An oligopeptide transport gene from Candida albicans

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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 evading the host immune system (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...
identified in the prokaryote Lactococcus lactis (Hagetinger 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 dipetides 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 dipetides (Milewski et al., 1988; McCarthy 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 (2% glucose, 1% Bacto-yeast extract; 0.1% Bactopeptone) or on YNB medium (2% glucose, 0.5% Bacto-yeast extract; 2% Bactopeptone; and 1 5% 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 SalI site of pRS202, a UR3/2, μ-based plasmid (Christanson et al., 1992).

Peptide medium consisted of minimal medium supplemented with auxotrophic requirements minus the amino acid leucine plus 100 μM of one of the following peptides: Lys-Leu (KL), Lys-Leu-Gly (KLG), Lys-Leu-Gly-Leu (KLGL), Lys-Leu-Leu-Gly (KLLG), or Lys-Leu-Leu-Leu-Gly (KLLL). 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-ALA-Eth, KLALEth; and Lys-Leu-Leu-Eth, KLLL. 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). KLG-[3H]L 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 C18 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 mg; 10 μmol) was dissolved in 108 μl of a 0.092 μmol μl⁻¹ solution of N-hydroxysuccinimide (10 μmol) in dry dioxane. Dicyclohexylcarbodiimide (10 μmol in 57 μl) in dry dioxane was added and the reaction mixture was stirred for 1 h at ambient temperature. Leu (0.65 mg; 5 μmol) 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 μl water/dioxane (4:1, v/v) containing N-methylmorpholine (50 μl) 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 μl water, injected onto a Waters Bondapack C18 column (7.8 × 300 mm) and eluted isocratically using 5% acetonitrile in water, containing 0.025% TFA. Product eluting at the KLG position was collected, evaporated, redissolved in water (200 μl) 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
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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 4–5 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 HyHyb buffer (Gibco-BRL) followed by two washes of 1× SSC/0.1% SDS at 65 °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′-GCATGGATTGTCCTGACTGG-3′) and FT2 (5′-CCAATACCAAACAAAA-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, 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; 85 μCi mmol^{-1}(314.5 MBq mmol^{-1})]. Competition experiments were done in the presence of either 1 mM L, KL, KLG, KLLG or KLKL. 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 tritiated 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 5×10^8 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

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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, BstXI; H, HincII; P, PvuII; Ba, BamHI; K, KpnI.

![Partial restriction map of the 3.8 kb fragment from pOPT1](image)

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, HincII digest; 2, PvuII-BamHI digest; 3, PvuII-KpnI digest. Size markers are in bp.

![Southern blot analysis of hybridization of a probe of OPT1 to genomic DNA](image)

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 HincII, BamHI/PvuII and PvuII/KpnI. The resulting fragments were separated on a 1% agarose gel and Southern blotting was performed as described in Methods. The PvuII–KpnI and PvuII–BamHI digests were each predicted to yield one band while the HincII 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 HincII (lane 1), 790 bp for PvuII–BamHI (lane 2) and 1163 bp for PvuII–KpnI (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 percent 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 FYXXINXGSLS (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 KLLLG 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
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Fig. 3. Nucleotide and predicted amino acid sequences of OPT1. The predicted amino acids are italicized and numbered to the left of the figure while nucleotides are numbered to the right. The 5' and 3' splice sites as well as the conserved branch point of the intron are boxed. The codon CUG (CTG in the DNA) encodes serine not leucine in C. albicans (Omaha et al., 1993).
Fig. 4. Comparison of oligopeptide transporters. The proteins Opt1, lsp4, SCYJL212C and YSCP9677 were aligned using the program PileUp. Conserved residues are in upper case and nonconserved residues are in lower case. The amino acids in each respective protein are numbered to the right.

Table 1. Sensitivity of *S. cerevisiae* PB1X-9B transformants to various ethionine-containing peptides

<table>
<thead>
<tr>
<th><em>S. cerevisiae</em> transformants</th>
<th>Nitrogen source</th>
<th>Zone of inhibition (mm)</th>
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<tbody>
<tr>
<td></td>
<td>Eth</td>
<td>AEth</td>
</tr>
<tr>
<td>pRS202</td>
<td>33</td>
<td>None</td>
</tr>
<tr>
<td>pOPT1</td>
<td>33</td>
<td>None</td>
</tr>
<tr>
<td>pRS202</td>
<td>33</td>
<td>None</td>
</tr>
<tr>
<td>pOPT1</td>
<td>35</td>
<td>None</td>
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*Transformants tested harboured either the parent vector (pRS202) or pRS202 containing the 3.8 kb fragment (pOPT1).
† A small and diffuse halo with no distinct zone of growth inhibition was observed.
‡ The halo observed was hazy.

392 nitrogen sources such as proline derepress transport. Therefore, we simultaneously determined the effect of nitrogen source on oligopeptide transport activity by supplying either ammonium sulfate or proline as the nitrogen source. When grown on a medium containing ammonium sulfate, PB1X-9B(pOPT1) was only able to utilize the peptide KLLG as a source of leucine whereas PB1X-9B(pRS202) did not utilize any of the peptides tested. When grown on a medium containing 0.1% proline, PB1X-9B(pOPT1) was also able to utilize the tetrapeptide KLLG as a sole source of leucine, although the growth was much more robust than the growth on the ammonium sulfate medium. No growth was observed on KL, KLG and KLLG for PB1X-9B(pOPT1) or PB1X-9B(pRS202).

Sensitivity of *S. cerevisiae* transformants to toxic peptides

*S. cerevisiae* PB1X-9B is sensitive to the toxic amino acid ethionine but is resistant to ethionine-containing di-, tri-, tetra- and pentapeptides. We used disk sensitivity assays to determine if cells transformed with pOPT1 were sensitive to toxic peptides and whether this sensitivity was dependent upon the nitrogen source. In those conditions where ammonium sulfate was used as a nitrogen source, no zone of growth inhibition was seen for the transformed strain in the presence of AEth, LEth or KLEth, whereas a 33 mm zone of inhibition was seen for ethionine alone (Table 1). A small and diffuse zone of growth inhibition (about 11–15 mm) was seen for KLLeth, KLAeth and KLLAeth. When 0.1% proline was used as a nitrogen source, a zone of complete growth inhibition was seen for the toxic peptides KLLeth, KLAeth and KLLAeth for PB1X-9B(pOPT1) but not for PB1X-9B(pRS202) (Fig. 5; Table 1). Neither strain exhibited sensitivity to the toxic dipeptide or
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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).

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 KLG 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).

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 KLLLG. 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 KLLLG. The tripeptide KLG exhibited decreased competition in comparison to KLLG or KLLLG (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 KLLLG were used as competitors.
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 KLLG, KLAeht or KLLAeht 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, KLAeht and KLLAeht 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 radiolabeled 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 KLLLG 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 PFR 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 KLLAeht and supported by the competition between KLLLG and KLG-[3H]L. However, KLLLG 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 CaPT2 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 KLLEth 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 KLLEth 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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