An oligopeptide transport gene from *Candida albicans*

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A *Candida albicans* oligopeptide transport gene, *OPT1*, was cloned from a *C. albicans* genomic library through heterologous expression in the *Saccharomyces cerevisiae* di-/tripeptide transport mutant PB1X-9B. When transformed with a plasmid harbouring *OPT1*, *S. cerevisiae* PB1X-9B, which did not express tetra-/pentapeptide transport activity under the conditions used, was conferred with an oligopeptide transport phenotype, as indicated by growth on the tetrapeptide Lys-Leu-Leu-Gly, sensitivity to toxic tetra- and pentapeptides, and an increase in the initial uptake rate of the radiolabelled tetrapeptide Lys-Leu-Gly-[³H]Leu. The level of oligopeptide transport was found to be influenced in the heterologous host by the source of nitrogen used for growth. The entire 3.8 kb fragment containing the oligopeptide transport activity was sequenced and an ORF of 2349 nucleotides containing a 58 nucleotide intron was identified. The deduced protein product of 783 amino acid residues contained 12 hydrophobic regions suggestive of a membrane transport protein. Sequence comparisons revealed that similar proteins are encoded by genes from *S. cerevisiae* and *Schizosaccharomyces pombe* and that *OPT1* is not a member of the ABC or PTR membrane transport families.

Keywords: *Candida albicans*, peptide transport, *Saccharomyces cerevisiae*

INTRODUCTION

Peptide transport, a phenomenon defined as the translocation of peptides across the plasma membrane in an energy-dependent manner, has been well documented in bacteria, plants, fungi and mammals (for reviews see Becker & Naider, 1995; Payne & Smith, 1994). Upon internalization, peptides are quickly hydrolysed into their amino acid components to serve as sources of amino acids or nitrogen. In addition to acquiring nutrients from the environment, peptide transport has been shown to play a role in recycling cell wall peptides and in transducing signals for group behaviours such as sporulation and competency in *Bacillus subtilis* and chemotaxis in *Escherichia coli*. Recently, it has been proposed that in *Salmonella typhimurium* peptide transporters aid the bacteria in evading the host immune response by transporting membrane-disrupting peptides away from the plasma membrane (Parra-Lopez *et al.*, 1993). Similarly, in *Streptococcus pneumoniae* the peptide transporters encoded by *plpA* and the *amiA* loci

play a role in virulence by modulating adherence to epithelial and endothelial cells (Cundell *et al.*, 1995).

Our laboratory has recently identified a family of di-/tripeptide transporters named the PTR (Peptide Transport) family. This family is characterized by several conserved motifs, has 12 putative transmembrane domains, and is driven by the proton-motive force. Members of the PTR family have been identified in a wide variety of eukaryotes and one prokaryote as well (Steiner *et al.*, 1995). Well-characterized members of the PTR family are the di- and tripeptide transporters from *Saccharomyces cerevisiae* (ScPTR2; Perry *et al.*, 1994) and from *Candida albicans* (CaPTR2; Basrai *et al.*, 1995). Both CaPTR2 and ScPTR2 are regulated by the nitrogen source and are inducible by micromolar amounts of amino acids; the proteins they encode have broad substrate specificities with a preference for peptides containing hydrophobic residues (Basrai *et al.*, 1992; Island *et al.*, 1987). Prior to the establishment of the PTR family, all peptide transporters cloned were from prokaryotes and were members of the ATP-binding cassette (ABC) superfamily (Higgins, 1992). Recently, transporters from the PTR family have been

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identified in the prokaryote *Lactococcus lactis* (Hagting *et al.*, 1994). However, in eukaryotes all peptide transporters thus far identified are members of the PTR family.

In addition to the di-/tripeptide transporter (*CaPTR2*) in *C. albicans*, three observations indicated the existence of another distinct peptide transport system. The first observation was that mutants resistant to the toxic peptide analogues bacilysin, polyoxin and nikkomycin Z (all demonstrated substrates of the di-/tripeptide transport system) were able to transport tetra- and pentapeptides at wild-type levels, and, conversely, mutants resistant to various toxic tetrapeptides were able to transport dipeptides at wild-type levels (Payne & Shallow, 1985; Milewski *et al.*, 1988; McCarthy *et al.*, 1985). Secondly, peptide-uptake experiments with radio-labelled compounds and chromophoric substrates demonstrated that dipeptides did not compete with tetra- and pentapeptides for entry into the cell, and vice versa tetra- and pentapeptides did not compete with labelled dipeptides (Milewski *et al.*, 1988; McCarthy *et al.*, 1985; Yadan *et al.*, 1984). Thirdly, sensitivity to toxic di- and tripeptides was influenced by the nitrogen source and micromolar amounts of amino acids while sensitivity to toxic tetra- and pentapeptides was not regulated by similar means (Basrai *et al.*, 1992). The conclusion from these studies was that *C. albicans* possessed two peptide transport systems: one for di- and tripeptides and another for oligopeptides. The size limitation of the oligopeptide transporter appeared to vary slightly from strain to strain, but in general tetra- and pentapeptides were transported well and tripeptides were transported to a lesser extent (for review see Becker & Naider, 1995). While the above studies provided strong physiological evidence for the presence of two peptide transport systems in *C. albicans*, final confirmation required genetic analysis. In an attempt to further define the molecular basis of peptide transport, we report here the cloning of a novel oligopeptide transporter gene from *C. albicans* that does not encode an ABC- or PTR-type transporter.

METHODS

Strains, vectors and media. The strains used in this study were *S. cerevisiae* PB1X-9B (*MATa ura3-52 leu2-3,112 lys1-1 his4-38 ptr2-2*) (Perry *et al.*, 1994) and *C. albicans* SC5314 (Fonzi & Irwin, 1993).

