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Ligand-activated proteolysis in NUTRIENT SIGNALING

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ridendo dicere severum

[Through what is laughable say what is somber]
F. Nietzsche. The Case of Wagner,
Turinese Letter of May 1888

Abstract

Cells respond to changing nutrient availability and make adjustments in physiological processes. Central for making proper adjustments is the ability to execute appropriate changes in patterns of gene expression. The budding yeast *Saccharomyces cerevisiae*, responds to the presence of extracellular amino acids by up regulating systems that internalize these nutrients. Extracellular amino acids are sensed by the amino acid transporter-like receptor Ssy1p that is localized to the plasma membrane. Ssy1p generates a signal that is transmitted via a pathway minimally composed of the core components Ssy1p, Ptr3p, and Ssy5p (SPS). The SPS sensor pathway ultimately up regulates genes encoding amino acid transporters, also known as amino acid permeases.

This thesis describes the elucidation of the mechanism connecting amino acid induced signals generated at the plasma membrane and gene regulation in the nucleus. A genetic selection for mutations that enable amino acid permease genes to be expressed even in the absence of a functional SPS sensor pathway identified the dominant *ASI13-1* mutation. The *ASI13-1* gene encodes a constitutively active form of the transcription factor Stp1p that lacks its regulatory N-terminal domain. Stp1p and its close homologue Stp2p are synthesized as latent cytoplasmic precursors. In response to extracellular amino acids, the SPS sensor induces the rapid endoproteolytic processing of Stp1p and Stp2p. The shorter forms of these transcription factors, lacking N-terminal inhibitory domains, are targeted to the nucleus, where they transactivate SPS-sensor target genes.

Several genetic approaches have been applied to identify mutations that affect the SPS sensor pathway. A novel genetic selection specifically designed for rare mutations that affect the SPS-sensing pathway identified the F-box protein Grr1p as an obligatory factor required for Stp1p and Stp2p processing. Genetic analysis suggests that Grr1p has a role in signal transduction within the SPS sensor.

The N-terminal domains of Stp1p and Stp2p contain two conserved motifs that are required for proper nuclear exclusion and proteolytic processing. These motifs function in parallel; mutations that abolish processing inhibit signaling, whereas mutations that interfere with cytoplasmic retention result in constitutive activation of SPS sensor-regulated genes independently of processing. The N-terminal domain of Stp1p is functionally autonomous and transferable to other transcription factors, where its presence confers regulated nuclear exclusion and SPS sensor-induced proteolytic processing.

Proteolytic processing of recombinant Stp1p in cell free lysates supports the notion of a SPS sensor activated protease. Analysis indicates that Ssy5p is a chymotrypsin-like serine protease that is activated via the SPS sensor pathway and is responsible for Stp1p and Stp2p processing. Mutations in the predicted catalytic triad of Ssy5p abolish Stp1p processing. A constitutive *SSY5* mutant promotes processing of Stp1p even in the absence of amino acids or Ssy1p and Ptr3p. Finally, Stp1p is processed when heterologously coexpressed together with activated Ssy5p in *Schizosaccharomyces pombe*, an organism that lacks the SPS sensor pathway.

Taken together, these results define a unique and streamline metabolic control pathway that directly routes nutrient signals initiated at the plasma membrane to transcriptional activation in the nucleus.

Table of contents

List of original publications

I. Forsberg, H., M. Hammar, C. Andréasson, A. Molinér and P.O. Ljungdahl. (2001). Suppressors of *ssy1* and *ptr3* null mutations define novel amino acid sensor-independent genes in *Saccharomyces cerevisiae*. *Genetics*. **158**:973-988.

II. Andréasson C. and P.O.Ljungdahl. (2002). Receptor-mediated endoproteolytic activation of two transcription factors in yeast. *Genes & Development*. **16**:3158-3172.

III. Andréasson C., E. P. A. Neve and P.O.Ljungdahl. (2004). Four permeases import proline and the toxic proline analogue azetidine-2-carboxylate into yeast. *Yeast*. **21**:193-199.

IV. Andréasson C. and P.O.Ljungdahl. (2004).The N-terminal regulatory domain of Stp1p is modular and, fused to an artificial transcription factor, confers full Ssy1p-Ptr3p-Ssy5p sensor control. *Molecular and Cellular Biology*. **24**:7503-7513.

Preface

With the clear exception of the opponent and the members of the graduation committee, the typical Swedish doctoral thesis lacks a defined circle of readers. In an attempt to reach a broader public, I have consequently aimed at making my thesis accessible to non-specialists. However, I have also incorporated sufficient details to satisfy the experts of the field. Chapter 1 provides a general introduction to the field of nutrient signaling. The text is written for the educated layman and free from technical language. In this chapter I define the field of nutrient signaling, discuss the terminology, and reflect over the possibilities and limitations of the scientific method. The following chapters are less reflective and more of a technical nature. Chapter 2 introduces amino acids as cellular nutrients for the model organism central for this study, the budding yeast *Saccharomyces cerevisiae*. In Chapter 3 I discusses how amino acids are internalized by yeast cells from the extracellular environment, and presents the many layers of regulation that control this process. Chapter 4 is a review of recent discoveries and presents the current mechanistic understanding of the SPS sensor signal transduction pathway. Finally, chapter 5 contains previously unpublished experimental details that are central to understanding of SPS sensor signal transduction pathway. For details regarding my scientific contribution, I refer the reader to my original publications reprinted in this thesis. However, results obtained in these primary research papers and my interpretation of the data is discussed throughout chapters 2 through 5.

Stockholm October 2004,
Claes Andréasson

Chapter 1

An introduction to nutrient signaling

The cell and its environment

From the mere existence of cells in the form of small lipid-encapsulated living entities, follows that cells must reside in an environment. The fact that a particular portion of space is defined as an environment solely based on its proximity to the cell makes it rather multiform. Cells can be found in as different environments as animal tissues and the cold and dry stratosphere. Despite the fascinating plethora of extreme and contrasting cell containing environments that can be envisaged, I will focus on what cellular environments have in common rather than what separates them. If the relevant cellular environments are limited to those that support effective cell proliferation, they begin to look more similar than contrasting. The environments contain water, dissolved compounds, and not surprisingly, other cells.

An environment must contain all compounds necessary for building the cell; however cellular metabolism limits the required complexity of compound-availability, since the cell can produce compounds derived from environmental precursors. Nearby cells can also make the environment more permissive for cell proliferation by sharing the metabolic burden and secrete required compounds. From a simplistic point of view, cell proliferation is entirely about accumulating compounds from the environment in an ordered and metabolically orchestrated manner.

Nutrients and cells

The compounds a cell uses for growth are called nutrients. The definition can be broadened to any compound that promotes growth. Cells have nutritional requirements in the sense that they need certain compounds to build the cell. Magnesium ions are for example frequently used as parts of proteins. Cellular deficiency for magnesium ions will result in halted growth and possible cell death. The need for magnesium ions constitutes an example of an absolutely essential nutrient, but many nutrients are not strictly required for growth and can be exchanged to other compounds fulfilling the same metabolic function. For example yeast cells can use the sugar glucose as the basic carbon donator molecule and energy source, but yeast cells can as well use another sugar, galactose. In fact, yeast cells do not need any sugar at all and can use many other compounds as sources of carbon and energy. The number of possible nutrients will therefore be greater than the number of nutrients the cell requires. The capacity to use alternative nutrients enables cells to deal with environments with changing nutritional availability. Thus cells with the least nutritional requirements and the greatest capacity to use diverse nutrients are capable of populating many different environments and environments with variable nutrient availability. Microbes are clear examples of organisms that often tolerate diverse or varying environments. Cells in environments with stable nutrient supplies, like within our bodies tend to be, have complicated nutritional requirements and little capacity to use alternative nutrients.

Genes and metabolism

The statement that cells have a capacity to use more nutrients than constantly available, calls for a discussion about the underlying mechanisms that enable and maintain this metabolic repertoire. According to current view, the ability to use alternative nutrients is nothing more than a reflection of the genes encoding enzymes that can manipulate them. Similarly, essential nutrients are those required due to the lack of genes encoding biosynthetic enzymes that make them. Moreover, also higher order of structure, for example organization of discrete enzymes into functional metabolic pathways, is regarded as a manifestation of gene-encoded information. Thus, genes have a central role in determining the metabolic capacity and requirements of the cell.

Regulated nutrient usage

The term regulated is very frequently used to describe the molecular basis of cellular function. I will try to illustrate some meanings of the term by considering something as distant from cells and their nutrients as heating a room in the cold winter. A radiator in the middle of the cold winter heats a room. The radiator regulates the temperature of the room by emitting heat. Experimentally this relation between the radiator and the temperature is very easy to establish. The temperature in the room will go down when the radiator is taken away. Likewise, putting in a second radiator will increase the temperature in the room. The regulation described in this example is not active. The radiator does not in any way respond to the temperature in the room, it just emits heat. Let us call this *passive* regulation. A second example includes a thermostat-controlled radiator that will emit heat only when the temperature in the room drops below a certain point. Experimentally removing the radiator will give the same effect as in the first example; the temperature in the room will drop. However introducing an extra thermostat-controlled radiator in the room will lead to a more rapid temperature rise in response to cold weather, rather than an increase in the general temperature. The radiator in this second example is *actively* regulating the temperature in the room in response to the temperature sensed by the thermostat.

How can these two meanings of the term regulated be applied to the cell's nutrient usage? Inbuilt passive regulation can be found in every enzyme investigated. Enzymes catalyze chemical reactions often by drastically facilitating a chemical change on a substrate molecule so it forms a product molecule. The mere lack of substrate can be said to be the simplest form of passive regulation. If there is no substrate around, surely no product will be made. This regulation is so common and obvious that it is hardly given any consideration at all. However, other examples of passive regulation attract more interest and mechanisms of passive regulation are often investigated and characterized. For example proteins are turned over as a consequence of their continuous synthesis and degradation. Degradation of a given protein is often a passive regulatory process and still attracts much interest.

When considering regulated nutrient usage, undoubtedly active regulation is more interesting. For instance, galactose-utilizing enzymes in yeast are only produced in the presence of galactose and absence of glucose. Glucose being a preferred carbon source for this microbe, supporting more effective metabolism and cell growth than galactose. Thus, active regulation enables cells to more effectively take us of the environment than passive regulation. Furthermore, mechanistically active regulation requires a more sophisticated system than passive regulation.

Sensing nutrients

The active regulation of a cellular process occurs in response to a change in physical state of a parameter. In nutrient regulation this state is often a change in nutrient availability (concentration) inside or outside the cell. This requires a device that monitors nutrient availability and that in response to the appropriate nutrient level, or change in nutrient level, generates a signal that regulates downstream metabolism. Analogously I already have introduced such a device in my example with the thermostat-controlled radiator and called this device a sensor. Thus, a sensor is a device that receives and responds to a signal or stimuli. The actual boundaries of the sensor can be arbitrarily defined; the whole radiator, the thermostat circuit, or the metal-piece expanding when temperature increases is merely a matter of possible delimitations.

Cellular nutrient sensors are not metal pieces, but usually proteins that have a structure that enables them to bind the relevant nutrient. The better such a sensor binds the nutrient the lower levels of nutrients can be sensed. Molecular structures of a cell that binds other molecules are usually called receptors, and receptors bind ligands. Consequently in the case of nutrients receptors, the ligand will be a nutrient. Merely having an affinity for a nutrient will not make a sensor, the binding must also generate a response or signal that regulates metabolism. Thus, the binding of a nutrient must change the properties of the sensor, for example by a conformational change that exposes a normally inaccessible surface of the sensor protein to which other proteins can bind.

A point must be made that sensing does not always need to be directly coupled to the nutrient to be effective. A particular nutrient can be imported into the cell, metabolized to generate a derivative compound and this secondary compound may in turn be sensed. These kind of indirect circuits of nutrient sensing are very common, and mechanistically they appear to function equally effectively as directly controlled circuits.

Nutrients can be sensed both inside and outside the cell. This distinction becomes important in the light of the limited ability most nutrients have to enter the cell. The cell is completely surrounded by a lipid plasma membrane that effectively prevents entry of water and water-soluble molecules. Most nutrients are water-soluble and thus cannot enter the cell without assistance. Transporter proteins that span the plasma membrane facilitate nutrient uptake into the cell by binding specific nutrients outside the cell and releasing them in the cytoplasm. Some transporters use energy for this activity and others work as highly selective pores that allows passive transport of restricted sets of nutrients.

Numerous nutrient sensors that work within the cell have been described. This location appears quite rational since the sensor monitors the actual nutrient status of the cell, i.e., the sum of the cell's production, consumption, and import of the nutrient. Such sensors generate signals that reflect the need for the particular nutrient. However intracellular nutrient sensors can only indirectly measure nutrient availability in the environment. In contrast, a sensor in the plasma membrane, facing the outside of the cell, will directly monitor nutrient availability but is unable to assess nutrient status of cells and their nutritional needs.

My example of a radiator heating a room in the cold winter can be extended to point out some effects of having a sensor inside the cell in contrast to having a sensor outside the cell. Weather like nutrient availability can change, and most habitants of the colder parts of the world have experienced the effects of a sudden temperature drop. The heated room becomes temporarily cold, despite the fact that the radiator in the long run is able to maintain the room properly heated. The exact mechanisms giving rise to this temperature drop in the room is probably more complex than I can imagine, but from a simplistic point of view the following scenario is likely. When the temperature drops outside, cold air seep into the room and the temperature of the room drops. The thermostat controlling the radiator senses this. However, the distribution of heat generated and the limited effect of the radiator will cause local temperature zones in the room; the temperature being highest near the radiator itself. This local high-temperature zone is sensed by the thermostat, which over and over again will turn the radiator off despite the fact that the overall temperature in the room is lower than desired. The relevant point from this example is that the internal sensor is only indirectly measuring the main factor affecting the temperature in the room, namely the temperature outside. Since the system has no direct information about the temperature outside, it must await consequences in the room before it acts, and this makes the system intrinsically slow. Cleaver engineers can counteract these temperature effects in many different ways, and modern buildings do not suffer the same problems as old ones, but to make use of the example I would like to limit the extension of the model by introducing an outdoor thermostat. This outdoor sensor directly measures the temperature outside, and allows the radiator to be turned on long before the room has experienced a drop in temperature. It also hinders the indoor thermostat from repetitively turning the radiator off despite the need for more heat. An outdoor sensor makes the system faster and more robust. Translated to nutrients, a sensor outside the cell provides a fast system capable of priming intracellular metabolic events so that cells more effectively will use the nutrients actually present in their growth environment.

Nutrient signaling

Nutrient sensors must be capable of detecting nutrient levels and to generate appropriate signals. I have already focused on the first function of the sensor, the detection task, but the subsequent step, signal generation, also requires discussion. I previously mentioned that signal generation often correlates with a conformational change of the sensor molecule itself. The conformational transformation may for example allow other proteins to bind the sensor. This example only states that the signal is an actual measurable entity, but does not state anything about the consequences of the signal. A protein that binds a nutrient-ligand and changes conformation with no consequences for the cell can hardly be called a nutrient sensor. Thus, what is and is not signaling or signaling events is in practice an outcome of the observer's success in finding a relevant consequence for the cell. This is not a trivial task. Sensors may detect nutrients and generate signals that affect the cell in a perfectly rational manner if the observer examines the conditions under which the cell was selected during evolution. In contrast, the same signaling event, and outcome, may appear completely irrational for the cell when it is studied under laboratory conditions. It should be noted that in certain cases what appears to be fully reasonable signaling pathways in the laboratory might solely exist in the observers mind and not be a consequence of adaptive evolution.

Often little critical consideration is awarded to the question if the observed signaling is relevant to the cell or not. If cells behave in a reasonably consistent way when challenged with a given stimulus, signaling pathways are often postulated and the mechanisms addressed. Relevance is often formulated retroactively, with the obvious risk that the studied signaling system is a laboratory artifact. This non-reflective approach becomes an even bigger issue when the stimulus is obscure, the outcome diffuse, and only fragments of the postulated signaling pathway are identified. A clear and detailed understanding of a proposed signaling pathway should reduce the risk that the pathway is merely an irrelevant artifact unintentionally invented by the investigator.

Despite the problems associated with identifying relevant signaling events, the number of characterized signaling pathways is ever growing. This thesis focuses on a particular kind of signaling, nutrient signaling. Nutrient signaling refers to nutrient-induced signaling, or possibly, nutrient-activated signaling. This means that nutrient signaling requires a nutrient, a nutrient sensor, and a signaling mechanism that ultimately regulates the cell, giving rise to reproducible and measurable outcomes.

Signaling molecules and mechanisms

Understanding mechanisms of nutrient signaling can in practice be summarized as a two-step process. First, the signaling components must be identified, and then, the components need to be linked mechanistically. The investigator will almost always be biased in his pursuit of signaling components simply as a consequence of the choice of experimental techniques. Most likely, the pursuit will involve the identification of genes (if skilled in genetics) or a protein (if skilled in protein purification). The end result will likely be the same, since a gene usually encodes a protein and a protein most certainly is be encoded by a gene. Therefore, from an epistemological perspective, the mechanisms of nutrient signaling are the stories of proteins. This includes details about their shapes, enzyme activities, their cellular localization, whom they interact with, and what modifications they carry. Some of these details can be said to allow proteins to exist in different states. This concept of differential states is built into the idea of signaling. Proteins involved in signaling are consequently often expected to have at least two states, a non-signaling and a signaling state. A protein may for example receive a phosphate group on a hydroxyl containing amino acid side chain by a protein kinase. The phosphorylated protein constitutes the signaling state and the unphosphorylated species a non-signaling state. Thus, the final states themselves are often regarded as the mechanism of signaling, in contrast to rare cases where the actual process of change between states is the signaling mechanism. For example the catalysis by the protein kinase is not important for signaling *per se*. Instead, the final phosphorylated state obtained is fundamental.

A protein that changes state and thereby in turn induces a change of state of a second protein fulfills the criteria of a sensor. The protein receives a signal and transmits it further by changing state. Thus, in essence signaling pathways can be regarded as a series of sensors, and it further emphasizes the subjective element required to judge what is stimulus, sensor, and signal transduction. However, the genes and corresponding proteins identified in the laboratory are not usually found due to their ability to change states, but rather as entities with an induced metabolic change or output (phenotype). They may for example physically associate with another known, or presumed, component of the pathway, they may be required for signal transduction to occur, or they may modulate the amplitude of signal transduction.

The scientific literature is full of assumptions regarding the components of signaling pathways, perhaps due to blurred distinctions. For example, a kinase required for a given phosphorylation event is often assumed to catalyze the phosphorylation of a signaling component after having received a signal. However, the change of state that causes the phosphorylation may not at all involve the kinase. The protein substrate may for example change its state (perhaps changing shape) that permits binding and consequential phosphorylation to occur. It is therefore valuable not to assume that required components of a signal transduction pathway are true signaling components.

