Acid proteinase secreted by *Candida tropicalis*: functional analysis of preproregion cleavages in *C. tropicalis* and *Saccharomyces cerevisiae*

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The 40 kDa secreted aspartyl proteinase (Sapt1) of *Candida tropicalis* is a pepsin-like enzyme encoded by the *SAPT1* gene. According to the deduced amino acid sequence, Sapt1 has a putative preproregion of 60 amino acids preceding the mature enzyme. Maturation and processing of Sapt1 was analysed in *C. tropicalis* and *Saccharomyces cerevisiae* strains expressing wild-type or mutated forms of *SAPT1*. In *S. cerevisiae*, the glycosylated 46 kDa proenzyme was converted to the mature 40 kDa form of Sapt1 by *KEX2*-dependent proteolytic cleavage following the Lys⁵⁹-Arg⁶⁰ sequence. The replacement of Lys⁵⁹-Arg⁶⁰ by Lys⁵⁹-Gly⁶⁰ revealed that the precursor can be processed by an autocatalytic cleavage. This alternative processing pathway to produce mature Sapt1 is less efficient than the Kex2-mediated pathway. Finally, it was shown that in *C. tropicalis* and *S. cerevisiae* the removal of the proregion was a prerequisite for the secretion of Sapt1.

Keywords: *Candida tropicalis*, *Saccharomyces cerevisiae*, secreted aspartyl proteinase, Kex2 endopeptidase, secretion

INTRODUCTION

The yeasts of the genus *Candida* are commensals of the gastrointestinal and genitourinary tracts capable of causing severe candidiasis in immunocompromised patients (Horn *et al.*, 1985; Kwon-Chung & Bennett, 1992; Wingard *et al.*, 1979). Medically important species of *Candida* produce one dominant secreted aspartic proteinase (Sap) *in vitro* when cultured in media containing protein as the sole nitrogen source (for a review see Ogrydziak, 1993). Sap2, Sapt1 and Sapp1 refer to the proteinases secreted by *C. albicans*, *C. tropicalis* and *C. parapsilosis*, respectively (Monod *et al.*, 1994). These well-characterized enzymes have been suggested to be potential virulence factors of these yeasts (Odds, 1988).

Recently, *C. albicans* has been shown to secrete other Saps. The *C. albicans* WO-1 switching strain can secrete Sap2 and two additional Saps in the opaque form (White *et al.*, 1993), and seven genes (*SAP*) encoding distinct aspartic proteinases of *C. albicans* have been cloned and sequenced (Hube *et al.*, 1991; Miyasaki *et al.*, 1994; Monod *et al.*, 1994; Morrow *et al.*, 1992; White *et al.*, 1993; Wright *et al.*, 1992). During *in vitro* serum-induced germ tube

formation *C. albicans* produces mRNAs that hybridize with the genes *SAP4*, *SAP5* and *SAP6* (Hube *et al.*, 1994). The existence of *SAP* multigene families in *C. tropicalis* and *C. parapsilosis* has also been demonstrated (Monod *et al.*, 1994). However, only one gene of *C. tropicalis* (*SAPT1*; Togni *et al.*, 1991) and two of *C. parapsilosis* (*SAPP1* and *SAPP2*; de Viragh *et al.*, 1993) have been cloned and sequenced to date.

A comparison of the N-terminal amino acid sequences of six Saps (Sap1, Sap2, Sap3, Sapp1, Sapp2 and Sapt1) determined by Edman degradation, together with the amino acid sequences deduced from the corresponding *SAP* genes, indicates that these enzymes are translated as a preproenzyme, approximately 60 amino acids larger than the mature form. All the precursors show similar processing sites. The 60 amino acid prepropeptide has a signal sequence of 14–21 amino acids with one to four putative signal peptidase cleavage sites (Von Heijne, 1986) and two Lys-Arg sequences, one of which is immediately before the N-terminus of the mature form of the enzyme. The Arg-Lys sequences are known to be cleaved by the Kex2 proteinase in *Saccharomyces cerevisiae* (Julius *et al.*, 1984).

In this paper we report studies on the secretion of Sapt1 by *C. tropicalis* and *S. cerevisiae* strains carrying the wild-

Abbreviations: ER, endoplasmic reticulum; Sap, secreted aspartyl proteinase.

type or mutated forms of *SAPT1*. With these yeasts we have determined the structures of the intracellular precursors of Sapt1, and evaluated the role of the processing sites in the secretion of this enzyme. The different steps of maturation and secretion of Sapt1 deduced here may be used as a model for the other *Candida* Saps.

METHODS

Organisms. *C. tropicalis* ATCC 750 is a wild-type pathogenic strain. *C. tropicalis* DSY59 (*ade2/ade2, sapt1Δ::λ/sapt1Δ::λ*) (Sanglard *et al.*, 1992) was chosen as the recipient for transformation. The strains of *S. cerevisiae* used in this study are listed in Table 1 and the characteristics of *sec* mutants are described in Table 3. *Escherichia coli* DH5α (Hanahan, 1983) was chosen as a carrier for recombinant plasmids. *E. coli* CJ236 (Kunkel *et al.*, 1987), *E. coli* TG1 (Gibson, 1984) and bacteriophage M13 mp18 were used for site-directed mutagenesis.

