Transport Activity Dependent Regulation of the Yeast General Amino Acid Permease

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Abstract

The general amino acid permease Gap1p of *Saccharomyces cerevisiae* scavenges amino acids from the extracellular medium for use as nitrogen sources in starvation conditions. Because unlimited uptake of both naturally occurring amino acids and amino acid analogs is toxic, Gap1p is active at the plasma membrane only when amino acid levels are low. Gap1p is down regulated when amino acids are abundant by two distinct post-translational mechanisms. Gap1p is regulated post-translationally to respond quickly and efficiently to changing amino acid concentration. An increase in amino acids causes accumulation of Gap1p in the vacuole and inactivation of Gap1p located at the plasma membrane. Conversely, a decrease in amino acid levels allows for redistribution of Gap1p from internal membranes to the cell surface.

Here I examine the mechanism of amino acid regulation of Gap1p. Previous studies of Gap1p sorting have focused on the *trans*-acting factors required for the distribution of Gap1p between the plasma membrane and internal compartments. To complement this body of work, these studies focus on the *cis*-elements required for Gap1p sorting. We find that post-translational regulation of Gap1p requires the catalytic activity of Gap1p, indicating that sorting and activity of Gap1p are controlled in *cis*. Gap1p therefore can serve as an amino acid sensor to control its activity in response to nutrient levels.

This finding suggested that post-translational regulation of Gap1p could apply to other transporter proteins in yeast. I examined the activity and localization of a related transporter protein, the histidine-specific permease Hip1p in response to various amino acids, and found that although Hip1p is down regulated only in response to histidine, this regulation is less tightly controlled than the regulation of Gap1p. This observation supports previous assertions that the function of Gap1p in the cell is distinct among yeast amino acid transporters.

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Chapter 1

Introduction

Regulation of Intracellular Protein Trafficking

Newly synthesized membrane proteins are directed to their final destinations in the eukaryotic cell via an exchange of membrane-bound compartments known as vesicle trafficking. This well-characterized and conserved process relies on movement of vesicles along specific routes between organelles. Proteins destined for the plasma membrane follow the secretory pathway from insertion into the ER membrane, to modification in the Golgi. At the *trans*-Golgi, cargo proteins are packaged into secretory vesicles that fuse with the plasma membrane, a process known as exocytosis (Reviewed in Jahn and Sudhof, 1999). Lysosomal resident proteins, such as various proteases, are directed from the Golgi to the lysosome via an intermediate compartment, the late endosome (Reviewed in Pryor and Luzio, 2009). Both secretion and lysosomal trafficking are balanced by retrieval pathways that direct vesicles back to their compartments of origin. These retrieval pathways include endocytosis, the internalization of plasma membrane proteins (Reviewed in Conner and Schmid, 2003), and retrograde transport, the movement of cargo from the endosome to the Golgi (Reviewed in Bonifacino and Rojas, 2006).

Regulation of many plasma membrane proteins occurs at the level of secretory vesicle trafficking. Regulated vesicle trafficking of plasma membrane proteins occurs in two ways. Proteins can be down-regulated by increasing delivery to the lysosome relative to the rate of secretion both by increasing endocytosis and by sorting directly to the lysosome from the *trans*-Golgi. Proteins destined for the lysosome can also be recycled from the late endosome and redirected to the plasma membrane. Cells maintain a balance of lysosomal trafficking and protein recycling to allow for rapid and sensitive control of proteins in response to changing environmental conditions, much faster than

could be achieved through transcriptional regulation and trafficking of newly synthesized proteins.

Understanding the mechanisms of regulated protein trafficking has implications for the treatment of many diseases resulting from the breakdown of this process. One example is type II diabetes, in which insulin-stimulated trafficking of the major glucose transporter in fat and muscle cells, GLUT4, is defective. Under basal conditions, GLUT4 is localized to internal compartments. A signaling cascade activated by insulin binding to its cell surface receptor stimulates the trafficking of GLUT4 to the plasma membrane. Fat and muscle cells in type II diabetics are unable to increase plasma membrane GLUT4 levels in response to insulin, which results in elevated blood glucose levels (Watson and Pessin, 2006; Blot and McGraw, 2008). Regulation of protein trafficking is also emerging as a potential factor in several neurological diseases. Neurotransmitter transporters such as the dopamine transporter (DAT) modulate neuronal response to their substrates by affecting the rate at which neurotransmitter molecules are removed from the synaptic cleft and repackaged into synaptic vesicles. Several studies have suggested that the rate of internalization of DAT from the synaptic membrane plays a significant role in the control of dopaminergic signaling (Melikian and Buckley, 1999; Kahlig et al., 2006). Alteration of dopaminergic signaling has been implicated in Parkinson's disease, schizophrenia, and addiction (Mortensen and Amara, 2003).