The classical use of genetics generates another widespread obstacle in the pursuit of signaling molecules. Inactivation of a gene by mutation is powerful in the sense that it eliminates the gene product from the cell. If the signaling pathway stops functioning, the gene can be regarded as required for the signaling to occur by direct or indirect means. If signaling still persists in its absence, the gene is often assumed not to participate in signaling. It should be emphasized that the lack of requirement is not the same as lack of involvement. In fact, the affected gene may very well encode a protein directly involved in transmitting the signal. For example, *bona fide* requirement of a component can be masked by another component with a similar, but not always identical role or activity. Alternatively, the amplitude of the signal may be severely weakened but still sufficient to provoke a sufficient detectable output.

The targets of nutrient signaling

The targets of nutrient signaling varies enormously. For example, nutrients regulate the motility of a bacterium by activating movement of its propelling tail (flagella). In other instances, nutrient signaling affects nutrient metabolism, such as transport of the nutrient into the cell. Common targets of nutrient signaling are genes and the identification of gene-targets is aided by well-developed methods to study gene function and expression (transcription). An enzyme called RNA polymerase performs the transcription of genes and this general factor is in eucaryotic cells recruited to relevant genes by the action of gene-specific proteins called transcription factors. Transcription factors can, directly or indirectly, bind both the gene-constituent DNA and the enzyme RNA polymerase, thereby linking the gene to the enzyme required for its transcription. Naturally, the active regulation of transcription factors is an effective and frequently used way of controlling gene expression.

The designation of the targets of signaling suffers from the same issues associated with identification of signaling itself. This can be exemplified by the fact that many signaling pathways regulate gene expression. The genes can be regarded as the end-point of the signaling and share many common requirements (general transcription machinery, etc), but the fact that genes encode proteins with activities and functions makes the matter controversial. Again, the investigator must define the borders of a given signal transduction pathway.

Model systems for nutrient signaling

Addressing mechanisms of nutrient signaling requires a model system to study. A system must be regarded as accessible if it behaves reproducible, is robust, has a clear readout, and is readily manipulated experimentally. Depending on the goal of the research, different organisms can be chosen that offer unique examples of nutrient signaling. I have already discussed expected differences in nutrient utilization between cells in stable environments, like many animal cells, contra cells in unstable conditions, like many microbes. Undoubtedly, the biology of the chosen organism will reflect evolutionary pressures and therefore limit the repertoire of signaling networks. However, despite expected differences in initiation and out-come of signaling, the mechanisms in many cases are more general.

The budding yeast *Saccharomyces cerevisiae* offers many attractive systems to study signaling in eucaryotic cells in general and nutrient signaling in particular. This unicellular organism is best known as the fermenting microbe in bread, beer and wine production but it has many characteristics making it a good choice for investigation. Yeast being a microorganism that can survive or proliferate in many different environments makes it a likely candidate to possess various nutrient signaling systems. Practically it is easy to propagate and it readily grows in various liquid and solid media. Yeast cells have a well defined developmental repertoire and grow as haploids (one of each chromosome per cell) or diploids (two of each chromosomes per cell) that can undergo meiosis to form haploid spores. Under certain environmental conditions both haploid and diploid cells form thread-like pseudo-hyphae. The ability to grow both as haploid and diploid, and a complete sexual cycle, is helpful when assessing function and identity of genes with their respective mutant versions. The total number of genes is modest and the size of the whole gene assembly (genome) is small. But most importantly, many years of molecular and cell biological research has generated detailed knowledge about yeast cells, assigned functions to many genes, and a generated methods of sophistication at a level more advanced than in any other model organism.

Chapter 2

Amino acids in yeast nutrient signaling

Defining amino acids

The chemical definition of amino acids is any molecule containing an amino and a carboxylic acid group, but the term is usually used in biochemistry for compounds where these two functional groups are attached to the same alpha carbon. Additionally, the proteinogenic imino acid proline is usually referred to as an amino acid, despite its lack of a primary amino group. In addition to the common 20 L-α-amino acids found in proteins some other 500 amino acids have been described in nature. Many of these have been found to be important metabolic intermediates, e.g., true signaling molecules, and others are constituents of peptide antibiotics. The discussion within this thesis regarding amino acids will focus on the common 20 proteinogenic amino acids.

Making use of amino acids

Cells use amino acids in protein biosynthesis and must either import or synthesize all of the 20 amino acids found in proteins. They are considered nutrients when their import facilitates cell proliferation. The lack of biosynthesis of a particular amino acid makes import necessary and the amino acid is thus essential for the cell. Even organisms that can synthesize all amino acids often use them as nutrients, as amino acids are important precursors for synthesis of other nitrogen containing compounds.

Yeast can synthesize all 20 proteinogenic amino acids when supplied with a non-amino acid nitrogen source, e.g., ammonium. Despite their biosynthetic capacity, yeast cells still prefer amino acids as nitrogen sources, and consistently use a sophisticated and complex system of transporters to internalize them. The complexity of this system (discussed in chapter 3) supports the notion that the import of amino acids into cells must be regulated. That is to say that amino acids contain valuable nitrogen, but can also cause problems for the cell, especially if imported in a way leading to unbalanced or to high internal concentrations of a particular amino acid. Thus, the intracellular levels of any one given amino acid must be regulated. Consequently, the system regulating internal pool sizes needs to respond to both the demand and the supply of the any particular amino acid by increasing or decreasing import, synthesis, and consumption. Amino acid metabolism therefore include intricate systems for coordinating these processes.

Amino acids are stored inside the yeast cell in the form of discrete intra-cellular pools (Klionsky et al., 1990). Two main pools are formed; a large pool with low metabolic turnover and a smaller pool with high metabolic turn over. The larger pool resides inside the vacuole, while the smaller pool is cytoplasmic. Interestingly, basic amino acids are sequestered in the vacuole and acidic amino acids are excluded from this compartment. The molecular system responsible for the formation of discrete pools is not known. The first proteins that transport amino acids across the vacuolar membrane have only recently been identified (Russnak et al., 2001).

Different amino acids function variably well as nitrogen sources. Glutamine and glutamate are central and accessible components in nitrogen metabolism, while utilization of proline is not as straightforward and requires metabolic conversion to glutamate before it can be further utilized. The quality of an amino acid as a nitrogen source has been defined as the cell's capacity to convert it to glutamine and glutamate (Magasanik and Kaiser, 2002). However, the complexity of regulated synthetic pathways that affect different amino acids makes their experimental categorization into simple groups such as preferred or non-preferred somewhat complicated. Traditionally, growth rates of cells supplied with an amino acid as the sole nitrogen source have been used as an experimental criterion for classification of the compounds nitrogen quality (Table 1 presents examples). However this categorization is inadequate since the over-all nitrogen status, glutamine and glutamate levels, of the cell is not always practically reflected in growth rates. A second experimental criterion for defining the nitrogen quality of amino acids is based on the level to which systems for use of alternative nitrogen sources are derepressed when growing cells with a given amino acid as the sole nitrogen source (Magasanik and Kaiser, 2002). However this experimental criterion is hampered by the subjectivity in the interpretation of the dimension and extent and such a response. Nevertheless, in direct comparisons, many different amino acids can be said to have different qualities as nitrogen sources. It therefore appears rational that cells regulate uptake of amino acids depending on availability of amino acids of different qualities. For example, the uptake of the poor nitrogen source proline is down regulated when cells are growing in the presence of the good nitrogen source glutamine. The mechanisms governing such regulation will be discussed below.

Table 1. Doubling times with various nitrogen sources

Compound	Doubling time (h)
Glutamine	2.7
Asparagine	2.8
Ammonium	2.9
Arginine	2.9
Aspartic acid	3.2
Glutamic acid	3.2
Serine	3.4
Valine	4.4
Alanine	4.7
Phenylalanine	5.2
Tyrosine	5.6
Leucine	5.7
Isoleucine	6.1
Proline	6.7
Threonine	6.8
Citrulline	6.9
Ornithine	13.4
Tryptophan	11.7
Cysteine	>20 †
Glycine	>20
Histidine	>20 †
Lysine	>20
Methionine	>20

Doubling times of yeast cells growing at 24°C with indicated compound as sole source of nitrogen. After (Chen and Kaiser, 2002). †, Indicates that the compound was at least partially toxic at ≥3 mM.

Amino acids in gene regulatory nutrient signaling

Amino acids initiate nutrient signaling events that regulate cell metabolism. Many of the identified targets of amino acid signaling are genes and I would like to discuss three examples of signaling pathways that regulate gene expression in response to amino acid availability. The three pathways were chosen because they constitute clear examples of nutrient signaling responding directly to amino acids, indirectly to amino acids and an important pathway still lacking a sensor.

Direct sensing of amino acids: Proline and Put3p

Cells can use the amino acid proline as the sole source of nitrogen. Proline is imported from the environment and is converted to glutamate in two steps by the sequential action of a pair of enzymes; proline oxidase and Δ^1-pyrroline-5-carboxylate dehydrogenase. The genes encoding these proline utilization enzymes, *PUT1* and *PUT2*, are only efficiently transcribed if cells are grown with proline as the sole nitrogen source, and if the transcription factor Put3p is functional (Brandriss, 1987; Marczak and Brandriss, 1991). Put3p is a transcriptional activator that belongs to the $Zn(II)_2Cys_6$ binuclear cluster family of transcription factors and contain N-terminal DNA binding and dimerization capacities. The C-terminal domain is highly acidic and required for transcriptional activation (des Etages et al., 1996).

Two signals appear to regulate the activation potential of Put3p. The first signal is nitrogen source dependent, and affects the phoshorylation state of the protein. Phosphorylation of Put3p correlates with *PUT3* dependent gene expression (Saxena et al., 2003). However, this protein modification cannot explain why Put3p responds specifically to proline. Moreover, the actual stimulus, sensor, and signaling pathway are unknown. The second signal is the cellular level of proline (Sellick and Reece, 2003). Intriguingly, Put3p directly binds proline and as a consequence is a receptor of proline. Binding of proline, or an unmodified pyrrolidine ring, makes Put3p a more effective transcriptional activator, presumably due to a structural conformation change in the activation domain. Put3p is therefore a direct sensor of proline that actively regulates transcription of genes required for utilization of proline as a nitrogen source.

The regulation of Put3p activity is an example of a clear and simple system that cells use to detect and regulate metabolism in response to amino acids. The system appears to have an inbuilt logic in the sense that high-level expression of *PUT1* and *PUT2*, the genes encoding proline-catabolizing enzymes, is only observed when a high level of proline is actually present. Thus, the enzymes are expressed when they are needed. The level of intracellular proline detected is mainly determined by two factors: (1) The concentration of proline in the environment, and (2) the efficiency of proline import. Proline transport is down regulated in the presence of preferred nitrogen sources (Courchesne and Magasanik, 1983) and thus an additional layer of rational regulation is added. Consequently, proline utilization will not be effective when the cell has access to a preferred nitrogen source.

The example of Put3p is clear in its simplicity. The integration of a proline receptor, with sensor function, and a transcriptional activator places common parts of nutrient signaling mechanisms in proximity. The proximity sets aside doubts concerning relevance that more complicated systems generate; certainly, proline availability will actively regulate cell metabolism via Put3p outside the laboratory, and the system must be a consequence of adaptive evolution!

Indirect sensing of amino acids: The general amino acid control system

Yeast cells respond to starvation of a single amino acid by simultaneously and globally derepressing many genes encoding components in several amino acid biosynthetic pathways. (Hinnebusch, 1986) (Hinnebusch, 1992). This phenomenon is called general amino acid control and contrasts to other more specific regulatory systems that respond by controlling discrete pathway specific components. The central regulator in the general control system is a transcription factor called Gcn4p that binds specific elements within promoters of numerous genes encoding diverse amino acid biosynthetic enzymes (Arndt and Fink, 1986; Hope and Struhl, 1985). The level of Gcn4p present in the cell is the determining factor for the transcription of the target genes and this level is actively regulated by synthesis and degradation in response to amino acid starvation (Hope and Struhl, 1985). I will restrict the discussion to the classical model for translational regulation of Gcn4p and must refer the reader to a recent review for more details regarding the regulation of Gcn4p stability (Irniger and Braus, 2003).

The general control pathway can be briefly outlined as follows (Hinnebusch, 1997). Cells starved for an amino acid will exhibit impaired charging of the relevant tRNAs. Consequently, uncharged tRNA molecules accumulate. Gcn2p is a protein kinase that binds and is activated by uncharged tRNAs (Wek et al., 1995). Active Gcn2p phosphorylates translation initiation factor 2 (eIF2) α-subunit, and the phosphorylated form of eIF2 has a decreased ability to form the ternary initiation complex eIF2·GTP·Met-$tRNA_i^{Met}$ (Dever et al., 1992). This compromises the ability of ribosomes to reinitiate translation. The *GCN4* transcript bears four small upstream open reading frames (uORFs) that normally function to restrict translation of Gcn4p (Mueller and Hinnebusch, 1986). In non-starved cells, ribosomes initiate at uORF 1 and subsequently reinitiate at uORF 4 and therefore cannot initiate at *GCN4*. Consequently, no Gcn4p is synthesized. Under amino acid starvation conditions, the impaired capacity of ribosomes to reinititate translation enables the scanning ribosomes to pass uORF 4 and reinitiate at the *GCN4* start codon, leading to translation of Gcn4p.

The translational control of Gcn4p offers a classical mechanism for amino acid starvation detection and signaling. However three characteristics of the system raise concerns whether this signaling system is best described as a single dedicated amino acid sensor system. First, many steps are involved in transduction of the signal from stimuli to transcriptional activation. Second, many Gcn4p target genes appear to have little or no involvement in amino acid biosynthesis. Third, purine limitation activates the system via the same Gcn2p dependent mechanism. Thus, the general control system may best be described as a system that enables cells to better cope with general stress. Furthermore, it may be intellectually constructive to regard the general control system as two signal transduction pathways rather than only one. From this perspective, the Gcn2p protein kinase functions as a sensor of uncharged tRNAs and transmits signals to the translational machinery via eIF2. The second more specialized signaling event responds to impaired translation initiation, which induce expression of Gcn4p. Putting these two signaling events together may reflect a function of the system during adaptive evolution. In this context the mechanism must be regarded as an example of indirect sensing of amino acids.

Nitrogen regulation

The rational behind nitrogen regulation (also called nitrogen catabolite repression), i.e., the repression of genes required for utilization of non-preferred nitrogen sources when preferred nitrogen sources are available, has already been introduced. Recall that a preferred nitrogen source is a compound that is readily converted to glutamate and glutamine, which in turn function as the starting substrate molecules for the synthesis of most nitrogen containing compounds in the cell (Magasanik, 1992). Two recent reviews properly deal with the subject of nitrogen regulation (Cooper, 2002; Magasanik and Kaiser, 2002) and form the basis for my discussion.

A set of four related GATA transcription factors perform the task of activating the appropriate promoters at the right time. Two of these factors, Gat1p (also known as Nil1p) (Coffman et al., 1996) and Gln3p (Mitchell and Magasanik, 1984), bind many different and overlapping promoters and activate transcription in the situation of nitrogen starvation. As expected, many of the activated target genes are involved in uptake and utilization of non-preferred nitrogen sources. The target genes *DAL80* (Chisholm and Cooper, 1982) and *GZF3* (also known as *DEH1* and *NIL2*) (Soussi-Boudekou et al., 1997) encode additional GATA factors with a negative effect on transcriptional activation. These GATA factors compete with Gat1p and Gln3p binding and thereby attenuate the expression of nitrogen regulated promoters.

The relative levels of the positively and negatively acting GATA factors are determined in part by an intricate auto-regulatory system where all GATA factor-encoding genes except *GLN3* appear to contribute to the expression of GATA factor genes. It is believed that this rather complex mechanism responds to different nitrogen regimes by rapidly adjusting the steady state levels of transcriptional activators and repressors.

An added level of complexity exists since the quality of the nitrogen source is known to affect the nuclear accumulation of the activating GATA factor Gln3p. When cells are supplied with a preferred nitrogen source, Gln3p is localized in the cytoplasm and therefore is prohibited from gaining access to the promoters (Beck and Hall, 1999). When cells are transferred to a non-preferred nitrogen source, Gln3p relocalizes to the nucleus and binds to promoters of relevant genes (Bertram et al., 2000). The nuclear exclusion of Gln3p correlates with the extent to which this protein is phosphorylated (Beck and Hall, 1999). Highly phosphorylated forms localize to the cytoplasm and dephosporylated/hypophosphoryled forms localize to the nucleus. Thus, the quality of the nitrogen source appears to be sensed and initiates signaling that regulates phosphorylation of Gln3p, and presumably Gat1p, that in turn control their accumulation in the nucleus.

Several components of the machinery controlling phosphorylation and nuclear targeting of Gat1p and Gln3p have been described. I will only very superficially introduce some of them and their functions, for the simple reason that detailed understanding of the sensing or signaling events in nitrogen regulation is lacking. However the field is currently making good progress in identifying the players and their functions. Mutants of *URE2* were first identified and described in the doctoral thesis of Francois Lacroute 1969. The *URE2* gene product is a cytoplasmic protein that can bind Gln3p (Blinder et al., 1996), and prevents Gln3p from promoting transcription when cells are growing with a preferred nitrogen source (Beck and Hall, 1999). The activity of TOR kinases (Tor1p and Tor2p) influences the ability of Gln3p and Gat1p to activate gene expression. The drug rapamycin inhibits TOR kinase, which results in the hypophosphorylation of Gat1p and Gln3p (Beck and Hall, 1999). Recall that in their

hypophosphorylated state these transcription factors accumulate in the nucleus, and consequently in rapamycin treated cells nitrogen regulated genes are constitutively expressed even under preferred nitrogen conditions. The mechanistic link between TOR kinase activity and the phosphorylation state of Gln3p and Gat1p is currently unclear, but involves additional factors including several members of the serine/threonine protein phosphatase 2A (PP2A) family (Beck and Hall, 1999; Bertram et al., 2000).

The many mechanistic models of nitrogen regulation that have been proposed are nicely presented and well scrutinized in (Cooper, 2002). From my perspective, they all suffer from a lack of a precisely defined stimulus and accompanying sensor that controls kinase and phosphatase interactions. Despite this lack of a defined stimulus, the availability of amino acids is an important factor regulating the pathway, i.e., nitrogen regulation is form of amino acid signaling. Consistently, many amino acid metabolic genes are subject to GATA factor regulation. (Magasanik and Kaiser, 2002) argue that intracellular levels of glutamate and glutamine are the stimuli sensed by the pathways specific for Gat1p and Gln3p respectively. This makes sense since the experimental growth conditions routinely used to turn on nitrogen regulation (glutamine, asparagine and commercial Bactopeptone) will lead to high levels of intracellular glutamine. Consequently, glutamate levels are high under these conditions.