Growth media. *C. tropicalis* ATCC 750, *C. tropicalis* DSY59 and non-transformed *S. cerevisiae* strains were maintained on Sabouraud agar medium (Diagnostic Pasteur). Transformed *C. tropicalis* and *S. cerevisiae* strains were maintained on minimal medium (MM) plates [0.67% (w/v) Yeast Nitrogen Base (YNB, Difco), 2% (w/v) glucose, 1.5% (w/v) agar] containing 20 µg ml⁻¹ of appropriate supplements. Non-inducing liquid cultures were carried out in Sabouraud medium (Diagnostic Pasteur) or supplemented liquid MM. Liquid *SAPT1* induction media were bovine serum albumin (BSA) medium [0.2% (w/v) BSA,

1.7% (w/v) YNB without amino acids and ammonium salts (Difco), 2% (w/v) glucose, pH 4.0] for *C. tropicalis* and galactose medium [0.67% (w/v) YNB, 2% (w/v) galactose] for *S. cerevisiae* strains. Secreted proteolytic activity was monitored using BSA plates [BSA medium solidified with 1.5% (w/v) agar] for *C. tropicalis* and supplemented glucose/galactose plates [0.67% (w/v) YNB, 2% (w/v) glucose, 2% (w/v) galactose, 2% BSA, 1.5% (w/v) agar, pH 4.0] for *S. cerevisiae* strains. Induced cultures were incubated for 4 d at 30 °C and proteolytic activity was observed as a clear zone around the colonies in the absence of any staining procedure.

Plasmid constructions and site-directed mutagenesis. The plasmid pDS22 (Sanglard *et al.*, 1992), containing the *SAPT1* gene, was used to transform *C. tropicalis* DSY59. The following mutagenesis strategy for *SAPT1* was chosen. The 1297 bp *HindIII*-*KpnI* fragment (Fig. 1) was excised from pMTL21-E4 (Togni *et al.*, 1991), which is a plasmid containing the whole *SAPT1* gene, and cloned in the bacteriophage M13 mp18, thus generating M13 mp18-HK1.3. This plasmid was mutated using the U-DNA mutagenesis kit (Boehringer), which is based on the method developed by Kunkel *et al.* (1987). Synthetic oligonucleotides used in site-directed mutagenesis are described in Table 2 and Fig. 1. The presence of mutations was verified by nucleotide sequencing. The 1200 bp *BglII*-*KpnI* fragment of pDS22 was excised and replaced with the different mutated 1200 bp *BglII*-*KpnI* fragments from M13 mp18-HK1.3, generating the plasmids listed in Table 1.

Table 1. *S. cerevisiae* strains and plasmids used in this study

Strain or plasmid	Genotype and characteristics*	Reference or source
<i>S. cerevisiae</i>		
GRF18	α , <i>leu2-3</i> , <i>leu2-112</i> , <i>his3-11</i> , <i>his3-15</i>	Sengstag & Hinnen (1987)
RSY10	α , <i>sec1-1</i> , <i>leu2-3</i> , <i>leu2-112</i> , <i>ura3-52</i>	R. Haguenaue-Tsapist
RSY9	α , <i>sec7-1</i> , <i>leu2-3</i> , <i>leu2-112</i> , <i>ura3-52</i>	R. Haguenaue-Tsapist
HMSF176	α , <i>sec18-1</i> , <i>leu2-3</i> , <i>leu2-112</i>	R. Haguenaue-Tsapist
RSY12	α , <i>sec53-6</i> , <i>leu2-3</i> , <i>leu2-112</i> , <i>ura3-52</i>	R. Haguenaue-Tsapist
RSY455	α , <i>sec61-3</i> , <i>leu2-3</i> , <i>his4</i> , <i>ura3-52</i>	R. Haguenaue-Tsapist
Tr 1625	α , <i>ura3-52</i> , <i>kex2::leu2-3</i> , <i>leu2-112</i>	A Hinnen‡
Plasmids		
pDS22	pMK16-derived plasmid carrying the <i>SAPT1</i> gene	Sanglard <i>et al.</i> (1992)
pDS22-46	Derivative of pDS22 mutated with oligonucleotide 1	This study
pDS22-97	Derivative of pDS22 mutated with oligonucleotide 2	This study
pDS22-178	Derivative of pDS22 mutated with oligonucleotide 3	This study
pDS22-275	Derivative of pDS22 mutated with oligonucleotide 4	This study
pDS22-833	Derivative of pDS22 mutated with oligonucleotide 5	This study
YE51	<i>E. coli</i> - <i>S. cerevisiae</i> shuttle vector	Rose & Broach (1991)
YE51- <i>SAPT1</i>	YE51-derived plasmid carrying the <i>SAPT1</i> gene	This study
YE51- <i>SAPT1</i> -46	Derivative of YE51- <i>SAPT1</i> mutated with oligonucleotide 1	This study
YE51- <i>SAPT1</i> -97	Derivative of YE51- <i>SAPT1</i> mutated with oligonucleotide 2	This study
YE51- <i>SAPT1</i> -178	Derivative of YE51- <i>SAPT1</i> mutated with oligonucleotide 3	This study
YE51- <i>SAPT1</i> -275	Derivative of YE51- <i>SAPT1</i> mutated with oligonucleotide 4	This study
YE51- <i>SAPT1</i> -833	Derivative of YE51- <i>SAPT1</i> mutated with oligonucleotide 5	This study
YE51- <i>SAPT1</i> -97/178	Derivative of YE51- <i>SAPT1</i> mutated with oligonucleotide 2 and 3	This study
YE51- <i>SAPT1</i> -178/275	Derivative of YE51- <i>SAPT1</i> mutated with oligonucleotide 3 and 4	This study
YE51- <i>URA3-SAPT1</i>	Derivative of YE51- <i>SAPT1</i> , carrying the <i>URA3</i> gene	This study

* For details of oligonucleotides 1 to 5, see also Table 2.