To better understand the mechanisms of regulated protein trafficking, this thesis explores the trafficking behavior of a model yeast transporter protein, the general amino acid permease Gap1p. Gap1p is tightly regulated to allow cells to maintain optimum amino acid levels and confer the greatest competitive advantage in limiting amino acid

conditions. By examining the regulated sorting of Gap1p, we aim to expand general knowledge of regulated protein trafficking.

Amino Acid Transporters

The Amino Acid/Polyamine/Organocation Superfamily

Nutrient transporters are a large, diverse group of proteins that are ubiquitous across species and responsible for import or export of small molecules. To better understand this type of protein, an effort has been made to classify the various nutrient transporters by several properties, including substrate recognition, mechanism of action, and sequence similarity (Saier et al., 2006; Saier et al., 2009). One of the largest groups is the Amino Acid/Polyamine/Organocation (APC) superfamily (Jack et al., 2000). APC superfamily proteins are a subset of secondary carrier-type transporters that utilize an electrochemical gradient across membranes to facilitate transport of a variety of substrates besides amino acids, including sugars, neurotransmitters, and other metabolites. APC proteins range in size from 250-800 residues and span the membrane 10-14 times (Saier, 2000). The proposed mechanism for transport through these proteins is an alternating access model, wherein the substrate is bound on one side of the membrane and is translocated across the membrane to the other side through a conformational shift in the protein (Jardetzky, 1966).

Due to the extremely hydrophobic nature of transporter proteins, few crystal structures have been solved. Thus far, the greatest success for generating three-dimensional structural information on transporter proteins has come from bacterial proteins that are homologous to transporters in higher organisms. Among the first transporter proteins to be crystallized were the lactose permease LacY of *E. coli*, of the related Major Facilitator Superfamily (MFS) (Abramson et al., 2003) and the leucine transporter LeuT of *Aquifex aeolicus* (Yamashita et al., 2005). More recently, structural data has been obtained for two APC proteins. The arginine:agmatine antiporter AdiC of

E. coli has been crystallized in both an open-to-out empty conformation and an arginine-bound conformation (Gao et al., 2009; Gao et al., 2010). The proton symporter ApcT of *Mechanocaldococcus jannaschii*, a broad specificity amino acid transporter, was crystallized in an empty, cytoplasmic facing conformation (Shaffer et al., 2009).

Thought there is relatively little sequence similarity among these transporters, they nonetheless share a common fold in which the transmembrane domains (TMD) arrange in two pseudo-symmetrical bundles with residues in both bundles forming the substrate binding pocket. One TMD in each bundle, TMD1 and TMD6 in the amino acid transporters, has a short, non-helical section in the center that makes contacts with the substrate. These unwound sections allow for significant movement of TMD1 and TMD6 during the transport cycle (Singh et al., 2008; Gao et al., 2010). Symporters also require import of an ion down its concentration gradient to provide the driving force for amino acid transport. LeuT imports two molecules of sodium per one molecule of leucine; the sodium binding sites have been identified (Yamashita et al., 2005). Proton symport has been proposed to occur by the protonation of an acidic amino acid residue in or near the binding pocket during transport, but as yet there is no consensus on the location or identity of the residue(s) involved (Shaffer et al., 2009).