This notion is supported by experimental results obtained with a leaky *gln1-1* mutant (Mitchell and Magasanik, 1984). *GLN1* encodes glutamine synthetase that catalyzes the formation of glutamine from glutamate and ammonium. The *gln1-1* mutant has decreased intracellular levels of glutamine when the cells are supplied with ammonia or glutamate as the sole nitrogen source. *GDH2* is a Gln3p activated gene that encodes NAD^+-linked glutamate dehydrogenase. The level of NAD^+-linked glutamate dehydrogenase is very low in wildtype cells grown with ammonia, glutamate, or glutamine as the sole nitrogen source. In contrast, the level of NAD^+-linked glutamate dehydrogenase was found to be high in *gln1-1* cells grown with ammonia or glutamate, but very low in cells grown with glutamine. Thus manipulating the intracellular level of glutamine, but not the levels of ammonium and glutamate, appears to affect Gln3p activity. However the interpretation of such experiments is clouded by possible indirect effects caused by metabolism, and that the identity of the stimulus (stimuli?!) and its sensor remain to be defined.

Chapter 3

Uptake of amino acids

Introduction

Regulated uptake of amino acids appears to have played a central role for the fitness of yeast during evolution, as judged from the complex and abundant systems they possess to internalize amino acids. The previous discussion emphasized the important role of amino acids in nitrogen metabolism and as compounds for protein synthesis. Both these functions are expected to benefit from relatively constant intracellular levels of amino acids. Cells can be expected to regulate amino acid uptake to selectively import desirable amino acids when such amino acids are present in excess, and scavenge any amino acids under starvation conditions. This idea, formulated quite early, was supported by evidence suggesting that yeast possess a single high capacity general amino acid transporter, which functions under starvation conditions to take up all amino acids, and a constitutively expressed set of low capacity transporters, each with narrow substrate specificities. However, more recent advances, including the complete sequencing of the yeast genome, indicate the presence of several more amino acid transporters. In almost every case investigated, the functional expression of amino acid transport proteins are subject to layers of active regulation. Despite the greater degree of complexity, the essence of the early ideas of amino acid uptake remain unchallenged, i.e., amino acid uptake, like transport of most other nutrients, appears to be up regulated during starvation conditions.

The amino acid permease family

Yeast possesses some twenty distinct transporters that import amino acids into the cell acting as proton symporters (Table 2). The majority of these proteins can be categorized into the Amino Acid Permease (AAP) protein family based on sequence homology. This family has homologues in fungi and bacteria and is a part of the Amino Acids-Polyamine-Choline (APC) superfamily of transporters (Saier, 2000). Like most of the APC superfamily members, the amino acid permeases have 12 membrane-spanning domains and a topology resulting in the N- and C-terminal tails facing the cytoplasm (Gilstring and Ljungdahl, 2000). Each amino acid permease has its own substrate specificity profile composed of distinct affinities for different amino acids. The number of amino acids recognized by different amino acid permeases varies but the substrate specificity for most permeases is generally broader than initially believed (Iraqui et al., 1999; Regenberg et al., 1999). The general amino acid permease, Gap1p, appears to represent one extreme with a capacity to transport most, if not all, amino acids including non-proteinogenic ones like D-isomers, citrulline and many toxic amino acid analogues. The broad substrate specificity is consistent with its believed function as a scavenger of amino acids under nitrogen limited conditions. In contrast to the commonly held belief, Gap1p is a high affinity permease for most of its natural substrates (Boles and Andre, 2004).

Table 2. Amino acid transporters in yeast

ORF	Gene	Substrates	Transcription	Inactivated
AAP Cluster I				
YCL025c	*AGP1*	Most amino acids	SPS, N^-	
YBR068c	*BAP2*	Many amino acids	SPS, N^+	–N
YDR046c	*BAP3*	Many amino acids	SPS	
YDR508c	*GNP1*	Many amino acids	SPS, N^+	
YBR069c	*TAT1*	Val	SPS	
YOL020w	*TAT2*	Aromatic amino acids	SPS	–N
AAP Cluster II				
YKR039w	*GAP1*	All amino acids	N^-	+N /AA
YGR191w	*HIP1*	His		
YLL061w	*MMP1*	S-methylmethionine		
YPL274w	*SAM3*	S-adenosylmethionine		
AAP Cluster III				
YNL270c	*ALP1*	Arg		
YEL063c	*CAN1*	Arg	N^-	
YNL268w	*LYP1*	Lys, Met		
AAP Unclustered				
YPL265w	*DIP5*	Many amino acids	SPS, N^-	
YOR248c	*PUT4*	Ala, Gly, Pro	N^-	+N /AA
YDR160w	*SSY1*			
YFL055w	*AGP3*	Asp, Glu, Met		
YBR132c	*AGP2*	Carnitine	N^+	
Other APC non AAP				
YGR055w	*MUP1*	Met	SPS	
YHL036w	*MUP3*	Met		
YDL210w	*UGA4*	GABA, putrescine	N^-	

Known amino acid transporters of yeast. Members of the amino acid permease family (AAP) are clustered according to (Nelissen et al., 1997). Substrate specificity as reported by (Regenberg et al., 1999) and references therein, with the exception of *MMP1*, *SAM3* (Rouillon et al., 1999), *AGP2* (van Roermund et al., 1999), *MUP1*, *MUP3* (Isnard et al., 1996), and *UGA4* (Andre et al., 1993). SPS; transcription induced by the SPS sensor (Didion et al., 1998; Eckert-Boulet et al., 2004; Forsberg et al., 2001; Iraqui et al., 1999; Klasson et al., 1999; Kodama et al., 2002). N^-; transcription repressed by a preferred nitrogen source. N^+; transcription facilitated by a preferred nitrogen source (Abdel-Sater et al., 2004; Didion et al., 1996; Iraqui et al., 1999; Regenberg et al., 1999).-N; permease inactivated when cells are starved for nitrogen (Beck et al., 1999; Omura et al., 2001). +N/AA; inactivated in response to preferred nitrogen sources or amino acids (Chen and Kaiser, 2002).

Agp1p, a broad-range-specificity permease, has a lower affinity than Gap1p for most amino acids (Iraqui et al., 1999; Regenberg et al., 1999). The high affinity proline permease Put4p is another permease believed to be important under starvation conditions (Vandenbol et al., 1989). Put4p provides an example of a narrow substrate range transporter, in addition to proline, it only appears to transport alanine and glycine (Regenberg et al., 1999). Other amino acid permeases, believed to have narrow substrate specificities, include the transporters of basic amino acids; the histidine permease (Hip1p) and the arginine permeases (Alp1p and Can1p), and the lysine permease (Lyp1p). Most amino acid permeases appear to have medium-range-substrate specificities, this class includes Bap2p, Bap3p, Gnp1p, Tat1p, Tat2p, and Dip5p (Regenberg et al., 1999).

Experiments with amino acid uptake

At first glance, the determination of which amino acid transporter imports what amino acid appears straightforward. However in reality it is an experimentally complicated issue to address. There are four minimal requirements for defining amino acid uptake activity. (1) The gene encoding the transporter should be transcribed. (2) The transporter should reach the plasma membrane. (3) The transporter should have a detectable affinity for the substrate. (4) Other competing amino acid should not be present. Experimental conditions impairing any of these four basic requirements for detecting amino acid transport activity will pose serious problems for the investigator. Such problems are often encountered. For example, the many amino acid transporters in yeast cause a basal uptake that can mask uptake specific for a given amino acid. The field is still lacking a yeast mutant with all amino acid permease genes deleted. Notably, this has already been accomplished for the hexose transporter gene family (Wieczorke et al., 1999). Additionally, laboratory yeast strains often carry mutations causing amino acid auxotrophies, which requires the addition of amino acids to growth media, and almost all standard laboratory media contain abundant quantities of amino acids. Finally, the functional expression of amino acid permeases is both transcriptionally and post-transcriptionally controlled by the nitrogen status of the cell.

Two approaches have been used to study amino acid uptake in yeast. First, early studies were usually based on kinetic characterization of amino acid transport activities before the genes or relevant transporters were known. Second, later the sequencing of the yeast genome made focus switch from activities to genes and mutant phenotypes. These two different approaches have possibly made an impact on the past and present view of amino acid transport in yeast. Early studies tended to focus on identification of transporters responsible for high affinity uptake, while later studies highlight also low affinity uptake. Since any given amino acid transporter is likely to transport fewer substrates with high affinity than substrates transported with low affinity, this may have contributed to the current view that most amino acid transporters in yeast have a relatively broad substrate range.

My own research regarding proline uptake represents a good example (Paper III). Proline uptake into yeast cells has traditionally been regarded to occur via the action of two permeases when cells are grown with non-preferred nitrogen sources like proline or allantoin. The permeases responsible are the high affinity proline permease Put4p and the general amino acid permease Gap1p. (Lasko and Brandriss, 1981) reported that high affinity proline uptake is abolished in *put4* mutants and practically all proline uptake, with the exception of very low affinity uptake, is gone in a *gap1 put4* double mutation strain. Despite this, *gap1*Δ *put4*Δ cells grow quite readily when supplied with proline as the sole nitrogen source (Paper III, figure 2A). The same cells are also sensitive to the

toxic proline analogue azetidine-2-carboxylic acid (AzC) when cells are grown with allantoin as the sole nitrogen source, indicating that they still can internalize this proline-like compound (Paper III, figure 1B). The barely measurable low affinity uptake of proline (Lasko and Brandriss, 1981) is still sufficient to provide a clear growth phenotype. It turns out that Agp1p is partly responsible for import of proline supplied as the sole nitrogen source, and that both Agp1p and Gnp1p can import proline when overexpressed (Paper III, figure 2). The matter is even more complex when considering that Gnp1p is not even transcribed on media with proline as the sole nitrogen source (Paper III, figure 2B). The example ends with a frustrating observation: *gap1*Δ *put4*Δ *agp1*Δ *gnp1*Δ cells are still able to grow very slowly with proline as the sole nitrogen source. Therefore, additional routes exist of proline to enter into cells.

Transcriptional regulation of amino acid permease genes

Transcription provides an obvious opportunity to regulate amino acid uptake. Each of the about 20 amino acid transporters in yeast is encoded by a distinct gene, consequently the expression of each permease has the potential of being subject to independent regulation at the promoter level. The understanding of the many mechanisms regulating the transcription of different amino acid permease genes is far from complete, but a few promoters have been characterized in some detail and at least some mechanisms have been elucidated.

Nitrogen regulation appears to play a principle role in the transcription of many amino acid permease genes. *GAP1* and *PUT4* are for example subject to strict nitrogen regulation and their corresponding transcripts levels are only high under conditions of nitrogen limitation (Jauniaux and Grenson, 1990; Jauniaux et al., 1987). The expression of *CAN1* presents an additional example of a permease gene subject to nitrogen regulation. The *CAN1* locus is differentially transcribed to give rise to different length transcripts. When cells are grown on a non-preferred nitrogen source a short transcript is generated, whereas a long transcript is detected in addition to the short transcript in cells grown in the presence of a preferred nitrogen source (Cox et al., 2000). However, the change in total transcript level is only somewhat reduced when cells are grown under the preferred nitrogen condition, and cells remain sensitive to the toxic arginine analogue canavanine transported by Can1p (Broach et al., 1979). Thus, even though *CAN1* is nitrogen regulated this regulation cannot be the sole determinant of the functional expression of Can1p. Consequently, other factors than Gat1p and Gln3p must facilitate *CAN1* transcription. Such parallel regulation of transcription appears to be a common theme for many amino acid permease genes. The broad-specificity permease *AGP1* is subject to double regulation. When cells are grown on the more preferred nitrogen source ammonium the transcript levels are very low or absent (Forsberg et al., 2001; Forsberg and Ljungdahl, 2001a; Regenberg et al., 1999) Abdel-Sater, 2004) (Paper III). The addition of low levels of leucine to the ammonium-based media potently induces expression of this gene (Forsberg et al., 2001; Forsberg and Ljungdahl, 2001a; Regenberg et al., 1999). Cells grown in the absence of inducing amino acids and in medium containing the non-preferred nitrogen source proline do not express high levels of *AGP1* (Paper III). When these cells are challenged with inducing amino acids the levels of transcript are even higher than with ammonium grown cells (Regenberg et al., 1999). Thus *AGP1* is mainly regulated by the presence or absence of inducing amino acids (Forsberg and Ljungdahl, 2001a; Iraqui et al., 1999; Regenberg et al., 1999), but nitrogen regulation also modulates transcript levels (Abdel-Sater et al., 2004; Regenberg et al., 1999) (Paper III). A similar regulation can be observed for *GNP1* that encodes a close relative to Agp1p. *GNP1* is induced by the presence of amino acids, but

in contrast to *AGP1,* the levels of transcript are higher when cells are grown with ammonium rather than with proline (Regenberg et al., 1999) (Paper III).

A general principle emerging from these studies is that amino acid permease genes are subject to nutrient regulation by multiple signaling pathways. The regulatory cues are nitrogen status of the cell and the presence of inducing amino acids in the extracellular environment. This thesis focuses on the regulatory mechanisms responsible for amino acid induced expression of a distinct set of amino acid permease genes.

Regulation of amino acid permease sorting and degradation

The investigator is likely to explain the existence of transcriptional regulation of amino acid permease genes by discussing the effects this has on uptake of amino acids. However this rational requires that gene expression directly is coupled to transport activity. Many amino acid permeases are subject to active post-transcriptional control of expression. For example, the trafficking of certain transporters to the plasma membrane, as well as their targeting to the vacuole and consequent degradation are known control points (see below). Hence, its is quite possible to set up growth conditions where an amino acid permease gene is transcribed but the resulting transporter never reaches the plasma membrane. Let us use the general amino acid permease Gap1p as an example (recently reviewed (Magasanik and Kaiser, 2002))!

As previously discussed, *GAP1* is transcribed in cells grown with non-preferred nitrogen sources, such as proline, and repressed when cells are grown with preferred nitrogen sources, such as glutamine. Consistently, Gap1p activity, measured as rates of radiolabeled citrulline accumulation, is high in proline grown cells and very low in glutamine grown cells. Intermediate quality nitrogen sources, such as ammonium or glutamate, do not effectively repress *GAP1* transcription, however Gap1p transport activity is very low (Jauniaux and Grenson, 1990; Stanbrough and Magasanik, 1995). The addition of ammonia to proline grown cells leads to the rapid inactivation of Gap1p activity (Grenson, 1983). The molecular mechanism responsible for the post-transcriptional inactivation of Gap1p in response to ammonium or glutamate has been found to affect the sorting of the permease in the secretory pathway. Transfer of cells from glutamate medium to urea medium (a less preferred nitrogen source than glutamate) results in the redistribution of Gap1p from intracellular compartments to the plasma membrane leading to a dramatic increase in Gap1p activity (Roberg et al., 1997). Conversely, the inactivation of Gap1p activity in response to addition of ammonium correlates with Gap1p degradation and a diminished level of Gap1p in the plasma membrane (Hein and Andre, 1997; Springael and Andre, 1998). A model based on intracellular protein transport within the secretory pathway has been proposed. According to this model, Gap1p constantly travels between compartments of the late secretory and endocytic pathways, and nitrogen availability regulates delivery to the plasma membrane (Magasanik and Kaiser, 2002). The regulating mechanism that directs Gap1p to the plasma membrane remains unidentified.

A recent observation suggest that regulated sorting of Gap1p may not be a consequence of nitrogen status of the cell but rather a direct consequence of intracellular levels of amino acids (Chen and Kaiser, 2002). It was found that the plasma membrane of growing cells is rapidly depleted of Gap1p when cells are exposed to amino acids, including non-proteinogenic amino acids and analogues. Furthermore, the experimental manipulation of intracellular levels of amino acids affected Gap1p sorting to the plasma membrane in a manner consistent with amino acids being the direct cause of the down regulated Gap1p activity. According to this model, ammonium-induced down regulation

of Gap1p should take place indirectly only after intracellular amino acid levels rise as a consequence of increased biosynthesis.

The regulated sorting of amino acid permeases in the late secretory pathway does not appear to be restricted to Gap1p. The high affinity proline permease Put4p exhibits similar nitrogen-associated regulation as Gap1p (Jauniaux et al., 1987). Additionally, nitrogen-starvation leads to the down-regulation and vacuolar degradation of Bap2p (Omura et al., 2001) and Tat2p (Beck et al., 1999; Schmidt et al., 1998). Likely, many more amino acid permeases are subject to similar regulation in the late secretory pathway.

Many key genes affecting the trafficking of Gap1p have been identified. *RSP5* encodes an ubiquitin E3 ligase that catalyses attachment of ubiquitin on lysines residues of proteins and is a key player in regulating amino acid permease trafficking (Hein et al., 1995; Huibregtse et al., 1995). Rsp5p interacts with two homologous proteins, Bul1p and Bul2p, and this complex is required for ubiquitylation and down-regulation of Gap1p (Helliwell et al., 2001; Soetens et al., 2001). Genes required for ubiquitin-associated sorting in the late endosomal pathway are also required for Gap1p sorting. *BRO1* and *DOA4* encode proteins that physically interact and localizes to the endosome (Luhtala and Odorizzi, 2004). Doa4p is an ubiquitin isopeptidase that removes ubiquitin from proteins that are being transported to the vacuole (Papa and Hochstrasser, 1993). Bro1p is likely to have a role in recruitment of Doa4p to the endosomal membrane (Luhtala and Odorizzi, 2004). Both *bro1* and *doa4* mutants exhibit phenotypes similar to *rsp5* and *bul1 bul2* mutants; Gap1p is stabilized at the plasma membrane in each of these mutants (Springael et al., 1999; Springael et al., 2002). Mutations inactivating *NPR1*, that encodes a kinase, has the opposite effect; Gap1p is directly sorted to the vacuole without ever reaching the plasma membrane (De Craene et al., 2001). However the exact mechanistic role for Npr1p is still elusive.

The first paper in this thesis describes a genetic screen that isolated mutations resulting in an increased uptake capacity of leucine. One class of mutations (class II) isolated in this Amino Acid Sensor Independent (*asi)* selection included all but one of the components described in the preceding paragraph; *bro1* (*asi6*), *doa4* (*asi7*), r*sp5* (*asi9*), and *bul1* (*ASI12*). Additionally, mutations in genes encoding other components of the late endosomal pathway *vps20* (*asi10*), *vps36 (asi11*) and the ubiquitin-activating enzyme *uba1* (*asi8*) were isolated. These findings indicate that mutations in the late endosomal pathway can lead to increased levels of amino acid permeases in the plasma membrane, and are consistent with the model that permeases constantly shuttle between late Golgi, endosomal compartments, and the plasma membrane. Consequently, the failure to target amino acid permeases to the vacuole enables them to recycle back to the plasma membrane. The identity of the specific amino acid permeases affected by the mutations has not been addressed.

Chapter 4

The Ssy1p-Ptr3p-Ssy5p amino acid sensing system

A recent and simple model

The Ssy1p-Ptr3p-Ssy5p (SPS) system has the capacity to sense extracellular amino acids and to initiate events that derepress the expression of genes encoding several amino acid permeases and the dipeptide transporter Ptr2p. The identification of this system and the components that comprise it is very recent, and important discoveries are made on an almost monthly basis in at least three different laboratories. Being aware of the many limitations and short half-life of early models, I would still like to present a current and simple model to place the components of the SPS system in context. I feel that this model will serve as a helpful introduction for the reader and document some ideas that would be interesting to test experimentally in the future. The model incorporates all presently available data, but still must be considered speculative and merely represents my best guess as to how signals are initiated and transmitted. I have not included references in the description of the model since the details are discussed throughout this chapter. The graphically oriented reader may find figure 1 helpful.