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Table 2. Oligonucleotide primers used for site-directed mutagenesis

Oligo-nucleotide	Oligonucleotide sequence	Codon: wild-type → mutant	Amino acid: wild-type → mutant
1	ATT GCT CTT ⁴⁶ CCT TTT GCT	GCT → CCT	Ala ¹⁶ → Pro ¹⁶
2	ATT GAG AAA ⁹⁷ GGA ACC GAC A	AGA → GGA	Arg ³³ → Gly ³³
3	ATT CAA AAG ¹⁷⁸ GGA AGT GAT G	AGA → GGA	Arg ⁶⁰ → Gly ⁶⁰
4	TT GTC ATT ²⁷⁵ GTC ACC GGT TC	GAC → GTC	Asp ⁹² → Val ⁹²
5	TT GTT TTG ⁸³³ GTC TCT GGT AC	GAC → GTC	Asp ²⁷⁸ → Val ²⁷⁸

Fig. 1. (a) Restriction map of the 4.0 kb *EcoRI* fragment carrying the *SAPT1* gene. (b) Localization of the oligonucleotides (1–5) used for site-directed mutagenesis. (c) Representation of the proteins encoded by the plasmids listed on the left and constructed as described in Table 1. *S. cerevisiae* strains were transformed with YEp51 derivatives and the *C. tropicalis* strain was transformed with pDS22 derivatives. The signal sequence is indicated as a solid black box, the proregion is symbolized by an open rectangle and the mature *SAPT1* sequence by diagonal lines. The arrows indicate the positions of the potential processing sites. The asterisks mark the regions mutated by site-directed mutagenesis. The one-letter amino acid code indicates the sequence of the protein encoded by each plasmid.

The YEp51 expression plasmid (Rose & Broach, 1991) was used to transform *S. cerevisiae*. The 1182 bp *SAPT1* coding region was amplified by PCR using the primers GCGTCGACAAA-ATGGCTACCCATTTTC (26-mer) and GCGGATCCATTC-AATTATACTTTGAC (27-mer), allowing the *SAPT1* gene to be flanked with *SalI* and *BamHI* restriction sites. The PCR was performed following standard protocols (Innis *et al.*, 1990). The

amplified fragment was cloned in the *SalI* and *BamHI* site of YEp51 and of bacteriophage M13 mp18 DNA, generating YEp51-*SAPT1* and M13 mp18-*SAPT1*, respectively, for site-directed mutagenesis. After mutagenesis, the *SAPT1* amplified region was excised from M13 mp18-*SAPT1* using *SalI* and *BamHI* and cloned into YEp51, generating the YEp51-*SAPT1*-derived plasmids listed in Table 1.

In order to construct a plasmid for the transformation of the *S. cerevisiae* strain Tr 1625 (Table 1), the *URA3* gene was introduced into the YEp51 plasmid as follows. The 1.2 kb *HindIII* fragment containing the entire *URA3* gene was isolated from YEp24. A 0.1 kb *HindIII* fragment of YEp51 was excised and replaced by the 1.2 kb *HindIII* *URA3* fragment, generating YEp51-*URA3-SAPT1* (Table 1).

Yeast transformations and plasmid preparation. *S. cerevisiae* was transformed by the lithium acetate method (Ito *et al.*, 1983). *C. tropicalis* cells were protoplasted and transformed as described by Sanglard *et al.* (1992).

Yeast plasmids were extracted from cells of *C. tropicalis* and *S. cerevisiae* grown overnight at 30 °C in 50 ml supplemented MM. Cells were harvested, washed with 1 M sorbitol, suspended in 10 ml of SCEM medium (1 M sorbitol, 100 mM sodium citrate, 25 mM EDTA, 15 mM β -mercaptoethanol, pH 5.8) in the presence of 5 U lyticase (Sigma) and incubated at 30 °C for 30 min. After lysis of sedimented protoplasts following the standard alkaline lysis protocol for bacterial plasmid preparations, yeast plasmids were purified using anion-exchange resin columns (Qiagen system, Kontron, Switzerland). One half of the recovered plasmid DNA was analysed on agarose gel following digestion with restriction enzymes.

In vitro translation. *C. tropicalis* total RNA was extracted following the guanidium thiocyanate phenol/chloroform protocol (Chomczynski & Sacchi, 1987) after breaking sedimented cells with a mortar and pestle under liquid nitrogen. *In vitro* translation was performed for 90 min at 37 °C in a final volume of 50 μ l containing 5 μ g total RNA, 25 μ l rabbit reticulocyte lysate and 50 μ Ci (1.85 MBq) Trans-³⁵S-Label (ICN).

Sapt1 production and detection. *C. tropicalis* cells were grown at 30 °C in YPD medium to an OD₆₆₀ of 1.0, sedimented and resuspended to an OD₆₆₀ of 1.0 in BSA medium and grown for 3 d at 30 °C. Sapt1 produced by *C. tropicalis* cells was purified by ion-exchange chromatography as previously described (Togni *et al.*, 1991). *S. cerevisiae* cells were grown at 30 °C in supplemented MM medium to an OD₆₆₀ of 1.0, sedimented and resuspended to an OD₆₆₀ of 1.0 in supplemented galactose medium and grown for 3 d at 30 °C. *S. cerevisiae* secreted proteins were recovered by trichloroacetic acid (TCA) precipitation. The proteins from *C. tropicalis* and *S. cerevisiae* samples were resuspended in Laemmli gel-loading buffer (Laemmli, 1970) at 1:100 of the original volume, separated by SDS-PAGE according to the method of Laemmli (1970) and analysed by Coomassie blue staining and immunoblotting. Immunoblotting was performed by passive transfer of proteins from the SDS-PAGE gel onto a nitrocellulose membrane according to Monod *et al.* (1991), with a 1:1000 dilution of the antiserum.

