To the Graduate Council:

I am submitting herewith a dissertation by Vanny Narita entitled "Molecular, Genetic, and Functional Analysis of Ptr3p, A Novel Protein Involved in Amino Acid and Dipeptide Regulation of Di/Tri-peptide Transport System in *Saccharomyces cerevisiae.*" I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Philosophy, with a major in Biochemistry, Cellular, and Molecular Biology.

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and recommend its acceptance:

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Molecular, Genetic, and Functional Analysis of Ptr3p, A Novel Protein Involved in Amino Acid and Dipeptide Regulation of Di/Tri-peptide Transport System in *Saccharomyces cerevisiae*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Vanny Narita

August 2002

Dedication

I would like to dedicate this work to my loving parents, Rosiah and Suthamrin Salam, whose love and support have kept me motivated through this journey of life. I also dedicate this dissertation to my husband, Amri Putradjaja, who has always been by my side through everything. I love you.

In memory of my father in law, Prof. Dr. H. Moeslim Taher, SH. and my best friend, Dini Andriani.

Acknowledgements

I would like to thank my committee members, Dr. Cynthia Peterson, Dr. Mary Ann Handel, Dr. Bruce McKee, and Dr. Gary Stacey for molding me into not only a mature scientist but also a strong and confident person. Thank you for being tough, analytical, and persistent throughout my graduate studies, especially during the notorious “comprehensive exam” because without it I would not have become what I am today. I would like to thank Dr. Thomas Chen and Dr. Ranjan Ganguly, with whom I spent my undergraduate work and Dr. John Koontz, my undergraduate advisor for subtly supporting in my development as a scientist. I am grateful to Dr. Elizabeth Howell for her wonderful protein structure classes, and Dr. Rebecca Prosser and Dr. Engin Serpersu for their attention during my comprehensive exam.

I also thank Dr. Fred Naider at the Department of Chemistry at the College of Staten Island, City University of New York for his discussion, suggestion, and careful observation of my work. His “eagle eyes” profoundly helped me to focus my efforts. I sincerely appreciate Dr. Dong Xu at Graduate School of Genome Science and Technology and Oak Ridge National Laboratory, Oak Ridge, for his colloboration and fruitful analysis.

I am blessed to have experienced the joyful companionship of all members of Becker lab; Dr. David Barnes as my mentor when I joined the lab for the first

time; Dr. Melinda “lab mom” Hauser for being compassionate throughout my graduate studies and for sharing stories of her super-cute kids with me; Dr. Keith “I-Mac” Henry for his attention to my work and for sharing a strong interest in cutting-edge technology; Dr. Byung-Kwon “model developer” Lee for his ideas and the dream of the “USA-Korea-Indonesia” connection; Gaynelle “the best secretary ever” Russel for her support and help especially when I was stranded on Java Island; Sarah “innocent mouseketeer-1” Kauffman and Summer “Mega Island” Galloway for their up to date news; Keith “don’t worry it’s coming from me” Goldstein, Taylor “mouseketeer-2” Sherril, and Greg “mouseketeer-3” Mascheck for sharing their dry humor and for raising the testosterone level in the lab; all graduate students, including Josh Sharp, Cagdas Son, Ayca-Akal Strader, Nathan Verberkmoes, Amy Wiles, Yong-Hun Lee, and Houjian Cai for their support and effort in keeping up the spirit of “Becker lab teamwork”; other previous and current lab members, including Brandon Belcher, Michael Brown, Monica Burts, Jeff Wiles, and Dr. Steve Wright; for the infamous Becker lab neighbor, Nancy “Happy Tuesday,” thanks for being crazy and freaky when dropping by the lab.

I would like to thank my best teacher, friend, and “dad” Dr. Jeffrey M. Becker for his guidance and attention throughout my graduate studies. The high point of his teaching method is the freedom he gives his students to work on

their projects. His open-mindedness allowed us to be independent researchers under his guidance. His social skills gave us the best example of how to be professional scientists. I have always had confidence in him to discuss my problems, both professional and personal. His support and attention during difficult times had a tremendously positive effect that I believe is one of the key factors that made me a stronger person.

My tremendous appreciation goes to my late friend Dini Andriani for teaching me the value of friendship and patience. I miss her, and may she rest in peace. I also thank my buddies Simon “we can do it” Ciptadi and Rick “check this out” Bilbrey. I enjoy playing video games with them and beating them up in Tetris. They made me insane, and I think it really refreshed my brain. Thanks to both of them for all the help and support. They are like my brothers, and I wish them both a bright and wonderful future. Together with my husband, the four of us always had a good time, especially during the floor-thumping competition. I would also like to thank the family of Dr. Richard Bilbrey for treating my husband and me as one of their own. We enjoyed the delicious meals and fun gatherings throughout the years.

I am grateful to my husband’s big family and especially to my mother in law Hj. Saleha and my father in law the late Prof. Dr. H. Moeslim Taher, SH., whose support, understanding, and sacrifice gave me a strong motivation to

continue my studies. Mr. Taher taught me the beauty of knowledge and determination. I miss our morning discussions of politics, religion, and education. His experience as a politician and educator made him one of the most charismatic persons I have ever known. It is a great honor for me to be his daughter, and I hope he is proud to be my dad as well. I will always remember our last long conversation on the phone, and God knows I will do my best to keep all of my promises.

My greatest appreciation goes to my dad H. Suthamrin Salam, my mom Hj. Rosiah, my sister Rika Savitri, my brothers Yoshi Prakasa and Rahmat Hidayat, and the big Salam family. Without their love and support, I would never have succeeded. Dad and mom taught me the importance of being kind, gentle, humble, and open-minded. At the same time they encouraged me to seek knowledge and to appreciate nature. I can only imagine the effort and the sacrifices they made in raising me, and I think they are the best and most loving parents in the world.

Finally, the greatest thing about Knoxville is that it is the place where I met my loving husband, Amri Putradjaja. He is one of the most talented and fun persons I have ever met. Our life is always remarkable because our interests are so diverse, yet our main philosophies of life are indistinguishable. I appreciate our debates and discussions that revitalize and energize me. He always gives me

love, support, and comfort whenever I need. May God bless our marriage, and from the bottom of my heart I thank Amri for his presence and companionship.

Abstract

The yeast *Saccharomyces cerevisiae* utilizes nutrient sensor activity at the plasma membrane to regulate growth. Amino acids are sensed by the membrane protein Ssy1p, and a signal is transduced to the novel intracellular regulatory protein Ptr3p. This leads to an upregulation of the expression of amino acid permease genes and *PTR2*, the gene encoding the di/tri-peptide transporter Ptr2p. Using a reporter gene assay, this study found that various amino acids induced *PTR2* expression to different levels. Peptide and amino acid induction required Ptr3p, while Ssy1p was required for amino acid induction, but not peptide induction. Ptr3p-mediated, amino acid/dipeptide-induced expression of *PTR2* did not involve transcriptional regulation of *CUP9,* a gene encoding a repressor of *PTR2* expression. A functional chimeric protein formed by the fusion of GFP to the N-terminus of Ptr3p was found to be associated with the plasma membrane in a punctate pattern. A two-hybrid and co-immunoprecipitation analysis showed that Ptr3p interacted with the cytoplasmic, N-terminal 282 amino acid domain of Ssy1p further supporting the suggestion that Ssy1p/Ptr3p interaction was involved in generating a signal after amino acid sensing.

We also showed that *STP1* and *STP2,* genes previously hypothesized to encode pre-tRNA splicing proteins and bind to the *BAP2* promoter as transcription factors, were required for regulation of the di/tri-peptide transport

system. The data provide the first evidence about the involvement of *STP1* and *STP2* in the di/tri-peptide transport system suggesting their function as transcription factors of *PTR2*. In contrast to the *PTR2* expression pattern, in medium containing a rich nitrogen source *STP1* and *STP2* are expressed at higher levels in comparison to their expression in medium with a poor nitrogen source. *STP2* likely acts downstream of *PTR3* as shown by epistasis studies, and *PTR3* does not regulate the expression of *STP1* or *STP2*.

To provide insights into the function of Ptr3p, we performed a two-hybrid analysis to identify proteins that may interact with Ptr3p. We identified proteins involved in transport, transcription processes, and unknown functions suggesting that Ptr3p plays role in numerous pathways. We used two different computational algorithms to predict that Ptr3p contains two putative nuclear localization signals (NLS), and alanine substitution of one of these NLS decreased its function as a regulator of *PTR2*. PROSPECT, a 3-dimensional protein structure algorithm, also calculated that Ptr3p has a RING finger and a seven-bladded β-propeller motif. Alanine site-directed mutagenesis of some conserved residues in the ring finger motif reduced the function of Ptr3p. Mutations within the predicted β-propeller motif indicated that this motif may act as an autoinhibitory domain, because their substitution resulted in a more functional mutant than the wild type Ptr3p. Finally, we identified functional

domains of Ptr3p. The expression of Ptr3p residues 1-250 or 1-500 partially complemented a *ptr3*Δ phenotype, and residues 250-678 appeared to be as functional as full length Ptr3p. These results add further to an understanding of the role of Ptr3p in the amino acid induced signal transduction pathway.

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PART I

GENERAL INTRODUCTION

CHAPTER I

A REVIEW OF THE PEPTIDE TRANSPORT LITERATURE

The ability of organisms to engage in carrier-mediated transport of small peptides (two to five amino acids) is ubiquitous. Peptide transporters with obvious structural similarities have been documented in bacteria, fungi, plants and animals. Peptide transport is important in terms of sources for amino acids, carbon, and nitrogen, whether these nutrients being scavenged from the environment by free-living microorganisms or absorbed from body fluids by metazoans. Additionally, in mammalian systems, these transporters have also been documented as a means for absorption of peptidomimetic drugs, including β-lactam antibiotics, angiotensin converting enzyme (ACE), and renin inhibitors (Leibach and Ganapathy 1996; Yang *et al*. 1999).

The common baker's yeast *Saccharomyces cerevisiae* provides a suitable model eukaryote for the study of peptide transport. The sequence of its genome is complete, making it amenable to genetic analysis. Furthermore, the organism can be easily maintained and manipulated for use in physiological assays. This introduction will summarize the function and regulation of the peptide transport systems of *S. cerevisiae* and will integrate this information into the rapidly

emerging field of peptide transport in other organisms. Reviews on the subject of proton-coupled peptide transport in higher eukaryotes have been published recently (Daniel and Herget 1997; Fei *et al.* 1998; Meredith and Boyd 2000).

Function of the peptide transport systems of *S. cerevisiae*.

Distinction between dipeptide and oligopeptide transport and multiplicity of peptide transport families.

S. cerevisiae has two genetically and physiologically distinct proton-coupled peptide transport systems. The PTR (**P**eptide **TR**ansport) system is specific for di/tri-peptides and consists of at least three inter-dependent genes. *PTR2* encodes an integral membrane protein (Ptr2p) involved in the physical translocation of peptides across the plasma membrane. *PTR1* regulates the transcription of *PTR2* and is essential for peptide transport. *PTR3* is another regulatory element, and while not absolutely essential, enhances the expression of *PTR2*.

Tetra- and pentapeptides are transported by members of the OPT (**O**ligo**P**eptide **T**ransport) family, first described in the human pathogen *Candida albicans* (Lubkowitz *et al.* 1997). At least 3 different members of the OPT family exist in *S. cerevisiae*. The best studied of these members is Opt1p which transports

both the opioid pentapeptide enkephalin and glutathione. No regulatory elements for the OPT system with homology to *PTR1* or *PTR3* of the PTR system have been identified. The OPT family is much smaller than the PTR family, and to date, members have been limited to fungi and plants. The OPT family is distinct from the PTR family with no homology between the two families at both the DNA and protein sequence levels. Despite the clear differences in substrate specificity between these two families, there are features common to both groups. Transport is dependent upon the proton-motive force and is enhanced by growth on a poor nitrogen source and by the presence of micromolar quantities of amino acids in the growth medium.

With the rapid discovery of di/tri-peptide and oligopeptide transporters in many diverse organisms, some confusion has arisen with the nomenclature for the various groups. The family of di/tri-peptide transporters has been called the PTR family (Steiner *et al.* 1995) and the POT (**P**roton-dependent **O**ligopeptide **T**ransport) family (Paulsen and Skurray 1994; Saier 2000). The POT term was proposed before the identification of proteins in eukaryotes that transport authentic oligopeptides, namely those consisting of four or more amino acids. Furthermore, within this family, individual members have been named "OPT" as well, such as the *Drosophila* "OPT 1," (Roman *et al.* 1998) and the *C. elegans* "OPT1," "OPT2" and "OPT3" (Fei *et al.* 2000), providing further confusion as to the nature of the substrate that is being transported. For the purposes of the

present discussion, the term "PTR family" refers to transporters demonstrated to translocate only di/tri-peptides. The name "OPT family" is reserved for those transporters that have been demonstrated to translocate peptides of four or more amino acids.

Historically, the first reported peptide transporters were described in prokaryotes as ABC transporters (**A**TP-**B**inding **C**assette), also referred to as traffic ATPases. The common term used is ABC transporters. The bacterial transport systems include Dpp and Opp from *E. coli* and *Salmonella typhimurium* (reviewed by Payne and Smith 1994) that are relatively specific for dipeptides and peptides from two to six amino acids, respectively. Eukaryotic members of this superfamily include the pharmacologically important multi-drug resistance protein (P-glycoprotein) and cystic fibrosis transmembrane conductance regulator (CFTR). Much later, bacteria were found to also possess PTR family transporters, including the DtpT transporters of *Lactococcus lactis* and *Bacillus subtilis* (Hagting *et al.* 1994: Saier *et al.* 1999). Several as yet uncharacterised *E. coli* transporters, including YhiP, DtpT, f493 and YjdL have also been identified (Saier 1999). The proton-coupled permeases are not traffic ATPases and are encoded by a single gene, rather than the operon organization of Dpp and Opp. In addition, the tripeptide permease Tpp of *S. typhimurium* may represent a third class of transporter that is energized by the proton-motive force, but is neither a traffic ATPase or a PTR family member (Smith *et al.* 1999). Recently, two

additional families of proton-motive force driven peptide transporters have also been described (Saier 2000). The PAT (**P**eptide-**A**cetyl-CoA **T**ransporter) family members are found in bacteria and eukaryotes. While largely uncharacterised, members of this family are thought to transport acetyl coA in the endoplasmic reticulum and golgi of humans and small peptides into *E. coli*. The PUP (**P**eptide-**U**ptake **P**ermease) family is restricted thus far to *E. coli* and *Rhizobium meliloti* and is involved in the uptake of thiazole ring-containing peptide antibiotics.

The PTR System.

The ability of *S. cerevisiae* to transport intact di/tri-peptides was investigated in the 1970's (Naider *et al.* 1974; Becker and Naider 1977, Becker and Naider 1980 for review). Multiple transport systems were not detected, but evidence for regulation was described. The uptake of peptides was substantially greater in cells grown on a poor nitrogen source, such as proline, when compared to cells grown on a rich nitrogen source, such as ammonium sulfate (Becker and Naider 1977; Nisbet and Payne 1979). Subsequent work added another level of regulation by showing that peptide transport activity could be further induced by micromolar concentrations of amino acids in the nitrogen-poor growth medium (Island *et al.* 1987). Genetic complementation analysis coupled with N-methyl-N-nitro-N-nitrosoguanidine mutagenesis documented that peptide

transport involved at least three components, identified as *PTR1*, *PTR2* and *PTR3* (Island *et al*. 1991). The transcription of *PTR2* is regulated by *PTR1*, and *PTR1* is essential for peptide transport. *PTR2* expression is also regulated by *PTR3*. However unlike *PTR1*, *PTR3* is not absolutely essential, but is required for the optimal expression of *PTR2*. The isolation and characterization of *PTR1* and *PTR3* have been completed and are detailed in a later section of this introduction (Regulation of the peptide transport systems, p. 25). *PTR2* isolation and characterization are detailed below.

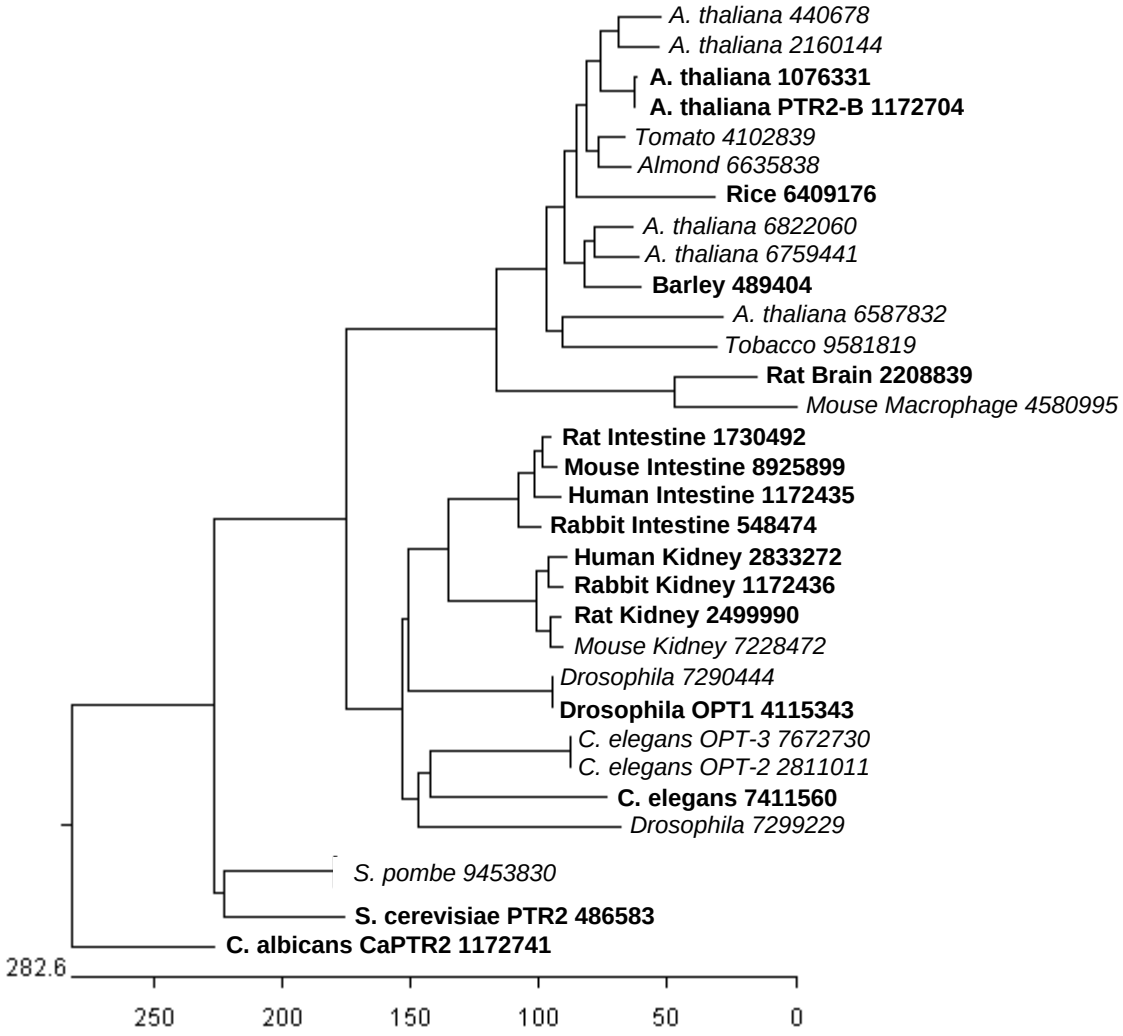
PTR2.

Ptr2p, the protein encoded by *PTR2*, is necessary and sufficient to translocate di/tri-peptides into the yeast cell. First described by Perry (Perry *et al*. 1994), it encodes an integral membrane protein of 601 amino acids containing predicted 12 membrane-spanning domains. The transporter is specific for di/tri-peptides, with a preference for peptides containing hydrophobic amino acids. Energetically, transport is coupled to the proton-motive force with a transport optimum at pH 5.5.

Using the sequence for Ptr2p as the query in a BLAST search (Altschul *et al*. 1997), over 100 high quality hits on the sequence query in a plethora of organisms including bacteria, other fungi, plants, mammals, *Drosophila* and *Caenorhabditis elegans* were found. A phylogenetic tree of approximately thirty

non-redundant hits was constructed to determine the degrees of relatedness among the family members (Fig. 1). Interestingly, most of the family members were segregated into groups based on the type of organism (i.e. mammal, invertebrate, plant or fungus). Within the mammalian members, further segregation occurred based on tissue of origin (intestine, kidney or brain). It is also of interest to note the redundancy of peptide transporters in various species with PTR family members. *Arabidopsis thaliana* and *C. elegans* have several distinct *PTR2* homologues. In humans, the characterized PTR family homologs are limited to hPEPT1 and hPEPT2, found primarily in small intestine and kidney, respectively. Additional uncharacterised human orthologues to the rat brain transporter have been identified by bioinformatic analysis of the human EST database (Botka *et al.* 2000). Northern blot analysis of the putative PTR family members hPHT1 and hPHT2 revealed broad tissue distribution. However, no expression was detected in the brain. Detailed analysis of the sequence alignment of these PTR family members reveals a number of highly conserved regions across this family of proteins occurring primarily within the membrane spanning domains (Fig. 2). The most highly conserved is the "FYING" motif, named for the conserved F247, Y248, I251, N252 and G254 residues in the fifth transmembrane domain (TMD). In a few members of the PTR family, the Y248 residue is not conserved (data not shown) and so this consensus sequence has also been called the "FING" region (Steiner *et al.* 1995). Other conserved

Fig. 1. PTR family tree (Hauser *et al*., 2001). Approximately thirty non-redundant hits from a BLASTP search were used to construct the tree using the CLUSTAL method (Lasergene 99, DNASTAR Inc., Madison, WI). Family members that have been physiologically characterized are indicated in bold, while italics indicates theoretical translations. The NCBI GI number is listed next to each member.


A. thaliana 440678
A. thaliana 2160144
A. thaliana 1076331
A. thaliana PTR2-B 1172704
Tomato 4102839
Almond 6635838
Rice 6409176
A. thaliana 6822060
A. thaliana 6759441
Barley 489404
A. thaliana 6587832
Tobacco 9581819
Rat Brain 2208839
Mouse Macrophage 4580995
Rat Intestine 1730492
Mouse Intestine 8925899
Human Intestine 1172435
Rabbit Intestine 548474
Human Kidney 2833272
Rabbit Kidney 1172436
Rat Kidney 2499990
Mouse Kidney 7228472
Drosophila 7290444
Drosophila OPT1 4115343
C. elegans OPT-3 7672730
C. elegans OPT-2 2811011
C. elegans 7411560
Drosophila 7299229
S. pombe 9453830
S. cerevisiae PTR2 486583
C. albicans CaPTR2 1172741
282.6
250
200
150
100
50
0

sequences include the WQIPQY in TMD 10 and EXCERFXYYG in TMD 1. Based on sequence conservation described above, site directed mutagenesis of highly conserved regions has been used to pinpoint residues important for transporter function in PTR family members. Ala-scanning mutagenesis of F247, I251, N252 and G254 of the "FYING" motif in *S. cerevisiae* results in decreased ability of strains carrying these mutations to grow on dipeptides, decreased sensitivity to toxic dipeptides, and virtually eliminated transport of radiolabeled dileucine (Wiles and Becker, unpublished observation). Mutagenesis of the low-affinity human intestinal homologue, hPept1, and its renal high-affinity counterpart, hPept2, has also been undertaken. The residue Y167 in the intestinal transporter is homologous to Y248 in the "FING" motif region of the *S. cerevisiae* Ptr2p. Mutagenesis of this residue (Y167A, Y167 F, Y167S, Y167H) rendered the protein unable to transport peptide (Yeung et al. 1998), suggesting an essential role for this residue in peptide transport. Mutagenesis of two other highly conserved residues in hPepT1 (W294 and E595; W362 and E480 in the *S. cerevisiae* protein, respectively) also rendered the protein inactive (Bolger *et al.* 1998). A residue that is vital for transporter function of hPept1, but has no counterpart in yeast, is H57. Mutagenesis of this residue (H57A, H57N, H57Q) eliminated peptide transport by hPEPT1 expressed heterologously in *Xenopus* oocytes (Meredith and Boyd 1996; Terada *et al.* 1996; Fei *et al.* 1997).

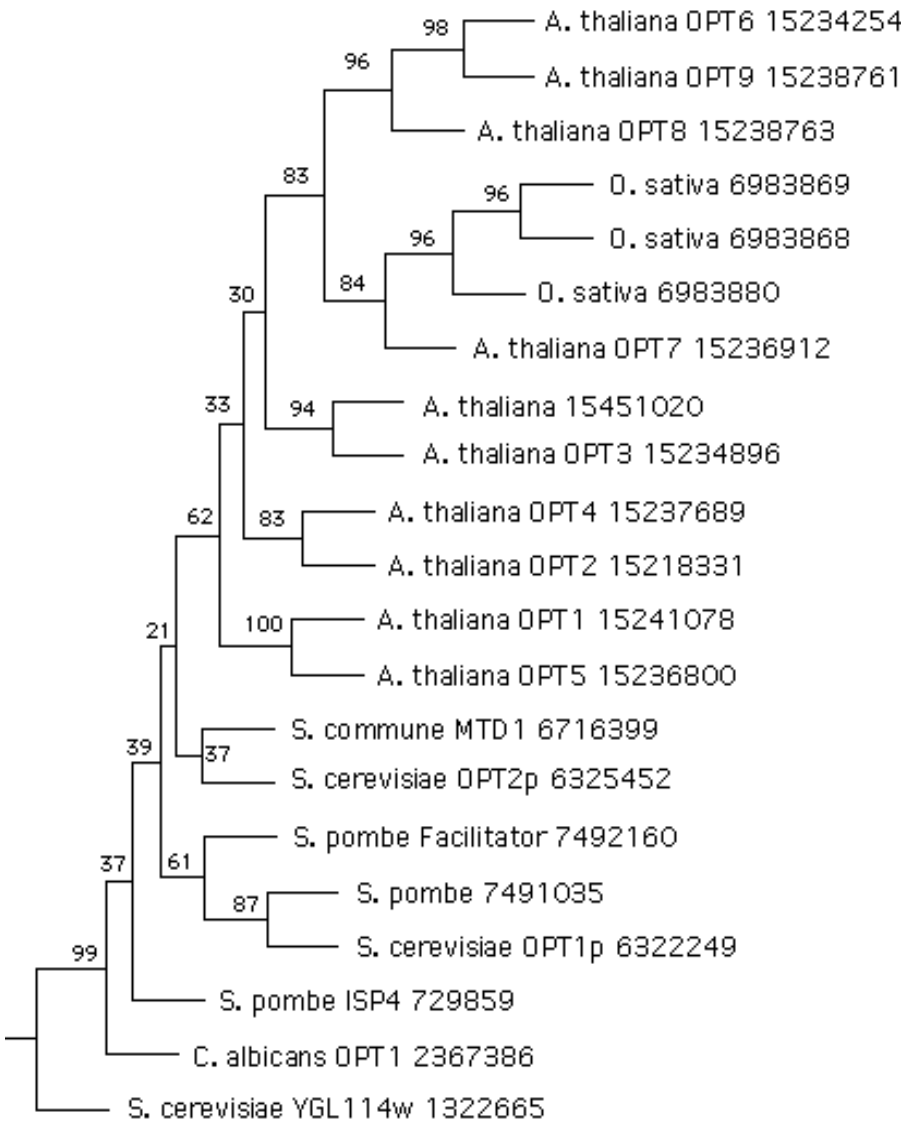
Fig. 2. Highly conserved regions in nine representative members of the PTR family (Hauser *et al.*, 2001). The consensus sequence determined using the CLUSTAL method (Lasergene 99, DNASTAR Inc., Madison, WI) is indicated above each alignment. Residues that match the consensus are boxed. The bars above the consensus indicate the relative location of the consensus sequences in relation to the putative transmembrane domains. The number in the left-hand column corresponds to the first residue of the consensus sequence of each protein. The NCBI GI number is listed next to each member.

```
     NE.CERF.YYG.        Consensus

 88  VELSERFSYYGL        S. cerevisiae PTR2 486583
 34  AELLERAAFYGV        Rat Brain 2208839
 54  NECCERLAYYGI        A. thaliana 1076331
 54  NECCERLAYYGI        A. thaliana PTR2-B 1172704
 54  TECCERLAFYGI        Almond 6635838
 23  NEFCERFSFYGM        C. elegans OPT-3 7672730
 22  NEFCERFSYYGM        Human Intestine 1172435
 52  NEFCERFSYYGM        Rabbit Kidney 2833272
 42  NEFCERFNYYGM        Drosophila OPT1 4115343


     FF..FYF.IN.GSL.     Consensus

243  VFMFFYFMINVGSLS     S. cerevisiae PTR2 486583
195  FFNWFYWSINLGAIL     Rat Brain 2208839
200  FFNWFYFSINIGALV     A. thaliana 1076331
200  FFNWFYFSINIGALV     A. thaliana PTR2-B 1172704
200  FFNWFYFSINIGALV     Almond 6635838
153  FFSFFYFAINGGSLF     C. elegans OPT-3 7672730
162  FFSIFYLAINAGSLL     Human Intestine 1172435
183  YFSGFYLAINAGSLI     Rabbit Kidney 2833272
172  FFSLFYFAINAGSLI     Drosophila OPT1 4115343


     ..WQIPQY.           Consensus

466  VCWQIPAYV           S. cerevisiae PTR2 486583
450  IWWQIPQYV           Rat Brain 2208839
473  VLWQIPQYF           A. thaliana 1076331
472  VLWQIPQYF           A. thaliana PTR2-B 1172704
472  IFWQIPQYF           Almond 6635838
575  ILWSLPQYI           C. elegans OPT-3 7672730
581  MALQIPQYF           Human Intestine 1172435
608  IAWQLPQYA           Rabbit Kidney 2833272
593  ILWQLPQIV           Drosophila OPT1 4115343
```

The OPT System.

A second peptide transport activity, genetically and physiologically distinct from the PTR system, exists in fungi. First described in the yeasts *C. albicans* and *Schizosaccharomyces pombe* (Lubkowitz *et al*. 1997; Lubkowitz, *et al*. 1998), OPT family members translocate tetra- and pentapeptides across the plasma membrane. Like the PTR system, oligopeptide transport is coupled to the proton-motive force (Hauser *et al*., 2000), and activity of the *C. albicans* transporter has been found to be sensitive to nitrogen source and low concentrations of amino acids in the growth medium (Lubkowitz *et al*. 1997). By sequence homology to CaOpt1p additional members of the OPT family have been identified, including three homologues in *S. cerevisiae.* Unlike the ubiquitous PTR2 family, the OPT family thus far appears limited to fungi and plants (Fig. 3), with the fungal members more closely allied to each other than to the plant members. Recently, nine oligopeptide transporter (OPT) orthologs (AtOPT1 to AtOPT9) in *Arabidopsis* were identified (Koh *et al.*, 2002). Based on hydropathy analysis, the family members are predicted to have 12 – 14 transmembrane domains. Sequence comparisons showed that the AtOPTs form a distinct subfamily when compared to the fungal OPTs (Figure 3). Hauser et al. (2001) found three regions of high conservation (SPYXEVRXXV, NPG-PFXXKEH, and GLNXXTEXIXGY) upon comparing the sequences of seven OPT family members. However, only the second of these motifs is highly conserved, with an

Fig. 3. OPT family tree (Stacey and Becker, personal communication). Twenty protein sequences from plants and fungi were aligned using ClustalX (1.81; Thompson *et al.*, 1997). Phylogenetic trees were generated using the neighbor-joining algorithm in Phylip3.57c (Felsenstein, 1993). Numbers on the branches indicate bootstrap values out of 100. Sequences are annotated with NCBI GI numbers.


A. thaliana OPT6 15234254
A. thaliana OPT9 15238761
A. thaliana OPT8 15238763
O. sativa 6983869
O. sativa 6983868
O. sativa 6983880
A. thaliana OPT7 15236912
A. thaliana 15451020
A. thaliana OPT3 15234896
A. thaliana OPT4 15237689
A. thaliana OPT2 15218331
A. thaliana OPT1 15241078
A. thaliana OPT5 15236800
S. commune MTD1 6716399
S. cerevisiae OPT2p 6325452
S. pombe Facilitator 7492160
S. pombe 7491035
S. cerevisiae OPT1p 6322249
S. pombe ISP4 729859
C. albicans OPT1 2367386
S. cerevisiae YGL114w 1322665
98
96
83
96
96
84
30
33
94
62
83
21
100
39
37
37
61
87
99

invariant glutamine residue, when all current members of the OPT family are compared (Figure 4). In addition, this comparison revealed an additional conserved region, the KP motif, K[L/F][G/A][H/M/T]YMK[I/V/L][P/D/S]PR. The final invariant amino acid, glycine, found in all OPT members is found in the sequence FFIIGA. These various motifs are found in regions predicted to be hydrophilic suggesting that they are probably critical to function.

Multiplicity of OPT genes in S. cerevisiae.

Of the three homologs identified in *S. cerevisiae*, the best characterized is encoded by the gene OPT1, while the second is encoded by the ORF YPR194c. The third homologue (ORF YGL114w) has been identified only by sequence homology. The OPT family homologs characterized in *Saccharomyces* (Opt1p and Ypr194cp) are different from those in *C. albicans* and *S. pombe* in that the *S. cerevisiae* members are not expressed during vegetative growth conditions. No mRNA or phenotypic expression of either OPT1 or YPR194c was initially discovered. Functional genomic analysis such as serial analysis of gene expression (SAGE) (Velculescu et al. 1997) and DNA microarray analysis (Chu et al. 1998) suggests that these genes are expressed during sporulation. To see expression of OPT1 and the product of YPR194c in log phase cells, it was necessary to place the genes under the control of the constitutive ADH promoter (Lubkowitz et al. 1998). Under these conditions it was determined that the protein encoded by YPR194c

Fig. 4. OPT conserved domains (Stacey and Becker, personal communication). ClustalX (1.81; Thompson *et al.*, 1997) alignment of twelve OPT sequences from plants and eight OPT sequences from fungi indicated the presence of four conserved amino acid residues. Numbers at the top indicate the positions of the three domains with respect to the consensus sequence. Sequences are annotated with NCBI GI numbers.