Yeast amino acid transporters

The genome of *Saccharomyces cerevisiae* contains 24 members of the APC superfamily (Nelissen et al., 1997; Jack et al., 2000). They range in size from 500-600 residues in length. The exception is Ssy1p, a non-transporting amino acid sensor, which has an extended hydrophilic N terminus that is required for its action (Didion et al., 1998;

Jorgensen et al., 1998). These proteins can be grouped based on the regulation of their activity. The basic amino acid permease Can1p (Hoffmann, 1985), the histidine permease Hip1p (Tanaka and Fink, 1985), and the lysine permease Lyp1p (Sychrova and Chevallier, 1993) are constitutively expressed regardless of nutrient conditions. A second class consists of transporters that are induced by the presence of amino acids. Members of this class include Agp1p, Bap2p, Bap3p, Gnp1p, Tat1p, and Tat2p (Ljungdahl, 2009). As most of these transporters are have high affinity for their substrates but low capacity, it is thought that these transporters are responsible for providing amino acids to be used in protein synthesis. A third class is active only when nutrient availability, specifically nitrogen, is poor. The two members of this class are the proline-specific permease Put4p (Lasko and Brandriss, 1981), and the general amino acid permease Gap1p, a transporter of all naturally occurring amino acids and many analogs (Grenson et al., 1970; Jauniaux and Grenson, 1990). Gap1p, which is the focus of this work, was found to be most active in proline medium and less active in ammonium medium (Grenson et al., 1970). Initially, this difference was ascribed to transcriptional regulation, as mRNA levels of the *GAP1* transcript varied with nitrogen source (Jauniaux and Grenson, 1990), but as I will describe, other mechanisms of nitrogen regulation have been found to play a role in the control of Gap1p activity.

Nutrient Regulation

Amino acid sensing

Transcriptional regulation of high affinity, low capacity amino acid transporters that are induced by the presence of amino acids relies on the SPS amino acid sensor. The SPS sensor is made up of three components: the transporter-like sensor Ssy1p (Didion et al., 1998; Iraqui et al., 1999), the sensor component Ptr3p (Klasson et al., 1999), and the serine protease Ssy5p (Abdel-Sater et al., 2004a; Jorgensen et al., 1998). Ssy1p, although homologous to nutrient transporters, is unable to take up amino acids itself. This was determined by the observation that although uptake of branched amino acids depends on Ssy1p, overexpression of *SSY1* did not allow uptake in cells deleted for the four branched chain amino acid transporters (Didion et al., 1998). Ptr3p is hyperphosphorylated by casein kinase I in response to amino acids and interacts with Ssy1p (Liu et al., 2008). Ssy5p is responsible for the cleavage of two transcription factors, Stp1p and Stp2p (Andreasson and Ljungdahl, 2002). Stp1p and Stp2p are synthesized in a latent form that is prevented from entering the nucleus by the Asi complex (Boban et al., 2006; Zargari et al., 2007).

Amino acid induced signaling begins with the recognition of extracellular amino acids by Ssy1p. This binding induces a conformational change in Ssy1p, potentially similar to the conformational change that allows transporter proteins to translocate their substrates (Poulsen et al., 2008; Wu et al., 2006). The long N terminal signaling domain of Ssy1p facilitates interaction with Ptr3p and Ssy5p (Forsberg and Ljungdahl, 2001). Amino acids lead to the phosphorylation of Ptr3p and activation of Ssy5p protealytic activity (Liu et al., 2008; Abdel-Sater et al., 2004a). Ssy5p then cleaves Stp1p and Stp2p,

to generate the active form, which is able to enter the nucleus (Andreasson and Ljungdahl, 2002; Andreasson and Ljungdahl, 2004).

Stp1p and Stp2p activate the transcription of several transporter genes, including *AGP1, BAP2, BAP3, GNP1, TAT1,* and *TAT2* (Abdel-Sater et al., 2004b; Didion et al., 1998; Nielsen et al., 2001; De Boer et al., 1998; Ljungdahl, 2009). Though Stp1p and Stp2p were thought to be functionally redundant, new data has suggested that they may have originated from a whole genome duplication event and, over time, evolved to have subtly different capacities for activation at various concentrations of amino acids (Wielemans et al., 2010).

Nitrogen assimilation

The regulation of Gap1p is a small part of a larger system of nitrogen regulation in the cell. In yeast, nitrogen metabolism depends on the interplay between three compounds: glutamate, glutamine, and ammonium (Cooper, 1982). These compounds form the starting material for amino acids and nucleotides. Free ammonium is incorporated into glutamate or glutamine for storage. The yeast cell stores 85% of its total cellular nitrogen in the amino group of glutamate and the remaining 15% in the amide group of glutamine. The set of reactions that relates these compounds is diagrammed in Figure 1.

