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Studies of the Di/tripeptide Transporter in *Saccharomyces cerevisiae*: The N-terminal Cytoplasmic Domain of Ptr2p is Involved in Post-Translational Regulation

Steven Clinton Minkin Jr.
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Steven Clinton Minkin Jr. entitled "Studies of the Di/tripeptide Transporter in *Saccharomyces cerevisiae*: The N-terminal Cytoplasmic Domain of Ptr2p is Involved in Post-Translational Regulation." 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 Doctor of Philosophy, with a major in Microbiology.

Jeffrey M. Becker, Major Professor

We have read this dissertation and recommend its acceptance:

Daniel Roberts, Timothy Sparer, Barry Bruce, Steven Wilhelm

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Steven Clinton Minkin Jr.

August, 2008

Dedication

This work is dedicated to my family. To my parents, Steven (Sr.) and Melissa Minkin, and my sister Molly. To my grandparents, Papa Doc and Grandma Minkin, Daddy Doug and Mama Faye Coulter, and Mamaw and Papaw Elliott. To my aunts, uncles, and cousins. Throughout my life, my family has been my source of strength. Their constant encouragement and support made this work possible. Words could never adequately express my gratitude. A special dedication goes to my father, Steven (Sr.), who as a renowned geologist endowed me with a love of science at an early age. He looked on with pride as I pursued my Ph.D., but unfortunately he passed away before I reached the finish line.

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great friends throughout the university. A very wise man once said, ”It takes a village to make a community.” Truer words have never been spoken.

Abstract

Throughout nature cells use peptides as a source of nutrition. For microbes, an ability to utilize peptides is especially important in nitrogen-poor environments, as peptides can be catabolized for their use as a nitrogen source. The yeast *Saccharomyces cerevisiae* imports di/tripeptides from the environment using the peptide transporter Ptr2p. Cellular levels of Ptr2p are highest under poor-nitrogen conditions. Here we report that the addition of a rich nitrogen source to the growth medium results in a down-regulation of Ptr2p, wherein plasma membrane Ptr2p is ubiquitinated, endocytosed, and delivered to the vacuole for destruction. We report evidence that the N-terminal portion of Ptr2p that is cytoplasmic is involved in mediating this effect. Mutations to known phosphorylation sites (Y37, S39, and S45) and suspected ubiquitination sites (K27 and K34) on Ptr2p's N-terminal region greatly impair both the normal turnover of Ptr2p and its down-regulation in response to a rich nitrogen source. The data presented support the notion that Ptr2p turnover from the cytoplasmic membrane requires phosphorylation and ubiquitination of a discrete N-terminal cytoplasmic domain.

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Chapter I:

Background and Introduction

I. A.

Discovery of Peptide Transport

Some of the first evidence hinting at the existence of a peptide transport system came from studies of the mammalian gut in the early twentieth century, when scientists observed that peptides were cleared from the intestinal lumen more rapidly than equivalent amounts of the corresponding free amino acids (Nolf 1907; Messerli 1913). This suggested that dietary protein was absorbed as peptides. However, despite these early observations, the prevailing view up until the 1950's was that peptides were hydrolyzed into free amino acids by extracellular peptidases before absorption at the brush border membrane, much like oligosaccharides are broken down into monosaccharides before absorption.

Strong evidence suggesting the existence of a peptide transport system came almost accidentally in 1949, when Simmonds and Fruton described a "microorganism exhibiting a growth requirement for peptides"(Simmonds and Fruton 1949). When a non-sterile saline solution containing the dipeptide L-leucylglycine became turbid after several weeks unattended at the lab bench, Simmonds and Fruton decided to investigate. When an inoculum from this turbid solution was streaked for isolation, only one colony

type grew. Microscopic and biochemical tests placed this isolate within the bacterial genus *Alcaligenes*, and for convenience Simmonds and Fruton named the isolate "SF". Interestingly, subsequent tests revealed that SF required the dipeptide L-leucylglycine for growth, and could not grow on a mixture of free L-leucine and glycine. They didn't know why SF required peptide for growth, but offered several hypotheses: perhaps SF converted the free amino acids into compounds that didn't support growth, maybe the intact peptides were incorporated directly into bacterial proteins, or perhaps amino acids in the peptide were more efficiently incorporated into bacterial proteins by an unknown "transpeptidation" reaction. An ability to import peptides, coupled with an inability to import the particular amino acids, was not mentioned as a possible explanation.

By the 1950's, several studies documented the fact that peptides supported the growth of many bacterial species better than the mix of corresponding amino acids (Gale 1945; Kihara and Snell 1952; Peters, Prescott et al. 1953; Meinhart and Simmonds 1955; Miller, Neidle et al. 1955; Shelton and Nutter 1964), but the mechanism behind this phenomenon was still unknown. Advances in understanding came with the development of direct techniques measuring peptide absorption, and indirect techniques measuring peptide utilization. The use of radioisotopes allowed researchers to follow radiolabeled peptides in the course of an experiment. Alternatively, peptide utilization could be investigated indirectly by observing specific biological responses to peptides (using peptides to satisfy amino acid auxotrophies, or using toxic peptides).

The earliest direct evidence of peptide transport was provided by David Kessel in 1963, in a work entitled, *On the distinction between peptidase activity and peptide transport*. (Kessel and Lubin 1963). Kessel was studying mutants of a glycine-requiring

strain of *Escherichia coli* (*E. coli* $W8_{33}$) and was able to demonstrate that peptide transport and peptidase activities were "separable functions of a bacterial cell". At the time, it was known that cells could use peptides to satisfy amino acid auxotrophies, but it was unclear if peptides were transported intact across the cell membrane, or if peptides were simply hydrolyzed outside of the cell, releasing the amino acids for cellular uptake. Kessel documented a peptidase mutant of *E. coli* which was unable to grow with glycylglycine as the sole source of glycine, yet this mutant was still able to concentrate glycylglycine inside the cell to a hundred times the concentration in the medium. Competition studies with different peptides showed that this transport system had surprisingly broad substrate specificity. This work clearly demonstrated the existence of a peptide transport system in *E. coli*, and provided an explanation for the special growth-supporting properties of peptides observed in previous studies (e.g. Simmonds and Fruton 1949).

From these early observations, many more studies quickly followed, searching for, and characterizing, peptide transport systems in other bacteria, including *Streptococcus faecium*, *Leuconostoc mesenteroides*, *Bacteroides ruminicola*, *Lactobacillus casei*, *Fusiform necrophorus*, *Mycoplasma laidlawii*, and *Clostridium perfringens* (Leach and Snell 1960; Brock and Wooley 1964; Hauschild 1965; Yoder, Beamer et al. 1965; Pittman, Lakshmanan et al. 1967; Pecht, Giberman et al. 1972; Wahren and Holme 1973). The observations in bacteria clearly distinguished peptide transport from amino acid transport, and they highlighted the importance of peptide transport in nature. Studies with *B. rumincola* and *M. laidlawii* showed that these organisms could absorb certain amino acids only if they were administered in peptide form (Pittman, Lakshmanan et al.

1967; Pecht, Giberman et al. 1972). Surprisingly, *F. necrophorus* is completely dependent upon peptide transport for proline acquisition, as this organism can neither synthesize proline nor import free proline from the environment (Wahren and Holme 1973).

Many of these studies in bacteria examined the substrate specificity/recognition of these peptide transport systems. Similar to transport systems for other nutrients (amino acids, sugars, metal ions, and other small molecules), they found that peptides were transported across the membrane by a system which had the hallmarks of a carrier-mediated system (structural specificity, competition for entry among substrates, saturation kinetics, and capacity for loss of function through genetic manipulation) (Cohen and Rickenberg 1956; Leach and Snell 1959; Payne 1971). Also, studies in *E. coli* pointed toward the existence of separate transport systems for dipeptides and oligopeptides, and differing structural requirements for transport (number of amino acids, presence of α-amine or C-terminal carboxy group, and stereospecificity) (Payne and Gilvarg 1968; Gilvarg 1971).

At the same time (1960's and 70's), peptide transport was being investigated in eukaryotic systems. Peptide transport in the bakers yeast, *Saccharomyces cerevisiae*, was demonstrated in 1973. At the time, *E. coli* was the dominant model system for study of peptide transport, and Becker et al showed that the transport system in *S. cerevisiae* was different than that of *E. coli*, as it displayed different substrate specificity (Becker, Naider et al. 1973). (Unlike *E. coli*, the *S. cerevisiae* strain was unable to transport a series of lysyl homopeptides or Gly-Met-Gly.) Shortly afterwards, peptide transport was

demonstrated in other fungi including *Neurospora crassa* and *Candida albicans,* (Wolfinbarger and Marzluf 1975; Lichliter, Naider et al. 1976).

Also, around this time the idea of intestinal absorption of peptides was resurrected. Until the 1950's, it was commonly assumed that dietary protein was broken down completely into free amino acids before absorption. This idea was reinforced by the observation of protease and peptidase activity in the luminal fluid of the small intestine, and by the demonstration of active transport mechanisms for amino acids in the small intestine (Gibson and Wiseman 1951). This made it unnecessary to postulate the existence of a peptide transport system, but several observations challenged this notion. It was consistently observed that amino acids were absorbed faster when in peptide form than in free form (Nolf 1907; Messerli 1913; Craft, Geddes et al. 1968; Matthews, Craft et al. 1968; Lane, Silk et al. 1975). Also, intestinal-uptake competition studies provided compelling evidence for a peptide transport system; amino acids competed with each other for absorption, as did peptides, but peptides and amino acids did not compete with each other for absorption (Cheng, Navab et al. 1971; Rubino, Field et al. 1971).

Decisive proof of intestinal peptide transport came from studies of Hartnup disease and cystinuria (Asatoor, Cheng et al. 1970; Hellier, Perrett et al. 1970). Hartnup disease impairs the uptake of neutral amino acids. Free L-tryptophan and free L-leucine are poorly absorbed by Hartnup patients, but these amino acids are rapidly absorbed when administered as dipeptides, thus indicating that there must be a mechanism for the translocation of dipeptides across the gut. Similarly, cystinuric patients have impaired uptake of cysteine and basic amino acids, but these amino acids are readily absorbed in dipeptide form, thus reinforcing the idea of a peptide transport system.

In the late 1970's, peptide transport was demonstrated in the scutellum of germinating barley (Sopanen, Burston et al. 1977). The scutellum is the absorptive organ that transfers nutrients from the endosperm (food store) to the seedling. Sopanen et al hypothesized that the scutellum might possess peptide transport activity, because earlier research suggested that complete hydrolysis of stored protein to free amino acid was unlikely to occur in the endosperm (the only peptidases found in the endosperm did not act upon di- and tripeptides). Experiments demonstrated that the scutellum did indeed transport small peptides, and it was the first demonstration of peptide transport in plants.

In the 1980's, the first peptide transporter genes were identified and cloned from *E. coli* and *S. typhimurium*, (Hogarth and Higgins 1983; Andrews and Short 1985; Hiles, Powell et al. 1987) and in the 1990's the first eukaryotic peptide transporter gene was cloned from *S. cerevisiae* (Perry, Basrai et al. 1994). Since then, peptide transport genes have been identified in virtually every organism investigated, from archea to bacteria to man. Advances in biological science have allowed in-depth studies into the structure and function of peptide transporters. Peptide transport is now recognized as an important phenomenon found throughout nature (Daniel, Spanier et al. 2006).

This dissertation focuses on a particular di- and tripeptide transporter, Ptr2p, from *S. cerevisiae*. Therefore, this review will focus primarily on related peptide transporters from the PTR (Peptide TRansport) family, also known as the POT (Proton Oligopeptide Transport) family. In depth discussion, including recent research, will be covered in the "PTR Family" section.

I. B.

Significance of Peptide Transport

Peptide transport is a general phenomenon that has been widely documented in archea, bacteria, fungi, plants, and animals (Payne and Smith 1994; Stacey, Koh et al. 2002; Daniel, Spanier et al. 2006). Virtually every organism examined possesses one or more peptide transporters. It is not surprising considering a selection pressure to import and utilize peptides, an abundant and ubiquitous source of amino acids, has existed since the earliest days of cellular life. This widespread distribution of peptide transporters underscores both the importance and evolutionary antiquity of peptide transport in nature.

Several families of peptide transporters exist (see "Types of Peptide Transporters" section) which are structurally unrelated and have separate evolutionary origins. This type of convergent evolution, in which different proteins evolve the ability to perform a similar function, also highlights the importance of peptide transport in nature.

Throughout the living world, peptide transport is an important mechanism for nutrient absorption, and this helps explain why peptide transporters are so abundant. However, peptide transport plays a role in many other important activities in nature, besides nutrient uptake.

In the microbial world, peptide transport is involved in many diverse processes including nutrient absorption, cell wall recycling, cell to cell communication, and toxin sensitivity and resistance. Microbial use of peptide transporters for nutrient uptake is very widespread. Peptides, generated by proteolysis, are abundant and ubiquitous in

nature. In order to utilize peptides, an organism must possess peptidases (intracellular and/or extracellular) to break down peptides into free amino acids. For microorganisms that do not secrete extracellular peptidases, peptide utilization requires peptide transport into the cytoplasm. Upon entry into the cytoplasm, peptides are rapidly degraded into free amino acids by intracellular peptidases.

In addition to being an essential mechanism of peptide utilization for organisms that lack extracellular peptidases, peptide transport is also a particularly efficient means of amino acid absorption (Daniel 2004). Numerous studies have documented the fact that peptides support the growth of many microbial species better (smaller generation time) than the mix of corresponding amino acids (Gale 1945; Kihara and Snell 1952; Peters, Prescott et al. 1953; Meinhart and Simmonds 1955; Miller, Neidle et al. 1955; Shelton and Nutter 1964), establishing peptide transport as an important mechanism of nutrient absorption.

For microbes, the nutritional importance of peptide transport is particularly underscored by the existence, and sensitivity, of peptide-specific chemotaxis systems (Manson, Blank et al. 1986; Abouhamad, Manson et al. 1991). The dipeptide-specific chemotaxis system of *E. coli* is more sensitive than the chemotaxis systems for amino acids. There is always a stronger chemotactic response towards dipeptides than to equimolar amounts of the corresponding free amino acids, and the system responds efficiently to peptides composed entirely of amino acids that cannot serve as attractants for *E. coli* (such as valine, leucine, isoleucine, phenylalanine, and proline). Interestingly, the chemoreceptor for this chemotaxis system is a periplasmic component of the dipeptide permease itself (Dpp).

In addition to the scavenging of exogenous peptides, peptide transporters also allow microbes to reabsorb self-peptides. An example of this in bacteria is the recycling of cell wall material (Goodell and Higgins 1987). To accommodate cellular expansion during growth, the rigid cell wall (peptidoglycan) must be continually broken down and reassembled. The break-down process releases unusual peptide components of the cell wall. (These peptides are different from protein-derived peptides, in that they contain D-amino acids and a γ-peptide bond.) Peptide transporters allow the cell to absorb these released peptides, so they can be reused in the creation of new cell wall material.

In microbes, peptide transport is also involved in cell-to-cell communication. Quorum sensing systems in Gram-positive bacteria use peptide signaling molecules (referred to as autoinducing peptides, or AIPs), which require peptide transporters for cellular import and/or export (Reading and Sperandio 2006). Similarly, many mating systems in the microbial world (and elsewhere, from mollusks and arthropods, to amphibians and mammals) use peptide pheromones, which require peptide transporters for secretion (Kuchler, Sterne et al. 1989; Casselton 1997; Altstein 2004).

There are many peptide (or peptidomimetic) antibiotics/toxins which are substrates for microbial peptide transporters. In different systems, peptide transporters can confer sensitivity or resistance to antimicrobial compounds. Disruption of a peptide transporter can often render a microorganism resistant to a particular antibiotic/toxin, because the agent can no longer gain entry into the cell (Mehta, Kingsbury et al. 1984; Yadan, Gonneau et al. 1984). Conversely, peptide transporters can confer resistance by acting to sequester the toxic compound or pump it out of the cell (Parra-Lopez, Baer et al. 1993).

Peptide transport has not been studied as extensively in plant systems as it has been in bacterial, fungal, and animal systems. Nonetheless, it has become increasingly clear that peptide transport is of great importance in the plant world. The most thorough studies of peptide transport in plants have been in *Arabidopsis thaliana* (thale cress) and *Hordeum vulgare* (barley). After completion of the *Arabidopsis* genome, it was discovered that *Arabidopsis* had ten times the number of peptide transporters than any other sequenced organism (as of 2002) (>80) (Stacey, Koh et al. 2002). In *Arabidopsis*, peptide transporters are expressed in all tissues, and disruption of peptide transport results in delayed flowering and arrested seed development (Song, Steiner et al. 1996; Song, Koh et al. 1997). Peptide transporters are expressed in the roots and aid in nutrient uptake (Steiner, Song et al. 1994). Like in Arabidopsis, studies in barley (*Hordeum vulgare*) have demonstrated a developmental role for peptide transport. Scutellar peptide transport is important for nutrient mobilization from the endosperm (food store) to the seedling during germination (Sopanen, Burston et al. 1977).

In animals, peptide transport is also involved in many diverse processes. **(**For a thorough review of peptide absorption and utilization in animals see (Gilbert, Wong et al. 2008)). As elsewhere in nature, peptide transport in animals is important for nutrient absorption. An intestinal PTR-type peptide transporter, PepT1, plays a large role in animal nutrition. After digestion of food in the stomach, dietary protein is broken down into peptides and free amino acids by hydrolases in the small intestine. These nutrients are absorbed by permeases in the epithelial cells of the brush border membrane. Free amino acids enter by a number of specialized transporters which are specific for one, or a few, of the 20 different amino acids. Di- and tripeptides are transported by PepT1. In

contrast to the specificity seen with amino acid transporters, PepT1 can potentially transport all 400 possible dipeptides, 8000 possible tripeptides, and related peptidomimetic compounds.

Nutrient absorption in the intestine by way of peptide transport is more efficient than absorption of free amino acids. Two or three amino acids (in peptide form) can be transported into the cell by PepT1 for same amount of energy required to transport one free amino acid (Daniel 2004). Experiments in different animals, including man, have consistently shown that intestinal uptake of amino acids in the form of di- and tripeptides is a faster route of uptake per unit time than the uptake of free form amino acids. (Silk, Hegarty et al. 1982; Frenhani and Burini 1999).