. 240 . . . 250 . .
. . 700 . . .
. . 790
Consensus
LNPGPFN-KEHVLITIFAN
D-KLGHYMKIPPRSMF
WFFL-GA
A. thaliana OPT1 15241078
A. thaliana OPT2 15218331
A. thaliana OPT3 15234896
A. thaliana OPT4 15237689
A. thaliana OPT5 15236800
A. thaliana OPT6 15234254
A. thaliana OPT7 15236912
A. thaliana OPT8 15238763
A. thaliana OPT9 15238761
O. sativa 6983869
O. sativa 6983868
O. sativa 6983880
C. albicans OPT1 2367386
S. pombe ISP4 729859
S. pombe 7491035
S. pombe 7492160
S. commune MTD1 6716399
S. cerevisiae OPT1p 6322249
S. cerevisiae OPT2p 6325452
S. cerevisiae YGL114w 1322665

conferred roughly the same phenotypic characteristics as the *C. albicans* and *S. pombe* OPT transporters: growth on tetra- and pentapeptides, sensitivity to toxic peptides and ability to transport radiolabeled KLGL peptide.

Enkephalin transport by Opt1p.

Reports in the literature suggested that endogenous opioids are translocated across the vascular endothelium via a saturable transport system (Banks and Kastin 1997), but no specific protein carrier had been identified. Recently, we have discovered that Opt1p (Hauser *et al.* 2000) transports the opioids leucine enkephalin (YGGFL, leu-enkephalin) and methionine enkephalin (YGGFM, met-enkephalin) into *S. cerevisiae*. A strain expressing Opt1p under the constitutive ADH promoter grew on leu-enkephalin or met-enkephalin as a sole source of leucine or methionine, respectively, to satisfy auxotrophic requirements. These same cells also accumulated intact radiolabeled leu-enkephalin with an apparent K_m of 310 μM. It was verified that extracellular hydrolysis of the peptide did not occur, although it was rapidly hydrolysed inside the cell. Like the PTR system, leu-enkephalin transport is driven by the proton-motive force, with a pH optimum of 5.5. Additionally, transport is sensitive to temperature and to uncouplers of cellular energetics as well as the sulfhydryl reagent pCMBS.

Inhibition of leu-enkephalin transport.

Opt1p does not transport free tyrosine, or di- and tripeptides such as leucyl-leucine and GGF as determined by competition with radiolabeled leu-enkephalin for uptake. The tetrapeptides GGFL, KLGL, and the pentapeptide met-enkephalin competitively decrease leu-enkephalin transport, suggesting that the amino terminal tyrosine is not required for substrate recognition. Only weak inhibition of transport was seen in the presence of amidated leu-enkephalin or the amidated tetrapeptide Tyr-MIF-1, a known substrate for the opioid transport activity of the mammalian central nervous system (Barrera *et al.* 1989), suggesting that the carboxyl terminus is required for substrate recognition by Opt1p.

Peptide opioid receptor antagonists, such as DADLE (Tyr-D-Ala-Gly-Phe-D-Leu enkephalin) and DPDPE (Tyr-D-Penicillamine-Gly-Phe-D-Penicillamine enkephalin) also antagonize transport of leu-enkephalin. Interestingly, naloxone and naltrexone, which are non-peptide opioid receptor antagonists, also inhibit uptake of leu-enkephalin in yeast, suggesting that the receptor and the Opt1 transporter recognize similar molecular features. We have also determined that naloxone is transported by Opt1p with a very low affinity, further confirming its interaction with the transporter (Hauser and Becker, unpublished observation). Naloxone and naltrexone do not inhibit the function of Ptr2p, suggesting that the

inhibitors are interacting directly with Opt1p and not simply disrupting membrane integrity.

Glutathione transport.

Recently, Opt1p has also been found to be a high-affinity glutathione transporter (Bourbouloux *et al.* 2000), the first described for any eukaryote. Glutathione is the most abundant non-protein thiol compound present in almost all prokaryotic and eukaryotic cells. It plays numerous roles including control of redox potential, protection against oxidative stress, detoxification of endogenous and exogenously derived toxins, protein folding, storage, and transport of organic sulfur (Meister and Anderson, 1983; May *et al*, 1998). The uptake of GSH (γ-Glu-Cys-Gly) was measured in several yeast strains with selected transporter gene disruptions (*ptr2*Δ, *ypr194c*Δ, *opt1*Δ,) individually and in combination. Only strains containing a functional copy of *OPT1* were able to transport GSH. Kinetic analysis yielded an apparent K_m of 52 μM for GSH by Opt1p. Both oxidized glutathione and the glutathione conjugate GS-NEM competed for GSH uptake. As expected, di- and tripeptides did not compete (Bourbouloux *et al.* 2000). Recent work in our laboratory has shown that leu-enkephalin also competes with GSH uptake, and visa versa (Donhardt and Becker, unpublished observation), although our results suggest a slightly higher K_m for GSH as compared to that reported by Bourbouloux and coworkers. Taken together, these data suggest that

Opt1p is a multifunctional protein that participates in glutathione transport and oligopeptide transport.

Regulation of the peptide transport systems.

Regulation of peptide transport in prokaryotes.

In prokaryotes, little is known about regulation of peptide transport systems. The *dpp* operon, for example, is negatively regulated by phosphate limitation in *E. coli*, but not regulated by nitrogen source, amino acids, or peptides (Smith and Payne, 1992). The explanation for why phosphate limitation regulates *dpp* expression remains unclear. The expression of *opp* genes, on the other hand, was repressed by the amino acid leucine (Austin *et al.*, 1989). Lrp, which is a leucine-responsive regulatory protein, modulated this negative leucine regulation on *opp* transcription. Interestingly, the expression of the high-affinity transport of branched-chain amino acids operons, *livJ* and *liv*KHMGF, were repressed by Lrp, but leucine relieves the Lrp negative regulation (Calvo and Matthews, 1994). The mechanism of Lrp regulation was proposed to be the result of intracellular leucine sensing. Intracellular leucine binds to Lrp, and the Leu-bound Lrp goes through a conformational change that alters its ability to interact with DNA or other proteins (Calvo and Matthews, 1994). Another regulation involves a repression by GcvB, which encodes a small-untranslated RNA, of *opp* and *dpp*

(Urbanowski *et al.*, 2000). The mechanism is unknown, but *opp* regulation occurs likely at the translational level, while *dpp* regulation seems to be at the mRNA level.

Another type of peptide transport regulation is observed in the expression of the *Tpp* operon in *S. typhimurium*. The *Tpp* transcription is not regulated by nitrogen or carbon source, but is induced by anaerobic growth conditions and amino acids like leucine (Gibson *et al.*, 1987). However, unlike the *Opp* regulation, which occurs as a response to intracellular amino acid, the modulation of *Tpp* expression is a result of extracellular amino acid sensing. A two-component sensor, coded by the *ompB* genes, is required for this type of regulation to occur (Gibson *et al*., 1987; Stock *et al.*, 1989). The extracellular leucine was sensed by this sensor and regulatory signals were transduced leading to induction of *Tpp*. The detailed signal transduction mechanism of this pathway is not known.

Regulation of peptide transport in eukaryotes.

Peptide transport systems identified in eukaryotes belong either to the OPT family, transporters that translocate peptides of four or more amino acids or the PTR family, transporters that translocate di/tri-peptides. The OPT transporters identified in fungal genera *Candida albicans* (Lubkowitz *et al*., 1997), *Saccharomyces*

cerevisiae (Lubkowitz *et al.*, 1998) and *Schizosaccharomyces pombe* (Lubkowitz *et al.*, 1998) are regulated by poor nitrogen source. The *C. albicans* transporter activity was sensitive to nitrogen source and low concentrations of amino acids in the growth medium. In 1991, Payne *et al.* reported that the addition of peptide mixtures to the culture medium increased the transport of oligopeptides and di/tri-peptides. In the mammals, another peptide transport system was observed in the bi-directional tranport of peptides across the blood-brain barrier. Whether this transport system belongs to the OPT family and under a similar regulation is under investigation. However, it is suggested that alcohol regulates the tranport of the pentapeptide methionine enkephalin since peptide transport system 1 (PTS-1) modulates the uptake of an opiate methionine enkephalin (Plotkin *et al.*, 1997). Other types of regulation are not known.

Di/tri-peptide transport in plants was initially observed in the scutellum, an absorptive tissue surrounding the endosperm, of barley and wheat seeds (Sopanen *et al.*, 1977; Higgins and Payne, 1980; Salmenkallio and Sopanen, 1989). Photoaffinity cross linking with a dipeptide analog identified two candidate proteins (66 and 41 kd) involved in this transport system (Hardy and Payne, 1991). The 66-kd protein is identical to the recently identified di/tri-peptide transport HvPTR1 protein expressed in the scutella of germinating barley grain (Waterworth *et al.*, 2000). Sequence analysis of the full-length HvPTR1 cDNA (2260 bp) revealed an open reading frame with 58% identity to the *Arabidopsis*

thaliana peptide transporter AtPTR2-B (West *et al.*, 1998). Peptide transport in plants should provide a significant source of nutrients for plant development. For example, HvPTR1 expression was detectable only during early germination, while inhibition of AtPTR2-B expression by its antisense message caused a late flowering and arrested seed development (Song *et al.*, 1997).

In humans, the PTR family homologs that have been characterized are limited to hPEPT1 and hPEPT2, found primarily in the small intestine and kidney, respectively. Regulation of di/tri-peptide transport in mammals has been less studied at the molecular level than in yeast. The expression of PEPT1 seems to be under developmental regulation in a tissue- and cell-type specific manner. For example, besides the small intestines, hPEPT1 was also observed in the liver, showing that it may function to clear the products of peptide hormone degradation (Nussberger *et al.*, 1997). The intestinal expression of PEPT1 was induced postpartum, possibly by suckling, and again at the time of weaning (Shen *et al.*, 2001). Its expression was also observed in the colon only during the first week of life.

Expression studies with rat and rabbit homologs of hPEPT1 confirm their function in mediating small peptide uptake. The rat homolog of hPEPT1 was specifically expressed in the brush border epithelial cells of the intestine (Wenzel *et al.*, 1996). The rat PEPT1 was the predominant carrier of β-lactam antibiotics in the rat small intestine, showing that peptide absorption by the intestine is an

important phenomenon for oral drug delivery (Fricker and Drewe, 1996; Walter *et al.*, 1996). The rabbit PEPT1 expression was abundant in the small intestine, supporting its role as a small peptide transporter in the small intestine (Fei *et al.*, 1994). PEPT1 expression was increased after a brief fasting or malnourishing conditions (Lee *et al.*, 1999; Ihara *et al.*, 2000). Furthermore, the up-regulation of rat PEPT1 was affected by selective amino acids (Phe, Arg, and Lys) and dipeptides (Gly-Sar, Gly-Phe, Lys-Phe, and Asp-Lys) through transcriptional activation of the PEPT1 gene (Shiraga *et al.*, 1999). Thus, functional expression of PEPT1 is affected by dietary intake.

In the human intestinal cell line Caco-2, hPEPT1 mRNA half-life, transcription, and protein levels are also induced after growth in peptide containing medium (Walker *et al.*, 1998). A direct response of Caco-2 cells to peptides indicates that peptide induction of hPEPT1 is independent of regulation by neurons or hormones (Walker *et al.*, 1998). However, hormonal regulation of hPEPT1 exists. Insulin, for example, did not change the hPEPT1 mRNA, but the amount of hPET1 protein was increased at the apical membrane. The mechanism appears to be increased translocation of the transporter from a preformed cytoplasmic pool (Thamotharan *et al.*, 1999). EGF treatment, on the other hand, negatively regulates peptide uptake in Caco-2 cells by decreasing the number of hPEPT1 protein in the apical membrane (Nielsen *et al.*, 2001).

In another study, protein kinase C (PKC) activity resulted in a decrease in the peptide uptake rate in Caco-2 cells (Brandsch *et al.*, 1994). Protein synthesis was not required in the PKC-dependent negative regulation of peptide transport. Furthermore, flavonoids that inhibit epidermal growth factor-receptor tyrosine kinase activity stimulate PEPT-1-mediated uptake of the beta-lactam antibiotic cefixime in Caco-2 cells (Wenzel *et al.*, 2001). Thus, protein kinase activity may directly regulate the intestinal peptide transport.

LLC-PK1, an hPept2 homologue, was described in porcine kidney cells (Wenzel *et al.* 1998) and demonstrated to be regulated by PKC- and protein kinase A (PKA) -dependent signalling pathways via a change in substrate affinity (Wenzel *et al.* 1999). Although PKC and PKA sites are present in the loop between the eighth and ninth TMDs, it is unclear whether the kinases act directly on the transporter or exert their effects indirectly. Interestingly, the Ptr2p transporter of *Saccharomyces* lacks this loop region. The regulation of expression of *PTR2* has an absolute requirement for *PTR1* (Alagramam *et al.* 1995) and can also be enhanced by *PTR3* (Barnes *et al.* 1998). Regulation of di/tri-peptide transport system will be discussed in further details in a section below.

In rat choroids plexus, PEPT-2 was shown to mediate the uptake of neuropeptides (Teuscher *et al.*, 2001). This transporter was recently found to be expressed in the peripheral nervous system (Groneberg *et al.*, 2001a) and lung (Groneberg *et al.*, 2001b), where PEPT-2 may serve as uptake system for products

of protein degradation, for removal of biologically active short-chain peptides and non-peptides such as peptidomimetics. In the kidney cells, a calmodulin antagonists affected the peptide transport activity, indicating that calmodulin modulated the peptide uptake in the kidney (Brandsch *et al.*, 1995).

Regulatory elements of peptide transport in S. cerevisiae.

The *S. cerevisiae* di/tri-peptide transport is regulated by at least 2 genes, *PTR1* and *PTR3*. *PTR1* regulates the transcription of *PTR2* and is essential for peptide transport. *PTR3* is another regulatory element, and while not absolutely essential, enhances the expression of *PTR2*. Regulatory elements including those with homology to *PTR1* or *PTR3* of the PTR system have not been identified in the OPT system making it difficult to initiate study of peptide regulation in this system. Therefore, instead of the newly discovered OPT family, the PTR system was used as a model system to study the regulation of peptide transport in *S. cerevisiae*. In this dissertation, I focused on regulation of di/tri-peptide transport, which is discussed in detail below.

Regulation of di/tri-peptide transport system.

The yeast PTR system is regulated in response to signals, which include nitrogen source and the presence of small amounts of amino acids (Barnes *et al.*, 1998; Island *et al.*, 1987). Transport was found to be up-regulated when cells were

grown in a poor nitrogen source such as allantoin or proline, and down-regulated in cells grown on a rich nitrogen source such as ammonium sulfate (Becker and Naider, 1977; Nisbet and Payne, 1979). Peptide transport is also subject to a high level of induction by micromolar amounts of various amino acids irregardless of the nitrogen source (Island *et al.*, 1987). Previous northern analysis of *PTR2* expression indicated that amino acid regulation of peptide transport occurred at the level of gene expression (Perry *et al.*, 1994). Finally, di/tri-peptide uptake was accelerated by extracelullar dipeptides (Turner *et al.*, 2000). Regulation of di/tri-peptide tranport is mediated by at least two genes: *PTR1* and *PTR3.*

These genes were discovered along with *PTR2* by a genetic complementation analysis coupled with N-methyl-N-nitro-N-nitrosoguanidine mutagenesis (Island *et al.* 1991). The transcription of *PTR2* is regulated by both *PTR1* and *PTR3.* However, while *PTR1* is essential for peptide transport, *PTR3* is not absolutely essential but is required for the optimal expression of *PTR2.* The isolation and characterization of *PTR1* and *PTR3* have been completed and are detailed below.

PTR1.

The *ptr1* allele mutant is characterized by its total inability to utilize small peptides to satisfy auxotrophic requirements, resistance to the growth inhibiting

effects of toxic peptides and inability to accumulate radiolabeled dipeptides at wild type levels (Island *et al.* 1991). In addition, *ptr1* strains display subtle defects in sporulation and slightly decreased growth rates (Alagramam, unpublished observation; Bartel *et al.* 1990). Cloning of the *PTR1* gene by functional complementation of a *S. cerevisiae ptr1* strain deficient in peptide transport reveals that *PTR1* encodes a soluble protein of 1950 amino acids, which is identical to Ubr1p (Alagramam *et al.* 1995). The protein Ubr1p encoded by *UBR1* was previously termed N-recognin (or E3), and was shown to be an essential component of the N-end rule pathway, a ubiquitin-dependent protein degradation system (Bartel *et al.* 1990). This system targets proteins carrying a degradation signal called the N-degron (Bachmair *et al.* 1986), which comprises a destabilizing N-terminal residue and a lysine residue (Bachmair and Varshavsky 1989, Varshavsky 1996). Further, Dohmen (Dohmen et al. 1991) found that Ubr1p / Ptr1p acts in concert with a specific ubiquitin-conjugating enzyme encoded by the gene *UBC2/RAD6* in the degradation of N-end rule substrates. Ubiquitin-dependent proteolysis is an important regulatory mechanism that controls many cellular processes. In this pathway, ubiquitin (Ub) is activated for transfer to substrate protein by ATP-dependent attachment to a ubiquitin-activating enzyme (E1). Ub is then transferred to a Ub-conjugating enzyme (E2), which then transfers Ub to substrate proteins through an interaction with a Ub-protein ligase (E3), ultimately conjugated in an isopeptide linkage via a lysine

residue of a substrate protein. Within the PTR system, the substrate for ubiquitin-dependent protein degradation system mediated by Ubr1p / Ptr1p is Cup9p, a protein previously shown to be involved in detoxification of copper in *S. cerevisiae* (Varshavsky 1996). This protein was identified in a genetic screen since mutations in *CUP9* bypassed a requirement of *PTR1* for peptide import and *PTR2* expression. Cup9p is a homeodomain-containing protein that binds to the *PTR2* promoter and, in the presence of Ubr1p / Ptr1p, is released from the promoter relieving the repression of *PTR2* expression (Byrd *et al.* 1998).

Several domains have been identified in Ubr1p / Ptr1p. A basic residue-rich domain located between amino acids 1081-1220 is involved in binding to Ubc2p / Rad6p (Xie and Varshavsky 1999). Furthermore, the RING-H2 domain located between amino acids 1176-1367 is absolutely required for ubiquitylation of N-end rule substrates, but not for binding of Ubr1p to Ubc2p / Rad6p or to substrates. Premature truncation of the last 15 amino acids containing an acidic tail of Ptr1p abolishes both peptide transport and ubiquitin-mediated degradation of an engineered N-end rule substrate (Anderson and Becker, unpublished observation). This suggests that this domain may be necessary for protein-protein interaction between Ubr1p / Ptr1p and Cup9p or the ubiquitin conjugating protein Ubc4p. Ubr1p / Ptr1p has two substrate binding sites, one for N-terminal type 1 with basic residues and the other for N-terminal type 2 with bulky hydrophobic residues (Kwon *et al.* 1999). *In vitro* studies showed that

binding of dipeptides with destabilizing N-terminal residues at these sites allosterically activated the degradation of Cup9p (Turner *et al.* 2000). The data suggest that imported peptides bind to Ubr1p / Ptr1p and accelerate the Ubr1p / Ptr1p-dependent degradation of Cup9p, thus relieving the Cup9p repression on the *PTR2* expression. However, since dipeptides are thought to degrade quickly as they are transported into the cell, the question whether intact dipeptide really binds to Ptr1p *in vivo* is critical to determine whether this positive feedback mechanism occurs in the cytoplasm. Finally, *UBR1/PTR1* is not required for the expression of *PTR3*, another regulatory protein in di / tri-peptide transport system (Barnes *et al.* 1998). Whether *PTR1* and *PTR3* regulate the di / tri-peptide uptake within the same or different pathway (s) is unknown.

PTR3.

In contrast to *ptr1* and *ptr2* strains, which exhibited a total inability to utilize small peptides to satisfy amino acid auxotrophic requirements, the *ptr3* strain was characterized by its partial ability to transport di / tri-peptides and by a decreased sensitivity to the toxic amino acid analogs ethionine and *m*-fluorophenylalanine (Island *et al.* 1991). Cloning of the *PTR3* gene by functional complementation of a peptide transport deficient *S. cerevisiae ptr3* strain revealed that *PTR3* encoded a soluble 678 amino acid protein with no similarity to any previously identified coding sequences (Barnes *et al.* 1998). Furthermore, Ptr3p

did not contain any signature sequences indicative of previously characterized functional motifs. Since mutations in *PTR3* did not interfere with nitrogen regulation of peptide transport, *PTR3* is specifically required for amino acid induction of peptide transport activity (Barnes *et al.* 1998). This gene is also required for amino acid-induced expression of some amino acid permeases such as *BAP2*, the gene encoding the branched amino acid permease. Moreover, *PTR3* does not function strictly as an activator since it is also necessary for repression of *GAP1* (General Amino Acid Permease). Genetic analysis showed that *PTR3* functions with *CUP9* and *PTR1* in regulating *PTR2* expression (Barnes *et al.* 1998). This finding indicates that the ubiquitin-mediated degradation of a transcriptional repressor is linked with the amino acid sensing mechanism.

A gene required for amino acid sensing involved in regulating amino acid permeases has been identified. This gene, *SSY1*, encodes Ssy1p, which is a homologue of an amino acid permease (Didion *et al.* 1998, Iraqui *et al.* 1999). Cells containing the *ssy1*Δ allele are resistant to toxic peptides and amino acid analogues (Didion *et al.* 1998). Ssy1p does not transport amino acids, but functions as a receptor for amino acids. An amino-terminal, cytoplasmic extension found in Ssy1p, but not amino acid permeases, appears to be involved in transducing a signal to intracellular target(s) subsequent to binding of amino acid to an extracellular domain of Ssy1p. Characterization of single null mutant *ssy1*Δ or *ptr3*Δ and the corresponding double mutant shows identical phenotypes

indicating that Ptr3p interacts with Ssy1p for regulating expression of both peptide transporters and amino acid permeases (Didion *et al*. 1998, Barnes *et al*. 1998, Klasson *et al*. 1999). Genetic interactions among *SSY1*, *PTR3*, and *SSY5* further suggested that the products of these genes form an amino acid sensor machinery named SPS, which stands for Ssy1p-Ptr3p-Ssy5p (Forsberg and Ljungdahl, 2001).

We recently tested whether, in addition to amino acids, extracellular dipeptides regulate the expression of *PTR2*. We showed that both the amino acids and dipeptides induced *PTR2* expression (Hauser *et al*., 2001). *SSY1* was critical for amino acid induction, but not as crucial for dipeptide induction. *PTR3* is required for both amino acid and peptide induction, although it appears to play a greater role in the latter case. The discovery of a dual role for *PTR3* in induction by either amino acids or dipeptides, suggests another layer of regulation of peptide transport. It is uncertain whether dipeptides must be transported into the cell or if they are detected by a dipeptide sensor similar to Ssy1p. *PTR2* is required for induction by dipeptides, but is not necessary for amino acid induction. Recently, *in vitro* binding assays showed that dipeptides accelerate peptide transport by binding to Ptr1p / Ubr1p (Turner *et al*. 2000). This result may support the requirement for *PTR2* in dipeptide induction since the peptides must be transported by Ptr2p in order to enter the cells and interact

with Ubr1p / Ptr1p. However, biochemical evidence indicates that dipeptides are hydrolysed as soon as they are internalized (Payne and Smith, 1994).

The biochemical function of Ptr3p remains a mystery, although there are some hints to its cellular role. There are no characterized homologs of Ptr3p in the entire protein database, although an uncharacterised Ptr3p homologue has been identified through the *C. albicans* genome sequencing project (Narita and Becker, unpublished observation). A small region (amino acids 511-575) of Ptr3p shows protein sequence homology to several amino acid permeases as well as to the transcription factor Gcn4p (Klasson *et al*. 1999). The function of this portion of Ptr3p is not known, although the region may serve as a regulatory amino acid binding site. Use of the PEST-Find algorithm (Rechsteiner and Rogers 1996) revealed that Ptr3p contains a sequence that closely matches the consensus for a PEST motif (Barnes and Becker, unpublished observation) indicating its role as a signal for ubiquitin-mediated protein degradation, similar to Cup9p. Obviously, further study is required to unravel the function of Ptr3p and the protein interactions involved in regulating peptide transport. This dissertation will mainly focus on studying the di / tri-peptide transport through dissecting the function of Ptr3p. Studies on the amino acid sensing system in *S. cerevisiae* provide some hints on the function of this novel protein.

Conclusion.

The recent expansion in the identification of new peptide transporters in both the PTR and OPT families has served to underscore how little is known about these important proteins. While we have knowledge of the gross characteristics of these families of transporters, such as tissue expression, energetic coupling, and substrate affinity and specificity, little is known about more subtle aspects of their function, such as control of expression. In many cases, most notably within *A. thaliana*, *C. elegans* and humans, multiple PTR family orthologues have been identified within the same organism. Why is this redundancy of transporters needed? Obvious possibilities are different kinetic properties (i.e. high vs. low affinity transporters) and differential tissue expression, but the regulation of their tissue-specific or developmental expression has not been investigated. In this respect, work in *S. cerevisiae* will be useful since many genes involved in the regulation of expression of *PTR2* have been identified and their roles are being elucidated. Perhaps some of what this system yields can be generalized to other organisms.

The restriction of OPT family members to fungi, plants and bacteria raises an interesting question. Do animals lack the ability to transport oligopeptides, or are the DNA sequences of potential metazoan transporters so different from those of the current OPT family members that they can not be identified by sequence homology? If no OPT family orthologues or other oligopeptide

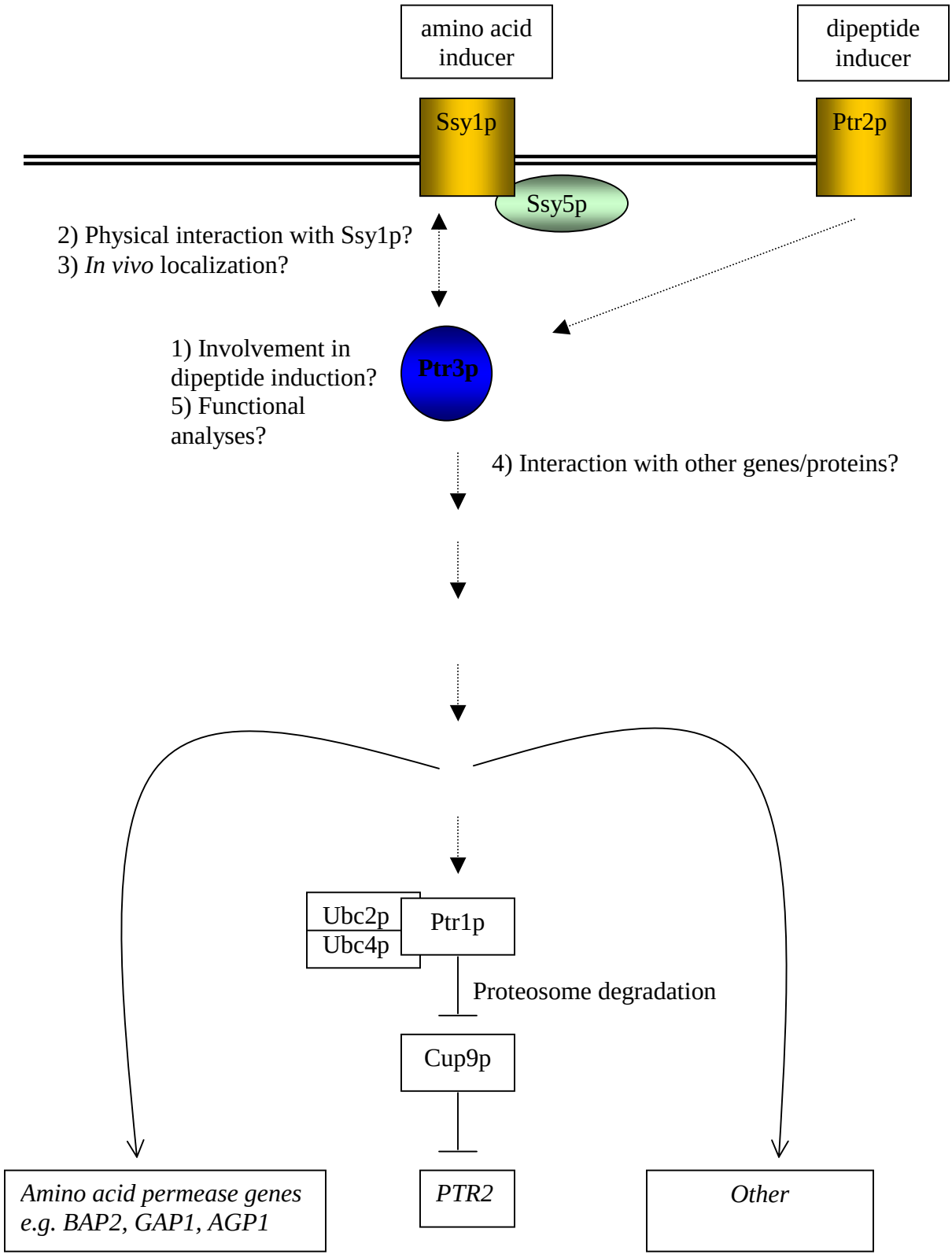
transporters can be identified in animals, specifically in mammals, then the fungal OPT family members provide a good target for peptidomimetic drug delivery with a minimum of host side-effects. Clearly, a more extensive search for mammalian homologs is warranted, as well as further investigation into the regulation of OPT family member expression.

The lack of any sort of sequence homology between the PTR and OPT families is also quite surprising. From an evolutionary standpoint, these proteins did not evolve from a common ancestor, but still converged on a common solution to complete similar tasks. It would be of interest to determine the strategies employed by each of these transporters to selectively recognize either di/tri-peptides or oligopeptides. The multiplicity of peptide transporters and the regulation of their expression and activity present a fascinating and challenging biological system for further study.

As discussed above, little is known about the detailed mechanism of peptide transport regulation. Previously, a novel regulatory component of the PTR system in *S. cerevisiae*, *PTR3*, was cloned and analyzed, providing a starting point to unravel regulatory pathways of peptide transport (Barnes *et al.*, 1998). Although it was proposed that Ptr3p is involved in amino acid induction of peptide transport as a component of an amino acid sensor machinery together with Ssy1p and Ssy5p (Klasson *et al.*, 1999), the biochemical function of Ptr3p is unknown. In this study several studies were proposed, and the data obtained

should give useful information to define Ptr3p function (Fig. 5). First, the involvement of Ptr3p in both amino acid and dipeptide induction of peptide transport should be analyzed. Evidence of physical interaction between Ptr3p and the amino acid sensor Ssy1p, as well as the cellular localization of Ptr3p, would form a foundation to determine the function of Ptr3p. Further insights into the function of Ptr3p should be gained by establishing a connection between *PTR3* and other genes known to modulate peptide transport. These data would constitute a rough draft of the regulatory network within peptide transport regulation pathways. Finally, a computational algorithm together with site directed mutagenesis studies could be utilized to identify any motif or functional domain in Ptr3p. Such information would provide hints on how Ptr3p regulates the peptide transport process. In this dissertation, I report the molecular, genetic, and functional analysis of Ptr3p, a novel regulatory protein of peptide transport in *S. cerevisiae.*

Fig. 5. Proposed studies on Ptr3p. To gain information on the function of Ptr3p in induction of peptide transport system, we analyze: 1) The involvement of Ptr3p in both amino acid and dipeptide induction of peptide transport, 2) Physical interaction between Ptr3p and the amino acid sensor Ssy1p, 3) Cellular localization of Ptr3p, 4) Interaction between *PTR3* and other genes or proteins, and 5) Functional analyses on Ptr3p, which include computational algorithms together with site directed mutagenesis studies to identify any motif or functional domain in Ptr3p.


amino acid inducer
dipeptide inducer
Ssy1p
Ptr2p
Ssy5p
2) Physical interaction with Ssy1p?
3) *In vivo* localization?
1) Involvement in dipeptide induction?
5) Functional analyses?
Ptr3p
4) Interaction with other genes/proteins?
Ubc2p
Ubc4p
Ptr1p
Proteosome degradation
Cup9p
Amino acid permease genes e.g. BAP2, GAP1, AGP1
PTR2
Other

regulation pathways. Finally, a computational algorithm together with site directed mutagenesis studies could be utilized to identify any motif or functional domain in Ptr3p. Such information would provide hints on how Ptr3p regulates the peptide transport process. In this dissertation, I report the molecular, genetic, and functional analysis of Ptr3p, a novel regulatory protein of peptide transport in *S. cerevisiae.*

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PART II

ROLE OF PTR3P IN AMINO ACID AND DIPEPTIDE INDUCED REGULATION OF DI/TRI-PEPTIDE TRANSPORT IN *S. CEREVISIAE*

CHAPTER I

INTRODUCTION

The yeast *S. cerevisiae* is known to utilize nutrient sensors acting at the plasma membrane to initiate proper growth responses. Some members of the sugar and amino acid transport gene families act as nutritional sensors. For example, Snf3p and Rgt2p, whose structures are similar to hexose transporters, have been suggested to transduce nutritional signals regarding extracellular glucose availability (Ozcan *et al.*, 1998). Another sensory protein, Ssy1p, acts as a receptor in an amino acid-inducible signal transduction pathway. Ssy1p is required for amino acid induction of the di/tri-peptide transporter (Didion *et al.*, 1998) and amino acid permeases such as the branched amino acid permease *BAP2* (Grauslund *et al.*, 1995; Didion *et al.*, 1996) and the general amino acid permease *GAP1* (Jauniaux and Grenson, 1990).

Ssy1p is a transmembrane protein that both hydropathy analysis and subcellular fractionation studies show to be localized at the plasma membrane (Didion *et al.*, 1998; Iraqui *et al.*, 1999a; Klasson *et al.*, 1999). It was hypothesized that Ptr3p transduced an amino acid-responsive signal from Ssy1p to downstream effectors including Ptr1p/Ubr1p and Cup9p, that resulted in a change in *PTR2* expression (Iraqui *et al.*, 1999b; Klasson *et al.*, 1999). Ptr3p was

physically associated with the cell membrane according to subcellular fractionation by gradient centrifugation (Klasson *et al.*, 1999), and genetic interactions suggested that Ptr3p was a component of the proposed amino acid sensor Ssy1p-Ptr3p-Ssy5p complex named SPS (Klasson *et al.*, 1999; Forsberg and Ljungdahl, 2001a).

Specific systems mediating transport of intact peptides across the plasma membrane in an energy-dependent manner have been observed in most cells examined (Ganapathy and Leibach, 1991; Payne and Smith, 1994; Becker and Naider, 1995). Once internalized, these peptides are rapidly hydrolyzed by intracellular peptidases and may then serve as a source of nitrogen or amino acids for growth. In *S. cerevisiae*, the transport of peptides containing two or three residues (dipeptide/tri-peptide transport) has been well characterized physiologically and genetically (Becker and Naider, 1995). The products of at least three genes, *PTR1*, *PTR2*, and *PTR3* were shown by mutagenesis and complementation analysis to mediate and regulate the di/tri-peptide transport process (Island *et al.*, 1991).