When cells are grown in medium containing ammonium sulfate as a sole nitrogen source, glutamate and glutamine can be synthesized from the starting materials ammonium and α-ketoglutarate. Free ammonia is imported from the extracellular environment by three ammonia transporters, Mep1p, Mep2p, and Mep3p. Although they

are highly similar in sequence, these three transporters have different affinities for nitrogen, and can be utilized differentially depending on the level of ammonia present in the medium. Mep2p can also serve as an ammonium sensor (Marini et al., 1994; Marini et al., 1997). The carbon skeleton for glutamate and glutamine is provided by α-ketoglutarate, which is produced by enzymes of the tricarboxylic acid (TCA) cycle. During growth on fermentable carbon sources such as glucose, the TCA cycle is repressed in favor of glycolysis and α-ketoglutarate is synthesized only for use in nitrogen incorporation (Gancedo, 1998). Ammonia and α-ketoglutarate are combined to form glutamate by the $NADP^+$-dependent glutamate dehydrogenase Gdh1p (Grenson et al., 1974). The resulting glutamate molecule can be combined with an additional molecule of ammonia to form glutamine. This reaction is catalyzed by the glutamine synthetase Gln1p and is coupled to hydrolysis of ATP (Mitchell and Magasanik, 1983; Mitchell, 1985). This sequence of reactions occurs when cells are growing in abundant glucose and ammonia.

When glutamate is present as a sole nitrogen source, glutamate synthesis is not required, but glutamine is still needed. Synthesis of glutamine requires a pool of free ammonia to combine with glutamate. The NAD^+-dependent glutamate dehydrogenase Gdh2p generates ammonia and α-ketoglutarate (Miller and Magasanik, 1990). Gln1p then can generate glutamine from ammonia and glutamate, as described. If glutamine is present as the sole nitrogen source, two molecules of glutamate can be generated from one molecule of glutamine and one molecule of α-ketoglutarate by the glutamate synthase Glt1p (Cogoni et al., 1995).

While glutamate, glutamine, and ammonia can be fed directly into this core set of reactions, cells can also generate ammonia from a number of other nitrogenous compounds, including amino acids, nucleotides, urea, ornithine, and γ-aminobutyric acid (GABA) (Cooper, 1982).

Nitrogen catabolite repression

Glutamine and asparagine are preferred nitrogen sources for *S. cerevisiae* – glutamine because it can be readily converted to glutamate or be deaminated to generate free ammonia, and asparagine due to the presence of an asparaginase that converts asparagine to ammonia and aspartate, which is converted to glutamate (Cogoni et al., 1995; Sinclair et al., 1994). In the presence of these preferred nitrogen sources, the cell has no need for enzymes to metabolize less-preferred nitrogen sources. Nitrogen catabolite repression (NCR) refers to the repression of metabolic pathways for non-preferred nitrogen sources when preferred nitrogen sources are available (Hofman-Bang, 1999; Magasanik and Kaiser, 2002). The genes inhibited by NCR include not only the enzymes necessary for catabolism of non-preferred nitrogen sources, but also transporters responsible for their uptake, for example Gap1p (Stanbrough et al., 1995; Stanbrough and Magasanik, 1996).

NCR is carried out by the actions of four GATA type transcription factors, so classified because they all contain a characteristic zinc finger region that binds a GATAA motif upstream of regulated genes. The first to be discovered was Gln3p, identified by a mutation that, in a *gln1* strain, was completely unable to grow in the absence of glutamine. Gln3p activated the transcription of *GLN1* when cells were shifted from

glutamine medium to glutamate medium (Mitchell and Magasanik, 1984). Nil1p/Gat1p was identified by homology to Gln3p and found to activate expression of NCR target genes in the presence of ammonium, but not glutamate or glutamine (Coffman et al., 1995; Stanbrough et al., 1995). Dal80p is a transcriptional repressor that inhibits the expression of enzymes responsible for the degradation of urea and allantoin (Chisholm and Cooper, 1982; Cunningham and Cooper, 1991). An additional repressor, Nil2p/Gzf3p/Deh1p was identified by homology to Gln3p, Nil1p, and Dal80p. Mutants of Nil2p were found to up-regulate NCR targets in cells growing in glutamine, asparagine, or ammonium media (Rowen et al., 1997; Soussi-Boudekou et al., 1997; Stanbrough et al., 1995). Dal80p and Nil2p are thought to inhibit expression of target genes by competing for binding of GATAA activating sequences (Hofman-Bang, 1999).