N-terminal sequence of proteins secreted by *S. cerevisiae* harbouring YEp51-SAPT1-178. *S. cerevisiae* cells carrying YEp51-SAPT1-178 were induced for Sapt1 production as described above. The supernatant was separated from yeast cells and Pepstatin A (40 μ g ml⁻¹) was added in order to inhibit Sapt1 enzymic activity. The secreted Sapt1 forms were purified in presence of Pepstatin A (40 μ g ml⁻¹) by ion-exchange chromatography as previously described (Togni *et al.*, 1991) for Sapt1 produced by *C. tropicalis*. The recovered proteins were then separated by Tricine-SDS-PAGE and transferred onto PVDF-Immobilon membranes (Millipore). After staining with Coomassie blue, the protein bands were cut out and analysed with a gas-phase sequencer 470A (Applied Biosystems) with on-line PTH-derivative analyser 120A.

Antibodies. Polyclonal antibodies were raised in rabbits as previously described (Monod *et al.*, 1991) by use of purified Sapt1 as the immunogen. Monoclonal antibody FX7-10 was obtained from a fusion experiment using mice immunized against *C. tropicalis* DSM 4238 SAPT1 proteinase and *C. albicans* ATCC 48867 Sap2 proteinase. This antibody was kindly provided by Dr M. Borg-von Zepelin (Göttingen).

Protein labelling and immunoprecipitation. *C. tropicalis* cells were grown at 30 °C in YPD medium to an OD₆₆₀ of 1.0, sedimented and resuspended to an OD₆₆₀ of 1.0 in BSA medium. Typically, a 50 ml culture was grown for 24 h at 30 °C before radiolabelling. A sample of 1 ml was transferred into a microfuge tube and 50 μ Ci (1.85 MBq) Trans-³⁵S-Label was added. After 120 min incubation at 30 °C, protein synthesis was stopped with 100 mM sodium azide. The proteins in the supernatant were precipitated with 200 μ l of 100% (w/v) TCA and incubated for 5 min on ice, centrifuged and resuspended in 50 μ l gel-loading buffer. For immunoprecipitation of intracellular proteins, the cells were pelleted, resuspended in 200 μ l 1.8 M NaOH, 1.2 M β -mercaptoethanol, and incubated for 5 min on ice. After addition of 200 μ l 10% (w/v) TCA, the mixture was incubated for 5 min on ice, centrifuged and resuspended in 50 μ l Laemmli gel-loading buffer. The samples were heated to 95 °C for 5 min. *S. cerevisiae* proteins were labelled as described for *C. tropicalis*, except that cells were grown in supplemented MM medium and induction was performed by culture in supplemented galactose medium for 9 h. The *sec* mutants were grown for 1 h at the restrictive temperature of 37 °C before radiolabelling.

Immunoprecipitation was performed as previously described (Franzoso *et al.*, 1991). Immunoprecipitated proteins were suspended in 50 μ l gel-loading buffer and heated to 95 °C for 5 min. Protein samples (10 μ l) were separated by SDS-PAGE using a 9% (w/v) polyacrylamide gel and analysed by fluorography (Chamberlain, 1979).

Tunicamycin, brefeldin A and endoglycosidase H treatments. Tunicamycin (10 μ g ml⁻¹) was used to prevent protein glycosylation (Doms *et al.*, 1989) and brefeldin A (5 μ g ml⁻¹) was used to block the translocation of the proteins through the Golgi apparatus (Duksin *et al.*, 1982). Sapt1 synthesis was derepressed as described above and the drugs were added 1 h before radiolabelling the culture samples. Endoglycosidase H digestion was carried out on immunoprecipitated labelled material following the protocol described by Monod *et al.* (1989).

RESULTS

Expression of SAPT1 in *S. cerevisiae*

S. cerevisiae GRF18 and *sec* mutants were used as tools to investigate the secretion pathway of Sapt1 in order to identify the intermediate precursors of this protein and to study the effect of point mutations of putative processing sites in the preprosequence. Several reports have shown that *S. cerevisiae* can secrete heterologous extracellular gene products in enzymically active forms (Sato *et al.*, 1986; Tatsumi *et al.*, 1989; Yamashita *et al.*, 1987). Notably, their N-termini were processed correctly by the use of the native pre and prepro sequences. To construct the plasmid for the expression of Sapt1 in *S. cerevisiae*, a 1182 bp fragment containing the entire prepro and mature coding region was amplified by PCR and inserted into YEp51, just downstream of the inducible *S. cerevisiae*

Table 3. Effect of *sec* mutation on Sapt1 secretion by *S. cerevisiae*

Strain	Mutation	Defective step in secretion at restrictive temperature (37 °C)	Accumulation of Sapt1 forms at restrictive temperature (37 °C)
RSY455	<i>sec61</i>	Translocation across the ER	PPE
RSY12	<i>sec53</i>	Glycosylation in the lumen	PE*, PE and M
HMS176	<i>sec18</i>	ER to Golgi apparatus transfer	PE*
RSY9	<i>sec7</i>	Secretory vesicle formation	PE* and M
RSY10	<i>sec1</i>	Exocytosis	PE* and M

Fig. 2. Visual assay of secreted proteolytic activity in *S. cerevisiae* carrying *SAPT1* and mutated forms of *SAPT1*. The plasmids harboured by each strain are indicated. *C. tropicalis* strains were grown on BSA agar and *S. cerevisiae* strains on glucose/galactose agar. Proteolytic activity was monitored after 4 d incubation at 30 °C.