PTR2 encodes a transporter protein consisting of 601 amino acids predicted to contain 12 membrane-spanning domains on the basis of hydropathy analysis (Perry *et al.*, 1994). *PTR1* (or *UBR1*) encodes a 1951 amino acid protein that functions as a regulator of *PTR2* expression (Alagramam *et al.*, 1995) through

its involvement in the proteolytic destruction of a *PTR2* repressor Cup9p via the N-end rule ubiquitin pathway (Byrd *et al.*, 1998). *PTR3* encodes a protein of 678 amino acids, which functions to regulate the peptide transport system in a complex manner. Unlike *ptr2* and *ptr1* strains that exhibited negligible levels of peptide transport activity, *ptr3* mutants (Island *et al.*, 1991) and *ptr3*Δ strains (Barnes *et al.*, 1998) are leaky. *ptr3* strains maintained the ability to use peptides as a source of amino acids for growth but gained resistance to toxic dipeptides in the presence of ammonium. Although Klasson *et al.* (1999) showed that a Ptr3p region consisting of amino acid 511-575 shares some homology to several amino acid permeases, there are no other regions of homology to any protein in the current database reported to date.

The yeast PTR system is regulated in response to signals that include nitrogen source and the presence of small amounts of amino acids (Barnes *et al.*, 1998; Island *et al.*, 1987). Transport was found to be up-regulated when cells were grown in a poor nitrogen source such as allantoin or proline, and down-regulated in cells grown on a rich nitrogen source such as ammonium sulfate (Becker and Naider, 1977; Nisbet and Payne, 1979). Peptide transport is also subject to a high level of induction by micromolar amounts of various amino acids irregardless of the nitrogen source (Island *et al.*, 1987). There is no obvious relationship between the physical-chemical properties of amino acids and their

ability to act as inducers for di/tri-peptide transport. Previous northern analysis of *PTR2* expression indicated that amino acid regulation of peptide transport occurred at the level of gene expression (Perry *et al.*, 1994). Finally, di/tri-peptide uptake was accelerated by extracelullar dipeptides, which once taken up by the cells were suggested to gain access to allosteric binding sites in Ptr1p/Ubr1p thereby activating ubiquitin-mediated degradation of the *PTR2* repressor Cup9p (Turner *et al.*, 2000). The Cup9p degradation then leads to up-regulation of *PTR2* expression as reported by a *lac-Z* reporter gene assay (Hauser *et al.*, 2001).

We were interested in studying further the involvement of Ptr3p in the amino acid and dipeptide regulation of peptide transport. In this paper, we report on the effect of amino acid supplementation on di/tri-peptide transport by gene reporter assays, we analyze whether extracellular amino acids and dipeptides regulate the expression of *PTR2* through the same or different mechanisms, and we study Ptr3p localization and its physical interaction with Ssy1p.

CHAPTER II

MATERIALS AND METHODS

Strains and media.

Yeast strains used are listed in Table 1. The strains used in this study were grown routinely on YEPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar). Strains transformed with a plasmid were cultured on minimal medium lacking uracil or leucine (0.67% Difco yeast nitrogen base with ammonium sulfate, without amino acids, 2% glucose, 0.2% casamino acids). For peptide toxicity assays, cells were inoculated into medium lacking ammonium sulfate (0.67% Difco yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose) supplemented with 0.1% proline as a nitrogen source, and leucine (228 μM) as inducer (proline medium). For two-hybrid analysis, cells were grown in minimal dropout media containing 2% glucose (Glu) or 2% galactose plus 1% raffinose (Gal) and are designated by the component that is left out (e.g. SC (Glu)–ura –leu –his –ade medium has 2% glucose and lacks uracil, leucine, histidine, and adenine). 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) minimal dropout plates contained 40 mg / ml X-Gal and phosphate buffer at pH

Table 1. *S. cerevisiae* strains used.

Strain	Genotype	Reference
FY2	*MATa ura3-52*	Winston et al. (1995)
BWS-201	*MATa ura3-52 ptr3::Loxp*	Barnes et al. (1998)
*ssy1*Δ	*MATa ura3-52 ssy1::Loxp*	This work
PB1X-2AΔ	*MATa ura3-52 leu2-3 lys1-1 his4-32 ptr2*::*LEU2*	Perry et al. (1994)
BBY-47	*MATa ura3-52 leu2-3,112 lys2-801 trp1-1 his3-Δ200 gal ubr1-Δ1::LEU2*	Bartel et al (1990)
BLS-201	*MATa ura3-52 cup9::Loxp*	Barnes et al. (1998)
BLS-206	*MATa ura3-52 ptr3::Loxp cup9::Loxp*	Barnes et al. (1998)
PJ69-4A	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*	James et al. (1996)

7.0. DNA was introduced into yeast by LiAc-mediated transformation as described (Gietz *et al.*, 1995). Construction of *ssy1*Δ strain in the FY2 background was accomplished by the method of Guldener *et al.* (1996), which results in the replacement of a target ORF with the dominant selectable kan^r marker. Briefly, bipartite oligonucleotide primers (*SSY1kan*-F and *SSY1kan*-R; Table 2) were used to PCR-amplify the kan^r marker from the pUG6 plasmid resulting in 40 bp extension of the *SSY1* ORF flanking a functional kan^r cassette. PCR amplification using these primers yielded a 1.7 kb fragment that contained the kan^r cassette flanked by the 40 nucleotides of *SSY1*. Transformants were identified on the basis of resistance to G418 (300 μg / mL) and were verified by PCR and Southern analysis (data not shown).

Plasmids.

All plasmids were created by *in vivo* ligation between the insert gene and the empty vector. Plasmids and nucleotides used in this study are shown in Table 2 and 3. The plasmid pUTR-*PTR3* was created by polymerase chain reaction (PCR) amplification of *PTR3* gene including its 5' and 3' UTR using primers pRS316-3-F and pRS316-3-R, and the resultant product was ligated into pRS316. A unique BglII site was introduced by a site directed mutagenesis right before *PTR3* start codon using primer SDM3-(START) creating plasmid pUTR3(1-678). The plasmid p*GFP-PTR3* was created by polymerase chain reaction (PCR)

amplification of the GFP cassette using primers sGFP3-F and SGFP3-R, and the resultant product was ligated into the pUTR3(1-678) such that a fusion protein was created with GFP at the N-terminus and under the *PTR3* endogenous native promoter. The plasmid pLACZ-2 was created by PCR amplification of the *PTR2* promoter and *PTR2* and the resultant product was ligated into the *lac-Z/URA3*/2μ-based vector pYLZ6 (Hermann *et al*., 1992) such that the reporter *lac-Z* genes were under the control of native *PTR2* promoter. The plasmids pGBDU-C1 and pGAD-C1 used for two-hybrid analysis were obtained from James *et al.* (1995). pGBD-*PTR3* was created by PCR amplification of the ORF (YFR209W) using primers pGBD-PTR3-F and pGBD-PTR3-R, and the resultant products was ligated into the *URA3*/2μ-based vector pGBDU-C1 such that the gene was fused with the Gal Binding Domain (GBD) gene and under the control of an ADH promoter. pGAD-N*SSY1* (YDR160w) was created by PCR amplification of the a cassette encoding only the first 282 amino acids of Ssy1p using primers pGAD-NSSY1-F and pGAD-NSSY1-R. The resultant products were cloned into the *LEU2*/2μ-based vector pGAD-C1 such that the genes were fused to the Gal Activating Domain (GAD) gene and were under the control of an ADH promoter. pHis_6-*PTR3* was created by PCR amplification of the ORF (YFR209W) using primers His_6-*PTR3*-F and His_6-*PTR3*-R, and the resultant products was ligated into pDB20 such that the gene was under the control of an ADH

Table 2. Plasmids used.

Plasmids	Description	Reference
pLACZ-2	*PTR2* promoter *lac-Z* fusion in pYLZ6	This work
pGFP-*PTR3*	GFP and *PTR3* fusion in pUTR3(1-678)	This work
pGFP-N-FUS	the GFP fusion vector in p416*MET*25	Niedenthal et al. (1996)
pAJ226	*RAT1* fused with GFP in pTD150	Johnson, A. (1997)
pVT-*RGS4*-GFP	*RGS4* fused with GFP in pRSETB-GFP	Srinivasa et al. (1998)
pAJ232	*RAT1* (NLSΔ) fused with GFP in pTD150	Johnson, A. (1997)
pGBDU-C1	two-hybrid expression vector containing GBD	James et al. (1995)
pGBD-*PTR3*	GBD-*PTR3* in pGBDU-C1	This work
pGAD-C1	two-hybrid expression vector containing GAD	James et al. (1995)
pGAD-*NSSY1*	GAD-*NSSY1* in pGAD-C1	This work
pHis6-*PTR3*	His6-*PTR3* in pDB20 under an ADH promoter	This work
pGAD-FLAG-*NSSY1*	FLAG-*NSSY1* in pGAD-C1	This work

Table 3. Nucleotides used.

Oligonucleotides	Sequence
PTR2-F	5’-TTCCGCACCATTCCAAAACTACAT-3’
PTR2-R	5’-GCAGCACAGAAAACTCCCGTCAAC-3’
ACT-F	5’-GCCGGTTTTGCCGGTGACGAC-3’
ACT-R	5’-GGAAGATGGAGCCAAAGCGTG-3’
SSY1kan-F	5’-AAACTGCTTAAAAATAGGGAAGTTCCTTGAGGAATCA GCTGAAGCTTCGTACGC-3’
SSY1kan-R	5’-ATAATAATACTAATAATAGTACATATAACCCTAAGCAT AGGCCACTAGTGGATCTG-3’
pRS316-3-F	5’-GATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTAA CGCCCGATCTCAAGATTT-3’
pRS316-3-R	5’-AGCTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTTG CACCCCATCTAAACGAAA-3’
SDM3-(START)	5’-GGTACGAAATACAGATCTGAT AGGCGTTCATGC-3’
sGFP3-F	5’- CACATACATAGGTACGAAATACAGATCTGATAGGCGT TCATGGCTAGCAAAGGAGAAGAA-3’
sGFP3-R	5’- GGCAAAAAAGTACTCCTTTTCTTCGTCCAGATCATGG CAGCCGGATCCTTTGTATAG–3’
pGBD-PTR3-F	5’-GAGTAGTAACAAAGGTCAAAGACAGTTGACTGTATCG CCGCACGCACACTCACACATACAT-3’
pGBD-PTR3-R	5’-TGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTC ATACCAGAACCTTAAACATACG-3’
*pGAD*NSSY1-F	5’-CGATGATGAAGATACCCCACCAAACCCAAAAAAAGAG ATCAAGAGGCCAAAAGTTAT-3’
*pGAD*NSSY1-R	5’-ACGATTCATAGATCTCTGCAGGTCGACATCGATGGATC CCCCAGTTGCCCTGGACCTATTG-3’
His6Ptr3p-F	5’-CCAAGCTTCGAGCGGCCGCCAGTGTGATGGATATCC-3’
His6Ptr3p-R	5’-AGTCCAAAGCTTCGACGGCCGCCAGTGTGATGGAA C-3’
FLAGN*SSY1*	5’-TTCAATAGGTCCAGGGCAACTGGGGGATCCATCGACT ACAAGGACGACGATGACAAGGGTACCGTCGACCTGCAG AGATCTATGAATCGTAGATACTGA-3’

promoter. pGAD-FLAG-NSSY1 was created by in vivo ligation using the FLAGNSSY1 oligonucleotide. Plasmids were transformed into yeast by the LiAc-mediated transformation as described (Agatep *et al.*, 1998), and transformants were selected by growth on minimal medium lacking uracil or leucine.

Northern analysis.

Yeast total RNA was isolated by phenol-chloroform extraction as previously described with the following modifications (Schmitt *et al.*, 1990; Perry *et al.*, 1994). All phenol-chloroform extractions were performed with 15 ml spin phase-lock gel tubes (5 Prime-3 Prime, Inc., Boulder, CO). Total RNA was quantified by monitoring absorbance at 260 nm, and 25 μg per lane was loaded into a formaldehyde-reducing gel as described (Perry *et al.*, 1994). Gels were run for 3 h at 75 V in running buffer containing 10% MOPS and 10% formaldehyde and then vacuum-transferred to a nylon hybridization membrane (Bio Trans, ICN Pharmaceuticals, Costa Mesa, CA) in 10xSSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The RNA was UV cross-linked with the Stratalinker UV source as specified by the manufacturer (Stratagene, La Jolla, CA). Blots were rinsed with 4x SSC and prehybridized for 4 h at 42^0C in Northern Max™ prehybridization / hybridization buffer (Ambion, Austin, TX). Radioactive probes were prepared as follows. The following DNA fragments were obtained by PCR (the oligonucleotides used to prime the reactions are indicated in parentheses; 1

μg of genomic DNA isolated from yeast strain FY2 served as the template): a 1.7 kb fragment of *PTR2 (PTR2-F* and *PTR2-R*), a 1.0 kb fragment of *CUP9* (*CUP9-F* and *CUP9-R*), a 1.8 kb fragment of *BAP2* (*BAP2-F* and *BAP2-R*), a 2.8 kb fragment of *UGA35* (*UGA35-F* and *UGA35-R*), and a 0.92 kb fragment of *ACT1* (*ACT1-F* and *ACT1-R*). PCR products were analyzed by restriction analysis to ensure their identity and gel purified. DNA fragments were labeled with [α-^{32}P]dATP (3000 Ci/mmol; ICN Pharmaceuticals, Costa Mesa, CA), using a megaprime DNA labeling system (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Hybridizations were carried out in Northern Max™ prehybridization/hybridization buffer (Ambion, Austin, TX) containing 10% dextran sulfate sodium salt at 42^{0}C overnight (10^6 cpm/ml). Blots were rinsed once in 1x SSC plus 0.1% SDS at 65^{0}C for 20 min, twice in 0.1xSSC plus 0.1% SDS at 65^{0}C for 20 min. Data analysis was performed by electronic, filmless autoradiography (Instant-*Imager*, Packard BioScience Company, Meriden, CT), and quantification of signal by Instant*Imager* analysis software (Packard BioScience Company, Meriden, CT). After a background correction, signal strengths were normalized to the levels of actin mRNA present in RNA preparations.

Reporter assay.

All cells were grown overnight in minimal medium lacking uracil and ammonium sulfate (0.67% Difco yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose) supplemented with 0.1% proline as a nitrogen source, and appropriate concentration of amino acid or dipeptide supplement to induce the di/tri-peptide transport. Under these growth conditions proline used as a nitrogen source does not serve as an amino acid inducer. The following day, amino acid or dipeptide supplement was added, and the cells were grown for another 2 hours. Then, standard liquid β-galactosidase assay for yeast cells was performed (Kippert, 1995). To test the amino acid inducing potential (Fig. 1), the mean Miller Unit values for each data set were normalized to the miller unit values of β-galactosidase assay of wild-type (FY2) cells under leucine induction. For other analyses, data points and error bars reflect the mean and standard deviation of a minimum of four independent measurements.

Toxicity assay.

Functionality of any Ptr3p fusions and fragments were measured by their ability to complement the *ptr3*Δ phenotype using peptide toxicity assays. This assay has been shown many times to mirror the results of peptide uptake experiments and expression of *PTR2* (Island *et al.*, 1987; Barnes *et al.*, 1998). When toxic dipeptides do not inhibit growth, uptake of radiolabeled dipeptide and *PTR2* are greatly

lowered. Cells were grown overnight in medium lacking uracil and ammonium sulfate (0.67% Difco yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose) supplemented with 0.1% proline as a nitrogen source, and leucine (228 µM) as inducer (proline medium) and suspended in sterile water at 5.0 X 10^6 cells per ml. Fractions (1 ml) were added to 0.8% Noble agar and plated on MM containing Leu and Trp supplements to induce peptide transport. A total of 0.38 mmol of L-ethionine (Eth) or L-alanyl-L-ethionine (Ala-Eth) was added to a disk made of Whatman 3MM filter paper. L-ethionine was obtained from SIGMA (St. Louis, MO), and Ala-Eth was synthesized as previously described (Naider *et* al., 1974). Disks containing each compound were placed on the surface of the plates. After 48 h of incubation, zones of inhibition were measured for all plates. Figures shown are representation of three independent measurements.

Fluorescent Microscopy.

Cells containing pSGFP-*PTR3* grown in minimal medium with or without leucine/tryptophan amino acid inducer, or Arg-Leu dipeptide inducer, were used for fluorescence microscopy studies. Controls for cellular localization of GFP fusion proteins (see Table 2) include nuclear localized Rat1p-GFP and cytoplasmic localized Rat1p(NLSΔ)-GFP, which are encoded by gene fusion in plasmids pAJ226 and pAJ232, respectively (gifts from Dr. Arlen W. Johnson at University of Texas at Austin) and plasma membrane localized Rgs4p-GFP,

which is encoded by gene fusion in plasmid pVT-RGS4-GFP (a gift from Dr. Maurine E. Linder at Washington University School of Medicine at St. Louis). Intact cells were observed and photographed with an Olympus microscope. Images were captured to Adobe Photoshop (Adobe Systems, Inc., San Jose, CA) with a Hamamatsu C5810 color chilled camera.

Yeast two-hybrid.

The yeast two-hybrid analysis was conducted by utilizing His, Ade, and *lac-Z* assays as described (James *et al.*, 1995). Strain PJ69-4A containing pGBD-*PTR3*, pGBD3(1-250), pGBD3(1-500), or pGBD3(500-678) was transformed with pGAD-N*SSY1*. The selection for interacting clones was performed in media containing glucose and lacking histidine (plus 2 mM 3-aminotriazole) or lacking adenine. Then, the colonies were further tested for *lac-Z* expression by plating them on minimal dropout media containing galactose/raffinose and X-Gal. Strong (89T and 89L plasmids) and zero (pGBT9.C and pGAD424.C plasmids) interaction controls (Bartel *et al.*, 1996) were a kind gift from the Stanley Fields laboratory (Daniel Lockshon, University of Washington, Seattle, WA).

Co-immunoprecipitation.

Whole cell lysates of His_6Ptr3p or FLAG-Nssy1p were prepared in 20 mM Tris-HCl, pH 6.5, 150 mM NaCl, 5 mM MgCl2, 2% Triton X-100 by glass bead lysis. Immunoprecipitations were conducted as described in protocol Seize™ Primary

Immunoprecipitation Kits (Pierce, Rockford, IL). For His_6Ptr3p immunoprecipitations, 1 ml of cell extracts were precleaned with protein A/G plus agarose by rotating at 4^0C for 1 hour. The supernatant was transferred to a fresh tube. His_6 antibody (SIGMA, St. Louis, MO) was added in the presence of 20 µl fresh protein A/G plus-agarose and incubated at 4^0C for 4 hours. The extract was then washed with washing buffer (Pierce, Rockford, IL). Proteins were separated on SDS-PAGE gel under the reduced condition, transferred to Hybond-ECL (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), blocked with 5% milk, incubated with HRP-conjugated India-His reagent (SIGMA, St. Louis, MO) at a 1:1000 dilution (16 h, 4 °C), and then detected using ECL detection kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). For co-immunoprecipitation, protein A/G sepharose coupled with His_6 was incubated with cell extracts containing pHis_6*PTR3* or empty vector (pDB20-His_6). After washing the agarose beads were incubated with cell lysates expressing FLAG-Nssy1p (overnight, 4 °C), washed, and proteins were separated on SDS-PAGE gel under the reduced condition. Blots were incubated with a monoclonal FLAG antibody (1:1000 diluted), HRP-conjugated anti-mouse IgG (1:2000 diluted), and then detected using ECL detection kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

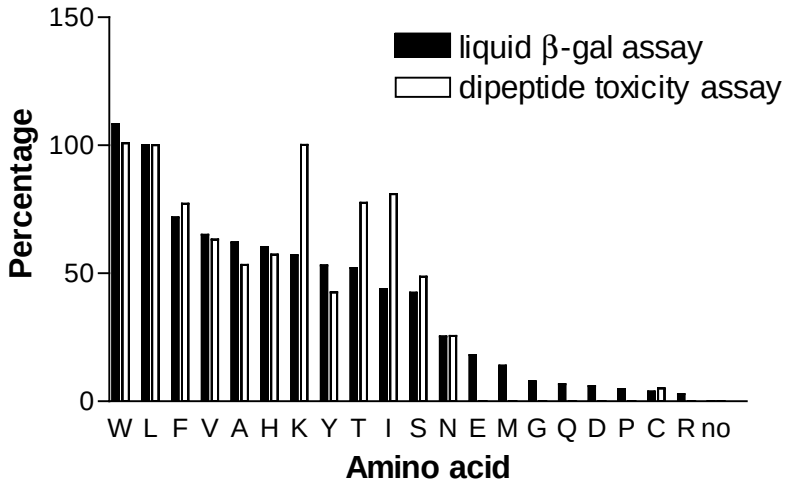
CHAPTER III

RESULTS

Various amino acids induce *PTR2* to different levels.

The effect of amino acid supplementation on expression of *PTR2* as measured by liquid β-galactosidase assay was compared to the response to toxic dipeptides as an assay for peptide transport (Island *et al.*, 1987; Fig. 1). Responses measured by the toxic peptide assay or the reporter gene assay to most amino acids were similar. However, the dipeptide toxicity assay showed a higher inducing effect for K, T, and I as compared to induction measured by the liquid β-galactosidase assay. On the other hand, while the dipeptide toxicity assay was not sensitive enough to show detectable levels of induction for the amino acids E, M, G, Q, D, P, and R, the liquid β-galactosidase assay could detect quantitatively low levels of induction of *PTR2* expression. Although most non-polar amino acids were good inducers, we could not deduce a correlation between the physical/chemical properties of an amino acid and its induction potential. Both assays confirmed that L and W were the best amino acid inducers. These amino acids were used in all subsequent amino acid induction treatments.

Fig. 1. Comparison of effects of different amino acids on induction of peptide toxicity and reporter gene expression. FY2 (wild-type) strain containing the pLACZ-2 reporter plasmid was grown in minimal medium with and without amino acids (as indicated by their one-letter abbreviation) as inducers of the peptide transport system and then assayed by dipeptide toxicity or liquid β-galactosidase assays (see Materials and Methods). One-hundred percent was normalized to the expression of *PTR2* induced by leucine as measured by either gene induction or response to toxic dipeptide.


150
100
50
0
Percentage
liquid β-gal assay
dipeptide toxicity assay
W L F V A H K Y T I S N E M G Q D P C R no
Amino acid

Amino acid induction of *PTR2* expression requires Ssy1p.

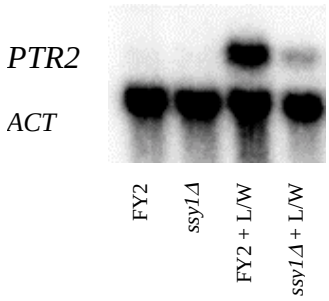
Extracellular amino acids are sensed by an amino acid sensor protein Ssy1p, which transduces the signal to Ptr3p and other downstream effectors such as Ssy5p, Ubr1p/Ptr1p, and Cup9p leading to an increase in *PTR2* expression (Iraqui *et al.*, 1999a; Klasson *et al.*, 1999; Forsberg and Ljungdahl, 2001a). To determine the extent to which induction required Ssy1p and to measure this effect quantitatively, we used a *lac-Z* reporter gene under control of the native *PTR2* promoter. Measurement of the expression patterns by both a liquid β-galactosidase assay and northern analysis were comparable as expression levels were low in FY2 and *ssy1*Δ strains, and an increase in expression was readily apparent in the wild-type FY2 strain upon the addition of amino acids as determined by either assay (Fig. 2). Most notably, amino acid induction was highly dependent on the presence of Ssy1p as indicated in both assay systems.

Dipeptide sequence is important for induction of *PTR2* expression.

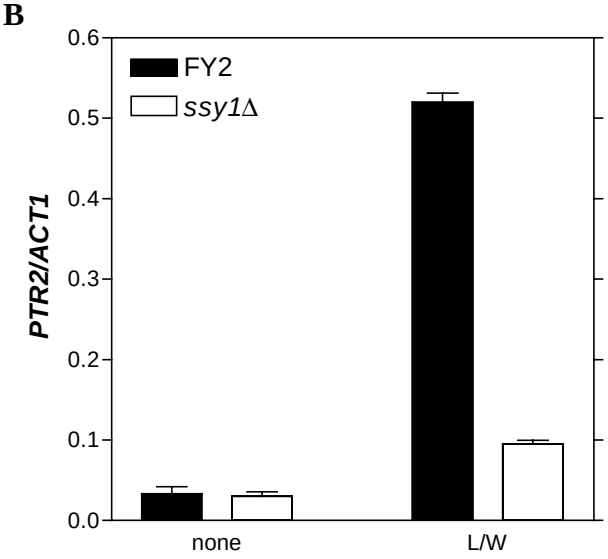
We have shown previously that the dipeptide W-A induced *PTR2* expression (Hauser *et al.*, 2001). To study physical/chemical properties of dipeptide inducers, assays were performed with various dipeptides and the *lac-Z* reporter gene, and the results are presented in Fig. 3. First, we showed that the dipeptide R-E induced *PTR2* expression to a greater extent than did the amino acids L and W (Fig. 3A, compare L/W to R- E lanes). The components of R-E, represented by

Fig. 2. *PTR2* expression patterns determined by *PTR2* northern analysis (A and B) and liquid β-galactosidase assay on the *lac-Z* reporter gene under the native *PTR2* promoter (C). For each analysis, FY2 (wild-type) and *ssy1*Δ null mutant strains were grown in minimal medium without and with amino acids (indicated by +L/W, leucine and tryptophan) as inducers of the peptide transport system. For northern analysis, the data are represented as well by signal strengths (cpm) relative to the levels of actin mRNA (*ACT1*) levels (B).

A


PTR2
ACT
FY2
ssy1Δ
FY2 + L/W
ssy1Δ + L/W

B


PTR2/ACT1
0.6
0.5
0.4
0.3
0.2
0.1
0.0
FY2
ssy1Δ
none
L/W

C

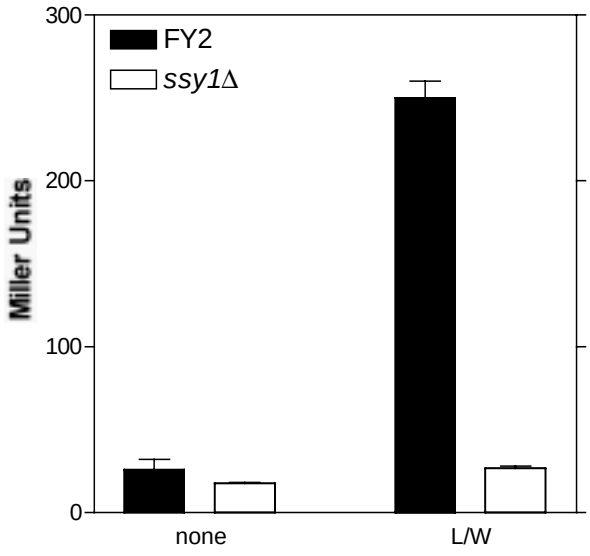
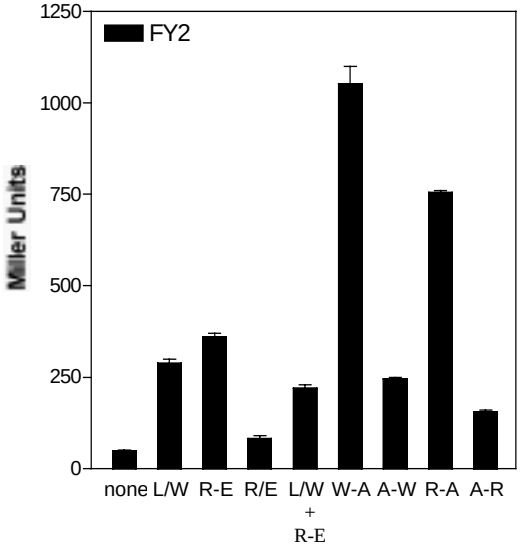
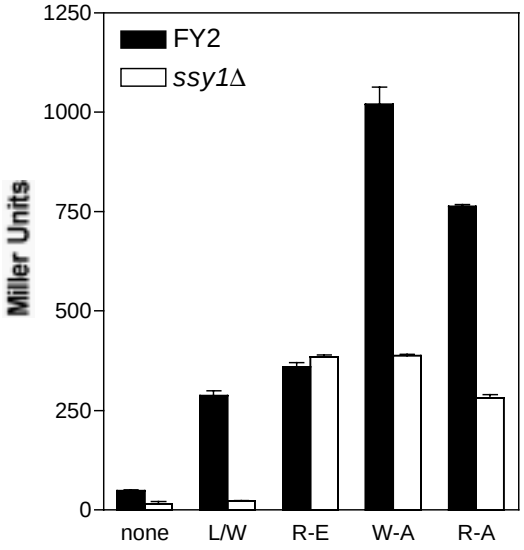

Miller Units
300
200
100
0
FY2
ssy1Δ
none
L/W

Fig. 3. Effects of dipeptides and amino acids on *PTR2* expression. Each strain containing the pLACZ-2 reporter plasmid was grown in minimal medium without or with amino acids or dipeptide and then assayed for reporter gene expression. Amino acids and dipeptides added to the media are indicated in the abscissas with dipeptide shown with a "dash" (for example R-E is arginyl-glutamic acid) and amino acids by a "slash" (for example L/W is leucine and tryptophan). (A) Wild-type FY2, (B) Comparison between FY2 and *ssy1*Δ, (C) Comparison between FY2 and *ptr3*Δ, and (D) Comparison between FY2 and *ptr2*Δ.

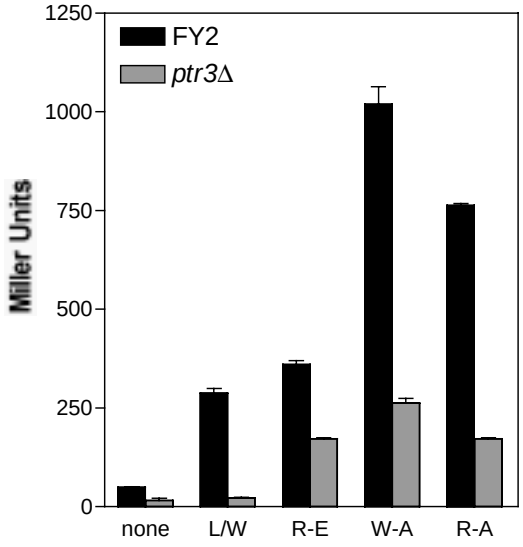
A.


1250
1000
750
500
250
0
Miller Units
FY2
none
L/W
R-E
R/E
L/W + R-E
W-A
A-W
R-A
A-R

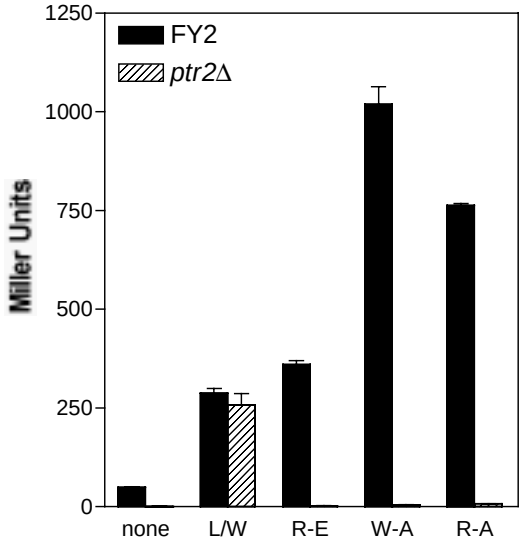
B.


1250
1000
750
500
250
0
Miller Units
FY2
ssy1Δ
none
L/W
R-E
W-A
R-A

C.


1250
1000
750
500
250
0
Miller Units
FY2
ptr3Δ
none
L/W
R-E
W-A
R-A

D.


1250
1000
750
500
250
0
Miller Units
FY2
ptr2Δ
none
L/W
R-E
W-A
R-A

R and E, did not induce di/tri-peptide transport to the same extent as that induced by the dipeptide R-E (compare R/E to R-E). A mixture of dipeptide and amino acids showed that the induction by amino acids and dipeptide was not additive. In fact, it was about 30% lower than amino acids or dipeptide induction alone (lane L/W+R-E compared to L/W or R-E). To determine whether the sequence of the amino acid components in a peptide affected its ability to induce, we chose the four peptides Trp-Ala (W-A), Ala-Trp (A-W), Arg-Ala (R-A), and Ala-Arg (A-R). The dipeptide W-A induced *PTR2* expression about 3-fold higher than that induced by amino acids L and W (Fig. 3A, compare W-A to L/W), but dipeptide A-W only induced *PTR2* to about 60% of that induced by amino acids L and W (compare A-W to L/W). Dipeptide R-A induced *PTR2* expression about 2-fold higher than that induced by amino acids L and W (compare R-A to L/W), but dipeptide A-R only induced to about 40% of that induced by amino acids L and W (compare FY2+A-R to FY2+L/W).

Dipeptide induction of *PTR2* expression is not totally dependent on Ssy1p.

To examine whether dipeptides may also act as inducers of *PTR2* expression via the amino acid induction pathway, specifically through the amino acid sensor Ssy1p, gene reporter assays were performed in wild-type and *ssy1*Δ strains. In the absence of Ssy1p, the induction by amino acids L and W was decreased to the level of *PTR2* expression without any inducer (Fig. 3B). In contrast, the dipeptide

R-E induced *PTR2* expression about the same in the absence or presence of Ssy1p. W-A induced *PTR2* to the same level as that induced by R-E in *ssy1*Δ cells, although R-A mediated induction was lower. However, Ssy1p was required for maximal induction of *PTR2* by W-A and R-A as shown by 2-fold higher level of β-galactosidase in FY2 cells compared to *ssy1*Δ cells induced by either W-A or R-A. These data indicate that Ssy1p is not required for, but may modulate, induction by some dipeptides.

Ptr3p is required for optimal dipeptide induction of *PTR2* expression.

To analyze whether *PTR3* is involved in dipeptide induction, gene reporter assays were performed in the wild-type and *ptr3*Δ strains. *PTR3* deletion reduced induction both by amino acids and dipeptides. In *ptr3*Δ cells the level of amino acid induction was decreased to that of the wild-type cells without any inducer (Fig. 3C). Induction by the dipeptide R-E in *ptr3*Δ cells was at the same level as that induced by R-A in *ptr3*Δ cells, although W-A-induced *PTR2* expression was somewhat higher. Optimal induction required *PTR3* as shown by a 3-4 fold higher level of *lac-Z* in FY2 cells compared to *ptr3*Δ cells induced by either R-E, W-A, or R-A. The results suggest that Ptr3p is necessary for optimal dipeptide-mediated induction of *PTR2* expression.

Dipeptide induction of *PTR2* expression requires Ptr2p.