Three of the four GATA factors are themselves regulated by quality of nitrogen source, adding additional control to gene expression in various nitrogen conditions (Figure 2). Gln3p is constitutively expressed, but is post-translationally regulated by Ure2p. Ure2p is required for repression of Gln1p in the presence of glutamine; deletion of *URE2* allows expression of Gln3p-dependent genes in preferred nitrogen sources (Courchesne and Magasanik, 1988). In glutamine, Ure2p binds Gln3p and sequesters it in the cytoplasm (Blinder et al., 1996; Beck and Hall, 1999). *NIL1* transcription requires Gln3p or Nil1p, and is thus subject to NCR (Coffman et al., 1996). In glutamine, Gln3p activation of *NIL1* transcription does not occur; nonetheless, Nil1p can auto-activate at a low level. Under these conditions, Nil2p antagonizes Nil1p to prevent an increase in Nil1p levels (Rowen et al., 1997; Georis et al., 2009). The end result is very low expression of other NCR genes (Figure 2A). In proline, Gln3p is released from Ure2p

and is active in the nucleus (Beck and Hall, 1999; Bertram et al., 2000). It activates expression of *NIL1, DAL80*, and other nitrogen-regulated genes. Dal80p then antagonizes the activation of genes by Nil1p, including *NIL1* and *DAL80* (Coffman et al., 1997). In this way, Dal80p prevents a perpetual positive feedback loop of Nil1p activated gene expression and sets a maximum level of expression for NCR target genes (Figure 2B).

Both Gln3p and Nil1p activate *GAP1* transcription. As a result of the differing regulatory patterns of Gln3p and Nil1p, Gap1p is synthesized in ammonia, glutamate, or other non-preferred nitrogen sources, but is completely repressed in glutamine (Stanbrough and Magasanik, 1995).

The RTG transcription factors

Regulation of glutamate synthesis occurs not only at the level of nitrogen incorporation, but also in generation of α-ketoglutarate, the carbon skeleton required for many amino acids. It occurs through retrograde (RTG), or mitochondrial, signaling, originally identified as the response of nuclear genes in response to mitochondrial dysfunction. In cells that have lost mitochondrial DNA (*rho*^{0}, or "petite"), RTG signaling increases synthesis of enzymes involved in mitochondrial biogenesis and production of TCA cycle intermediates (Liu and Butow, 2006). Further study revealed that some, but not all, *rtg* mutants were glutamate auxotrophs, indicating that RTG signaling responds to deficiencies in both catabolism (production of ATP) and biogenesis (glutamate synthesis). Two basic helix-loop-helix/leucine zipper transcription factors, Rtg1/3p, were identified as the main activators of α-ketoglutarate synthesis for use in glutamate

production. Among their targets are early TCA cycle enzymes: the pyruvate carboxylase *PYC1,* citrate synthases *CIT1* and *CIT2*, the aconitase *ACO1*, and the isocitrate dehydrogenase subunits *IDH1/2* (Liu and Butow, 1999).

In analogous fashion to Gln3p and Nil1p, ability of Rtg1p and Rtg3p to activate gene expression is controlled by their localization. Rich nutrient conditions cause the sequestration of Rtg1/3p in the cytoplasm by the negative regulator Mks1p (Edskes et al., 1999; Tate et al., 2002; Dilova et al., 2002). The positive regulator Rtg2p, a cytoplasmic ATP binding protein, is required for Rtg1/3p-dependent gene expression when RTG signaling is active. Rtg1/3p reach the nucleus in an *rtg2mks1* double mutant. Rtg2p and Mks1p have also been shown to physically interact in vitro. Thus, Rtg2p allows Rtg1/3p-dependent gene expression by antagonizing Mks1p (Liu et al., 2003). How Rtg2p inactivates Mks1p in non-preferred nitrogen sources, but not in glutamate or glutamine, is poorly understood (Ferreira Junior et al., 2005). One hypothesis is that the ATP binding domain of Rtg2p functions as an ATP sensor, and Rtg2p binds Mks1p when ATP levels are low (Liu and Butow, 2006).

