Intra- and intermolecular events direct the propeptide-mediated maturation of the *Candida albicans* secreted aspartic proteinase Sap1p

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Pathogenic yeasts of the genus *Candida* secrete aspartic proteinases (Sap) which are synthesized as preproenzymes. Expression of the *C. albicans* *SAP1* gene lacking the propeptide-coding region in the methylotrophic yeast *Pichia pastoris* does not lead to the secretion of the enzyme into the culture supernatant, but results in an accumulation of recombinant protein in the cell. Co-expression in this system of the unattached propeptide from Sap1p, as well as from other Saps, restored Sap1p secretion. A deletion analysis revealed that only a 12 aa sequence in the propeptide, corresponding to a highly conserved region in all Sap propeptides, was necessary and sufficient to produce a large amount of Sap1p in culture supernatant. No Sap1p was secreted when Sap1p was produced with a propeptide carrying an F to D mutation in the identified 12 aa sequence. However, the simultaneous production of equivalent amounts of Sap1p and His-tagged Sap1p (H₆-Sap1p) with a mutated and a non-mutated propeptide, respectively, led to the secretion of both proteins in a ratio of approximately 1:2. The restoration of Sap1p secretion occurred at the expense of secretion of H₆-Sap1p since the total activity was comparable to that of strains producing only H₆-Sap1p with a non-mutated propeptide. In contrast, the proteolytic activity of strains secreting Sap1p and H₆-Sap1p both with a functional propeptide was twice that of strains producing either Sap1p or H₆-Sap1p alone, and the two enzymes were found in an equivalent amount in the culture supernatant. Altogether, these results show that the propeptide can only function once and that the maturation of recombinant *C. albicans* secreted aspartic proteinase Sap1p is directed through a combination of intra- and inter-molecular pathways.

Keywords: *SAP1* gene, protein secretion, signal peptides, recombinant proteins, *Pichia pastoris*

INTRODUCTION

The secreted aspartic proteinases (Saps) of the opportunistic pathogens of the genus *Candida* are of particular interest because of their role as virulence factors (Fallon *et al.*, 1997; Borg-von Zepelin *et al.*, 1998; Schaller *et al.*, 1998, 1999). In *C. albicans*, they are encoded by a gene family with at least nine members,

SAP1 to *SAP9* (Monod *et al.*, 1994, 1998). To date, only one gene from *C. tropicalis* (*SAPT1*; Togni *et al.*, 1991) and two from *C. parapsilosis* (*SAPP1* and *SAPP2*; de Viragh *et al.*, 1993) have been cloned and sequenced, although preliminary investigations support the existence of more *SAP* genes in both species (Monod *et al.*, 1994). The *Candida* Saps, like many other microbial secreted proteinases, are synthesized as precursors in a preproprotein form. The prepeptide, or signal peptide, of 16–18 aa residues is necessary for entry into the secretory pathway by transporting the protein across the membrane of the endoplasmic reticulum (Pfeffer &

Abbreviations: Sap, secreted aspartic proteinase; H₆-Sap1p, His₆-tagged Sap1p.

Rothman, 1987). With the exception of Sap7p, which has a putative 195 aa propeptide, the *Candida* Saps have a relatively short propeptide (32–58 aa) which contains one to four KR sequences, one of which is located immediately before the N-terminus of the mature part of the proenzyme.

The propeptide of many secreted proteinases has been found to be essential and specific for assisting correct folding as well as secretion of the mature domain of the enzyme (for a review, see Eder & Fersht, 1995). Upon completion of folding, the propeptide is cleaved and removed to generate the active enzyme through an autoproteolytic reaction or, as in the *Candida* Saps, through an exogenous proteolytic reaction in the Golgi apparatus via the membrane-bound proteinase Kex2p (Togni *et al.*, 1996; Newport & Agabian, 1997), which specifically cleaves peptides after KR sequences (Julius *et al.*, 1984). Two different conformations of the mature part of a protein can be obtained with two different prosequences (Shinde *et al.*, 1997). Indeed, the mature part of subtilisin E was shown to be folded in an altered conformation through a mutated prosequence, resulting in differences of secondary structure, thermostability and substrate specificity.

