Cloning and sequencing of two *Candida parapsilosis* genes encoding acid proteases

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Candida parapsilosis secretes an inducible acid protease (ACP) when cultivated in the presence of bovine serum albumin as the sole nitrogen source. In order to clone the ACP gene (*ACP*) of *C. parapsilosis*, a genomic library was screened with *C. tropicalis* ACP as the probe. Two different ORFs, *ACPR* and *ACPL*, were found to hybridize with the *C. tropicalis* ACP. *ACPR* contained a DNA sequence in agreement with the N-terminal amino acid sequence of *C. parapsilosis* ACP isolated from culture supernatants. *ACPR* was shown to be expressed and functional in a *C. tropicalis* acid protease mutant (*acp*) and with SDS-PAGE the protein product showed the same mobility as the ACP secreted by *C. parapsilosis*. These results imply that *ACPR* encodes the *C. parapsilosis* ACP. The deduced amino acid sequence of *ACPR* is similar to the amino acid sequence of proteases of the pepsin family. As in the case of the *C. tropicalis* and *C. albicans* ACP, the 5' extremity of *ACPR* revealed a propeptide containing two Lys-Arg amino acid pairs that have been identified as peptidase processing sites in several yeast-secreted peptides and protein precursors. As judged from the deduced amino acid sequences, the *ACPL* product would be similar to that of *ACPR*; however, a protein corresponding to *ACPL* was not found in supernatants from *C. parapsilosis* liquid cultures. In addition, *ACPL* did not complement the *C. tropicalis acp* mutant. We conclude that *ACPL* is a pseudogene or serves an as yet unidentified function.

Introduction

Candida albicans, *C. tropicalis* and *C. parapsilosis*, three opportunistic yeast species of medical interest, secrete acid proteases (ACP) *in vitro*, when proteins are the sole nitrogen source in the medium (Rüchel *et al.*, 1983). Individual ACPs from the three species have been characterized at the protein level (Remold *et al.*, 1968; MacDonald & Odds, 1980; Rüchel, 1981; Negi *et al.*, 1984; Rüchel *et al.*, 1986; Shimizu *et al.*, 1987; Ray &

Payne, 1990). *C. tropicalis* and *C. albicans* ACP are 41–43 kDa glycoproteins with a pI of 4·5. *C. parapsilosis* ACP has a lower apparent molecular mass of 33 kDa and a higher pI of 5·7 (Rüchel *et al.*, 1986). *Candida* acid proteases have optimal activity between pH 4·0 and 4·5 and are inactivated below pH 2·5 and above pH 6·0. These enzymes have a broad substrate specificity including keratin, denaturated collagen, haemoglobin and bovine serum albumin. They are inactivated by pepstatin and consequently belong to the pepsin family. Results of immunological studies suggest the existence of common and specific domains among the acid proteases of the three species (Rüchel *et al.*, 1986). Recently, the genes coding for ACP of *C. albicans* and *C. tropicalis* were isolated and sequenced (Hube *et al.*, 1991; Togni *et al.*, 1991). The protein sequences deduced from the two cloned genes both include regions of homology to the active sites of proteases of the pepsin family.

C. parapsilosis like other *Candida* species, causes fungemia, but is more prevalent in patients with solid tumours (Horn *et al.*, 1985; Komshian *et al.*, 1989).

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Abbreviations: ACP, extracellular acid protease; *ACP*, extracellular acid protease gene; PAS, periodic acid–Schiff reagent. *ACPR* and *ACPL* designate the two *C. parapsilosis* open reading frames described in this work.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession numbers Z11918 (*ACPL*) and Z11919 (*ACPR*).

Studies on *Candida* ACP are expected to elucidate the pathogenicity of these organisms. For *C. albicans* and *C. tropicalis*, proteolytic activity may be associated with tissue invasion, since ACP is expressed at the surface of the fungus during invasion (Rüchel *et al.*, 1991) and preliminary studies for *C. albicans* have shown a correlation between proteolytic activities and virulence (MacDonald & Odds, 1983; Kwon-Chung *et al.*, 1985; Ross *et al.*, 1990). Although *C. parapsilosis* secretes a large amount of ACP *in vitro*, this species is much less invasive than *C. albicans* and *C. tropicalis* (Bistoni *et al.*, 1984). This discrepancy could be related to the expression of ACP in macrophages after phagocytosis of yeast since *C. albicans* and *C. tropicalis*, but not *C. parapsilosis*, express ACP on the surface of yeast cells in macrophages after phagocytosis (Borg & Rüchel, 1990).

We report the cloning and the nucleotide sequence of two tandemly arranged *C. parapsilosis* acid protease genes, *ACPR* and *ACPL*. *ACPR* encodes the *C. parapsilosis* ACP found in the supernatant of liquid cultures, and *ACPL* is a putative pseudogene or serves a function as yet unidentified. The cloned genes will help clarify the contribution of fungal secreted proteases to virulence and can be used for molecular studies of ACP expression following phagocytosis.