TOR

NCR and RTG genes are both regulated by the target of rapamycin (TOR) pathway. The TOR pathway is an evolutionarily conserved signaling cascade that regulates biogenesis and cellular growth (Wullschleger et al., 2006; Rohde et al., 2008). When nutrients are abundant, the TOR kinases stimulate ribosome biogenesis, protein synthesis, and nutrient import. However, upon nitrogen starvation or treatment with the

drug rapamycin, anabolic processes are downregulated and catabolic processes and autophagy are induced.

Because rapamycin treatment induces trafficking of both Gln3p and Nil1p from the cytoplasm to the nucleus, TOR has been implicated in the repression of Gln3p and Nil1p when nutrients are abundant (Beck and Hall, 1999). Ure2p-bound Gln3p is phosphorylated. Beck and Hall (1999) observed that rapamycin treatment caused Gln3p to shift from a phosphorylated to a dephosphorylated form that did not interact with Ure2p. They further implicated the TOR-regulated phosphatase Sit4p as responsible for Gln3p dephosphorylation. Nitrogen limitation or rapamycin treatment activates Sit4p by releasing it from an inhibitory complex with TorC1 and Tap42p. Rapamycin-dependent Gln3p dephosphorylation was reported to depend on Sit4p (Beck and Hall, 1999). There are caveats to this model, however. Gel mobility shift experiments suggested that Gln3p is dephosphorylated in response to rapamycin treatment but not in response to nitrogen starvation, despite both conditions resulting in Gln3p activation. Moreover, the localization of Gln3p after rapamycin treatment was observed to be transient; 30 minutes post-rapamycin addition, the majority of Gln3p is nuclear, but after 60 minutes, Gln3p is cytoplasmic, despite no apparent change in its phosphorylation state (Cox et al., 2004).

It has been suggested that Gln3p localization is also controlled by protein trafficking in the late secretory pathway, although a consensus has not been reached as to whether this effect is direct or indirect. The TOR-regulated protein kinase Npr1p, along with the E3 ubiquitin ligase complex Rsp5p/Bul1p/Bul2p, was reported to negatively regulate Gln3p (Crespo et al., 2004). However, Npr1p is required for trafficking of permeases, including Gap1p and Mep2p (Lorenz and Heitman, 1998; De Craene et al.,

2001). Trafficking of certain permeases (including Gap1p) also requires ubiquitination, as will be explained later in this chapter. Thus it appears that Npr1p control of Gln3p occurs through the modulation of nitrogen source uptake (Feller et al., 2006). In another model, class C and class D genes required for Golgi-endosome vesicular transport were reported to be required for Gln3p activation in nitrogen starvation, but not during rapamycin treatment. In the same study, Gln3p was peripherally localized to Golgi membranes (Puria et al., 2008). Thus it was proposed that during nitrogen starvation, Gln3p-Ure2p complexes are sorted from the Golgi to the endosome, where Sit4p dephosphorylates Gln3p, while in rapamycin treatment, Sit4p is released from TorC1 and bypasses the requirement for Gln3p trafficking (Puria and Cardenas, 2008).

TOR also controls α-ketoglutarate formation in two ways. TOR hyperphosphorylation of Mks1p allows Mks1p to repress Rtg1/3p (Dilova et al., 2002). Rapamycin-induced upregulation of Rtg1/3p occurs through partial dephosphorylation of Mks1p, preventing its interaction with Rtg1/3p (Liu et al., 2001; Dilova et al., 2004). The TOR component Lst8p, which was originally identified as a regulator of Gap1p trafficking, negatively regulates Rtg1/3p-dependent transciption (Roberg et al., 1997a; Liu et al., 2001; Chen and Kaiser, 2003). Oddly, the specific effect of Lst8p on RTG signaling is unclear, given that certain *lst8* mutants require Rtg2p for activation of RTG, but others do not (Chen and Kaiser, 2003). Rapamycin induction of RTG also depends on specific nutrient conditions; cells growing in glutamine are rapamycin-responsive, while cells growing in glutamate or any amino acid readily converted to glutamate are not (Dilova et al., 2004). RTG may therefore be subject to additional regulation independently of TOR.