Underscoring the importance of peptide transport to human nutrition are observations from disease patients. As mentioned before, normally people absorb essential amino acids in the diet by a dual mechanism of uptake of free amino acids and peptides. But in patients with impaired uptake of certain amino acids, as with Hartnup disease or cystinuria, nutrition is largely dependent upon uptake of peptides (Asatoor, Cheng et al. 1970) (Hellier, Perrett et al. 1970; Palacin, Goodyer et al. 2001).

Peptide transport is of great importance to animals besides its role in nutrient absorption. For example, in all jawed vertebrates, almost every nucleated cell in the body possesses a particular peptide transporter (TAP), which plays a major role in the adaptive immune system. Viruses and certain bacteria replicate in the cytosol of the host cell. In order to alert the immune system ($CD8^+$ T cells) to the presence of a cytosolic pathogen, peptides derived from the pathogen must be presented on the host cell surface in the context of major histocompatibility complex (MHC) class I molecules. MHC class

I molecules are synthesized in the endoplasmic reticulum (ER) membrane with the peptide binding site facing the ER lumen. Hence, in order to load cytosolicly-derived peptides onto MHC class I molecules, the peptides must be transported across the ER membrane, from the cytosol to the ER lumen. This process requires the action of the ABC-type peptide transporter, TAP (Transporter associated with Antigen Processing). Disruption of TAP impairs antigen presentation, and results in immune deficiency against cytosolic pathogens and cancerous cells (McCluskey, Rossjohn et al. 2004). Underscoring the importance of TAP to adaptive immunity are the numerous strategies that pathogens have evolved to inactivate this peptide transporter, and the frequency with which TAP disruption is observed in malignant tumors (McCluskey, Rossjohn et al. 2004; Scholz and Tampe 2005; Abele and Tampe 2006; Koppers-Lalic, Verweij et al. 2008).

Incidentally, a separate peptide transport system may also play an important role in immune self-tolerance. Recent research has suggested that a novel peptide transport mechanism (TAP-independent) is involved in the loading of endogenous self-peptides onto MHC class II molecules (Dani, Chaudhry et al. 2004).

Besides intestinal PEPT1 and the ubiquitous TAP, peptide transporters are at work throughout body mediating the trafficking of peptides. PEPT1 is also expressed in the kidney, liver, bile duct, and pancreas (Bockman, Ganapathy et al. 1997; Thamotharan, Lombardo et al. 1997; Shen, Smith et al. 1999; Knutter, Rubio-Aliaga et al. 2002). The peptide transporter PEPT2 is expressed in the lung, brain, kidney, mammary gland, pituitary gland, eye, testis, prostate, uterus, and ovary (Berger and Hediger 1999; Shen, Smith et al. 1999; Groneberg, Doring et al. 2001; Groneberg, Nickolaus et al. 2001;

Daniel and Kottra 2004; Groneberg, Rubio-Aliaga et al. 2004; Biegel, Knutter et al. 2006; Lu and Klaassen 2006). PEPT2 has been studied most thoroughly in the kidney, where it mediates the absorption of di- and tripeptides (and peptidomimetic drugs) from glomerular filtrate (Daniel and Rubio-Aliaga 2003). In the brain, PEPT2 acts to remove hydrolysis products of neuropeptides. PEPT2 in the choroid plexus acts as an efflux pump (from the cerebrospinal fluid to the blood) and may be involved regulation of neuropeptide levels in the cerebrospinal fluid (Berger and Hediger 1999). The physiological role of PEPT2 in other tissues is not as well defined, although it appears that its primary function is removing small peptides from extracellular fluids. (For an excellent review of peptide transport in animals, see (Gilbert, Wong et al. 2008).)

Due to the ubiquity and broad substrate specificity of peptide transporters, peptide transport is important pharmacologically, and peptide transporters have been explored for their potential in drug delivery. PEPT1 and PEPT2 can mediate the uptake of peptidomimetic drugs including many β-lactam antibiotics, ACE-inhibitors, pro-drugs such as valcyclovir, and others. The intestinal absorption of many orally administered peptidomimetic drugs is mediated by PEPT1. The use of PEPT2 in the lung for delivery of respiratory drugs is also being explored (Groneberg, Fischer et al. 2004). The absorption of many drugs can be improved by covalent addition of an amino acid to make the compound more peptide-like, and thus a better substrate for a peptide transporter. (For a thorough review of the pharmacological importance of peptide transporters see (Brandsch, Knutter et al. 2008))

I. C.

Types of Peptide Transporters

Analysis of the genes coding for peptide transporters has revealed different types of peptide transporters with separate evolutionary origins. Broadly, peptide transporters can be segregated into two main groups based upon their mechanism of energy coupling; primary active transporters (ATP-hydrolysis) or secondary active transporters (proton-symport). These groups of transporters can be further subdivided into several families based upon sequence homology. (For a review of families of transmembrane transporters, including peptide transporters, the reader is directed to (Saier 2000).)

The ABC (ATP-Binding Cassette) superfamily of primary active transporters use ATP hydrolysis to drive the transport process. The ABC superfamily is among the largest families of proteins in nature, and ABC transporters are found in every living organism (Davidson and Maloney 2007). Proteins are classified as ABC transporters based upon the sequence of their conserved ATP binding domain, which includes the characteristic Walker A and Walker B motifs. ABC proteins mediate the transport of a wide range of substrates ranging from metal ions and lipids, to carbohydrates and large polypeptides. There are 48 families of ABC transporters within the ABC superfamily, several of which are known to transport peptides, including the TAP, STE, MDR, Pep1E, Pep2E, Pep3E, and PepT families (Saier 2000). Transporters within the ABC superfamily are the only known primary active transporters that can transport peptides.

The secondary active peptide transporters rely on the proton motive force (PMF) to drive transport, and they can be divided into four families: the PTR (or POT) family, the OPT family, the PAT family, and the PUP family.

Transporters of the PTR (Peptide TRansport) family (also known as the POT family; Proton Oligopeptide Transport) (TC 2.A.17) are found in Gram positive and Gram negative bacteria, fungi, plants, and animals. While most PTR-family members transport di and tripeptides, certain PTR family members have been shown to catalyze the transport of nitrate and histidine. (Frommer, Hummel et al. 1994; Yamashita, Shimada et al. 1997; Zhou, Theodoulou et al. 1998). This family of peptide transporters will discussed in depth in the "PTR family" section.

The OPT (OligoPeptide Transport) family of transporters (TC 2.A.67) transports oligopeptides that are four to six amino acids long (Lubkowitz, Hauser et al. 1997; Lubkowitz, Barnes et al. 1998). OPT family members have been functionally characterized in several yeast species (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Candida albicans*). OPT family members have also been characterized in plants (*Arabidopsis thaliana*, *Oryza sativa*, and *Brassica juncea*), and distantly related members may be present in bacteria and archea (Saier 2000; Cagnac, Bourbouloux et al. 2004; Osawa, Stacey et al. 2006; Stacey, Osawa et al. 2006; Wiles, Cai et al. 2006).

The PAT (Peptide Acetyl-CoA Transporter) family of the Major Facilitator Superfamily (MFS) (TC 2.A.1.25), and the PUP (Peptide Uptake Permease) family (TC 9.A.18), are not as well characterized. Two members of the PAT family have been characterized. One is a putative acetyl-CoA transporter in the ER and golgi membranes of man, and the other is thought to transport small peptides in *E. coli* (Lindquist, Weston-

Hafer et al. 1993; Kanamori, Nakayama et al. 1997). Homologues of these two PAT transporters have been identified in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Neisseria gonorrhoeae*, *Rickettsia prowazekii*, and *Haemophilus influenzae*. Two characterized transporters, BacA of *Rhizobium meliloti* and SbmA of *E. coli*, define the PUP family of transporters (Glazebrook, Ichige et al. 1993; Salomon and Farias 1995). They are thought to catalyze the transport of thiazole ring-containing peptide antibiotics. The PUP family may be distantly related to ABC transporters, and their mechanism of energy coupling is unknown.

This dissertation work studied the PTR-type peptide transporter in Saccharomyces cerevisiae, and this review will focus on the di/tripeptide transporters of the PTR family.

I. D.

The PTR Family

Members of the PTR family of peptide transporters are found throughout nature, from bacteria to man (Daniel, Spanier et al. 2006). PTR transporters rely on the proton motive force (PMF) to energize transport. They transport di and tripeptides in symport with protons, and the peptide/H^+ stoichiometry varies depending upon the charge of the peptide (Daniel 2004). Although the usual substrates for PTR family members are di- and tripeptides, many related peptidomimetic compounds are also transported (Brandsch, Knutter et al. 2008), and some PTR family members are known to transport nitrate and histidine (Frommer, Hummel et al. 1994; Yamashita, Shimada et al. 1997; Zhou,

Theodoulou et al. 1998). This section will address the history of the PTR family, substrate specificity, conserved features of the PTR family, structure and transport mechanism, and the significance and distribution of PTR-type peptide transporters in nature.

Broad analysis of membrane transporters has revealed that certain characteristics of transmembrane transport are virtually immutable and others are readily altered by evolution. In general, when comparing different members of a particular transporter family, the mechanism of transport and energy coupling are highly conserved, membrane topology and polarity are conserved to an intermediate degree, and the mechanisms of transporter regulation are poorly conserved (Saier 2000). So, in this section reviewing the PTR family, the highly conserved features (e.g. transport mechanism and topology) will be discussed, including studies of Ptr2p. In the specific "Ptr2p" section, Ptr2p specific regulation will be addressed. The regulation of other PTR family members varies widely in nature and will not be addressed in this review.

History

Although the study of peptide transport has faint roots near the beginning of the 20th century, most advances in the field didn't come until the latter half of the century, with major momentum picking up around the 1960's and 70's. The first peptide transporters to be cloned and characterized were ABC-type peptide transporters from bacteria (Hogarth and Higgins 1983; Andrews and Short 1985; Hiles, Powell et al. 1987). In 1994, the first eukaryotic peptide transporter gene, *PTR2*, was cloned from *Saccharomyces cerevisiae* by the Becker lab (Perry, Basrai et al. 1994). Soon after, other

eukaryotic peptide transporter genes were cloned from *Candida albicans*, *Arabidopsis thaliana*, and rabbit intestine (Fei, Kanai et al. 1994; Steiner, Song et al. 1994; Basrai, Lubkowitz et al. 1995). These newly identified eukaryotic peptide transporters displayed no sequence similarity (amino acid) with the previously identified ABC-type peptide transporters, and they displayed no similarity with any other proteins in the sequence databases. However, a sequence (amino acid) comparison of these non-ABC-type peptide transporters revealed there was a high degree of similarity among them. This new family was termed the PTR family (Peptide TRansport) (Steiner, Naider et al. 1995), or alternatively the POT (Proton Oligopeptide Transport) family (Paulsen and Skurray 1994). The "PTR" family may be a better designation because members of this family transport di- and tripeptides, not larger oligopeptides.

Structure

PTR family members have several characteristic sequence motifs which are highly conserved, and members are predicted to have 12 alpha-helical transmembrane domains (TMs) with a large intracellular loop between TM6 and TM7. The highly conserved motifs reside in the predicted transmembrane domains; the "EXCERFXYYG" motif in TM1, the "FYING" motif in TM5, and the "WQIPQY" motif in TM10 (Hauser, Narita et al. 2001). Of these three motifs, the FYING motif is the most highly conserved. It makes sense that the highly conserved sequences lie within the transmembrane domains. The most highly conserved features of a transporter family deal with the transport mechanism, and one would predict that the essential components of the transport mechanism would be found within the transmembrane portion of the transporter.

A comparison of the PTR-type transporters from closely related *Saccharomyces* species (*S. paradoxus*, *S.mikatae*, and *S. castellii*) further illustrates this point. The vast majority of variation between them is found in the predicted intracellular loops and tail regions.

The predicted transmembrane topology of the PTR family (12 TMs) is based upon a Kyte-Doolittle prediction, and it is substantiated by several lines of empirical evidence (Figure 1). (All figures are located in the appendix.) Membrane topology has been experimentally investigated in a bacterial PTR member from *Lactococcus lactis*, DtpT, and a human PTR member, PEPT1 (Hagting, vd Velde et al. 1997; Covitz, Amidon et al. 1998).

DptT transmembrane topology was investigated by analyzing a series of 42 DptT-alkaline phosphatase fusion proteins (DtpT-PhoA). The localization (intracellular or extracellular) of PhoA was determined for the series of fusion proteins, allowing the transmembrane topology to be deduced. The experiments confirmed the presence of 12 TMs, with cytoplasmicly oriented amino and carboxy termini (Hagting, vd Velde et al. 1997). The topology model was validated by SCAM (Single Cysteine Accessibility Method) experiments. PEPT1 topology was investigated in a similar fashion with the use of epitope insertions. The results were also consistent with the 12 TM model (Covitz, Amidon et al. 1998).

There is no crystal structure available for any PTR family member or closely related membrane protein, so there is very little information available about the 3-D structure of PTR-type transporters. The only structural data for a PTR family member, beyond membrane topology, comes from a transmission electron microscopy (TEM) study of *ydgR* of *E. coli* (Weitz, Harder et al. 2007). *ydgR* exists as a monomer and has

transport properties similar to mammalian PTR-type di/tripeptide transporters. *ydgR* was purified, functionally reconstituted into liposomes, and analyzed by TEM. TEM revealed that *ydgR* proteins have a crown like structure with a central density, and a diameter of ~8nm. This diameter is similar to that reported for the "red permease", a 12 TM lactose permease fusion protein from *E. coli.* (Zhuang, Prive et al. 1999; Weitz, Harder et al. 2007).

Mechanism

Although a high resolution structure is not available, several studies have investigated the transport mechanism of PTR-type transporters. Experiments with PEPT1 (mammals), PEPT2 (mammals), and Ptr2p (yeast) have shown that the N-terminal half of the transporter is involved in substrate recognition/selection(Terada, Saito et al. 2000; Hauser, Kauffman et al. 2005). Other studies, using computer simulations and systematic SCAM, have investigated the arrangement of transmembrane domains in attempts to elucidate the structure of the aqueous substrate translocation pathway(Bolger, Haworth et al. 1998; Yeung, Basu et al. 1998; Kulkarni, Haworth et al. 2003; Kulkarni, Haworth et al. 2003; Kulkarni, Davies et al. 2007; Links, Kulkarni et al. 2007).

Mammals possess two similar PTR-type peptide transporters, PEPT1 and PEPT2, which share about ~50% amino acid sequence identity. PEPT1 is a low affinity/high capacity transporter, and PEPT2 is a high affinity/low capacity transporter. PEPT1 and PEPT2 also differ substantially in substrate recognition. Terada *et al.* (Terada, Saito et al. 2000) exploited these differences in substrate affinity and recognition to investigate the function of the PEPTs. Terada *et al.* created two chimeric proteins (PEPT N1C2 and

PEPT N2C1) by mixing and matching the N-terminal and C-terminal halves of PEPT1 and PEPT2. (The PEPTs were split between TM6 and TM7, in the large intracellular loop region.) Studies of these chimeric proteins revealed that the N-terminal half of the protein is responsible for substrate recognition, and that domains mediating substrate affinity are located in both the N- and C-terminal halves of the protein. The notion, that domains responsible for substrate recognition reside in the N-terminal half of the transporter, is supported by experiments in yeast, looking at highly conserved residues in TM5.

The fact that the "FYING" region of TM5 is invariably conserved from yeast to man implies that this region is important to the function of the transporter. Working with Ptr2p (*Saccharomyces cerevisiae*), Hauser *et al.* (Hauser, Kauffman et al. 2005) conducted alanine-scanning mutagenesis of TM5 (including the FYING region), creating 22 single mutants replacing each residue in TM5. The experiments revealed that mutations within TM5 impact substrate specificity. For example, certain Ptr2p mutations (M250A, N252A, and L258A) increased trimethione transport and decreased dileucine transport, while other mutations (Q241A, N242A, and A260G) had an almost opposite effect, decreasing trimethione uptake without decreasing dileucine uptake. The FYING mutants (F247A, Y248A, I251A, N252A, and G254A) were the most severely compromised in their ability to transport peptide. A helical wheel plot of TM5 predicted that the highly conserved FYING residues all localize to the same half of the helix.

Working with human PEPT1, Kulkarni *et al.* (Kulkarni, Haworth et al. 2003) did systematic SCAM of TM5, creating 21 single cysteine mutations replacing each residue of TM5. They tested the solvent accessibility of each residue, and their results suggest

that TM5 lines a putative aqueous channel. This notion is consistent with the FYING studies of Hauser *et al.*, as one would predict that TMs lining an aqueous channel would have intimate interactions with the substrate, possibly determining which substrates are accepted.

The Kulkarni group has also done other studies investigating the structure of human PEPT1, including similar SCAM studies on TM3 and TM7 and computer simulations looking at pairwise energy of adjacent transmembrane domains and amphipathicity (Bolger, Haworth et al. 1998; Yeung, Basu et al. 1998; Kulkarni, Haworth et al. 2003; Kulkarni, Davies et al. 2007; Links, Kulkarni et al. 2007). Based upon their data, they have proposed a crude speculative model of TM arrangement and the aqueous substrate translocation pathway (Links, Kulkarni et al. 2007). To briefly summarize their findings, their studies suggest that: TMs 1, 3, 5, 7 and 10 may interact with the substrate and form the aqueous substrate translocation pathway; TM3, TM5 and TM7 are tilted relative to the translocation pathway; the extracellular portions of TM5 and TM7 are amphipathic with a few solvent-accessible residues facing the putative translocation pathway; the intracellular portions of TM5 and TM7 are much more solvent accessible; three residues (S174, Y167 and N171) in the intracellular portion of TM5 may directly bind to substrate; TM7 and TM8 may form a salt bridge (a charge-pair interaction between R282 and D341) stabilizing the pre-transport state of the protein; and TM7 may shift after substrate binding.

Energy Coupling

PTR-family transporters rely on the proton motive force (PMF) for transport, carrying protons in symport with peptides. Electrophysiology studies of PEPT1 expressed in *Xenopus laevis* oocytes have shown that the proton/peptide stoichiometry of transport varies depending upon the charge of the peptide (Mackenzie, Fei et al. 1996; Mackenzie, Loo et al. 1996; Steel, Nussberger et al. 1997; Kottra, Stamfort et al. 2002; Irie, Terada et al. 2005). Basic and neutral dipeptides are carried in symport with one proton, while acidic dipeptides are transported with two protons (one extra proton to neutralize the negative side chain charge of the dipeptide). Experiments also showed that transport is reversible for PEPT1 and PEPT2 when membrane voltage and substrate gradients are reversed, although the substrate binding affinity is less on the cytosolic face of the transporter (Kottra, Stamfort et al. 2002).