Many species of amino acids present outside the cell are able bind the poorly expressed Ssy1p receptor located in the plasma membrane. Ssy1p is an amino acid permease like molecule that can shift between an open conformation capable of binding extracellular amino acids and a closed conformation unable to bind amino acids outside the cell. The binding of amino acids available in the extracellular environment stabilizes the open conformation. The number of Ssy1p molecules in open conformation present in the plasma membrane decides the effectiveness of signal transduction to the next component, the WD40 repeat protein Ptr3p. Ptr3p associates peripherally with the cytoplasmic side of the plasma membrane and is able to associate with the extended N-terminal domain of Ssy1p. Consistent with its pattern of mobility on SDS-page, it is tempting to speculate that Ptr3p is phosphorylated. Presumably, the yeast casein kinases Yck1p and Yck2p catalyze the phosphorylation of Ptr3p when Ssy1p is stabilized by amino acids in its open conformation. Phosphorylated Ptr3p could then transiently associate with the inactive C-terminal protease domain of Ssy5p. Somehow this association facilitates the activation of the serine protease activity of Ssy5p. However activation requires the prior degradation of phosphorylated Ptr3p via the ubiquitin-proteasome system and the SCF^{Grr1p} ubiquitin E3 ligase complex. Active Ssy5p protease processes the two transcription factors Stp1p and Stp2p that are sequestered outside the nucleus due to the presence of inhibitory domains within their N-termini. The C-terminal transcription factor-domains of Stp1p and Stp2p can consequently enter the nucleus and bind promoters that contain the sequence element UAS_{aa}. The zink-fingers of Stp1p and Stp2p mediate binding to DNA. Stp1p and Stp2p are likely to bind as dimers to direct or indirect repeats constituting the UAS_{aa} motif, and it is pssible that they can form heterodimers on at least some promoters. The affinity of Stp1p or Stp2p for the different versions of UAS_{aa} determines the priority of genes that are expressed. However Stp1p and Stp2p are probably not good activators of transcription themselves, but are likely to associate with promotyer specific transcriptional cofactors such as Abf1p, Dal81p, and the GATA factors. Consequently, depending on their association with different cofactors, the efficiency of amino acid induced transcription of a given promoter will vary.

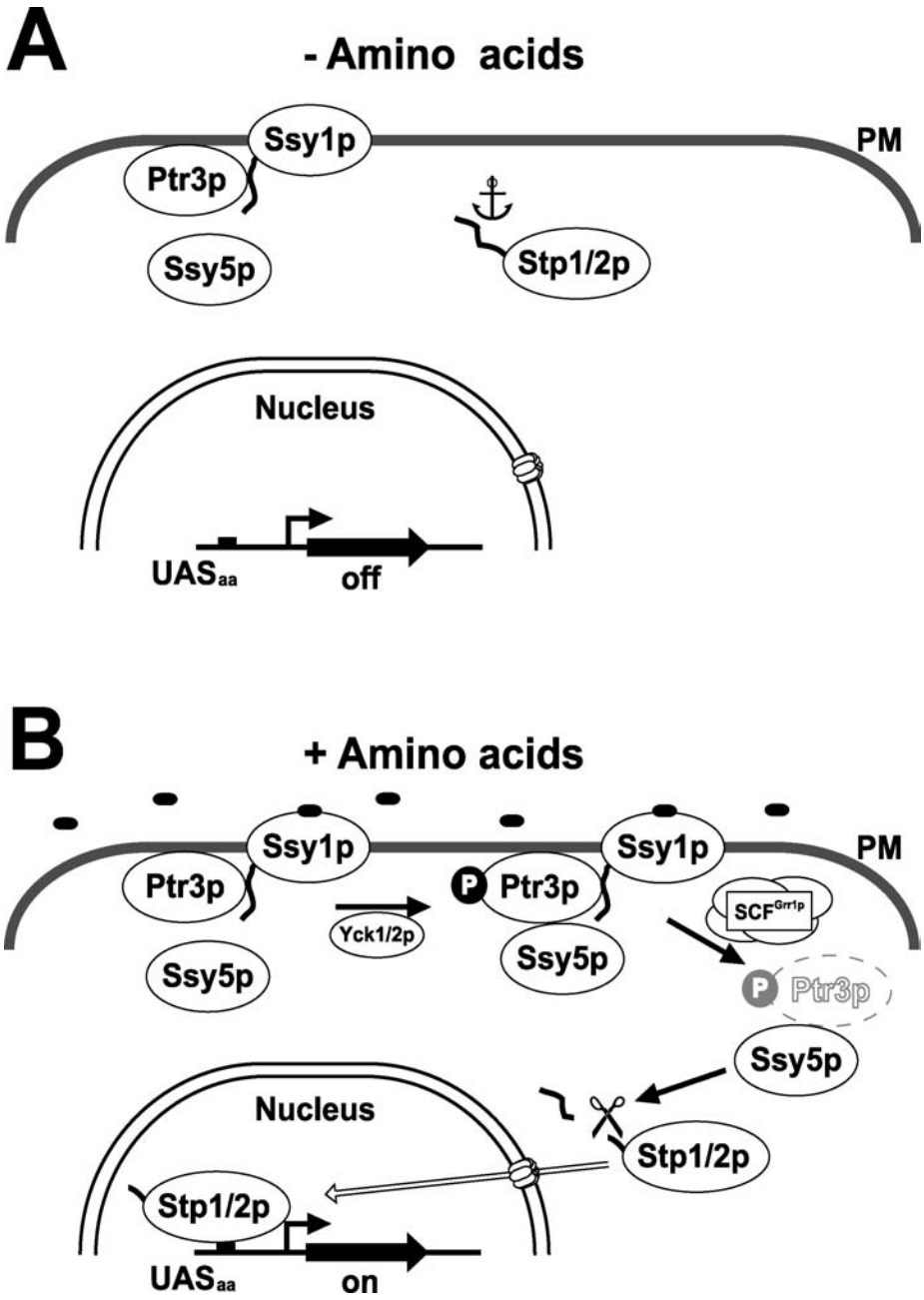

Figure 1. Schematic representation of a speculative model of SPS sensor signaling. A. No signaling in the absence of extracellular amino acids. B. Signaling in the presence of extracellular amino acids. See text for details.

The first evidence for amino acid signaling

The first experiments suggesting the existence of a system that upregulate gene expression in response to the presence of certain amino acids in the growth medium came from the study of dipeptide transport (Island et al., 1987). Cells were observed to exhibit an increased sensitivity to a toxic dipeptide analogue when media was supplemented with micromolar amounts of certain amino acids. The added amino acids stimulated peptide uptake. Subsequent cloning of the peptide transporter gene *PTR2* revealed that the stimulatory amino acid leucine activated uptake by reducing *PTR2* transcription (Perry et al., 1994). A similar observation was made for the *BAP2* gene encoding a broad-specificity branched chain amino acid permease (Didion et al., 1996). Several amino acids including leucine increased transcription of *BAP2* when present at micromolar levels in the growth medium. However none of these experiments was helpful in determining how leucine was affecting the expression of these genes or if this even occurred via the same mechanism.

Isolation of mutants with defects in amino acid signaling

Genetic approaches have been central to the understanding of the mechanisms behind amino acid induced gene expression. The genetic screen yielding Sensitive to Sulfonylurea on YPD (*ssy)* mutations provides an early and quite complete example of how mutants with defects in amino acid signaling can be obtained (Jorgensen et al., 1998). Sulfonylurea herbicides inhibit acetohydroxyacid synthase, encoded by the *ILV2* gene, resulting in a defect in synthesis of branched chain amino acids. Consequently, cells must import isoleucine and valine for growth on sulfonylurea herbicide containing medium. Mutants with a defect in uptake of branched chain amino acids cannot grow on complex medium supplemented with sulfonylurea due to the reduced level of functional expression of specific transporters. Sulfonylurea sensitive mutations were not expected to be found within transporter encoding genes for the reason that uptake of isoleucine and valine are mediated by multiple transporters. Consequently, mutations in one transporter would not affect amino acid uptake via the others. The genetic screen yielded 21 recessive mutations that were found to fall into five complementation groups. The *ssy1*, *ssy3*, and *ssy5* all share the characteristic that they give rise to reduced uptake of many amino acids and demonstrated resistance to several toxic amino acid analogues. Cells harboring *ssy2* mutations had similar but milder phenotypes and *ssy4* cells appeared to have defects in the uptake of a much narrower range of amino acids.

Mutations in the three loci (*SSY1*, *PTR3* (*SSY3*), and *SSY5*) found in the *ssy* screen were isolated in other laboratories as resistance to toxic peptide analogues (Peptide Transport, *ptr*) (Island et al., 1987), high levels of histidine (Super-high Histidine Resistant, *shr*) (Ljungdahl et al., 1992), and to multiple toxic amino acid analogues (Amino Acid Permease Factor, *apf*) (Bernard and Andre, 2001a). A general principle that unifies mutations in these three loci appears to be that simple loss of function alleles cause severe uptake defects of multiple amino acids and peptides without observable growth impairments if cells are able to synthesize all amino acids.

A sensor of extracellular amino acids

The *SSY1* gene product is intriguing by itself. The polypeptide encoded by this gene is a distant member of the yeast APC amino acid permease family. It has 12 putative membrane spanning domains and shares sequence homology with other amino acid permeases (Jorgensen et al., 1998). Ssy1p demonstrates unique features not present in the other amino acid permeases. It has an unusually long (about 280 amino acid residues) long cytoplasmically oriented N-terminal extension and it contains two extended extracellular loops between transmembrane segments five/six and seven/eight. It was quickly recognized that Ssy1p was not capable of catalyzing measurable amino acid uptake (Didion et al., 1998). Branched chained amino acids were known to be imported mainly by Bap2p, Bap3p, and Tat1p. However *ssy1* cells had severe uptake defects of branched chain amino acids. The pleiotropic affect could not readily be explained by the simple model that Ssy1p is transporter of branched chain amino acids. An alternative hypothesis was that Ssy1p is required for the transcription of other amino acid permeases including the above-mentioned transporters of branched chain amino acids. This hypothesis has since proved correct, the leucine induced expression of *BAP2*, *BAP3*, and *TAT1* require *SSY1*. Furthermore, L-leucine and D-leucine were shown to induce the *BAP2* promoter driving the expression of β-galactosidase in a strain largely defective in uptake of branched chain amino acids or D-isomers. This observation suggested that leucine is sensed outside the cell and that the amino acid permease-like molecule Ssy1p is a sensor that functions in the plasma membrane.

In a parallel study in the laboratory of Bruno André, Ssy1p was identified as a unique member of the amino acid permease family. They were intrigued by two unusual properties; first, Ssy1p is unusually long for an amino acid permease and second, the *SSY1* gene has a very low codon bias index suggesting it is poorly expressed (Iraqui et al., 1999). These two properties are shared with the hexose transporter family members Rgt2p and Snf3p, two transporter-like molecules that functions as sensors of extracellular glucose (for a recent review see (Boles and Andre, 2004)). They found that *SSY1* is required for amino acid induced expression of *AGP1* encoding a broad specificity amino acid permease. This induction was found to depend on extracellular rather than intracellular levels of amino acids as demonstrated by nice experiments taking advantage of knowledge of how to manipulate tryptophan uptake and metabolism. The addition of tryptophan to the medium induced the expression of the *AGP1* promoter in a *SSY1* dependent manner, even in cells where tryptophan uptake was severely impaired by mutation. Additionally cells with 70 fold higher levels of intracellular tryptophan, the consequence of a feedback resistant mutation in *TRP2* (encodes anthranilate synthase), do not exhibit induced *AGP1* promoter activity.

Also, in a parallel study, *shr10* mutations that enable cells to grow in the presence of toxic concentration of histidine (Ljungdahl et al., 1992) were found to be allelic with *SSY1* (Klasson et al., 1999). Results obtained in the Ljungdahl laboratory initially suggested that this permease-like molecule was a vacuolar transporter, since the *shr10* cells exhibit altered vacuolar pools of many amino acids. The idea that the N-terminal extension contained vacuolar localization determinants was rapidly revised when an epitope tagged Ssy1p was shown to localize to the plasma membrane. In this study the extended N-terminus of Ssy1p was shown to be important for function since in-frame insertions of epitopes in the N-terminus abolished function without altering overall levels of expressed protein. Also, overexpression experiments with the N-terminus alone supported a role for this domain in amino acid sensing (Bernard and Andre, 2001a; Forsberg and Ljungdahl, 2001a).

Recently, more direct evidence that Ssy1p truly functions as a sensor of extracellular amino acids was obtained when mutations in *SSY1* that appear to constitutively induce amino acid permease gene expression were isolated (Gaber et al., 2003). These mutations initiate signaling even in the absence of inducing amino acids. These findings suggest that Ssy1p indeed participates in signal generation and that the uptake of amino acids is not required for the process. Mutations in *SSY1* that exhibit altered inducer specificity have been isolated. This mutant still activates the *AGP1* promoter in response to leucine but not in response to the normally potent inducer phenylalanine (Bernard and Andre, 2001a). Together these results clearly implicate Ssy1p in amino acid detection and suggest that it functions as a receptor.

An emerging signal transduction pathway

The genetic approaches that led to the isolation of *SSY1* mutants also identified additional components of a potential signal transduction pathway. Mutations in *PTR3* and *SSY5* give rise to similar mutant phenotypes as mutations in *SSY1*. However, since these genes do not encode proteins with obvious conserved structural motifs, their corresponding roles on the protein level were harder to predict. With the development of better bioinformatic tools, it is now quite clear that Ptr3p exhibits homology to the seven bladed propeller-like proteins of the WD40 type (unpublished observation) and that Ssy5p exhibits weak structural similarity with chymotrypsin like proteases (see chapter 5). The cloning and characterization of *PTR3* was first accomplished as a consequence of genetic studies on peptide uptake (Barnes et al., 1998). Mutations in *PTR3* were found to be defective in amino acid induced transcription of the peptide transporter gene *PTR2* and the broad specificity amino acid permease gene *BAP2*. The paper describing the selection and cloning of the *ssy* mutations, published the same year, demonstrated that *ssy1*, *ptr3*, and *ssy5* cells exhibited identical defects of uptake of branched chain amino acids (Jorgensen et al., 1998). These studies indicated that the gene products affect a common regulatory mechanism. Similarly, the next publication described results demonstrating that strains carrying *ssy1* and *ptr3* mutations exhibited identical phenotypes, and *SSY1* and *PTR3* were required for proper expression of several genes on many different media (Klasson et al., 1999). This latter report demonstrated that Ptr3p is a peripherally associated membrane protein that resides on the cytoplasmic side of the plasma membrane. Ptr3p can be released from the membrane by mild treatments like EDTA or high salt. When analyzed by SDS-PAGE, Ptr3p was found to be post-translationally modified; distinct faster and slower migrating forms of Ptr3p were evident (Forsberg and Ljungdahl, 2001a). The faster migrating form is prevalent in cells grown in the absence of amino acids or when *SSY1* is inactivated, while the slower migrating form accumulates when amino acids are present in the growth medium (SC) or *SSY5* is inactivated (SD or SC). The molecular nature of these two species of Ptr3p is unknown but it is likely that they arise as a consequence of phosphorylation. However the appearance of the slower migrating form does not strictly correlate with the time frame of signaling (Forsberg and Ljungdahl, 2001a). Thus the role of the modification is currently unclear.

Strains carrying *ssy5* mutations exhibit identical phenotypes as *ssy1* and *ptr3* mutants (Bernard and Andre, 2001a; Forsberg and Ljungdahl, 2001a; Jorgensen et al., 1998), and subsequent analysis has shown that Ssy5p is also required for signal transduction from Ssy1p. Ssy5p may associate with the plasma membrane since a chimera of human SOS fused to the N-terminus of Ssy5p was shown to complement a *cdc25-2* mutation (Forsberg and Ljungdahl, 2001a). The complementation of the *cdc25-2* mutation indicates that the SOS fusion protein reaches the cytoplasmic side of the

plasma membrane, however it should be noted that the assay does not provide information about the amounts of Ssy5p protein present at the plasma membrane. I will avoid a further discussion about past results obtained from experiments with epitope tagged versions of Ssy5p (Forsberg and Ljungdahl, 2001a) since I have concerns that this procedure changes the normal function of the protein, and that the immuno-detectable portion of the N-terminally epitope tagged protein appear to be a proteolytic fragment constitutively generated during biogenesis. Recently obtained data indicate that Ssy5p is a chymotrypsin-like serine protease with an activity that is activated by inducing amino acids. I discuss these experiments separately in chapter 5.

The SPS sensor complex hypothesis

Genetic and biochemical data suggests that Ssy1p, Ptr3p, and Ssy5p may be components of a sensor complex within the plasma membrane. This hypothesis is based on several lines of evidence. First, *ssy1*, *ptr3*, and *ssy5* cells exhibit identical phenotypes (discussed above) suggesting that they are involved in the same pathway. Second, overproduction of the N-terminus of Ssy1p interferes with signaling suggesting that components interacting with the N-terminus are sequestered away from the signaling pathway (Bernard and Andre, 2001a; Forsberg and Ljungdahl, 2001a). Third, Ssy1p, Ptr3p and possibly Ssy5p colocalizes to the plasma membrane (discussed above). Fourth, the N-terminus of Ssy1p interacts with Ptr3p, and Ptr3p interacts with Ssy5p in two-hybrid experiments (Bernard and Andre, 2001a). However, convincing evidence of a complex formation, such as a purification of a sensor complex, is still lacking. Despite this obvious short coming, I will refer to the pathway initiated by extracellular amino acids as the Ssy1p-Ptr3p-Ssy5p (SPS) sensor pathway (or short; SPS sensor). I regard the use of this expression as reference to the pathway where Ssy1p, Ptr3p and Ssy5p are involved in core signaling events. Hence, my use of the term SPS sensor does not echo a belief of a strict requirement for the function of a protein complex in the process of sensing or signaling.

Grr1p and the ubiquitin-proteasome pathway

Many yeast genes are regulated in response to glucose availability. A subset of genes is coordinately repressed when cells are grown in medium containing glucose while others are activated. Mutations in the *GRR1* gene lead to the inappropriate expression of glucose repressed genes (Bailey and Woodword, 1984). *GRR1* encodes a long protein with leucine rich repeats and an F-box (Flick and Johnston, 1991). F-box containing proteins are components of ubiquitin E3 ligase complexes that catalyze the attachment of ubiquitin, a small polypeptide of 72 amino acid residues, to lysine residues on substrate proteins (Kipreos and Pagano, 2000). This protein modification, termed ubiquitylation, is often the first step in targeting a protein for degradation via the ubiquitin-proteasome pathway (for a review of the yeast ubiquitin-proteasome system see (Smith et al., 1996)). The F-box proteins function as adaptors that bind to discrete substrate proteins and correctly position them with respect to the E3 enzyme. Grr1p is the F-box protein in the SCF^{Grr1p} ubiquitin E3 ligase complex that is required for ubiquitinylation of a diverse set of substrates, including G1 cyclins (Skowyra et al., 1997), and the degradation of Mth1p and Std1p, two proteins required for proper glucose regulation (Flick et al., 2003) (Moriya and Johnston, 2004).