C. albicans and *S. cerevisiae* cells were maintained on YEPD medium (2%, w/v, dextrose; 1%, w/v, yeast extract; 2%, w/v, peptone; and 1.5%, w/v, agar). The minimal medium used for most studies was made by adding 10 ml 10 × filter-sterilized YNB (Yeast Nitrogen Base; Difco) with ammonium sulfate and without amino acids to 90 ml sterile water containing 2 g glucose and auxotrophic supplements (Sherman *et al.*, 1986). For those experiments where proline was used as a sole nitrogen source, YNB without amino acids and without ammonium sulfate was supplemented with 0.1% proline. The mutant strain *S. cerevisiae* PB1X-9B was grown in Synthetic Complete medium (SC), which consisted of minimal medium with histidine, uracil, lysine and leucine. *S.*

cerevisiae PB1X-9B transformed with pRS202-based plasmids was grown on SC lacking uracil (SC-Ura).

The *C. albicans* library used for cloning *OPT1* was kindly provided by Gerry Fink (MIT, Boston, USA) (Liu *et al.*, 1994). The library was created by partially digesting *C. albicans* strain 1006 genomic DNA (Goshorn & Sherer, 1989) with *Sau3A* and cloning the resulting fragments (> 4 kb) into the *Sall* site of pRS202, a *URA3/2* μ -based plasmid (Christianson *et al.*, 1992).

Peptide medium consisted of minimal medium supplemented with auxotrophic requirements minus the amino acid leucine plus 100 μ M of one of the following peptides: Lys-Leu (KL), Lys-Leu-Gly (KLG), Lys-Leu-Leu-Gly (KLLG), Lys-Leu-Gly-Leu (KLGL) or Lys-Leu-Leu-Leu-Gly (KLLLG). Abbreviations for toxic peptides and amino acids used in this study are as follows: ethionine, Eth; Ala-Eth, AEth; Leu-Eth, LEth; Lys-Leu-Eth, KLEth; Lys-Leu-Leu-Eth, KLEth; Lys-Leu-Ala-Eth, KLAeth; and Lys-Leu-Leu-Leu-Eth, KLLLEth. All amino acids were in the L configuration.

Enzymes, chemicals and reagents. Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase and alkaline phosphatase were purchased from New England BioLabs or Promega and were used according to the manufacturers' specifications.

Synthesis of radioactive Lys-Leu-Gly-[³H]Leu (KLG-[³H]L). KLGL was prepared by conventional automated solid-phase peptide synthesis on an Applied Biosystems model 433A synthesizer. The peptide was cleaved from the resin with trifluoroacetic acid (TFA) and purified using a *C*₁₈ reversed-phase column (19 × 300 mm) to > 99% homogeneity with a 5–20% linear gradient of acetonitrile in water over 60 min. The product was verified using mass spectrometry [(*M*⁺ + 1) = 430.2; calculated = 429.6].

KLG-[³H]L was prepared by solution-phase peptide synthesis as follows. BOC-Lys(BOC)-Leu-Gly-OH (5.2 mg; 10 μ mol) was dissolved in 108 μ l of a 0.092 μ mol μ l⁻¹ solution of *N*-hydroxysuccinimide (10 μ mol) in dry dioxane. Dicyclohexylcarbodiimide (10 μ mol in 57 μ l) in dry dioxane was added and the reaction mixture was stirred for 1 h at ambient temperature. Leu (0.65 mg; 5 μ mol), dissolved in 1 ml water, was added to radioactive leucine [American Radiolabelled Chemicals; specific activity 60 Ci mmol⁻¹ (2.22 TBq mmol⁻¹); concentration 1 mCi ml⁻¹ (37 MBq ml⁻¹) in 2% ethanol]. This solution was evaporated to dryness, redissolved in 250 μ l water/dioxane (4:1, v/v) containing *N*-methylmorpholine (50 μ mol) and the solution containing the activated tripeptide was added. The resulting reaction mixture was stirred for 6 h at ambient temperature, 5.5 ml TFA was then added, and after 5 min the reaction mixture was evaporated to dryness. The residue was redissolved in 500 μ l water, injected onto a Waters μ Bondapak *C*₁₈ column (7.8 × 300 mm) and eluted isocratically using 5% acetonitrile in water, containing 0.025% TFA. Product eluting at the KLGL position was collected, evaporated, redissolved in water (200 μ l) and analysed by HPLC and on silica thin layers using a butanol/acetic acid/water (4:1:5, by vol.) mobile phase. TLC plates were exposed to film overnight at -80 °C and developed to show one radioactive spot with the mobility of the desired tetrapeptide. KLG-[³H]L was > 97% pure according to HPLC. Specific radioactivity was 90 mCi mmol⁻¹ (3.33 GBq mmol⁻¹). The peptide was diluted with nonradioactive KLGL as required.

DNA manipulations. Small-scale plasmid DNA preparations from *E. coli* transformants were performed as described in Sambrook *et al.* (1989). Plasmid DNA from *S. cerevisiae*

transformants was isolated as described previously by Ward (1990). Whole-cell DNA from *C. albicans* was obtained by the procedure described by Ausubel *et al.* (1990).

Yeast transformations were done using the procedure described by Gietz *et al.* (1991) and plates were incubated at 30 °C for 4 d or longer.