GAL10 promoter. This recombinant plasmid and YE51 were introduced in *S. cerevisiae* GRF18 and several *sec* mutants (Table 3). At non-restrictive temperature, the transformants harbouring YE51-*SAPT1* formed a clear zone around the colonies when grown on glucose/galactose plates containing 0.4% BSA (Fig. 2) and secreted a 40 kDa protein reacting against polyclonal anti-Sapt1 antiserum when grown in liquid galactose medium (data not shown). This finding indicated that the strains transformed with the YE51-*SAPT1* plasmid secreted the M form (see Fig. 4) of Sapt1.

Precursors of Sapt1 in *S. cerevisiae*

Two intracellular proteins were isolated by immunoprecipitation of radiolabelled products from *S. cerevisiae* GRF18 cells harbouring YE51-*SAPT1*. Their estimated molecular masses on SDS-PAGE were 40 kDa and 46 kDa, respectively (Fig. 3a). The lower band had the same mobility as the protein purified from the culture medium and therefore corresponds to the M form. Accumulation of this 46 kDa protein was not affected when cells were treated with brefeldin A (Fig. 3a), a drug

that blocks the translocation of the proteins through the Golgi apparatus, or in the *sec18* mutant at non-permissive temperature (Fig. 3b). However, *S. cerevisiae* cells treated with tunicamycin, a drug known to prevent protein glycosylation, like the *sec53* mutant at non-permissive temperature, accumulated a 2 kDa smaller precursor (Fig. 3a, b). Therefore, we conclude that the 46 kDa precursor of Sapt1 is an *N*-glycosylated protein located in the endoplasmic reticulum (ER). This observation is consistent with the presence of an *N*-glycosylation site at the position Asn⁵⁰ in the propeptide sequence. The labels PE* and PE will refer henceforth to the glycosylated 46 kDa and the unglycosylated 44 kDa proenzymes, respectively (Fig. 4).

The mutants *sec1* and *sec7*, which are defective in secretory vesicle formation and exocytosis respectively, accumulated the 46 and the 40 kDa proteins at non-permissive temperature (Fig. 3b), suggesting that the processing step leading to the M form formation took place in the Golgi apparatus.

In order to determine the size of Sapt1 preproenzyme, total RNA was extracted from *C. tropicalis* and translated *in vitro*. The immunoprecipitation of translated products permitted isolation of a protein with an estimated molecular mass of 45 kDa (Fig. 3c), corresponding to the Sapt1 preproenzyme (PPE form), which is the non-processed precursor. Accumulation of PPE was observed in protein extracts of the *S. cerevisiae sec61* mutant grown at the non-permissive temperature (data not shown).

Effect of point mutations on *SAPT1* expressed in *S. cerevisiae*

By introducing point mutations of the putative processing sites in the 60-amino-acid precursor peptide a systematic study was undertaken to analyse the role of cleavage sites in maturation and secretion of Sapt1. *S. cerevisiae* GRF18 was used as recipient for transformation with YE51 plasmid carrying mutated forms of the *SAPT1* gene. To determine the presence of proteolytic activity, each transformant was grown on glucose/galactose medium containing 0.4% BSA (Fig. 2).

The strain harbouring YE51-*SAPT1*-46 formed only a faint halo of clearing on glucose/galactose plates as compared to colonies carrying YE51-*SAPT1* (Fig. 2), indicating that the mutation of the signal sequence

Fig. 3. Detection by immunoprecipitation of precursors and mature form of Sapt1. These experiments were performed with cell extracts of induced cultures of *S. cerevisiae* GRF18 and *sec* mutants and with products from *in vitro* translation of total RNA of induced *C. tropicalis* cells. Inductions were performed as indicated in Methods. Proteins were radiolabelled by addition of 50 μ Ci (1.85 MBq) Trans- 35 S-Label to 1.0 OD₆₆₀ of yeast cells or to 5 μ g of total RNA. Immunoprecipitated proteins were separated by SDS-PAGE (9% polyacrylamide) and detected by fluorography. The positions and apparent molecular masses of marker proteins (phosphorylase *b*, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 42 kDa; carbonic anhydrase, 31 kDa) are shown on the right of each gel. (a) Effects of tunicamycin (10 μ g ml⁻¹) and brefeldin A (5 μ g ml⁻¹) on Sapt1 in *S. cerevisiae* GRF18 carrying the YEp51-SAPT1 plasmid. Drug treatments were performed for 1 h at 30 °C. (b) Intracellular Sapt1 precursors in *S. cerevisiae* GRF18 carrying the YEp51-SAPT1 plasmid in the presence (+) or absence (-) of tunicamycin (10 μ g ml⁻¹) and accumulation at the restrictive temperature of 37 °C of Sapt1 precursors by *S. cerevisiae* *sec* mutants (*sec1*, *sec7*, *sec18* and *sec53*) harbouring the YEp51-SAPT1 plasmid. (c) Detection by immunoprecipitation of proteins from *S. cerevisiae* GRF18 cells carrying the YEp51-SAPT1 plasmid (lane 1, PE; lane 2, PE*) and of products from *in vitro* translation of total RNA from a 26 h induced culture of *C. tropicalis* ATCC 750 (lane 3, PPE). Before protein extraction *S. cerevisiae* GRF18 cultures were treated (lane 1) or not (lane 2) with tunicamycin (10 μ g ml⁻¹) for 1 h. (d) Detection by immunoprecipitation of intracellular Sapt1 precursors in *S. cerevisiae* GRF18 transformed with (lane 1, PE*) YEp51-SAPT1 and (lane 2, PPE*; lane 3, PPE) YEp51-SAPT1-46. Cell extracts were treated (+) or not (-) with endoglycosidase H (Monod *et al.*, 1989).

cleavage sites reduced secretion of Sapt1. In liquid galactose medium this strain secreted the 40 kDa M form of Sapt1 (data not shown). The analysis of the Sapt1 precursors in cell extracts revealed the presence of an

additional 47 kDa protein (PPE*, Fig. 3d). The 47 kDa PPE* was converted to PPE by endoglycosidase H treatment (Fig. 3d), indicating that the PPE* was glycosylated.