A couple of previous observations led the laboratory of Bruno André to investigate the possibility that Grr1p may be involved in SPS sensor signaling. First, glucose induced signaling from the transporter like plasma membrane sensors Rgt2p

and Snf3p is defective in *grr1* mutants (Ozcan et al., 1993; Ozcan and Johnston, 1995; Vallier et al., 1994). Second, *grr1* mutants exhibit defects in uptake of aromatic amino acids and leucine (Flick and Johnston, 1991). Indeed it was found that the *AGP1* promoter is not amino acid induced in *grr1* mutants (Iraqui et al., 1999). The generality of the requirement for *GRR1* in SPS sensor signaling was established when it was found that amino acid induced *PTR2* expression also was defective in *grr1* mutants (Bernard and Andre, 2001b). The requirement for other components of the SCF^{Grr1p} complex (*CDC34*, *CDC53*, *HRT1*, and *SKP1*) was also investigated, and all of these components appear to be required for proper amino acid induction of *AGP1* and *PTR2* (Bernard and Andre, 2001b). The level of free ubiquitin within intracellular pools was also found to affect SPS sensor induced transcription of the *AGP1* promoter.

It should be noted that mutations in *GRR1* had not been isolated in genetic screens that identified the SPS sensor components. Only recently were *grr1* mutants found in an unbiased genetic selection relying on amino acid uptake based assays, but only after all previously isolated SPS sensor component genes had been duplicated (Paper IV). Apparently, the extreme slow growth of *grr1* mutants has made their identification difficult. Additionally, paper IV documents that *grr1* mutants exhibit similar phenotypes as strains carrying *ssy1*, *ptr3*, and *ssy5* mutations, and that *GRR1* is absolutely required for SPS-sensor signaling.

Amino acid sensor independent (asi) mutants

As a consequence of the decreased expression of many amino acid permease genes, SPS sensor mutants are unable to efficiently transport leucine. Mutations in *LEU2* (encoding β-isopropylmalate dehydrogenase) result in a strict requirement of leucine uptake from the medium. Thus, *leu2* auxotrophic strains carrying mutations that inactivate the SPS sensor pathway will only grow under conditions that permit non-specific leucine uptake. Under such conditions the presence of other amino acids easily out compete low affinity leucine uptake. Consequently, such double mutants are unable to grow. This synthetic non-growth phenotype, observed for *ssy1 leu2* mutants and *ptr3 leu2* mutants, formed the basis to select suppressing mutations that restored leucine uptake (Paper I). This genetic approach was designed to identify downstream components of the SPS sensor pathway. The Amino Acid Sensor Independent (*asi*) mutations obtained defined two phenotypic classes. Class I mutations were found to constitutively activate the transcription of SPS sensor regulated amino acid permease genes. Class II mutations stabilized residual amino acid uptake systems at the plasma membrane by impairing their targeting to the vacuole.

The *asi* class I genes included *ASI1*, *ASI2*, *ASI3*, *TUP1*, *SSN6*, and *ASI13*. Tup1p and Ssn6p are previously characterized repressors of transcription that act on many genes (Smith and Johnson, 2000). However Northern analysis indicated that the isolated *tup1* and *ssn6* mutations did not derepress all SPS sensor promoters. The expression of *BAP2* and *GNP1* was derepressed in *tup1* or *ssn6* mutants while *AGP1* was not. This suggests that the repressing activity of Tup1p and Ssn6p is not directly regulated by the SPS sensor pathway, indicating that they are promoter specific. In contrast, mutations in *ASI1*, *ASI2*, *ASI3*, and *ASI13* appeared to derepress all three SPS sensor dependent promoters assayed. *ASI1*, *ASI2*, and *ASI3* have not previously been characterized. The isolated *asi1*, *asi2*, and *asi3* mutations were recessive and gave rise to identical phenotypes, suggesting that these components are all required parts of a common pathway that normally functions to negatively regulate SPS sensor signals.

Asi1p and Asi3p encode homologous proteins with five predicted transmembrane domains in the N-terminal half of the protein. The C-terminal halves of the proteins are predicted to face the cytosolic side of the membranes and contain a highly conserved

RING-like motif of the very C-terminus. RING domains are structural elements stabilized by cysteine and histidine residues that serve to coordinate metal ions (normally Zn^{2+}). Their function is believed to be to mediate protein-protein interactions and their presence is characteristic for ubiquitin E3 ligases. Interestingly, the RING-like domain present in Asi1p and Asi3p differ from the archetypical RING domain definition in that a cysteine residue replaces a conserved histidine residue. The possibility that Asi1p and Asi3p are ubiquitin ligases remains an open question. Asi2p also encodes a integral membrane protein with a topology distinct from Asi1p and Asi3p.

Stp1p and Stp2p – severed, free and nuclear

Based on its dominant nature, the *ASI13-1* allele was assumed to encode a constitutively active downstream component of the SPS sensor pathway (Paper I). Consistently, the allele strongly suppressed all plate phenotypes exhibited by *ssy1* or *ptr3* mutants. The transcript levels of three SPS sensor regulated genes (*AGP1*, *BAP2*, and *GNP1*) were even higher in *ASI13-1* suppressed *ssy1* and *ptr3* mutant cells, than in amino acid induced wildtype cells. The cloning of *ASI13-1* revealed that the mutation resided in *STP1* (Paper II).

STP1 was originally identified as a high-copy facilitator of tRNA processing (Wang and Hopper, 1988). The processing effect appeared to be specific for certain classes of tRNA molecules, thus the name Species Specific tRNA Processing (*STP*). It is now clear that this effect is the consequence of *STP1* regulating amino acid uptake, which in turn affects intracellular amino acid pool sizes and indirectly the tRNA maturation process. Consistent with this idea, mutations in *STP1* (*bap1*) were isolated due to diminished branched chain amino acid uptake (Tullin et al., 1991). Additional alleles of *STP1* were isolated as *ssy2* mutations (Jorgensen et al., 1998). Based on the observation that *stp1* null mutants are defective in leucine induction of the *BAP2* promoter, *STP1* was proposed to encode a transcription factor (Jorgensen et al., 1997). This notion is consistent with the presence of three putative zink-finger domains and that a β-galactosidase fusion of Stp1p localizes to the nucleus (Wang et al., 1992). The assignment of Stp1p as a transcription factor was further supported by the lack of *BAP3* expresson in an *stp1* null mutant strain (De Boer et al., 1998). Stp1p was shown to bind the promoter of *BAP2* (–628 to –376 bp relative to the start codon) in gel shift experiments (Nielsen et al., 2001). *STP2* encodes a close homologue of Stp1p and was originally given its name based on this similarity. However, *STP2* was first found in a genetic screen as a multi copy suppressor that enabled growth on minimal proline medium in the presence of a sulfonylurea inhibitor of branched chain amino acids synthesis (de Boer et al., 2000). In this study, the presence of either *STP1* or *STP2* was shown to be required for leucine activation of the *BAP3* promoter, and using a gel shift assay it was found that Stp2p binds the *BAP3* promoter (-495 to –392 bp relative to the start codon). Similar redundancy was later reported for transcriptional activation of the *BAP2* promoter (where Stp1p appears to play the major role), and again Stp2p binding of the promoter was demonstrated (Nielsen et al., 2001).

A number of transcription factors in addition to Stp1p and Stp2p that affect expression of different SPS sensor regulated promoters have been isolated. Initially this seemingly complex situation made it hard to appreciate the important role of Stp1p and Stp2p (Forsberg and Ljungdahl, 2001b). The cloning of the constitutive *ASI13-1* allele of *STP1* was very instrumental in deciphering the outline of the SPS sensor pathway (Paper II). The *ASI13-1* mutation is a large in-frame deletion removing 58 codons corresponding to the N-terminus of Stp1p. The removal of the N-terminal domain constitutively activated expression of SPS sensor regulated genes suggesting that the N-

terminal domain has a negative regulatory role. Experiments were designed to test whether signals initiated by the SPS sensor directly affected the negative activity within the N-terminus. If such a link could be found this would constitute a possible mechanism that transmits the amino acid induced signal from the SPS sensor at the plasma membrane to the nucleus. Epitope tags inserted at the N- and C-termini of Stp1p revealed the existence of an endoproteolytic processing event that rapidly removed a ~10 kD portion of the N-terminus of the protein in response to addition of leucine to the growth medium. The removal of this inhibitory domain correlated in time with previously characterized SPS sensor regulated promoter activation and exhibited the expected product-precursor relationship. The processing was found to be dependent on an intact SPS sensor. Later work demonstrated that processing is also defective in strains harboring *grr1* null mutations (Paper IV).

Several independent approaches were use to investigate whether the lack of processing in strains carrying mutations inactivating the SPS sensor and *GRR1* was an indirect consequence of diminished amino acid uptake. First, cells were grown with leucine as the sole nitrogen source and the processing of Stp1p was monitored (Paper II). It was found that even though the mutant cells imported leucine at sufficient rates for robust growth, processing did not occur. A second more conclusive experiment took advantage of the constitutively signaling *SSY1-102* mutant allele (Gaber et al., 2003). Stp1p processing was observed in the cells harboring *SSY1-102* even in the absence of added amino acids (Paper IV).

A direct test of the requirement of Stp1p processing in SPS sensor signaling was enabled by the isolation of a processing defective mutation of *STP1*, *stp1-102* (F66A) (Paper IV). Stp1p-102p was expressed at normal levels but was not processed. The *stp1-102* allele does not complement *stp1*Δ phenotypes, however, it is able to complement *stp1*Δ phenotypes when secondary mutations are placed in *cis* (point mutations in the negative regulatory domain) or *trans* (*asi1*Δ), suggesting that the inactivity of Stp1-102p is specifically due to the processing defect.

Previous observations indicated that Stp1p cannot be the sole downstream component of the SPS sensor pathway. Examining the transcription from the *BAP2* and *BAP3* promoters suggested that *STP1* or *STP2* can, at least partially, substitute for each other (de Boer et al., 2000; Nielsen et al., 2001). Furthermore, *stp1*Δ mutations do not manifest the same phenotypes as *ssy1*Δ, *ptr3*Δ, or *ssy5*Δ mutations. However, simultaneous inactivation of both *STP1* and *STP2* did result in identical plate-phenotypes (Paper II), supporting the hypothesis that Stp1p and Stp2p are redundant and able to function in parallel. Stp2p was found to undergo SPS sensor dependent proteolytic processing in response to extracellular amino acids, and removal of codons 2-74 created a dominant active allele of *STP2* that effectively suppresses *ssy1*Δ phenotypes. Thus, Stp1p and Stp2p are partially redundant transcription factors that both receive and transmit signals from the SPS sensor.

The SPS sensor induced signals generated at the plasma membrane must somehow be transmitted to the nucleus. The first suggestion that Stp1p and Stp2p themselves may constitute this spatial link came from an experiment with human SOS-protein fusions. An N-terminal fusion of the human SOS protein to either full-length Stp1p or the N-terminal region of Stp1p complemented of a *cdc25-2* temperature sensitive mutant (Paper II). This indicated that at least some part of the population of chimeras are targeted to the plasma membrane and suggests that full-length Stp1p is able to associate with the plasma membrane in an N-terminal dependent manner. However, the association apparently occurs in the absence of Ssy1p (Paper II), or even in cells carrying *ssy1*Δ *ptr3*Δ *ssy5*Δ triple mutations (unpublished observation), excluding the possibility that the interaction occurs exclusively via these components. A

series of immunolocalization experiments provided clear support for the notion that Stp1p enters the nucleus as a consequence of proteolytic removal of the N-terminal regulatory domain (Paper II). Stp1p was only observed in the nucleus after addition of leucine to the media or when the negative regulatory domain had been removed. In contrast, Stp1p was never observed in the nucleus in an *ssy1* null mutant strain.

The negative regulatory domain of Stp1p and Stp1p

The negative regulatory domains of Stp1p and Stp2p have central roles in controlling the transactivation capacity of these transcription factors. This regulation likely occurs by controlling the nuclear accumulation of Stp1p and Stp2p. Hence, the simplest model states that the SPS sensor regulates Stp1p and Stp2p solely by severing away the negative regulatory domain, and as a consequence the processed transcription factors accumulate in the nucleus. This model is supported by an experiment where the N-terminus of Stp1p ($Stp1p_{1-125}$) was fused to an artificial transcription factor consisting of the DNA binding domain of bacterial LexA, and the VP16 activation domain (AD) (paper IV). Lex-AD is a constitutive transcription factor that localizes to the nucleus due to a nuclear localization signal within lexA (Rhee et al., 2000). However, when the $Stp1p_{1-125}$-LexA-AD chimera is expressed, promoter activation requires an intact SPS sensor and the presence of inducing amino acids. Consistently, immunoblot analysis indicated that the chimera is proteolytically processed in response to SPS sensor signals. Thus, the first 125 amino acid residues of Stp1p contain all activities required to actively regulate a normally constitutive transcription factor via the SPS sensor. This suggests that the SPS sensor regulates Stp1p and Stp2p exclusively by proteolytic processing and that their negative regulatory domains are nuclear exclusion determinants.

The net accumulation of Stp1p or Stp2p in the nucleus is most likely a function of their import and export in or out of the nucleus. In the simplest model, only import of Stp1p and Stp2p is regulated (Figure 2A). The function of the negative regulatory domain must consequently be to effectively prohibit such import, for example by anchoring Stp1p and Stp2p to a cytosolic determinant. A more complicated mechanism involves export as well. Accordingly, Stp1p and Stp2p constantly shuttle in and out of the nucleus (Figure 2B). The rate of import and export determines if the transcription factors accumulate inside or outside of the nucleus. In this model, the function of the negative regulatory domain would be to facilitate export, presumably by binding export receptors or other exported cargo. Both models are fully compatible with all data, and examples of both anchoring and shuttling transcription factors are found in the literature (Schwoebel and Moore, 2000). Hence, experiments that distinguish between these two models are needed.

Results from a few experiments link the activity of the negative regulatory domain of Stp1p and Stp2p to Asi1p, Asi2p, and Asi3p function. Inactivation of any of the *ASI* genes makes SPS sensor regulated promoters constitutively active, even in the absence of a functional SPS sensor (Paper I). This constitutive activation requires Stp1p or Stp2p since *stp1*Δ *stp2*Δ cells, that are resistant to the toxic amino acid analogue AzC (AzC is imported via SPS sensor regulated permeases Agp1p and Gnp1p (Paper III)), do not become sensitive to AzC when an *asi1*Δ mutation is introduced (Paper IV). Furthermore, Stp1p is expressed as a full-length protein in cells harboring *asi1* mutations. Consistently, *asi1*Δ *stp1*Δ *stp2*Δ mutant cells are resistant to AzC but gain sensitivity when the processing defective full-length Stp1-102p is expressed. Thus, normally inactive full-length Stp1p potently activates promoters in *asi1* mutants. This is likely the cause of a malfunctioning negative regulatory domain in cells harboring *asi1*,

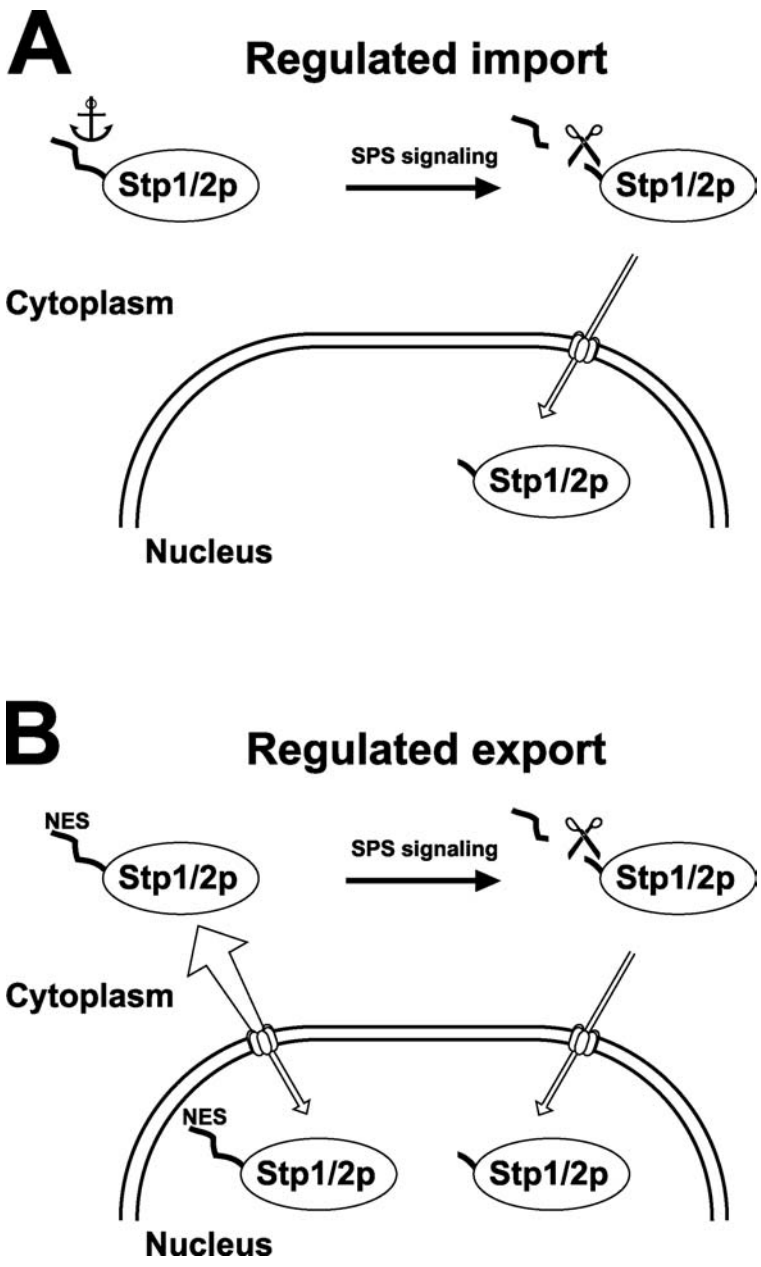

Figure 2. Schematic representation of two distinct models of the mechanism regulating nuclear accumulation of Stp1p and Stp2p (Stp1/2p). A. Regulated import. The N-terminal negative regulatory domains of Stp1/2p anchor the transcription factors outside the nucleus. B. Regulated export. The N-terminal negative regulatory domains contain a nuclear export signal (NES) that facilitates efficient export of full-length Stp1/2p. See text for further details.

asi2, or *asi3* mutations, since full-length $Stp1p_{1-125}$-LexA-AD gain transactivation capacity in cells carrying *asi1* mutations (Paper IV). This may for example be due to a defect in the function of the negative regulatory domain outside the nucleus (e.g., anchoring) or due to a defect in recognizing full-length Stp1p that illegitimately gains access to the nucleus (e.g., nuclear export). The possibility exists that in the absence of inducing amino acids the majority of full-length Stp1p and Stp2p are anchored outside the nucleus, however, a small portion may escape anchoring and enter the nucleus. Thus, the Asi -proteins may have a direct or indirect role in keeping the nucleus devoid of full-length Stp1p (e.g., export or degradation). Unfortuitously, the current experimental data is not comprehensive enough for detailed understanding of either the function of the Asi-proteins or how inactivation of *ASI*-genes converts normally inactive Stp1p and Stp2p to active transcription factors.