Previously, extracellular dipeptides containing a basic or hydrophobic N-terminal residue were proposed to provide a positive feedback loop for di/tri-peptide uptake by being transported and subsequently binding to allosteric sites on intracellular Ptr1p/Ubr1p, thereby activating ubiquitin-mediated degradation of the *PTR2* repressor Cup9p (Turner *et al.*, 2000). To test whether Ptr2p was required for dipeptide induction of *PTR2* expression, *ptr2*Δ cells were tested for their *PTR2* expression using a *lac-Z* reporter gene under the native *PTR2* promoter (Fig. 3D). In contrast to amino acid induction, which was similar in wild-type FY2 and *ptr2*Δ cells (Fig. 3D, L/W, closed and hatched bar), dipeptide induction was abolished in a *ptr2*Δ background. The activities in the *ptr2*Δ background with no addition (none) and dipeptide addition (R-E, W-A, or R-A) were less than 10 Miller units making them undetectable (R-E) or barely detectable (W-A and R-A) as shown in Fig. 3D.

***PTR3* is not required for *CUP9* expression.**

Cup9p is known to be a transcriptional repressor of *PTR2* (Byrd *et al.*, 1998). Since *ptr3*Δ was shown to be epistatic to *cup9*Δ by genetic analysis (Barnes *et al.*, 1998) and both amino acid and dipeptide induction of *PTR2* expression involve Cup9p degradation, we carried out an experiment to determine whether *PTR3* was involved in regulation of *CUP9* expression. All cells (wild-type FY2 and *ptr3*Δ

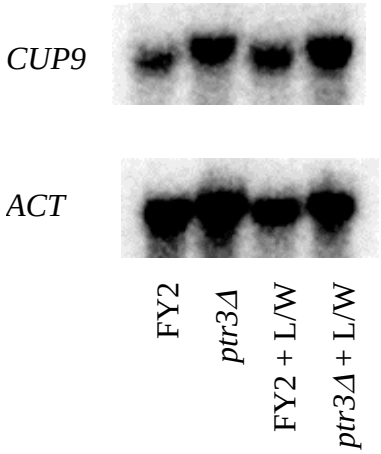
strains) were grown in medium lacking ammonium sulfate (0.67% Difco yeast nitrogen base without amino acids and ammonium sulfate supplemented with 2% glucose and 2 μg/mL of uracil with 0.1% proline as a nitrogen source). Leucine (228 μM) and tryptophan (98 μM) were added to induce peptide transport. *CUP9* expression was not affected by amino acid induction in the presence of Ptr3p (Fig. 4, lanes 1 and 3). In the absence of Ptr3p, amino acids increased *CUP9* expression by only 30% (lanes 2 and 4). A similar increase of 30% in *CUP9* expression occurred when the wild-type is compared to the *ptr3*Δ in the absence of amino acid induction (lanes 1 and 2). These data indicate that *PTR3* does not greatly affect *CUP9* expression in the presence or absence of inducing amino acids. The same expression patterns were observed for the dipeptide induction treatment (data not shown). However, we note that deletion of *PTR3* leads to a small increase of *CUP9* expression with amino acid induction as measured by northern analysis (lanes 3 and 4).

GFP-Ptr3p is functional.

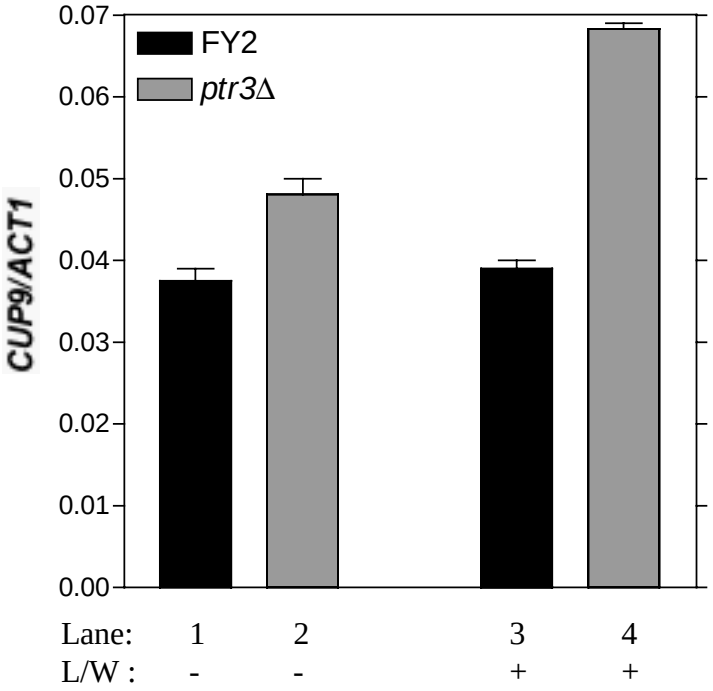
Previous sucrose gradient centrifugation showed that Ptr3p was localized at the plasma membrane as a peripheral protein in the presence or absence of Ssy1p (Klasson *et al.*, 1999). However, because Ptr3p was easily removed from the plasma membrane by a mild detergent, it was hypothesized that Ptr3p dissociates from the plasma membrane after amino acid-induced signaling.

Fig. 4. The effect of *PTR3* on *CUP9* expression patterns determined by northern analysis. FY2 (wild-type) and *ptr3* null mutant strains were grown in minimal medium with (indicated by +L/W) or without amino acids as inducers of the peptide transport system. *CUP9* expression was determined by northern analysis (A). These data are represented in (B) as signal strengths (cpm) relative to the levels of actin mRNA (*ACT1*) normalized to the expression observed in each condition.

A


CUP9
ACT
FY2
ptr3Δ
FY2 + L/W
ptr3Δ + L/W

B


0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
FY2
ptr3Δ
CUP9/ACT1
Lane: 1 2 3 4
L/W : - - + +

Therefore, Ptr3p localization *in vivo* under various physiological conditions was tested as described below. A fusion of GFP to the N-terminus of Ptr3p was engineered and expressed in yeast cells from a high-copy-number plasmid. The GFP-Ptr3p fusion protein retained its Ptr3p function as shown by a toxic peptide assay (Fig. 5). The zone of inhibition by the toxic dipeptide Ala-Eth (A-E) in *ptr3*Δ cells containing p*GFP-PTR3* (Fig. 5B) was similar to that of *ptr3*Δ cells containing pUTR-*PTR3*, which expressed *PTR3* under its native promoter (Fig. 5C), or to that of wild-type *PTR3* cells (data not shown). *ptr3*Δ cells containing p*GFP* alone were not able to transport the toxic dipeptide A-E (Fig. 5A) as indicated by the absence of the zone of inhibition surrounding the disk containing A-E. *ptr3*Δ cells with the p*GFP* plasmid were not sensitive to the toxic amino acid Eth (Fig. 5A). This pleiotropic effect of the *ptr3*Δ allele was previously reported (Island *et al.*, 1987). However, toxicity to Eth was fully restored in cells expressing *PTR3* or *GFP-PTR3* (Fig. 5B and C). These data indicate that the fusion GFP-Ptr3p complemented the *ptr3*Δ phenotype.

GFP-Ptr3p is localized in a punctate pattern associated with the plasma membrane.

GFP-Ptr3p in both *ptr3*Δ and *ssy1*Δ cells was associated with the plasma membrane in a punctate manner (Fig. 6E and F). Confocal microscopy also

Fig. 5. Toxic peptide sensitivity as a measure of GFP-Ptr3p function. GFP-Ptr3p in *ptr3*Δ cells was tested for the ability to complement the *ptr3*Δ phenotype by measuring the zone of inhibition in a lawn of cells due to transport of the toxic amino acid ethionine (E) and the toxic dipeptide alanyl-ethionine (A-E). The disk on the left contained E, whereas the disk on the right contained A-E. The toxic components were spotted on the disks (white circles) on the petri plates. (A) *ptr3*Δ cells containing p*GFP* alone. (B) *ptr3*Δ cells containing p*GFP-PTR3.* (C) *ptr3*Δ cells containing pUTR3(1-678), which expressed *PTR3* under its native promoter.

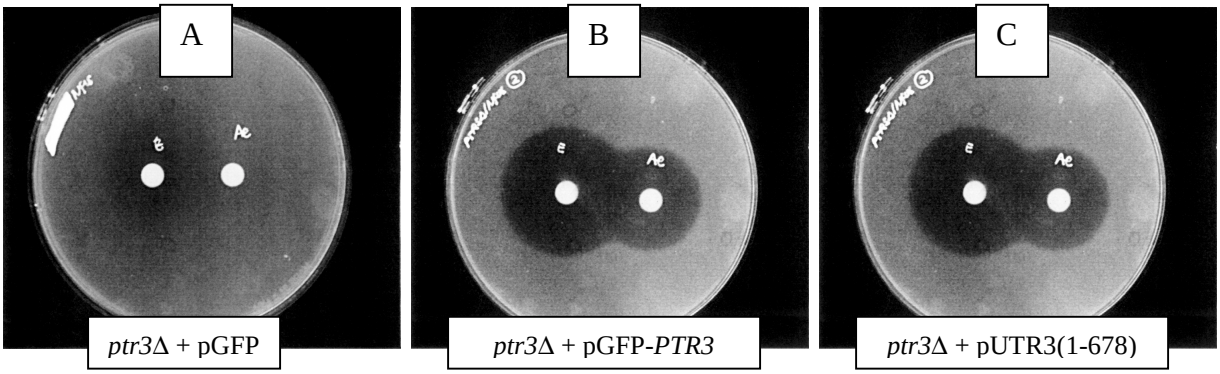
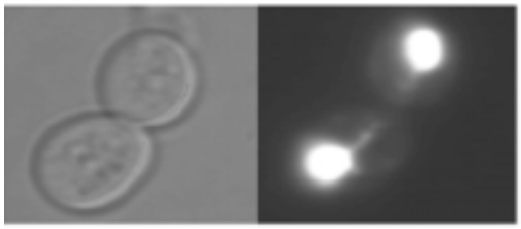
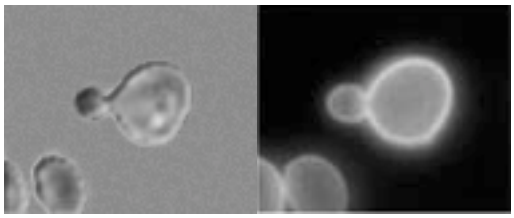

A
B
C
*ptr3*Δ + pGFP
*ptr3*Δ + pGFP-*PTR3*
*ptr3*Δ + pUTR3(1-678)

Fig. 6. *In vivo* localization of GFP-Ptr3p. On the left half of each panel (A-F) are Differential Interference Contrast (DIC) images, and on the right half of each panel (A-F) are images of GFP fluorescence. Arrows (panels E and F) indicate discrete areas of localization. (A) FY2 cells containing nuclear localized Rat1p-GFP control. (B) FY2 cells containing plasma membrane localized Rgs4p-GFP control. (C) FY2 cells containing cytoplasmic localized Rat1p(NLSΔ)-GFP control. (D) *ptr3*Δ cells containing GFP alone. (E) *ptr3*Δ cells containing GFP-Ptr3p. (F) *ssy1*Δ cells containing GFP-Ptr3p.

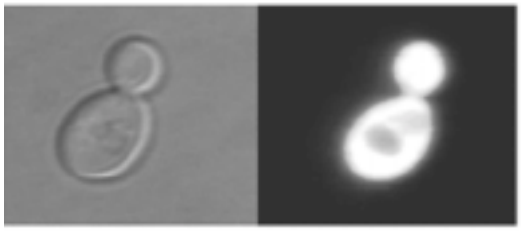
A



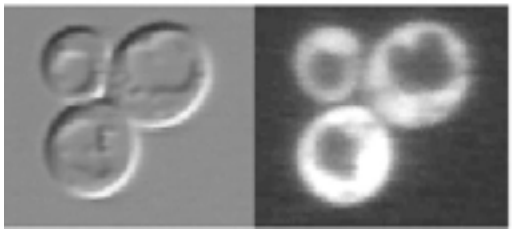
B



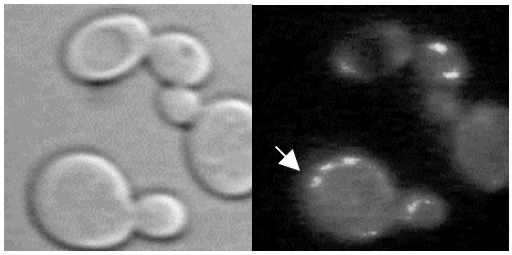
C



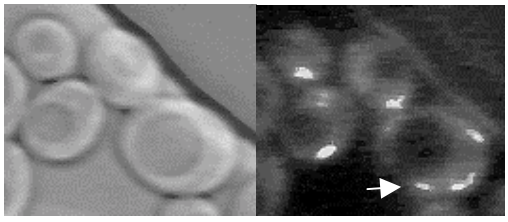
D



E



F



showed punctate localization at the plasma membrane (data not shown). The punctate localization was due to the Ptr3p moiety since it was not observed when GFP was expressed alone (Fig. 6D), nor was it observed when GFP was fused to control proteins known to be localized to the nucleus, plasma membrane, and cytoplasm (Fig. 6A, B, and C). For example, the control plasma membrane localized protein GFP-Rgs4p (Fig. 6B) showed a distinct ring pattern of fluorescence, consistent with the plasma membrane localization, and GFP expressed alone showed a cytoplasmic localization with exclusion from vacuoles readily apparent (Fig. 6D). Addition of leucine and tryptophan or Arg-Glu to induce di/tri-peptide transport did not affect GFP-Ptr3p localization (data not shown). The deletion of *SSY1* gene did not affect the punctate localization of GFP-Ptr3p (Fig. 6F).

Ptr3p interacts strongly with the N-terminal domain of Ssy1p.

Because Ssy1p is localized to the plasma membrane, and Ptr3p is peripherally associated with the cytosolic face of the plasma membrane as shown by subcellular fractionation studies, it has been suggested that they interact (Klasson *et al.*, 1999). We focused on testing the 282 amino acid N-terminus of Ssy1p as a Ptr3p interaction domain because of its predicted cytoplasmic location and its lack of similarity to other yeast amino acid permeases. To test whether the N-

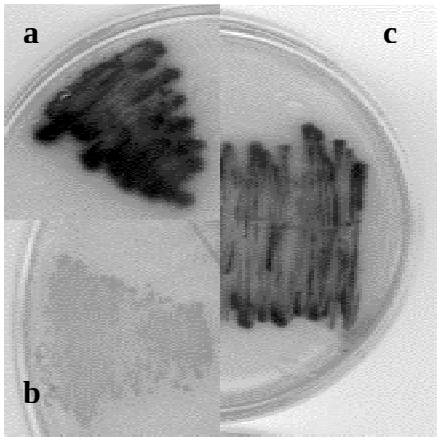
terminal domain of Ssy1p interacts with Ptr3p, we performed a protein-protein interaction analysis by the two-hybrid assay (James *et al.*, 1996). As the bait, full length Ptr3p was fused to the Gal Binding Domain (GBD) protein. The fusion GBD-Ptr3p retained its Ptr3p function (about 50% of the wild-type Ptr3p) shown by a toxic peptide assay (data not shown). Yeast expressing GBD-Ptr3p and pGAD-NSsy1p tested positive for His and Ade prototrophy indicating a physical interaction between Ptr3p and Ssy1p (N-terminal, 282 residues). Neither GBD-Ptr3p nor GAD-NSsy1p alone autoactivated the His or Ade systems or induced *lac-Z* (Fig. 7B). The interaction of Ptr3p and NSsy1p was strong as shown by the level of activation of *lac-Z* by both the X-Gal plate and liquid β-galactosidase assays (Fig. 7A and B). Similar to a strong interaction control (Fig. 7A), the blue color showed up after PJ69-4A cells containing *pGBD-PTR3* and p*GAD-NSSY1* were incubated for two hours on the X-Gal plate (Fig. 7, panel A, sector c). Liquid β-galactosidase assays showed that the GBD-Ptr3p interacted two-times stronger than the strong interaction control (Fig. 7B).

Ptr3p interacts with the N-terminal domain of Ssy1p *in vitro.*

To study the interaction between Ptr3p and the N-terminal tail of Ssy1p *in vitro*, co-immunoprecipitation was performed. Cell extracts transformed with pHis$_6$*PTR3* or empty vector (pDB20-His$_6$) were incubated with protein A/G

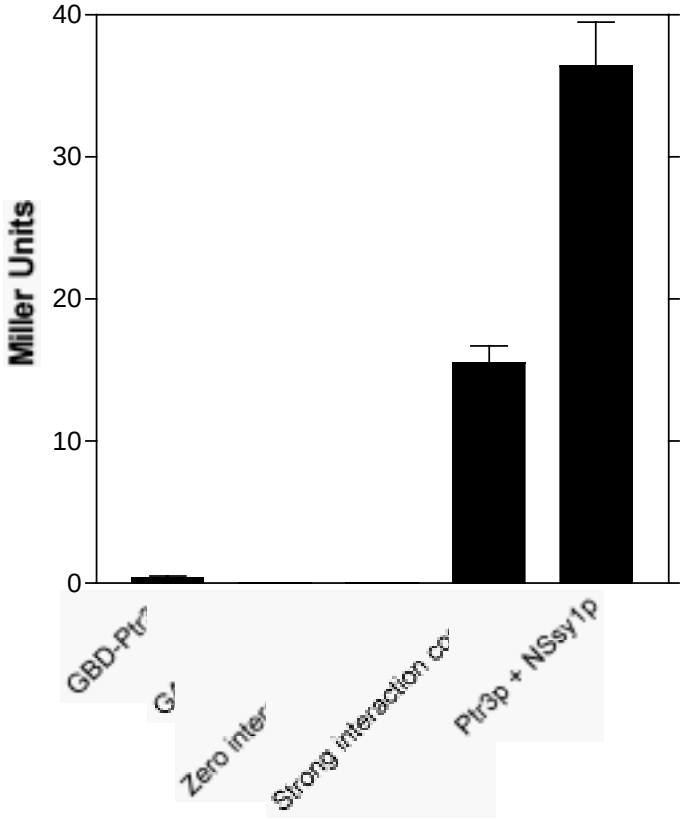
Fig. 7. Two hybrid assay and co-immunoprecipitation of Ptr3p and Ssy1p (residues 1-282). (Panel A) An X-gal plate assay showing the strength of interaction between Ptr3p and the N-terminal domain of Ssy1p (c) compared with strong (a) and zero (b) interaction controls. (Panel B) A liquid β-galactosidase assay of cells expressing GBD-Ptr3p, GAD-NSsy1p, a strong interaction control (89T and 89L plasmids), a zero interaction control (pGBT9.C and pGAD424.C plasmids), and both GBD-Ptr3p and GAD-NSsy1p. (Panel C) Co-immunoprecipitation between His_6-Ptr3p and GAD-FLAG-NSsy1p. Cell extracts expressing FLAG-NSsy1p were incubated with proteinA/G agarose-His6-antibody previously loaded with cell extracts containing pHis_6*PTR3* (lane 1 and 2) or empty vector pDB20-His_6 (lane 4 and 5). After washing and elution, bound proteins were run on SDS-PAGE, immunoblotted with FLAG antibody, and detected as described in Materials and Methods.

A.



a = strong interaction control
b = zero interaction control
c = PJ69-4A containing p*GBD-PTR3* and p*GAD-NSSY1*

B.



C.

Cell extracts containing: pHis$_6$-*PTR3* pDB20-His$_6$

Eluate (μg): 2.0 1.0 0.0 2.0 1.0 0.0

Lane: 1 2 3 4 5 6

agarose coupled with His_6 antibody. Cell extracts containing pGAD-FLAG-NSSY1 were added to the agarose and incubated at 4 °C overnight. After extensive washing, bound proteins were eluted by boiling in SDS sample buffer, separated on 10% SDS-PAGE, and analyzed by Western blotting using a FLAG antibody. As shown in Fig. 7C, lanes 1 and 2 showed bands with molecular weight corresponding to the expected molecular weight of GAD-FLAG-NSsy1p (72 kDa). No band corresponding to the approximate molecular weight of GAD-FLAG-NSsy1p was observed in lanes 4 and 5. The data show that Nssy1p is captured by Ptr3p bound to the agarose column confirming two-hybrid results indicating that Ptr3p interacts with the N-terminal domain of Ssy1p.

CHAPTER IV

DISCUSSION

We report here the involvement of the Ptr3 protein in both amino acid and peptide induction of the di/tri-peptide transport system in *S. cerevisiae*. Ptr3p, previously known to regulate the amino acid induction of *PTR2* expression, was now found to be involved in dipeptide induced *PTR2* expression. *PTR3* deletion reduced induction by dipeptides (Fig. 3C), suggesting that Ptr3p is important for optimal dipeptide induction. Therefore, Ptr3p may function as a shared regulatory protein between the amino acid and dipeptide induction pathways. To analyze the difference between amino acid and dipeptide induction, their effects were observed in the presence or absence of the amino acid sensor Ssy1p, the novel regulatory protein Ptr3p, and the di/tri-peptide transporter Ptr2p. In contrast to amino acid induction, dipeptide induction of *PTR2* expression did not require Ssy1p. In the absence of Ssy1p, the induction by amino acids L and W was reduced to the level of *PTR2* expression without any inducer, but the dipeptide induction in the absence of Ssy1p (Fig. 3B) was decreased (W-A or R-A) or not changed (R-E).

While Ptr2p deletion had little effect on amino acid induction of *PTR2* expression, the absence of Ptr2p abolished the dipeptide induction effect on the

expression of *PTR2*. Turner *et al.* (2000) suggested that extracellular dipeptides may be taken up by Ptr2p, and then play regulatory roles by binding to di/tri-peptide regulatory proteins such as Ptr3p or Ptr1p/Ubr1p. An *in vitro* binding assay indicated that dipeptides accelerated the di/tri-peptide uptake by binding to Ptr1p/Ubr1p thereby activating ubiquitin-mediated degradation of the *PTR2* repressor Cup9p. However, the question of how fast dipeptides are degraded in the cells remains to be solved to prove that intact dipeptide actually binds to Ptr1p/Ubr1p *in vivo*. Since the cells are able to sense extracellular nutrients such as amino acids and sugars, the above data might indicate that Ptr2p functions not only as a di/tri-peptide transporter, but also as a dipeptide sensor. Such a dual function has been shown for GLUT2, a human homolog of Snf3p, the major facilitative glucose transporter isoform expressed in hepatocytes, pancreatic β-cells, and absorptive epithelial cells (Thorens *et al*., 1988). GLUT2 protein has been suggested recently to transduce signals from the plasma membrane to the nucleus and to propagate glucose-regulated gene expression in humans (Guillemain *et al.*, 2000). The large cytoplasmic oriented hydrophilic loop between transmembrane VI and VII of GLUT2 is responsible for the glucose-induced gene expression after glucose sensing (Guillemain *et al.*, 2000). The loop is unique among glucose tranporters for GLUT2, and overexpression of the loop resulted in a dominant negative effect on the glucose-mediated gene expression

in wild-type cells. In addition, the loop rapidly moves from the cytoplasm to nucleus in response to extracellular high glucose concentration, and moves in the opposite direction when the cells are grown in low levels of glucose. Further investigation is needed to determine whether dipeptide induction is triggered by internal or external intact dipeptides, and whether Ptr2p can act as both a transporter and a dipeptide sensor protein.

We observed that the components of these dipeptides did not induce *PTR2* expression to the same extent as did the dipeptides, suggesting that the induction was dependent on intact peptide. The dipeptide sequence appeared to be important for induction of *PTR2* expression. The data indicated a requirement for a bulky or hydrophobic N-terminal residue of a dipeptide to bind to Ubr1p/Ptr1p, (Turner et al, 2000). The expression of *PTR2* induced by both R-E and amino acid addition was not additive. Instead, it was lower than amino acid or R-E induction alone (Fig. 3A) suggesting antagonism that might involve competition for components of amino acid and dipeptide sensing pathways.

To analyze further the involvement of Ptr3p in amino acid and dipeptide induction of di/tri-peptide transport, subcellular localization of GFP-Ptr3p was performed in several different conditions. A functional GFP fusion to the N-terminus of Ptr3p localized in a punctate association with the plasma membrane. The localization pattern was not affected by the addition of amino acid and

dipeptide inducers or in the absence of Ssy1p. However, these experiments do not exclude the possibility that regulatory events following Ssy1p/Ptr3p interaction require that Ptr3p disengage from the membrane and localize in some other compartment(s) (Klasson *et al.*, 1999). In this case, Ptr3p may act similarly to Std1p, which interacts with the C-terminal domain of the glucose sensors, Snf3p and Rgt2p, to transduce signals from the plasma membrane to the nucleus (Schmidt *et al.*, 1999). The punctate association may indicate the presence of unrecognized compartmentalization of a sensory complex at the cell membrane. In fact, a punctate localization of Std1p has been reported (Schmidt *et al.*, 1999). A similar punctate pattern localization in the *ssy1*Δ strain indicated that additional interactions were responsible for Ptr3p localization. Preliminary results of a two-hybrid library screen indicated that Ptr3p interacted with portions of membrane transporters Vap1p, Thi7p, and Ygr125wp (Narita and Becker, unpublished observation). Further experiments will be required to verify these associations and the localization of a sensory complex.

Previous genetic analysis strongly suggested that Ptr3p interacted with the N-terminal 280 amino acid cytoplasmic portion of Ssy1p (Klasson *et al.*, 2000). Support for Ssy1p-Ptr3p interaction was shown in a comprehensive study on yeast protein-protein interaction by the Yeast PathCalling™ screen (Uetz *et al.*, 2000; GeneScape Portal Web Site, http://portal.curagen.com/). The function of

N-terminal residues of Ss1p may be comparable to that of the carboxy-terminal extensions of glucose transporter homologs Snf3p and Rgt2p, which interact with Std1p or Mth1p (Schmidt *et al.*, 1999) and generate a signal for induction of proper transcription of several hexose transporter genes after sensing either low or high extracellular glucose concentration (Ozcan *et al.*, 1998). By two hybrid and co-immunoprecipitation experiments, we provide the first evidence that Ptr3p and the N-terminal domain of Ssy1p interact *in vivo* and *in vitro.* The results suggest that upon amino acid sensing, the N-terminal domain of Ssy1p may signal amino acid availability through interaction with Ptr3p. Consequently Ptr3p propagates a signal cascade to downstream targets such as Ssy5p, which was shown to interact with Ptr3p by two hybrid analysis (Bernard and Andre, 2001a), leading to the expression of *PTR2* and amino acid permeases such as *BAP2.* The signaling is likely to cause changes in conformations and covalent modifications of Ssy1p, Ptr3p, and Ssy5p since the presence of the other two components and extracellular amino acids altered electrophoretic mobility of these proteins (Forsberg and Ljungdahl, 2001a).

Recent discoveries of additional proteins involved in inducing transcription of amino acid and peptide permeases indicate that amino acid sensing is connected to several different pathways. One pathway may involve components of the SCF^{GRR1} ubiquitin-ligase complex involved in cyclin

degradation. The SCF^{GRR1} ubiquitin-ligase complex is essential for transcription of genes under Ssy1p control, which upon amino acid addition leads to a signaling pathway in which Grr1p, an F-box protein, and Uga35p, a transcription factor of Cys_6-Zn_2 family, regulate expression of amino acid permeases (Iraqui *et al.*, 1999a; Bernard and Andre, 2001b). Another mechanism may involve an indirect Ssy1p control of the functional expression of amino acid permeases (Forsberg *et al.*, 2001b). Two independent ubiquitin-mediated processes negatively modulate amino acid uptake: a novel membrane-bound ubiquitin ligase system (Asi1p, Asi2p, Asi3p) that affects the transcription of amino acid permease genes and a ubiquitin ligase system (Rsp5p, Bul1p, Bro1p) that affects traffic of amino acid permeases in the late endosomal vacuolar protein sorting pathways. Finally, other genes that control *BAP2* expression such as Leu3p, Tup1p, Stp1p, and Stp2p transcription factors may also provide additional pathways (Nielsen *et al.*, 2001). The elucidation of the above signal transduction pathways presents a challenging problem, and the regulation among these pathways portrays a fascinating system by which cells respond to their nutritional environment.

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PART III

STP1 AND *STP2* REGULATE THE EXPRESSION OF *PTR2*, A DI/TRI-PEPTIDE TRANSPORT GENE, IN *S. CEREVISIAE*

CHAPTER I

INTRODUCTION

The yeast *Saccharomyces cerevisiae* takes up peptides containing two to three amino acids from the extracellular environment to serve either as nitrogen sources or amino acids for protein biosynthesis once inside the cell. Transport across the plasma membrane is active, driven by the proton gradient, via a single di/tri-peptide transporter that is encoded by *PTR2* (Perry *et al.,* 1994). Ptr2p is an integral membrane protein of 601 amino acids containing 12 membrane-spanning domains that is necessary and sufficient to translocate di- and tri-peptides into the yeast cell (Perry *et al.*, 1994). The transporter is specific for di- and tri-peptides, with a preference for peptides containing hydrophobic amino acids.

It was noted that the uptake of di/tri-peptides was substantially greater in cells grown on a poor nitrogen source, such as proline, when compared to cells grown on a rich nitrogen source, such as ammonium sulfate (Becker and Naider, 1977; Nisbet and Payne, 1979). Subsequent work added another level of regulation by showing that peptide transport activity could be further increased by micromolar concentrations of amino acids in a nitrogen-poor or nitrogen-rich growth medium (Island *et al.*, 1987). Two genes, *PTR1/UBR1* and *PTR3*, have

been identified as encoding regulatory proteins of the di/tri-peptide transport system (Island *et al.*, 1987; Alagramam *et al.*, 1995; Barnes *et al.*, 1998).

Amino acid induction of peptide transport appears to be regulated by a sensory pathway mediated in part by the Ssy1p-Ptr3p-Ssy5p (SPS) complex (Didion *et al.*, 1998; Klasson *et al.*, 1999; Forsberg and Ljungdahl, 2001). Upon binding of extracellular amino acids to Ssy1p, a membrane protein, a signal is transduced through Ptr3p and Ssy5p to downstream effectors including Ptr1p/Ubr1p, activating ubiquitin-mediated degradation of the *PTR2* repressor Cup9p and resulting in induced expression of *PTR2* (Alagramam *et al.*, 1995; Byrd *et al.*, 1998). Extracellular dipeptides also accelerate di/tri-peptide uptake. Once taken up by the cells, dipeptides appear to gain access to allosteric binding sites of Ptr1p/Ubr1p thereby activating ubiquitin-mediated degradation of the Cup9p (Turner *et al.*, 2000) and up-regulation of *PTR2* expression (Hauser *et al.*, 2001).

Recently, *STP1* and *STP2*, genes previously hypothesized to encode pre-tRNA splicing proteins (Wang and Hopper, 1998), were identified as transcription factors for *BAP2*, a branched amino acid permease whose expression is also induced by extracellular amino acids through the SPS complex (Grauslund *et al.*, 1995; Didion *et al.*, 1996; Nielsen *et al.*, 2001). In this communication, using response to toxic amino acids and peptides, direct

radiolabeled dipeptide uptake, and a reporter gene assay, we provide evidence that *STP1* and *STP2* are involved in regulating the di/tri-peptide transport system through *PTR2* expression. Single and double deletions of *STP2* and *PTR3* showed an epistatic relationship indicating that *STP2* may act downstream of *PTR3*. The data presented strongly suggest that *STP1* and *STP2* are additional regulatory proteins of the di/tri-peptide transport system that mediate the amino acid or dipeptide induction of *PTR2* expression as *PTR2* transcription factors.

CHAPTER II

MATERIALS AND METHODS

Strains and media.

Yeast strains used are listed in Table 1. The strains used in this study were grown routinely on YEPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar). Strains transformed with a plasmid were cultured on minimal medium lacking uracil (0.67% Difco yeast nitrogen base with ammonium sulfate, without amino acids, 2% glucose, 0.2% casamino acids). For peptide toxicity assays, cells were inoculated into medium lacking ammonium sulfate (0.67% Difco yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, with auxotrophic marker amino acids) supplemented with 0.1% proline as a nitrogen source, and leucine (228 μM) and tryptophan (98 μM) or Arg-Glu (1 μM) as inducer (proline medium). Plasmids were introduced into yeast by LiAc-mediated transformation as described previously (Gietz *et al.*, 1995; Agatep et al., 1998). RG-*stp2*Δ and RG-*stp1*Δ strains were obtained from Research Genetics (Birmingham, AL). These deletion mutant strains were maintained on the basis of resistance to G418 (300 μg / mL) and were verified by PCR (data not shown). Construction of *stp2*Δ and *ptr3*Δ*stp2*Δ strains in the FY2 background was

Table 1. *S. cerevisiae* strains used.

Strain	Genotype	Reference
FY2	*MATα ura3-52*	Winston et al. (1995)
*ptr3*Δ or BWS-201	*MATα ura3-52 ptr3::Loxp*	Barnes et al. (1998)
*stp2*Δ	*MATα ura3-52 stp2::Loxp*	This work
*ptr3*Δ*stp2*Δ	*MATα ura3-52 ptr3*Δ*15 stp2::Loxp*	This work
RG-*stp1*Δ	*MATα his3*Δ*1 leu2Δ0 lys2Δ0 ura3Δ0 stp1::Loxp*	Research Genetics (strain #14297)
RG-*stp2*Δ	*MATα his3*Δ*1 leu2Δ0 lys2Δ0 ura3Δ0 stp2::Loxp*	Research Genetics (strain #16603)
*ptr2*Δ or PB1X-2AΔ	*MATa ura3-52 leu2-3 lys1-1 his4-32 ptr2*::*LEU2*	Perry et al. (1994)

accomplished by the method of Guldener *et al.* (1996), which results in the replacement of a target ORF with the dominant selectable kan^r marker. Briefly, bipartite oligonucleotide primers (*STP2kan*-F and *STP2kan*-R; Table 2) were used to PCR-amplify the kan^r marker from the pUG6 plasmid resulting in 40 bp extension of the *STP2* ORF flanking a functional kan^r cassette. PCR amplification using these primers yielded a 1.7 kb fragment that contained the kan^r cassette flanked by the 40 nucleotides of *STP2*. Transformants were identified on the basis of resistance to G418 (300 μg/mL) and were verified by PCR (data not shown).

Toxicity assay.

Functionality of any deletion strain was measured by its ability to uptake the toxic amino acid ethionine (Eth) or the toxic dipeptide Ala-Eth. Cells were grown overnight in proline medium and suspended in sterile water at 5.0 X 10^6 cells per ml. Portions (1 ml) were added to 0.8% Noble agar and plated on proline medium containing Leu and Trp or Arg-Glu supplements to induce peptide transport. A total of 0.38 mmol of L-ethionine (Eth) or L-alanyl-L-ethionine (Ala-Eth) was added to a disk of Whatman 3MM filter paper. L-ethionine was obtained from SIGMA (St. Louis, MO), and Ala-Eth was synthesized as previously described (Naider *et al.*, 1974). Disks containing each compound were placed on the surface of the plates. After 48 h of incubation, zones of inhibition

Table 2
Plasmid and nucleotides used.