Substrate specificity

The broad substrate specificity of PTR-type peptide transporters is truly remarkable. Hannelore Daniel put it well, describing the di/tripeptide transporters of the PTR family as "specifically nonspecific" or "nonspecifically specific" (Daniel, Spanier et al. 2006). PTR family members transport virtually any of the 400 dipeptides and 8000 tripeptides (with few exceptions). Many peptidomimetic drugs are also transported, including β-lactam antibiotics, angiotensin converting enzyme (ACE) inhibitors, prodrugs such as valcyclovir, and others. (For a thorough review of drugs transported by human PEPTs, see (Brandsch, Knutter et al. 2008))

There is a strong preference for di and tripeptides composed of L- amino acids, although some peptides containing D-amino acids are also transported. Tetrapeptides and larger peptides are not transported by PTR transporters, and most PTR transporters do not transport free amino acids, although some PTR family members are known to transport nitrate and histidine (Frommer, Hummel et al. 1994; Yamashita, Shimada et al. 1997; Zhou, Theodoulou et al. 1998).

PTR Family Members in Nature

PTR-family members have been identified in organisms throughout nature, including humans, mice, rats, rabbits, fish, worms, insects, plants, yeast, Gram-positive bacteria, and Gram-negative bacteria (Daniel, Spanier et al. 2006). In both plants and animals, PTR-family members are found in a large variety of tissues. (See "Significance of Peptide Transport" section)

I. E.

Ptr2p Background

Discovery

In 1994, the *PTR2* gene was cloned from *Saccharomyces cerevisiae*. *PTR2* was the first eukaryotic peptide transporter gene cloned, and it is the founding member of the PTR family of peptide transporters (Perry, Basrai et al. 1994). A few years earlier, the Becker lab had isolated a series of spontaneous mutants unable to transport di/tripeptides,

by selecting for resistance to toxic di/tripeptides (e.g. alanyl-ethionine or leucyl-fluorophenylalanine) (Island, Perry et al. 1991). They segregated these di/tripeptide transport-deficient mutants into three complementation groups, which they dubbed PTR1, PTR2, and PTR3. *PTR2* was isolated from a genomic DNA library by functional complementation of a di/tripeptide transport-deficient (Ptr2-) mutant.

PTR2

The *PTR2* gene is a 1,803 bp open reading frame (ORF) which codes for the di/tripeptide permease Ptr2p, a protein of 601 amino acids with a calculated molecular mass of ~68 kD (Perry, Basrai et al. 1994). *PTR2* is located on the right arm of chromosome 11. (This is indicated by *PTR2*'s alias/genomic-annotation, YKR093W: Y for yeast chromosome, K for 11th chromosome, R for right arm, 093 for 93rd ORF, and W for Watson strand.)

Function

Ptr2p functions at the plasma membrane, transporting di and tripeptides into the cell in symport with protons. Like other PTR-type peptide transporters, Ptr2p has broad substrate specificity, transporting virtually any of the 400 dipeptides and 8000 tripeptides (with few exceptions) and a variety of peptidomimetic compounds.

Ptr2p expression is highest when *S. cerevisiae* is grown on poor nitrogen sources (e.g. proline or allantoin) and in the presence of inducing amino acids (especially leucine and tryptophan) (Bekcer and Naider 1977; Moneton, Sarthou et al. 1986; Island, Naider

et al. 1987). Ptr2p expression is repressed by rich nitrogen sources such as ammonium sulfate or glutamate.

Genetic regulation

As mentioned at the beginning of this section, di/tripeptide transport deficient mutants isolated by Island et al fell within three complementation groups; PTR1, PTR2, and PTR3. *PTR2* encodes the di/tripeptide permease itself, and *PTR1* and *PTR3* regulate *PTR2* transcription. The *PTR1* gene was found to encode an E3 ubiquitin ligase (Alagramam, Naider et al. 1995), and *PTR3* was found to encode a component of SPS sensor of extracellular amino acids (Barnes, Lai et al. 1998; Klasson, Fink et al. 1999; Forsberg, Gilstring et al. 2001).

PTR1/UBR1

Ptr1p (a.k.a. Ubr1p) is an E3 ubiquitin ligase which catalyzes the transfer of ubiquitin to selected substrates (Alagramam, Naider et al. 1995). Ubiquitin-attachment serves as a signal, targeting the substrate for destruction. (Poly-ubiquitination of cytoplasmic proteins targets them to the 26s proteasome, and ubiquitination of membrane proteins targets them to the vacuole/lysosome.) Ubr1p promotes *PTR2* gene expression by ubiquitinating the transcriptional repressor Cup9p, leading to its destruction (Byrd, Turner et al. 1998). Cup9p is a homeodomain protein which prevents transcription of *PTR2* by occupying the promotor region (between -488 and -897 upstream of the *PTR2* start codon). Ubiquitin-attachment (via Ubr1p) to Cup9p leads to its degradation, thus de-repressing *PTR2* transcription. In this way, Ptr1p/Ubr1p is required for *PTR2* gene

expression, and is therefore required for di/tripeptide transport in *S. cerevisiae*. However, a *ptr1 cup9* double mutant is fully capable of peptide transport.

Peptide regulation

A remarkable positive feedback loop exists within the Ubr1p-Cup9p-Ptr2p circuit. Ubr1p is the recognition component of the N-end rule proteolytic pathway, an important and ubiquitous proteolytic pathway which operates in all organisms examined from bacteria to mammals (Varshavsky 1996). The N-end rule pathway recognizes target proteins bearing an "N-degron". An N-degron has two essential components, a destabilizing N-terminal residue and an internal lysine residue. N-terminal residues which are basic or hydrophobic are considered destabilizing, and the internal lysine residue is the site of ubiquitin attachment. Ubr1p recognizes the N-terminal destabilizing residue of the N-degron and ubiquitinates the substrate. Dipeptides bearing an N-terminal destabilizing residue can act as competitive inhibitors, preventing the degradation of N-end rule substrates. Unexpectedly, Ubr1p recognizes an internal degron in Cup9p which resides in the C-terminal half of the protein. Turner et al tested whether dipeptides with destabilizing N-terminal residues could also act as competitive inhibitors for Cup9p degradation, despite its unusual internal degron. Surprisingly, the dipeptides had the opposite effect, they accelerated Cup9p degradation. Their studies demonstrated the existence of a positive feedback loop, whereby dipeptides accelerate their own uptake by allosteric activation of Ubr1p, which increases Ubr1p-dependent Cup9p degradation, resulting in increased *PTR2* expression (Turner, Du et al. 2000).

PTR3

Ptr3p is required for amino acid-induced expression of *PTR2* (Barnes, Lai et al. 1998). Ptr3p is a component of the SPS (Ssy1p-Ptr3p-Ssy5p) extracellular amino acid sensor complex, which resides at the plasma membrane. The SPS sensor is known to positively regulate the expression of *PTR2*, many amino acid transporter genes, and several amino acid metabolizing genes (Forsberg, Gilstring et al. 2001; Forsberg and Ljungdahl 2001). The permease-like Ssy1p is the sensor component which recognizes amino acids in the environment. When Ssy1p binds amino acids, Ssy1p's N-terminal signaling domain recruits kinases Yck1 and Yck2, which then hyperphosphorylate Ptr3p. Ptr3p hyperphosphorylation activates the proteolytic activity of Ssy5p. Ssy5p removes the N-terminal inhibitory sequences of the transcription factors Stp1p and Stp2p, which allows them to enter the nucleus and activate target gene expression (including *PTR2*) (Bernard and Andre 2001; Forsberg, Hammar et al. 2001; Abdel-Sater, El Bakkoury et al. 2004; Poulsen, Lo Leggio et al. 2006; Liu, Thornton et al. 2008).

Genome-wide screen for genes affecting peptide transport

In 2005, Cai et al conducted a genome wide screen (in *S. cerevisiae*) to identify genes involved in the regulation of di/tripeptide transport (Cai, Kauffman et al. 2005). Cai screened the single deletion mutant library for mutants which showed increased or decreased peptide utilization compared to wild-type. The single deletion mutant library consists of 4826 mutant strains, each missing a single non-essential gene. The strain background was auxotrophic for leucine. In the screen, mutant strains were grown in minimal medium supplemented with histdyl-leucine (His-Leu) as the sole leucine source,

making peptide transport essential for growth. 103 single deletion mutants were identified in this screen and they fell into two categories, the "*cup9*" category and the "*ptr1*" category. When *CUP9* is deleted, *PTR2* expression is increased relative to wild-type, and there is enhanced growth on the His-Leu transport-selective media (46 strains fell into this category). When *PTR1* is deleted, *PTR2* expression is undetectable, and cells are unable to grow using His-Leu as the sole leucine source (57 strains fell into this category). Cai verified the 103 candidate genes by a similar growth assay on solid media, and by testing for sensitivity to the toxic dipeptide alanyl-ethionine.

The 103 genes Cai identified are involved in a wide array of biological processes, and are thought to affect peptide transport both directly and indirectly by affecting *PTR2* transcription, *PTR2* mRNA stability, or Ptr2p localization.

Ptr2p Structure

Ptr2p is predicted to have 12 transmembrane domains (TMs), a large intracellular loop between TM6 and TM7, and cytoplasmicly oriented N- and C- termini (Figure 1). This topology model is based upon a Kyte-Doolittle prediction, and validated by topology experiments conducted with related PTR-type peptide transporters from *Lactococcus lactis* and human (See "PTR family" section) (Hagting, vd Velde et al. 1997; Covitz, Amidon et al. 1998). The assumed intracellular localization of Ptr2p's N- and C-termini is further supported by the fact they are both phosphorylated (Ficarro, McCleland et al. 2002; Li, Gerber et al. 2007), and phosphorylation of membrane proteins is only thought to occur within cytoplasmic domains.

In a phosphoproteomics experiment, Ficarro et al. identified 383 phosphorylation sites in the yeast proteome by mass spectrometry (Ficarro, McCleland et al. 2002). This data set included 4 phosphorylation sites in Ptr2p (S39, S45, S594, and S597), located within the relatively acidic N- and C-terminal cytoplasmic domains. Using this as a starting point, we set out to determine the role of phosphorylation in Ptr2p. Our investigation of these phosphorylation sites led us to study the post-translational regulation of Ptr2p. Using analysis of mutants, GFP-tagged Ptr2p, and other methodologies, we report that the N-terminal region of Ptr2p (including identified phosphorylation sites) is important for nitrogen-induced turnover of Ptr2p from the plasma membrane.

I. F.

Experimental Plan for the Dissertation

Background

The transport of di- and tripeptides in Saccharomyces cerevisiae is mediated by the peptide transporter, Ptr2p (Perry, Basrai et al. 1994). Di- and tripeptide transport in S. cerevisiae is under nitrogen control (Bekcer and Naider 1977; Moneton, Sarthou et al. 1986; Island, Naider et al. 1987). Peptide transport rates are greatest when yeast are grown with “non-preferred” sources of nitrogen (e.g. allantoin), and peptide transport rates are minimal when yeast are grown with “preferred” sources of nitrogen (e.g. ammonium sulfate). The mechanism(s) underlying this nitrogen regulation is unknown.

Ptr2p is known to be phosphorylated and ubiquitinated (Ficarro, McCleland et al. 2002; Hitchcock, Auld et al. 2003; Li, Gerber et al. 2007). These two post-translational modifications have been shown to be involved with the down-regulation of other yeast membrane proteins (Dupre, Urban-Grimal et al. 2004; R. Haguenauer-Tsapis 2004).

Starting Hypothesis:

The down-regulation of Ptr2p in response to preferred nitrogen sources is mediated by the phosphorylation and ubiquitination of Ptr2p.

Chapter II:

Materials and Methods

Strains, Media, and Growth Conditions

The peptide-transport deficient *S. cerevisiae* strain BY4742-16009 (*MATa*, *ptr2 his leu lys ura*) was obtained from Invitrogen Corporation (Carlsbad, CA, USA). *PTR2* was expressed from plasmid pMS2 (Hauser, Kauffman et al. 2005), a CEN-based plasmid with the *URA3* selectable marker. In this construct, *PTR2* is under the control of its native promoter, and FLAG and His tag sequences were fused in-frame to the 3' end of the coding region. For fluorescence microscopy studies, *PTR2*-GFP was expressed from a pMS2-derived plasmid, pMS4, in which two copies of GFP were fused in frame between the FLAG- and His-tags at the 3' end of the coding region (Cai, Kauffman et al. 2005). Yeast were transformed by the method of Gietz (Gietz & Woods 2002). Transformants bearing pMS2 (or pMS4) constructs were routinely maintained under uracil selection on agar medium containing 0.17% (w/v) yeast nitrogen base (YNB) without amino acids or ammonium sulfate, 2% (w/v) glucose, 20 mg histidine ml^{-1}, 30 mg leucine ml^{-1}, 30 mg lysine ml^{-1}, 20 mg tryptophan ml^{-1}, and 0.1% (w/v) allantoin as the N-source. This medium (both broth and agar form) is referred to as "PTR2-inducing medium" and was used in all experiments, unless otherwise specified. All media components were obtained from Difco (Sparks, MD, USA) and amino acids were obtained from Sigma (St. Louis, MO, USA). Cultures were incubated at 30° C, and liquid cultures were grown with shaking at 250 rpm.

Table 1: Oligonucleotide primers for *PTR2* site-directed mutagenesis and *PTR2* sequencing.

	Primer sequence
Mutagenesis primers	
Y37F-S39A-S45A	GCGTTCCGTGGCGTTGGCGACGTCGTCGGCAACAAACGAATCCTTCAGCG
S39A	GTTGTAGCGTTCCGTGGAGTTGGCAACGTCGTCGGCAACATACGAATCCTTCAGCG
S45A	GTTGTAGCGTTCCGTGGCGTTGGCAACGTCGTCGCTAACATACGAATCCTTCAGCG
S39A-S45A	GTTGTAGCGTTCCGTGGCGTTGGCAACGTCGTCGGCAACATACGAATCCTTCAGCG
S594A-S597A	CTTGTAGTCATATTTGGTGGTGGCTCTTAGAGCTTCCATGGGTTCTAATATTTC
S594A-S597A-T598A-T599A	CTTGTAGTCATATTTGGCGGCGGCTCTTAGAGCTTCCATTGGTTCTAATATTTC
K27R	CGCTAACATACGAATCCTTCAGCGTTACAGCCTGGGTCCTCTCTTCTTCGATGACCGGG
K34R	CGCTAACATACGAATCCCTCAGCGTTACAGCCTGGGTCTTCTCTTCTTCGATGACCGGG
K27R-K34R	CGCTAACATACGAATCCCTCAGCGTTACAGCCTGGGTCCTCTCTTCTTCGATGACCGGG
S39D-S45D	GTTGTAGCGTTCCGTGTCGTTGGCGACGTCGTCGTCAACATACGAATCCTTCAGCG
S594D-S597D	CTTGTAGTCATATTTGGTGGTGTCTCTTAGATCTTCCATTGGTTCTAATATTTC
Sequencing primers	
PTR2 599F	CCACTGGTATGATTAAAGC
PTR2 669R	GCCCGATTTTAAAACTTTG
PTR2 1203F	CCCAATTTTTGAAAAATTCG
PTR2 1275R	CCAAACATGAAACCGAAG

Site-directed mutagenesis

Mutations were made to *PTR2* in plasmid pMS2 using double-stranded PCR mutagenesis as described previously (Lee, Naider et al. 2006). Primers encoding the mutations were used to amplify pMS2, and the methylated wild-type template was digested with Dpn1. Products of the mutation reactions were transformed into E.coli DH5α max efficiency cells (Invitrogen, Carlsbad, CA), and transformants were selected by plating on agar with 50 μg ampicillin ml^{-1}. Mutations were confirmed by sequencing at the University of Tennessee Molecular Biology Resource Facility. All primers were purchased from Sigma/Genosys (Woodlands, TX) and Integrated DNA Technologies Inc. (Coralville, IA). The mutagenesis primers and sequencing primers are listed in Table 1.

Metabolic Labeling

Cells were grown overnight in PTR2-inducing medium, and harvested in mid-log phase ($5x10^6$ cells ml^{-1}), and resuspended at $5x10^6$ cells ml^{-1} in "no-phosphate medium". [The composition of the "no-phosphate medium" was identical to the "PTR2-inducing medium" described above, except that potassium phosphate was replaced with 8mM MES (2-(N-morpholino)ethanesulfonic acid) buffer, pH 5.5.] Cells were allowed to grow in the no-phosphate medium for 2 hours, at 30° C with shaking. Cells were then harvested and resuspended into fresh no-phosphate medium at 10^8 cells ml^{-1}. Then 1mCi of $^{32}PO_4$, per 10^8 cells, was added and cells incubated at 30° C for 20 minutes. Aliquots of cells were then snap-frozen in a dry-ice/ethanol bath.

Membrane Preparation

Cells from 100 ml cultures were harvested by centrifugation (4000 rpm for 5min) and washed once with 25ml cold water. All procedures from this point were carried out at 4° C. Cells were resuspended in 500 µl of buffer 1 (1x PBS, pH 7.5, 5mM EDTA, with phosphatase inhibitors: 310 mM NaF, 3.45 mM $NaVO_3$, 50 mM sodium pyrophosphate, 10mM glycerophosphate, and with protease inhibitors: 10 µg pepstatin A ml^{-1}; 10 µg leupeptin ml^{-1}; and 17 µg PMSF ml^{-1}). Cells were lysed using 600 µl of glass beads added to the resuspended cells in 1.8ml microfuge tubes followed by vortexing for 3, 1-minute bursts, with one minute cooling on ice in between each burst. The cells were placed on ice for 15 minutes, and then the cells received another set of 3, 1-minute bursts. The cell lysate was centrifuged at 700g for 3 min to pellet the glass beads and unbroken cells. The supernatant was transferred to a clean tube and centrifuged at 20,000g for 30

minutes at 4°C to pellet membranes. Membrane pellets were resuspended in 125 µl of Buffer1 with 20% (v/v) glycerol. Protein concentration in the membrane preparation was determined by the Bradford method.