For Southern analyses, whole-cell DNA was digested with restriction enzymes and electrophoresed on 1.0% agarose gels. Southern blotting was done as described in Sambrook *et al.* (1989). Hybridization was performed at 60 °C for 12 h in a Hybrite (Gibco-BRL) followed by two washes of 1 × SSC/0.1% SDS at 42 °C and two washes of 0.1 × SSC/0.1% SDS at 60 °C. The probe used for Southern blots was generated via PCR using the primers LC2 (5' GCATGGATTGTT-CCTGACTGG 3') and FT2 (5' CCAATACCAACAAA-TGAGGC 3'). The product was 408 bp in length and its position within the *OPT1* ORF is depicted in Fig. 1. The Southern blot displayed in Fig. 2 was processed using the program Adobe Photoshop.

For plasmid-curing experiments, *S. cerevisiae* transformants were grown nonselectively in YEPD broth for about 40 generations. Cells were then plated on YEPD plates to obtain isolated colonies, which were picked, washed with water, resuspended at 5×10^6 cells ml⁻¹ in sterile water and spotted onto the appropriate peptide medium.

The nucleotide sequence of the 3.8 kb insert in plasmid pOPT1 was generated through automated cycle sequencing using an ABI 373A automated sequencer (Smith *et al.*, 1986). The insert of pOPT1 was digested with either *TaqI* or *Sau3A*, subcloned into M13, and ssDNA was isolated as a template for sequencing from randomly chosen plaques. The sequenced fragments were assembled using the software DNASTAR and the remaining gaps were filled using properly placed primers. Primers were purchased from Bioserve Biotechnologies. Final assembly was performed using Autoassembler from ABI.

Growth and transport assays. Growth assays to determine the phenotype of the cells were done as described by Island *et al.* (1991). Briefly, 3 µl culture from a suspension of 5×10^6 cells ml⁻¹ was spotted onto the surface of the medium and plates were incubated at 30 °C for 4–7 d.

Uptake of KLG-[³H]L was determined using a protocol for uptake of dipeptides as described by Basrai *et al.* (1995) with a few modifications. *S. cerevisiae* cultures were grown overnight to exponential phase in SC–Ura medium. Cells were harvested by centrifugation, and resuspended in 2% glucose at a cell density of 2×10^8 cells ml⁻¹. Cell suspension (250 µl) was added to an equal volume of an uptake assay reaction mixture and incubated at 30 °C. The final concentrations of the components in the uptake assay solution were: 2% (w/v) glucose; 10 mM sodium citrate/potassium phosphate buffer (pH 5.0); and KLG-[³H]L [150 µM; 8.5 mCi mmol⁻¹ (314.5 MBq mmol⁻¹)]. Competition experiments were done in the presence of either 1.5 mM L, KL, KLG, KLLG or KLLLG. At various time-points, 90 µl portions were removed and filtered through a membrane. The yeast cells retained on the filter were washed twice with ice-cold distilled water, once with room temperature distilled water, and the residual radioactivity was measured by liquid scintillation. There was no peptide adsorption to the cell surface or sticking to filters since at 0 °C the counts were at background level. The uptake results, calculated on the basis of 50% counting efficiency (determined using L-[³H]lysine as a standard, and the specific activity of the peptide), were expressed as nmol peptide uptake min⁻¹ (mg cell dry weight)⁻¹.

Sensitivity assays. Sensitivity to ethionine, a toxic methionine analogue, and ethionine-containing peptides was determined by the method described by Island *et al.* (1987). Cells were grown overnight to the exponential phase of growth in SC, washed, and then resuspended at 5×10^6 cells ml⁻¹ in sterile water. One millilitre of this cell suspension was added to 3 ml molten Noble agar (0.8% final concentration) and overlaid on 20 ml of the same medium used to prepare the inoculum. A disk (6 mm diameter; Difco) was placed on the plate and 0.38 µmol of each of the compounds to be tested was applied to the disks. Zones of inhibition were measured after 24–48 h incubation at 30 °C. Each test comprised at least three independent assays and the results represented in the Tables are means of the values obtained. Maximum variation between the zones of inhibition measured for each test was ≤ 3 mm. A value of 7 mm for the diameter of the zone of inhibition represents a minimal growth inhibition value as the disk diameter was 6 mm. Photodocumentation of sensitivity assays was done with a Umax Scanner and processed by Adobe Photoshop.

RESULTS

Cloning of an oligopeptide transporter

We have recently cloned di- and tripeptide transporters of *C. albicans* (CaPTR2) (Basrai *et al.*, 1995) and *Arabidopsis thaliana* (AtPTR2-A and AtPTR2-B) (Steiner *et al.*, 1994; Song *et al.*, 1996) through heterologous expression in *S. cerevisiae*. Unlike *C. albicans*, *S. cerevisiae* has been found to transport only a limited number of tetra- and pentapeptides under a limited number of growth conditions (reviewed by Becker & Naider, 1995). Therefore, as our primary screen, we transformed the *S. cerevisiae* di-/tripeptide transport mutant PB1X-9B with a high copy number *C. albicans* genomic library and screened for the ability of *S. cerevisiae* to grow on a normally nonutilized tetrapeptide as a sole source of auxotrophic supplements.

A pRS202-based *C. albicans* genomic library was transformed into *S. cerevisiae* PB1X-9B and 32000 *URA3*⁺ transformants were obtained. Transformants were pooled into six groups of approximately 5200 transformants each and subsequently plated onto a medium containing 50 µM KLLG as the sole source of leucine and lysine and ammonium sulfate as a nitrogen source. A double auxotrophic selection was employed to preclude the possibility of cloning the *C. albicans* *LEU2* or *LYS1* homologues. *S. cerevisiae* PB1X-9B cannot utilize the tetrapeptide KLLG as a sole source of lysine or leucine when grown on a medium containing a rich nitrogen source such as ammonium sulfate (unpublished observation). Oligopeptide-transport (OPT)-positive colonies appeared after 5–7 d incubation at 30 °C.