Fig. 4. List of the Sapt1 forms, with their code and molecular size. A model of Sapt1 is given at the top, indicating the signal sequence (black box), the proregion (open rectangle), the mature sequence (diagonal lines), the putative processing sites (arrows) and amino acids in these regions analysed in this study (one-letter code).

The proteolytic haloes around the colonies of yeasts harbouring YEp51-*SAPT1*, YEp51-*SAPT1*-97 and YEp51-*SAPT1*-178 were not significantly different from one another, indicating that mutation of one of the two Lys-Arg sites did not affect the activity of the secreted enzyme. The strain carrying the YEp51-*SAPT1*-97 plasmid (mutation of the Lys³²-Arg³³ site) showed the same intracellular precursors and secreted form of Sapt1 as the strain carrying the YEp51-*SAPT1* plasmid (data not shown). In contrast, two proteins were present in the supernatant of the culture of cells transformed with the YEp51-*SAPT1*-178 plasmid, which encoded proteins lacking the Lys⁵⁹-Arg⁶⁰ site immediately upstream of the N-terminus of the mature form of Sapt1 (Fig. 5a). The larger protein migrated as a protein with a molecular mass of 44 kDa and its initial seven N-terminal amino acid residues were determined to be Thr-Asp-Lys-Val-Val-Ser-Leu. This protein corresponded to the predicted Sapt1 form cleaved at the Lys³²-Arg³³ site, 27 amino acids upstream of the N-terminal amino acid of the M form; it was therefore named the M + 27* form (Fig. 4). The smaller protein (MA form) migrated as a protein slightly larger than the M form. The initial 10 N-terminal amino acid residues were determined to be Ile-Gln-Lys-Gly-Ser-Asp-Val-Pro-Thr-Thr, indicating that the MA form is four amino acid residues longer than the M form. The two proteins M + 27* and MA were present at the same time. However, over the course of time the upper band was diminished and the signal of the lower band intensified, suggesting that cleavage was taking place in the growth medium (Fig. 5a). This shift was observed even in the absence of cells (Fig. 5b). In order to verify that this cleavage was the consequence of an autocatalytic activity by Sapt1, pepstatin A was added to the supernatant and the reaction mixture was incubated at 30 °C. Pepstatin A

Fig. 5. Forms of Sapt1 secreted by *S. cerevisiae* GRF18 carrying the YEp51-*SAPT1*-178 plasmid. (a) In lanes 1, 2 and 3, the proteins were recovered from the supernatant of an induced culture at different incubation times (lane 1, 10 h; lane 2, 30 h; lane 3, 50 h) and analysed by Western blotting. Their apparent molecular masses were compared to that of the 40 kDa M form (lane 4). The fast-migrating band (lane 2) was identified as a degradation product of the enzyme. Similar results were obtained with other proteinases, such as alkaline proteinase secreted by *Aspergillus fumigatus* (Monod *et al.*, 1991). (b) In lanes 5 and 6, samples of cell-free supernatant from the 10 h induced culture were incubated at 30 °C in the presence (lane 5) or absence (lane 6) of pepstatin A (40 µg ml⁻¹) and the proteins were compared to the Sapt1 form present in the 50 h induced culture (lane 7). When testing for inhibition of autocatalytic cleavage, pepstatin A (40 µg ml⁻¹) was added to the supernatant of *S. cerevisiae* cultures and the incubation at 30 °C was continued for 10 h before TCA precipitation.

is a specific inhibitor of enzymes belonging to the pepsin family. Under these conditions, the intensity of the upper M + 27* form remained unaffected (Fig. 5b), confirming that the smaller MA form was an autocleavage product of the M + 27* Sapt1.

Colonies of cells carrying the double mutation YEp51-*SAPT1*-97/178 formed no haloes (Fig. 2) and no Sapt1 was detected in the growth medium of a culture of yeast cells carrying this plasmid. The same results were obtained with *S. cerevisiae* GRF18 harbouring the YEp51-*SAPT1*-178/275 plasmid and the *S. cerevisiae* Tr 1625 *kex2* strain transformed with the YEp51-*URA3-SAPT1* plasmid. Immunoprecipitation of radiolabelled proteins from these three strains revealed the presence of the 46 kDa PE* form (data not shown).

The transformants harbouring the YEp51-*SAPT1*-275 and YEp51-*SAPT1*-833 plasmids, which encoded Sapt1 proteins with mutated active sites, were also unable to hydrolyse BSA (Fig. 2). These mutants showed the same intracellular precursors and secreted form of Sapt1 as the strain carrying the YEp51-*SAPT1* plasmid (data not shown).