It has been clearly established by many examples *in vitro* and/or *in vivo* that the propeptide can also mediate the folding of the proteinase when added as a separate polypeptide chain (Ohta *et al.*, 1991; Fabre *et al.*, 1992; Fukuda *et al.*, 1994; McIver *et al.*, 1995; van den Hazel *et al.*, 1994). *In vitro*, the propeptide covalently attached to the mature domain of prosubtilisin E has been found to function intermolecularly as folding catalyst, although a parallel intramolecular pathway has not been ruled out (Zhu *et al.*, 1989; Hu *et al.*, 1996). However, whether the maturation of secreted proteinases occurs through inter- or intramolecular events has never been investigated *in vivo*. This question instigated our detailed analysis of the function of the Sap propeptide in the secretion of the mature enzyme. Our study on *C. albicans* Sap1p was carried out using the methylotrophic yeast *Pichia pastoris* expression system, which allows the production of substantial amounts of recombinant fungal proteinase (Borg-von Zepelin *et al.*, 1998). We show here that recombinant Sap1p maturation is guided by the propeptide in a combination of intra- and intermolecular processes, and that only a 12 aa sequence in the propeptide is necessary and sufficient to completely ensure the secretion of active enzyme.

METHODS

Organisms and plasmids. *Escherichia coli* DH5 α was used for transformation and propagation of the recombinant plasmids. The *E. coli*-*P. pastoris* shuttle vector pPICZ α A was provided with the *P. pastoris* expression system from Invitrogen. The low-copy-number plasmid pKJ113 has been previously described (Borg-von Zepelin *et al.*, 1998). The *P. pastoris* strains GS115 and KM71 (Invitrogen) were used as transformation hosts.

Construction of the expression plasmids. Expression plasmids were constructed by cloning a SAP PCR product in the multiple cloning site of pKJ113 and pPICZ α A. Custom-made primers were provided by Microsynth (Balgach, Switzerland). PCR buffers and AmpliTaq polymerase were from Perkin Elmer Applied Biosystems. The buffer composition was 10 mM Tris/HCl (pH 8.3), 50 mM KCl with 1.5 mM MgCl₂, containing 0.2 mM each dNTP and 2.5 U polymerase per reaction. The PCR was carried out in a GeneAmp PCR system 2400 (Perkin Elmer Applied Biosystems) with a first denaturation step of 5 min at 94 °C followed by 25 cycles of annealing at 55 °C for 30 s, elongation at 72 °C for 30 s and denaturation at 94 °C for 30 s. PCR was completed by a final elongation step at 72 °C for 10 min. DNA from plasmids containing individual SAP genes was used as template. The PCR products were purified using a PCR purification kit (Roche Diagnostics) and were digested by restriction enzymes for which a site (*Xho*I, *Bam*HI or *Not*I) was previously designed at the 5' extremity of the primers. The digested PCR products were then cloned into the appropriate sites of the multiple cloning site of the *E. coli*-*P. pastoris* shuttle vector.

The following pairs of sense and antisense primers were used to construct plasmids pSB10, pSB11, pSB42 and pSB249 (Tables 1 and 2), respectively, restriction sites underlined: GATGCTCGAGCAGCTAAAAGATCCCCAGGT and CA-AAGGATCCTAGGTAAGAGCAGCAATGTT; GAGC-CTCGAGAAAGCCATCCCAGTTACTTTAAAT and CAA-AGGATCCTAGGTAAGAGCAGCAATGTT; TTC-CTCGAGAAAAGATCTCCAGCTAAAAGATCC and GT-AGCGGCCGCTCTATCTTTTAACTTTACCTTCTTG; GCACTCGAGAAAAGATCTCCAGCTAAAAGATCCCCAGG and GCAGGATCCCTAGTGATGGTGATGGTG-ATGGGTAAGAGCAGCAATGTT. Plasmidic SAP1 DNA (Hube *et al.*, 1991; Monod *et al.*, 1994) was used as template. pKJ113 was used to generate pSB10 and pSB11, and pPICZ α A was used to generate pSB42 and pSB249.