Curing of the plasmid by growth in nonselective conditions as well as shuttling the plasmid through *E. coli* and back into *S. cerevisiae* PB1X-9B demonstrated that the OPT activity was plasmid-borne. Subsequently, two different plasmids, denoted pOPT1 and pOPT24, containing inserts of 3.8 and 4.3 kb, respectively, were recovered from a representative sample of OPT⁺ colonies. Initial restriction mapping demonstrated that the

Fig. 1. A partial restriction map of the 3.8 kb fragment from pOPT1. The location and orientation of the ORF are indicated as well as the location of the probe used in Southern blots. Restriction sites are as follows: B, *Bst*XI; H, *Hinc*II; P, *Pvu*II; Ba, *Bam*HI; K, *Kpn*I.

Fig. 2. Southern blot. Analysis of hybridization of a probe of *OPT1* to genomic DNA isolated from *C. albicans* SC5314 was performed as described in Methods. Lanes: 1, *Hinc*II digest; 2, *Pvu*II–*Bam*HI digest; 3, *Pvu*II–*Kpn*I digest. Size markers are in bp.

smaller of the two plasmids, pOPT1, overlapped entirely with the larger plasmid, pOPT24. Therefore, the plasmid pOPT1 (Fig. 1) was used in all subsequent experiments.

Southern blotting

Southern blot analysis was done to ensure that *OPT1* was derived from *C. albicans* genomic DNA and to determine if there were other homologous genes. Genomic DNA was isolated from *C. albicans* SC5314 and digested with the restriction enzymes *Hinc*II, *Bam*HI/*Pvu*II and *Pvu*II/*Kpn*I. The resulting fragments were separated on a 1% agarose gel and Southern blotting was performed as described in Methods. The *Pvu*II–*Kpn*I and *Pvu*II–*Bam*HI digests were each predicted to yield one band while the *Hinc*II digest was predicted to yield two bands. As seen in Fig. 2, each digest produced their predicted bands: bands of 617 bp and > 2700 bp for *Hinc*II (lane 1), 790 bp for *Pvu*II–*Bam*HI (lane 2) and 1163 bp for *Pvu*II–*Kpn*I (lane 3).

Nucleotide and deduced amino acid sequence of *OPT1*

Sequence analysis revealed the presence of two ORFs, separated by a type II intron, and encoding a hydrophobic protein of 783 amino acids with an apparent

molecular mass of 88 kDa and a pI of 7.1 (Fig. 3). The first ORF contained 1626 nucleotides while the second ORF contained 723 nucleotides excluding the stop codon. The intron separating the two ORFs was 58 nucleotides in length and contained the highly conserved 5' splice site (GCATGT), 3' splice site (TAG) and branch point (TACTAAC) (Rymond & Rosbash, 1992). The two ORFs and intron constitute the gene *OPT1*. The size and hydrophobic nature of the predicted protein product of *OPT1* are suggestive of a membrane-bound protein with at least 12 putative transmembrane domains of 20–24 amino acid residues.

A search of the database using the BLAST algorithm (Altschul *et al.*, 1990) identified two ORFs from *S. cerevisiae* and one ORF from *Schizosaccharomyces pombe* as having significant homology. The ORFs SCYJL212C and YSCP9677 from *S. cerevisiae* were identified during the genome sequencing project and were not assigned any function. The remaining ORF, *ISP4* from *Schiz. pombe*, was identified as a gene of unknown function that was up-regulated as a result of inducing meiosis through nitrogen starvation (Sato *et al.*, 1994). However, whether this induction was meiosis-specific or due simply to nitrogen starvation was not determined. The predicted protein products of the putative homologues were aligned (Fig. 4) using the PileUp program (Feng & Doolittle, 1987) from the Genetics Computer Group (GCG) software (Devereux *et al.*, 1984) and percentage identity and similarity were calculated using the GCG program Bestfit. The protein Isp4p from *Schiz. pombe* exhibited the highest homology with 48% identity and 70% similarity. The two proteins from *S. cerevisiae* exhibited lower homology with 40% identity and 63% similarity for SCYJL212C and 34% identity and 59% similarity for YSCP9677.

The PTR family of peptide transporters is characterized by the signature motif FYXXINXGSL (Steiner *et al.*, 1995), whereas the ABC transporters are characterized by the ATP-binding Walker motifs (Higgins, 1992). The predicted protein product of *OPT1* did not contain the PTR signature motif or the ABC Walker motif. Furthermore, a comparison of *OPT1* with the PTR di-/tripeptide transporter *CaPTR2* using the GCG program Bestfit revealed only 18% identity between the two transporters. These data indicated that *OPT1* is not a member of the PTR or ABC families of membrane transporters.

Peptides as growth substrates

To determine the size constraints of peptide utilization mediated by pOPT1, we tested the ability of *S. cerevisiae* PB1X-9B (a mutant in the di-/tripeptide transporter) harbouring either pRS202 (the parent vector) or pOPT1 (pRS202 containing the 3.8 kb insert with the *OPT1* gene) to grow on KL, KLG, KLLG and KLLL as a sole source of leucine. Previously it has been shown that di-/tripeptide transport activity in *C. albicans* and *S. cerevisiae* is regulated by nitrogen: rich nitrogen sources such as ammonium sulfate repress transport, while poor