Western Blot Analysis and Autoradiography

Membrane preparations and affinity-enriched Ptr2p samples were electrophoresed on SDS-PAGE gels (10% acrylamide) and blotted onto Immobilon-P membranes. Blots were blocked with 5% non-fat dry milk for one hour at room temperature, and then probed with the primary antibody solution (1/15,000 dilution of Anti-FLAG M2 antibody) (Eastman Kodak Company, New Haven, CT). Blots were washed twice with 5% non-fat dry milk and then twice with TBST. The blots were then probed with secondary antibody (goat α-mouse IgG-HRP) (Promega, Madison, WI). Bands were visualized with West Pico chemiluminescent detection system (Pierce Biotechnology, Rockford, IL). ^{32}P signal from western blots was detected using a phosphor screen (GE Healthcare, UK). All bands were quantified using Quantity-One ® 1-D image analysis software (BioRad, Hercules, CA)

Ptr2p Enrichment

Ptr2p was enriched using ANTI-FLAG® M2 affinity gel (Sigma, St. Louis, MO). Membrane preparations were first solublized overnight in Lysis Buffer (50 mM Tris HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% (v/v) TRITON™ X-100) with protease inhibitors and phosphatase inhibitors as used for membrane preparation, with gentle end-over-end mixing. Solublized membranes were centrifuged at 20,000g for 60

minutes at 4° C to remove insoluble material. The supernatant was then added to packed and prepared ANTI-FLAG® M2 affinity gel. Membranes incubated with the affinity gel overnight at 4°C with gentle end-over-end mixing. The affinity gel was then pelleted and washed three times with 500µl TBST (50 mM Tris HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.4). Samples were eluted from the affinity gel by boiling with 2x non-reducing sample buffer [125mM Tris HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.004 % (w/v) bromophenol blue] for 5 minutes. The affinity gel was pelleted by centrifugation (8,000 g for 30 seconds at room temperature) and the supernatant containing the eluted proteins was removed and stored at -20° C.

Growth Assays

Growth assays were conducted using a modified PTR2-inducing solid medium (as described above), which lacked leucine (a "leucine dropout medium"). Leucyl-leucine was supplied at 160µM to satisfy the leucine auxotrophy of the cell, making peptide transport essential for cell growth. Growth indicates leucyl-leucine transport. Plates were incubated at 30° C.

Toxic Peptide Halo Assays

Toxic peptide halo assays used plates with 25 ml of bottom agar (PTR2-inducing media). Cells were grown overnight to mid-log phase ($5x10^6$ cells ml^{-1}), washed twice and resuspended in cold 2% glucose to a concentration of 10^6 cell ml^{-1}. 1ml of cell suspension was combined with 3 ml of molten (55°C) 0.1% Noble agar, and this mixture was overlaid onto the bottom agar plates and allowed to solidify. 6mm sterile filter discs

(BD Biosciences, San Jose, CA) were placed on the surface of the plates, and either 5 µl or 10 µl of 40mM alanyl-ethionine (toxic dipeptide) was added to each disc. Plates were incubated at 30° C. Formation of a halo (zone of clearing) indicates sensitivity to the toxic dipeptide, and indicates cells are capable of peptide transport.

Uptake Assays

100ml yeast cultures were grown overnight in PTR2-inducing medium. Cells were harvested at $5x10^6$ cells ml^{-1}, washed twice with 25 ml cold water, and resuspended in cold 2% (w/v) glucose to a concentration of $2x10^8$ cells ml^{-1}. After incubating at 30° C for 10 minutes, a 60µl portion of the cell suspension was combined with 60 µl of 2x uptake medium [2% glucose, 400 µM leucyl-leucine, 1µM 3H-leucyl-leucine (1µCi ml^{-1}), 20 mM sodium citrate/phosphate buffer, pH 5.5] and incubated at 30° C for 1 minute. After the 1 minute incubation, the assay was terminated by filtration of the cells over 0.45 µm HAWP membrane filters (Millipore, Bedford, MA) followed by washing with ice cold water (4 ml). Retained radioactivity was determined by liquid scintillation spectrometry. Uptake assays were repeated a minimum of three times.

Microscopy

Cells expressing various versions of Ptr2p-GFP were grown overnight in PTR2-inducing media to a concentration of $5x10^6$ cells ml^{-1}. Cells were then concentrated by centrifugation and examined by fluorescence microscopy using a 470- to 490-nm excitation wavelength and 515-nm emission filter fitted to an Olympus (Lake Success,

NY) BX50 microscope. Images were taken with a MicroFire camera (model S99809; Olympus).

Time-Lapse Microscopy

Time-lapse microscopy experiments were conducted using Y2 Yeast Cell Microfluidic Plates and the "ONIX™ micro" flow-control system (CellASIC Company, San Leandro, CA). This system is optimized for yeast, and captures cells in an elastic trap with a height of ~ 5μm, thus keeping the yeast cells in a single optical plane for viewing. The system allows for rapid medium exchange without subjecting the cells to high shear stress. Cells were grown overnight to mid-log phase ($5x10^6$ cells ml^{-1}) in PTR2-inducing medium. Cells were then loaded into the microfluidic plates and perfused with PTR2-inducing medium. Cells were observed for 15 minutes under inducing conditions, and then the medium was switched to an identical medium supplemented with 40mM ammonium sulfate to induce Ptr2p down-regulation. The down-regulation process was observed for 120 minutes. Cells were viewed under the 40x objective lens on a Leica (Bannockburn, IL) DMIRB inverted fluorescence microscope. Images were captured once a minute for 135 minutes using a Q-Imaging (Surrey, BC, Canada) Retiga 1300 digital camera. Videos files (".wmv") were created from the series of still images using "Windows Movie Maker" version 2.1.4026.0 (Microsoft, Redmond, WA). Each image is displayed for 0.25 seconds. Thus each second of the movie corresponds to 4 minutes of real time. Videos are included in the Plate 1 CD-ROM, and are entitled "WT-full", "WT-cropped", "N3-full", and "N3-cropped". Two135-minute time-lapse experiments were conducted; one observing wild-type Ptr2p-GFP ammonium-induced down-regulation

(WT), and the other observing Y37F-S39A-S45A Ptr2p-GFP ammonium-induced down-regulation (N3). The "WT-full" and "N3-full" videos show the full images from these experiments. To focus in on particular representative cells, the "WT-cropped" and "N3-cropped" videos were generated using cropped images from the same experiment.

Chapter III:

Results

Phosphorylation site mutants are functional

Four sites of phosphorylation in Ptr2p were identified in a phosphoproteomics experiment: S39, S45, S594, and S597 (Ficarro, McCleland et al. 2002). These serine residues are located on N- and C-terminal regions of Ptr2p (Figure 1). In efforts to determine the role of phosphorylation in Ptr2p function, these four residues were mutated to alanine or aspartic acid. Alanine mimics unphosphorylated serine, which cannot be phosphorylated but is similar in size to serine. Aspartic acid mimics phosphoserine in size and charge. These substitutions are used often in studies where the role of serine phosphorylation is tested (Hicke, Zanolari et al. 1998; Marchal, Haguenauer-Tsapis et al. 2000). Initially, two quadruple mutants were generated; S39A-S45A-S594A-S597A and S39D-S45D-S594D-S597D. Mutations were made on a plasmid-borne copy of *PTR2*, where *PTR2* is under the control of its native promotor. These plasmid constructs were transformed into *ptr2Δ* cells.

The functionality of the mutant transporters was assayed by measuring the uptake of ^{3}H-leucyl-leucine (Figure 2). Both quadruple mutations increased the peptide transport activity of the cell, with the serine-to-alanine mutant showing the strongest increase. Interestingly, the mutations to alanine (mimic of unphosphorylated serine) and to aspartic acid (mimic of phosphoserine) did not have opposing phenotypes. The serine

to alanine mutant (S39A-S45A-S594A-S597A) was selected for further investigation because it displayed the strongest mutant phenotype.

S39A-S45A mutation in Ptr2p increases cellular peptide transport capacity

To determine whether specific residues among the four residues mutated were responsible for the mutant phenotype, two double mutants were generated and evaluated; S39A-S45A (N-terminal double mutant) and S594A-S597A (C-terminal double mutant). Peptide transport activity was assayed by measuring ^{3}H-leucyl-leucine uptake. Cellular peptide transport was measured in a range of substrate concentrations: 1mM, 200μM, 50μM, 25μM, and 10μM. The experiments revealed that the S39A-S45A mutation increased the apparent peptide transport capacity of the yeast cells as measured by the V_{max} (Figure 3). In all three mutants, the K_m of transport was not greatly affected. However, for the N-terminal double mutant (S39A-S45A) and the quadruple mutant (S39A-S45A-S594A-S597A), the V_{max} for cellular peptide transport was increased roughly three-fold. The V_{max} for the C-terminal double mutant (S594A-S597A) was not significantly altered.

In order to determine if the phenotype caused by the S39A-S45A double mutation was primarily the result of a single mutation (S39A or S45A) or was the combined effect of the two individual mutations, single S39A and S45A mutants were generated. Peptide transport activity was assayed by measuring ^{3}H-leucyl-leucine uptake. (Figure 4). The single mutations each increased peptide transport rates above wild-type, but not to the level of the S39A-S45A double mutant. Hence, the S39A-S45A mutant phenotype of

increased cellular peptide transport is the combined effect of both single mutations, rather than being to due to a single mutation.

S39A-S45A mutations led to impaired Ptr2p down-regulation

Western blot analysis revealed that S39A-S45A mutation increased Ptr2p accumulation relative to wild-type (Figure 5A). *PTR2* expression levels should be equal among the different mutants, therefore the S39A-S45A mutation may have interfered with the normal turnover, or down-regulation, of Ptr2p. To test this hypothesis directly, a nitrogen repression experiment was devised. Peptide transport expression is highest under poor-nitrogen conditions, where the primary source of nitrogen in the growth medium is "non-preferred" (e.g., an organic N-source such as allantoin or proline)(Bekcer and Naider 1977). Here we report that the addition of a rich nitrogen source (i.e. ammonium sulfate) to the growth medium results in a rapid down-regulation of Ptr2p. Ptr2p levels, as assayed by western blot, are dramatically decreased three hours after addition of ammonium sulfate to cells growing in *PTR2*-inducing conditions (Figure 5A). The S39A-S45A mutation interfered with Ptr2p down-regulation, which explains the increased Ptr2p accumulation and peptide transport activity of the mutant cells. As expected, the S594A-S597A mutation did not appear to affect Ptr2p down-regulation.

This down-regulation process was impaired in the presence of cycloheximide, indicating that Ptr2p down-regulation is a protein synthesis-dependent process (Figure 5B). Ptr2p down-regulation was also impaired in a *pep4Δ* yeast strain. *pep4Δ* yeast are deficient in vacuolar proteolysis. (Pep4p is required for enzymatic activation of vacuolar

proteases.) The fact that Ptr2p degradation was impaired in *pep4Δ* cells is strong evidence that Ptr2p is degraded in the vacuole (Figure 5C).

Ptr2p-GFP

To determine the subcellular localization of Ptr2p, a Ptr2p-GFP fusion protein was generated (Cai, Kauffman et al. 2005). Fluorescence microscopy revealed a difference in the pattern of subcellular localization between wild-type and the S39A-S45A mutant (Figure 6). For the wild-type yeast cells growing under Ptr2p inducing conditions, Ptr2p-GFP was detected at the plasma membrane, endocytic/secretory vesicles, and in the vacuole. Addition of ammonium sulfate caused a rapid change in the pattern of Ptr2p-GFP localization. Within a few minutes, there was an increased localization of Ptr2p-GFP within endocytic vesicles and in the vacuole, and there was decreased localization at the plasma membrane. Forty minutes after addition of ammonium sulfate, Ptr2p-GFP was almost completely undetectable at the plasma membrane, and the Ptr2p-GFP signal was almost exclusively localized in the vacuole. After three hours, the vast majority of Ptr2p-GFP signal had vanished and only a faint Ptr2p-GFP signal in the vacuole remained.

The localization of the S39A-S45A mutant was different than that of the wild-type. Under Ptr2p inducing conditions, there was much greater Ptr2p-GFP accumulation at the plasma membrane and there was very little intracellular localization. (This is in contrast with wild-type Ptr2p, which showed substantial intracellular localization even under inducing conditions.) Twenty minutes after addition of ammonium sulfate, there was little difference in the pattern of Ptr2p-GFP localization. After forty minutes, there

was increased Ptr2p-GFP signal within endocytic vesicles, but there was still strong localization at the plasma membrane. Three hours after addition of ammonium sulfate, there was increased intracellular localization, but there was still strong Ptr2p-GFP signal from the plasma membrane (Figure 6). These results indicate that the S39A-S45A mutant phenotype is the result of impaired turnover of Ptr2p from the plasma membrane.

Additional Phosphorylation Sites

Recently, a new phosphoproteomics experiment identified three additional phosphorylation sites in Ptr2p; Y37, T598, and T599 (Li, Gerber et al. 2007).These newly identified sites were in close proximity to the previously identified phosphorylation sites (Figure 1). Equipped with this new phosphorylation data, a new series of mutants were generated including an N-terminal triple mutant (Y37F-S39A-S45A), a C-terminal quadruple mutant (S594A-S597A-T598A-T599A), and a heptamutant (Y37F-S39A-S45A-S594A-S597A-T598A-T599A) in which all identified phosphorylation sites were mutated. To test the effect of these new mutations, an ammonium sulfate repression experiment was conducted (Figure 7). The results confirmed the role of Ptr2p N-terminus in post-translational regulation. The N-terminal triple mutant showed impaired down-regulation, while the C-terminal quadruple mutant was similar to wild-type.

To determine if additional phosphorylation sites exist within Ptr2p, ^{32}P metabolic labeling experiments were conducted. Cells growing in log phase under Ptr2p inducing conditions were labeled with ^{32}P, Ptr2p was purified/enriched using affinity resin, and radioactivity of Ptr2p was measured from a western blot. *PTR2* mutants were compared with wild-type (Figure 8). As expected, the mutation of known phosphorylation sites

diminished the radioactivity of Ptr2p. However, the heptamutant (Y37F-S39A-S45A-S594A-S597A-T598A-T599A), in which all identified phosphorylation sites were mutated, was still labeled by ^{32}P, indicating that additional, unidentified phosphorylation sites exist within Ptr2p. The band intensities were quantified for the chemiluminescent α-FLAG blot and the corresponding autoradiograph. The ratio of the ^{32}P signal to the chemiluminescent signal (^{32}P/α-FLAG) serves as a crude estimate of the level of Ptr2p phosphorylation. The ^{32}P/α-FLAG ratios (normalized to wild-type) are as follows: Wild-type = 1, Y37F-S39A-S45A (N3) = 0.6, S549A-S597A-T598A-T599A (C4) = 0.5, Y37F-S39A-S45A-S594A-S597A-T598A-T599A (heptamutant) = 0.4. The heptamutant ^{32}P/α-FLAG ratio of 0.4 suggests that phosphorylation within the seven identified sites constitutes a little more than half of overall Ptr2p phosphorylation.

Ptr2p Ubiquitination

The aforementioned data indicated that mutation of known phosphorylation sites in Ptr2p's N-terminal cytoplasmic region impaired normal down-regulation of Ptr2p, greatly delaying Ptr2p turnover from the plasma membrane. Previous studies on some yeast transporters had indicated that ubiquitination was involved in down regulation (Hicke 1997). Ubiquitin, a small 76-amino acid protein, is covalently attached to lysine residues on target proteins as a signal for degradation. Attachment of ubiquitin to yeast plasma membrane proteins targets them to the endocytic pathway and ultimately to the vacuole for destruction (Hicke 1997). Furthermore, in a proteomics experiment in 2003

carried out to identify ubiquitinated yeast proteins, Ptr2p was identified, but the site of ubiquitin attachment was not resolved (Hitchcock, Auld et al. 2003).

One the most well characterized ubiquitination events for a membrane protein in yeast, is the ubiquitination of Gap1p, the general amino acid permease (Soetens, De Craene et al. 2001). In Gap1p, ubiquitin is attached to Lys9 and Lys16 which reside in the N-terminal portion of Gap1p. We found a similarity between this ubiquitinated motif in Gap1p and a motif in Ptr2p. In Gap1p, the two ubiquitinated lysine residues are separated by six residues, the first lysine (Lys9) is preceded by glutamic acid, and the second lysine (Lys16) is preceded by leucine. In Ptr2p, Lys27 and Lys34 fit this same pattern (EKxxxxxLK). Additionally, these two lysine residues (27 and 34) are located in close proximity to the known phosphorylation sites Y37, S39, and S45, which displayed the mutant phenotype of impaired down-regulation.

To investigate these potential ubiquitin-attachment sites, Lys27 and Lys34 were converted to arginine by site-directed mutagenesis. Arginine is a similar basic amino acid which cannot be ubiquitinated. The K27R-K34R mutant was analyzed in the same manner as the previous mutants; radiolabeled-dipeptide uptake assays, western blot, and GFP-fluorescence microscopy (Figures 6 and 9). In these tests, the K27R-K34R mutant behaved similarly to S39A-S45A mutant; showing increased plasma membrane localization and impaired down-regulation.

The ubiquitination of Ptr2p was assayed by western blot (Figure 10). Ptr2p was enriched from cells grown under inducing conditions (T_0) and from cells harvested sixty minutes after addition of ammonium sulfate to the growth medium (T_{60}). Enriched Ptr2p samples were then analyzed by western blot. The results showed that Ptr2p

ubiquitination was greatly impaired by the N-terminal mutations (either Y37F-S39A-S45A or K27R-K43R). Under inducing conditions, the level of ubiquitinated Ptr2p was very low for the wild-type yeast, and even lower for the mutants. Sixty minutes after the addition of ammonium sulfate, the level of ubiquitinated Ptr2p was greatly increased. For the N-terminal mutants, ubiquitination of Ptr2p was also increased sixty minutes after addition of ammonium sulfate, but the level of Ptr2p ubiquitination was greatly reduced compared to that of the wild-type.

From the western blot, the estimated molecular weight of ubiquitinated wild-type Ptr2p is ~110-120 kD, suggesting that five or six molecules of ubiquitin (8.5 kD each) are attached to Ptr2p (with a theoretical mass of ~115 and 123 kD respectively). The fact that K27R-K34R Ptr2p was still ubiquitinated indicates that Ptr2p is normally, or can be, ubiquitinated on sites other than K27 and K34. Whether or not K27 and/or K34 are actually sites of ubiquitin attachment on Ptr2p remains to be resolved (by methods involving mass spectrometry), but it is clear that the mutation of N-terminal residues (Y37F-S39A-S45A or K27R-K34R) led to a decrease in the overall ubiquitination of Ptr2p. Also, the tighter banding patterns of the ubiquitinated Ptr2p mutants suggest that their ubiquitinated forms may be under-ubiquitinated compared to wild-type.