Plasmids expressing N- or C-terminally truncated Sap1p propeptide were constructed after PCR amplification of pSB42 fragments with appropriate homologous primers. The PCR products were used to replace the corresponding part encoding the full-length propeptide in pSB42. Subsequently, plasmids expressing Sap1p propeptide truncated at both extremities were constructed in a similar way using previously constructed plasmids encoding Sap1p propeptide truncated at one extremity (see Fig. 4). Plasmids expressing other Sap propeptides were constructed as pSB42 using different SAP DNA as a template (Togni *et al.*, 1991; de Viragh *et al.*, 1993; Monod *et al.*, 1994). All cloned fragments were further sequenced to confirm the absence of possible PCR induced errors.

In vitro mutagenesis. For construction of pSB269, the following pairs of sense and antisense primers were used to amplify two contiguous fragments of pSB10: TTGGAGCTC-GCTCATTCCAAT and AGTGACGTCACCTGGGGATC-TTTTAGCTGG; GGTGACGTCACCTTTAGACTTTGAT-GTC and TTAGCGGCCGCTCTAGGTAAGAGCAGCA-ATGTT. Subsequently, the 0.8 kb *Sac*I-*Not*I fragment of pKJ113 was excised and replaced by adding end-to-end the two PCR products digested by *Sac*I/*Aat*II and *Aat*II/*Not*I.

***P. pastoris* transformation.** *P. pastoris* was transformed by electroporation with 5–10 μ g linearized plasmid DNA digested by *Sma*I using pKJ113 constructs, and *Sac*I or *Bst*XI using pPICZ α A constructs. The His⁻ Mut⁺ *P. pastoris* strain GS115 was transformed with pKJ113 constructs, and transformants

were selected on histidine-deficient medium [1 M sorbitol, 1% (w/v) glucose, 1.34% (w/v) yeast nitrogen base (YNB) without amino acids, 4×10^{-5} % (w/v) biotin, amino acids (0.005%, w/v, each of L-glutamic acid, L-methionine, L-lysine, L-leucine and L-isoleucine), 2% (w/v) agarose]. Subsequently, the transformants were screened for insertion of the construct at the AOX1 site on minimal methanol plates [1.34% (w/v) YNB without amino acids, 4×10^{-5} % (w/v) biotin, 0.5% (v/v) methanol, 2% (w/v) agarose]. Transformants unable to grow on media containing only methanol as a carbon source were retained for further investigations. They were assumed to contain the construct at the correct yeast genomic location by integration events in the AOX1 locus displacing the AOX1 coding region. The prototrophic *P. pastoris* strains were transformed with pPICZ α A constructs and transformants were selected on YPDS medium containing 100 μ g zeocin ml $^{-1}$ (Invitrogen).

Production of Sap1p in *P. pastoris*. All selected transformants were grown to near saturation (OD $_{600}$ 10) at 30 °C in 10 ml glycerol-based yeast medium [0.1 M potassium phosphate buffer pH 6.0, containing 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) YNB without amino acids, 1% (v/v) glycerol and 4×10^{-5} % (w/v) biotin]. Cells were harvested by centrifugation and resuspended in 2 ml of the same medium with 0.5% (v/v) methanol instead of glycerol and incubated for 2 d. Thereafter, the supernatants and the cell pellets were separated by centrifugation and retained for protein analysis.

Protein extract analysis. The proteins in 5 or 10 μ l of *P. pastoris* culture supernatant were loaded without further treatment onto SDS-PAGE gels (Laemmli, 1970). Cell protein extracts of 0.1 ml culture were prepared following the method of Yaffe & Schatz (1984). The pelleted cells were 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% TCA, the mixture was incubated for 5 min, centrifuged, and the pellet was resuspended in 50 μ l 2 $\times$ SDS-PAGE loading buffer and neutralized at pH 7.0 by adding 5–10 μ l 1 M Tris base. The samples were heated to 95 °C for 3 min before loading onto SDS-PAGE gels (12% acrylamide).