	GATCATGTGGCTAAAAATTCATATGGCTTTATGCTGTGGCTTCAGCTCTACTGATAATGATATCTTTATT																																-410		
	CCTATACATAAAATATTAAGCCACTTGATTATTTGCTCATAGGGCCAAAAAAGATGCAGAACCATCTAAAGTTTGTGTTTGTGCTATT																																-312		
	TTGTGCTAGTGAGATTAAATAGTTATCTTTTCATGACAAAAATCCTTTAGACTACTTTTATCCATTGTTTGGTTTACGATTATCAATCGTCAT																																-214		
	AGTTCAATTTGTAAAAATTTTATCTTTTCAATCCCAACCTTTTAAATAGTCAGTAATTTCTCTCATAGGAATTTCAAGTTTCCACTTTTTTTGTCTT																																-116		
	CCTTCTATTTCTTTTATAAGTTTACTGTTTCGTGAAATATTATTCAATTGTATTATTTTTACTAAGTCAACCCTATTGATTCCATTCTTAACACT																																-18		
	TATTATAAGTACTTACT																																-1		
1	ATG	GAC	AAA	ATA	AGG	GCA	GTA	ATT	AGT	GGA	GGT	GAG	AAA	CCT	CCC	GTT	GAC	ACT	GAC	AAC	GAT	CAC	AAC	ACA	GAC									75	
	M	D	K	I	R	A	V	I	S	G	G	E	K	P	P	V	D	T	D	N	D	H	N	T	D										
26	TTT	GAG	GCT	GAC	AGA	AAA	ATG	CCA	GAT	TTG	GAT	ATT	GTA	GTT	TCC	AAA	TCA	CAA	GAA	TTT	GAC	CCA	GTC	ACC	TCC									150	
	F	E	A	D	R	K	M	P	D	L	D	I	V	V	S	K	S	Q	E	F	D	P	V	T	S										
51	CAC	TTG	GTT	AAT	GAT	ATT	ATG	GAA	GAT	GAA	TAT	GCT	GCT	GTC	CAT	GTT	GAA	GAT	GAT	TCT	CCT	TAT	CCA	GAA	GTT									225	
	H	L	V	N	D	I	M	E	D	E	Y	A	A	V	H	V	E	D	D	S	P	Y	P	E	V										
76	AGA	GCA	GCT	GTT	CCT	TCT	ACT	GAC	GAC	CCA	ACT	TTA	CCT	CAA	AAT	ACC	ATT	AGA	GCC	TGG	GTT	ATT	GGT	TTG	ATA									300	
	R	A	A	V	P	S	T	D	D	D	P	T	L	P	Q	N	T	I	R	A	W	V	I	G	L	I									
101	TTG	ACT	ACG	GTT	GGT	TGT	GGT	ATG	AAT	ATG	TTG	TTC	AGT	TTC	CAT	AGT	CCC	TCA	TTT	GCT	ATC	ACC	ACT	TTT	GTC									375	
	L	T	T	V	G	C	G	M	N	M	L	F	S	F	H	S	P	S	F	A	I	T	T	F	V										
126	ACA	TCC	ATT	TTG	GCT	TGG	CCA	ATT	GGG	AAC	TTT	TGG	GCA	TGG	ATT	GTT	CCT	GAC	TGG	AAG	ATT	TTT	GGT	GCT	TCG									450	
	T	S	I	L	A	W	P	I	G	N	F	W	A	W	I	P	D	W	K	I	F	G	A	S											
151	TTA	AAT	CCA	GGT	CCA	TTC	AAC	GTT	AAA	GAA	CAT	ACT	ATC	ATC	ACT	ATT	ATG	GCC	AAC	GTT	TCT	TTT	GGT	ACT	GGT									525	
	L	N	P	G	P	F	N	V	K	E	H	T	I	I	T	I	M	A	N	V	S	F	G	T	G										
176	GCC	GCA	TAT	GCC	ACA	GAT	ATC	TTG	CTT	GCA	CAA	AAT	ATG	TTT	TAT	AAA	TCA	AAT	TTT	GGT	TGG	GGG	TAC	AAT	TTA									600	
	A	A	Y	A	T	D	I	L	L	A	Q	N	M	F	Y	K	S	N	F	G	W	G	Y	N	L										
201	TTA	CTT	ATC	TGG	AGT	ACC	CAA	TGT	ATT	GGG	TTT	GCT	TTT	GGA	GGT	GTT	ATG	AGA	AGA	TTT	GTT	GTT	GAC	AGT	CCA									675	
	L	L	I	W	S	T	Q	C	I	G	F	A	F	G	G	V	M	R	R	F	V	V	D	S	P										
226	GGG	GCC	ATC	TGG	CCC	CTG	AAT	TTG	GTC	ACC	GCA	ACA	TTC	TTG	ACT	AAT	ATG	CAC	ATT	AAC	GAA	AAC	CAC	ACT	GCT									750	
	G	A	I	W	P	S	N	L	V	T	A	T	F	L	T	N	M	H	I	N	E	N	H	T	A										
251	AAT	GGC	TGG	AAA	ATT	TCT	CGT	CTT	GCA	TTT	TTC	GTG	ATC	GTG	TTT	GTT	GCC	TCA	TTT	GTT	TGG	TAT	TGG	TTC	CCA									825	
	N	G	W	K	I	S	R	L	A	F	F	V	I	V	F	V	A	S	F	V	W	Y	W	F	P										
276	GGT	TAT	ATT	TTC	CAG	GCT	TTA	TCG	TAT	TTT	TCT	TGG	ATC	ACC	TGG	ATT	AAA	CCA	AAC	AAT	GTC	ATT	ATC	AAT	CAA									900	
	G	Y	I	F	Q	A	L	S	Y	F	S	W	I	T	W	I	K	P	N	N	V	I	I	N	Q										
301	GTT	TTC	GGT	TCT	TCA	TCT	GGG	TTA	GGT	ATG	ATT	CCT	AAC	AAC	ATT	GCC	TTG	GAC	TGG	AAC	CAA	ATT	GCA	GGG	TAT									975	
	V	F	G	S	S	S	G	L	G	M	I	P	N	N	I	A	L	D	W	N	Q	I	A	G	Y										