Growth Assays and Toxic Peptide Halo Assays

As complementary methods of confirming the functionality of mutant forms of Ptr2p, *PTR2* mutants were subjected to growth assays and toxic peptide halo assays. The yeast strains used in this study are auxotrophic for leucine, and cannot grow without it. In the growth assays, cells were plated on a minimal medium supplemented with leucyl-

leucine as the sole source of leucine. Growth on this medium requires peptide transport, and indicates a functional version of Ptr2p (Figure 11).

Toxic peptide halo assays test for peptide transport by measuring the sensitivity to toxic peptides. Cells which are capable of peptide transport import the toxic peptide. After entry into the cytoplasm, the toxic amino acid moiety is released from the peptide, poisoning the cell. Cells incapable of peptide transport are insensitive to toxic peptides. To test the functionality of mutant versions of Ptr2p, sensitivity to alanyl-ethionine was tested. (Ethionine is a toxic methionine analog.) All *PTR2* mutant strains tested in this study were sensitive to alanyl-ethionine, confirming the functionality of Ptr2p (Figure 12).

Time Lapse Microscopy

In order to get a better view of the Ptr2p down-regulation process, time-lapse microscopy was conducted with wild-type Ptr2p-GFP and Y37F-S39A-S45A Ptr2p-GFP. Cells were observed in a CellASIC flow chamber, (designed specifically for yeast) under 400x total magnification, with pictures taken once a minute for 135 minutes. The time-lapse microscopy began by observing cells for 15 minutes in *PTR2*-inducing medium. After this, down-regulation was triggered by switching the medium in the flow chamber to an identical medium supplemented with ammonium sulfate. The down-regulation process was then observed for 120 minutes. The resulting time-lapse video provides an excellent visualization of the Ptr2p down-regulation process, and confirms the earlier microscopic observations (Figures 13 and 14, and Plate 1).

Chapter IV:

Discussion

The results presented in this dissertation show that the N-terminal cytoplasmic region of Ptr2p plays an important role in post-translational regulation. Mutations to known phosphorylation sites and suspected ubiquitination sites in this region greatly impaired the normal turnover of Ptr2p from the plasma membrane. While it is clear that ubiquitination of Ptr2p triggers turnover of the transporter from plasma membrane and delivery to the vacuole for destruction, it remains unknown whether reversible phosphorylation of residues in Ptr2p regulate ubiquitination, and whether K27 and K34 are the actual sites of ubiquitin attachment. We have not been able to detect a change in phosphorylation (by gel-shift or metabolic labeling) during changes in regulation, which suggests that phosphorylation is constitutive. However, with limits to the precision of our measurements (a problem compounded by the existence of additional unidentified phosphorylation sites), we are unable to rule out the possibility that reversible phosphorylation of an N-terminal residue (e.g. Y37, S39, or S45) regulates Ptr2p ubiquitination. Also, despite the considerable amount of indirect evidence, without mass-spectrometric identification we are unable to say conclusively that K27 and K34 are sites of ubiquitin attachment.

Despite the lack of definitive data on certain mechanistic details of Ptr2p down-regulation, comparison of Ptr2p with other yeast plasma membrane transporters reveals many interesting similarities. When considered in this context, our observations support a

model of Ptr2p down-regulation, albeit speculative. In this section, Ptr2p will be compared with other yeast transporters with respect to phosphorylation, ubiquitination, and post-translational regulation in general.

Ubiquitination

Ubiquitin is a 76 amino acid protein found in all eukaryotic cells, which serves as a degradation signal for the substrate proteins on which it is covalently attached (For reviews of ubiquitin structure and function, see (Hicke 1997; Horak 2003; Dupre, Urban-Grimal et al. 2004; Pickart and Eddins 2004; Smalle and Vierstra 2004; Urbe 2005; Nandi, Tahiliani et al. 2006; Herrmann, Lerman et al. 2007). In addition to the well-known role of ubiquitination in targeting cytosolic proteins to the proteasome for degradation, ubiquitination of plasma membrane proteins targets them for internalization and sorting to the vacuole/lysosome for destruction. Ubiquitin is attached to substrates by E3-ubiquitin ligases. These E3 ligases covalently attach the C-terminus of ubiquitin, through an amide isopeptide bond, to the ε-amino group of an internal lysine residue in the target protein. Ubiquitin can also be covalently attached to lysine residues in other ubiquitin molecules, creating poly-ubiquitin chains. There are seven different lysine residues in ubiquitin (K6, K11, K27, K29, K33, K48, and K63), which allows for considerable variation in the architecture of poly-ubiquitin chains. The most common ubiquitin-attachment sites within the ubiquitin molecule are K29, K48, and K63 (Dupre, Urban-Grimal et al. 2004; Urbe 2005).

For cytosolic proteins to be efficiently targeted to the 26s proteasome, they must receive a polyubiquitin chain. The polyubiquitin chain must be at least four subunits long

with K48 ubiquitin-ubiquitin linkages (Glickman and Ciechanover 2002). For membrane proteins, mono-ubiquitination or K63-linked poly-ubiquitination of lysine residues triggers endocytosis and sorting to the vacuole for proteolytic degradation (Hicke and Dunn 2003; Horak 2003; R. Haguenauer-Tsapis 2004). The notion that ubiquitin serves as a signal triggering internalization of yeast membrane proteins is supported in large part by the following observations: 1) With yeast mutants defective in endocytosis, such as *end3Δ* and *end4Δ* mutants, an accumulation of ubiquitin-conjugated membrane proteins is observed (Kolling and Hollenberg 1994). 2) Mutation of target lysine residues within a membrane protein will often impair internalization (Staub, Gautschi et al. 1997; Soetens, De Craene et al. 2001). 3) In-frame fusions of ubiquitin to mutant membrane proteins which lack their usual target lysine residues restores internalization (Terrell, Shih et al. 1998; Roth and Davis 2000; Blondel, Morvan et al. 2004).

In yeast, the only E3 ubiquitin ligase shown to modify plasma membrane proteins is the HECT-type E3 ubiquitin ligase, Rsp5p (Rotin, Staub et al. 2000; Hicke and Dunn 2003; Horak 2003). Rsp5p has several identified functional domains: the HECT catalytic domain, three WW substrate recognition domains, and the C2 domain which appears to direct Rsp5p localization to the plasma membrane and endosomes (Wang, Yang et al. 1999; Dupre, Urban-Grimal et al. 2004). Not only is Rsp5p required for ubiquitination of yeast membrane proteins, it is also involved in endocytosis of target proteins in a manner independent of its role in ubiquitin attachment. It has been shown that deletion of the Rsp5p C2 domain impairs internalization of the general amino acid permease (Gap1p) (Springael, De Craene et al. 1999) and the uracil permease (Fur4p)(Wang, McCaffery et al. 2001) without impairing the ubiquitination of these proteins. The mechanism by which

Rsp5p recognizes and interacts with membrane protein substrates is still unclear. Rsp5p WW domains are known to interact with PPxY motifs in cytosolic target proteins, such as RNA polymerase II(Chang, Cheang et al. 2000). However, plasma membrane proteins known to be Rsp5p substrates generally don't have PPxY motifs. So the mechanism of Rsp5p interaction with membrane proteins remains unclear, and likely involves adaptor proteins. Possible candidate Rsp5p adaptor proteins include Bul1p and Bul2p. The Bul proteins contain PPxY motifs and are known to physically interact with Rsp5p via WW domains (Yashiroda, Oguchi et al. 1996; Yashiroda, Kaida et al. 1998). Some membrane proteins require Bul1p and Bul2p (putative Rsp5p adaptors) for Rsp5p-dependent ubiquitination and internalization (e.g. Gap1p)(Soetens, De Craene et al. 2001), and others do not (e.g. Fur4p)(Dupre, Urban-Grimal et al. 2004).

The ubiquitin ligase which acts upon Ptr2p has not been experimentally determined, but Rsp5p is a prime candidate. If Ptr2p was shown to be ubiquitinated by a different E3 ubiquitin ligase, it would be the first example of Rsp5p-independent ubiquitination of a yeast plasma membrane protein in the literature. It should be tested whether or not Ptr2p ubiquitination is Rsp5p-dependent, and whether or not the Bul proteins are required. Judging from the literature of Gap1p and Fur4p, if Ptr2p down-regulation is found to be dependent on Rsp5p, it is quite possible that Rsp5p's contribution to Ptr2p down-regulation is more complex than the simple act of ubiquitin transfer.

D/EXKS/T motif and EKxxxxxLK motif

From yeast membrane proteins studied thus far, a couple of substrate consensus motifs for ubiquitination have emerged. Putative ubiquitin-attachment sites for the plasma membrane transporters Ste6p(Kolling and Losko 1997), Fur4p(Marchal, Haguenauer-Tsapis et al. 2000), and Tat2p(Beck, Schmidt et al. 1999), and α-pheromone receptor Ste2p(Hicke, Zanolari et al. 1998; Terrell, Shih et al. 1998), have been identified and the target lysine residues reside within a (D/E)xK(S/T) motif (Dupre, Urban-Grimal et al. 2004). Lys 27 of Ptr2p is found within such a motif, EE**K**T. Lys 27 of Ptr2p also resides in a region with sequence similarity to Gap1p, the EKxxxxxLK motif. Unlike Ptr2p, neither of the ubiquitinated Lys residues in the Gap1p EKxxxxxLK motif fit into a (D/E)xK(S/T) motif. Thus, Ptr2p appears to have similarity to transporters with and without (D/E)xK(S/T) motifs. The fact that Ptr2p lysine residues 27 and 34, which showed such a strong mutant phenotype of impaired ubiquitination and down-regulation, reside within these ubiquitination consensus motifs adds strong support to the notion that K27 and K34 are indeed ubiquitin acceptor sites.

Acidic Stretches

Both the N and C-terminal cytoplasmic regions of Ptr2p are rich with acidic residues (Figure 15). Several studies of other yeast transporters including Ste6p (Kolling 2002), Mal61p (Medintz, Wang et al. 2000), Zrt1p(Gitan, Shababi et al. 2003), and Fur4p(Marchal, Haguenauer-Tsapis et al. 1998) have identified acidic stretches required for efficient ubiquitination. This is consistent with our observations of Ptr2p, as the

putative sites of ubiquitin-attachment (K27 and K34) are found within the acidic N-terminal cytoplasmic domain.

PEST sequences

"PEST" sequences are regions of a protein rich with proline (P), glutamic acid (E), serine (S), and threonine (T) (and aspartic acid to a lesser extent) which are usually flanked by positively charged residues. PEST sequences are thought to be degradation signals, and are usually associated with short-lived nuclear or cytoplasmic proteins (Rogers, Wells et al. 1986; Rechsteiner and Rogers 1996). The involvement of PEST-like sequences in the down-regulation of several yeast membrane proteins has been reported, including Fur4p(Marchal, Haguenauer-Tsapis et al. 1998; Marchal, Haguenauer-Tsapis et al. 2000), Ma161p(Medintz, Wang et al. 2000; Gadura and Michels 2006), and Ste3p(Roth, Sullivan et al. 1998; Roth and Davis 2000). In these studies, it was shown that mutations within the PEST-like region impaired down-regulation and ubiquitination, and with Fur4p and Ste3p the site of ubiquitin-attachment was shown to be near or within the PEST-like sequence, respectively. Ptr2p contains three potential PEST-like sequences located in the N and C-terminal cytoplasmic regions. Residues 4-16 and 48-72 in the N-terminus, and 554-582 in the C-terminus were identified by the PESTfind webtool as potential PEST sequences, with residues 48-72 showing the highest prediction score (http://www.at.embnet.org/toolbox/pestfind/). This is consistent with observations of Fur4p and Ste3p, as the predicted sites of ubiquitin-attachment within Ptr2p are located near a PEST-like region.

Overall, the notion that Ptr2p is regulated by the canonical ubiquitin degradative pathway for membrane proteins is supported by the following evidence: 1.) As mentioned above, Ptr2p possess ubiquitination consensus motifs in common with other yeast membrane proteins. 2.) Ptr2p mutants impaired in internalization are also under-ubiquitinated. 3.) Ptr2p is degraded in the vacuole, as evidenced by studies in *pep4Δ* strain deficient in vacuolar proteolysis.

In yeast, there are only two known exceptions to this ubiquitin-vacuole system of membrane protein degradation, ERAD and Ctr1p. Misfolded or mutant proteins in the endoplasmic reticulum can be subject to endoplasmic reticulum-associated degradation (ERAD), a process which involves retro-translocation of the substrate from the ER membrane to cytosol and targeting to the proteasome for destruction (Meusser, Hirsch et al. 2005). The other documented exception to the ubiquitin-vacuole system for membrane protein degradation in yeast is the copper transporter, Ctr1p. Under normal circumstances Ctr1p is a substrate for ubiquitin-linked endocytosis and vacuolar degradation, but upon exposure of yeast to high levels of copper in the medium (>10μM), Ctr1p is degraded *in situ* at the plasma membrane(Ooi, Rabinovich et al. 1996).

Phosphorylation

Phosphorylation has been linked to ubiquitination for several yeast membrane proteins. Phosphorylation is one of the most common post-translational modifications of proteins, and is estimated to affect 30% of the yeast proteome (Ptacek, Devgan et al. 2005). Reversible phosphorylation is believed to regulate, at least in part, most processes within a cell. Phosphorylation is most commonly observed on serine, threonine, and

tyrosine residues, but also occurs on histidine, lysine, and arginine residues (Matthews 1995).

With recent advances in proteomics, phosphorylation data on many yeast transporters has been obtained (Ficarro, McCleland et al. 2002; Ray and Haystead 2003; Chi, Huttenhower et al. 2007; Li, Gerber et al. 2007). However, for most yeast transporters, the role of phosphorylation remains unclear. Phosphorylation is thought to stabilize some transporters at the plasma membrane (e.g. the general amino acid permease, Gap1p)(De Craene, Soetens et al. 2001), while it is thought that phosphorylation of other transporters triggers their internalization (e.g. the uracil permease, Fur4p)(Marchal, Haguenauer-Tsapis et al. 1998; Marchal, Haguenauer-Tsapis et al. 2000). Experiments have demonstrated that phosphorylation of yeast membrane proteins occurs in different cellular locations. For example, Gap1p was shown to be phosphorylated along the secretory pathway (De Craene, Soetens et al. 2001), and Fur4p was shown to be phosphorylated mainly at the cell surface (Volland, Garnier et al. 1992).

As with cytoplasmic proteins, phosphorylation may regulate the catalytic activity or localization of membrane proteins. Phosphorylation of the plasma membrane H^+/ATPase, Pma1p, regulates its catalytic activity (Eraso, Mazon et al. 2006; Lecchi, Nelson et al. 2007). For other yeast transporters such as the uracil permease (Fur4p), the maltose permease (Mal61p), the **a**-pheromone efflux transporter (Ste6p), and the general amino acid permease (Gap1p), phosphorylation may affect subcellular localization (Marchal, Haguenauer-Tsapis et al. 1998; De Craene, Soetens et al. 2001; Kolling 2002; Gadura and Michels 2006).

The evidence that phosphorylation may regulate the turnover or subcellular localization of yeast plasma membrane permeases is based primarily on mutational analyses. In these studies phosphoacceptor residues are replaced with alanine (to mimic the unphosphorylated state) or aspartic acid or glutamic acid (to mimic the phosphorylated state). Many experiments also use kinase deficient cells to examine the role of phosphorylation. These studies have led many to propose that phosphorylation regulates ubiquitination. For example, it was shown that Fur4p is phosphorylated at the plasma membrane (Volland, Garnier et al. 1992). Later experiments showed that cells deficient in the activity of certain kinases, or serine to alanine mutations of the phosphoacceptor sites in a PEST-like region impair Fur4p ubiquitination and endocytosis (Marchal, Haguenauer-Tsapis et al. 1998; Marchal, Haguenauer-Tsapis et al. 2000). These observations led the researchers to proclaim that "The modification of uracil permease by phosphorylation at the plasma membrane is a key mechanism for regulating endocytosis of this protein. This modification in turn facilitates its ubiquitination and internalization."(Marchal, Haguenauer-Tsapis et al. 2000) This may be true in a sense, but such language can be misleading. Their experiments established that the phosphorylated residues are important for ubiquitination and internalization, and they are justified in claiming that phosphorylation *facilitates* Fur4p ubiquitination/internalization. But claiming that phosphorylation *regulates* ubiquitination/internalization of Fur4p implies that phosphorylation only occurs as part of the down-regulation process and that phosphorylation leads directly to ubiquitination/internalization. It could be that phosphorylation of Fur4p at the plasma membrane is constitutive, and that the phosphorylated motif is recognized by the ubiquitin-ligase. The true *regulatory* event

might not be at the level of phosphorylation, it might be at level of the ubiquitin ligase and/or its association with Fur4p. Their data seems consistent with the notion that phosphorylation truly *regulates* ubiquitination/internalization, but it has not been proven. To be fair, you *could* say that phosphorylation of Fur4p regulates ubiquitination/internalization, but only in the sense that if you block phosphorylation (in artificial experimental conditions) you block ubiquitination/internalization. But again, such language is misleading.

Similar uncertainty also exists with phosphorylation studies on other yeast plasma membrane proteins including Ste6p (Kelm, Huyer et al. 2004). With Fur4p and Ste6p it has been shown that impairing phosphorylation (by mutation of phosphoacceptor sites, or through the use of kinase mutants) results in changes in regulation, but it has not been shown that the phosphorylation state of the transporter changes with changes in regulation. The data on Fur4p and Ste6p is consistent with phosphorylation regulating ubiquitination/endocytosis, but does not prove it.

The notion that phosphorylation regulates ubiquitination/internalization rests on a firmer foundation with Gap1p and the pheromone receptors, Ste2p and Ste3p. With these plasma membrane proteins it has been shown that phosphorylation changes with changes in regulation, and that impairment of normal phosphorylation impairs regulation(Roth and Davis 1996; Hicke, Zanolari et al. 1998). In contrast to Fur4p and Ste6p, impairment of Gap1p phosphorylation increases down-regulation (De Craene, Soetens et al. 2001).