Untagged Sap1p was separated from His $_6$ -tagged Sap1p (H $_6$ -Sap1p) by filtration of the *P. pastoris* culture supernatant through a nickel chelating resin column (ProBond, Invitrogen) equilibrated with 10 mM phosphate buffer, pH 7.0. After washing the column with the same buffer, adsorbed H $_6$ -Sap1p was eluted with 300 mM imidazole buffer, pH 6.0. All fractions with detectable enzymic activity were retained and pooled. Sap1p was purified from filtered *P. pastoris* culture supernatant as previously described (Borg-von Zepelin *et al.*, 1998). Protein concentrations were measured by the method of Bradford (1976).

SDS-PAGE gels were stained with Coomassie brilliant blue R-250 (Bio-Rad). Immunodevelopment of Western blots was performed using an antiserum (α -Sap2) raised in rabbits and cross-reacting with Sap1p (Borg-von Zepelin *et al.*, 1998), and alkaline-phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad), or using an anti-polyhistidine peroxidase conjugate monoclonal antibody (Sigma).

Proteolytic assays. The proteolytic activity of Sap isoenzymes was measured with 0.02% (w/v) resorufin-labelled casein as a substrate (Roche Diagnostics) in sodium citrate buffer (50 mM; pH 4.5) in a total volume of 0.25 ml. After incubation at 37 °C for 60 min, the undigested substrate was precipitated by TCA acid (5% final concentration) and separated from the supernatant by centrifugation. The absorbance of the super-

natant was measured in the alkaline range at 574 nm after adding 250 μ l 1 M Tris/HCl at pH 10. For practical purposes, one unit of Sap1p activity was defined as that producing an absorbance of 0.001 per min.

RESULTS

Importance of the propeptide for secretion of recombinant Sap1p by *P. pastoris*

In a preliminary experiment, *P. pastoris* was used to express the gene *SAP1* with and without the nucleotide sequence encoding the propeptide. *SAP1* DNA fragments (bp 59–1176 and bp 152–1176 of the open reading frame) were cloned into pKJ113 downstream of the *PHO1* signal peptide sequence, generating the plasmids pSB10 and pSB11, respectively (Table 1). Plasmid pKJ113 is a low-copy-number *E. coli* vector which contains the *P. pastoris* pHIL-S1 expression cassette. The pHIL-S1 constructs insert into the *P. pastoris* genome via homologous recombination at the AOX1 site and carry, in addition to the cloned coding sequence of interest, the *HIS4* gene for selection after transformation of *P. pastoris* GS115 with linearized plasmidic DNA. The resulting His $^+$ Mut $^-$ transformants were selected and screened for proteinase production. *C. albicans* Sap1p was recovered as a single protein and an active enzyme in the *P. pastoris* culture supernatants in all transformants with a yield of approximately 350 U ml $^{-1}$ (50 μ g ml $^{-1}$) when its encoding nucleotide sequence was expressed behind that of its propeptide. Direct expression of the *SAP1* gene without its propeptide did not lead to secretion of recombinant protein into the culture supernatant of *P. pastoris* transformants. An accumulation of Sap1p in the cell was demonstrated by Western blotting of total protein extracts immunodeveloped with anti-Sap antibody (Fig. 1). One strain secreting and one strain accumulating Sap1p were selected, and labelled SB10 and SB11, respectively, in subsequent investigations (Table 1).

Rescue of Sap1p secretion by unlinked propeptides

The nucleotide sequence encoding the Sap1p propeptide was cloned into plasmid pPICZ α A downstream from the α -factor signal peptide sequence generating the plasmid pSB42 (Table 2). This construction was performed in order to provide an access into the secretory pathway for the propeptide as a separate protein. *P. pastoris* SB11, which does not secrete Sap1p, was transformed with linearized pSB42 DNA, and clones resistant to zeocin were further tested for proteinase production. *C. albicans* Sap1p was recovered as a single protein and an active enzyme in all transformant culture supernatants with a yield comparable to that of strain SB10. Therefore, co-expression in *P. pastoris* of a non-covalently attached propeptide could rescue the lack of *cis*-expressed propeptide.