Methods

Strains and plasmids. Ten strains of *C. parapsilosis* were isolated from patients at the Centre Hospitalier Universitaire Vaudois (CHUV) and were maintained on Sabouraud agar medium. *C. tropicalis* ATCC 750 *ade2/ade2*, *acpΔ::λ/acpΔ::λ* (strain DSY59) is homozygous for the disrupted ACP gene (Sanglard *et al.*, 1992). The plasmid used to transform *C. tropicalis* DSY59, pMK16, was obtained from the Squibb Institute for Medical Research (Princeton, New Jersey, USA) and was described in Kurtz *et al.* (1987). *C. albicans* Ca74 is a clinical strain isolated at the CHUV (Togni *et al.*, 1991). *E. coli* LE392 was used for the propagation of bacteriophage λ EMBL3 (Promega). All plasmid subcloning experiments were performed in *E. coli* strain DH5α using the plasmid pMTL21 (Chambers *et al.*, 1988).

Liquid cultures. Yeast were grown at 30 °C in Sabouraud liquid medium or, to promote production of ACP, in 1.2% (w/v) yeast carbon base (YCB) medium supplemented with 0.2% BSA and adjusted to pH 4.0 with 1 M-HCl. The latter medium was sterilized by filtration.

ACP production on solid medium. The medium contained 1.2% (w/v) YCB, 0.2% BSA and 1.5% (w/v) agar. Nine hundred millilitres of a solution containing YCB and agarose was adjusted to pH 4.0 with 1 M-HCl, and sterilized by autoclaving at 120 °C. BSA (2.0% w/v, 100 ml) was sterilized by filtration and added to cooled YCB-agarose to form a milky medium which was poured into Petri dishes. Cultures were incubated for 4 d at 30 °C and ACP activity was observed as a clearing zone around the colonies. Agar plates were subsequently stained with amido black 0.1% in acetic acid/methanol/water (10:25:65, by vol.) and destained with 10% (v/v) acetic acid to confirm proteolytic activity around the colonies.

Enzyme purification and N-terminal amino acid sequence. Supernatants were separated from yeast cultures (500 ml) by centrifugation

at 5000 g. Ammonium sulphate was dissolved in the supernatants at room temperature to 65% saturation. The precipitated proteins were collected by centrifugation and resuspended in distilled water at 1/100 of the original volume. Insoluble material was removed by centrifugation at 5000 g for 5 min and the supernatant was dialysed against 15 mM-sodium citrate (pH 5.6) for 2 h. After reduction of the volume to 5 ml by ultrafiltration (Ultracent-30 system, BioRad), the enzyme solution was chromatographed on a column of polyacrylamide P60 gel in 20 mM-sodium citrate buffer (pH 5.6). Fractions with enzymic activity were retained and pooled.

Protease activity was determined with azoalbumin as substrate as described (Sanglard *et al.*, 1992). Protein concentration was measured by Lowry's method with BSA as the standard. SDS-PAGE was performed as described by Laemmli (1970), using 9% (w/v) polyacrylamide gels. Gels were either stained with Coomassie Brilliant Blue R-250 or, for glycoproteins, with the periodic acid-Schiff reagent (PAS) (Zacharius *et al.*, 1969). Determination of the N-terminal amino acid sequence of ACP was performed as described previously by Jaton-Ogay *et al.* (1992).

Immunological methods. Preparation of anti-ACP immune sera and immunoblotting were performed as described by Monod *et al.* (1991).

Construction of the genomic library. A genomic DNA library was prepared using DNA from the *C. parapsilosis* isolate CHUV E18. Genomic DNA (Sanglard *et al.*, 1992) was partially digested with *Sau3A*, and DNA fragments of 12–20 kb were isolated from low melting agarose (BioRad) (Sambrook *et al.*, 1989). These fragments were inserted into bacteriophages using the λ EMBL3 *Bam*HI arm cloning system (Promega).

Screening of the genomic library. Approximately 50000 recombinant plaques of the genomic library were immobilized on nylon membranes (Zeta-Probe, BioRad). The filters were hybridized with ³²P-labelled *C. tropicalis* ACP probes (Togni *et al.*, 1991), in a solution containing 5 × SSC, 7% (w/v) SDS, 10 × Denhardt's, and 20 mM-sodium phosphate (pH 7.0), at 50 °C for 24 h. The membranes were exposed to X-ray film after a first wash in 3 × SSC, 5% (w/v) SDS, 25 mM-sodium phosphate, pH 7.0, and a second wash in 1 × SSC, 1% (w/v) SDS, at 50 °C. Positive plaques were purified and the DNA was isolated as described (Grossberger, 1987). Agarose gel electrophoresis of restricted recombinant bacteriophage λ EMBL3 DNA and Southern blotting were performed according to standard protocols (Sambrook *et al.*, 1989). DNA probes were labelled by random primer extension using a Boehringer kit and [α³²P]-dCTP (Amersham).

DNA sequencing. Double stranded DNA subcloned into plasmid pMTL 21 (Chambers *et al.*, 1988) was sequenced using a Sequenase version 2.0 sequencing kit (USB) following the supplier's instructions. DNA was annealed with the reverse primer (Biofinex, Praroman), SL primer (USB) or synthetic oligonucleotide primers (Microsynth, Windisch).

DNA transformation. *E. coli* was transformed using competent cells with standard protocols (Sambrook *et al.*, 1989). *C. tropicalis* was transformed by protoplasting as described by Sanglard *et al.* (1992).

Protein comparisons. Identity and similarity scores (%) between *Candida* acid proteases, were calculated using the algorithm of Needleman & Wunsch (1970) implemented in the GCG Wisconsin program.

