase isoenzyme from *Candida parapsilosis*. *FEMS Yeast Res.* **6**, 1018–1026.

Naglik, J., Albrecht, A., Bader, O., and Hube, B. 2004. *Candida albicans* proteinases and host/pathogen interactions. *Cell Microbiol.* **6**, 915–926.

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

Nombela, C., Gil, C., and Chaffin, W.L. 2006. Non-conventional protein secretion in yeast. *Trends Microbiol.* **14**, 15–21.

Nosek, J., Holesova, Z., Kosa, P., Gacser, A., and Tomaska, L. 2009. Biology and genetics of the pathogenic yeast *Candida parapsilosis*. *Curr. Genet.* **55**, 497–509.

Parr, C.L., Keates, R.A.B., Bryksa, B.C., Ogawa, M., and Yada, R.Y. 2007. The structure and function of *Saccharomyces cerevisiae* proteinase A. *Yeast* **24**, 467–480.

Petersen, T.N., Brunak, S., von Heijne, G., and Nielsen, H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* **8**, 785–786.

Trofa, D., Gacser, A., and Nosanchuk, J.D. 2008. *Candida parapsilosis*, an emerging fungal pathogen. *Clin. Microbiol. Rev.* **21**, 606–625.

van den Hazel, H.B., Kielland-Brandt, M.C., and Winther, J.R. 1993. The propeptide is required for *in vivo* formation of stable active yeast proteinase A and can function even when not covalently linked to the mature region. *J. Biol. Chem.* **268**, 18002–18007.

Vinterová, Z., Šanda, M., Dostál, J., Hrušková-Heidingsfeldová, O., and Pichová, I. 2011. Evidence for the presence of proteolytically active secreted aspartic proteinase 1 of *Candida parapsilosis* in the cell wall. *Protein Sci.* **20**, 2004–2012.

Xia, Z., Turner, G.C., Hwang, C.S., Byrd, C., and Vashavsky, A. 2008. Amino acids induce peptide uptake via accelerated degradation of *CUP9*, the transcriptional repressor of the *PTR2* peptide transporter. *J. Biol. Chem.* **283**, 28958–28968.