Five transformants were retained for restriction fragment analysis. Genomic DNA of the yeasts was digested with *SacI*, *DraI* and *HindIII*, electrophoresed and probed

Table 1. *P. pastoris* GS115 and KM71 transformants generated in this study

The *SAP1* translation products produced with a full length propeptide, without propeptide, with a non-functional propeptide (F27→D mutation) and with a C-terminal His₆ tag are abbreviated as Pre-Pro-Mat, Pre-Mat, Pre-*Pro*^{*}-Mat and Pre-Pro-Mat-H₆, respectively.

Strain	Transformed original strain	Plasmid	<i>SAP1</i> translation product	Secreted proteolytic activity (U ml ⁻¹)
SB10	GS115	pSB10	Pre-Pro-Mat	352
SB11	GS115	pSB11	Pre-Mat	0
SB269	GS115	pSB269	Pre- <i>Pro</i> [*] -Mat	0
SB249	KM71	pSB249	Pre-Pro-Mat-H ₆	324

Fig. 1. Detection of Sap1p from *P. pastoris* SB10, SB11 and SB269. The proteins in 5 µl of supernatant (S) without further treatment and in cell extracts (C) from 5 µl of methanol-induced culture were separated by SDS-PAGE and analysed by Western blotting.

with the larger *Bam*HI–*Bgl*III fragment of plasmid pPICZαA containing the *Streptoalloteichus hindustanus* BLE gene for resistance to zeocin (Gatignol *et al.*,

1988; Drocourt *et al.*, 1990) (Fig. 2). The band pattern of transformants secreting Sap1p, which showed a hybridizing fragment of identical size (3.8 kb) for each of the restriction digests, was consistent with the correct integration of pPICZαA constructs at the locus of the *AOX1* promoter of the SB11 strain (data not shown). In our genetic construction, DNA encoding Sap1p propeptide and DNA encoding Sap1p without its propeptide are in tandem under the control of *pAOX1* (Fig. 2).

In a similar experiment, we tested whether the secretion defect of Sap1p in *P. pastoris* SB11 could also be rescued by providing other secreted proteinase propeptides as a separate protein (Table 2, Fig. 3). Sap1p was recovered as a single protein and an active enzyme in the *P. pastoris* culture supernatants with a yield varying from 128 to 320 units, corresponding to 16 to 40 µg ml⁻¹, using all Sap propeptides tested. The specific activities of the Sap1p enzymes secreted by the different strains were the same.

Using pSB42, we constructed a series of plasmids encoding the Sap1p propeptide deleted at the N- or the C-terminus, or both (Fig. 4) in order to determine the

Table 2. Sap propeptides used as a separate protein to rescue Sap1p secretion in strain SB11, and Sap1p activity of SB11 transformants

Plasmid (encoded propeptide)	Sap propeptide aa sequence	Sap1p activity of SB11 transformants (%) [*]
pSB42 (Sap1p propeptide)	SPAKR..SP↓GFVTLDFFDIKT.....FVNATGQEGKV..KR	100
pSB104 (Sap2p propeptide)	TPTTTKR..SAGFVALDFSVVKT.....PKAFPVNTGQEGKTS..KR	82 ± 4
pSB105 (Sap3p propeptide)	TPTTFNN..SPGFVALNFDVIKT.....HKNVTGPQGEINTNVNV..KR	61 ± 5
pSB106 (Sap6p propeptide)	APVKR..SPGFVTLDFFNVKRSLVVPDDPTAESKRSPLFLDLDPQTQIPVDDTGRNVGVDKR	78 ± 4
pSB107 (Sapp2p propeptide)	GAAIPD.PAKRDDNP↓GFVALDFEVTRK.....PLDVNATSELS..KR	40 ± 1
pSB108 (Sapt1p propeptide)	GLTIPDGIEKRTDK..VVSLEDFTVIRK.....PFNATAHRLIQ..KR	44 ± 4

^{*} The percentage values refer to the activity of SB11 transformed with pSB42 encoding the Sap1p propeptide. The value of 100% corresponds to 320 U ml⁻¹. The values shown are mean activities with standard deviation obtained from three experiments. No proteolytic activity was detected in SB11 culture supernatant.

The arrow indicates the F residue substituted by a D in strain SB269.