Results

Purification and properties of the *C. parapsilosis* ACP

All 10 strains of *C. parapsilosis* examined exhibited comparable proteolytic activities on BSA agar plates,

Fig. 1. (a) PAS positive staining of *C. parapsilosis* E18 (lane 1), *C. tropicalis* ATCC 750 (lane 2) and *C. albicans* Ca74 (lane 3) ACP. (b) As (a) but the gel was stained with Coomassie Brilliant Blue R-250. Proteins were precipitated from the culture supernatant with 65% saturated ammonium sulphate and separated by SDS-PAGE (9% gels, w/v). M, molecular mass markers: phosphorylase B, 97 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; bovine carbonic anhydrase, 31 kDa.

thus, protease purification and characterization was performed only with one strain (CHUV E18).

In a typical experiment, a total of 15000 U of proteolytic activity was produced in one litre of BSA medium after 4 d growth at 30 °C. The *C. parapsilosis* ACP was purified as described in Methods, with a yield of 50%. A single peak of proteolytic activity was obtained after P60 gel chromatography that corresponded to the major peak from the gel column. The purified enzyme showed a single protein band in SDS-PAGE gel at 37 kDa, corresponding to the molecular mass of the protease obtained by P60 gel chromatography. Staining with PAS reagent indicated that this enzyme was a glycoprotein (Fig. 1a). The enzyme was a major protein secreted by the fungus as shown by the protein profile of the 65% saturated ammonium sulphate precipitate of the culture supernatant. The initial 15 amino acid residues of the N-terminus were determined to be Asp-Ser-Ile-Ser-Leu-Ser-Leu-Ile-Asn-Glu-Gly-Pro-Ser-Tyr-Ala. The ACP of *C. parapsilosis* was related to ACP of *C. albicans* and *C. tropicalis*. Using a polyclonal antibody raised against the *C. parapsilosis* ACP, crossreactivities with the ACP from *C. albicans* and *C. tropicalis* could be observed in immunoblotting experiments (data not shown).

Cloning and sequencing of the putative *C. parapsilosis* ACP gene

A 2 kb *Bgl*II-*Eco*RI fragment, containing the entire *C. tropicalis* ACP gene (Togni *et al.*, 1991) was used as the probe to screen the *C. parapsilosis* λ EMBL3 genomic library. Four hybridizing clones were identified. Restriction enzyme digestion of purified DNA revealed that these clones carried similar but not identical DNA sequences. However, all four clones contained a 7.6 kb *Hind*III fragment which hybridized to the probe (data not shown). This fragment, for which a restriction map is shown in Fig. 2, was subcloned in pMTL 21 (Chambers *et al.*, 1988), generating the plasmid pMTL 21-H7. Further analysis revealed that the 7.6 kb *Hind*III fragment contained two distinct segments that hybridized with the probe, separated by a 3 kb intervening sequence. Nucleotide sequencing of the two segments revealed two long ORFs of 1206 and 1185 bp, with opposite directions of transcription. The genes for these two ORFs were named *ACPR* and *ACPL* (R and L are relative to their right and left positions on Fig. 2). Their sequences, including the 5' and 3' flanking regions, are shown in Fig. 3. Both ORFs were preceded at -81 (*ACPR*) and -70 (*ACPL*) bp (relative to the first ATG codon) by a TATAAAT sequence which constitutes a possible TATA box (Corden *et al.*, 1980).

Amino acid sequences deduced from *ACPR* and *ACPL*

The amino acid sequences deduced from *ACPR* and *ACPL* showed similarity with other proteases, in particular within three regions which are highly conserved in all members of the pepsin family. Two of these regions contained the two reactive aspartic acid residues of the active site for these enzymes. Both *C. parapsilosis* ORFs suggested the existence of a signal peptide with putative signal peptidase cleavage sites (von Heijne, 1986), indicated by arrows on Fig. 3. Furthermore, pairs of Lys-Arg residues which are known to be proteolytic processing sites in other yeast secretion signal sequences (Julius *et al.*, 1984; Davidow *et al.*, 1987) were found at about position 30 and 60 relative to the Met 1 residue. These sites are also conserved in *C. albicans* and *C. tropicalis* ACP prosequences (Hube *et al.*, 1991; Togni *et*

Fig. 2. Restriction map of the 7.6 kb *Hind*III fragment obtained from positive recombinant bacteriophages. The two ORFs described in this work are represented by horizontal arrows. The two segments for which the sequence is reported in this work are dotted. The fragments inserted in pMK16 to generate pDS20 and pDS21 (Sanglard *et al.*, 1992) are indicated below the map. Symbols: A, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; Hc, *Hinc*II; K, *Kpn*I; N, *Nsi*I; S, *Sac*I; X, *Xho*I; Xb, *Xba*I.