In vivo expression of Sapt1 by *C. tropicalis*

The kinetics of Sapt1 production by *C. tropicalis* ATCC 750 in induction medium was monitored by radiolabelling and immunoprecipitation of proteins after different incubation times. The analysis of supernatant samples

Fig. 6. Kinetics of Sapt1 production by *C. tropicalis*. After different times (1, 3, 6, 10, 26 h) of growth in Sapt1 induction medium, 1 ml cell suspension was mixed with 50 μ Ci (1.85 MBq) Trans- 35 S-label and incubated for 120 min at 30 °C. Proteins from cell extracts (a) and medium supernatant (b) were immunoprecipitated using 5 μ l polyclonal anti-Sapt1 specific rabbit antiserum, separated by SDS-PAGE using a 7.5% polyacrylamide gel and analysed by fluorography (Chamberlain, 1979). The arrows indicate the position of the M form of Sapt1. The molecular size markers are the same as for Fig. 3.

permitted observation of the 40 kDa M form after 6 h incubation, with the highest signal intensity observed after 26 h, at the end of the observation interval (Fig. 6). The analysis of intracellular precursors of Sapt1 was not possible with the antiserum (Fig. 6) or with the monoclonal antibodies (data not shown) because of the presence of several interfering reacting products. Therefore, we decided to investigate the role of the preproregion by the analysis of the effects of *SAPT1* gene point mutations on *C. tropicalis* growth, proteolytic activity on BSA plates and Sapt1 secretion in BSA liquid medium. *C. tropicalis* DSY59, which is a *sapt1*, *ade2* mutant, was transformed with pDS22 carrying mutations in the *SAPT1* gene. The presence of the plasmid in each strain was verified by extraction of the plasmids from yeast cells and analysis on agarose gels after digestion with restriction enzymes. The transformants were stable when maintained on MM plates.

On BSA plates clear zones were observed only around colonies harbouring pDS22, pDS22-46 or pDS22-97 (Fig. 2), indicating that the mutation of the signal sequence

Fig. 7. Summary of the Sapt1 maturation pathway. The figure is explained in the discussion. To the left of the figure are indicated the name and the apparent molecular mass of each intermediate. Open arrows signify an inter-organelle translocation step and closed arrows an intra-organelle processing step. Gene names are placed so as to indicate which Sapt1 precursor accumulates in sec mutants. The asterisk represents the Asn-linked carbohydrate chain.

cleavage site and the Lys³²-Arg³³ site did not affect the enzymic activity of the yeast on solid medium. These strains were able to grow in BSA liquid medium and to secrete the M form of Sapt1 (data not shown). Only a faint clearing zone was observed around the colonies carrying pDS22-178 (Lys⁵⁹-Arg⁶⁰ site mutated). However, neither this strain, nor the *C. tropicalis* *sapt1* mutant, grew in BSA liquid medium. The transformants harbouring pDS22-275 or pDS22-833, which encoded Sapt1 proteins with mutated active sites, grew neither on BSA plates nor in liquid BSA medium, presumably because the inability to hydrolyse BSA meant that they lacked a nitrogen source.

DISCUSSION

Secreted proteinases are normally translated as larger precursors which are subsequently processed during

Table 4. Effects of *SAPT1* point mutations on Sapt1 secretion by *S. cerevisiae*

Plasmid		Characteristics	SAPT1 forms produced	
			Intracellular	Secretion
YEp51	Parent plasmid		Not detected	Not detected
YEp51-SAPT1	YEp51 carrying SAPT1		PE* and M	M
YEp51-SAPT1-46	Signal sequence cleavage site mutated (Ala ¹⁶ → Pro ¹⁶)		PPE*, PE* and M	M
YEp51-SAPT1-97	Lys ³² -Arg ³³ site mutated (Arg ³³ → Gly ³³)		PE* and M	M
YEp51-SAPT1-178	Lys ⁵⁹ -Arg ⁶⁰ site mutated (Arg ⁶⁰ → Gly ⁶⁰)		PE*	M + 27* and M + 4
YEp51-SAPT1-97/178	Both Lys-Arg sites mutated (Arg ³³ → Gly ³³ , Arg ⁶⁰ → Gly ⁶⁰)		PE*	Not detected
YEp51-SAPT1-275	Active site mutated (Asp ⁹² → Val ⁹²)		PE* and M	Inactive M
YEp51-SAPT1-833	Active site mutated (Asp ²⁷⁸ → Val ²⁷⁸)		PE* and M	Inactive M
YEp51-SAPT1-178/275	Lys ⁵⁹ -Arg ⁶⁰ site (Arg ⁶⁰ → Gly ⁶⁰) and active site mutated (Asp ⁹² → Val ⁹²)		PE*	Not detected
YEp51-URA3-SAPT1	Non-mutated Sapt1		PE*	Not detected

secretion. The nucleotide sequence of the entire *SAPT1* gene suggested that the enzyme is translated as a preproform, 60 amino acids larger than the mature Sapt1. A putative precursor of Sap from *C. albicans* was described by Homma *et al.* (1992, 1993) and Benerjee *et al.* (1991), but the processing mechanism was not investigated. In *S. cerevisiae*, transcription of *SAPT1* resulted in secretion of the active enzyme identical to that secreted by *C. tropicalis*. The analysis of *S. cerevisiae* GRF18 and *sec* mutant products (results summarized in Table 3) as well as the drug effects on Sapt1 secretion (Fig. 3a) allowed us to propose a secretion mechanism for this enzyme shown in Fig. 7. Only the PE* precursor was observed in cell extracts of *S. cerevisiae* GRF18. After translocation into the Golgi apparatus, the glycosylated 46 kDa PE* precursor form undergoes proteolytic cleavage by the Kex2 endopeptidase, generating the 40 kDa M form. The M form of Sapt1 is sorted into secretory vesicles and finally released into the extracellular space (Fig. 7).