The yeast genome contains more than 120 protein kinase genes (Hunter and Plowman 1997). Protein kinases are involved large regulatory networks, and affect

virtually every process in a living cell. As would be expected, many of the identified yeast kinases have been shown to affect the phosphorylation of yeast transporters. For example, phosphorylation of Gap1p is dependent upon the Npr1p kinase(De Craene, Soetens et al. 2001), and phosphorylation of Fur4p and Pdr5p is dependent upon the redundant casein kinase 1 isoforms, Yck1p and Yck2p(Decottignies, Owsianik et al. 1999; Marchal, Haguenauer-Tsapis et al. 2000). However, in most cases where kinase involvement has been shown, direct phosphorylation of the transporter by the kinase has not been demonstrated, and it is likely that the identified kinase indirectly affects phosphorylation.

Two phosphoproteomics experiments have identified seven phosphorylated residues in Ptr2p (Y37, S39, S45, S594, S597, T598, and T599)(Ficarro, McCleland et al. 2002; Li, Gerber et al. 2007). The kinase(s) which phosphorylate these residues in Ptr2p have not been identified. The NetPhosK 1.0 server produces neural network predictions of kinase specific phosphorylation sites within proteins (http://www.cbs.dtu.dk/services/NetPhosK/) (Blom, Sicheritz-Ponten et al. 2004). NetPhosK kinase predictions for the experimentally-determined phosphorylation sites in Ptr2p are as follows: Y37- INSR kinase, S39 and S45-CKII, S597 and T598- PKC. NetPhosK does not make kinase predictions for S594 or T599.

Model of Ptr2p Down-Regulation

The model of Ptr2p down-regulation that emerges from our experiments and from comparison of Ptr2p with other yeast membrane proteins is fairly simple, and speculative. When a rich nitrogen source (e.g. ammonium sulfate) is added to PTR2-induced yeast

cells, down regulation of Ptr2p is triggered. An E3 ubiquitin ligase (probably Rsp5p) is recruited to the N-terminal cytoplasmic domain of Ptr2p, with the possible assistance of adaptor proteins. Phosphorylated residues in the N-terminal cytoplasmic domain (Y37, S39, and S45) appear to be important for E3 recognition of Ptr2p. Once the E3 ubiquitin ligase is recruited to the N-terminus, adjacent lysine residues (probably K27 and K34, and perhaps others) are ubiquitinated. Ubiquitination of Ptr2p triggers internalization and sorting to the vacuole for proteolysis.

Conclusion

This research project started with clear goals, to answer the question; "What is the role the phosphorylation in Ptr2p?" One might assume that answering such a short, simple question would be straight forward, or easy. Yet, it was not. We cannot definitively say whether or not reversible phosphorylation of a residue(s) in our favorite protein regulates subcellular localization or catalytic activity. But alas, we are not alone as scientists who can't answer this question, despite language that might, at a passing glance, suggest otherwise. Closer inspection of the literature reveals that much of the data on the role of phosphorylation in yeast membrane proteins is just as inconclusive or indirect as ours on Ptr2p. We refrain from claiming phosphorylation regulates ubiquitination/internalization of our protein, yet other groups with similar data do not refrain. In the literature, the term "regulate" can be vague, misinterpreted, or used inaccurately. In the most accurate sense, to say (with certainty) that phosphorylation of a protein "regulates" activity/localization, one must first be able to demonstrate that phosphorylation status changes with changes in regulation, and furthermore, that the

change in phosphorylation is the *cause* of the change in activity/localization and doesn't merely *correlate* with the change. Although there is a plethora of compelling evidence with different systems, few approach the high bar of metaphysical certitude.

Having said that all of that, it is possible to answer questions regarding the role of phosphorylation with a degree of certainty. It takes a lot of work though, and one must at least be able to show that phosphorylation changes with changes in regulation and that impairment of phosphorylation affects regulation. If the phosphorylation status of a particular residue actually changes with changes in regulation, it may be quite difficult to detect. This is especially true if there are other phosphorylated residues in the protein, or if the particular phosphorylated species is short lived or of low abundance. 2-D gel electrophoresis may assist in resolving changes in phosphorylation of residues in multiply phosphorylated proteins. Detecting a short-lived phosphorylated species may rely on artificially accumulating the particular phosphorylated form, using phosphatase inhibitors, kinase inhibitors, phosphatase mutants, or kinase mutants. Experiments examining protein phosphorylation can be tedious; they must be designed and interpreted carefully.

While it takes a considerable amount of work to clearly elucidate the role of phosphorylation for individual proteins, the field is clearly headed in that direction, and the pace is accelerating. Advances in proteomics have been tremendous. Not long ago, identification of any phosphorylation sites within your favorite protein would always require a lot of work, and would always merit a publication. Today, phosphoproteomics experiments using mass spectrometry are identifying hundreds, or thousands, of phosphorylation sites throughout the proteome in a single experiment(Ficarro, McCleland et al. 2002; Ray and Haystead 2003; Brinkworth, Munn et al. 2006; Chi, Huttenhower et

al. 2007; Li, Gerber et al. 2007). Our research shows that this type of phosphorylation data is not comprehensive (due to biases at different points in the experimental technique), but nonetheless, it is providing a wealth of data, accelerating research across the board. While tremendously useful, proteomics will not tell us everything, and it will certainly not replace "low throughput" scientific research and lab groups which slave away on individual proteins.

Our Ptr2p Research and the Simple Story

When growing under poor nutrient conditions, *S. cerevisiae* up-regulates many plasma membrane transporters, including Ptr2p, which increases the cell's ability to scavenge various alternative nutrients from the environment. These various transporters are a great asset to a yeast cell trying to satisfy its nutritional requirements, but their assistance comes at a price, namely energy. When nutrient conditions are rich, the service of many of these nutrient transporters is no longer required, and the needless presence of a transporter at the plasma membrane is an energy liability for the cell. As models of efficiency, yeast cells quickly adapt by selectively retrieving these transporters from the membrane and sending them to the vacuole for destruction. The work presented in this dissertation has shown that N-terminal cytoplasmic domain of Ptr2p is important for ammonium-induced down-regulation. Thus, we have made advances in understanding how the yeast cell responds after it has decided "No more peptides... I've had enough!".

Appendix

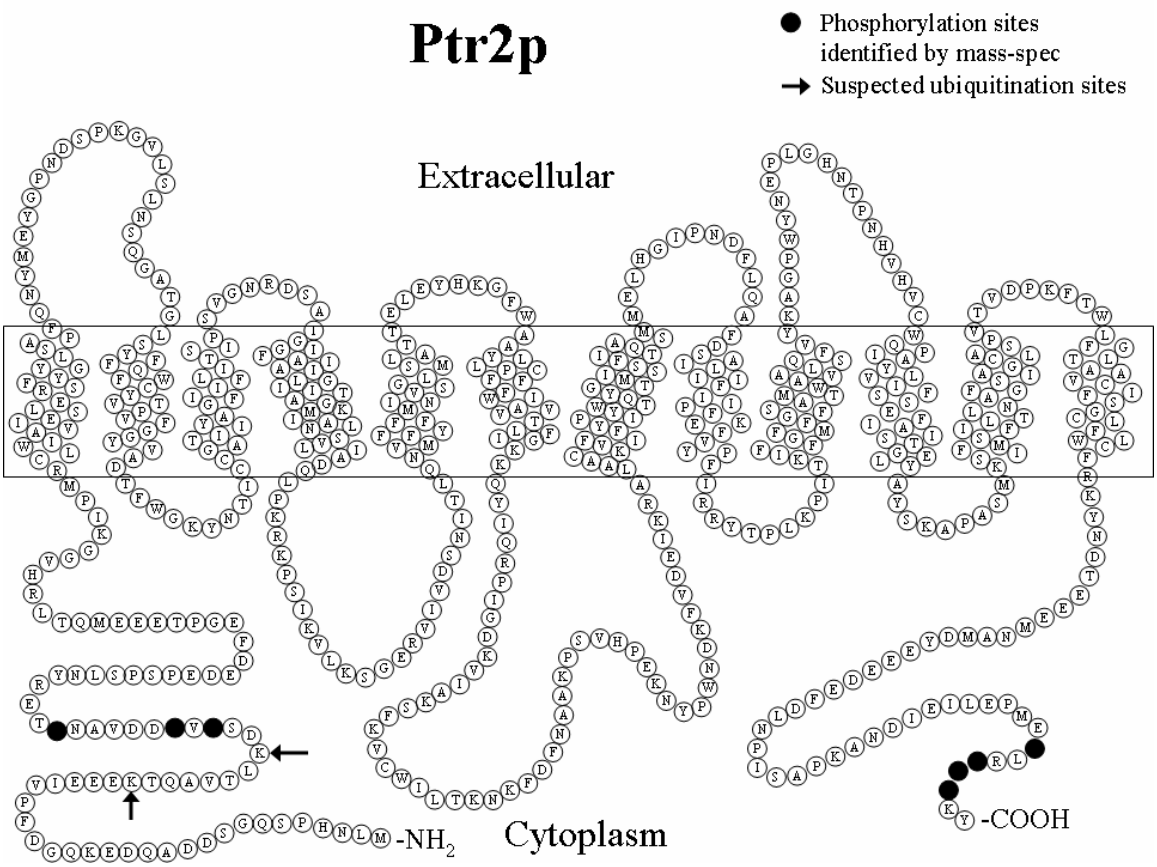

Figure 1: Ptr2p Model. This transmembrane topology model of Ptr2p is based upon a Kyte-Doolittle prediction. Phosphorylation sites (Y37, S39, S45, S594, S597, T598, and T599) and suspected ubiquitin attachment sites (K27 and K34) are shown.

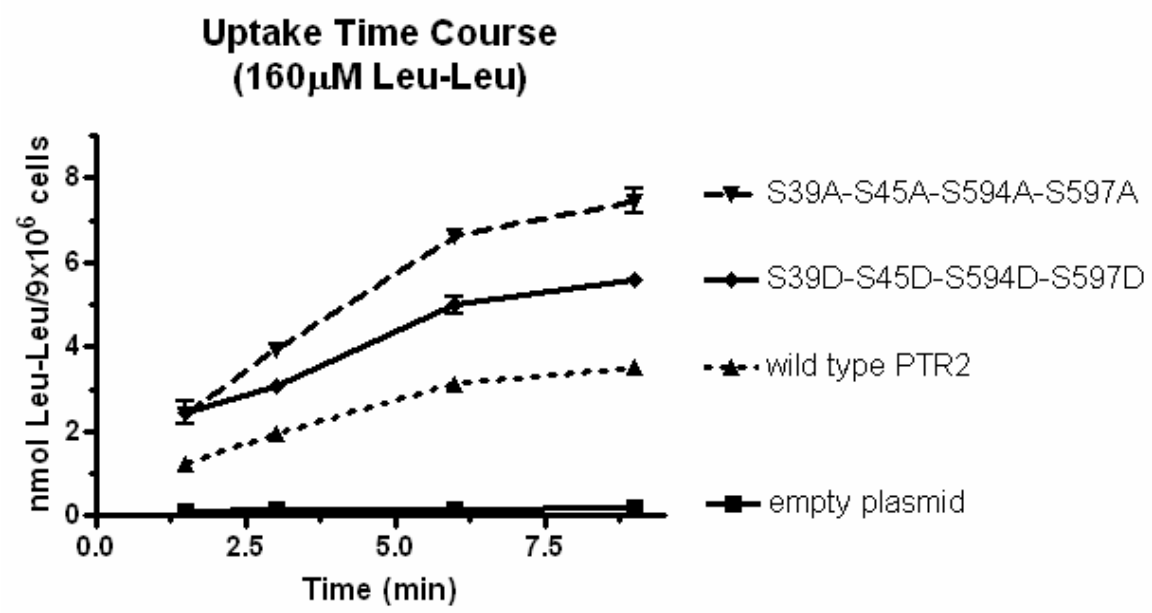

Figure 2: Phosphorylation-site mutations increase cellular peptide transport activity. ^{3}H-leucyl-leucine uptake (160μM Leu-Leu) is measured over a short time-course (1.5, 3, 6, and 9 minutes). Wild-type Ptr2p is compared with S39A-S45A-S594A-S597A Ptr2p and S39D-S45D-S594D-S597D Ptr2p, and empty plasmid is included as a negative control. Both quadruple mutants show increased peptide transport activity relative to wild-type, with the serine to alanine mutant showing the greatest increase. ($n=3$, $P<0.001$ for 1-way ANOVA)

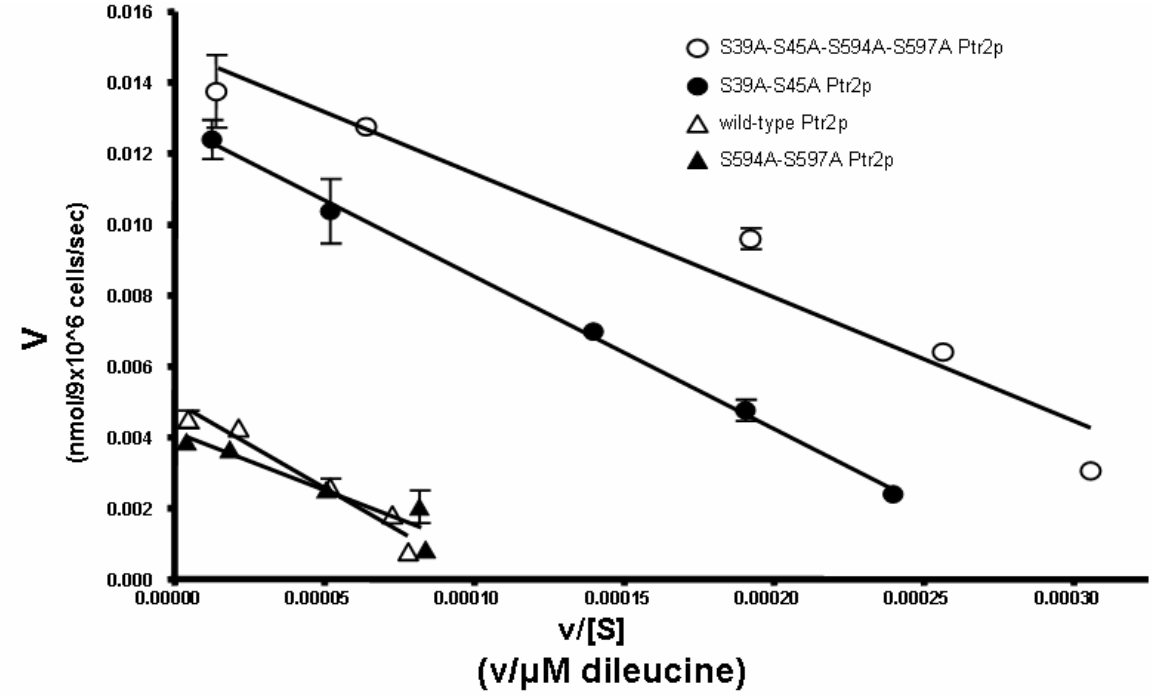

Figure 3: Kinetic Analysis of ^{3}H-leucyl-leucine transport. ^{3}H-leucyl-leucine uptake over a 45 second time period was measured for wild-type and mutants (S39A-S45A-S594A-S597A, S39A-S45A, and S594A-S597A) in a range of substrate concentrations (1mM, 200μM, 50μM, 25μM, and 10μM). This figure is an Eadie-Hofstee plot of the transport data; transport velocity (v) is plotted against velocity/substrate concentration (v/[S]). In this type of plot, V_{max} is determined by the Y-intercept, and the slope of the line corresponds to the negative K_m. The data shows that the S39A-S45A mutation causes a large (2.5-3 fold) increase in cellular dipeptide uptake V_{max}. The K_m of the transport system was not greatly affected. Data analysis by Michealis-Menton, Lineweaver-Burke, and Eadie-Hofstee methods all yielded similar results. (n=4, $P<0.001$ for 1-way ANOVA)

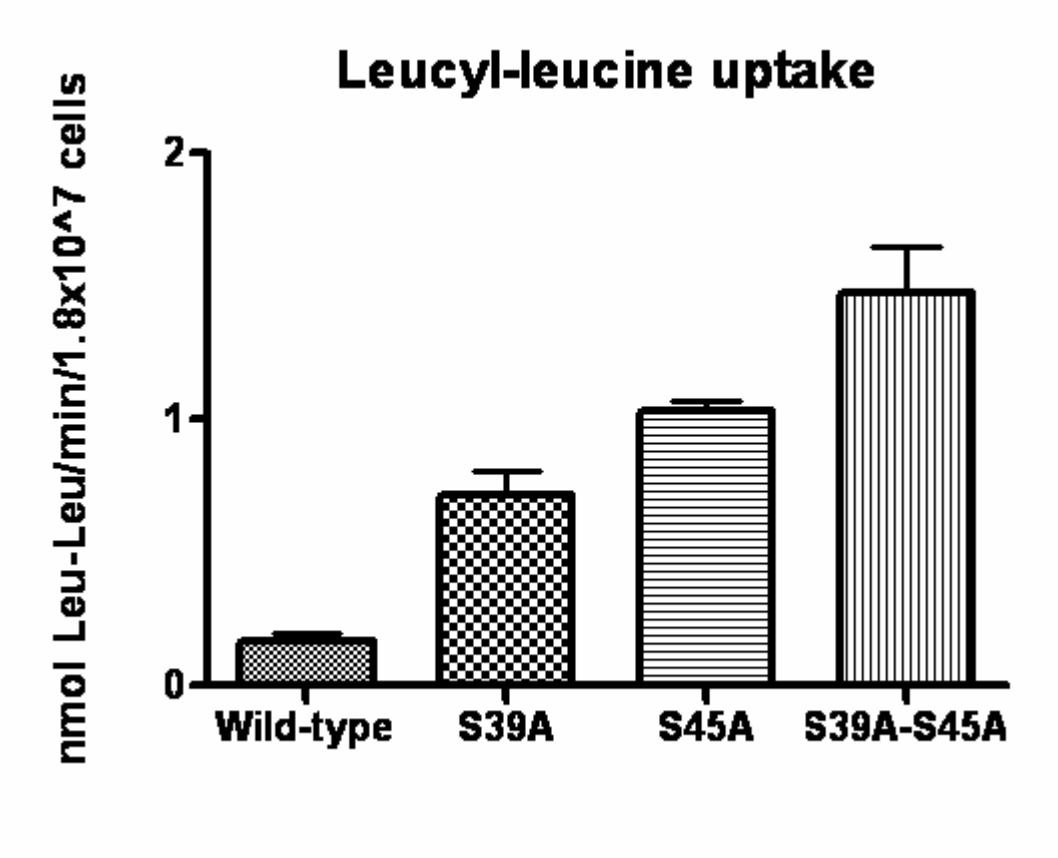

Figure 4: S39A and S45A Mutant Uptake Assay. The uptake of ^{3}H-leucyl-leucine (200μM) over 60 seconds was measured for wild-type Ptr2p, single mutants S39A Ptr2p and S45A Ptr2p, and the double mutant S39A-S45A Ptr2p. The S39A-S45A Ptr2p mutant shows the greatest increase in transport activity (n=4, $P<0.001$ for 1-way ANOVA).