Following the results of the propeptide deletion analysis, a mutation was introduced in plasmid pSB10 to replace the phenylalanine (F) residue 27 of the *SAP1* translation product, which is conserved in different Sap propeptides (Table 2), by an aspartic acid (D) residue. The newly generated plasmid was called pSB269 (Table 1). Direct expression of the mutated *SAP1* gene in *P. pastoris* GS115 did not result in the secretion of Sap1p into the culture supernatant. However, a Sap1p product with an electrophoretic mobility higher than that of the mature form of Sap1p was detected in all transformants, from among which strain SB269 was selected (Table 1, Fig. 1).

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Propeptide	Sap1p activity (%)
SPAKRSPGFVTLDFDVIKTPVNATGQEGVKR	100
KRSPGFVTLDFDVIKTPVNATGQEGVKR	97 ± 4
PGFVTLDFDVIKTPVNATGQEGVKR	94 ± 7
GFVTLDFDVIKTPVNATGQEGVKR	84 ± 6
FVTLDFDVIKTPVNATGQEGVKR	57 ± 1
VTLDFDVIKTPVNATGQEGVKR	0
SPAKRSPGFVTLDFDVIKTPVNATGQ	98 ± 5
SPAKRSPGFVTLDFDVIKTPVN	100 ± 8
SPAKRSPGFVTLDFDVIKTPV	99 ± 5
SPAKRSPGFVTLDFDVIKTP	84 ± 7
SPAKRSPGFVTLDFDVIKT	80 ± 10
SPAKRSPGFVTLDFDVIK	67 ± 1
SPAKRSPGFVTLDFDVI	28 ± 3
SPAKRSPGFVTLDFDV	0
SPAKRSPGFVTLDFD	0
GFVTLDFDVIKTPVN	98 ± 7
GFVTLDFDVIKTPV	94 ± 5
GFVTLDFDVIKTP	80 ± 3
GFVTLDFDVIKT	84 ± 9
GFVTLDFDVIK	37 ± 3
GFVTLDFDVI	19 ± 1
FVTLDFDVIKTPVN	71 ± 4
FVTLDFDVIKTPV	63 ± 4
FVTLDFDVIKTP	46 ± 1
FVTLDFDVIKT	38 ± 11
FVTLDFDVIK	24 ± 5
FVTLDFDVI	10 ± 1

Fig. 4. N- and/or C-terminally truncated Sap1p propeptides encoded by pSB42 derivatives, and Sap1p activity of SB11 transformants. The first line shows the full-length Sap1p propeptide encoded by pSB42. The Sap1p activity of SB11 transformed with pSB42 (320 U ml⁻¹) was set to 100%. The values shown are the mean activity with standard deviation obtained from three experiments.

SB10 transformants was twice that of the SB10 strain. Untagged Sap1p could be separated from H₆-Sap1p by filtration of the *P. pastoris* culture supernatant through a nickel chelating resin column (Fig. 5, lanes 11 and 12). No H₆-Sap1p was detected in the culture supernatant after filtration through the column (Fig. 5, lane 12). Adsorbed H₆-Sap1p could be subsequently released with imidazole for quantification.

When *P. pastoris* SB11 producing Sap1p without propeptide and SB269 producing Sap1p with a mutated propeptide were transformed by plasmid pSB249, Sap1p and H₆-Sap1p were again both secreted in the culture supernatants of all transformants, but in a ratio close to 1:2 (54:100) (Fig. 5, lanes 4–9). Therefore, H₆-Sap1p encoded by pSB249 permitted a restoration of secretion of Sap1p made without propeptide and with a mutated propeptide in the SB11 and SB269 transformants,