The mutation of the signal peptidase cleavage site does not affect the ability of the mutated protein to cross the ER membrane and to be processed correctly, but it leads to a decrease of Sapt1 secretion as deduced from BSA plate analysis (Table 4). Previous studies of *S. cerevisiae* acid phosphatase secretion (Monod *et al.*, 1989) showed that signal sequence cleavage was not necessary for the entry of proteins into the secretory pathway, but was important for efficient secretion. The mutation of the Lys³²-Arg³³ site alone does not affect Sapt1 maturation and secretion (Table 4). Modification of the Lys⁵⁹-Arg⁶⁰ site, which just precedes the N-terminal amino acid of the 40 kDa M form of Sapt1, did not affect secretion of the Sapt1 protein. However, this form was four amino acids longer than the M form, as observed by the N-terminal amino acid sequencing of this MA form of Sapt1. The MA protein is produced by an autocatalytic cleavage step since pepstatin A, a specific inhibitor of aspartic proteinases, prevented the conversion of the M + 27* form into the MA form. Similar results were obtained by Lin *et al.*

(1993), who cloned the *SAPT1* gene into a T7 expression vector of *E. coli*. The purified unprocessed precursor of Sapt1, corresponding to the unglycosylated 45 kDa PPE form of our study, was converted to the Sapt1 mature form by autocatalytic processing under acidic conditions. The N-terminal sequence analysis revealed a mixture of two sequences corresponding to proteins one and two amino acids longer than the M form. In the experimental conditions of Lin *et al.* (1993), autocatalytic cleavage of unglycosylated PPE *in vitro* can only be initiated by self-hydrolysis. However, further hydrolysis of unprocessed Sapt1 molecules by processed ones can not be excluded. The amino acid position of the autocatalytic cleavage site appears to be substrate dependent since it is different for unglycosylated PPE and M + 27 forms.

No Sapt1 secreted protein was found during the expression by *S. cerevisiae* GRF18 of the *SAPT1* gene with both the Lys-Arg sites mutated, nor during expression of the unmutated *SAPT1* gene by *S. cerevisiae* Tr 1625, which is a *kex2* mutant. In the present case, to obtain the Sapt1 mature form, Kex2 can not be substituted by any other endopeptidase such as Yap3 (Egel-Mitani *et al.*, 1990; Ash *et al.*, 1995). These results, obtained with *S. cerevisiae*, suggest that the removal of the proregion is required for the secretion of the Sapt1.

Neither the antiserum nor the monoclonal antibodies permitted the analysis of intracellular precursors of Sapt1 in *C. tropicalis* because of the presence of several cross-reacting components. Therefore, we analysed the role of the putative processing sites present in the preproregion by studying the effect of *SAPT1* point mutations on proteolytic activity secreted by *C. tropicalis*. The results, summarized in Table 5, are consistent with those obtained with *S. cerevisiae*. Growth in BSA liquid medium was observed only with strains able to produce the 40 kDa form of Sapt1, like the wild-type *C. tropicalis* ATCC 750 and the *sapt1* mutant carrying pDS22, as well as the strains producing the Sapt1 precursor with a mutated signal sequence cleavage site or Lys³²-Arg³³ site. In contrast, the

Table 5. Effects of *SAPT1* point mutations on Sapt1 secretion by *C. tropicalis*

Plasmid	Characteristics	Effects on <i>C. tropicalis</i> Sapt1 secretion	
		BSA plates	BSA liquid medium
pDS22	Non-mutated Sapt1	Halo	Living cells; mature Sapt1 secretion
pDS22-46	Signal sequence cleavage site mutated (Ala ¹⁶ → Pro ¹⁶)	Halo	Living cells; mature Sapt1 secretion
pDS22-97	Lys ³² -Arg ³³ site mutated (Arg ³³ → Gly ³³)	Halo	Living cells; mature Sapt1 secretion
pDS22-178	Lys ⁵⁹ -Arg ⁶⁰ site mutated (Arg ⁶⁰ → Gly ⁶⁰)	Faint halo	Death; Sapt not detected
pDS22-275	Active site mutated (Asp ⁹² → Val ⁹²)	–	Death; Sapt not detected
pDS22-833	Active site mutated (Asp ²⁷⁸ → Val ²⁷⁸)	–	Death; Sapt not detected

Lys⁵⁹-Arg⁶⁰ mutation blocked growth in this Sapt1 induction medium. Mutation of Lys⁵⁹-Arg⁶⁰ led either to a decrease of the secretion of active Sapt1 or to the production of a Sapt1 with reduced enzymic activity (Fig. 2). In both cases the growth of this mutant in BSA liquid medium is dramatically affected because the yeast lacks a nitrogen source. Our results suggest that an endopeptidase similar to Kex2 is present in *C. tropicalis*.

The processing of proenzymes to the mature form is achieved either by autocatalytic cleavage (Hiramatsu *et al.*, 1989; Miyazaki *et al.*, 1989; Silen *et al.*, 1989) or by specific endopeptidases (Vos *et al.*, 1989). In *S. cerevisiae* the non-mutated form of Sapt1 is processed by the specific endopeptidase Kex2. Examination of precursors and their derived peptides has shown that most proteolytic processing occurs after di- or tetrabasic amino acid sequences, and several endopeptidases, structurally related to Kex2, have been described as being involved in this specific cleavage. The widespread distribution of this stepwise cleavage mechanism could be explained by its efficiency (for a review see Van de Ven *et al.*, 1993). The autocatalytic cleavage observed when the Lys⁵⁹-Arg⁶⁰ site was mutated indicates that the yeast possesses an alternative, less efficient way to produce the mature form of Sapt1. Sapt1 is a unique case where the processing of a proenzyme can be achieved either by a specific endopeptidase or by an autocleavage.

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