ACPL

AceI

1 GTCTCACACACACACACGCGAGATGCCCGTTGGCTGTGATTCGACCTTGTCAATGACCATATAGAAACAATAGAAACAATAAAGGATGTGATAGTGC 100

101 TCTCACATTTGCTCAGGCGATGTGGTCCCAAGCAAGCGCTTGACAGATCTCTAAATTTGACAGATTCTCTCCACGCGAGAAACGCGACTGCTGCAATGGAAACACA 200

201 AAATCAAAAAATGCCTTCCCACTTTCCCGCTCCACGGCTCTAATTTGTTGTTTTCAGGTTTACAATTTGAACTGGCAATGATTAAGACTGCTGAAGTGA 300

301 TACAATAACAAGACAATGGATTACACGGGCTGTGGCTTAAGCCGCCACATATACCAAGATAGTACTAAACGGGAATCACTTCTGCTCATGGGCTCGTA 400

401 CGGCACCAAGCTGACAAACACCAATTACAAATTCAGTAAATATAAATACCCCATGTGCTCTCTCTTTTCATCCACTGGTCAATGCCATCCATTGCGCAA 500

501 ATAAAAAACGCTATCCATACAATAGTTGGCATCTTGTACCTTAACCTGCTGAATTTTACTACCTACCTGCTGCTGCTTTTGTGCACAAAGTGACGCTATT 600

M V A I V T L T R Q V L L T G I L L A L F A Q G G A I T

20

601 CCAGAGAGGGCTGCTAAAAAGAGCAGCAATCTCGGTTTGTGCTTGGACTTTGATGTGCTTAGGAAACCAATGAATTCAGCGAGGGCTTCTCTCGTG 700

P E E A A K R D N P G F V A L D F D V L R K P L N L T E A L L R

701 AAAAGAGCACTCTCTGTTGCTGTGATCAATGAAGCTGCTATGATCATTAAGTTTTCAGTGGCTTCAAAACAAACAGCAGCAACCGCTATTAT 800

E K R D S I S L I N E G P S Y A S K I V S V G S N K Q Q V T I I

60 80

801 TGACTACTGTTGCGAGCTCTTTGGGTAGTGATTCAAAATGCCCAATGTGGAAAAAGGCTTGATTCGAAGCACTCAGGAGACCTTACCACCATCATGCTCT 900

T G S S D F W V I V D S N A Q C G K G V D C K S S G T F T P S S

100 120

901 TCTTCAACAAGAATTTAGGAGCGCTTTCATCTTCGATATGGGGATGCTTACATCCGAAGTATGATGGGTAAAGATACCGTCAACAATCAATGGAG 1000

S S Y K N L G A A A T T I R Y G D G S T S Q G T T W G K D T V T I N G

140

1001 TCTCAATCACTGGACAATAATTCGACTGTCACTCAAACTCAGTGTACAAGGTATATTGGGATTTGGCTACACCAGCAATGAAGCAATTTACGACAC 1100

V S I T G Q Q Q I A D V T Q T S V D Q G I L G I Y T S N E A V D Y T

160

1101 GAGTGGCTGTCAAACTCCAACTACGACAAGCTTCCCGTACTTTGA AAAAGCAAGAAAGATCAGAACCAATGCTTACTCATTATATTTGAACCTT 1200

S G R Q T T P N T G D N V P V T L K K Q G K I R T N A Y S L Y L N S

200 220

1201 CTTCTCACTGAGCTGACCAATCACTTTTGGTGTGTAATGCCAAGTACTTCGCAAGTTGGTTGCCGAGCAATTTACCTGCTGACACGGCTTGA 1300

P S A E T G T T I I F G G V D N A K Y S G K I V A E Q V T L S Q P L

240

1301 CTATTTCTGCTTCCGCTCAATTTGAAGGTTTCATCTTTTATTTGGAGATGGTCTTTGTGTTA TCTGGAACGACACTCACTTACTTCCAAAGTGA 1400

T I S L A S L N L K G S S F S F G D G A L L A S G T L T A Y F P P S D

260 280

1401 CTTTGGCCGACAACTTGCTGAATAAGCTGTGCTGCTTTGTTCAGAGTGTAGACAACTCTGTACTTATTTAGTATCAACGGATCACTCTGGC 1500

F A A Q L A D K A G A R L V Q V A R D Q Y L Y F I D C N T D T S G

300 320

1501 ACAACGGTGTTTAACTTTGGAAATGGGGCAAGATTACCCTTCCAAACAGAGTACGTTTATCAAAATGGGATGGTCACTTGTCTTCTGGGATTCACAC 1600

T T V F N F G N C A K I T V P N T E Y V Y Q G D G T C L W G I Q

340 380

1601 CATCAACGACTATTTTACGTGACAACTTTTGAGACACTTTTACTCTCTTTACAACCTGGATGCAACCAACATCTCAATCGCTCAAGTCAAGTA 1700

P S D D T I L T G D N F L R H A Y Y L L Y N L D A N T I S I A Q V K Y

360 400

1701 CACCACGACGACGACATTTCTGCGGTTTAAGAGAGAAGAGAAAAAGTCTCTTTCTAGCTCGTTGGATTATAAACAGTTCAGTTCAAATACGATTTCGT 1800

T D S S I S A V

420

1801 TTAATTCGGAACATGGCTCACTCAACCGCACTTTGATATTGAGATGCAACAAAATCTCTTCGCTCAACTATACGCAATAGGAT 1885

NaII

ACPL

	<i>AclI</i>		<i>EcoRI</i>
1	AGCTTACTGCTACTTACGAAAGGCGAAAAACAGACCCCAATTATTAACATCGATGAGGTCGTAAGAGTCACAGAGAGCTTTAGAAAGCTTTGAAATAG		100
101	ACACATCTTACCAGTGGCGGCTGGCTGCTTTTGGCAGCAGTGGGGTGACACCAATCTTCAAGTCTTAACCTTGTAACACCGTTGTTGTGCTGTTTTCACACTT		200
201	CCTCGGAGGAGCTCTCTTCACTTCTATTAACCACTTCTTTGGCTGAATTTTGGCCCATGCGCATATCCATTGAAATAATTGAGCCAATCAAGTGAAACAAC		300
301	GAAGAGAGTGGAAAGAAATGCTTTTGCAGGAGTCAATTTATGGCAGTGAATCTCTTTTGCAGAGAGTCTCATTTGCCACCGTTGGTGTTTACACCGGTGC		400