Plasmid or oligonucleotides	Description or sequences	Reference
PLACZ-2	*PTR2* promoter *lac-Z* fusion in pYLZ6	This work
PMSI	*PTR2* in pYCP50 under native *PTR2* promoter	Perry et al. (1994)
STP2KAN-F	5'- CAAGAGAGCAAAAAGTTGGGCAGCT GAAGCTTCGTACGC-3'	This work
STP2KAN-R	5'-CGTAAAATACCTGAAACCGCCGCATA GGCCACTAGTGGATCTG-3'	This work
PTR3-F	5'-CATACATAGGTACGAAATAC-3'	This work
PTR3-R	5'-CCACTTTTGTCTATGAGAGTG-3'	This work
STP1-F	5'-CGTTCAAAAATGCCCTCTACC-3'	This work
STP1-R	5'-TTTTCGATGGCGTGCTTCTT-3'	This work
STP2	Forward and reverse primers were obtained from Research Genetics.	-
ACT1	Forward and reverse primers were obtained from Research Genetics.	-
PTR2	Forward and reverse primers were obtained from Research Genetics.	-

were measured for all plates. Pictures shown are representation of three independent measurements.

Uptake assays.

Transformed cells were grown overnight to mid-exponential phase in proline medium. The uptake assay was initiated by combining equal volumes of pre-warmed (30 °C) cells and 2X uptake assay mixture (2% glucose, 20 mM sodium citrate / potassium phosphate, pH 5.5, 500 µM Leu-Leu (SIGMA, St. Louis, MO), 320 µM L-leucyl-L-[3H]leucine (16 mM, 10 mCi / mmol). L-Leucyl-L-[3H]leucine was synthesized by standard solution-phase techniques. All compounds were dissolved in either water or sodium citrate / potassium phosphate buffer (pH 5.5). After 10 minutes, aliquots (90 µl) were removed and washed four times by vacuum filtration with 1 ml ice-cold water onto a membrane filter (HAWP, Millipore, Bedford, MA). The membranes were counted by liquid scintillation spectrometry, and results were reported as nmol / mg dry weight. Data points and error bars reflect the mean and standard deviation of a minimum of four independent measurements.

Reporter assay.

All cells were grown overnight in proline medium supplemented with leucine (228 µM) and tryptophan (98 µM) or dipeptide Arg-Glu (1 µM) to induce di / tri-peptide transport. The following day, amino acid or dipeptide supplement was

added, and the cells were grown for another 2 hours. Then, standard liquid β-galactosidase assay for yeast cells was performed (Kippert, 1995). Data points and error bars reflect the mean and standard deviation of a minimum of four independent measurements.

RNA isolation and northern blot analysis.

Total RNA from approximately 2-3x10^8 cells was extracted using the PureScript RNA isolation kit from Gentra Systems. 15μg of total RNA was denatured with glyoxal (Ambion, Austin, TX), and run on a 1% agarose gel. The RNA was transferred to a Magnacharge positively-charged nylon membrane (MSI, Westboro, MA) using a downward alkaline transfer (modified from Chomczynski, P., 1992). Probe templates were amplified from wild-type genomic DNA, using gene-specific primers as described in Table 1 and Taq polymerase (SIGMA, St. Louis, MO). These PCR products were purified with Edge BioSystems' Quick Step PCR purification kit. Purified PCR products were labeled with ^{32}P using Ambion's Strip-EZ DNA kit. Blots were prehybridized overnight at 42°C in Ambion's NorthernMax prehybridization/hybridization buffer, supplemented with 100μg/ml sheared salmon sperm DNA. Probe hybridization was carried out overnight at 42°C in Ambion's NorthernMax prehybridization/hybridization buffer, supplemented with 10% Dextran Sulfate, and approximately 2-3 x 10^6 CPM/ml of probe. After hybridization, the blots

were washed for 30 minutes at 50°C in the following buffers: 3X SSC, 0.05% sodium pyrophosphate, 0.5% SDS; 1X SSC, 0.05% sodium pyrophosphate, 0.5% SDS; 0.2X SSC, 0.05% sodium pyrophosphate, 0.5% SDS. The hybridized filters were then placed on damp Whatman paper (0.2X SSC, 0.05% sodium pyrophosphate, 0.5% SDS), and wrapped with plastic film to prevent them from drying out during exposure. The filters were exposed to a storage phosphor screen, and analyzed on a Molecular Dynamics Storm phosphorimager at a resolution of 100μm. After exposure, the filters were stripped using Ambion's Strip-EZ DNA kit at 68°C. After stripping, the filters were re-exposed to a phosphor screen to ensure that the signal was effectively removed.

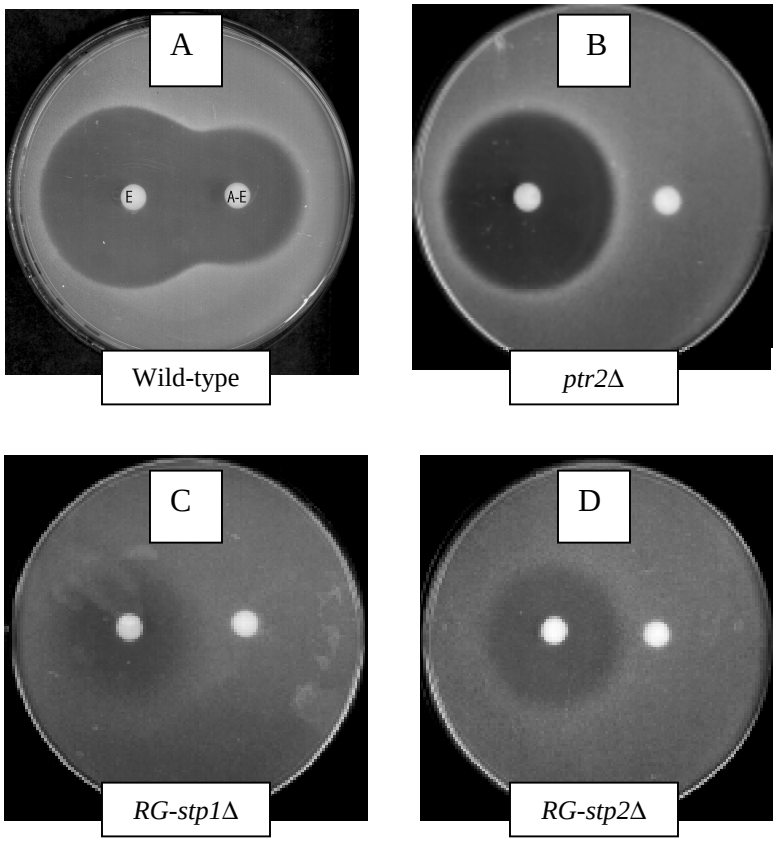
CHAPTER III

RESULTS

*stp1*Δ and *stp2*Δ cells have decreased ability to uptake toxic amino acid and dipeptide.

To determine whether cells lacking *STP1* or *STP2* had a functional di/tri-peptide system, cells carrying a deletion of *STP1* or *STP2* (RG-*stp1*Δ or RG-*stp2*Δ cells, respectively) were tested by measuring their responses to toxic dipeptide. The wild-type cells (Fig. 1A) formed approximately a 3.5 cm diameter zone of inhibition to the toxic amino acid Eth and a 2.1 cm diameter zone to the toxic dipeptide Ala-Eth, indicating that the wild-type cells were able to uptake amino acid and dipeptide. In the presence of Arg-Glu as an inducer of peptide transport, similar responses to toxic dipeptide were observed (data not shown). The *ptr2*Δ cells, which are *ptr2* null mutant strains, formed a zone in the presence of Eth showing that these cells transported amino acids in the absence of Ptr2p, but did not form a zone of inhibition in the presence of Ala-Eth, showing that these cells did not have a functional di/tri-peptide transport system (Fig. 1B). The zone of inhibition by the toxic amino acid Eth in either RG-*stp1*Δ or RG-*stp2*Δ cells was decreased compared to that of the wild-type or *ptr2*Δ cells, suggesting

Fig. 1. Toxic peptide and amino acid sensitivity. Each strain was tested for its sensitivity or resistance to the toxic amino acid ethionine (E) and the toxic dipeptide alanyl-ethionine (A-E). In each petri plate, the disk on the left contained E, whereas the disk on the right contained A-E. (A) wild-type cells, (B) *ptr2*Δ cells, (C) RG-*stp1*Δ cells, and (D) RG-*stp2*Δ cells.

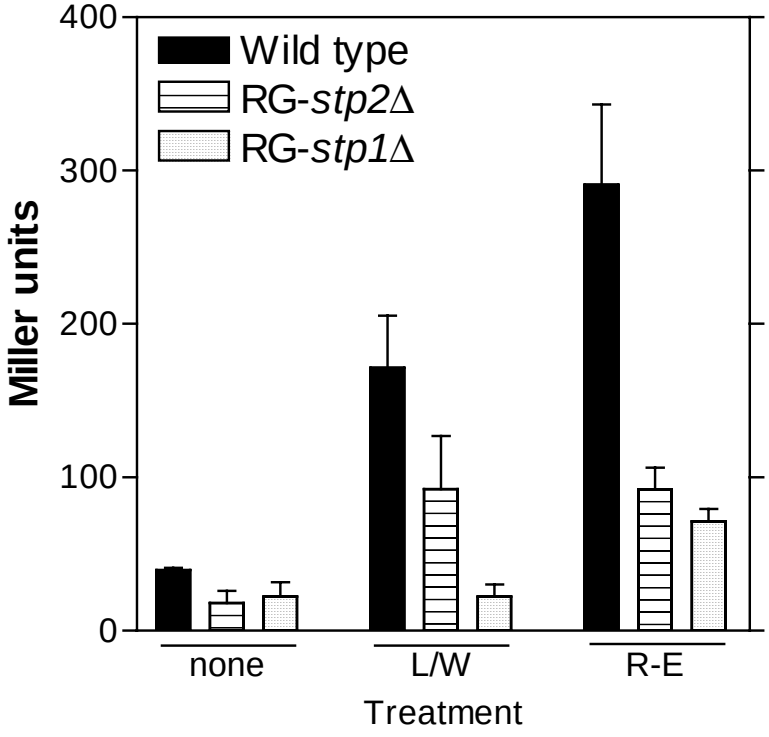

A
E
A-E
Wild-type
B
ptr2Δ
C
RG-stp1Δ
D
RG-stp2Δ

that RG- *stp1*Δ or RG-*stp2*Δ cells have reduced ability to uptake amino acid (Fig. 1C and 1D). The assays also showed that *STP1* and *STP2* are involved in di/tri-peptide uptake. The zone of inhibition by dipeptide Ala-Eth (A-E) in either RG-*stp1*Δ (Fig. 1C) or RG-*stp2*Δ (Fig. 1D) cells was not visible.

The level of radiolabeled Leu-Leu uptake was decreased in *stp1*Δ or *stp2*Δ cells.

The reduced toxicity to amino acid and dipeptide observed in the previous assay was difficult to measure quantitatively. Therefore, a direct assay of radiolabeled Leu-Leu uptake was performed. The radiolabeled Leu-Leu uptake showed that wild-type cells were able to transport Leu-Leu dipeptide (Fig. 2). In the presence of Arg-Glu as inducer of peptide transport instead of tryptophan and leucine, similar uptakes were observed (data not shown). The peptide uptake in *ptr2*Δ cells (Fig. 2) was not above background levels as measured by adsorption of peptide to cells at 0^0C (data not shown). The ability to uptake radiolabeled Leu-Leu dipeptide in RG-*stp1*Δ or RG-*stp2*Δ cells was greatly decreased compared to wild-type cells (Fig. 2). In contrast to abolished uptake in *ptr2*Δ cells, RG-*stp1*Δ or RG-*stp2*Δ cells still had about 7% and 15%, respectively, functional dipeptide transport in comparison to that of the wild-type. The results corroborate the lack of visible halos observed in the previous assays (Fig. 1C and 1D) and indicate that RG-*stp1*Δ or RG-*stp2*Δ cells have a limited ability to transport di/tri-peptide.

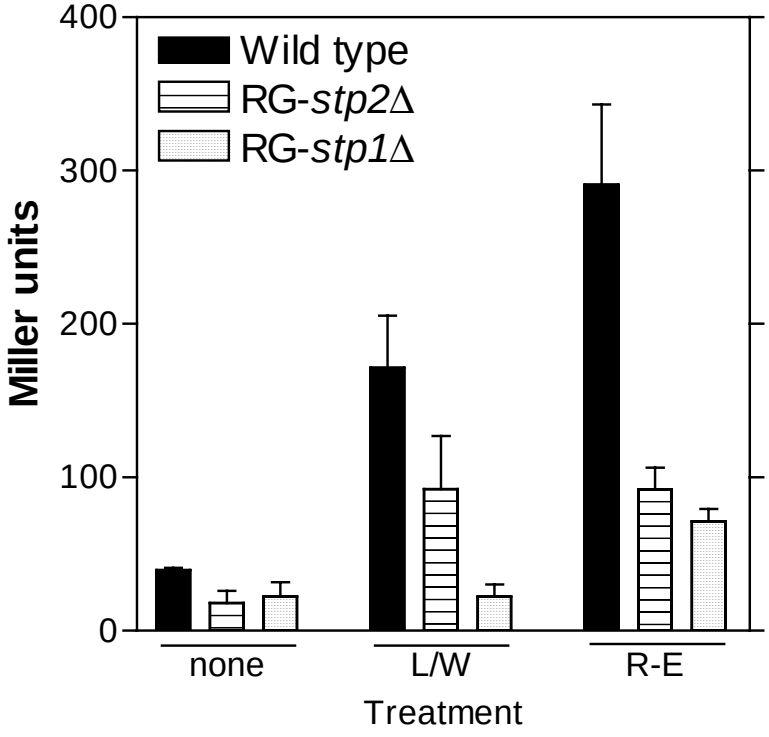
Fig. 2. Peptide transport assay. Accumulation of L-leucyl-L-[3H]leucine was measured after 10 minutes as described in Materials and Methods. Each *S. cerevisiae* strain was grown overnight in minimal medium lacking ammonium sulfate (0.67% Difco yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, with auxotrophic amino acids) supplemented with 0.1% proline as a nitrogen source, and leucine (228 μM) and tryptophan (98 μM) to induce di/tri-peptide transport.


400
300
200
100
0
Miller units
Wild type
RG-stp2Δ
RG-stp1Δ
none
L/W
R-E
Treatment

Amino acid and dipeptide induction of *PTR2* expression require *STP1* and *STP2*.

STP1 and *STP2* were identified as transcription factors for *BAP2*, a branched-chain amino acid permease whose expression is induced by extracellular amino acids (Nielsen *et al*., 2001). *PTR2* expression is induced by amino acids (Klasson *et al*., 1999) and dipeptides (Turner *et al.*, 2000; Hauser *et al.*, 2001). To test whether *STP1* and *STP2* are involved in di/tri-peptide transport, *PTR2* expression, induced by either amino acids or dipeptide in the presence or absence of *STP1* or *STP2*, was measured using a colorimetric assay that detected activation of the native *PTR2* promoter. In wild-type cells, the addition of amino acids L and W or dipeptide R-E caused a 4- or 6-fold induction of *PTR2* expression, respectively (Fig. 3, solid bars). Without any inducer, RG-*stp1*Δ or RG-*stp2*Δ cells expressed *PTR2* at about 50% of the level of the wild-type. In the presence of amino acid inducers, *PTR2* expression in RG-*stp2*Δ was about 60% of the wild-type expression, while RG-*stp1*Δ only expressed 20% of the wild-type level of *PTR2*. In the presence of R-E dipeptide inducer, RG-*stp1*Δ and RG-*stp2*Δ cells had *PTR2* expression levels about 30% of that of the wild-type. The results suggest that both Stp1p and Stp2p are necessary for optimal amino acid- or dipeptide-mediated induction of *PTR2* expression.

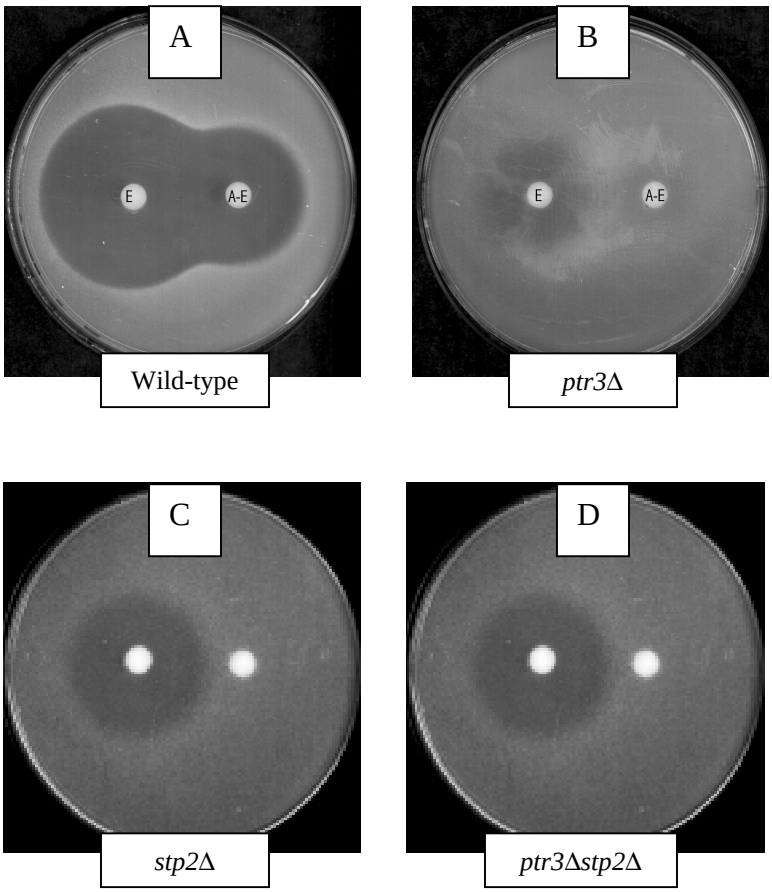
Fig. 3. *PTR2* expression patterns determined by liquid β-galactosidase assay on the *lac-Z* reporter gene under the native *PTR2* promoter. For each analysis, each strain was grown in minimal medium without and with inducer of the peptide transport system (indicated by +L/W [plus leucine and tryptophan] or +R-E [plus Arginyl-Glutamate]).


400
300
200
100
0
Miller units
Wild type
RG-stp2Δ
RG-stp1Δ
none
L/W
R-E
Treatment

_stp2_Δ and _ptr3_Δ_stp2_Δ cells have similar ability to uptake toxic amino acid and dipeptide.

Amino acid and peptide induction of *PTR2* expression are also regulated by *PTR3*. The product of this gene is a component of the amino acid complex sensor Ssy1p-Ptr3p-Ssy5p (Klasson *et al.*, 2000). *PTR3* acts upstream of *SSY5* and *CUP9,* which are genes involved in the regulation of *PTR2* expression (Andre *et al.*, 2001; Barnes *et al.*, 1998). To test the epistatic relationship between *STP2* and *PTR3*, *stp2*Δ and *ptr3*Δ*stp2*Δ strains were constructed in a FY2 wild-type background (see Materials and Methods), and their di/tri-peptide transport functions and *PTR2* expression levels were compared to those of *ptr3*Δ cells. To analyze the di/tri-peptide transport function of *ptr3*Δ, *stp2*Δ, and *ptr3*Δ*stp2*Δ cells, each strain was tested for its response to toxic dipeptide. The wild-type cells formed approximately a 3.5 cm diameter zone of inhibition to the toxic amino acid Eth and a 2.1 cm diameter zone to the toxic dipeptide Ala-Eth (Fig. 4, panel A and Fig. 1). The *ptr3*Δ cells formed a 1 cm diameter fuzzy zone of inhibition in the presence of Eth and a nearly invisible zone of inhibition in the presence of Ala-Eth (Fig. 4, panel B). The fuzzy zone was previously observed in identical assay, and was an indication of a leaky phenotype of *ptr3*Δ strain (Island *et al.*, 1991). The zones of inhibition by the toxic amino acid Eth or toxic dipeptide Ala-Eth in either *stp2*Δ or *ptr3*Δ*stp2*Δ cells were similar (Fig. 4, panels C and D).

Fig. 4. *STP2* and *PTR3* epistatic relationship as determined by toxic peptide sensitivity. Each strain was tested for the ability to uptake amino acid or dipeptide by measuring the zone of inhibition due to transport of the toxic amino acid ethionine (E) and the toxic dipeptide alanyl-ethionine (A-E). In each petri plate, the disk on the left contained E, whereas the disk on the right contained A-E. (A) Wild-type FY2 cells, (B) *ptr3*Δ cells, (C) *stp2*Δ cells, and (D) *ptr3*Δ*stp2*Δ cells.


A
E
A-E
Wild-type
B
E
A-E
ptr3Δ
C
stp2Δ
D
ptr3Δstp2Δ

These cells formed a 2 cm diameter fuzzy zone of inhibition in the presence of Eth. The zone of inhibition by dipeptide Ala-Eth (A-E) in either *stp2*Δ or *ptr3*Δ*stp2*Δ cells was not visible.

The level of radiolabeled Leu-Leu uptake was equivalent in *stp2*Δ and *ptr3*Δ*stp2*Δ cells.

A direct radiolabeled Leu-Leu uptake experiment was performed to complement the peptide toxicity assay. Fig. 5 shows that the wild-type cells were able to transport Leu-Leu dipeptide. In the presence of Leu and Trp or Arg-Glu as inducers of peptide transport, similar uptakes were observed (data not shown for Arg-Glu induced cells). The peptide uptake in *ptr3*Δ cells was reduced to about 20% of that of the wild-type. The level of radiolabeled Leu-Leu dipeptide uptake in *stp2*Δand *ptr3*Δ*stp2*Δ cells was similarly decreased to approximately 60% of that of the wild-type cells.

Amino acid and dipeptide mediated *PTR2* expression levels were comparable in *stp2*Δ and *ptr3*Δ*stp2*Δ cells.

We tested the level of *PTR2* expression induced by either amino acids or dipeptide in *ptr3*Δ, *stp2*Δ, and *ptr3*Δ*stp2*Δ cells by utilizing a *lac-Z* reporter gene under the native *PTR2* promoter. In wild-type cells, the addition of amino acids L and W or dipeptide R-E caused a 4 or 6 fold induction of *PTR2* expression,

Fig. 5. Peptide transport assay. Accumulation of L-leucyl-L-[3H]leucine in wild-type FY2, *ptr3*Δ, *stp2*Δ, or *ptr3*Δ*stp2*Δ strains was measured after 10 minutes as described in materials and methods. Each *S. cerevisiae* strain was grown overnight in minimal medium lacking ammonium sulfate (0.67% Difco yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, with auxotrophic amino acids) supplemented with 0.1% proline as a nitrogen source, and leucine (228 μM) and tryptophan (98 μM) to induce the di/tri-peptide transport .

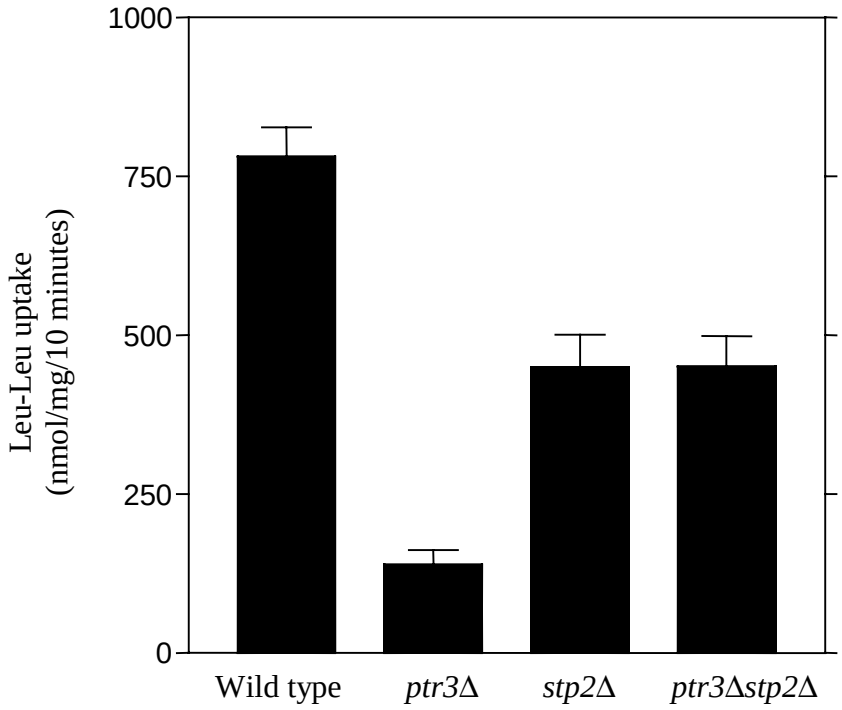
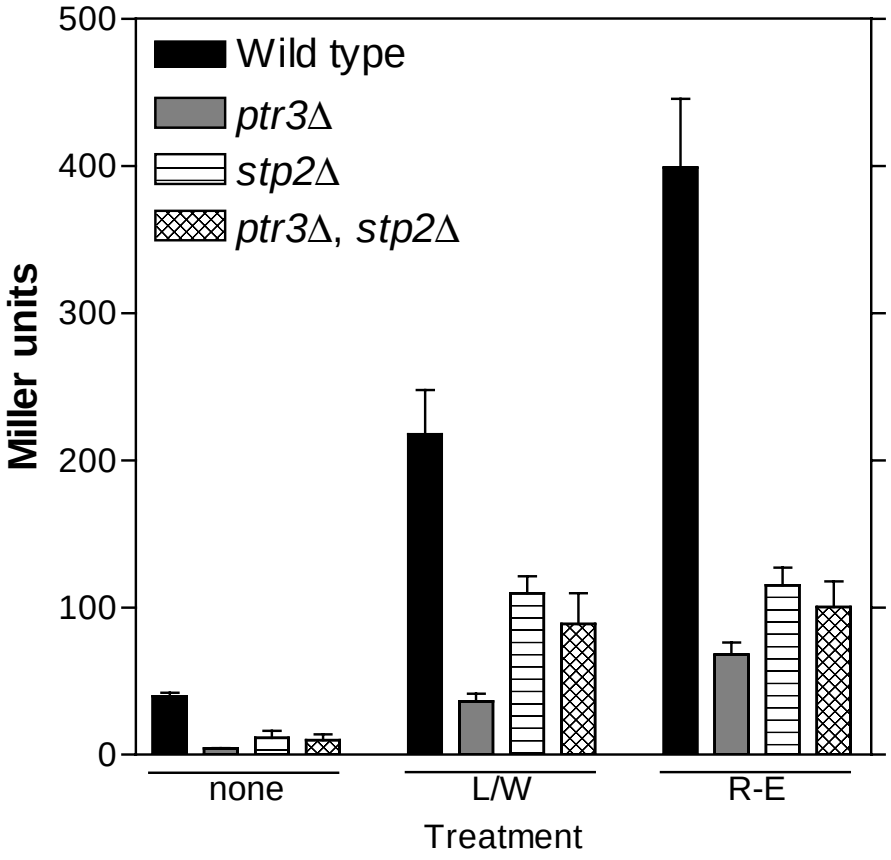

1000
750
500
250
0
Leu-Leu uptake
(nmol/mg/10 minutes)
Wild type
ptr3Δ
stp2Δ
ptr3Δstp2Δ

Fig. 6. *PTR2* expression patterns determined by liquid β-galactosidase assay of the *lac-Z* reporter gene. For each analysis, each strain was grown in minimal medium without and with inducer of the peptide transport system (indicated by +L/W [plus leucine and tryptophan] or +R-E [plus Arginyl-glutamate]).

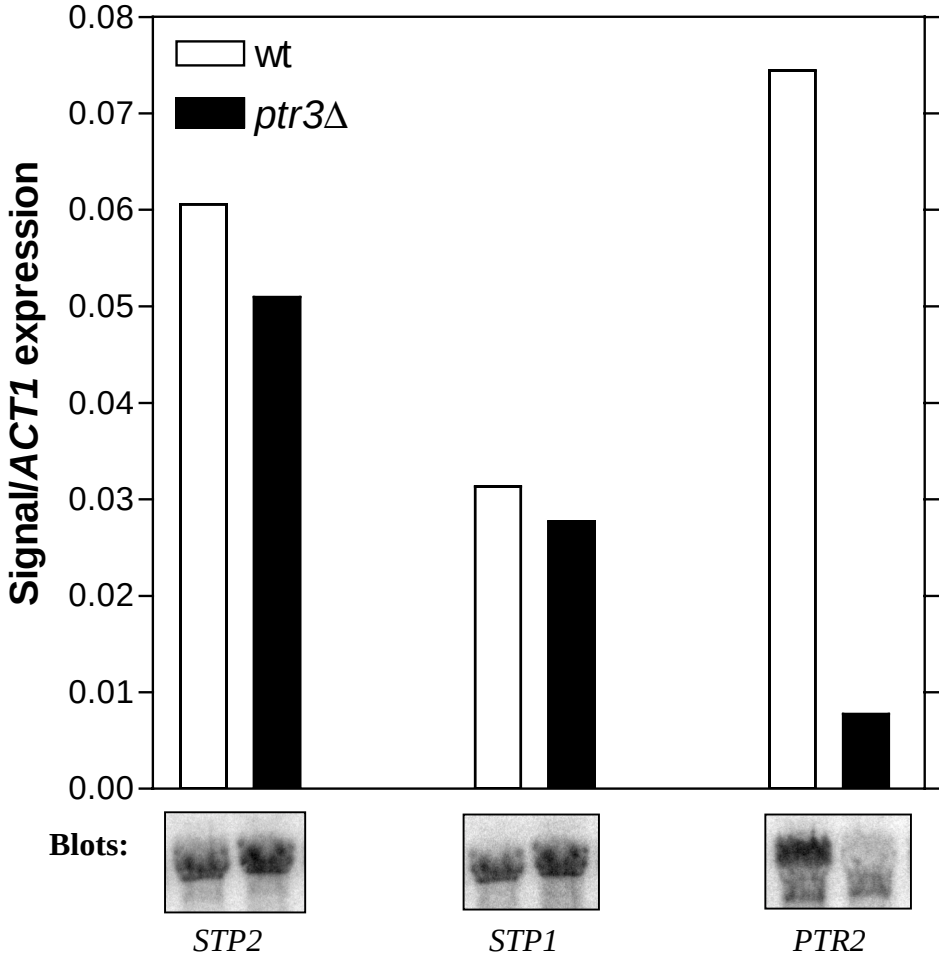

500
400
300
200
100
0
Miller units
Wild type
ptr3Δ
stp2Δ
ptr3Δ, stp2Δ
none
L/W
R-E
Treatment

respectively (Fig. 6, solid bars). These results were similar to those found in a separate experiment using the same cells and conditions (Fig. 3). Without any inducer, the absence of *PTR3* decreased the expression of *PTR2* to 10% of that of the wild-type, and *stp2*Δ and *ptr3*Δ*stp2*Δ cells had approximately 25% of the *PTR2* expression level compared to that of the wild-type. In the presence of L and W amino acid inducers, *PTR2* expression in *ptr3*Δ cells was decreased to 17% of that of the wild-type. Under the same conditions, both *stp2*Δ and *ptr3*Δ*stp2*Δ cells had about 50% *PTR2* expression compared to that of the wild-type. In the presence of R-E dipeptide inducer, *ptr3*Δ cells had *PTR2* expression levels about 20% of that of the wild-type, and similar *PTR2* expression levels were observed in *stp2*Δ and *ptr3*Δ*stp2*Δ cells.

PTR3 does not regulate _STP1_ or _STP2_ expression.

Since the results of the above epistatic analysis suggested that *STP2* acts downstream of *PTR3* and both amino acid and dipeptide induction of *PTR2* expression involved *PTR3* and *STP2*, we carried out an experiment to determine whether *PTR3* was involved in regulation of expression of *STP2* or its homolog *STP1*. As shown by a northern analysis (Fig. 7), *PTR3* regulated *PTR2* expression, because in the absence of *PTR3* the amino acid-induced expression of *PTR2* was greatly reduced. The expression of *STP1* or *STP2* was not induced or repressed

Fig. 7. *STP2*, *STP1*, and *PTR2* expression patterns in the presence or absence of *PTR3* as determined by northern analysis. For each analysis, the wild-type (FY2) strain was grown in minimal medium and compared to *ptr3*Δ (BWS-201 strain). Northern blots and quantitative representation of the signal strengths relative to actin (*ACT1*) expression are shown.

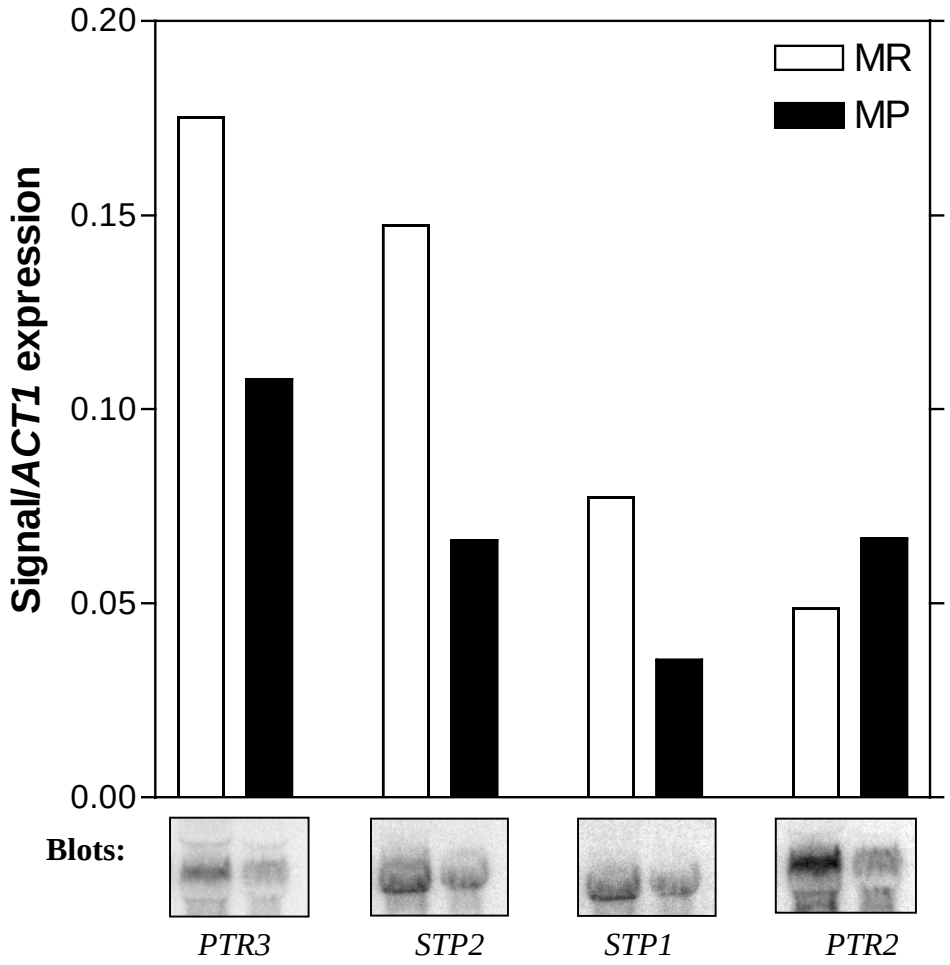

0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
wt
ptr3Δ
Signal/ACT1 expression
Blots:
STP2
STP1
PTR2

by the addition of amino acid, and *STP1* and *STP2* expression levels were not noticeably affected by amino acids L and W in the absence or presence of *PTR3.*

STP1 and _STP2_ are highly expressed in medium containing a rich nitrogen source.