Fig. 5. (a) Protein preparations from the culture supernatants of *P. pastoris* KM71, SB11, SB269 and SB10 transformed with pSB249. A 5 ml sample of *P. pastoris* culture supernatant was filtered through a 1.0 ml nickel chelating resin column as described in Methods. After washing of the column, the adsorbed H₆-Sap1p was eluted with imidazole buffer. All fractions with detectable enzymic activity were retained and pooled and the volume was adjusted to 5.0 ml for comparison with the culture supernatant filtrate. Lanes 1, 4, 7, 10: culture supernatant loaded without further treatment. Lanes 2, 5, 8, 11: H₆-Sap1p eluted from the nickel column. Lanes 3, 6, 9, 12: Sap1p in the culture supernatant after filtration through the column. Ten microlitres of each preparation was analysed by SDS-PAGE. A, gel stained with Coomassie brilliant blue; B, immunodetection of Sap1p using anti-polyhistidine monoclonal antibody. Only H₆-Sap1p was detected. (b) Total activities of each preparation.

respectively. However, this restoration occurred at the expense of secretion of H₆-Sap1p since the proteolytic activity of SB11 and SB269 transformants was similar to that of *P. pastoris* KM71 transformed with pSB249, but not higher. Furthermore, an accumulation of H₆-Sap1p product with an electrophoretic mobility higher than that of Sap1p was observed in cell extracts of SB11 and SB269 transformants but not in those of KM71 and SB10 transformants (Fig. 6).

DISCUSSION

When not covalently attached to the mature domain, only a 12 aa segment of the propeptide was necessary and sufficient to ensure complete Sap1p secretion. The

Fig. 6. Detection of Sap1p and H₆-Sap1p intracellular products from strains KM71 (1), SB11 (2), SB269 (3) and SB10 (4) transformed with pSB249. The proteins in cell extracts from 5 µl of methanol-induced culture were separated by SDS-PAGE and analysed by Western blotting using anti-Sap polyclonal antibody (a) and anti-polyhistidine monoclonal antibody (b). Cell extracts of SB10 (5) and SB11 (6) strains and 5 µl of SB249 culture supernatant (*) were added on the gels for comparison of molecular masses of Sap1p products.

amino acid sequences of the *Candida* Saps show considerable homology to each other and this 12 aa segment corresponds to a highly conserved region in all Sap propeptides (Table 2). Consistently, the secretion defect of Sap1p in *P. pastoris* SB11 could be rescued by other Sap propeptides expressed as separate proteins, albeit with reduced efficiency when the propeptides had reduced homology. The activity detected in the culture supernatant was proportional to the amount of secreted protein (Fig. 3). We conclude that all Sap1p molecules in the culture supernatants were enzymically active. In another set of experiments, we found that the 12 aa homologous sequence in the Sap6p propeptide was also sufficient for the maturation of Sap6p (unpublished data). The requirement for only a short segment of the propeptide appears to be a characteristic of Sap enzymes. In contrast, a relatively long domain in the propeptide is essential for assisting the correct folding and the secretion of the mature bacterial subtilisins, since pro-subtilisin molecules with unique point mutations distributed throughout the whole propeptide (in positions -76 to -13) did not yield active subtilisin (Lerner *et al.*, 1990; Inouye, 1991).

The fate of eukaryotic proproteinases mutated in their prosequence was previously investigated with the *Yarrowia lipolytica* alkaline proteinase and the *Rhizopus niveus* aspartic proteinase I (Fabre *et al.*, 1991; Fukuda *et al.*, 1996). All deletions affecting the proregion of the *Y. lipolytica* alkaline proteinase precursor resulted in the intracellular accumulation of unprocessed protein. The rates of synthesis of one mutated and one pro-deleted precursor of the *R. niveus* aspartic proteinase I were comparable in *Saccharomyces cerevisiae*, but both precursors were degraded in the endoplasmic reticulum. As a general rule, misfolded or unfolded proteins are selectively retained in the endoplasmic reticulum (Helenius *et al.*, 1992), where a more or less rapid degradation occurs depending on the protein. The amount of Sap1p product detected intracellularly when Sap1p was produced with a mutated propeptide or

without a propeptide was reduced, which suggests protein degradation (Fig. 1). It is likely that the non-functional folding of Sap1p results in the degradation of its mature domain, which consequently causes the lack of secretion of the Sap1p molecules. When *SAP1* was expressed without its propeptide sequence an accumulation of Sap1p product with the same electrophoretic mobility as secreted Sap1p was observed in the cell. In contrast, an accumulation of a Sap1p product with an electrophoretic mobility higher than Sap1p was observed when *SAP1* was expressed with a sequence encoding a mutated propeptide (Fig. 1). A similar His₆-tagged product was detected in cell extracts of pSB249 transformants of SB11 and SB269 in parallel with the rescue of Sap1p secretion (Fig. 6, lanes 2 and 3). It appears that Sap1p produced without a propeptide and Sap1p produced with a mutated propeptide are not degraded in the same way.