401	CCTTGGAAACATCATAAATAGGGAAGCAATCCCCAAGATTGGTGAGTTAAATGTCAAGTCACCAACTATCAATTATCTCCAATGACTACAAATGCCATC		500
		M T T I A I	
501	TTTACTAAAAACGTTTGTCTGCCATGCTTTTGTCTTTTGTCTGCCAAGTGCTGCTATTTCCAGCACCGCTAAAAGAGATGATAATCACTGGTTTGTGTG		600
	F T K N V L L A I A F A L F A Q G A A I P D P A K R D D N P G F V		
601	CTTTGGATTTTGAAGTACAAAGAACCCCTCTCGATGATGACTGCTATTCAGAACTCTTAAAGAGTCTTCCATCATCACCATTGACTTTGAGGAGAC		700
	A L D F E V T R K P L D V N A T S E L S K R S P S S P L Y F E G P		
701	TTTCATATGGTATTCGTGTTTCAGTTGGTTTCCAACAAACAGAAACAAGTGCTTTCAGACCGGTCTTCTAGTACTTTTGGGCTGTGATTCCTCTGCA		800
	S Y G I R L V S V G S N K Q E Q Q V V L D T G S S D F V V D S S A		
801	TCTTGTCAAAAAGGTAATTTGAAGCAATATGGAATTTTGACCCACATGCTCAACCTCTATCAAGTCATAGGCTCAGTTTACAGCATTTGGTTATGGGG		900
	S C Q K G N C K Q Y G T F D P H S S T S F K S L L G S S F S I Y G Y		
901	ACAAGAGTCTTCTGATTGGAACTTGGGGCAAGATACATTTATCTTGGTGCTATCTCCATTACCAANTCAACCGTTTGTGTGATGTACCAGCACTCGGT		1000
	D K S S I G T W G Q D T I Y L G G T S I T N Q R F A D V T S T S V		
1001	CAATCAAGGAATATAGGTCTTGGACGTGTGGAACCTTGAGAGTCGAAATCCCCCATATCGACACCTCCCTATCACTTTGAAAAGCAAGGAAAAATCAAG		1100
	N Q G I L G V C G R V E T E S A N P P Y D N V P I T L K K Q G K I K		
1101	ACCAATGCTTACTCATTTGTAATTCAGACACCAAGCGACTGACTTGGTAGCTATTTTGGTGCTGTGATAATGCGAGTATTTTGGAAAGACTCATGT		1200
	T N A Y S L L Y L L N S P G A T T G C T T I I F G C V D N A K Y S G K L I		
1201	AGGAGCCATTGGTTCTGGACCGATACTTGGCTGTAAATCAAAATCCCTTAACTACAAATGGCGATAACTCAACCGGGGTTTGGTGTTGTGTGGATTCT		1300
	E E P L L D T R Y L A V N L K S L N Y N G D N S N A G F G G V V V D S		
1301	CGGAACCAAAATTAGTTACTTTCGCTGACAGCATCGTTTAACGATCTCGCCACAAAGGTTGGTGCACTCTTGAGCCAGCTTGGTTTGGGAACAGAGCTTTAC		1400
	G T T I S Y L L P D S I Y N D N L A N K V G Y A Y L E P V G L G N E L Y		
1401	TTTATTGATTGTAATGCCAATCTCAAGGTAGTGTCTTCTTCACTTCGCATAATGCTGCTAAGATTACTGTTCCATTATCTGGAATTTGTCTCTCAAAGTA		1500
	F I D C N A N P Q G S A S F T F D N G A K I T V T P L S E F V L Q S		
1501	CTGCCAATGCTTCGCTGTGGGTTTCAAGAGTTCCGATAGACAAATTTCTTCCAAATTTGGGTGATAATTTTGGAGACAGCTTATGTGCTTTTCAA		1600
	T A N A C V W G L Q S R G Q R N V P L L L G D N F L R H A Y V V F N		
1601	TTTGGATAAAGAGACCGGTTCTCTCGCTCAGGTGAAGTACACTTCTCGCTCAAGTGTTCAGCAATTTAGAAACTTCCATTGTTTGGAAACCTTTTTCAT		1700
	L D K E T V S L A Q V K Y T S A S S V S A I		
1701	CAATCGTTATATTCAATAAATTTGTTGTTTATGACAGCAATCTGTAGTCTAAATTTTGGCCAAATTTGTGTGATGAAGACGGCGGATAGAAGAACAAATTTG		1800
1801	ATAGCGAATATACATGTTTCTGCTTTCTTCAAGTGTTATTTCAAAATGTAAGTTGTTGATTCATAAATATACAGACTGCTGATATATTTAAAT		1900
1901	ATACACAAATACAGGAAACACGACATCAAGAAAGATCGTTTATCAAAATGAGGCGATACC		1995

Fig. 3. Nucleotide sequence of *C. parapsilosis* *ACPR* and *ACPL*. The deduced amino acid sequence is shown below the nucleotide sequence. The N-terminal amino acid sequence of the mature *C. parapsilosis* ACP begins at residue 63 in *ACPR* (horizontal arrow). The 15 amino acid sequence from the N-terminus of the protein determined by amino acid sequencing is underlined. The two Lys-Arg peptidase processing sites found in the *ACPR* propeptide sequence and the homologous sites in *ACPL* are dotted. Putative cleavage sites within the signal sequence are indicated by vertical arrows. Aspartic acid residues homologous to those of the active site of pepsin-like proteins are marked by a filled triangle. Three domains showing strong homology with proteins which are members of the pepsin family are delineated with brackets. Possible TATA sequences are boxed.