Stp1p, Stp2p and nuclear partners

SPS sensor regulated promoters respond differently to nitrogen availability. Recall that leucine induces the transcription of *AGP1* more potently when cells are grown with the non-preferred nitrogen source proline than with ammonia. Inversely, *GNP1* is transcribed more effectively when cells are grown with ammonia and less effectively when cells are grown with proline (Paper III) (Regenberg et al., 1999). The current understanding of the nitrogen source dependence of amino acid induced signaling via the SPS sensor pathway is that other transcription factors participate to differentially control expression. These factors are expected to work either synergistically/antagonistically with Stp1p and Stp2p, or function in parallel.

Factors acting together with Stp1p and Stp2p are expected to function via the promoter elements bound by Stp1p or Stp2p. Inversely, factors that act in parallel are predicted to function via other upstream regulatory sequences. A key finding for distinguishing between these modes of action has been the isolation of the upstream activating sequence element that responds to amino acid induced SPS sensor signals. This UAS_{aa} element was first found in the *BAP3* promoter (-418 to -376 bp relative to start codon) and was demonstrated to be sufficient for leucine induction when transferred to an unrelated minimal promoter as a 42 bp fragment (De Boer et al., 1998). Later it was reported that mutations in a similar sequence element in the *BAP2* promoter dramatically reduced *STP1* dependent transcription (Nielsen et al., 2001). Also the *AGP1* promoter contains a UAS_{aa} like element that is required for SPS sensor dependent transcription and when transferred to an unrelated promoter as a 21 bp fragment, confers SPS sensor dependent transcription (Abdel-Sater et al., 2004). The UAS_{aa} element is likely to be the Stp1p and Stp2p binding site since gel shift experiments demonstrate that Stp1p and Stp2p bind promoter fragments containing intact but not mutated UAS_{aa} (de Boer et al., 2000; Nielsen et al., 2001). Furthermore, UAS_{aa} like elements are found in all amino acid permease promoters believed to be regulated by Stp1p and Stp2p (Eckert-Boulet et al., 2004) and many of these promoters can be cross-linked to Stp1p or Stp2p under SPS sensor inducing conditions (Lee et al., 2002) (Table 3).

The precise sequence of a functional UAS_{aa} is presently unclear. The typical UAS_{aa} appears to consist of two 5′–CGGC–3′ in either direct or inverse repeats separated by four to eight nucleotides. The UAS_{aa} in the promoter of *AGP1* is the inverted type but this configuration is not absolutely required for function since inversion of the second 5′–CGGC–3′ to 5′–GCCG–3′ still supports amino acid induction (Abdel-Sater et al., 2004). A recent report proposed the UAS_{aa}-like sequence [a/g]CGGCnnn[a/g]CGGC as a likely Stp1p binding motif (Harbison et al., 2004). This

motif was obtained by merging genome wide location data, phylogenetically conserved sequences, and prior knowledge. The study also identified two distinct sequences as over represented in promoter-probes bound by Stp1p (CCGTACGGC--GC) or Stp2p (TTGACGT[G/T][A/G]TT) by using a hypergeometric algorithm. However, the predictive value of such over represented sequences is presently unclear. The pair-wise configuration of 5′–CGGC–3′ suggests that Stp1p or Stp2p may bind as dimers to many promoters, opening the possibility of hetero- and homodimerization. However, despite the fact that both Stp1p and Stp2p carries three predicted zink-finger domains, no experiments have directly implicated these domains in DNA binding or protein-protein interactions.

Dal81p (also known as Uga35p) appears to be a factor that acts synergistically with Stp1p and Stp2p via UAS_{aa}. Dal81p encodes a general transcription factor believed to be recruited by promoter specific transcription factors (Coornaert et al., 1991). The first characterization of the *AGP1* promoter demonstrated that *DAL81* was required for full SPS sensor dependent activation of the promoter (Iraqui et al., 1999). Consistently, it was recently reported that *DAL81* is required for activation from an isolated UAS_{aa} obtained from the *AGP1* promoter (Abdel-Sater et al., 2004). Full activation of *BAP2* also requires *DAL81* (Bernard and Andre, 2001a). This suggests that Stp1p and Stp2p require Dal81p as a cofactor for potent transcriptional activation. Dal81p has been found to cross-link to many promoters subject to Stp1p and Stp2p activation (Lee et al., 2002) (Table 3).

Another potential cofactor for Stp1p and Stp2p is Abf1p. Based on the similarity between the UAS_{aa} sequence and known Abf1p binding sites, the Planta lab investigated the role for Abf1p (de Boer et al., 2000). Indeed they found that Abf1p was able to form a complex with an UAS_{aa} containing fragment from the *BAP3* promoter. Multiple mutant versions of the UAS_{aa} that do not bind Abf1p or Stp2p are consistently impaired in amino acid induced activation. Together these results suggest a role for Abf1p in activation of at least the *BAP3* promoter. A simple test for the requirement of Abf1p for the expression of SPS sensor dependent promoters is complicated by the fact that *ABF1* is an essential gene.

Table 3. UAS_{aa}-like motifs upstream of SPS regulated genes and experimentally confirmed promoter binding of Stp1p, Stp2p, and Dal81p

		Detected binding[2]		
Gene	**UAS_{aa}-like motif in promoter**[1]	Stp1p	Stp2p	Dal81p
AGP1	5′–GTGCCGTCTAAGCGGCAC–3′	+	+	+
BAP2	5′–AACGGCGACACGGCGC–3′			+
BAP3	5′–TAGCCGTGCATGCGGCTC–3′	+		+
DIP5	5′–TGGCCGTACGGCGTCGCTA–3′	+		+
GNP1	5′–CCGCCGTACGGTATGCGGCGC–3′	+		+
MUP1	5′–TTCGGCTCCGTAAGCCGGC–3′	+		+
PTR2	5′–GCGCCGAAGGCAGCGGCGA–3′			+
TAT1	5′–TCGCCGCGCCGGGACGGCCA–3′			+
TAT2	5′–CCCTAAAGAAGCTACGGCGC–3′			+

[1] UAS_{aa}-like motifs experimentally determined for *AGP1* (Abdel-Sater et al., 2004), *BAP2* (Nielsen et al., 2001), and *BAP3* (De Boer et al., 1998), or similar motif present within 1 kb upstream of indicated gene.
[2] Binding detected ($p \leq 0.05$) by genome-wide chromatin immunoprecipitation analysis (Lee et al., 2002).

In contrast to the potential cofactors Abf1p and Dal81p, the GATA-factors Gln3p and Gzf3p are likely to bind the *AGP1* promoter outside the UAS_{aa} element. Mutation of *GLN3* reduces the potent SPS sensor activation of *AGP1* but has no effect on the activation of an isolated UAS_{aa} sequence (Abdel-Sater et al., 2004). Expression from the *AGP1* promoter is similarly increased in *gzf3*Δ or *ure2*Δ mutants but expression from the isolated UAS_{aa} is not affected. Since these GATA-factors are responsible for nitrogen regulation (see chapter 2), and only accumulate in the nucleus when cells are grown with a non-preferred nitrogen source, these factors are likely responsible for the observed nitrogen dependency of the *AGP1* promoter (Abdel-Sater et al., 2004). However, since the expression from the *AGP1* promoter is strictly dependent on SPS sensor initiated signals, and consequently dependent on Stp1p or Stp2p, the GATA-factors themselves are not able to activate the promoter. Thus, a synergistic or hierarchical mechanism of activation of this promoter is likely to exist.

Accumulating evidence suggests that the *BAP2* promoter is regulated in parallel by the combined action of Stp1p and Stp2p, and the transcription factor Leu3p. Leu3p is activated by intracellular levels of the leucine biosynthetic precursor α-isopropyl malate and binds a defined sequence element in the promoters of several genes involved in leucine biosynthesis (Kohlhaw, 2003). SPS sensor activation of the *BAP2* promoter is not strictly dependent on Leu3p since it occurs in *leu3* cells (Didion et al., 1996). However, when the predicted binding site of Leu3p in the *BAP2* promoter is mutated, the expression is reduced at least 3-fold under SPS sensor inducing conditions (SC) and the basal level of expression under non-inducing conditions is abolished (amino acid free ammonium medium). Similarly, the basal level of expression is raised dramatically when Leu3p is made hyperactive by introducing a feed-back resistant isopropyl malate synthase (encoded by *LEU4*) mutation even under non SPS sensor inducing conditions (Nielsen et al., 2001). With respect to amino acid uptake, the parallel regulation of the *BAP2* promoter is unique, Leu3p binding sites are not found in the promoters of other amino acid permease genes (Nielsen et al., 2001).

As mentioned earlier, the global transcription repressor complex consisting of Tup1p and Ssn6p appears to have a role in regulation of at least some Stp1p and Stp2p activated genes. These factors may function to maintain the promoters repressed in the absence of Stp1p and Stp2p. Specifically, mutations in *TUP1* or *SSN6* have been shown to derepress the *BAP2* and *GNP1* genes is *ssy1* and *ptr3* cells (Paper I) (Nielsen et al., 2001).

Stp1p and Stp2p as sole effectors

Let us consider the evidence supporting the notion that Stp1p or Stp2p are the sole effectors of the SPS sensor pathway. First, detailed studies on the *BAP2* and *BAP3* promoter revealed that inactivation of both these genes reduce the transcription as potently as inactivating the SPS sensor (de Boer et al., 2000; Nielsen et al., 2001). Second, cells carrying *stp2* null mutations exhibit detectable expression from the *AGP1* promoter while cells harboring *stp1 stp2* double mutations do not (Paper II). Third, *stp1 stp2* double mutants share multiple and indistinguishable phenotypes with *ssy1* mutants (Paper II). Fourth, genome-wide transcriptional profiling demonstrates very similar profiles when *ssy1*Δ and *stp1*Δ *stp2*Δ strains are compared (Eckert-Boulet et al., 2004). Fifth, UAS_{aa} sequences are present in known regulated genes (Eckert-Boulet et al., 2004) (Table 3). Sixth, Stp1p or Stp2p cross-links to many known SPS sensor regulated promoters (Lee et al., 2002) (Table 3).

Despite this list of evidence, additional effector components cannot be excluded. For example, from a mechanistic point of view, other proteins activated or inactivated by the endoprotease activity of the SPS sensor are readily conceivable. Such prospective proteins do not necessarily need to be involved in transcription. However, a recent report suggests that additional factors or branching of the SPS sensor signal transduction pathway may exist (Abdel-Sater et al., 2004). The two key observations described in this study are (1) *gap1*Δ *stp1*Δ *stp2*Δ strains do not mimic amino acid uptake phenotypes of *gap1*Δ *ssy1*Δ strains, and (2) the *AGP1* promoter exhibits residual expression even when *STP1* and *STP2* are inactivated, but not when *SSY1* is inactivated. Thus, accordingly, branching of the signal transduction pathway could occur after Ssy1p detects amino acids. However, the residual *AGP1* expression detected in cells harboring *stp1 stp2* null mutations contrasts with earlier observations (above). Furthermore, I am unable to detect any residual β-galactosidase expression (unpublished observation) from the commonly used YCpAGP1-LacZ plasmid (Iraqui et al., 1999) in cells harboring *stp1*Δ *stp2*Δ double mutations grown in a comparable medium used in the report. These contrasting observations may be explained at the level of experimental conditions used, i.e., growth conditions or yeast strain background. The experiments in the published report are based on the yeast strain background Σ1228b with many known differences to the S288C background that is used in work from the Ljungdahl or Kielland-Brandt laboratories. It is therefore tempting to speculate that the Σ1228b background harbors a third functional Stp1p or Stp2p homologue.

Targets of Stp1p and Stp2p

Given that Stp1p and Stp2p are the sole effectors of the SPS sensor pathway, SPS sensor regulated genes are defined as Stp1p or Stp2p controlled genes. This definition appears straightforward but is difficult to address experimentally. For example, Stp1p and Stp2p may function in parallel with additional factors that activate transcription, making any contribution from amino acid activated Stp1p and Stp2p undetectable. Recall that the *BAP2* promoter offers such an example, since Leu3p and Stp1p and Stp2p function in parallel. Conversely, the transcription of other genes may dramatically be affected by adding amino acids to the growth medium, and this induction may require the SPS sensor pathway including *STP1* and *STP2* to merely enable their uptake. The general amino acid permease gene, *GAP1*, provides an illustrative example. *GAP1* is regulated by nitrogen availability. In the presence of amino acids in the growth media (like standard SC or YPD medium) transcription is repressed. When amino acids are absent, or cannot be taken up effectively, *GAP1* is effectively transcribed. SPS sensor pathway mutations result in severely reduced uptake capacity of many amino acids (discussed above). Consequently, transcription of *GAP1* is derepressed in SC grown cells when the SPS sensor pathway is inactivated (Klasson et al., 1999) (Paper I). Thus, since the SPS sensor pathway is required for efficient amino acid internalization, *GAP1* is indirectly regulated via the SPS sensor pathway. Direct regulation of a subset of important amino acid permease genes will give rise to many additional transcriptional effects. For a more comprehensive discussion about inducer-exclusion effects see (Eckert-Boulet et al., 2004).

Despite the problem of distinguishing direct from indirect effects and pinpointing genes where SPS sensor signaling has only a moderate effect, no less than three laboratories have attempted to understand the extent of the SPS sensor regulon by using genome-wide transcription analysis (Eckert-Boulet et al., 2004; Forsberg et al., 2001; Kodama et al., 2002). Many genes were found to be effected by addition of amino acids to the growth medium, and quite many observed effects required an intact SPS sensor pathway. However, direct regulation by Stp1p or Stp2p is likely to be more restricted.

Probable targets include the amino acid permease genes *AGP1*, *BAP2*, *BAP3*, *DIP5*, *GNP1*, *MUP*1, *TAT1*, and *TAT2*, and the peptide transporter gene *PTR2*. This notion is based on the following observations. First, transcript levels are strongly induced by amino acids and this requires an intact SPS sensor pathway. Second, all promoter regions contain UAS_{aa}-like elements (Table 3). Third, Dal81p cross-links to all of these promoters and most of the promoters also cross-link to Stp1p or Stp2p (Table 3). Fourth, detailed studies of the promoters of *AGP1*, *BAP2*, and *BAP3* provide evidence that they are activated directly via Stp1p or Stp2p (Abdel-Sater et al., 2004; Nielsen et al., 2001) (De Boer et al., 1998; de Boer et al., 2000). However, no data at this point suggests that these transporter genes are the only targets of the SPS sensor pathway. In conclusion, genes regulated by the SPS sensor pathway may be hard to experimentally identify if they do not exhibit a simple and rigorous requirement of the SPS sensor pathway for their transcription and consequently are not strongly induced by amino acids in the growth medium.

Unknown components

It is likely that additional components will be found that participate in SPS sensor signal transduction. It is reasonable to assume that SPS sensor signal-generation (or transduction within the SPS sensor) involves a change in state of minimally Ssy1p, Ptr3p, and Ssy5p. Both Ssy1p and Ptr3p appear to be subject to modifications consistent with their phosphorylation (Forsberg and Ljungdahl, 2001a). If Ssy1p and Ptr3p are phospho-proteins, yet unidentified kinases and phosphatases must be involved in signal transduction. Interestingly, kinase candidates have already been implicated in SPS sensor signaling in work on glucose signaling. The yeast casein kinases Yck1p and Yck2p are required, for glucose signaling from the transporter-like sensors Rgt2p and Snf3p (Moriya and Johnston, 2004), and a recent report suggested their involvement in SPS sensor signaling. They observed that the broad specificity amino acid permease gene *BAP3* was not efficiently transcribed under SPS sensor inducing conditions in a *yck1 yck2* double mutant strain (Spielewoy et al., 2004). Many components affecting latency and activity of Stp1p and Stp2p also remain unidentified. For example, components directing nuclear targeting and recruitment to relevant promoters will likely involve additional components than presently identified.

The currently known components, with a few exceptions, were initially identified as mutations selected using traditional genetic approaches. However powerful, genetic analysis is limited by the genetic predisposition of the studied organism. Mutations that abolish signaling without severe side effects for the cell will preferentially be isolated. For example, mutations in *SSY1*, *PTR3*, or *SSY5* were readily obtained since they are compatible with life and completely abolish signaling. In contrast, *STP1* and *STP2* were more difficult to identify since single mutations did not completely abolish signaling due to their overlapping functions. Conversely, *grr1* mutations completely abolish signaling but inactivation of the gene is associated with severe growth defects. Finally, despite their documented involvement (Bernard and Andre, 2001b), the central components of the SCF complex, encoded by essential genes, have still not been isolated in genetic screens aimed at identifying components of the SPS sensor pathway. In summary, many more components of the SPS sensor pathway are likely awaiting discovery. However, since the SPS sensor pathway is not required for life in the laboratory, such components are likely to have more general functions (for example to provide kinase activity) rather than being pathway specific components.

The mechanism of sensing

Given that we understand the major outlines of the whole SPS sensor signal transduction pathway, from the plasma membrane to the nucleus, surprisingly little is known about signal initiation. Signal initiation may either be regarded as a one-protein process, where only Ssy1p is involved, or a multi-protein process, where Ssy1p forms a complex with other required components. The SPS sensor complex hypothesis suggests, at least implicitly, that Ssy1p cannot detect extracellular amino acids without being part of a complex. An opposing view regards Ssy1p as an entity fully operational as a sensor but in need of downstream components for further signal transduction. Neither of these two views has been effectively falsified by any experiments. Some observations suggest that the expected sensor-complex components Ptr3p and Ssy5p are not in need of any complex to transmit signals. Overexpression of *PTR3* suppresses certain growth defects of *ssy1*Δ strains, but not of *ssy5*Δ strains. Similary, overexpression of *SSY5* suppresses particular growth defects of *ssy1*Δ and *ptr3*Δ strains (Bernard and Andre, 2001a) (With reservations that an N-terminal epitope tag may have been used in the experiments; see chapter 5.). Even more strikingly, introduction of an epitope tag at the N-terminus of Ssy5p makes the protein constitutively active and able to facilitate processing of Stp1p even in a heterologous expression system (see chapter 5). Thus, the interdependency of Ssy1p, Ptr3p, and Ssy5p is restricted, and at least Ptr3p and Ssy5p appear to be able to function autonomously without their respective postulated upstream components.