The proteolytic activity of the SB11 and SB269 transformants secreting two Sap1ps produced with and without a functional propeptide under the control of two identical promoters was similar to that of KM71 transformed with pSB249 (Fig. 5). The lack of increased proteolytic activity in these transformants producing two different proteins can only be explained by a single-turnover catalytic property of the propeptide, meaning that each propeptide only participates in a single protein-folding reaction (Ohta *et al.*, 1991; Baker *et al.*, 1992).

If folding of the Sap1p molecules were obligatorily assisted by their propeptide *in cis* (first-order reaction, Fig. 7a), only H₆-Sap1p would have been secreted by the SB11 and SB269 transformants. Therefore, as our experiments demonstrate, there must be an inter-molecular pathway to mature Sap1p without a priori exclusion of a possible intramolecular one. It was previously shown that prosubtilisin exists as dimers under non-denaturing conditions *in vitro* (Hu *et al.*, 1996). A model of enzyme maturation was postulated where the prosequence of one prosubtilisin molecule is

Fig. 7. Model for intra- and intermolecular events in Sap1P maturation in *P. pastoris* SB269 transformed with pSB249. The F27→D mutation in the Sap1p propeptide is indicated by an oblique line across the propeptide. (a) First-order reaction. The folding of an enzyme molecule is guided by its own propeptide. Using such a mechanism, only H₆-Sap1p is matured. (b) Second-order reaction. Assuming that Sap1p and H₆-Sap1p are synthesized in a ratio of 1:1, intracellular Sap1p homodimer-, Sap1p/H₆-Sap1p heterodimer- and H₆-Sap1p homodimer-formations should occur in a 1:2:1 ratio. In this model, the homodimers made of H₆-Sap1p with an active propeptide generate two molecules of secreted active enzyme. The homodimers made of Sap1p with a mutated and non-functional propeptide generate no active enzyme, while heterodimers generate one molecule of Sap1p. Therefore, a strict intermolecular mechanism would predict a ratio of H₆-Sap1p to Sap1p of 1:1 in the culture supernatant.

the template for the refolding of the mature sequence of the second one (second-order reaction). A strict intermolecular mechanism would predict a ratio of H₆-Sap1p to Sap1p of 1:1 in the culture supernatant of SB11 and SB269 transformants assuming that Sap1p and H₆-Sap1p are synthesized in a ratio of 1:1 (Fig. 7b). In fact, the ratio of H₆-Sap1p to Sap1p was experimentally determined to be close to 2:1. This result leads to the conclusion that maturation of Sap1p occurs in *P. pastoris* through both intra- and intermolecular pathways (Fig. 7). The observation of an intracellular accumulation of a H₆-Sap1p product in the SB11 and SB269 transformants (Fig. 6) is consistent with the prediction that H₆-Sap1p molecules do not mature without a propeptide or with a non-functional propeptide in heterodimers (Fig. 7).

This is the first report demonstrating that the maturation of an enzyme *in vivo* can be guided by the propeptide in an intermolecular process. The strategy developed in this work for Sap1p secretion could be used to test whether maturation of other proteinases via a combination of intra- and intermolecular pathways is a general pattern.

ACKNOWLEDGEMENTS

S. Beggah performed this work in partial fulfilment of the requirements for a PhD degree from the University of Lausanne (Switzerland). We thank J. Wyniger and C. Zaugg for technical assistance, and Dr M. Holdom, Dr H. Pooley, Dr E. Grouzmann and Dr D. Sanglard for critical review of the manuscript and assistance with the English. This work was

supported by the Swiss National Foundation for Scientific Research, grant 3100-043193.

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Received 13 April 2000; revised 24 July 2000; accepted 9 August 2000.