326	ATT	GGG	TCT	CCA	TTG	ATT	GCA	CCA	GCT	AGT	GTT	ATT	GCT	ACA	ATT	TTT	GGA	TCC	ATT	GTG	CTT	ATT	TTC	TGG	ATT									1050	
	I	G	S	P	L	I	P	P	A	S	V	I	A	T	I	F	G	S	I	V	L	I	F	W	I										
351	GTT	GTG	CCA	GCT	ATT	CAC	TAT	TCC	AAC	ACT	TGG	TAC	TCC	CAA	TAC	TTG	CCA	ATC	TCA	TCT	ACT	GGA	TCG	TTT	GAT									1125	
	V	V	P	A	I	H	Y	S	N	T	W	Y	S	Q	Y	L	P	I	S	S	T	G	S	F	D										
376	AGG	TTT	CAA	CAA	ACT	TAT	AAT	GTG	TCA	AAA	ATT	ATC	GAC	CAT	AAA	ACT	TTA	TCA	TTC	AAT	GAA	GCG	GAA	TAC	AAA									1200	
	R	F	Q	Q	T	Y	N	V	S	K	I	I	D	H	K	T	L	S	F	N	E	A	E	Y	K										
401	AAG	TAC	TCC	CCT	TTG	TTT	TTA	TCC	ACC	ACC	TTT	GCC	ATT	TCC	TAT	GGG	CTA	TCA	TTT	GCC	TCC	ATT	TTA	GCC	ACT									1275	
	K	Y	S	P	L	F	L	S	T	F	A	I	S	Y	G	L	S	F	A	S	I	L	A	T											
426	ATA	ACA	CAC	ACC	ATT	TGC	TTT	CAT	GGA	CGT	GAC	CTT	ATC	GCA	TCG	TTG	AAG	GCC	AAA	GAA	AAA	CCA	GAT	GTT	CAC									1350	
	I	T	H	T	I	C	F	H	G	R	D	L	I	A	S	L	K	A	K	E	K	P	D	V	H										
451	AAT	AGA	TTA	ATG	AAA	GCA	TAC	AAA	CGA	GTG	CCT	GAA	TGG	TGG	TAC	CTA	GTT	GTC	TTT	TTG	GTC	TTT	TTC	GGT	ATG									1425	
	N	R	L	M	K	A	Y	K	P	V	P	E	W	W	Y	L	V	V	F	L	V	F	F	G	M										
476	TCC	ATA	GCC	ACC	GTA	CGT	GCT	TGG	CCT	ACT	GAA	ATG	CCA	GTA	TGG	GGG	TTA	GTT	TTT	GCT	CTT	ATC	ATC	GCT	ATC									1500	
	S	I	A	T	V	R	A	W	P	T	E	M	P	V	W	G	L	V	F	A	L	I	I	A	I										
501	ATA	TTT	TTA	TTA	CCC	GTT	GCT	ATC	ATT	TAT	GCA	AAA	ACG	AAT	ATT	GCT	GTT	GGT	TTA	AAC	GTT	GTA	ACC	GAG	TTT									1575	
	I	F	L	L	P	V	A	I	Y	A	K	T	N	I	A	V	G	L	N	V	V	T	E	F											
526	ATC	GTG	GGC	TAC	GTA	CTA	GGT	GGA	CGT	CCC	CTA	TGT	ATG	ATG	TTG	TTC	AAG	GCATGT	ATTAGAATTG	CAGATCATAATCA														1656	
	I	V	G	Y	V	L	G	G	R	P	L	C	M	M	L	F	K																		
543	GTCAGTT	TACTAAC	CTAATTTTGAA	TAG	ACC	TTT	GGA	TAC	ATC	ACT	AAT	AAC	CAA	GCT	GTT	ACT	TTT	GTG	CAG	GAT	ATG													1735	
560	AAA	CTT	GGG	CAC	TAC	ATG	AAA	ATA	GAT	CCG	CGC	ACT	TTG	TTT	TGG	GCG	CAG	TTT	GCT	ACC	ATA	TGG	GGA	TCG										1810	
	K	L	G	H	Y	M	K	I	D	P	R	T	L	F	W	A	Q	F	A	A	T	I	W	G	S										
585	TTA	GTT	GAC	ATC	GCA	GTT	TTG	GAG	TGG	GCC	TAT	GGT	GCA	ATC	GAC	AAT	TTG	TGT	GCT	GCT	GAC	CAA	AAA	CAT										1885	
	L	V	Q	I	A	V	L	E	W	A	Y	G	A	I	D	N	L	C	A	A	D	C	K	N	H										
610	TAC	ACA	TGT	CCA	AAC	GGT	AAA	GTT	TTC	TTC	AAT	GCT	TCG	ATC	ATT	TGG	GGT	GTC	ATT	GGA	CCC	CAA	CGT	CAA	TTT									1960	
	Y	T	C	P	N	G	K	V	F	N	A	S	I	I	W	G	V	I	G	P	Q	R	Q	F											
635	TCA	CAT	GGG	CAG	ATT	TAT	TAT	GGG	TTA	CTT	TTC	TTT	TTC	ATC	ATT	GGT	GCT	GTG	ACC	CCT	GTC	ATC	AAT	TGG	TTG									2035	
	S	H	G	Q	I	Y	Y	G	L	L	F	F	F	I	I	G	A	V	T	P	V	I	N	W	L										
660	ATC	TTG	AAA	AAA	TGG	CCA	AAC	TCT	CCA	GTC	AAG	TAT	TTG	CAT	TGG	CCA	GTG	TTC	TTT	TCT	GGG	ACA	GGG	TAC	ATT									2110	
	I	L	K	K	W	P	N	S	P	V	K	Y	L	H	W	P	V	F	F	S	G	T	G	Y	I										
685	CCT	CCA	GCC	ACT	CCA	TAT	AAC	TAT	ACC	TCC	TAC	TGT	GCT	GTG	GGT	TTG	TTC	TTT	GGA	TGG	TGG	ATT	AAA	AAG	AAG									2185	
	P	P	A	T	P	Y	N	Y	T	S	Y	C	A	V	G	L	F	F	G	W	W	I	K	K	K										
710	TGG	TTT	CAC	TGG	TGG	TCT	AAA	TAC	AAC	TAT	TCC	TTG	TCT	GCG	GCG	TTG	GAT	ATT	GCT	TTG	GCA	TGG	TGC	TCG	TTG									2260	
	W	F	H	W	W	S	K	Y	N	Y	S	L	S	S	A	A	G	L	D	I	G	L	A