In complex, or alone, Ssy1p must somehow detect the levels of extracellular amino acids. It is hard to imagine such detection without the direct binding of the extracellular amino acid to Ssy1p. Binding of amino acids to the receptor Ssy1p does not rule out that Ssy1p possesses some transporting capacity coupled to signaling. However, mutations in Ssy1p appear to be able to initiate basal and constitutive signaling in practically amino acid free environments (Gaber et al., 2003) (Paper IV). Thus, any potential transport via Ssy1p cannot be a strict requirement for signaling. Alternative models for transporter-associated signaling conventionally involve transport of the inducers and secondary detection of effects generated by transport (Hyde et al., 2003). However, recall that even amino acids that are not transported effectively induce the SPS sensor pathway. Thus, a simple and reasonable model is consequently that Ssy1p functions as a receptor of extracellular amino acids and that the binding of amino acids stabilizes a conformation that transmits a signal.

Physiological role of the SPS sensor system

Molecular biological approaches are well suited to unravel mechanisms but are less likely to generate direct information about physiological function. For example, the central importance of cell division for life was postulated long before molecular biologists discovered the many intriguing mechanisms defining and controlling the cell cycle and mitosis. Genetics not only allows us to isolate mutations and thereby indirectly identify components of cellular machineries, but the experimental approach also allows us to perform analysis in cells lacking these components or processes. The only mechanistic function identified for the SPS sensor pathway is positively and actively regulating transactivation of genes in response to extracellular amino acids. The regulated targets comprise nearly half of all genes encoding amino acid permeases and the single dipeptide transporter encoding gene. Thus, the most striking phenotypes of a cell lacking a functional SPS sensor pathway are linked to severely impaired amino acid and dipeptide uptake capacity (Didion et al., 1998; Iraqui et al., 1999; Island et al., 1991; Klasson et al., 1999).

The SPS sensor system appears to be robust in the sense that it functions on a variety of laboratory media, at least when considering the use of different nitrogen sources. Therefore it is likely that the SPS sensor system has relevance in environments outside the laboratory. Hence, amino acids in a diverse set of environments are likely to be required for the expression of many amino acid transporters and the dipeptide transporter Ptr2p. This raises questions what physiological role this regulation has for the cell, and in extension, why the system has evolved? One explanation takes into account that it must be beneficial to express a set of amino acid transporters with broad specificity and low affinity under conditions where amino acids are abundant in the environment. Conversely, some disadvantage must be associated with the constitutive expression of the amino acid permeases regulated by the SPS sensor. An obvious disadvantage that is associated with expression of any protein is the large expenditure of energy required to synthesize the protein. However, the disadvantage ought to be more specific and directly related to amino acid uptake. The broad specificity of the amino acid permeases regulated by the SPS sensor may be the key to understanding why they are not expressed constitutively. They may be prone to import compounds other than proteinogenic amino acids under conditions where amino acids are not abundant. The alternative compounds are, according to this model, toxic for the cell. However, the model is not really consistent with the fact that Gap1p is expressed under amino acid free conditions and has the capacity to transport almost any compound with similarity to amino acids.

The above discussion is based on two extreme conditions, first, the amino acid devoid environment, and second, the amino acid rich environment. These two environments may reflect the experimental conditions used to investigate the SPS sensor rather than the natural yeast habitats. It is easy to see the advantages in amino acid poor environment, but it is harder to appreciate the physiological role in amino acid rich environments. In amino acid rich environments the primary function of the SPS sensor will not be to merely detect amino acids, but rather to modulate expression in response to levels of particular amino acids. There are three observations that are consistent with the view that the SPS sensor has evolved as a fine-tuning mechanism, rather than a master switch. First, the SPS sensor system must be genetically inactivated (*ssy1*, *ptr3*, *ssy5*, *stp1 stp2*, or *grr1* mutations) to completely avoid expression of the regulated amino acid permease genes (for example see paper III). Simple growth of cells on amino acid free media does not convincingly mimic mutant phenotypes. Second, the SPS sensor responds with different sensitivity to different amino acids (Bernard and Andre, 2001a; Gaber et al., 2003; Iraqui et al., 1999). Third, discrete promoters respond differently to SPS sensor initiated signals (see above). From the fine-tuning mechanism point of view, the SPS sensor does not provide one discrete advantage for the cell, but rather is a part of the many interacting systems required for optimization of growth.

Chapter 5

Ssy5p – a chymotrypsin-like serine protease

Introduction

Subsequent to establishing the endoproteolytic activation of Stp1p and Stp2p (Paper II), the major focus of my work has been to identify the processing protease. Right from the start, several lines of evidence suggested that the proteolytic processing activity was intrinsic to the SPS sensor. The set of experiments presented here demonstrates that Ssy5p is a chymotrypsin-like serine protease that is activated by SPS sensor signaling, and subsequently catalyzes the endoproteolytic processing of Stp1p and Stp2p.

Processing of Stp1p in cell-free lysates by a SPS sensor regulated protease activity

The previous results indicating that Stp1p and Stp2p are subject to endoproteolytic processing in response to SPS sensor signals (Paper II) did not distinguish if the signals activated a latent protease, or if signaling made Stp1p and Stp2p available as substrates for a constitutively active protease. We sought to distinguish between these possibilities by establishing an *in vitro* system for monitoring Stp1p processing. Stp1p carrying a combined C-terminal 2×HA-2×MYC-hexahistidine tag was expressed in *Escherichia coli*. An N-terminal hexahistidine-Protein A fusion was found to alleviate the insolubility problems enabling the isolation of the recombinant protein by cobalt affinity chromatography. Immunoblot analysis with antibodies recognizing the HA-epitope demonstrated that the purified protein migrated as a single full-length protein upon SDS-PAGE (Figure 3, lane 1).

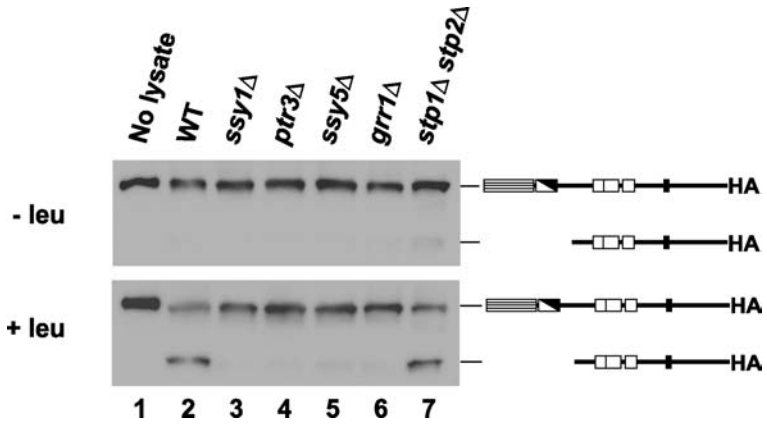

Figure 3. Processing of recombinant Stp1p in cell-free yeast lysates. Lysates were prepared from, non-induced (-leu) and leucine induced (+leu), CAY29 (WT), CAY91 (*ssy1*Δ), JAY7 (*ptr3*Δ), JAY15 (*ssy5*Δ), CAY 86 (*grr1*Δ), and CAY123 (*stp1*Δ *stp2*Δ) grown in SD supplemented with uracil. Purified recombinant Stp1p was incubated with cell-free yeast lysates (100 μg total protein) in 20 μl reactions for 30 minutes at 30°C. The reactions were mixed with sample buffer, heated, separated on 7.5% SDS-PAGE, and analyzed by immunoblotting (α-HA antibody, 12CA5).

Cell-free lysates were prepared from wildtype cells, and cells harboring null mutations in *SSY1*, *PTR3*, *SSY5*, *GRR1* and *STP1 STP2*. Cells were grown in amino acid free SD medium and SD medium supplemented with leucine 30 minutes prior to harvest. Recombinant Stp1p incubated with cell free lysates at 30° C for 30 minutes was analyzed by immunoblotting. Incubation of recombinant Stp1p with lysates prepared from cells grown in SD did not significantly change the electrophoretic mobility of Stp1p (Figure 3, upper panel, compare lane 1 with lanes 2 to 7). In contrast, a second faster migrating band was detected when Stp1p was incubated with lysates prepared from wildtype or *stp1*Δ *stp2*Δ cells grown in SD supplemented with leucine (Figure 3, lower panel, lanes 2 and 7). The apparent processing of recombinant Stp1p did not occur in lysates prepared from leucine induced *ssy1*Δ, *ptr3*Δ, *ssy5*Δ, or *grr1*Δ cells (Figure 3, lower panel, lanes 3 to 6). These results suggest that the SPS sensor pathway activates a protease competent to process recombinant Stp1p in cell free lysates.

Ssy5p is homologous to chymotrypsin-like serine proteases

All of the known protease candidates were found to be required in the *in vitro* assay. Extensive sequence homology searches with Ssy1p, Ptr3p, Ssy5p, and Grr1p using the MEROPS protease database (http://merops.sanger.ac.uk/) revealed that Ssy5p exhibited very limited sequence homology to known proteases (data not shown). Ssy5p was found to exhibit significant similarity to the cysteine and serine protease superfamily ($2.9e^{-7}$ T) when using the InterPro search tool (Mulder et al., 2003). The region encompassing the cysteine and serine protease signature spans the C-terminal part of the protein (residues 459 to 688) (Figure 4A, Protease).

Comparison of sequences from *Saccharomyces cerevisiae* Ssy5p and orthologues identified in *S. bayanus*, *S. mikatae*, *S. paradoxus*, *Ashbya gossypii*, and *Yarrowia lipolytica* revealed that the N-terminal domain (Figure 4A, NTD) of Ssy5p is poorly conserved between orthologues (4% identical residues). In contrast, the predicted protease domain (Figure 4A, Protease) exhibits a higher degree of conservation (10 % identical residues). There are no conserved cysteine residues within the protease homology domain suggesting that Ssy5p is not a cysteine protease. In contrast, many conserved serine residues are found. Strikingly, serine 640 is positioned in a GDSG motif that is highly conserved between orthologues and is identical to the active-site serine motif of many serine proteases (Rawlings and Barrett, 1994). A direct comparison of primary sequences from *Bos taurus* chymotrypsins and Ssy5p orthologues (data not shown) suggested that the catalytic H-D-S triad of chymotrypsin-like serine proteases (peptidase family S1) are present in Ssy5p (Figure 4A, H465, D545, and S640). Residues surrounding the putative catalytic triad appeared to be conserved between Ssy5p-orthologues and chymotrypsin (Figure 4B, white color).

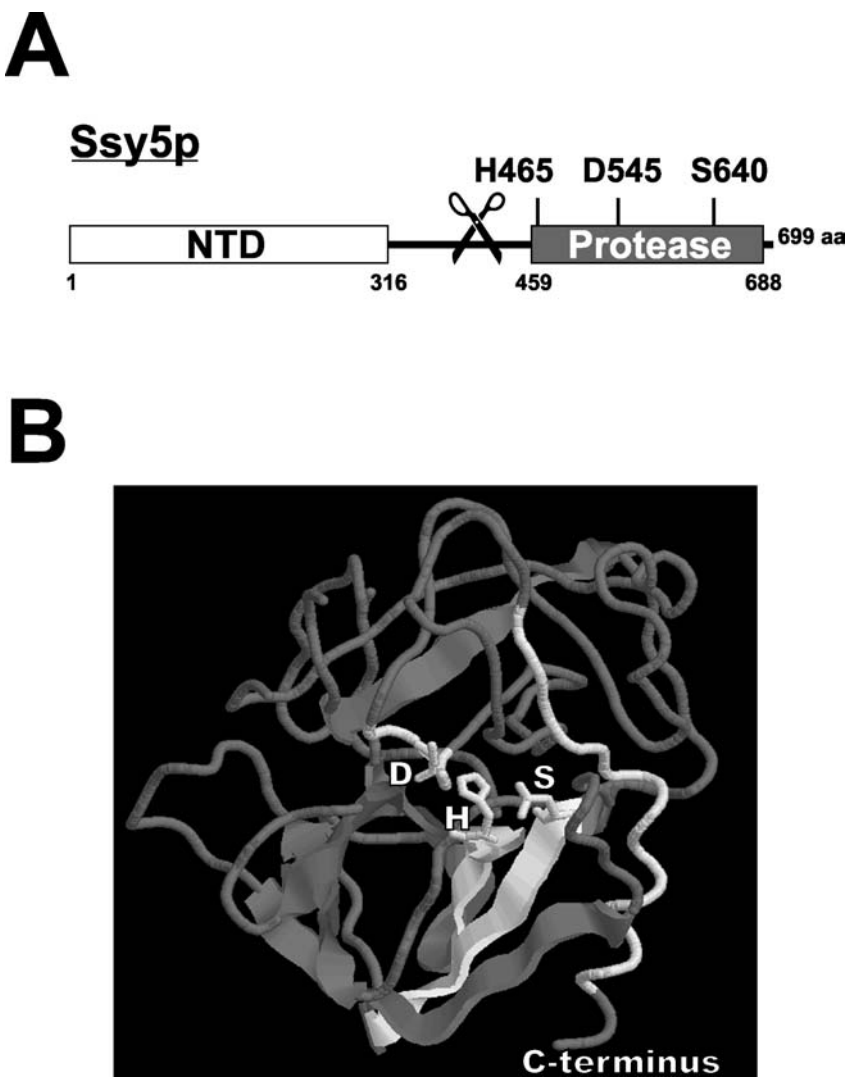

Figure 4. Ssy5p exhibits homology with chymotrypsin-like serine proteases. A. Schematic representation of the domains of Ssy5p. Amino acid residues 1-316 (NTD) exhibits weak sequence conservation (4% identical residues) when Ssy5p orthologues from *Saccharomyces cerevisiae*, *S. bayanus*, *S. mikatae*, *S. paradoxus*, *Ashbya gossypii*, and *Yarrowia lipolytica* are compared. Residues 459-688 (Protease) are more conserved between orthologues (10% identical residues) and exhibits weak sequence homology with serine proteases. Histidine residue 465, aspartate residue 545, serine residue 640 are conserved between Ssy5p and the catalytic triad of chymotrypsin like serine proteases. The putative position of endoproteolysis near amino acid residue 400 is indicated (scissor). B. A 3-dimensional representation of the structure of bovine chymotrypsin A (created using RasMol). Regions exhibiting sequence conservation between chymotrypsin and Ssy5p are colored white. The conserved catalytic triad (H, D, and S) and the C-terminus are labeled.

Ssy5p exhibits electrophoretic migration patterns consistent with endoproteolysis

We next introduced 6×HA epitope tags at the extreme N- and C-termini of Ssy5p. The functionality of epitope tagged proteins was tested in a strain carrying an *ssy5* null mutation. Serially diluted suspensions of cells harboring control plasmids or plasmids expressing N- and C-terminally tagged Ssy5p were applied to growth medium containing azetidine-2-carboxylic acid (AzC) and the branched chained amino acid synthesis inhibitor sulfonylurea (MM). Both epitope tagged alleles of *SSY5* complemented *ssy5* null mutant phenotypes (Figure 5A). Next, the levels of epitope tagged proteins was monitored. Immunoblot analysis of separated whole cell protein extracts revealed differences in the pattern of immunodetectable bands that were dependent upon the position of the epitope tags. The C-terminally tagged protein was predominantly expressed as a faster migrating product (~50 kD). A second weak band migrating above the 83 kD marker was also detected (Figure 5B, lanes 2 and 5). The N-terminally tagged protein was mainly expressed as a product migrating above the 62 kD marker. A faint band, migrating above the 83 kD marker was also observed for the C-terminally tagged protein (Figure 5B, lanes 3 and 6). These observations are consistent with the slower migrating species being the full-length epitope tagged Ssy5p (predicted 87 kD including epitope tag) and the faster migrating species are N- and C-terminal products from a single endoproteolytic processing event near amino acid residue 400 (Figure 4A, scissor). Thus, the behavior of Ssy5p appears to be very similar to chymotrypsin-like proteases. Chymotrypsin-like proteases are expressed as full-length inactive zymogens, that are autoproteolytically processed, resulting in an active C-terminal protease domain (Rawlings and Barrett, 1994). However, controlling the processing of Ssy5p cannot be the mechanism of activation used by the SPS sensor since it is constitutive in both the absence and presence of inducing leucine in the growth medium (Figure 5B, compare lanes 2 to 3 and 5 to 6). Thus a second mechanism of activation must exist.

The 6HA epitope tag at the N-terminus makes Ssy5p constitutively active

Concerned that the new epitope tagged versions of Ssy5p may only possess modest activity as compared to wildtype protein, we used a more quantitative *ssy5*Δ phenotype to assess complementation. Leucine induced expression of the *AGP1* promoter was measured in *ssy5*Δ strains harboring a P*AGP1-lacZ* reporter integrated in the genome and the levels of transcriptional induction was monitored in cells carrying plasmids expressing N- and C-terminally tagged Ssy5p. Expression was not detected in cells transformed with an empty vector (Figure 5C, Vector). In contrast, leucine induced β-galactosidase expression when wildtype Ssy5p or C-terminally tagged Ssy5 was expressed (Figure 5C, Ssy5p and 6HA-CT). Surprisingly, cells expressing the N-terminally tagged Ssy5p exhibited constitutive β-galactosidase activity even in medium lacking leucine. β-galactosidase activity was as high in these cells grown in amino acid free SD medium as in leucine supplemented medium (Figure 5C, 6HA-NT). Thus, N-terminally tagged Ssy5p constitutively activates the *AGP1* promoter.

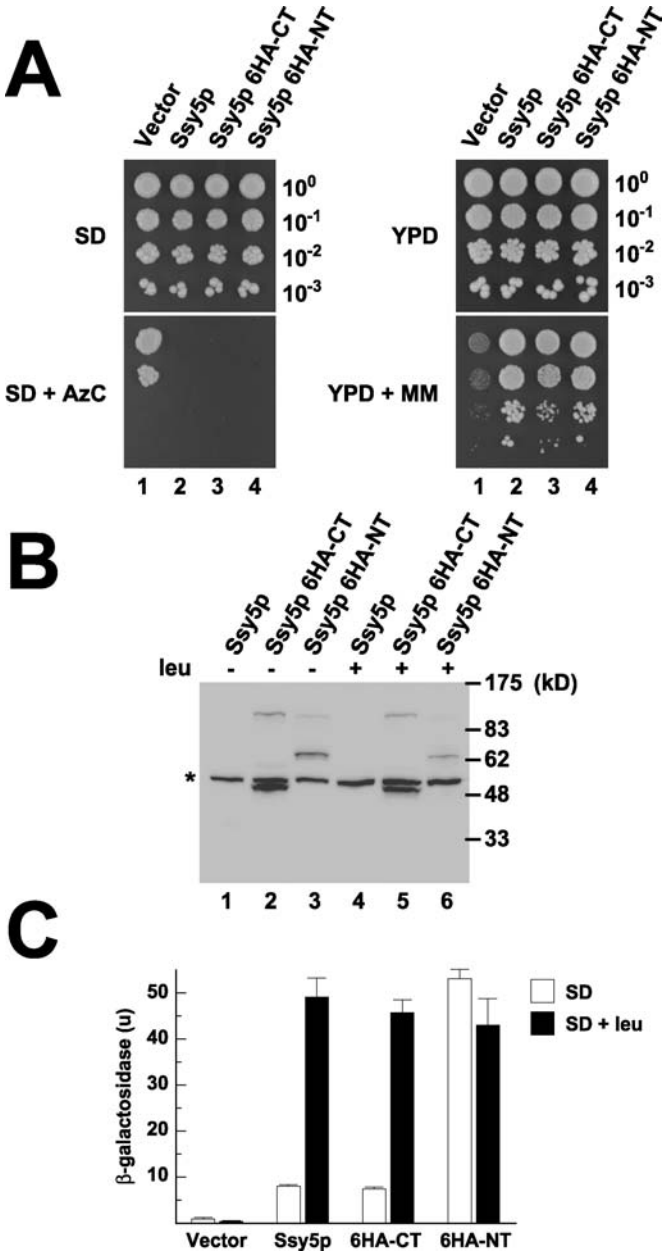

Figure 5. Ssy5p carrying N or C-terminal 6HA epitope tags. Phenotypic analysis of strain CAY265 (*MAT**a** ssy5*Δ*2 gap1*Δ::P*AGP1-lacZ ura3-52*) transformed with pRS316 (Vector), pFL001 (Ssy5p), pCA177 (Ssy5p 6HA-CT), pCA195 (6HA-NT). A. Transformants were spotted onto agar plates containing SD, SD containing AzC (SD + AzC), YPD, and YPD containing MM (YPD + MM). Plates were incubated at 30°C and photographed. B. Immunoblotting of whole-cell extracts (α-HA antibody, 12CA5) prepared from cells grown in SD (leu -) or SD supplemented with leucine (leu +) 30 minutes before harvest. An asterisk marks the position of an unrelated antigen that cross-reacts with the 12CA5 antibody. C. β-galactosidase activity measurements with *N*-lauroyl-sarcosine-permeabilized cells grown in SD or SD supplemented with leucine (SD + leu). Arbitrary activity units (u) are normalized to OD_{600}. Error-bars indicate 95% confidence intervals.