Fig. 4. Protein profiles of culture media of *Candida tropicalis* ATCC 750 (lane 1), *C. parapsilosis* E18 (lane 2), *C. tropicalis acp* mutant complemented by *C. tropicalis* ACP (lane 3) and by *ACPR* (lane 4). The proteins precipitated from the culture medium with 65% saturated ammonium sulphate were visualized by 9% (w/v) SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R-250. Molecular mass markers as used in Fig. 1.

al., 1991). The N-terminal amino acid sequence of mature *C. parapsilosis* ACP, obtained by Edman amino acid sequencing of the purified enzyme, could be found in the *ACPR* deduced amino acid sequence only. It was preceded by the second Lys-Arg tandem sequence, as previously found for ACP of *C. tropicalis* and *C. albicans*. In *ACPL* the amino acid sequence following the second Lys-Arg residue was different from that of *ACPR*, and no sequence identical to the N-terminus of the *ACPR* product could be found in the *ACPL* sequence. The amino acid sequencing of the N-terminus of *C. parapsilosis* ACP prepared after precipitation of the proteins from liquid culture supernatants with 100% saturation ammonium sulphate did not identify another exoprotease. These findings suggest that only the *ACPR* product was secreted in the medium in the presence of BSA as a nitrogen source.

Complementation of a *C. tropicalis acp* mutant with acid protease genes from *C. parapsilosis*

Both the *ACPR* and *ACPL* sequences were subcloned into pMK16, a plasmid able to replicate in a *ade2* *C. tropicalis* mutant (Sanglard *et al.*, 1992). The recombinant plasmids pDS20 and pDS21 carrying *ACPR* and *ACPL*, respectively (Sanglard *et al.*, 1992), were used to transform *C. tropicalis* DSY59, a strain homozygous for the disrupted *ACP*. A clearing zone on BSA-agar was obtained only for the yeasts transformed with pDS20. The expressed protease was isolated from the supernatant of a BSA liquid culture of these transformants and showed the same mobility on SDS-PAGE as ACP secreted by *C. parapsilosis* (Fig. 4). The *C. tropicalis acp* mutant transformed with pDS21 did not grow in BSA liquid medium.

Discussion

We report here the purification of *C. parapsilosis* ACP secreted by the yeast when BSA is the sole nitrogen source and the cloning, sequencing and functional characterization of the corresponding gene. The enzyme we isolated from *C. parapsilosis* E18 is similar to that characterized by Rchel (1986) in having a lower molecular mass than *C. albicans* and *C. tropicalis* ACP. The ACP isolated from other *C. parapsilosis* strains showed identical molecular mass to that of strain E18 (data not shown).

Evidence for the cloning of the *C. parapsilosis* ACP gene included the following. (i) The gene we identified as *ACPR* contained a DNA sequence corresponding to the N-terminal 15 aa sequence of purified *C. parapsilosis* ACP protein. (ii) When *ACPR* was expressed in a *C. tropicalis acp* mutant, ACP activity was restored, and (iii) the protease secreted from the transformed *acp* mutant showed the same mobility on SDS-PAGE as the protease secreted by wild type *C. parapsilosis*.

Inspection of the deduced N-terminal amino acid ACPL sequence beginning from the Met 1 residue suggests the existence of a precursor sequence similar to that of *C. albicans* and *C. tropicalis* ACP, and *ACPR* product. This precursor sequence has a secretion signal sequence of 14–21 amino acids with 4 putative signal peptidase cleavage sites (von Heijne, 1986) and two tandem Lys-Arg sequences which are proteolytic processing sites in *C. tropicalis* and *C. albicans* ACP (Togni *et al.*, 1991; Hube *et al.*, 1991), and also in the *ACPR* product. The presence of *ACPL* secretion signal sequence similar to those of other *Candida* ACP seems to indicate that *ACPL* has no defect in secretion capability. However, only one acid protease, the *ACPR* product, was found in supernatants of liquid cultures of *C. parapsilosis*, and *ACPL* could not be expressed in the *C. tropicalis acp* mutant. *ACPL* could be considered as a pseudogene, but it can not be excluded that *ACPL* may be expressed under not identified conditions. Indeed an acid protease gene of *C. albicans* WO-1, Op1a (99% homologous to *C. albicans* *ACP1* on Fig. 5), is transcribed only when the cells switch from a white to opaque form (Morrow *et al.*, 1992). This feature is strain dependent: in *C. albicans* 3153A this gene remains silent, although present in the genome. When, however, BSA is added to the culture medium, the stimulation of extracellular proteolytic activity in *C. albicans* is due to the expression of another acid protease gene functionally analogous to *ACPR*. Recent studies showed that *C. albicans*, like *C. parapsilosis*, contains at least two genes encoding secreted acid proteases (Wright *et al.*, 1992).