Fig. 5. Toxic peptide inhibition assay. Sensitivity to the ethionine-containing peptides KLLAeth (1), KLAeth (2) and KLEth (3) on a 0.1% proline medium was determined as described in Methods. (a) PB1X-9B(pRS202); (b) PB1X-9B(pOPT1).

tripeptide Aeth and KLEth when proline was used as a nitrogen source.

Transport of KLG-[^3H]L in *S. cerevisiae* transformants

To determine if *S. cerevisiae* transformants harbouring pOPT1 could accumulate a radiolabelled tetrapeptide, uptake assays were performed with the radiolabelled substrate KLG-[^3H]L with cells grown to exponential phase in SC-Ura with either ammonium sulfate or 0.1% proline as a nitrogen source. PB1X-9B(pOPT1) grown in SC with ammonium sulfate exhibited a significant uptake rate compared to no uptake by PB1X-9B(pRS202) (Fig. 6a). Furthermore, PB1X-9B(pOPT1) demonstrated a higher initial rate of uptake when compared to *C. albicans* SC5314 grown in the same medium. This higher initial rate can be explained by overexpression due to high copy number or alternatively by the lack of requisite regulatory elements which may be absent in the heterologous host. All three strains had a higher rate of initial uptake when grown in SC-Ura with 0.1% proline as a nitrogen source (Fig. 6b). PB1X-9B(pRS202) did accumulate the tetrapeptide KLGL under these conditions but apparently not to a large enough extent to support growth on KLLG or to exhibit sensitivity to KLEth or KLAeth (Table 1; Fig. 5).

Fig. 6. Peptide transport assay. Accumulation of KLG-[^3H]L was measured over a 12 min time course as described in Methods. *S. cerevisiae* PB1X-9B harbouring pRS202 (●) or pOPT1 (▽) and *C. albicans* SC5314 (○) were grown in SC-Ura medium with either ammonium sulfate (a) or 0.1% proline (b) as a nitrogen source.

To more rigorously determine the size constraints of the oligopeptide transporter, the accumulation of KLG-[^3H]L was measured in the presence of a 10-fold molar excess of the competitors L, KL, KLG, KLLG and KLLL. SC-Ura with ammonium sulfate was chosen as the growth medium because under these growth conditions PB1X-9B(pOPT1) accumulated KLG-[^3H]L whereas PB1X-9B(pRS202) did not (Fig. 6a). As seen in Fig. 7, L and KL do not compete with the uptake of KLG-[^3H]L whereas competition was seen with KLLG and KLLL. The tripeptide KLG exhibited decreased competition in comparison to KLLG or KLLL (Fig. 7), possibly due to a lower affinity, although this low level of KLG uptake is below the threshold to support full growth when used as an auxotrophic supplement. Uptake rates were calculated from a best-fit of the slope for each set of data. The uptake rate of KLG-[^3H]L in the presence of no competitor, L or KL was 0.24, 0.25 and 0.26 nmol min⁻¹ (mg dry weight)⁻¹, respectively. When KLG was used as a competitor, the uptake rate was 0.12 nmol min⁻¹ (mg dry weight)⁻¹, which was approximately 50% of the no competitor rate. The uptake rate approximated zero when KLLG and KLLL were used as competitors.

Fig. 7. Peptide transport competition experiment. Accumulation of KLG-³H]L (○) was measured in the presence of a 10-fold molar excess of the competitors L (●), KL (▽), KLG (▼), KLLG (□) or KLLL (■) over a 12 min time course.

DISCUSSION

Three lines of evidence support the cloning of an oligopeptide transport gene from *C. albicans*. First, the plasmid pOPT1 conferred the ability to utilize the peptide KLLG to satisfy the leucine auxotrophic requirement of *S. cerevisiae* PB1X-9B when grown on a medium with a rich or poor nitrogen source. Secondly, the *S. cerevisiae* strain PB1X-9B was not sensitive to the toxic peptides KLEth, KLAeth or KLLAeth when grown on a minimal medium with 0.1% proline as a nitrogen source but was sensitive when transformed with pOPT1 (Fig. 5; Table 1). Similarly, a very faint zone of growth inhibition was seen for KLEth, KLAeth and KLLAeth when PB1X-9B(pOPT1) but not PB1X-9B(pRS202) was grown in a medium containing ammonium sulfate. Finally, PB1X-9B(pRS202) had an initial uptake rate of zero for the radiolabelled substrate KLG-³H]L when grown on a medium containing ammonium sulfate whereas PB1X-9B(pOPT1) had a dramatically higher initial uptake rate (Fig. 6a). When the growth medium contained proline as a nitrogen source, the initial uptake rate was 2.5 times higher for PB1X-9B(pOPT1) than for PB1X-9B(pRS202) (Fig. 6b). Furthermore, KLLG and KLLL and to a lesser degree KLG competed with the uptake of KLG-³H]L (Fig. 7). The fact that leucine did not compete with KLG-³H]L for uptake excluded the possibility that OPT1 encoded a secreted protease. Therefore, we propose that we have cloned an oligopeptide transporter from *C. albicans* capable of transporting tetra- and pentapeptides and, to a lesser extent, tripeptides.