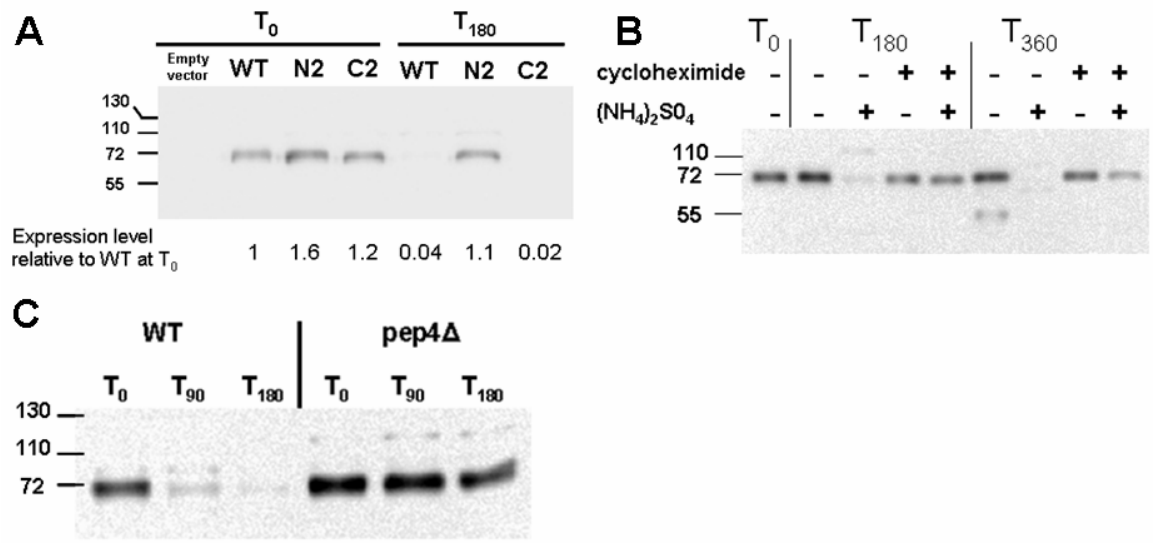

Figure 5: Down-Regulation. A: Ammonium Sulfate Repression of Ptr2p is impaired by the S39A-S45A mutation. This figure is an α-FLAG western blot. Samples were taken before, or three hours after, treatment with ammonium sulfate. Wild type (WT) is compared with the S39A-S45A mutant (N2) and the S594A-S597A mutant (C2). **B: Ptr2p down-regulation requires protein synthesis.** Ammonium sulfate induced down-regulation of wild-type Ptr2p is blocked by cycloheximide. **C: Ptr2p is degraded in the vacuole.** Ammonium sulfate induced destruction of Ptr2p is blocked in a *pep4Δ* strain, which is deficient in vacuolar proteolysis. Pep4p is a "master" vacuolar protease which is needed for the maturation of other vacuolar proteases.

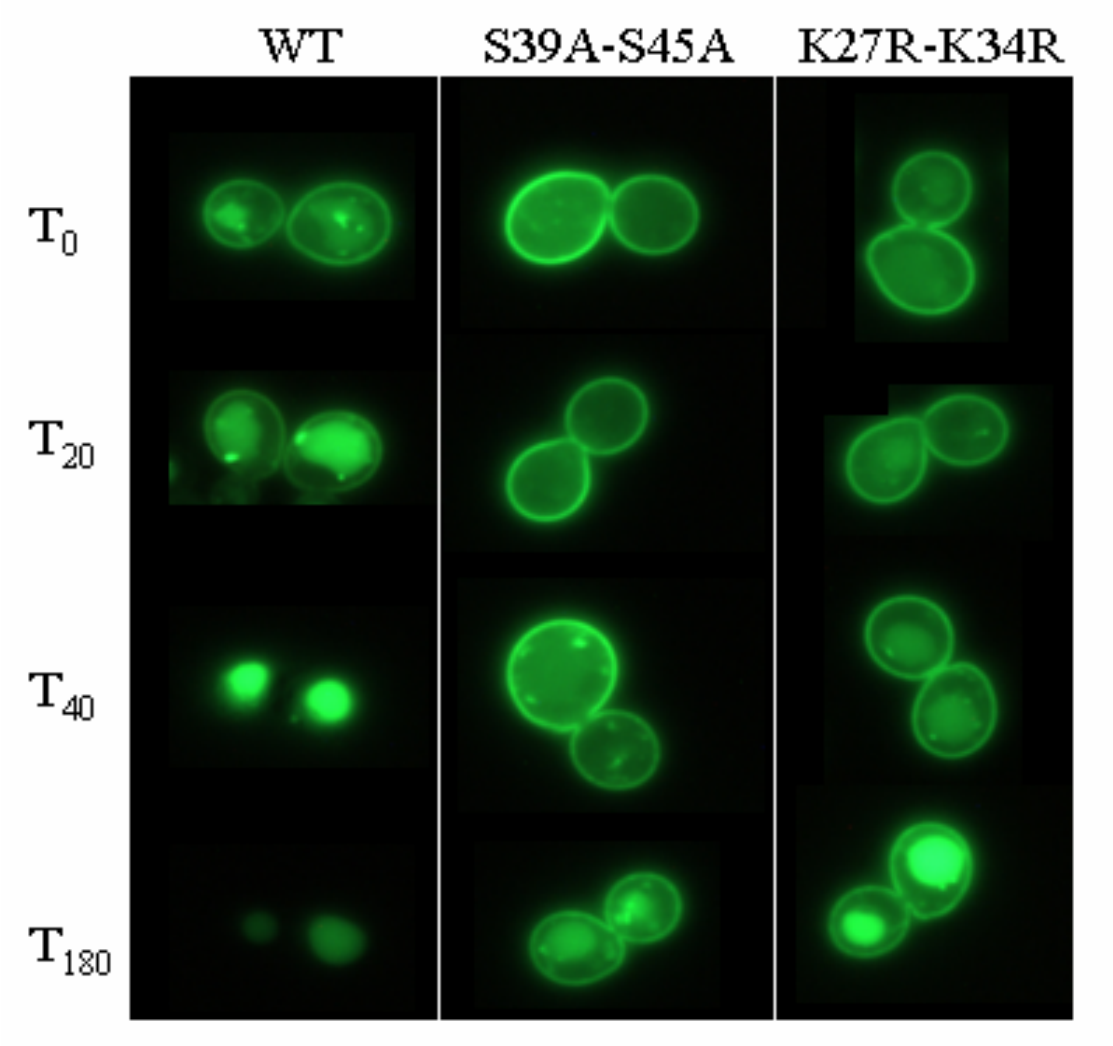

Figure 6: Ptr2p-GFP localization time course. This figure shows the localization of Ptr2p-GFP before, and after, a shift from inducing to non-inducing conditions (addition of ammonium sulfate at T_0). The localization of wild-type (WT) Ptr2p is compared with two mutants over a three hour time course. (Single cells were not followed over time. Rather, this figure was produced using images of cells that were representative of the greater population, to illustrate subcellular localization over the time course.) The two mutants exhibit impaired down-regulation; the mutant forms persist at the plasma membrane much longer after addition of ammonium sulfate.

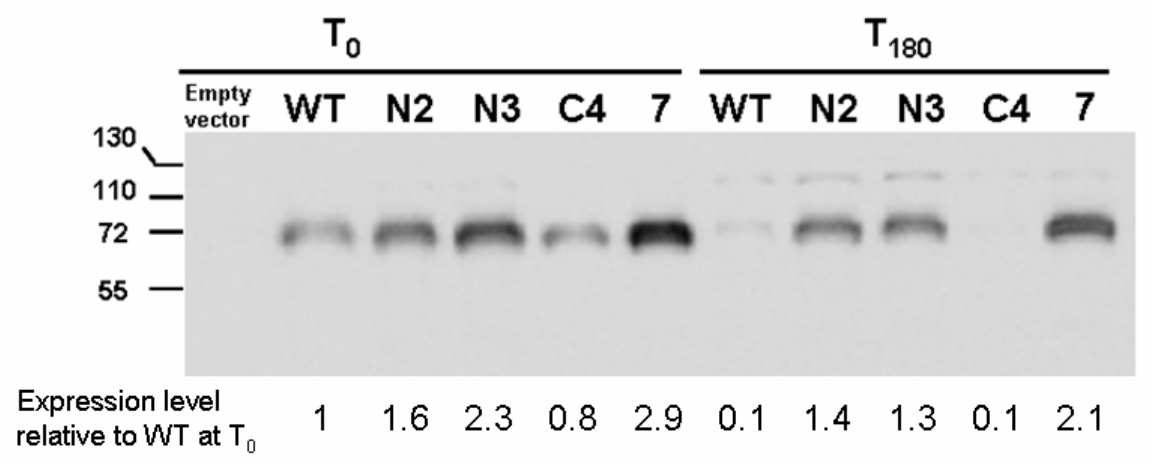

Figure 7: Ammonium Sulfate Repression of Ptr2p is impaired by the Y37F-S39A-S45A mutation. This figure is an α-FLAG western blot. Samples were taken before, or three hours after, treatment with ammonium sulfate. The Y37F mutation increases Ptr2p accumulation and impairs down-regulation, amplifying the S39A-S45A phenotype. C-terminal mutations did not have an affect in this experiment.

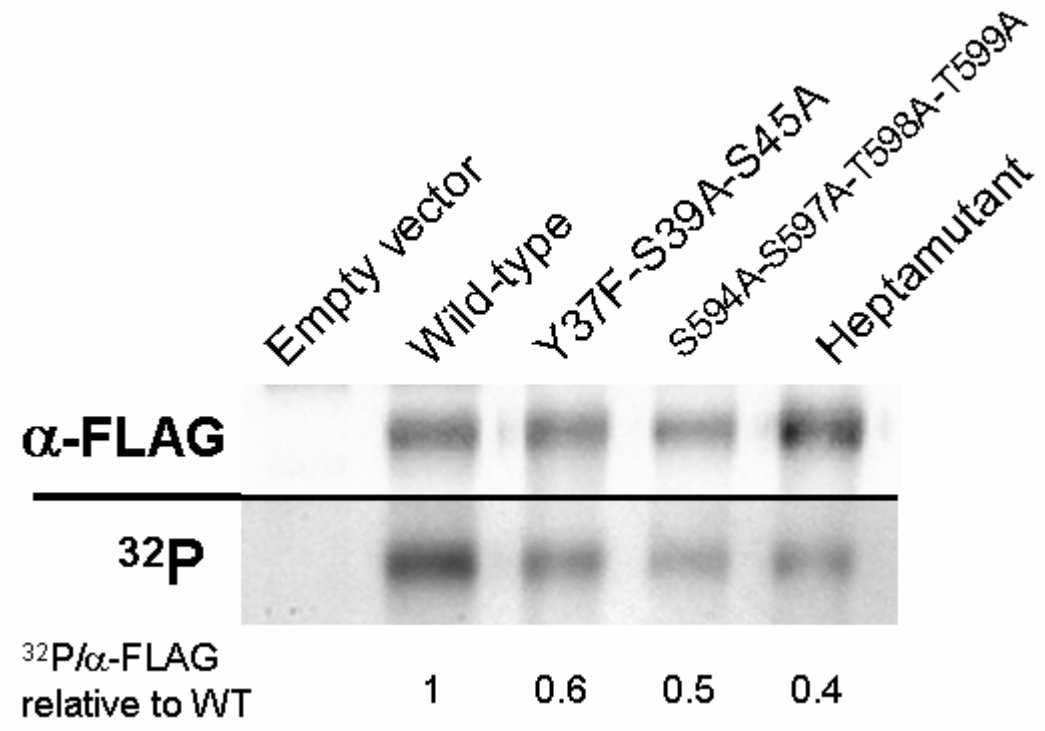

Figure 8: Ptr2p phosphorylation assayed by ^{32}P Metabolic Labeling. This figure shows a western blot image, and the corresponding autoradiograph, of immunoprecipitated Ptr2p. Wild-type (WT) Ptr2p is compared with various mutants; N-terminal triple mutant Y37F-S39A-S45A, C-terminal quadruple mutant S594A-S597A-T598-T599, and the heptamutant Y37F-S39A-S45A-S594A-S597A-T598-T599. Ptr2p levels are assessed with an α-FLAG 1° Ab and an HRP-conjugate 2° Ab. Phosphorylation of Ptr2p is measured by autoradiography. The heptamutant Ptr2p is still labeled by ^{32}P, demonstrating that other phosphorylation sites exist within Ptr2p, beyond the seven identified sites Y37, S39, S45, S594, S597, T598, T599. (For more accurate comparison, the gels were loaded with differing sample volumes in effort to obtain similar levels of purified Ptr2p between the wild-type and various mutants.)

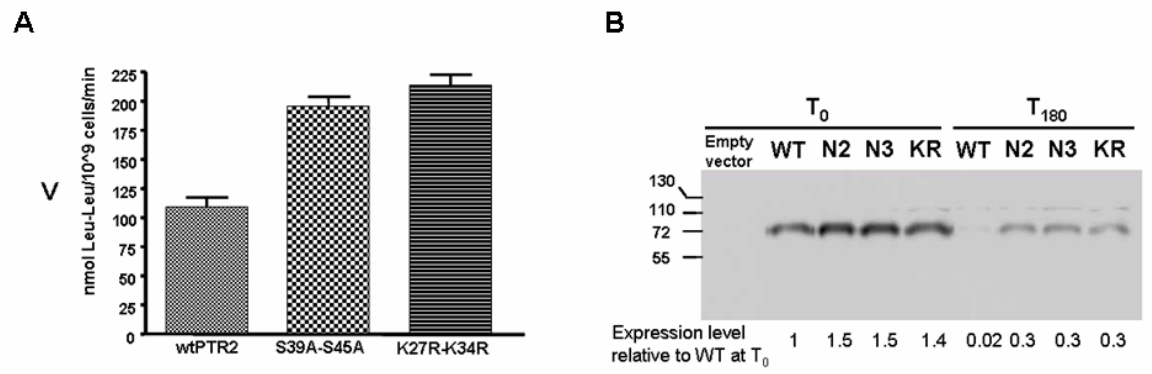

Figure 9: K27R-K34R mutation has an effect similar to the mutation of phosphorylation sites in the N-terminal cytoplasmic domain. **A.** An uptake assay, where yeast import of ^{3}H-leucyl-leucine (200μM) is measured. (n=4, $P<0.001$ for 1-way ANOVA) The K27R-K34R mutation increased ^{3}H-leucyl-leucine uptake, similar to the S39A-S45A mutant. **B.** An α-FLAG western blot image showing expression levels of wild-type Ptr2p (WT), the S39A-S45A mutant (N2), the Y37F-S39A-S45A mutant (N3), and the K27R-K34R mutant (KR). Similar to the N2 and N3 mutations, the K27R-K34R mutation impairs ammonium-induced down-regulation of Ptr2p.

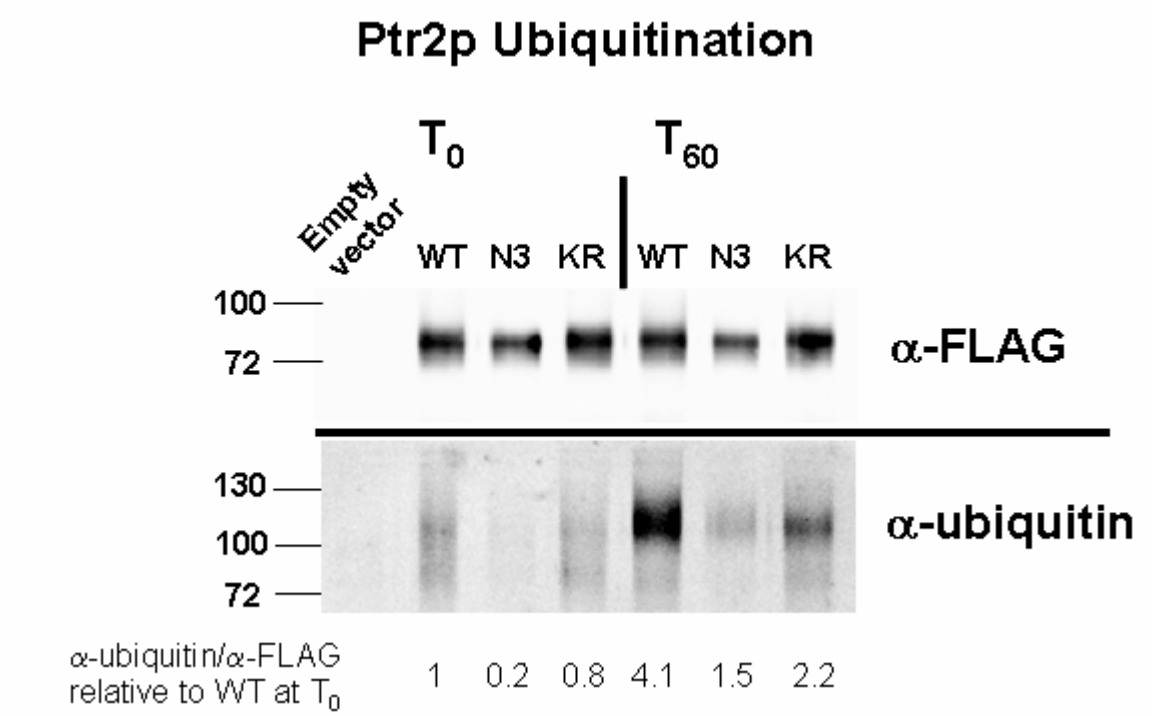

Figure 10: Ubiquitination of Ptr2p as measured by western blot before, or sixty minutes after, addition of ammonium sulfate. Blots with affinity-purified Ptr2p samples were probed with either α-FLAG or α-ubiquitin antibody. (Sample loads were adjusted to achieve comparable levels of Ptr2p.) Wild-type (WT) Ptr2p is compared to the Y37F-S39A-S45A mutant (N3) and the K27R-K34R mutant (KR). Ubiquitination of Ptr2p increases greatly after ammonium sulfate treatment, and mutations to the N-terminus of Ptr2p (Y37F-S39A-S45A or K27R-K34R) impair this effect.