Immunoblotting experiments with an anti-ACP polyclonal antibody suggested that *C. tropicalis*, *C. albicans*

ACPR MVAIVTLTRQVLLTIALFAFQAAGAIPEEAARKDDNPGFVALDFVLRLKPLNLTEALLRE 160
ACPL MTITIAIPTKNVLLAIFALFAQGAAIPD-PAKRDDNPGFVALDFEVRTRKPLDVNATSELS
ACP1 *C. albicans* M-----FLKNIFIALAIALLVDA---SPA-KR-SPGFVLDLDFVIKTVPN---ATTGQE
ACP2 *C. albicans* M-----FLKNIFIALAIALLVDA---TPPTTKR--SAGFVALDFSVVKTPKAFPVTTNGQE
ACP *C. tropicalis* MATIFLFTKNVFIALAFALFAOGLTIPOGLEKK--TDKVVSLLDFTVIRKP--FNATAHRL
.....*

ACPR ---KPSISLSLINEGPSYASKVSVGSNKQQQTVIDTGSSDFWVVDSNAQCGKGVD-- 114
ACPL ---KRPSPSSPLYEGPGSYGIRVSVGSNKQEQVVLDTGSSDFWVVDSNASQCKG-N--
ACP1 *C. albicans* GKV--KRQAIPTVLNNHVSYAADITIGSNKQKFNVIVDTGSSDLWVPDASVTCDKPPRPGQ
ACP2 *C. albicans* GKTSKRQAVPTVLNHNEQVITYAADITVGNSNQKLNVIVDTGSSDLWPDPVNDACQVITYSDQ
ACP *C. tropicalis* --IKRSDVPTLLINEGPSTAADIVSGSNQKLTIVDTGSSDLWVPDADACQVITYSQG
..*****.

ACPR ----CKSSGTFTPSSSSSYKNLGAAFTIRYGDGSTSQTGWGKDTVTTINGVSI TGQQIADV 170
ACPL ----CKQGTTFPHSSSTSFKSGLGSSF IGYGDKSSSIGTWGQDITIYLGGTSTINQRFADV
ACP1 *C. albicans* SADFCCKGKIYTPSSSTTSQNLSGPFY IGYGDSSSGCTLYKDTVFGGGSATIKQVFDNI
ACP2 *C. albicans* TADFCKQKGTYPDSSGSSAQDLNTFFPIGYGDGSSSGCTLYKDTVFGGGSVINKQVLADV
ACP *C. tropicalis* TNNFCKQEGFTDPSSSNLNQDFSIEYGLDTSQGSFYKTVFGGSI IKHQGFADV
.....*

ACPR TQTSVDQGI LGICYTSNEAVDYDTSGRGTPPNVPTLKKGKIRTNAVSLYNLSPSAE 230
ACPL TSTSVMQGI LGVGRVETES-----ANPDGVSSDPTLKKGKIRTNAVSLYNLSPGAA
ACP1 *C. albicans* TKTSIPQGI LGICYKTNEAAGD-----YDNVPVTLKNGQVIARNAYSLYNLSPNAA
ACP2 *C. albicans* DSTSIDQGI LGVGYKTNEAGGS-----YDNVPVTLKNGQVIARNAYSLYNLSPDAA
ACP *C. tropicalis* TTTSVDQGI MGIGFTA VEAEGYNL-----YSNPVPTLKKGQI INKNAYSCLDNLESDAS
.....*

ACPR TGTTIFFGV DNAKYSGKLVAEQVTL SQPLTISLASVNLKGSSFSFGD-GALLDSGTTITY 289
ACPL TGTTIFFGV DNAKYSGKLEEPLVLDRLAVNLKSLMYNGDNSNAG-GVVVDSGTTISY
ACP1 *C. albicans* TGQIIFFGV DKAKEYSGSLIAVPVTSDBRELIRISLGVAVGKNIN-GNIDVLLDSGTTITY
ACP2 *C. albicans* TGQIIFFGV DNAKYSGSLIALPVTSDRELRI SLSGEVSGKITINDMVDVLLDSGTTITY
ACP *C. tropicalis* TGKIIFFGV DNAKYTGCTLATPVTSSVELRVHLGSI NFGDGSVST-NADVDVLLDSGTTITY
.....*

ACPR FPSDFAAQLADKAGARLVQVARDQYLF IDCNTDTSGMTVFNFNGAKITVPNTYEY--- 346
ACPL LPDSIVNDLANKVGALEYLPGLGNELYF IDCNANPQGSASF TFDNGAKITVPLSEFY---
ACP1 *C. albicans* LQQDAQDI IDAFNAELKLDGGQHFTYVTCDS--GTVDVFNFDNNAKISVPASEFTAPL
ACP2 *C. albicans* LQQDLADI IKA FNKGLTQDSNGSFYEVCNLS--GDVVFNEGSKNAISVPASEFTASS
ACP *C. tropicalis* FSQSADTKFARIUGA--TWDSRNEYRLPSCVLS--GDVAVNFQCGVKITVPLSELII---
.....*