The predicted protein product of OPT1 did not show any significant homology to any members of the ABC superfamily or PTR family of transporters. Furthermore, a search of the Prosite (Bairoch, 1992) and Motifs (Devereux *et al.*, 1984) databases for protein motifs did not reveal any previously identified functional domains

common to transport proteins with the exception of potential glycosylation sites. However, the 12 putative transmembrane domains separated by hydrophilic regions as well as the expression of transport activity in a heterologous host are suggestive of an integral membrane transporter. Because three ORFs of significant homology as well as several expressed sequence tags (data not shown) were identified, the possibility exists that OPT1 constitutes the first identified member of a new family of transporters. We are currently testing this hypothesis in our laboratory by testing these ORFs for oligopeptide transport activity.

Opt1p is able to accommodate peptides of three to five residues; peptides of larger than five residues were not tested. As demonstrated by growth assays, halo assays and competition experiments, tetrapeptides were most readily transported by Opt1p. Pentapeptides did enter the cell as demonstrated by sensitivity to KLLAeth and supported by the competition between KLLL and KLG-³H]L. However, KLLL was not able to support growth when used as a source of leucine, possibly due to the inability of cellular peptidases to release leucine from this peptide. Similarly, KLG was able to compete slightly with KLG-³H]L for entry into the cell, but KLG did not support growth and KLEth was not toxic. Because the number of possible tri- and pentapeptides is large and our sample size small, we cannot conclude that Opt1p has a lower affinity for tri- and pentapeptides than for tetrapeptides.

Sequence analysis revealed the presence of a 58 nucleotide intron located within the 3' half of OPT1. The 5' splice site, 3' splice site and branch point are identical to previously reported type II introns within fungi (Rymond & Rosbash, 1992). It is interesting to note that the di-/tripeptide transporter CaPTR2 also contains a small type II intron that is located within the 3' half of the gene. It has been suggested that introns play a regulatory role. However, a comparison of the two introns did not reveal any apparent consensus sequences that might be suggestive of a common regulatory element or of a common ancestry.

To date, only one study has been published addressing the regulation of oligopeptide transport activity in *C. albicans*. Basrai *et al.* (1992) concluded that sensitivity to toxic oxalysine-containing tetra- and pentapeptides was not influenced by the nitrogen source or by the presence of amino acid inducers. However, our findings suggest that when expressed in *S. cerevisiae*, OPT1 is regulated by the nitrogen source. The discrepancy in the results may be explained by differences in the levels of regulation or substrate specificity between the two different strains used in the studies or, alternatively, by superimposition of a *S. cerevisiae* regulatory mechanism on the CaOPT1 gene expressed heterologously.

A search of the database using the BLAST algorithm identified three putative homologues of OPT1. The ISP4 gene from *Schiz. pombe* exhibited the highest homology and was identified by Sato *et al.* (1994) through a subtractive hybridization experiment using RNA iso-

lated from nitrogen-starved and non-nitrogen-starved cells. In *Schiz. pombe*, nitrogen starvation induces meiosis and therefore this nitrogen-starvation/meiosis-inducing screen identified genes that were either induced during meiosis or regulated by the nitrogen catabolite repression system. Based upon the high homology between *OPT1* and *ISP4* and the established role of nitrogen regulation in many peptide transport systems, we hypothesize that *ISP4* encodes an oligopeptide transporter that is regulated by the nitrogen source. The remaining two putative homologues were from *S. cerevisiae* and were identified during the genome sequencing project. Interestingly, few favourable conditions have been identified for oligopeptide transport activity in *S. cerevisiae*. As seen in Fig. 6, when PB1X-9B(pRS202) was grown in a medium containing proline it exhibited an initial KLG-[³H]L uptake rate that was comparable to the initial uptake rate in PB1X-9B(pOPT1) when grown in a medium with ammonium sulfate. However, under these conditions PB1X-9B(pRS202) exhibited no sensitivity to the toxic tetrapeptides KLEth and KLAeth and was not able to utilize the tetrapeptide KLLG as a sole source of leucine, whereas PB1X-9B(pOPT1) did grow on KLLG and exhibited slight sensitivity to the toxic peptides KLEth and KLAeth. This discrepancy in results could be explained by an uptake rate exhibited by PB1X-9B(pRS202) that may not necessarily be reflective of total peptide accumulation over the prolonged incubation times necessary for growth and sensitivity assays. We are currently trying to determine if these two putative *S. cerevisiae* *OPT1* homologues are responsible for this low-level oligopeptide transport activity.

In summary, we report the cloning of a second peptide transport gene from *C. albicans* through heterologous expression in *S. cerevisiae*. The existence of multiple peptide transport systems has been documented extensively in both prokaryotes and eukaryotes. The redundancy of this phenomenon underscores its importance. With the identification of two peptide transport genes in *C. albicans*, *CaPTR2* and now *OPT1*, the role of peptide transport in virulence, induction of secreted acid proteinases and cell wall recycling can now be explored in greater detail.

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