Because nitrogen source is known to affect *PTR2* expression, the effect of nitrogen source on expression of *STP1* and *STP2* was tested. Wild-type cells were grown in medium containing ammonium sulfate (0.1%) as a rich nitrogen source or medium containing proline (0.1%) as a poor nitrogen source. Appropriate amino acid prototrophic markers (K, H, L, or W) were added. A northern analysis showed that when the cells were grown in a poor nitrogen source, *PTR2* expression was increased by 35% compared to that of cells grown in a rich nitrogen source (Fig 8). Among the cells grown in the two conditions, *PTR3, STP1* or *STP2* were expressed highest when the cells were grown in a rich nitrogen source. When the cells were grown in a poor nitrogen source, *PTR3, STP1* or *STP2* expression was decreased to 64%, 47%, or 44%, respectively, compared to that of cells grown in a rich nitrogen source.

Fig. 8. *PTR3, STP1, STP2,* and *PTR2* expression patterns in rich or poor nitrogen source determined by northern analysis. For each analysis, wild-type cells (*ptr2*Δ cells containing pMSI, a plasmid encoding wild-type Ptr2p under *PTR2* promoter) were grown in minimal medium with rich (indicated by MR) or poor (indicated by MP) nitrogen source. Northern blots and quantitative representation of the signal strengths relative to actin (*ACT1*) expression are shown.


0.20
0.15
0.10
0.05
0.00
Signal/ACT1 expression
MR
MP
Blots:
PTR3
STP2
STP1
PTR2

CHAPTER IV

DISCUSSION

The present work reports on the involvement of *STP1* and *STP2* in the di/tri-peptide transport system of *S. cerevisiae*. We began the study by examining transport function and *PTR2* expression in the absence of *STP1* or *STP2*. By measuring response to toxic amino acid and dipeptide and direct dipeptide uptake, we showed that *STP1* and *STP2* were involved in di/tri-peptide transport. A liquid β-galactosidase reporter gene assay showed that these genes were required for optimal amino acid and dipeptide induced *PTR2* expression. Because the absence of Stp1p had a greater impact on amino acid induced *PTR2* expression than that in the absence of Stp2p, *STP1* may play a greater role in amino acid induction of di/tri-peptide transport than *STP2* (Fig. 3). We also noticed that the absence of Stp2p or Stp1p did not completely abolish the induction of *PTR2* expression. Thus, *STP1* and *STP2* are not essential, but required for optimal induction of expression of *PTR2*.

Because mutations in *SSY5* manifest phenotypes that are indistinguishable from those resulting from either single *ssy1* and *ssy5* mutations or *ssy5 ssy1* and *ssy5 ptr3* double mutations, Ssy5p and Ptr3p are hypothesized to be a component of the

Ssy1p-Ptr3p-Ssy5p amino acid sensory machinery (Hanna and Forsberg, 2001). We explored the relationship between *PTR3* and *STP1* or *STP2* and showed that although *PTR3* regulated amino acid-mediated *PTR2* expression, *PTR3* did not affect *STP1* or *STP2* expression (Fig. 5). Furthermore, through an epistasis analysis, we showed that *stp2*Δ and *ptr3*Δ*stp2*Δ strains had similar phenotypes indicating that *STP2* functions in the same pathway as *PTR3*, likely by acting downstream of *PTR3* (Fig. 4, 5, and 6). Thus, it is likely that *PTR3* acts upstream of *STP1* and *STP2* through a mechanism other than expression regulation. For example, *PTR3* may regulate the turnover of Stp1p and Stp2p or interact with Stp1p or Stp2 leading to the activation or inactivation of an Stp1p or Stp2p function. It is interesting to note that, in cells grown in the presence of amino acids, biochemical analysis found that Ptr3p appeared to acquire distinct as yet undetermined modifications (Forsberg and Ljungdahl, 2001). A detailed biochemical and genetic analysis of the amino acid sensory pathway will be required to identify all the members of the pathway and their physiological roles. However, potential members of this pathway have been identified. They include the global transcription factor coded by *ABF1*, which is required for amino acid induction of *BAP2* and *BAP3* (Della Seta *et al.*, 1990), and the product of *UGA35*, which is a common factor that is required for transcription induced by several compounds such as GABA and allophonate (Iraquie *et al*., 1999).

The pleiotrophic phenotype of *ptr3*Δ cells opens up the possibility that Ptr3p interacts with a wide range of regulatory proteins involved in the amino acid induction mechanism. These proteins may include: SCF^{GRR1} ubiquitin-ligase complex which is essential for transcription of genes under Ssy1p control (Iraqui *et al.*, 1999; Bernard and Andre, 2001a), a novel membrane-bound ubiquitin ligase system (Asi1p, Asi2p, Asi3p) that affects the transcription of amino acid permease genes, and a ubiquitin ligase system (Rsp5p, Bul1p, Bro1p) that affects amino acid permease traffic in the late endosomal vacuolar protein sorting pathways (Nielsen *et al.*, 2001). Whether Stp1p or Stp2p interacts with Ptr3p or any of these proteins above is unknown. A comprehensive study on yeast protein-protein interaction by the Yeast PathCalling™ screen (Uetz *et al.*, 2000; GeneScape Portal Web Site, http://portal.curagen.com) showed that Stp1p interacts with Cns1p, a component of the Hsp90 chaperone complex (Dolinski et al., 1998) and Ydl100cp, a protein with similarity to *E. coli* ArsA ATPase, a component of the arsenite-antimonite efflux pump (Boskovic *et al.*, 1996).

Northern analysis further showed that cells grown in ammonium sulfate as a rich nitrogen source have higher expression of *STP1* and *STP2* than those grown in proline as a poor nitrogen source. The induced expression levels in a rich nitrogen source were the opposite to the reduced expression of *PTR2* and several amino acid permease genes in cells grown in a rich nitrogen source, a

phenomenon called nitrogen catabolite repression (Coornaert *et al*., 1992; Magasanik, 1992; Barnes *et al*, 1998). Complementary studies analyzing the effects of extracellular amino acids, peptides, and nitrogen sources on the activation of the transcriptional machinery of di/tri-peptide transport system will be necessary to elucidate the regulation of di/tri-peptide transport in response to extracellular stimuli.

The involvement of *STP1* and *STP2*, which were previously hypothesized to encode pre-tRNA splicing proteins and function as transcription factors of a branched amino acid permease Bap2p, places an additional layer of regulation of the di/tri-peptide transport system. Does Stp1p or Stp2p function as pre-tRNA splicing proteins or transcription factors or in other roles in di/tri-peptide transport? Several clues to answer these questions can be obtained from similar regulation of di/tri-peptide and amino acid transport systems. For example, both *PTR2* and *BAP2* expression were induced by a micromolar amount of amino acid inducers, and these amino acid induction mechanisms were regulated by the same amino acid sensory complex consisting of Ssy1p, Ptr3p, and Ssy5p. It is possible that Stp1p and Stp2p, which were previously shown to bind directly to the UAS_{aa} (amino acid-dependent upstream activator sequence) found in the promoter of the *BAP2* promoter, also bind to the promoter of *PTR2*. The hypothesis was supported by the fact that the hypothetical Stp2p binding

sequence 5'-CGGCTC-3' in the UAS_{aa} of *BAP2* promoter (de Boer *et al.*, 2000) is present in the promoter of *PTR2* (Narita and Becker, unpublished observation). Further analysis supporting interaction of Stp1p and Stp2p at the *PTR2* promoter will be needed to test whether Stp1p and Stp2p are *PTR2* transcription factors.

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PART IV

STRUCTURAL AND FUNCTIONAL ANALYSIS OF PTR3P, A REGULATORY PROTEIN OF DI/TRI-PEPTIDE TRANSPORT IN *S. CEREVISIAE*

CHAPTER I

INTRODUCTION

The ability of the yeast *Saccharomyces cerevisiae* to respond to changing environmental conditions is essential for the viability of the cell. Sensing extracellular conditions, receptors on the cytoplasmic membrane transduce signals to intracellular molecules activating chemical changes of specific metabolic pathways culminating in a cellular response. One particular group of environmental sensors monitors nutrients, such as sugars, amino acids, and peptides, to allow the cell to respond to conditions of starvation or growth in a rich environment.

Amino acid and peptide transport in yeast occurs through a number of transport proteins. The amino acid permeases Gap1p and Agp1p, for example, transport a wide variety of amino acids for catabolic purposes, whereas a number of amino acid permeases transport only a specific amino acid such as branched chain amino acids, histidine, or arginine taken up by Bap2p, Hip1p, or Can1p, respectively (Regenberg *et al.*, 1999). Ptr2p is the only yeast di/tri-peptide transporter (Island *et al.*, 1991; Perry *et al.*, 1994). Genes encoding these amino acid and peptide transporters are differentially regulated by the nitrogen source and the presence of specific amino acids in the growth medium.

Several studies have been performed on an *S. cerevisiae* amino acid sensor, Ssy1p, a unique member of the amino acid permease family characterized by its long cytoplasmic N-terminal tail (Iraqui *et al.*, 1999; Jorgensen *et al.*, 1998). Binding of extracellular amino acids to Ssy1p does not result in amino acid transport; rather binding initiates a signal transduction pathway whose outcome is the induction of a number of amino acid permeases genes as well as the di/tri-peptide transporter gene (Iraqui *et al.*, 1999; Klasson *et al.*, 1999). Together with regulatory proteins Ssy5p and Ptr3p, Ssy1p is hypothesized to form an amino acid sensor machinery complex (Forsberg and Ljungdahl, 2001). All three components of the amino acid sensing complex (named SPS for Ssy1p-Ptr3p-Ssy5p) are required for amino acid induction of amino acid and peptide transport. (Forsberg and Ljungdahl, 2001).

Ssy5p was identified as a component of SPS since *ssy5*Δ mutants are in the same epistasis group as *ssy1*Δ and *ptr3*Δ mutants. All three mutants exhibited similar phenotypes such as increased resistance to toxic amino acids and increases in vacuolar pools of histidine and arginine (Forsberg and Ljungdahl, 2001). Ptr3p was originally identified as a regulatory protein for expression of the di/tri-peptide transporter gene, *PTR2*, as well as a number of amino acid permease genes (Barnes *et al.*, 1998; Forsberg and Ljungdahl, 2001). Ptr3p interacts with the N-terminal domain of amino acid sensor Ssy1p as shown by

two-hybrid analysis and co-immunoprecipitation (Narita and Becker, unpublished observation). Growing yeast cells engage distinct developmental pathways leading to vegetative growth or filamentous-like growth in response to nutrient availability. Involvement of Ptr3p in these pathways was indicated by the enhancement of haploid filamentous-like growth in the absence of Ptr3p (Klasson *et al.,* 1999). Furthermore, *PTR3* is involved in induction of *PTR2* by not only amino acids, but also dipeptides (Hauser *et al.*, 2001).

Some limitations to further study of the function of Ptr3p include the fact that functional motifs have not been identified in Ptr3p, and Ptr3p has no overall sequence similarity to other proteins in the database. Limited homology exists to a short sequence (54 amino acids) within Gcn4p and extracellular loops (about 51 amino acids) of some amino acid permeases (Klasson *et al*., 1999). Previous results showed that the regulatory events controlled by SPS may require that Ptr3p disengage from the membrane. After amino acid-induced activation, Ptr3p was proposed to localize to other regions of the cell to exert control on gene transcription (Klasson *et al*., 1999). Ssy5p is apparently proteolytically modified in a Ptr3p-dependent manner because in extracts derived from *ptr3* null mutants, Ssy5p migrated as a 76-kDa protein, while in those from the wild-type Ssy5p migrated as a 67-kDa protein (Forsberg and Ljungdahl, 2001). The Ptr3p-

dependent processing event is likely to occur within the C-terminal portion of Ssy5p, but the nature of this putative enzymatic role is not clear.

To better understand the function of Ptr3p, we performed several experiments. We report on the identification of proteins that may functionally interact with Ptr3p on the basis of a two-hybrid analysis. We expressed truncated forms of Ptr3p to identify functional domains. A 3-dimensional protein structure prediction algorithm, called PROSPECT (Xu and Xu, 2000), found a potential ring finger and a beta propeller motif within Ptr3p. Furthermore, two other different computational algorithms predicted that two putative nuclear localization signals (NLS) were present in Ptr3p. Alanine site-directed mutagenesis of the predicted conserved residues in each of these motifs and NLS were performed. Each Ptr3p mutant was tested for its ability to complement the *ptr3*Δ phenotype using peptide toxicity assays as a measure of a functional Ptr3p. On the basis of these experiments, we speculate on the role of Ptr3p in the amino acid-induced signal transduction pathway.

CHAPTER II

MATERIALS AND METHOD

Strains and media.

Yeast strains used are listed in Table 1. Cells were grown routinely on YEPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar). Strains transformed with a plasmid were cultured on minimal medium lacking uracil or leucine (0.67% Difco yeast nitrogen base with ammonium sulfate, without amino acids, 2% glucose, 0.2% casamino acids).

For two-hybrid analysis, cells were grown in minimal dropout medium containing 2% glucose (Glu) or 2% galactose plus 1% raffinose (Gal) and are designated by the component that was omitted (e.g. SC (Glu)–ura –leu –his –ade medium has 2% glucose and lacks uracil, leucine, histidine, and adenine). 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) minimal dropout plates contained 40 mg / ml X-Gal and phosphate buffer at pH 7.0. DNA was introduced into yeast by LiAc-mediated transformation as described elsewhere (Gietz *et al.*, 1995). For peptide toxicity assays, cells were inoculated into medium lacking uracil and ammonium sulfate (0.67% Difco yeast nitrogen base without amino

Table 1. *S. cerevisiae* strains used.

Strain	Genotype	Reference
FY2	*MATa ura3-52*	Winston *et al*. (1995)
BWS-201	*MATa ura3-52 ptr3::Loxp*	Barnes *et al*. (1998)
PB1X-2AΔ	*MATa ura3-52 leu2-3 lys1-1 his4-32 ptr2*::*LEU2*	Perry *et al*. (1994)
BBY-47	*MATa ura3-52 leu2-3,112 lys2-801 trp1-1 his3-Δ200 gal ubr1-Δ1::LEU2*	Bartel *et al*. (1990)
BLS-201	*MATa ura3-52 cup9::Loxp*	Barnes *et al*. (1998)
BLS-206	*MATa ura3-52 ptr3::Loxp cup9::Loxp*	Barnes *et al*. (1998)
PJ69-4A	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*	James *et al*. (1996)

acids and ammonium sulfate, 2% glucose) supplemented with 0.1% proline as a nitrogen source, and leucine (228 µM) as inducer (proline medium).

Plasmids.

All plasmids were created by *in vivo* ligation between the insert gene and the empty vector. All plasmids and nucleotide used are listed in Table 2. The plasmids pGBDU-C1, pGAD-C1, and genomic DNA libraries used for two-hybrid analysis were obtained from James *et al.* (1995). pGBD-*PTR3*, pGBD-*SSY5*, or pGBD-*NSSY1* were created by PCR amplification of the ORF YFR209W, YJL156C, or YDR160w encoding only the first 282 amino acids of Ssy1p, respectively, and cloning the resultant products into the *URA3*/2µ-based vector pGBDU-C1 such that the gene was fused with the Gal Binding Domain (GBD) gene and under the control of an ADH promoter. The plasmids pGAD-*PTR2*, pGAD-*PTR1*, and pGAD-*CUP9* were created by PCR amplification of the appropriate ORFs (YKR093w, YGR184c, and YPL177c, respectively) and cloning the resultant products into the *LEU2*/2µ-based vector pGAD-C1 such that the gene was fused with the Gal Activation Domain (GAD) gene and under the control of an ADH promoter. Plasmids pGBD-3(1-250), pGBD-3(1-500), or pGBD-3(250-678) encoding fusion protein GBD and Ptr3p residues 1-250, 1-500, or 250-678 respectively were created by PCR amplification of the ORF (YFR209W) using primer pairs GBD(1-250)-F/GBD(1-250)–R, GBD(1-250)-F/GBD(1-500)-R or

GBD(250-678)-F/GBD(250-678)-R, and the resultant products were ligated into pGBDU-C1. A similar strategy was used to create the pGAD-*PTR3* plasmids. All Ptr3p mutants were expressed from pUTR3(1-678) based plasmid that expressed *PTR3* under its 5'and 3' UTR. pUTR3(1-250) which contains *PTR3* fragment residues 1-250 was created by introducing a stop codon at residue 250 on pUTR3(1-678) through a site directed mutagenesis using SDM3-(1-250) oligonucleotide. Plasmids pUTR3(1-500) and pUTR3(250-678) containing *PTR3* fragment residues 1-500 and 250-678 were created by PCR amplification of the ORF (YFR209W) using primer pairs 1-UTR-F/250-UTR-R and 250UTR-F/678-UTR-R respectively, and the resultant products were ligated into pUTR3(1-678). Plasmids were transformed into yeast by the LiAc-mediated transformation as described (Agatep *et al.*, 1998), and transformants were selected by growth on minimal medium lacking uracil or leucine.

Yeast two-hybrid analysis.

The yeast two-hybrid screens were conducted by utilizing His, Ade, and *lac-Z* assays as described (James *et al.*, 1995). For library screening, strains containing pGBD-*PTR3* were transformed with Y2HL-C1, Y2HL-C2, and Y2HL-C3 genomic libraries (James *et al.*, 1995). These libraries had been amplified to obtain at least twenty million transformants for each library in DH10B electrocompetent *E. coli* to make sure that the libraries remained representative. Plasmids were

transformed into yeast by the LiAc-mediated transformation adjusted for 2 Hybrid System TRAFO Protocol (Agatep *et al.*, 1998). For each library about 295,800 transformants were screened representing approximately 5–fold coverage or 98% of the *S. cerevisiae* genome. The selection for interacting clones was performed in media containing glucose and lacking histidine (plus 2 mM 3-aminotriazole) or lacking adenine. Then, the colonies were further tested for *lac-Z* expression by plating them on minimal dropout media containing galactose/raffinose and X-Gal. Strong (89T and 89L plasmids) and zero (pGBT9.C and pGAD424.C plasmids) interaction controls (Bartel *et al.*, 1996) were a kind gift from the Stanley Fields laboratory (Daniel Lockshon, University of Washington, Seattle, WA). Library plasmids from yeast colonies, expressing the putative GAD-interacting proteins, were rescued by growing the colonies on 5-Fluoroorotic acid (5-FOA) minimal dropout media lacking leucine to cure the bait plasmid, then followed by transformation of yeast plasmid into DH5-α *E. coli*. The confirmed interacting fusion genes were sequenced and evaluated by the Basic Local Alignment Search Tool (Altschul *et al.*, 1990) through the *Saccharomyces* Genome Database (http://genome-www2.stanford.edu/cgi-bind/SGD). To test interaction of Ptr3p fragments, strain PJ69-4A containing pGAD-*PTR3*(1-250), (1-500), and (250-678) was transformed either with pGBD-*NSSY1* or pGBD*SSY5*.

Table 2. Plasmids, libraries, and nucleotides used.

Plasmid, library, or oligonucleotide	Description or sequence	Reference
Plasmids		
pGBDU-C1	two-hybrid expression vector containing GBD	James *et al.* (1995)
p*GBD-PTR3*	*GBD-PTR3* in pGBDU-C1 (functional)	This work
pGBD-*NSSY1*	GBD-*NSSY1* in pGBDU-C1	This work
pGBD-*SSY5*	GBD-*SSY5* in pGBDU-C1	This work
pGAD-C1	two-hybrid expression vector containing GAD	James *et al*. (1995)
p*GAD-PTR2*	*GAD-PTR2* in pGAD-C1	This work
p*GAD-PTR1*	*GAD-PTR1* in pGAD-C1	This work
p*GAD-CUP9*	*GAD-CUP9* in pGAD-C1	This work
pGAD-*NSSY1*	GAD-*NSSY1* in pGAD-C1	Narita and Becker (manuscript in preparation)
p*GBD-PTR3(1-250)*	*GAD-PTR3* in pGAD-C1 encoding fusion GAD and Ptr3p residues 1-250.	This work
p*GBD-PTR3(1-500)*	*GAD-PTR3* in pGAD-C1 encoding fusion GAD and Ptr3p residues 1-500.	This work
p*GBD-PTR3(250-678)*	*GAD-PTR3* in pGAD-C1 encoding fusion GAD and Ptr3p residues 250-678.	This work
pRS316		
pUTR3(1-678)	*PTR3* expressing residue 1-678 in pRS316 under *PTR3* 5' and 3' UTR	This work
pPtr3p(C52A)	C52A mutation in pUTR3(1-678)	This work
pPtr3p(C57A)	C57A mutation in pUTR3(1-678)	This work
pPtr3p(C59A)	C59A mutation in pUTR3(1-678)	This work
pPtr3p(C60A)	C60A mutation in pUTR3(1-678)	This work
pPtr3p(C52A;C60A)	C52A;C60A mutation in pUTR3(1-678)	This work
pPtr3p(C52A;C57A;C60A)	C52A;C57A;C60A mutation in pUTR3(1-678)	This work
pPtr3p(F544A)	F544A mutation in pUTR3(1-678)	This work
pPtr3p(N545A)	N545A mutation in pUTR3(1-678)	This work
pPtr3p(S546A)	S546A mutation in pUTR3(1-678)	This work
pPtr3p(S547A)	S547A mutation in pUTR3(1-678)	This work
pPtr3p(P548A)	P548A mutation in pUTR3(1-678)	This work
pPtr3p(RKK)	RKK(278-280)AAA mutation in pUTR3(1-678)	This work
pPtr3p(KKKKK)	K_5(345-358)A_5 mutation in pUTR3(1-678)	This work
pUTR3(1-678)	*PTR3* expressing residue 1-678 in pRS316 under *PTR3* 5' and 3' UTR	This work

Table 2. Continued.

pUTR3(1-250)	*PTR3* expressing residue 1-250 in pRS316 under *PTR3* 5' and 3' UTR	This work
pUTR3(1-500)	*PTR3* expressing residue 1-500 in pRS316 under *PTR3* 5' and 3' UTR	This work
pUTR3(250-678)	*PTR3* expressing residue 250-678 in pRS316 under *PTR3* 5' and 3' UTR	This work
pADH-3(1-250)	*PTR3* in pDB20 expressing residue 1-250 under an ADH promoter	This work
pGBD-3(1-250)	GBD-*PTR3* expressing fusion GBD-Ptr3p residue 1-250 in pGBDU-C1	This work
pGBD-3(1-500)	GBD-*PTR3* expressing fusion GBD-Ptr3p residue 1-250 in pGBDU-C1	This work
pGBD-3(250-678)	GBD-*PTR3* expressing fusion GBD-Ptr3p residue 1-250 in pGBDU-C1	This work
Libraries		
YL2H-C1, YL2H-C2, YL2H-C3	Genomic DNA two hybrid libraries	James *et al*. (1995)
Oligonucleotides		
PTR2-F	5'-TTCCGCACCATTCCAAAACTACAT-3'	
PTR2-R	5'-GCAGCACAGAAAACTCCCGTCAAC-3'	
CUP9-F	5'-GCTAACAACCTTCGAGAATAGTTACATTCG-3'	
CUP9-R	5'-CAATTCATATCAGGGTTGGATAGC-3'	
BAP2-F	5'-TCAACCGGCTCTAATGGGAAGTGT-3'	
BAP2-R	5'-GTGGTGGGGGCCGGAAATGAC-3'	
ACT-F	5'-GCCGGTTTTGCCGGTGACGAC-3'	
ACT-R	5'-GGAAGATGGAGCCAAAGCGTG-3'	
*p*GBD-PTR3-F	5'-GAGTAGTAACAAAGGTCAAAGACAGTTGACTGTATCGCCGCACGCACACTCACACATACAT-3'	
*p*GBD-PTR3-R	5'-TGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTCATACCAGAACCTTAAACATACG-3'	
*p*GAD-PTR2-F	5'-CCAAAAAAAGAGATCGAATTCCCCGGGGGATCCATCGCTGCTCCACTGCAAACAA-3'	
*p*GAD-PTR2-R	5'-CGGGGTTTTTCAGTATCTACGATTCATAGATCTCTGCTCTGTGAAACAAATAACAGCA-3'	
*p*GAD-PTR1-F	5'-CCAAAAAAAGAGATCGAATTCCCCGGGGGATCCATCGATTCCGTTGCTGATGATGATTTAGG 3'	
*p*GAD-PTR1-R	5'-CGGGGTTTTTCAGTATCTACGATTCATAGATCTCTGCAGCTACCAAATCTCTCGCTC-3'	

Table 2. Continued.

*p*GAD-CUP9-F	5’-CCAAAAAAAGAGATCGAATTCCCCGGGGGATCCATCGC TAACAACCTTCGAGAATAGTTACATTCG-3’
*p*GAD-CUP9-R	5’-GGTTTTTCAGTATCTACGATTCATAGATCTCTGCAGCTGT ATACCAGAACCTTAAAC-3’
C52A	5’-CGGGGTTTTTCAGTATCTACGATTCATAGATCTCTGCAG CAATTCATATCAGGGTTGGATAGC-3’
C57A	5’-GGTATAGTCAAAGACGCCTCTGTGCTTACGTG-3
C59A	5’-CTGCTCTGTGCTTACGGCTGGCTGCTGTATATCTG-3’
C60A	5’-GTGCTTACGTGTGGCGCCTGTATATCTGAATC-3’
C52A; C60A	5’-GCTTACGTGTGGCTGCGCTATATCTGAATCGC-3’
F544A	5’-CTGCTCTGTGCTTACGGCTGGCTGCGCTATATCTGAATC GC-3’
N545A	5’-GTGTATCACTAGTACAGCAGCCAATTCATCTCCACTG G-3’
S546A	5’-CTGATGTGTATCACTAGTACAGCATTCGCTTCATCTCCA CTGGTC-3’
S547A	5’-CAACAGCATTCAATGCATCTCCACTGGTC-3’
P548A	5’-CAGCATTCAATTCAGCTCCACTGGTCATAAAC-3’
RKK(278-280)AAA	5’-CTGATGTGTATCACTAGTACAGCATTCAATTCATCTGCAC TGGTCATAAACACC-3’
K_5(345-358)A_5	5’-CCAACTGTTTCCCCATGTACGCAGCAGCATTTCAGTTC AACACACACCC-3’
SDM3-(1-250)	5’-CTACTTCTCAAGCGTCAGCAGCGGCAGCGGCAAACTGG AGCCAACG-3’
1-UTR-F	5’-CCAAACAAATAGTGCATAAGATCTAGACGAAGAAAA GG-3’
500-UTR-R	5’-CCAAACAAATAGTGCATAAGATCTAGACGAAGAAAA GG-3’
250-UTR-F	5’-AGAACCTTAAACATACGTATAT ATTTAGATGCATTA
678-UTR-R	5’-CATAGGTACGAAATACAGATCTGATAGGCGTTCATG
GBD3(1-250)-F	5’-CGTATATATTTAGATGCATTATT TGTGGAATATTTG
GBD3(1-250)-R	5’-CCAAAAAAAGAGATCGAATTCCCCGGGGGATCCATCG CATCAATGCAGAAGCTGATCTCAGAGGAGGACCTGAACTG ATAGGCGTTCATGCA-3’
GBD3(1-500)-R	5’-GGTTTTTCAGTATCTACGATTCATAGATCTCTGCAGTGT TTGGTAGTTTGCTGTGGA-3’
GBD3(250-678)-F	5’-GGTTTTTCAGTATCTACGATTCATAGATCTCTGCAGGGT ATTGATGGGATCTCTGT-3’
GBD3(250-678)-R	5’-AAAAAAGAGATCGAATTCCCCGGGGGATCCATCGCATC AATGCAGAAGCTGATCTCAGAGGAGGACCTGAGAGATCCC ATCAATACCAT-3’

Prospect.

PROSPECT is a computer package for finding an optimal alignment between a protein sequence and a protein structural fold (Xu and Xu, 2000). PROSPECT guarantees to find the globally optimal sequence-structure alignment and does so in an efficient manner, when considering both alignment gap penalty and pairwise potential between residues that are spatially close. A neural network assessment for the reliability of the fold recognition is given by PROSPECT (Xu *et al*., in press). The atomic structures were generated using MODELLER (Sali and Blundell, 1993) based on the alignments obtained from threading. PROSPECT has been applied successfully to many proteins with unknown experimental structures (Xu *et al*., 2001).

Computational analysis of Ptr3p subcellular localization.

Prediction of subcellular location of Ptr3p was performed with two different programs. The first analysis was done at http://bioinfo.mbb.yale.edu/genome/yeast server, which predicts subcellular location of yeast proteins using Bayesian formalism (Alexandrov and Gerstein, in press). A complementary study was performed at http://psort.ims.u-tokyo.ac.jp/form2.html server, which uses PSORT II, a program to predict the subcellular localization sites of proteins from their amino acid sequences. The

reasoning algorithm was based on the *k*-nearest neighbors classifier (Horton and Nakai, 1996; Horton and Nakai, 1997).

Site-directed mutagenesis.

Single-stranded phagemid DNA of pUTR3(1-678) or pPtr3p(C52A;C60A) was prepared by infecting *Escherichia coli* strain CJ236 (*ung*,*dut*) carrying pUTR3(1-678) or pPtr3p(C52A;C60A) with the helper phage M13KO7 (Mead *et al.*, 1986). Oligonucleotide-directed mutagenesis of single-stranded phagemid DNA was constructed as described by Kunkel *et al.* (1991). The product of the mutagenesis reaction mixture was transformed into *E. coli* strain DH5α (Invitrogen Corporation, Carlsbad, CA), and transformants were selected on ampicillin-containing plates. Plasmids were then isolated from transformants using the Wizard Miniprep kit (Promega, Madison, WI). After sequence confirmation, constructs were transformed into yeast strain BWS-201 (*ptr3*-deletion strain) or wild-type FY2 strain, and transformants were selected by their growth in the medium lacking uracil. All primers were purchased from Sigma/Genosys (The Woodlands, TX). Sequences of the primers are shown in Table 2. DNA sequencing was carried out in the DNA sequencing facility located on the campus of the University of Tennessee.

Toxicity assay.

Functionality of any Ptr3p mutants was measured by their ability to complement the *ptr3*Δ phenotype using peptide toxicity assays. This assay has been used may times, and it mirrors the results of peptide uptake experiments and expression of *PTR2* (Island *et al.*, 1997; Barnes *et* al., 1998). When the toxic peptide Ala-Eth does not manifest toxicity, uptake of radiolabeled dipeptide is lowered greatly along with the expression of *PTR2*. Cells were grown overnight in medium lacking uracil and ammonium sulfate (0.67% Difco yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose) supplemented with 0.1% proline as a nitrogen source, and leucine (228 μM) and tryptophan (98 μM) as inducer and suspended in sterile water at 5.0 X 10^6 cells per ml. Fractions (1 ml) were added to 0.8% Noble agar and plated on minimal media containing Leu and Trp supplements to induce peptide transport. A total of 0.38 mmol of L-ethionine (Eth) or L-alanyl-L-ethionine (Ala-Eth) was added to a disk made of Whatman 3MM filter paper. L-ethionine was obtained from SIGMA (St. Louis, MO), and Ala-Eth was synthesized as previously described (Naider *et al.*, 1974). Disks containing each compound were placed on the surface of the plates. After 48 h of incubation, zones of inhibition were measured for all plates. Figures shown are representation of three independent measurements.

Uptake Assays.

Transformed cells were grown overnight to mid-exponential phase in proline medium. The uptake assay was initiated by combining equal volumes of pre-warmed (30 °C) cells and 2X uptake assay mixture (2% glucose, 20 mM sodium citrate / potassium phosphate, pH 5.5, 500 µM Leu-Leu (SIGMA, St. Louis, MO), 320 µM L-leucyl-L-[3H]leucine (16 mM, 10 mCi / mmol). L-Leucyl-L-[3H]leucine was synthesized by standard solution-phase techniques as described previously. For assays done in the presence of competitors, the 2X uptake assay mixture was supplemented with competitor (2X final concentration) prior to combining with the cells. At the appropriate time, aliquots (90 µl) were removed and washed by vacuum filtration four-times with 1 ml ice-cold water onto a membrane filter (HAWP, Millipore, Bedford, MA). The membranes were counted by liquid scintillation spectrometry, and results were reported as nmol / mg dry weight. Data points and error bars reflect the mean and standard deviation of a minimum of two independent experiments with samples taken in quadruplicate.

CHAPTER III

RESULTS

Identification of Ptr3p interacting proteins by two-hybrid screen.

To identify potential interactors of Ptr3p, we screened the Y2HL-1, Y2HL-2, and Y2HL-3 genomic libraries for clones that encoded GBD-Ptr3p-interacting proteins (see Materials and Methods). Control experiments showed that GBD-Ptr3p did not activate transcription of any of the reporter genes used in the library screen. Yeast strains expressing the full length Ptr3p fused to GBD were transformed with the YH2L-1, -2, and -3 libraries. Following histidine and adenine screening, we identified 37 clones in which the *lac-Z* reporter genes were transcribed, indicating the presence of plasmid-encoded proteins showing a positive two-hybrid complex with Ptr3p. Isolation and subsequent sequencing of these plasmids revealed they contained inserts representing the presence of 20 different ORFs (Table 3). *RPN10* (non-ATPase component of the 26S proteasome complex) and *RRP1* (protein involved in maturation of 25S rRNA) were considered as false positives because these highly basic proteins are found abundantly as interactors in many two-hybrid screens. Although expected to be identified on the basis of their putative formation of the Ssy1p-Ptr3p-Ssy5p amino acid regulatory complex, GAD-Ssy1p and Ssy5p were not found in this

Table 3**.** Ptr3p interactors found in two hybrid screening using Y2HL libraries.