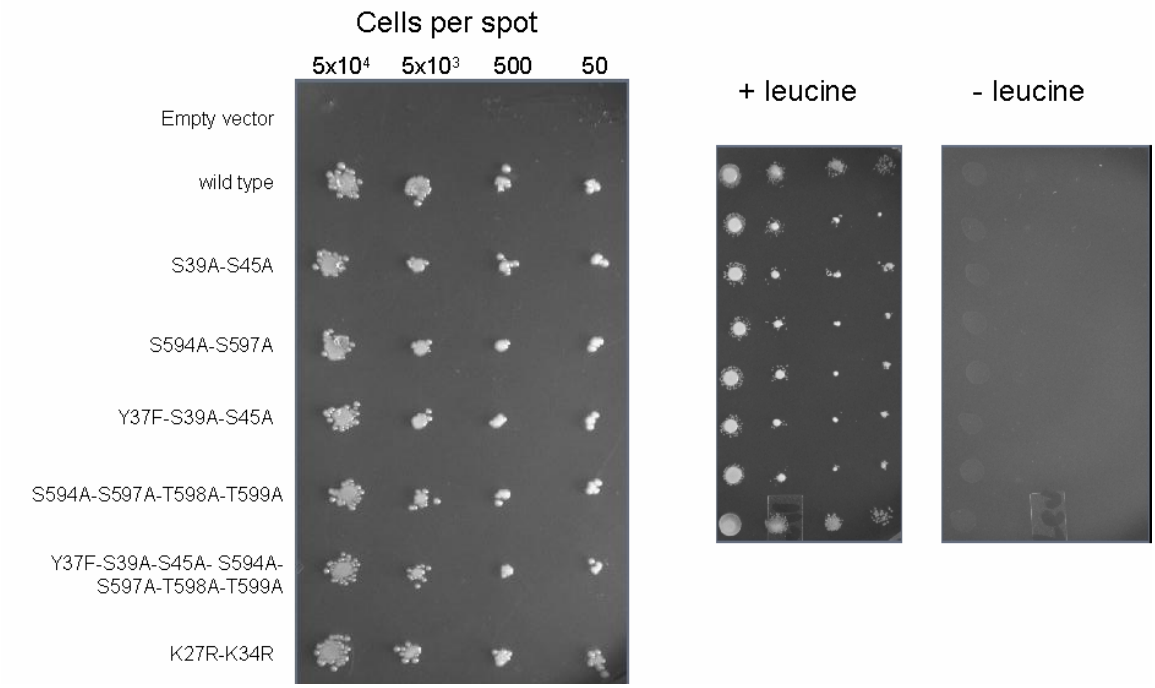

Figure 11: Growth Assay. Yeast expressing different *PTR2* mutants were grown on leucine-dropout medium supplemented with leucyl-leucine (160uM). The leucine-auxotrophic yeast strains must express functional Ptr2p in order to import leucyl-leucine and use it as a leucine source. Positive controls (+ leucine) and negative controls (- leucine) are included. As evidenced by growth, all mutant forms of Ptr2p are functional in peptide transport. Cells without Ptr2p (empty vector) fail to grow on the peptide-transport selective medium.

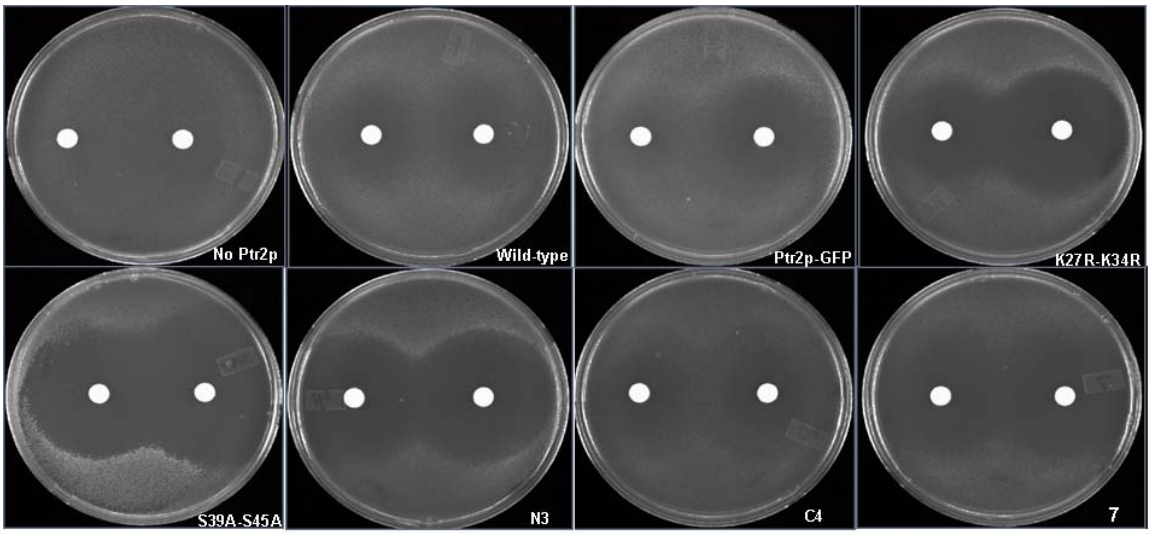

Figure 12: Toxic Peptide Halo Assay. Yeast are tested for sensitivity to alanyl-ethionine (a toxic dipeptide). Filter discs contain either 5µl (left) or 10µl (right) of 40mM alanyl-ethionine. A halo indicates sensitivity to the toxic peptide, and confirms peptide transport. Wild-type is compared the negative control (No Ptr2p, carrying empty vector) and with the following mutants: Ptr2p-GFP, K27R-K34R, S39A-S45A, Y37F-S39A-S45A (N3), S594A-S597A-T598A-T599A (C4), and Y37F-S39A-S45A-S594A-S597A-T598A-T599A (7). All mutants tested were functional in peptide transport.

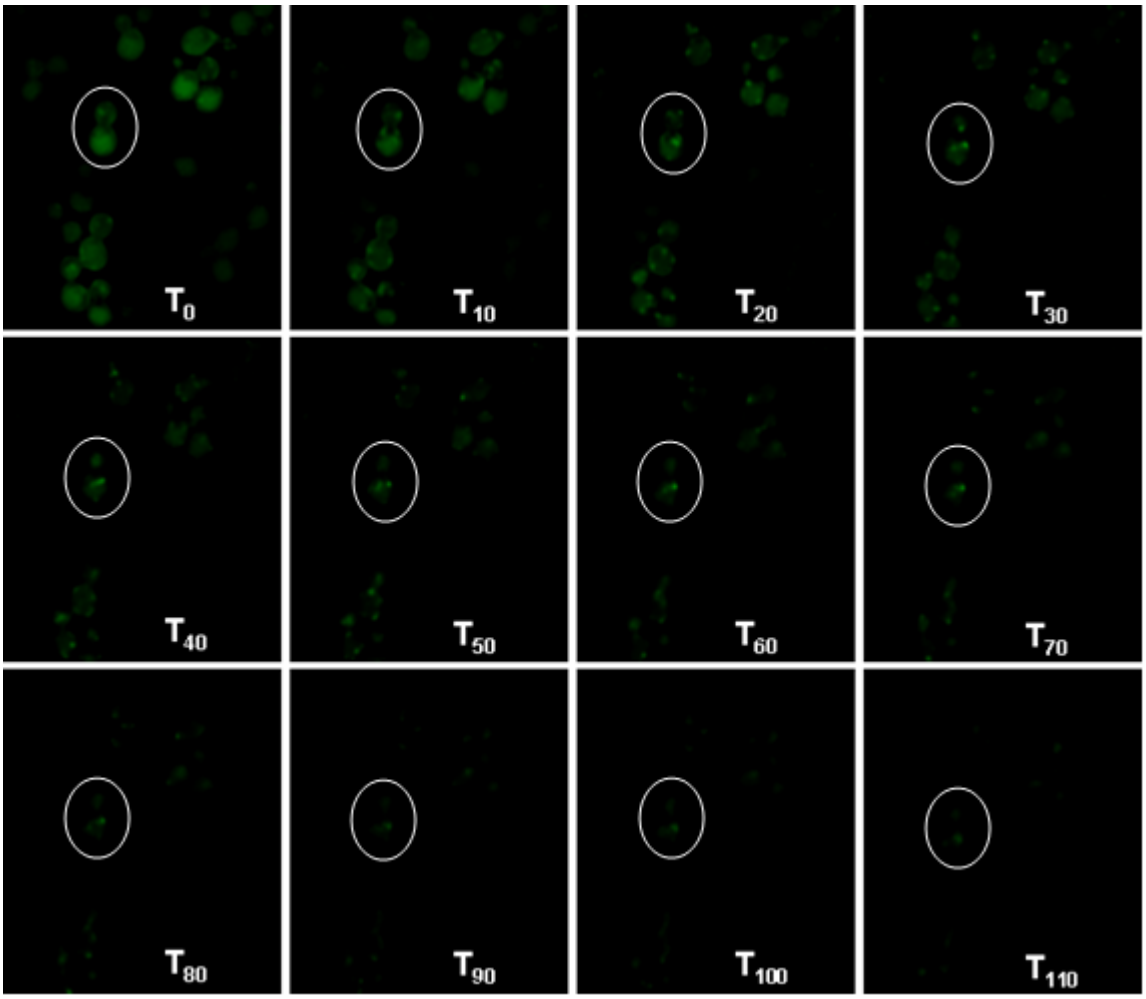

Figure 13: Time Lapse Microscopy-Wild-type. This figure was generated using a series of still images collected from a time-lapse microscopy experiment (see Plate 1 for complete video). Yeast cells expressing Ptr2p-GFP are viewed for 110 minutes. Cells were shifted from PTR2-inducing conditions to PTR2-repressing conditions at T_{15} (between images of T_{10} and T_{20}) by addition of ammonium sulfate. Ptr2p-GFP down-regulation is observed. Representative cells are highlighted.

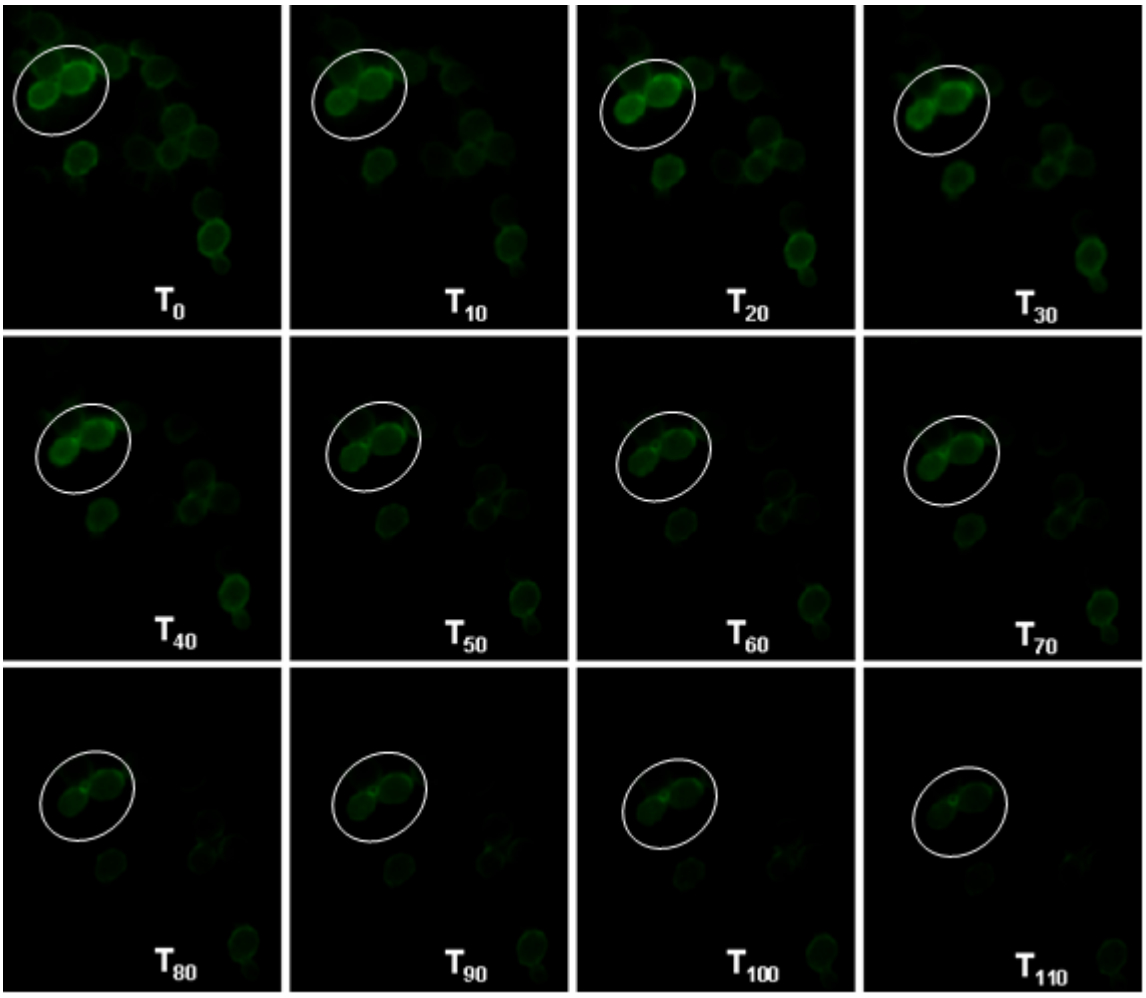

Figure 14: Time Lapse Microscopy-Y37F-S39A-S45A mutant. This figure was generated using a series of still images collected from a time-lapse microscopy experiment (see Plate 1 for complete video). Yeast cells expressing Y37F-S39A-S45A Ptr2p-GFP are viewed for 110 minutes. Cells were shifted from PTR2-inducing conditions to PTR2-repressing conditions at T_{15} (between images of T_{10} and T_{20}) by addition of ammonium sulfate. Impaired down-regulation of Y37F-S39A-S45A Ptr2p-GFP is observed. Representative cells are highlighted.

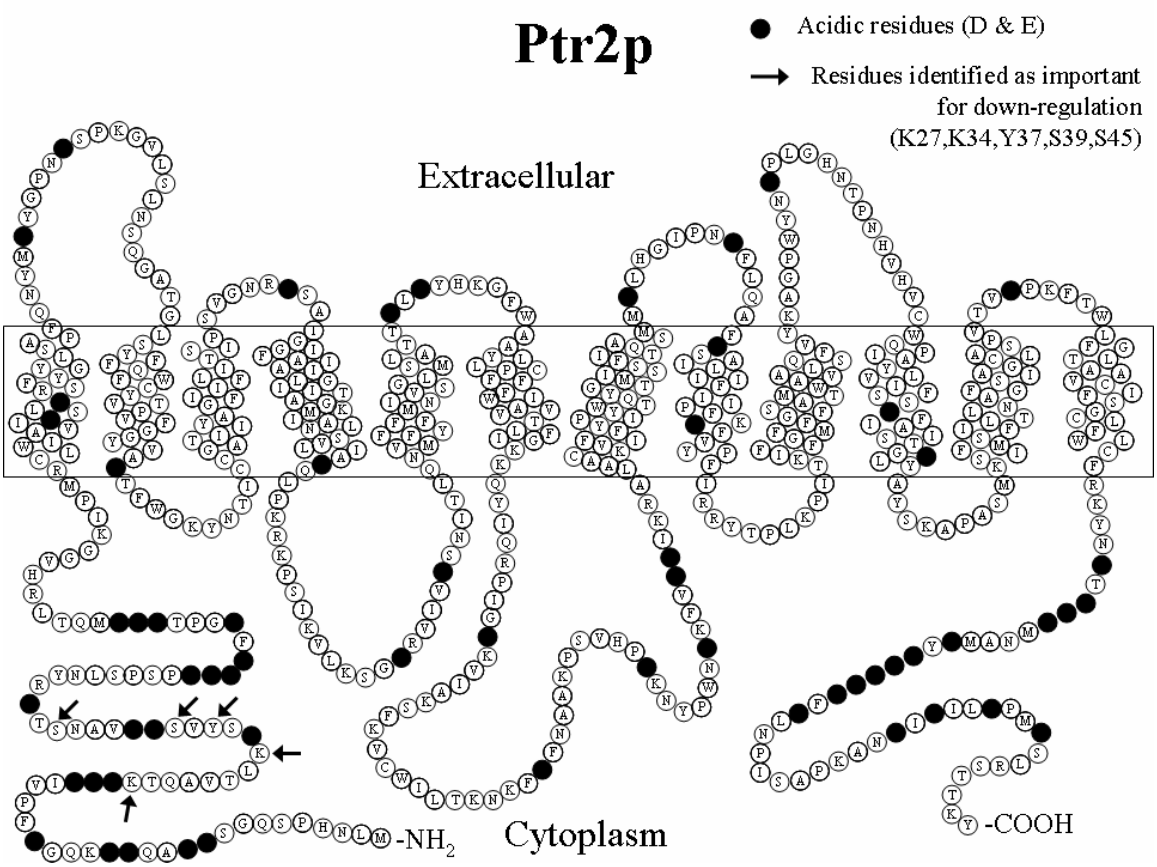

Figure 15: Ptr2p Model – Acidic Residues are shown in relation to N-terminal residues demonstrated to be important for ammonium-induced down-regulation.

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Vita

Steven C. Minkin Jr. was born in Knoxville, TN on October 25, 1977. From Knoxville he moved to Texas, and then to Lafayette, LA when he was three years old. He lived in Lafayette until 1986, when he moved to Idaho Falls, ID. In 1995, Steven graduated from Idaho Falls High School. From there, he went to the University of Tennessee, Knoxville and received his B.S. in Biochemistry, Cellular and Molecular Biology in 2001.