6HA-Ssy5p signals even in the absence of SSY1, PTR3, and GRR1

The isolation of the constitutive N-terminally tagged allele of *SSY5* (*SSY5**) allowed us to investigate if it was functional even in the absence of the other SPS sensor components. We monitored β-galactosidase expression from the *AGP1* promoter in *ssy5*Δ mutants carrying null alleles of *ssy1*, *ptr3*, and *grr1*. *SSY5** facilitated the expression from the *AGP1* promoter in all of these mutant strains (Figure 6A, compare lanes 6, 9, 12 and 5, 8, 11).

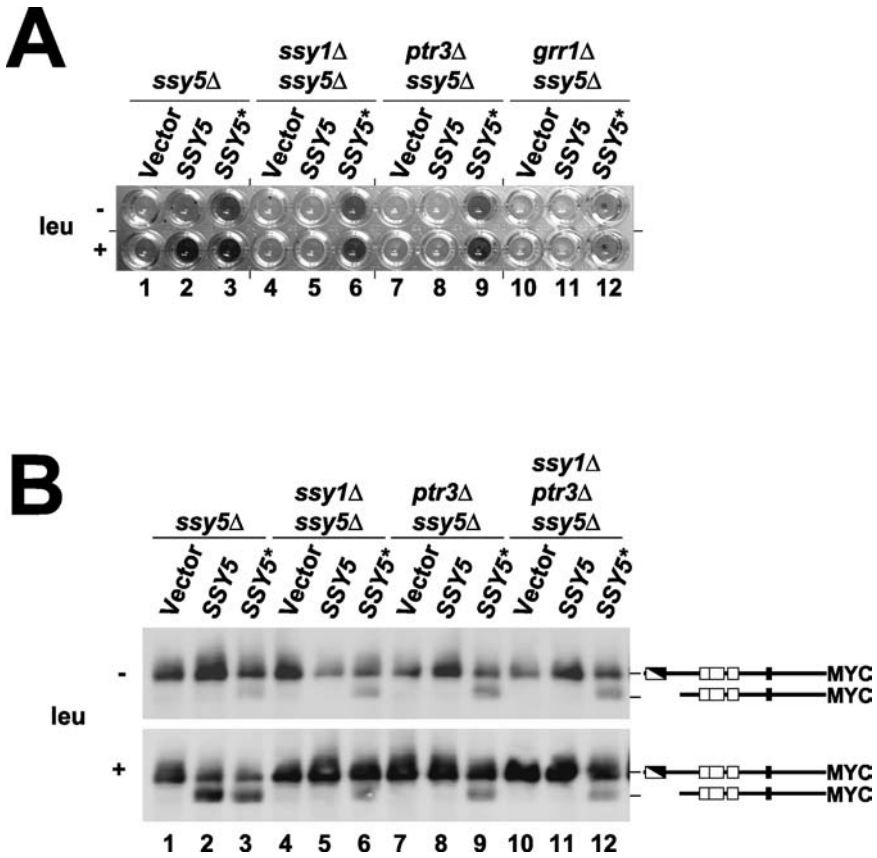

Figure 6. SSY5* bypasses SPS sensor and *grr1* mutations. A. Yeast strains HKY77 (*ssy5*Δ), HKY84 (*ssy1*Δ *ssy5*Δ), HKY85 (*ptr3*Δ *ssy5*Δ), and CAY274 (*grr1*Δ *ssy5*Δ) carrying pCA030 [P*AGP1-lacZ LYS2*] and pRS316 (Vector), pFL001 (*SSY5*), or pCA195 (*SSY5**) were grown in SD medium with (+) or without (-) leucine (leu). The levels of X-gal staining (blue precipitate results in dark wells) resulting from the expression of β-galactosidase from P*AGP1-lacZ N*-lauroyl-sarcosine-permeabilized cells were assessed. B. Immunoblotting of whole-cell extracts (α-MYC antibody, 9E10) prepared from strains HKY77 (*ssy5*Δ), HKY84 (*ssy1*Δ *ssy5*Δ), HKY85 (*ptr3*Δ *ssy5*Δ), and CAY285 (*ssy1*Δ *ptr3*Δ *ssy5*Δ), carrying pCA204 [*STP1-13×MYC LYS2*) and pRS316 (Vector), pFL001 (*SSY5*), or pCA195 (*SSY5**), grown in SD medium with (+) or without (-) leucine (leu) added 30 minutes before harvest. The immunoreactive forms of Stp1p present in the cell extracts are schematically represented at their corresponding positions of migration.

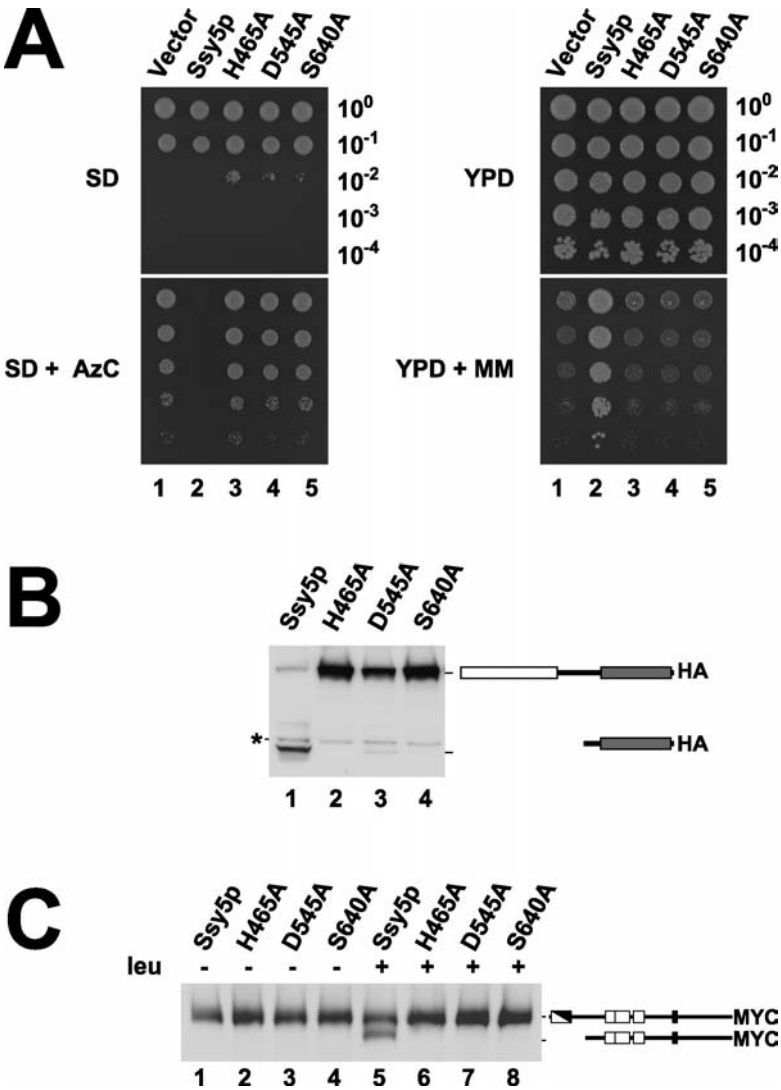

Figure 7. Mutations in the catalytic triad of Ssy5p abolish activity. A. Phenotypic analysis of strain CAY265 (*MAT**a** ssy5*Δ*2 gap1*Δ::P*AGP1-lacZ ura3-52*) transformed with pRS316 (Vector), pCA177 (Ssy5p), pCA215 (H465A), pCA216 (D545A), or pCA217 (S640A). Transformants were spotted onto agar plates containing SD, SD containing AzC (SD + AzC), YPD, and YPD containing MM (YPD + MM). Plates were incubated at 30°C and photographed. B. Immunoblotting of whole cell extracts (α-HA antibody, 12CA5) from cells grown in SD (strains as in panel A). An asterisk marks the position of an unrelated antigen that cross-reacts with the 12CA5 antibody. The immunoreactive forms of Ssy5p present in the cell extracts are schematically represented at their corresponding positions of migration. C. Immunoblotting of whole-cell extracts (α-MYC antibody, 9E10) prepared from strain HKY77 (*ssy5*Δ) carrying pCA204 [*STP1-13×MYC LYS2*) and pCA177 (Ssy5p), pCA215 (H465A), pCA216 (D545A), or pCA217 (S640A) grown in SD with (+) or without (-) leucine (leu) added 30 minutes before harvest. The immunoreactive forms of Stp1p present in the cell extracts are schematically represented at their corresponding positions of migration.

Ssy5p facilitates processing of Stp1p in the absence of amino acids or SSY1 and PTR3*

The constitutive nature of *SSY5** even in the absence of SPS sensor components prompted us to investigate if signaling induced by this allele is transmitted to Stp1p and Stp2p via normal proteolytic processing. Processing of 13×MYC tagged Stp1p was monitored in *ssy1*Δ, *ptr3*Δ, or *ssy1*Δ *ptr3*Δ mutant strains expressing Ssy5p* or controls. *SSY5** induced processing was observed in all strains, both in the absence and presence of leucine in the growth medium (Figure 6B, lanes 3, 6, 9 and 12). However, the addition of leucine to the growth medium facilitated more effective processing in SPS competent strains, but not in SPS sensor defective *ssy1*Δ, *ptr3*Δ, or *ssy1*Δ *ptr3*Δ mutant strains. Thus, Ssy5p* is not fully active without SPS sensor stimuli. However, the results suggest that Ssy5p* is able to function without signal transduction from the SPS sensor pathway, either by activating a protease downstream the SPS sensor or by directly processing Stp1p and Stp2p.

Mutation of the predicted catalytic triad of Ssy5p abolish protease activity

The structural similarity to chymotrypsin like proteases makes Ssy5p a likely candidate to directly process Stp1p and Stp2p. We tested the importance of the residues predicted to encode the catalytic triad of Ssy5p by individually substituting histidine 465, aspartate 545, and serine 640 with alanine residues in the context of the neutral C-terminally 6×HA tagged protein. As judged from uptake of AzC and branched chained amino acids all three proteins were non-functional (Figure 7A, compare panels 1 to 2 and 3 to 5 in both panels). This is consistent with the notion that the mutated residues constitute the catalytic triad of a serine protease. Next, Ssy5p expression levels in cells harboring the mutant alleles were investigated. The levels of full-length protein were dramatically increased in cells harboring all three mutant alleles and apparent processing was abolished (H456A and S640A) or severely reduced (D545A) (Figure 7B, compare lanes 1 and 2 to 4). This indicates that the mutant proteins are expressed but inactive. The observed accumulation of full-length mutant proteins furthermore supports the notion that Ssy5p is autoproteolytically processed. The residual processing detected for the D545A mutation is consistent with similar mutations in the catalytic triad of chymotrypsin (Rawlings and Barrett, 1994). Finally, we monitored Stp1p processing in strains expressing Ssy5p-H465A, Ssy5p-D545A, or Ssy5p-S640A as the sole source of Ssy5p protein. Consistent with their inability to complement *ssy5* null mutations, none of the three mutant proteins facilitated Stp1p processing in response to leucine induction (Figure 7C, compare lane 5 and lanes 6 to 8). Taken together, the data highlight the importance of H465, D545, and S640, and is consistent with their postulated role in catalysis as components of a catalytic triad of a serine protease.

Ssy5p processes Stp1p when heterologously coexpressed in Schizosaccharomyces pombe*

Given that Ssy5p is a serine protease involved in SPS sensor signaling, we asked if Ssy5p was the protease directly responsible for processing Stp1p and Stp2p. If so Ssy5p or constitutively active Ssy5p*, should be able to process Stp1p when these proteins are coexpressed in a heterologous system devoid of SPS sensor components. We previously had established that Ssy5p is poorly expressed in *E. coli* (unpublished observation) and consequently searched for a eucaryotic expression system.

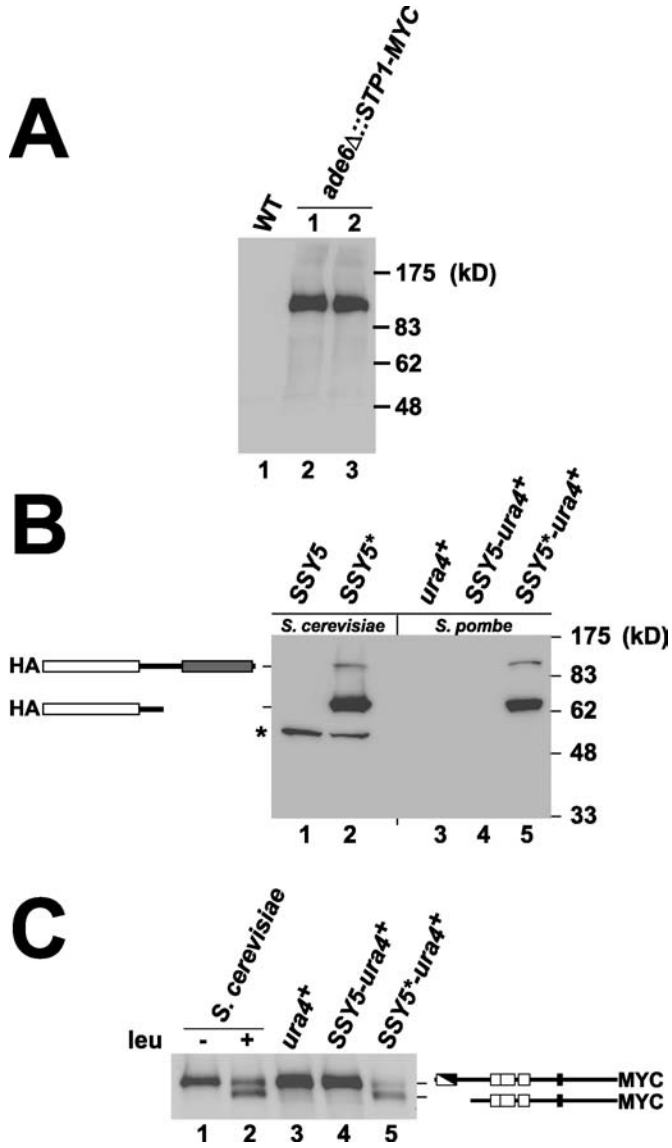

Figure 8. Stp1p is proteolytically processed in a Ssy5p* dependent manner when coexpressed in *Schizosaccharomyces pombe*. A. Immunoblotting of whole-cell extracts (α-MYC antibody, 9E10) from *S. pombe* strains FY995 (WT), or Stp1p-MYC-expressing derivatives CAY299 (1) and CAY300 (2) growing in YES. B. Immunoblotting of whole-cell extracts (α-HA antibody, 12CA5) from *S. cerevisiae* strain HKY77, carrying pCA204 [*STP1-MYC LYS2*] and either pFL001 (*SSY5*) or pCA195 (*SSY5**) grown in SD, and *S. pombe* strains CAY301 (*ura4*$^+$), CAY302 (*SSY5-ura4*$^+$), and CAY303 (*SSY5*-ura4*$^+$) grown in YES. The immunoreactive forms of Ssy5*p present in the cell extracts are schematically represented at their corresponding positions of migration. An asterisk marks the position of an unrelated antigen in *S. cerevisiae* that cross-reacts with the 12CA5 antibody. C. Immunoblotting of whole-cell extracts (α-MYC antibody, 9E10) from *S. cerevisiae* strain HKY77 carrying pCA204 [*STP1-MYC LYS2*] and pFL001 [*SSY5*] grown in SD with (+) or without (-) leucine (leu) added 30 minutes before harvest, and *S. pombe* strains from panel B grown in YES. The immunoreactive forms of Stp1p present in the cell extracts are schematically represented at their corresponding positions of migration.

Schizosaccharomyces pombe lacks orthologues of any SPS sensor component, including Stp1p and Stp2p, and thus appeared suitable for heterologous expression analysis. Stp1p-13×MYC coding sequence was integrated in the genome of *S. pombe* strain FY995 under the control of the *ade6*$^{+}$ promoter. Protein expression was monitored in two clones by immunoblot analysis of separated whole cell protein extracts. Stp1p-13×MYC was detected in both strains as bands migrating above the 83 kD marker (Figure 8A, compare lane 1 and lanes 2 and 3). Clone 1 was used to create three derived strains harboring different construct in the *psh3* locus; a control, where only the marker *ura4+* was integrated, and two strains expressing either wildtype Ssy5p or constitutive Ssy5p*. Immunoblot analysis of Ssy5p* expression demonstrated that the protein was expressed and apparently processed similarly as in *S. cerevisiae* (Figure 8B, compare lanes 2 and 5). This observation further supports the notion that Ssy5p is autoproteolytically processed. Next, we monitored Stp1p processing in the control strain and strains expressing Ssy5p and Ssy5p*. The control strain, harboring only *ura4*$^{+}$, and the Ssy5p expressing strain, expressed full-length Stp1p (Figure 8C, compare lane 1 and lanes 3 and 4). In contrast, proteolytically processed Stp1p was readily detected in cells expressing Ssy5p* (Figure 8C, compare lanes 2 and 5). This key observation provides the first evidence indicating that Ssy5p directly processes Stp1p. We regard the alternative possibility, that an endogenous Stp1p-processing protease in *S. pombe* becomes activated by Ssy5p*, but not the Ssy5p wildtype protein, as most unlikely.

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