ACPR --YQNGD--GTCLWG IQPSDD-----TILGDNFLRHAYLLYNLDANTISIAQVKYTSS 398
ACPL --LQSTA--NACVWLQSSDRQNPPI LGDNFLRHAY-VVFNLDKETVSLAQVKYTSASS
ACP1 *C. albicans* LSYANGQYPKQCQLLGLISD----ANILGDNFLRSAY-LVYDLDDDKISLAQVKYTSASN
ACP2 *C. albicans* QG-DGGQPYDKQLLFPVDN----ANILGDNFLRSAY-LVYDLDDDKISLAQVKYTSASS
ACP *C. tropicalis* --LKSDSDS-SICYFGISRND----GNILGDNFLRRAY-IVYDLDDDKISLAQVKYTSASS
.....*

ACPR ISAV-
ACPL VSAI-
ACP1 *C. albicans* IAALT
ACP2 *C. albicans* ISALT
ACP *C. tropicalis* ISAL-
..*

Fig. 5. Comparison of acid protease amino acid sequences from *C. parapsilosis* ACPR and ACPL, *C. albicans* and *C. tropicalis* ACP. The numbering is that of the *C. parapsilosis* ACPR product. Filled triangles identify aspartic acid residues corresponding to those found in the active site of proteases of the pepsin family. The N-terminus of the mature secreted protein is indicated by a horizontal arrow. Tandem Lys-Arg sequences known to be proteolytic processing sites are boxed. A potential N-linked glycosylation site sequence N-F-S in *C. albicans* ACP2 is underlined. Asterisks and points show identical and similar amino acids, respectively. ACPR, ACPL, *C. tropicalis* ACP (Togni *et al.*, 1991), *C. albicans* ACP1 (Hube *et al.*, 1991) and ACP2 (Wright *et al.*, unpublished) are available in the EMBL database with the accession numbers Z11919, Z11918, X61438, X56867 and M83663, respectively.

and *C. parapsilosis* ACP were closely related. However, the comparisons of the deduced amino acid sequences of the mature proteins show that they are far from being identical (Fig. 5). When ACP of two different species were compared, identity and similarity scores varied from 47.9 to 58.9% and from 62.2 to 70.1%, respectively. High identities were found in the two domains containing the two aspartate residues of the pepsin family active site, and in a conserved domain at the C-terminal part of the proteins (Fig. 5). The theoretical pI of *C. parapsilosis* ACP is 4.4. This value is slightly, but notably, higher than those of *C. albicans* ACP2 (4.0) and *C. tropicalis* ACP (4.1).

The polypeptide chains of mature *C. tropicalis*, *C. albicans* and *C. parapsilosis* acid proteases have calcu-

lated molecular masses of 35.7, 36.3 and 36.0 kDa, respectively. The size of *C. parapsilosis* ACP, estimated from electrophoretic mobility on SDS-PAGE, is 4 kDa smaller than that of *C. tropicalis* and *C. albicans* ACP. This discrepancy may be explained by the fact that the mature ACPs are glycoproteins, as shown by positive staining with PAS (Fig. 1a). The *C. albicans* and *C. tropicalis* ACPs were shown to be mannose proteins by MacDonald & Odds (1980) and Rchel *et al.* (1986), but glycosylation sites on the mature protein have not been investigated. Preliminary experiments on the expression of the *C. tropicalis* ACP in the *Saccharomyces cerevisiae* phosphomannomutase deficient mutant *sec53* (Kepes & Schekman, 1988) confirmed the presence of mannose, since the expressed protein showed a smaller size than

the native enzyme (data not shown). Inspection of the amino acid sequence of the mature protein reveals only one putative *N*-glycosylation site present in a *C. albicans* ACP at position 321 (Fig. 5). Consequently, the *C. parapsilosis* ACP, like the other *Candida* ACPs must contain *O*-glycosylated residues and not *N*-glycosylated ones. However, glycosylation of *Candida* ACP remains to be further investigated biochemically.

Aspartic proteases are found in retroviruses and in eukaryotic cells, but not in bacteria. Retroviral aspartic proteases are dimers of identical subunits of 95–125 aa, each containing an active aspartic residue (Rao *et al.*, 1991). Eukaryotic aspartic proteases consist of a polypeptide chain of about 320 aa which contains two active aspartic acid residues, one each in an N and C-terminal domain. These two domains have only limited sequence homology except in the vicinity of their active site (Tang & Wong, 1987). It is suggested that eukaryotic aspartic proteases evolved by duplication of an ancestral gene. Retroviral and eukaryotic aspartic proteases show a similar bilobal tertiary structure (Rao *et al.*, 1991). The two lobes, identical in the case of retroviral aspartic proteases, are different in eukaryotic proteases, and correspond to the N and C-terminal domains. These two lobes are separated by a deep cleft which contain the two aspartic residues of the active site. The three different *Candida* ACPs structurally resemble other eukaryotic aspartic proteases described. They have a polypeptide chain of about 340 aa residues and the locations of two conserved regions containing the two reactive aspartic acid residues are similar. Mammalian and plant aspartic proteases have three disulphide bridges in similar positions (Tang & Wong, 1987; Runeberg-Roos *et al.*, 1991). Like other fungal aspartic proteases (Horiuchi *et al.*, 1988; MacKay *et al.*, 1988) two disulphide bridges are apparently conserved in the three *Candida* secreted enzymes as attested by the four cysteine residues conserved in all acid proteases (Fig. 5).

Now that the genes encoding *Candida* ACP are available, experiments are planned to study the expression of these genes in macrophages. Further studies may show how differences in ACP translate to differences in virulence.

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