Gene name[a]	Protein function	Strength of interaction[b]
Permease		
VAP1 (47-168)	Amino acid permease that transports valine, leucine, isoleucine, tyrosine, and tryptophan	Weak
THI7 (457-554)	Thiamin transport protein	Weak
YGR125w (274-395)	Member of sulfate permease of membrane transporters	Strong
Transport		
YGL014w (809-888)	Protein with pumilio repeats that is involved with Mpt5p in relocalization of Sir3p and Sir4p from telomeres to the nucleolus	Weak
MTR4 (1022-1040)	Protein required for mRNA export from nucleus, member of the DEAD-box RNA helicase family	Very weak
Transcription factor etc.		
GRF10 (391-512)	Homeodomain protein required for expression of phosphate pathway and other genes	Very weak
CKI1 (1-22)	Choline kinase, first step in CDP-choline biosynthesis pathway	Weak
BRR2 (400-435)	RNA helicase-related protein required for pre-mRNA splicing	Very weak
FAA4 (346-475)	Acyl-CoA synthase (long-chain fatty acid CoA ligase); contributes to activation of imported myristate	Very weak
SAR1 (143-191)	Component of COPII coat of vesicles involved in endoplasmic reticulum to Golgi transport, GTP-binding protein of the arf family in the ras superfamily	Very weak
TSL1 (192-291)	Component of the trehalose-6-phosphate synthase/phosphatase complex; alternate third subunit with Tps3p	Very weak

Table 3. Continued.

BUD5 (13-133)	GTP/GDP exchange factor for Rsr1p/Bud1p involved in bud site selection, mutants bud at random sites	Very weak
YGL001C (107-227)	C-3 sterol dehydrogenase, C-4 decarboxylase, required for ergosterol biosynthesis	Very weak
RPN 10 (117-187) *False positive*	Non-ATPase component of the 26S proteasome complex	Very weak
RRP1 (181-321) *False positive*	Protein involved in maturation of 25S rRNA	Weak
CEF1 (1-101)	Pre-mRNA splicing factor, also required for transition into mitosis, member of the Cdc5p family	Very weak

Unclassified protein

YGL258w (10-57)	Protein of unknown function	Very weak
YPL158c (61-179)	Protein of unknown function	Strong
YOL019w (296-439)	Protein of unknown function	Weak
YGL164c (338-402)	Protein of unknown function	Very weak

[a] Interacting residues in two-hybrid constructs are indicated in the bracket after the gene name

[b] The strength of interaction was determined by X-gal plate assay.

two-hybrid screening. Ptr3p interacted with peptides from portions of three permeases encoded by the two-hybrid library GAD-plasmids. These peptides include an N-terminal region of Vap1p, an amino acid permease that transports valine, leucine, isoleucine, tyrosine, and tryptophan, a C-terminal region of Thi7p, a major facilitator superfamily transporter responsible for thiamine uptake, and a loop of Ygr125wp, a member of the sulfate permease family of membrane transporters. The interacting residues of these permeases were predicted to be on the cytoplasmic face of the plasma membrane. In addition to the three permeases identified, Ptr3p was shown to interact with portions of a number of proteins located in the nucleus, although many of the interactions were apparently weak according to the strength of the β-galactosidase signal on plates. Transporters involved in movement of proteins within the nucleus (YGL104w) and export from the nucleus (Mtr4p) and a number of transcription factors (Table 3) were detected. Furthermore, several unclassified proteins were identified in the two-hybrid screen, including Ypl158c protein, which interacts strongly with Ptr3p.

Putative nuclear localization signals were present in Ptr3p.

Previous sucrose gradient centrifugation (Klasson *et al.*, 1999) and GFP fused Ptr3p localization analysis (Narita and Becker, unpublished observation) showed that Ptr3p localized at the plasma membrane as a peripheral protein, and a

biochemical analysis showed that Ptr3p may be cleaved during signal transduction and then localize to different cellular compartments (Klasson *et al.*, 1999). The finding of some nuclear localized proteins in the two-hybrid screen supports the idea that Ptr3p may be modified and then move from the plasma membrane to the nucleus to interact with nuclear localized proteins. Computational predictions by both PSORT II and Bayesian formalism highly favor nuclear localization of Ptr3p (94.1% and 94.9% probability respectively; Table 4). Ptr3p contains two putative nuclear localization signals (NLS): KKKK (residues 345-358) and PMYRKKF (residues 275-281). That these two NLS act as a bipartite NLS was not predicted by the algorithm. According to the same computational analysis, the amino acid sensor Ssy1p and di/tri-peptide transporter Ptr2p were predicted to be membrane localized (Table 4). Other proteins (Ptr1p/Ubr1p, Cup9p, Stp1p, and Stp2p) known to be involved downstream in the amino acid induction pathway (Byrd *et al.*, 1998; Barnes *et al.*, 1998; Nielsen *et al.*, 2001) were predicted to localize at the nucleus.

3D modeling predicted that Ptr3p contains ring finger and β-propeller motifs. Since Ptr3p does not have significant sequence similarity to any proteins with known structures based on searches using algorithms such as BLAST, we used PROSPECT (Xu and Xu, 2000) to predict its structure based on structural fold recognition. For the 678 residues of Ptr3p, the result suggested this protein may

Table 4. Computational predictions of subcellular localization of proteins involved in di/tri-peptide transport.

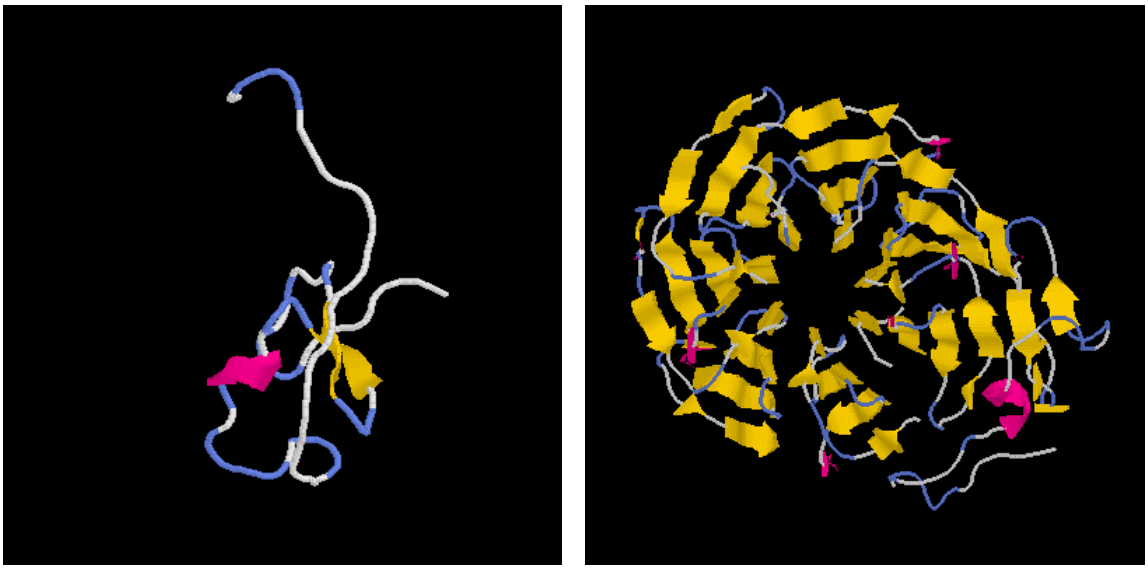
Proteins involved in peptide transport	**Bayesian formalism prediction**[a]		**PSORT II prediction**[b]	
	Subcellular localization	**Probability**	**Subcellular localization**	**Probability**
Ptr2p	Transmembrane	89.4%	Plasma membrane	73.9%
Ssy1p	Transmembrane	85.8%	Plasma membrane	78.3%
Ptr3p	Nucleus	94.9%	Nucleus	78.3%
Ssy5p	Nucleus	43.2%	Nucleus	60.9%
Ptr1p/Ubr1p	Nucleus	92%	Endoplasmic reticulum	44.4 %
Cup9p	Nucleus	91.4%	Nucleus	78.3%
Stp1p	Nucleus	97.6%	Nucleus	82.6%
Stp2p	Nucleus	45.2%	Nucleus	60.9%

[a] Bayesian formalism analysis was done at http://bioinfo.mbb.yale.edu/genome/yeast server, which predicts subcellular location of yeast proteins (Alexandrov and Gerstein, in press).

[b] PSORT II was performed at http://psort.ims.u-tokyo.ac.jp/form2.html server, which predicts the subcellular localization sites of proteins from their amino acid sequences. The reasoning algorithm was based on the *k*-nearest neighbors classifier (Horton and Nakai, 1996; Horton and Nakai, 1997).

have two major domains: one within residues 1-250 and the other residues 260-678. For the N-terminal domain, PROSPECT predicted a zinc finger motif of C3HC4 type (RING finger) around residues 52-61 identified by PROSITE (Hofmann *et al.*, 1999) (see Fig. 1, panel a). This motif often occurs at the DNA binding site of a transcription factor. The top hits with good confidence for the C-terminal domain were mostly signal transduction proteins such as 1qqg [pleckstrin homology (PH) and phosphotyrosine binding (PTB) targeting region of human insulin receptor substrate 1; Shirano *et al.*, 1999] and 1tbg (the heterotrimeric G-protein transducin; Noel *et al.*, 1993). A structural model based on 1tbg is shown in Fig. 1b. The model has a seven-bladed β-propeller fold. We have high confidence on the prediction of the seven-bladed β-propeller fold (the probability of the correct fold for this domain is >90%). The prediction of the seven-bladed β-propeller fold is also confirmed when we used an independent fold recognition program SAM-T98 (Karplus *et al.*, 1998). Based on the prediction, the C-terminal domain may be involved in protein-protein interaction, since many of the β-propeller structures that have been identified have known functions in governing protein-protein interactions, e.g., lectins (Beisel *et al.*, 1999) and the WD-40 domains in G-protein signaling molecules (Wall *et al.*, 1998).

Fig. 1. Structural model for the Ptr3p protein. For the N-terminal domain (within residues 1-250), PROSPECT confirmed a zinc finger motif of C3HC4 type (RING finger) around residues 52-61 (panel a), and the C-terminal domain (within residues 250-678) was predicted to have a seven-bladed β-propeller fold (panel b).

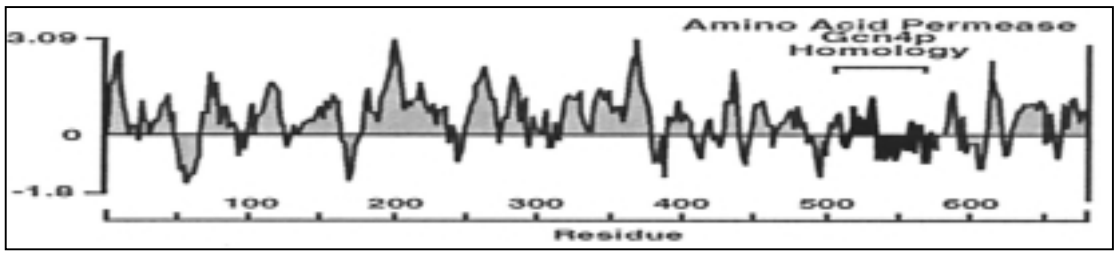


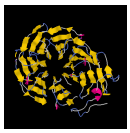
(a) (b)

Alanine substitution of conserved Cys residues in predicted ring finger motif reduced Ptr3p function.

The predicted ring finger motif in Ptr3p contains 3 conserved Cys residues (C52, C57, and C60; see Fig. 2). To test whether this motif was involved in the di/tri-peptide function of Ptr3p, site-directed mutagenesis of these conserved Cys residues were performed. Non-conserved Cys residue 59 was also mutated as a control. Single Ala substitution of C52, C59, and C60 in Ptr3p did not affect the ability of the Ptr3p mutant to complement a *ptr3*Δ phenotype (Table 5). Cells expressing Ptr3p C52A, C59A, and C60A mutants had similar sizes of halo of inhibition of toxic amino acid and dipeptide compared to that of wild-type Ptr3p cells. In contrast, a single Ala substitution of residue C57 reduced the ability of the cells expressing Ptr3p C57A to complement *ptr3*Δ strain. The zone of inhibition in *ptr3*Δ cells expressing Ptr3p C57A was reduced by 26% and 56% for Eth and Ala-Eth, respectively, compared to that of wild-type Ptr3p cells. The C52A;C60A double mutant and the triple C52A;C57A;C60A showed similar phenotypes to that of the C57A mutant. Because Ptr3p was suggested to interact with itself by two-hybrid analysis (Andre *et al.*, 2001), we tested whether any of the mutants affected the function of wild-type Ptr3p. None of these mutants caused a dominant negative phenotype because the halo of inhibition by toxic Eth and Ala-Eth in cells expressing both wild-type Ptr3p and mutant Ptr3ps was

Fig. 2. A map of predicted motifs and nuclear localization signals within Ptr3p. The mutated residues are underlined. The predicted ring finger motif lies within residues 52-CSVLTCGCCI-61. The position of Ptr3p's limited homology sequence to transcription factor Gcn4p and some permeases are shown. Each residue within 544-FNSSP-548 was substituted with alanine. The two nuclear localization signals are present in predicted beta propeller structure residues 275-PMYRKK-280 and 352-SSSSLSTTSQASKKKKK-368.



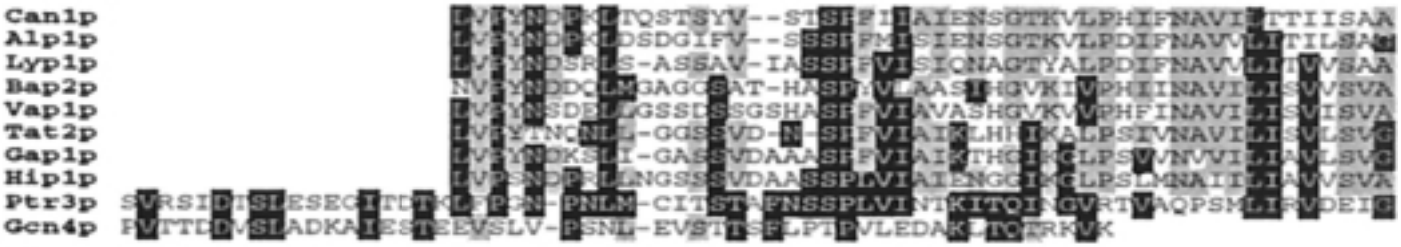


1 250 500 678

52-**C**SVLT**C**GC**C**I-61
Ring finger

275-PMY**RKK**-280
NLS

352-SSSSLSTTSQAS**KKKKK**-368
NLS



544-**FNSSP**-548
Hypothesized amino acid binding
Gcn4p/permease homology

similar to that of the FY2 cells expressing only the wild-type Ptr3p (Table 5).

Ala substitution of FNSSP residues resulted in Ptr3p conferring an increase in peptide transport.

Within the predicted seven-bladed β-propeller region of Ptr3p a limited homology sequence (residues 544-548, Fig. 2) with Gcn4p and an extracellular loop of some amino acid permeases was present (Klasson *et al*., 1999). We decided to perform a single alanine substitution within this region. Single Ala substitution of each residue generated a mutant Ptr3p conferring greater ability of the cell to response to toxic Eth and Ala-Eth (Table 5). Cells expressing F544A, N545A, S546A, S547A, and P548 have a 5%, 10%, 8%, 8%, and 2%, respectively, greater halo of inhibition in response to ethionine as compared to that of the wild-type cells (Table 5). An increased ability to complement *ptr3*Δ phenotype of each of these mutants was more prominent in their response to toxic Ala-Eth with increase of 76%, 80%, 68%, 72%, and 52% in halo size compared to that of the wild-type observed for cells expressing F544A, N545A, S546A, S547A, and P548A, respectively. None of these mutants affected the response to toxic Eth and Ala-Eth in FY2 wild-type cells because the halo of inhibition was similar as that of the FY2 cells (Table 5).

Table 5. Toxicity assays of Ptr3p mutants.

Plasmid/Mutant	Function in			
	*ptr3*Δ cells		wild-type FY2 cells	
	Diameter (mm) of zone of inhibition in response to:			
	Eth	Ala-Eth	Eth	Ala-Eth
pRS316	0.0 ± 1 *	0.0 ± 0 *	40 ± 1	23 ± 1
pUTR3(1-678)	39 ± 1	25 ± 2	41 ± 0	24 ± 0
Ring finger				
C52A	40 ± 1	28 ± 0	40 ± 1	25 ± 1
C57A	29 ± 1	11 ± 3	41 ± 0	24 ± 0
C59A	36 ± 1	25 ± 5	42 ± 1	29 ± 0
C60A	40 ± 0	21 ± 1	41 ± 1	28 ± 1
C52A;C60A	28 ± 4	12 ± 2	40 ± 1	23 ± 5
C52A;C57A; C60A	30 ± 1	17 ± 1	41 ± 1	26 ± 4
β-propeller				
F544A	41 ± 1	44 ± 2	40 ± 1	27 ± 1
N545A	43 ± 1	45 ± 1	42 ± 1	28 ± 1
S546A	42 ± 3	42 ± 1	41 ± 1	25 ± 1
S547A	42 ± 1	43 ± 1	40 ± 3	26 ± 1
P548A	40 ± 2	38 ± 4	40 ± 1	24 ± 2
NLS				
K_5(345-358)A_5	39 ± 1	25 ± 3	40 ± 3	24 ± 1
RKK(278-280)AAA	30 ± 0	10 ± 1	40 ± 1	28 ± 2

* 0.0 mm-zone of inhibition in *ptr3*Δ cells was actually fuzzy, which was previously observed in identical assay, indicating a leaky phenotype of *ptr3*Δ strain (Island *et al.*, 1991).

Ala substitution of predicted RKK nuclear localization signals resulted in a reduction in the function of Ptr3p.

The computational analysis by PSORT II and Bayesian formalism programs predicted the nuclear localization signals within Ptr3p (KKKKK (residues 345-358) and PMYRKKF (residues 275-281) with 94.1% and 94.9% probability, respectively; Table 4). To test whether the predicted nuclear localization signals affected the function of Ptr3p, site-directed mutagenesis of charged KKKKK and RKK residues were performed. Ala substitution of KKKKK(345-358)AAAAA in Ptr3p did not affect the ability of Ptr3p mutant to complement the *ptr3*Δ phenotype (Table 5). Cells expressing KKKKK(345-358)AAAAA mutant had similar sizes of halo of inhibition by toxic amino acid Eth and dipeptide Ala-Eth compared to that of wild-type Ptr3p cells. On the other hand, Ala substitution of RKK residues reduced the ability of the cells expressing this mutant to complement *ptr3*Δ phenotype. *ptr3*Δ cells expressing Ptr3p RKK(278-280)AAA had 23% and 60% reduced sizes of the zone of inhibition by toxic Eth and Ala-Eth, respectively, compared to that of wild-type Ptr3p cells. No dominant negative phenotype of Ptr3p RKK(278-280)AAA mutant was observed because its halo of inhibition by toxic Eth and Ala-Eth was similar to that of the FY2 cells.

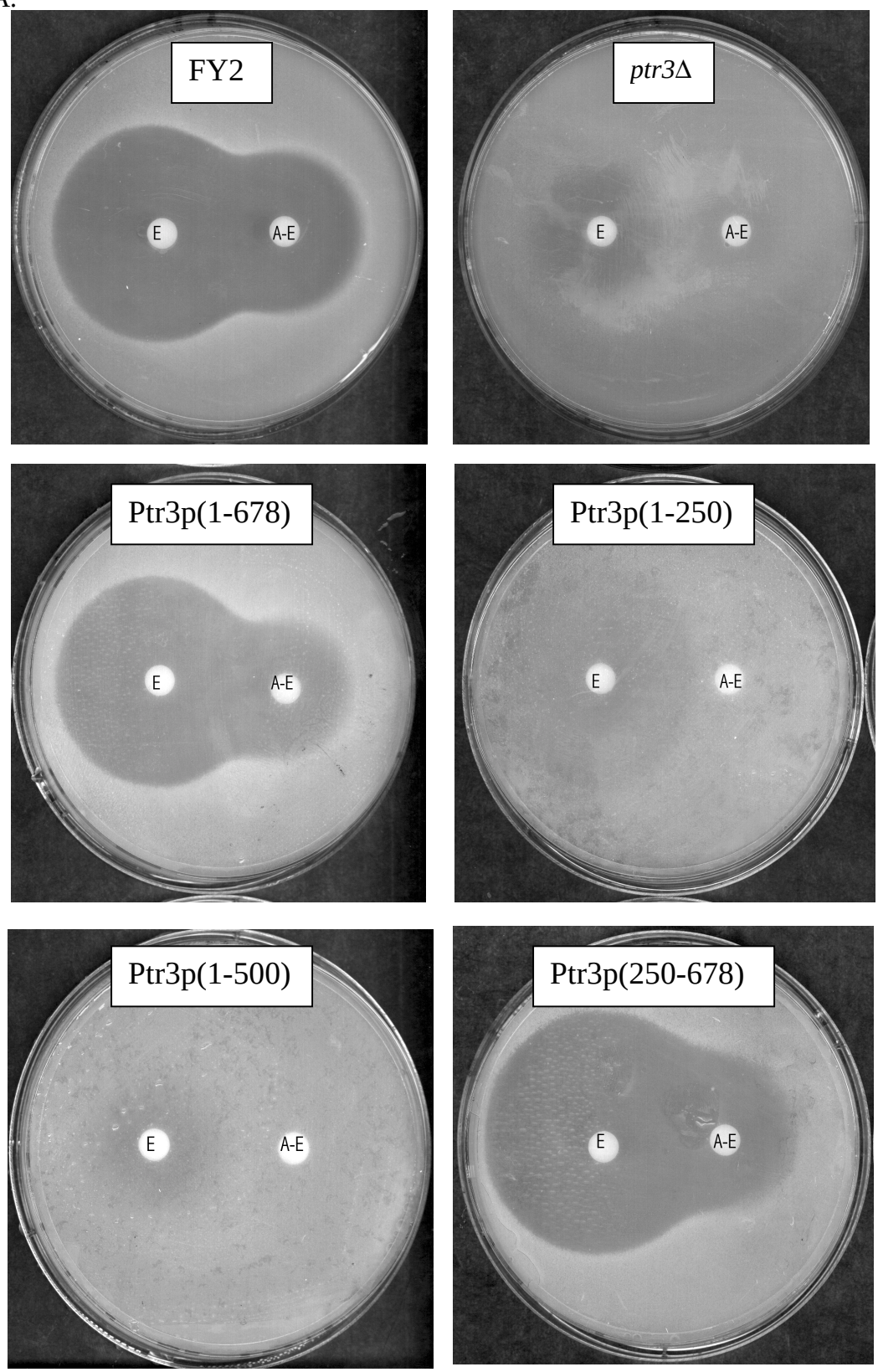
Functional domains of Ptr3p identified.

To identify Ptr3p functional domains, we analyzed the ability of a series of Ptr3p fragments under *PTR3* native promoter to complement the *ptr3*Δ phenotype. Ptr3p fragments constructed were Ptr3p spanning residues 1-250, 1-500, and 500-678. Ptr3p (1-250) was predicted to contain a ring finger motif based on a protein structure prediction program PROSPECT (Xu and Xu, 2000; Dong Xu, personal communication). Ptr3p (250-678) contains limited homology with transcription factor Gcn4p and extracellular loops of some amino acid permeases (Klasson *et al*., 1999). Whether each Ptr3p fragment could complement a *ptr3*Δ strain was tested by response to toxic dipeptide and direct radiolabeled Leu-Leu uptake.

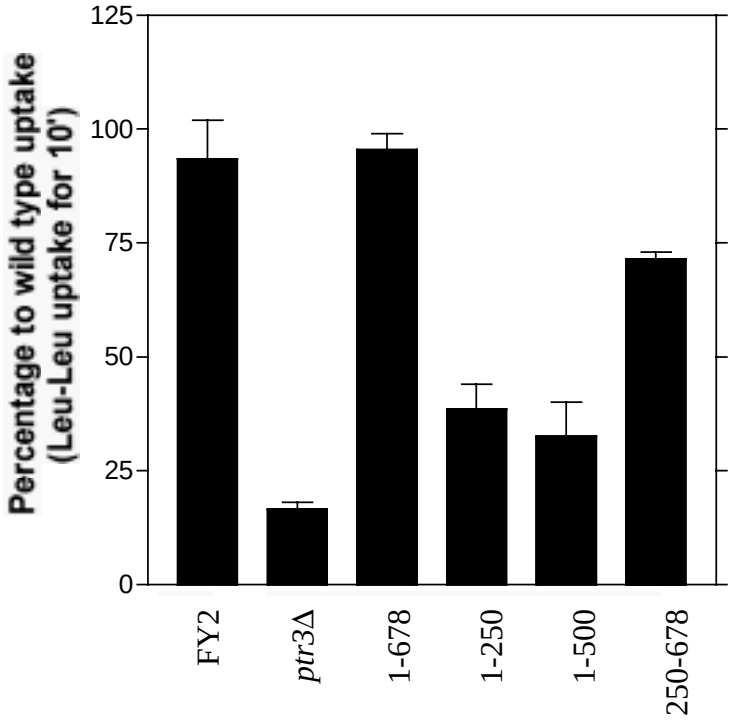
The zone inhibition by the toxic dipeptide Ala-Eth (A-E) in *ptr3*Δ cells expressing Ptr3p (250-678) (Fig. 3A sector f) was similar to that of full lawn Ptr3p (1-678) (Fig. 3A sector c) or wild-type FY2 cells (Fig. 3A sector a). However, *ptr3*Δ cells expressing Ptr3p (1-250) and Ptr3p (1-500) did not form zones of inhibition similar to the wild-type (Fig. 3A sector d and e, respectively). Instead they formed fuzzy halos similar to that of *ptr3*Δ cells (Fig. 3A sector b), which were hard to measure quantitatively. The radiolabeled Leu-Leu uptake assay (Fig. 3B) showed that Ptr3p (1-250) and Ptr3p (1-500) were able to restore uptake about 25% of that of the wild-type FY2 or *ptr3*Δ expressing Ptr3p (1-678). Ptr3p (250-

Fig. 3. Toxic peptide sensitivity (panel A) and direct dipeptide uptake (panel B) as measures of function of Ptr3p fragments. Ptr3p fragments in *ptr3*Δ cells were tested for the ability to complement the *ptr3*Δ phenotype by measuring the zone of inhibition by toxic amino acid ethionine and dipeptide Ala-Eth (A) or the ability to uptake ^{3}H-Leu-Leu (B).

A.


FY2
E
A-E
*ptr3*Δ
E
A-E
Ptr3p(1-678)
E
A-E
Ptr3p(1-250)
E
A-E
Ptr3p(1-500)
E
A-E
Ptr3p(250-678)
E
A-E

B.


Percentage to wild type uptake
(Leu-Leu uptake for 10')
125
100
75
50
25
0
FY2
ptr3Δ
1-678
1-250
1-500
250-678

678) was able to restore uptake of ^{3}H-Leu-Leu to about 70% of that of the wild-type or *ptr3*Δ expressing Ptr3p (1-678).

CHAPTER IV

DISCUSSION

To provide information on how the novel Ptr3p functions specifically in the amino acid and dipeptide induction of *PTR2* and some amino acid permeases genes, we identified Ptr3p-interacting proteins by a two-hybrid screen. We found that Ptr3p interacted with proteins of various functions, including proteins involved in transport, transcription activation, and proteins of unknown function (Table 3). However, most of the positive clones encoded peptides that interacted weakly with Ptr3p. Weak interaction may indicate that another protein(s) is needed to form an interacting complex. On the other hand, weak interaction as determined by two-hybrid analysis may be sufficient for Ptr3p to initiate signal transduction for various downstream targets.

It was interesting that Ptr3p interacted with domains of a number of permeases including the following: (1) a small portion of the first transmembrane domain and cytoplasmic N-terminal domain of Vap1p, an amino acid permease that transports valine, leucine, isoleucine, tyrosine, and tryptophan, (2) Thi7p, a major facilitator superfamily transporter responsible for thiamine uptake, at its cytoplasmic C-terminal domain, and (3) a protein encoded by *YGR125W*, a

member of the sulfate permease family of membrane transporters, at its middle cytoplasmic loop. Interactions of Ptr3p with cytoplasmic portion of Vap1p, Thi7p, and YGR125Wp raise a possibility that Ptr3p interacts with these permeases as part of a sensing mechanism.

Our two-hybrid screening did not pick up an Ssy1p-Ptr3p interaction, although we showed previously that the N-terminal domain of Ssy1p interacted strongly with Ptr3p by a two-hybrid assay between GBD-Ptr3p and GAD-N-terminus-Ssy1p residues 1-282 (Narita and Becker, unpublished observation). Ssy5p was also not observed in the library screen despite the genetic evidence for the formation of an Ssy1p-Ptr3p-Ssy5p complex (Forsberg and Ljungdahl, 2001). Several reasons have been proposed for not obtaining a two-hybrid positive result (Philip James, personal communication). First, the fusion protein may not fold properly such that the interacting region will not maintain its native structure in the clones of the two-hybrid libraries. Second, the fusion protein may not be internalized to the nucleus preventing it from interacting with bait. Third, the genomic DNA library may not be comprehensive enough or amplified exhaustively to represent every gene in the library.

Identification of unclassified proteins as candidates for Ptr3p-interacting proteins raise a possibility that Ptr3p-mediated signal transduction may involve several novel pathways. Strong interaction with residues 61-179 encoded by

YPL158c is particularly interesting because it may give clues on how Ptr3p functions and the role of the unknown Ypl158c protein in yeast metabolism. YPL158C was one of 113 genes expressed at a peak during M/G1 phase of the cell cycle (Spellman *et al.*, 1998) suggesting that Ptr3p-mediated regulation of peptide transport could be interconnected with the cell cycle.

The presence of some transcription factors in the two-hybrid screen indicates that Ptr3p may interact with these proteins in the nucleus. Putative nuclear localization signals (NLS), KKKK (residues 345-358) and PMYRKKF (residues 275-281) were predicted to be present in Ptr3p. Interestingly, the mutant Ptr3p containing the alanine substitution of KKKKK residues still maintained its function, but when RKK residues were substituted with alanine residues, the RKK mutant had a reduced Ptr3p function. The reduced activity in peptide transport of the RKK-Ptr3p mutant suggests that Ptr3p may move from the plasma membrane to the nucleus to function. Biochemical analysis also supports that Ptr3p could dissociate from its plasma membrane localization and may localize to different cellular compartments in the cell (Klasson *et al*., 1999). However, the N-terminus fusion of GFP-Ptr3p did not show a nuclear localization, but instead localized in a punctate pattern associated with the plasma membrane (Narita and Becker, unpublished observation). A shuttling mechanism has been observed in sugar sensing, where Std1p, a regulatory

protein that interacts with the the carboxy-terminal extensions of the glucose sensor Snf3p, moves to the nucleus and generates a signal for induction of several hexose transporter genes (Schmidt *et al.*, 1999; Ozcan, *et al.*, 1998). Std1p localized both in the nucleus and in a punctate pattern associated with the plasma membrane. It is possible that when Ptr3p moves from cytoplasmic membrane to the nucleus in the course of amino acid sensing, the protein may be cleaved or covalently modified before acting as a shuttling protein. This might be the reason for why the GFP-Ptr3p signal did not show nuclear localization.

The identification of a zinc finger motif of C3HC4 type (RING finger) in Ptr3p around residues 52-61 further suggests its potential as part of a transcriptional complex. This RING finger motif occurs frequently at the DNA binding site of a transcription factor. Surprisingly only the C57A mutant had a reduced Ptr3p function, while single Ala substitution of the other two conserved cysteine residues C52 and C60 did not affect the ability of Ptr3p to complement a *ptr3*Δ phenotype (Table 5). The C52A;C60A double or C52A;C57A;C60A triple mutant had a reduced Ptr3p function similar as that of C57A single mutant. However, there was no decreasing Ptr3p function when single, double, or triple conserved cysteine residues were substituted by alanine, suggesting that these cysteine residues may not form a RING finger motif. The reduced function of cysteine mutants may indicate that they are involved in other structural function

such as disulfide bond formation. Ptr3p residues 1 to 250, which contain the predicted RING finger motif and were shown by preliminary two-hybrid analysis to interact weakly with the N-terminus of Ssy1p and strongly with Ssy5p (data not shown), partially complemented a *ptr3*Δ strain suggesting that residues 1 to 250 of Ptr3p are not enough to exert fully a functional Ptr3p phenotype.

The identification of a high probability (>90%) seven-bladed β-propeller fold within the C-terminal portion of Ptr3p suggested that this domain may be involved in protein-protein interactions. Shown by both response to toxic peptide and uptake of radiolabeled Leu-Leu (Fig. 3A and B), Ptr3p residues 250 to 678 which contain the predicted seven-bladded β-propeller motif complemented a *ptr3*Δ strain suggesting that these residues contain a domain(s) sufficient for Ptr3p function. Several β-propeller structures govern protein-protein interactions, e.g., lectins (Beisel *et al*., 1999) and the WD-40 domains in G-protein signaling molecules (Wall *et al*., 1998). Within the predicted β-propeller at the C-terminal portion of Ptr3p, a limited homology sequence with Gcn4p and the extracellular loop of some amino acid permeases was present (Klasson *et al*., 1999). These sequences are hypothesized to serve as an amino acid binding site (Klasson *et al*., 1999). Alanine substitution of each of the conserved FNNSP residues resulted in a Ptr3p with a phenotype demonstrating up-regulation of

peptide transport suggesting that these residues may regulate Ptr3p function negatively as an autoinhibitory domain. It may also indicate that Ptr3p's ability to interact with another protein is regulated by amino acid binding to these residues.

Further experiments will be needed to analyze protein-protein interactions among Ssy1p, Ptr3p, and Ssy5p and their post-translational modification or conformational changes in response to amino acid and peptide sensing**.** It will also be necessary to study which residues in Ptr3p are responsible for its putative proteolytic property on Ssy5p, and whether this enzymatic capability is the key for Ptr3p to propagate amino acid signal transduction.

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PART V

SUMMARY, DISCUSSION, AND

SUGGESTIONS FOR FUTURE EXPERIMENTS

CHAPTER I

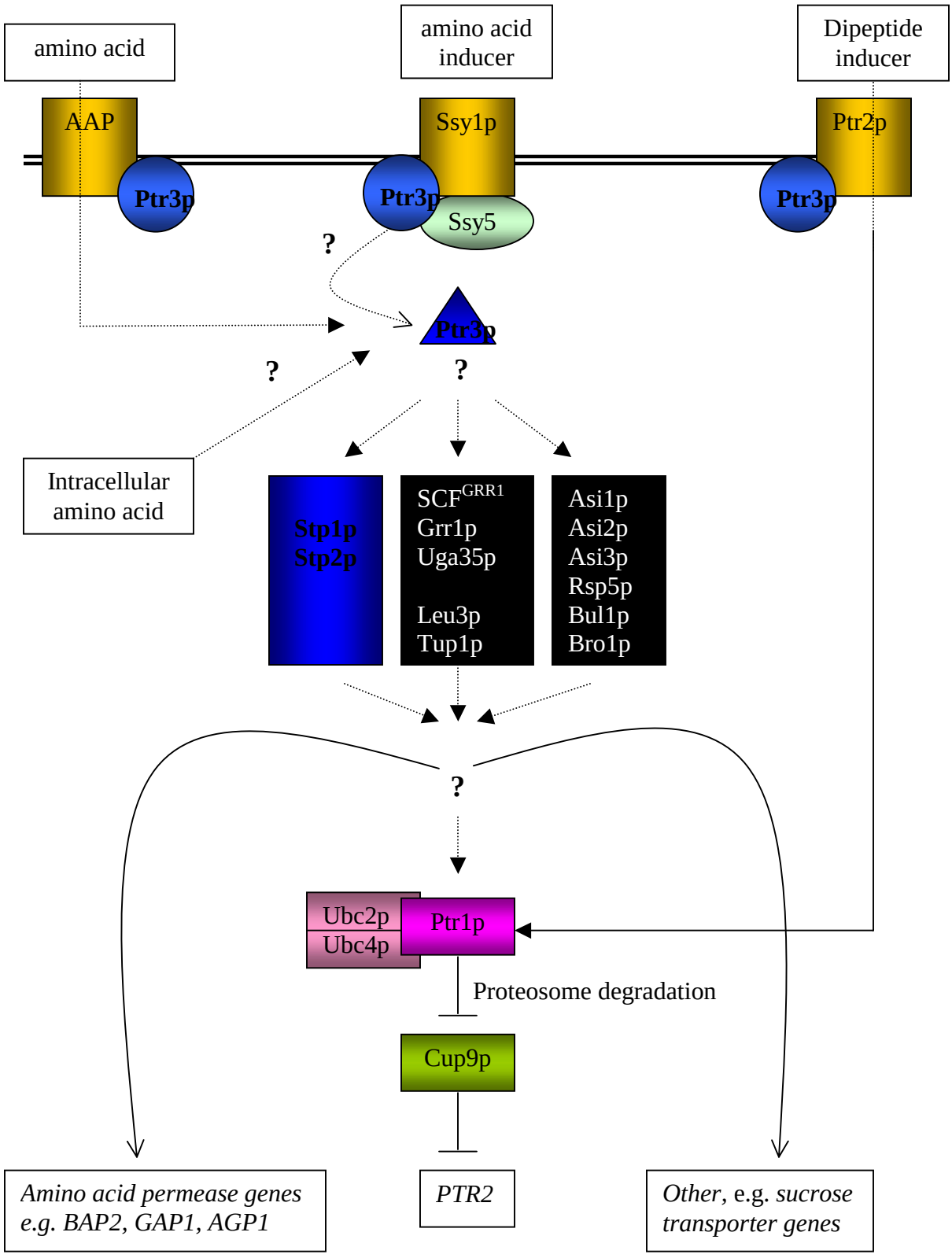
SUMMARY AND DISCUSSION

PTR3 modulates the amino acid and dipeptide induction of di/tri-peptide transport.

We have performed molecular, genetic, and structural analyses of Ptr3p, a novel protein involved in the regulation of the di/tri-peptide transport system in *Saccharomyces cerevisiae.* The new findings obtained from this study are used to develop further known and potential roles of Ptr3p, which are shown in Fig. 1 and summarized below.

Using a reporter gene assay to determine the activation of *PTR2* di/tri-peptide transporter gene, we found that Ptr3p mediated not only amino acid, but also dipeptide induction of *PTR2* expression. Ptr3p is likely to play a role in an early step of amino acid induction pathway because two-hybrid analysis and co-immunoprecipitation showed that Ptr3p interacted with the cytoplasmic, N-terminal 282 amino acid domain of the amino acid sensor Ssy1p suggesting that the Ssy1p/Ptr3p interaction was involved in generating signal after amino acid sensing (Chapter II). Interestingly, we found thatGFP-Ptr3p fusion protein was associated with the plasma membrane in a punctate pattern in the wild-type cell

Fig. 1. Known and potential roles of Ptr3p in amino acid and dipeptide mediated signal transduction. We found that Ptr3p is involved in amino acid sensing regulation by physically interacting with the N-terminal domain of amino acid sensor Ssy1p. Upon amino acid binding to Ssy1p, Ptr3p is hypothesized to dissociate from the sensor machinery Ssy1p-Ptr3p-Ssy5p and move to another subcellular compartment(s) (Klasson *et al.*, 1999). The nature of conformational changes or covalent modifications is currently unknown (two forms of Ptr3p are indicated by open circle and open triangle). We found that Ptr3p is involved in dipeptide induction of *PTR2* expression. Dipeptide is likely to provide positive feedback regulation by binding to allosteric sites of Ptr1p (Turner *et al.*, 2000). We discovered that *STP1* and *STP2* are involved in di/tripeptide transport, and Stp1p and Stp2p (in blue box) may act as candidates for subsequent Ptr3p-interacting proteins besides those listed in black boxes (Bernard and Andre, 2001b; Forsberg *et al.*, 2001; Nielsen *et al.*, 2001). Finally, we propose that intracellular amino acids (from intracellular pools or from amino acids transported into the cell) might provide another regulatory loop by binding to putative amino acid binding sites in Ptr3p, implying Ptr3p as part of intracellular amino acid sensing signaling perhaps through interactions with Ptr2p and amino acid permeases (AAP).


amino acid
amino acid inducer
Dipeptide inducer
AAP
Ssy1p
Ptr2p
Ptr3p
Ptr3p
Ptr3p
Ssy5
?
Ptr3p
?
?
Intracellular amino acid
Stp1p
Stp2p
SCF^GRR1
Grr1p
Uga35p
Leu3p
Tup1p
Asi1p
Asi2p
Asi3p
Rsp5p
Bul1p
Bro1p
?
Ubc2p
Ubc4p
Ptr1p
Proteosome degradation
Cup9p
Amino acid permease genes e.g. BAP2, GAP1, AGP1
PTR2
Other, e.g. sucrose transporter genes

and *ssy1*Δ mutant indicating that Ptr3p may have other functions besides working together with Ssy1p to generate intracellular amino acid sensing signals. Alternatively, the punctate localization may suggest a previously unknown sensory complex.

Insights into the function of Ptr3p should be further gained with information on the connection between *PTR3* and other genes known to modulate peptide transport. We identified *STP1* and *STP2*, genes previously hypothesized to encode pre-tRNA splicing proteins (Wang and Hopper, 1998) and bind to the *BAP2* promoter as transcription factors (Nielsen *et al.*, 2001), as new players in peptide transport regulation (Chapter III). *STP2* likely acts downstream of *PTR3* as shown by epistasis studies, but *PTR3* does not regulate the expression of *STP1* or *STP2*. In contrast to the *PTR2* expression pattern, in rich nitrogen source *STP1* and *STP2* are expressed at higher levels than those in a poor nitrogen source. The data provide the first evidence about the involvement of *STP1* and *STP2* in the di/tri-peptide transport system, suggesting their function as transcription factors of *PTR2* acting downstream of *PTR3*.

Ptr3p is expected to play roles in numerous pathways, because we identified by a two-hybrid screen Ptr3p-interacting proteins involved in transport including some transporters, transcription processes, and unknown functions (Chapter IV). Two different computational algorithms predicted that

Ptr3p contains two putative nuclear localization signals (NLS), and alanine substitution of one of these NLS decreased the Ptr3p function as a regulator of *PTR2*. PROSPECT, a 3-dimensional protein structure algorithm, also calculated that Ptr3p has a RING finger and a seven-bladed β-propeller motif. Alanine site-directed mutagenesis of some conserved residues in the ring finger motif reduced the function of Ptr3p. Mutations within the predicted β-propeller motif indicate that this motif may be important as an autoinhibitory domain of Ptr3p, because their substitution resulted in a more functional mutant than the wild-type Ptr3p. Finally, we identified functional domains of Ptr3p. The expression of Ptr3p residues 1-250 or 1-500 partially complemented *ptr3*Δ phenotype, and residues 250-678 were as functional as full length Ptr3p. These results add further to an understanding of the role of Ptr3p in the amino acid induced signal transduction pathway. However, the detailed mechanism of how Ptr3p participates in a signal transduction after extracellular amino acid sensing remains elusive.

Identification of hypothesized amino acid sensor machinery complex consisting of Ssy1p, Ptr3p, and Ssy5p.

Recent genetic and biochemical data indicate that Ptr3p together with the amino acid sensor Ssy1p and a newly discovered regulatory protein Ssy5p are

components of the SPS (Ssy1p-Ptr3p-Ssy5p) sensor at the plasma membrane. The presence of each protein is needed to form a functional sensor (Forsberg and Ljungdahl, 2001). Genetic analysis showed that deletion mutants of single or combination of *SSY1*, *PTR3*, and *SSY5* had identical phenotypes including inability to induce the transcription of amino acid permease genes and *PTR2* in cells grown in amino acid containing media. (Barnes *et al.*, 1998; Jorgensen *et al.*, 1998; Iraqui *et al.*, 1999; Klasson *et al.*, 1999; Forsberg and Ljungdahl, 2001). The N-terminal tail of Ssy1p (NSsy1p), which is expected to face the cytoplasm, is essential for sensor function. Some small, in-frame mutations within the N-terminal tail of Ssy1p abolished Ssy1p function (Klasson *et al.*, 1999). Overexpression of NSsy1p caused a dominant negative phenotype suggesting that the overproduced NSsy1p competes with the full-length of Ssy1p for binding of a necessary regulatory protein to transduce the amino acid sensing signals (Klasson *et al.*, 1999).

Biochemical analysis suggests that electrophoretic properties of Ssy1p, Ptr3p, and Ssy5p components depends on the presence of the other two components or amino acids in the growth media (Forsberg and Ljungdahl, 2001). Ssy1p migrates faster in the presence of amino acid-containing media, and both *PTR3* and *SSY5* are required for this electrophoretic phenotype. *A* slower migrating Ptr3p in amino acid containing media depends on *SSY1*, but not *SSY5*.

The nature of modification in these Ssy1p and Ptr3p species is not known. Interestingly, Ptr3p seems to have an enzymatic property since the proteolytic cleavage of Ssy5p's N-terminus is dependent on the presence of Ptrp3p. Two-hybrid analysis also showed that Ssy5p interacted with Ptr3p (Bernard and Andre, 2001a).

A striking similarity is observed between the amino acid and sugar sensing mechanism. *SNF3* and *RGT2* encode unique members of the hexose transporter (HXT) family that function as sugar sensor proteins and possess unusually long C-terminal domains. The cytoplasmically oriented C-terminal extensions of Snf3p and Rgt2p have important roles in signaling. Phenotypes associated with an *snf3* deletion can be partly suppressed when the C terminus of Snf3p is expressed as a soluble protein (Vagnoli *et al*., 1998), or as a hybrid with the Hxt2p hexose transporter (Ozcan *et al.*, 1998). Two homologous proteins, Std1p and Mth1p, which act as repressors of *SNF3* and *RGT2* target genes in the absence of signaling, physically interact with the C-terminal extensions of Snf3p and Rgt2p (Schmidt *et al*., 2000; Lafuente *et al*., 2000). Green fluorescent protein-Std1p fusion proteins are localized to the cell periphery and the cell nucleus; thus the possibility exists that Std1p mediates information directly from the cell surface to the nucleus (Lafuente *et al*., 2000). Similarly, Ptr3p may mediate amino acid sensing information directly from the cell environment through its

interaction with the N-terminal tail of Ssy1p to another subcellular compartment (Klasson *et. al.*, 1999).

In mammalian systems, there is no amino acid sensor protein yet identified. However, GLUT2, a human homolog of Snf3p, (Celenza *et al.* 1988), is the major facilitative glucose transporter isoform expressed in hepatocytes, pancreatic beta-cells, and absorptive epithelial cells, and has been suggested recently to transduce signals from the plasma membrane to the nucleus and to propagate glucose-regulated gene expression in human (Guillemain *et al.*, 2000). Unlike Snf3p or Rgt2p, GLUT2 protein is unique because besides its implied function as a glucose-sensor, it also functions as a glucose transporter. Thus, GLUT2 protein has a dual function. Furthermore, instead of an extension of the N-terminal tail like Ssy1p or the C-terminal like Snf3p and Rgt2p, the large cytoplasmic oriented hydrophilic loop between transmembrane VI and VII of GLUT2 is responsible for the glucose-induced gene expression after glucose sensing (Guillemain *et al.*, 2000). The loop is unique among mammalian glucose transporters for GLUT2, and overexpression of the loop resulted in a dominant negative effects on the glucose-mediated gene expression in wild-type cells. In addition, the loop rapidly moved from the cytoplasm to the nucleus in response to extracellular high glucose concentration and moved in the opposite direction when the cells were grown in low levels of glucose. The results indicate that this

loop competes to bind to protein(s) involved in regulation of gene expression after glucose sensing. Thus, the mechanism has some similarity with those proposed in yeast sugar or amino acid sensing.

The role of Ptr3p in amino acid and dipeptide-mediated signal transduction.

The fact that yeast amino acid sensors have only been recently discovered shows how little is understood regarding the molecular signals that enable yeast cells to adapt to changing environments. Another layer of regulation on amino acid and peptide transport was also added by dipeptide positive feedback regulation on peptide transport. *In vitro* binding assay indicated that dipeptides accelerated the di/tri-peptide uptake by allostericaly binding to Ptr1p/Ubr1p thereby activating ubiquitin-mediated degradation of the *PTR2* repressor Cup9p (Turner *et al.*, 2000). Ptr3p, previously known to regulate the amino acid induction of *PTR2* expression, was involved in dipeptide induced *PTR2* expression. *PTR3* deletion reduced induction by dipeptides, suggesting that Ptr3p is important for optimal dipeptide induction. Therefore, Ptr3p may function as a shared regulatory protein between the dipeptide and amino acid induction pathways, and a complex network of regulatory processes under these two induction systems should exist in a co-ordinated way.

Ptr3p was previously shown to localize at the plasma membrane by gradient centrifugation and GFP-fusion localization (Klasson *et al.*, 1999; Chapter II). Studies are underway to test a possibility that Ptr3p may dissociate from the SPS sensor and localize in some other compartment(s) after amino acid sensing. The reduced activity in peptide transport of a Ptr3p mutant at its predicted nuclear localization signal RKK residues suggests that Ptr3p may move from the plasma membrane to the nucleus to function. As discussed above, a shuttling mechanism is possessed by regulatory protein Std1p, which interacts with the C-terminal domain of the glucose sensors, Snf3p and Rgt2p and needs to move from the plasma membrane to the nucleus to transduce the sugar sensing signals (Schmidt *et al.*, 1999). Information on this shuttling mechanism can be gained by testing whether the predicted NLS RKK is actually a true NLS. GFP-fusion with Ptr3p containing the RKK alanine mutation did not affect its localization (data not shown), but this observation does not rule out RKK as a signal. The resolution of the GFP-Ptr3p fusion protein carried out by standard fluorescent microscopy may not be fine enough to determine subcellular movement in intracellular localization. Some experiments should include localization of GFP containing the predicted NLS RKK residues. If these RKK residues really confer a signal for nuclear localization, GFP containing the RKK amino acids might localize in the nucleus in contrast to the cytoplasmic localization of GFP alone.

Previously, in contrast to localization of GFP-Std1p at both plasma membrane and nucleus, N-terminal fusion of GFP with Ptr3p localized as a punctate pattern associated with the plasma membrane only. Nevertheless, experiments showing that Ptr3p may dissociate from the cytoplasmic membrane (Klasson *et al.*, 1999) and may move to other subcellular compartment(s) as truncated Ptr3p (Chapter 4; residues 250-678 complemented a *ptr3*Δ strain) may indicate that part of Ptr3p may migrate into the nucleus during signal transduction. Such movement of a portion of Ptr3p would not be detected by the analysis of the GFP-Ptr3p construct performed in this dissertation. Our data suggest that Ptr3p residues 250-678, which contain the predicted NLS RKK residues, retained properties allowing peptide transport activity similar to that of the wild-type Ptr3p. Therefore, GFP-fluorescence and biochemical gradient-centrifugation localization analysis of Ptr3p residues 250-678 might address the possibility that during signal transduction, the full length of Ptr3p is necessary to form the SPS sensor, but after amino acid sensing and perhaps some post-posttranslational modifications, a certain domain of Ptr3p may move to another subcellular compartment such as the nucleus.

The complexity of regulation is magnified by the fact that mutations in Ptr3p have a pleiotropic phenotype indicating that this novel protein is involved in several pathways. If Ptr3p moves to the nucleus to function or stays at the

plasma membrane to transduce the signals, what are the downstream targets of the SPS sensor? Specifically, what are the candidates of Ptr3p interacting proteins, which subsequently send signals to further downstream effectors? From epistatic analysis, genes involved in di/tri-peptide transport such as *CUP9* which encodes a repressor of *PTR2* and *STP2* which encodes previously hypothesized pre-tRNA splicing proteins (Wang and Hopper, 1988) and is a transcription factor that binds to the *BAP2* promoter (Nielsen *et al.*, 2001) have been shown to act downstream of *PTR3.* Thus, Cup9p or Stp2p are candidates for downstream Ptr3p interacting proteins within the amino acid induction pathway.

However, recent discoveries have found additional proteins from several different pathways involved in induced transcription of amino acid and peptide permeases. The list of Ptr3p interacting protein should include all of these proteins (Fig. 1). Proteins involved in ubiquitin pathways such as the SCF^{GRR1} ubiquitin-ligase complex (Grr1p, an F-box protein, and Uga35p, a transcription factor of Cys_6-Zn_2 family) may interact with Ptr3p during amino acid sensing pathway mediated by the SPS sensor because the SCF^{GRR1} ubiquitin-ligase complex is essential for transcription of genes under Ssy1p control (Iraqui *et al.*, 1999a; Bernard and Andre, 2001b). Ptr3p localized independently from Ssy1p indicating functions of Ptr3p other than a component of the SPS sensor. Proteins

involved in the functional expression of amino acid permeases under an indirect control of Ssy1p (Forsberg *et al*., 2001) may also interact with Ptr3p. They include Asi1p, Asi2p, and Asi3p, which together form a novel membrane-bound ubiquitin ligase system, and Rsp5p, Bul1p, and Bro1p affect amino acid permease traffic in the late endosomal vacuolar protein sorting pathways. Furthermore, because *PTR3* has also been shown to regulate the expression of amino acid permease genes, other proteins that regulate the expression of amino acid permeases genes may serve as Ptr3p interacting proteins. These include regulatory proteins of *BAP2* expression such as Leu3p, Tup1p, Stp1p, and Stp2p transcription factors (Nielsen *et al.*, 2001).

Obviously, to determine which proteins interact with Ptr3p requires many experiments in addition to a two-hybrid screen, whose limitation includes a one-to-one protein-protein interaction assay rather than a protein complex interaction. A pull-down experiment can be performed to discover members of putative Ptr3p-interacting complexes. Further biochemical and biophysical analysis to show post-translational modification(s) important for specific interaction after or before amino acid sensing could be worthwhile to pursue. The elucidation of the protein-protein interaction and the post-translational modification within the SPS sensor and the subsequent downstream targets should provide valuable information on how amino acid-induced signal

transduction occurs. Sets of experiments need to be designed carefully to capture the types of modification and interaction because the amino acid sensing appears to be a dynamic process. Three sensor states were discovered based on the rapid physical alterations and reduced levels of sensor components in response to amino acid availability (Forsberg and Ljungdahl, 2001a). The state I sensor is the complex present in cells grown in the absence of amino acids, where each of the SPS component is present at high level. State II is a series of transient complexes that are rapidly formed when cells grown in the absence of amino acids are induced by amino acids, and state III corresponds to a down-regulated complex, which is indistinguishable from those found in the complex of cells grown in media containing amino acids.

Discoveries of functional domains and motifs in Ptr3p.

The involvement of a *PTR3*-downstream gene encoding Stp2p, a previously hypothesized pre-tRNA splicing proteins (Wang and Hopper, 1988), in di/tri-peptide transport may add an unusual regulation on *PTR2* expression. Linking the roles of Stp1p and Stp2p as hypothetical pre-tRNA splicing proteins with their role as transcription factors should be important for dissecting regulatory pathways of di/tri-peptide and amino acid transport. In *E. coli* a repression on *opp* and *dpp* transport system by *gcvB* encoding a small-

untranslated RNA was recently identified (Urbanowski *et al.*, 2000) providing a putative similar regulatory protein in a prokaryote.

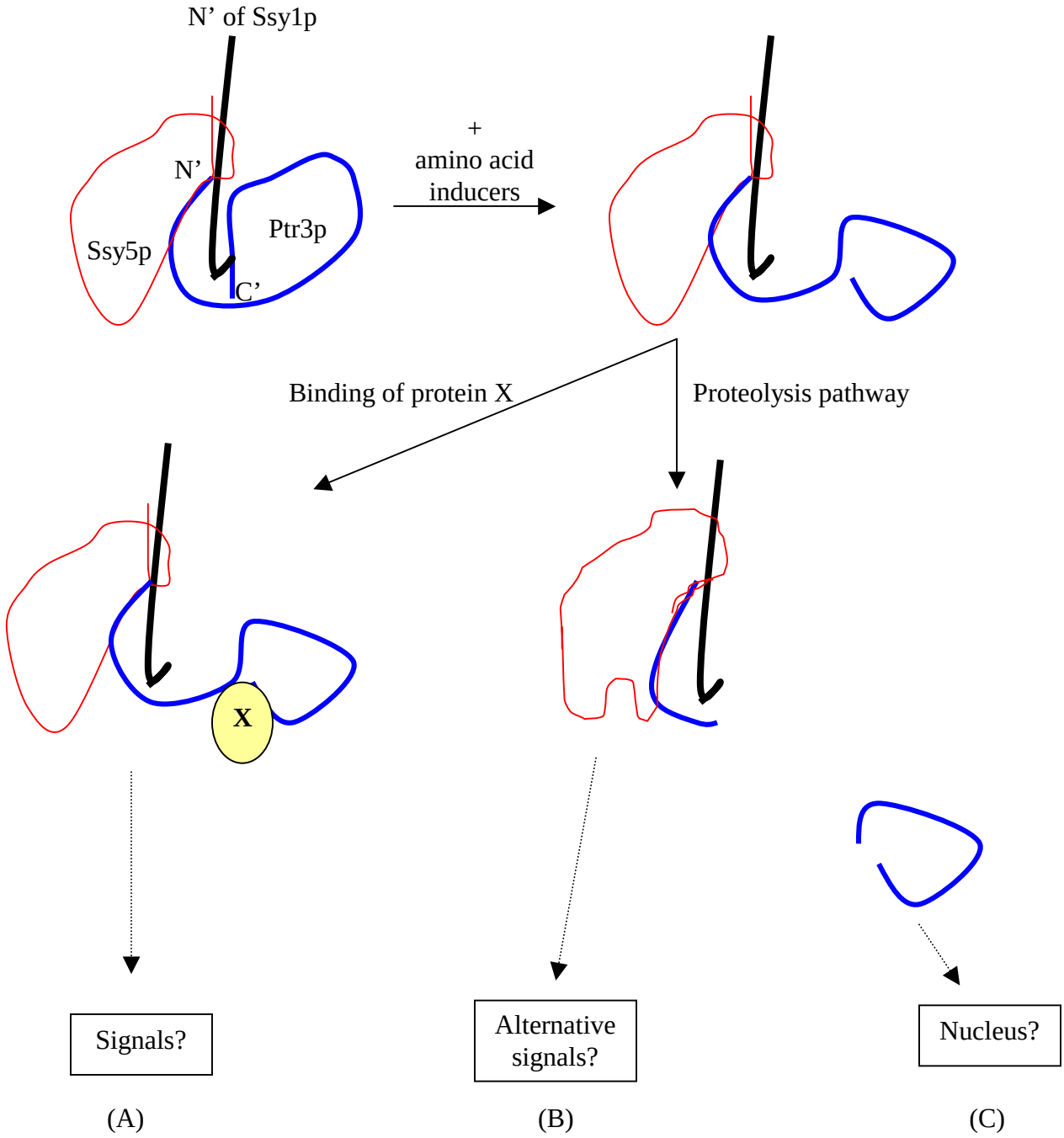
The ability of Ptr3p to interact with the cytoplasmic portion of other membrane proteins localized at the plasma membrane such as the C-terminal domain of Thi7p and the internal domain of YGR125Wp shown in two-hybrid assay raises a possibility that the ability of Ptr3p to interact with permeases may be part of its function in the amino acid sensing feedback mechanism. Ptr3p was also hypothesized to bind amino acids (Klasson *et al.*, 1999), and alanine mutation of these proposed amino acid binding residues led to an increased peptide transport activity compared to that of the wild-type cells (Chapter 4). Therefore, Ptr3p may also function as a component of an intracellular amino acid sensing mechanism, and this mechanism may be the type of regulation that connects amino acid and dipeptide induction of peptide transport in yeast cells.

If Ptr3p plays roles in different pathways, how does it function? The predicted ring finger motif within residues 1-250 and seven-bladed β-propeller motif within residues 250-678 indicate that different regions of Ptr3p exert specific functions. Interestingly, the leaky phenotype of *ptr3* null mutant or Ptr3p residues 1-250 suggests that alternative(s) pathways should exist. *SSY5* may provide a different amino acid induction pathway from that mediated by *PTR3* *b*ecause overexpression of *SSY5* can complement the *ptr3*Δ phenotype (Forsberg

and Ljungdahl, 2001). It is necessary to perform complementary analyses such as two-hybrid and co-immunoprecipitation is needed to show which regions of Ptr3p interact with Ssy1p and Ssy5p. Biochemical analysis to show the domain(s) responsible for the Ptr3p-dependent proteolytic products of Ssy5p should also be performed. Additionally, as discussed above localization study on each domain is needed to test the hypothesis of Ptr3p's movement from the plasma membrane to another subcellular compartment.

To study thoroughly the structural properties of Ptr3p a solved crystal structure of Ptr3p is needed. This may require the crystallization of each predicted domain independently. The circular dichroism analysis of the purified, predicted seven bladed β-propeller domain of Ptr3p may also give insight whether β-sheets, the builders of β-propeller fold, are present. Another demanding task will be providing answers on how signal transductions occur after the cells sense amino acid or dipeptide. For example, after amino acid binding to Ssy1p, what are the subsequent conformational changes or post-translational modification of Ptr3p that result in the activation of gene expression? Fig. 2 shows a proposed model of signal transduction pathways after amino acid sensing. Since Ptr3p may bind to amino acid, and dipeptide actually binds to Ubr1p / Ptr1p, regulation between extracelullar versus intracellular amino acid or dipeptide sensing should also exist. This type of regulation might

Fig. 2. A proposed model of Ptr3p-mediated signal transduction after amino acid sensing. In the absence of amino acid inducers, the C-terminal portion of Ptr3p autoinhibits its own function. Amino acid inducer binding to Ssy1p relieves the Ptr3p autoinhibition, and the C-terminal portion of Ptr3p, which is predicted to have a β-propeller structure, is exposed. One pathway may involve a subsequent binding of protein X to Ptr3p (A). Alternatively, Ptr3p may be proteolytically process and the C-terminal portion moves to another subcellular compartment such as nucleus (C). Ssy5p may be also enzymatically processed by Ptr3p in this pathway and generates alternative signals (B).


N' of Ssy1p
N'
Ssy5p
Ptr3p
C'
+
amino acid
inducers
Binding of protein X
Proteolysis pathway
X
Signals?
Alternative
signals?
Nucleus?
(A)
(B)
(C)

also inter-connect with the ubiquitination system. Previously, SCF^{GRR1} ubiquitin-ligase complex (Iraqui *et al.*, 1999a; Bernard and Andre, 2001b), a membrane-bound ubiquitin ligase system Asi1p, Asi2p, Asi3p, and a ubiquitin ligase system Rsp5p, Bul1p, Bro1p (Nielsen *et al.*, 2001) were shown to be involved in di/tri-peptide or amino acid transport. Regulation among these systems and how a novel protein such as Ptr3p may be involved in a wide variety of metabolic processes will be essential for understanding how the cells respond to nutrient availability.

Finally, with the explosion of genome sequencing, it is important to perform comprehensive searches of the SPS homologs in different organisms. Ssy1p, Ptr3p, and Ssy5p homologs have been recently identified in other fungal species (Narita and Becker, unpublished observation), and it is likely that the other two components of the SPS sensor have homologs in these newly sequenced fungal organisms. Further regulatory networks could be obtained by application of techniques utilizing genome-wide scale analysis such as microarray. Microarray analysis showed that Ssy1p regulated transcription of many non-amino acid or peptide permease genes (Forsberg *et al.*, 2001b). Furthermore, our microarray analysis also showed that Ptr3p was involved in various pathways including other nutrient transport such as sugar and sulfate uptake systems (Goldstein *et al.*, manuscript in preparation). Comprehensive

genome-wide two-hybrid analysis that can detect protein complex interaction will accelerate the completion of amino acid signal transduction pathways maps.

CHAPTER II

SUGGESTIONS FOR FUTURE EXPERIMENTS

Our recent work has provided a framework of amino acid and dipeptide-mediated signal transduction. Ptr3p seems to have several roles that are worthwhile to test within the pathways proposed. The first hypothesis to be tested should be that Ptr3p dissociates from SPS and moves to the nucleus after amino acid sensing. I suggest the following experiments to test this hypothesis:

1. The RKK NLS in Ptr3p, whose mutant was shown to be reduced in transport activity, needs to be tested. GFP containing a portion of Ptr3p with this RKK NLS could be designed and tested for localization. If this GFP mutant localizes to the nucleus, it indicates that the RKK NLS is sufficient to provide nuclear localization signal.
2. GFP containing both RKK and KKKKK NLS could be tested also for its localization to test the possibility of a bipartite NLS existence in Ptr3p.
3. Previously, the N-terminal GFP fusion to Ptr3p was shown to localize as a punctate pattern around the plasma membrane and was not detectable in the nucleus. Furthermore, fragmentation studies showed that Ptr3p residues 250-678 were functional similar to that of the wild-type suggesting that the

absence of nuclear localization may be due to a cleavage of Ptr3p. Thus, instead of the full length of Ptr3p, only residues 250-678 may move to the nucleus. One experiment to test this possibility is creating a GFP fusion to Ptr3p residues 1-250 and residues 250-678 with the expectation that residues 1-250 should still show a punctate pattern of localization, while residues 25-678 should show a nuclear localization. All the GFP fluorescence analysis should be done in several conditions such as in the presence or absence of amino acid or dipeptide inducer and using either good or poor nitrogen sources.

4. Complementation of the GFP analysis should be performed by a biochemical analysis such as a gradient centrifugation of protein extracts grown in the presence or absence of amino acid inducers to determine the subcellular localization of Ptr3p.

Related to the first set of studies above, the second set of experiments involves protein-protein interactions analyses. All precautions should be taken carefully to make sure that each experiment is done in the right condition because amino acid sensing transduction is likely to be a dynamic process. Thus, the hardest part seems to be determining the correct condition for growing the cells and extracting the proteins in order to obtain physiological Ptr3p conformation(s). The following are the suggested experiments:

1. Two-hybrid analysis on protein-protein interaction among Ssy1p, Ptr3p, and Ssy5p should be performed. Preliminary two hybrid studies showed that Ptr3p residues 1-250 interacted weakly with Ssy1p and strongly with Ssy5p, while residues 250-678 did not seem to interact with either Ssy1p or Ssy5p. Similar tests therefore need to be done to determine which Ptr3p residues interact with Ssy1p and Ssy5p.
2. Two-hybrid analysis should also be done for testing the interacting of Ptr3p with different portions of amino acid permeases and Ptr2p.
3. Co-immunoprecipitation analysis to confirm the two-hybrid results should be performed.
4. A Ptr3p pull-down assay of cells extracts grown in inducing or non-inducing condition could be done to complement previous two hybrid screen. The pull down assay experiment could be done also with different fragments of Ptr3p.
5. Biochemical and physical analysis such as mass spectrophotometry of the different fragments and full length of Ptr3p in different inducing condition is necessary to study post-translational modification(s) that are responsible for protein-protein interaction. Additionally, once the direct downstream Ptr3p interacting protein identified, experiments above should be performed to test interacting Ptr3p residues and their covalent modification(s).

6. Ptr3p interacting proteins may also be discovered by a genetic analysis, which after obtaining a constitutively active Ptr3p by a random mutagenesis, a repression of its activity by overexpression or deletion of certain genes indicate that the products of these genes may interact physically or genetically with Ptr3p. Pools of yeast cells containing deletion of every gene in the genome have been constructed and could be purchased from Research Genetics (Birmingham, AL).

The third set of experiments should address the structural analysis of Ptr3p. They are:

1. Circular dichroism and crystallization of residues 250-678 should be performed to test the predicted β-propeller structures.
2. The cross-linking experiment using an amino acid analog along with mass spectrophometry should be done to analyze amino acid binding to Ptr3p.
3. The proposed autoinhibitory domain within residues 250-678 may be also analyzed by placing a fluorescent chemical -reagent within these residues, and then quenching fluorescence experiment could be performed. The results should tell us whether this domain is buried or exposed during signal transduction.

Finally, because Ssy1p, Ptr3p, and Ssy5p homologs have been recently identified in other fungal species such as *Candida albicans* (Narita and Becker,

unpublished observation) and because the SPS-system affects filamentous growth of haploids (Klasson *et al.*, 1999), it will be valuable to test whether these components have a function during *C. albicans* invasion in pathogenesis. To answer this hypothesis, I suggest the following experiments:

1. Deletion of *SSY1*, *PTR3*, and *SSY5* homologs in the fungal pathogen *C. albicans* and then determining the pathogenicity of the mutated strain *C. albicans* should be performed.
2. Comparison of microarray analysis of *C. albicans* gene expressions between the wild-type and the deletion SPS strain may also give information on virulent phenotype.
3. Similar techniques could be performed to test the effect of SPS in other pathogenic fungus.

In summary, results of these experiments could provide valuable information on Ptr3p subsequent interaction, covalent modification, and conformation after sensing, and whether Ptr3p regulation should involve sensing intracellular amino acids by binding to these amino acids. Furthermore, because Ptr3p may also be involved in various pathways including other nutrient transport such as sugar and sulfate uptake systems (Goldstein *et al.*, manuscript in preparation), deletion of *PTR3* in this pathogenic fungal could delay the invasion process by limiting the nutrient availability. Identification of other

regulatory proteins in *S. cerevisiae*, C. *albicans*, and other fungal species should accelerate the elucidation of amino acid and dipeptide mediated signal transduction in either model system.

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