Intracellular protein trafficking

GAP1 is one of many genes that are transcriptionally regulated by the quality of nitrogen source. However, Gap1p is unique in that activity is post-transationally controlled by nitrogen availability. Independently, both Grenson (1983) and Stanbrough and Magasanik (1995) observed that the activity of Gap1p could be affected separately from its transcript level. Grenson found that cells shifted from a repressive nitrogen source to a non-repressive nitrogen source reactivated Gap1p rapidly and did not rely on newly synthesized Gap1p. Stanbrough and Magasanik found that a *ure2* mutant growing in gluamine showed high Gap1p expression, due to the deregulation of Gln3p, but nonetheless had low Gap1p activity. They observed similar results for *URE2* cells grown in glutamate. Further experimentation revealed that trafficking of Gap1p through the late secretory pathway is tightly regulated (Roberg et al., 1997b).

The use of mutants at various stages of the secretory and vacuolar protein sorting pathways has revealed many features of Gap1p trafficking. A summary of the genes and complexes to be described in this section is diagrammed in Figure 3.

Exocytosis

Plasma membrane sorting of proteins requires the targeting of secretory vesicles to sites of endocytosis. The exocyst complex is required for the docking and fusion of vesicles with the plasma membrane. Six components of the exocyst complex, Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, and Sec15p, were identified in the screen for yeast secretory pathway mutants by Schekman and colleagues (Novick et al., 1980). Temperature sensitive mutants of these genes accumulate post-Golgi secretory vesicles that are unable to fuse with the plasma membrane (Novick et al., 1980). One of these

temperature sensitive mutants, *sec6-4*, is used in Chapter 2 to block delivery of post-Golgi Gap1p containing vesicles to the plasma membrane. Two other components, Exo70 and Exo84, were identified after immunoprecipitation of the full exocyst complex (TerBush et al., 1996; Guo et al., 1999a).

The exocyst complex assembles on the bud tips of dividing cells (TerBush and Novick, 1995). Assembly of the complex is thought to define specific sites for vesicle docking and fusion. Sec3p is sorted to the plasma membrane independently of vesicular traffic and is thought to serve as the landmark for assembly of the complex (Boyd et al., 2004; Finger et al., 1998). The other components arrive at exocytic sites on secretory vesicles (Boyd et al., 2004). Sec15p binds to Sec4p, a Rab GTPase on the secretory vesicle (Guo et al., 1999b). This interaction brings the secretory vesicle in close proximity to the plasma membrane and allows for the formation of SNARE complexes that mediate membrane fusion.

Vacuolar protein sorting

Degradation of Gap1p occurs via sorting to the vacuole, which occurs through endocytosis or direct sorting from the Golgi. Like many plasma membrane proteins, Gap1p is endocytosed from the plasma membrane, and much of the initial research of Gap1p trafficking focused on that phenomenon (Grenson, 1983; Hein et al., 1995; De Craene et al., 2001). However, this thesis will focus on the trafficking of Gap1p between the *trans*-Golgi and the vacuole. In vacuolar protein sorting (VPS), cargo are identified and sorted directly to the vacuole. Examples include vacuolar resident proteins like the hydrolase carboxypeptidase Y (CPY), as well as unneeded plasma membrane proteins

such as Gap1p. VPS has been well studied in yeast; many of the genes involved were identified by the isolation of mutants that caused secretion of CPY (Bowers and Stevens, 2005). Cargo undergoing VPS reaches the vacuole by way of the late endosome, also referred to as the multivesicular endosome (MVE) or multivesicular body (MVB) due to their characteristic inward-budding morphology (Haigler et al., 1979).

Ubiquitination

Ubiquitination serves as the signal for sorting of cargo to the vacuole. Ubiquitin is a highly conserved protein that attaches to substrates via a covalent linkage between the ubiquitin molecule and a lysine residue of the substrate (Staub and Rotin, 2006). Ubiquitination occurs via a three-step process. First, the E1 enzyme, known as the ubiquitin-activating enzyme, forms a thioester bond with the ubiquitin moiety. This reaction is coupled to ATP hydrolysis. The E1 enzyme then transfers the ubiquitin to the catalytic cysteine of the E2 ubiquitin-conjugating enzyme. The E3 ubiquitin-ligase facilitates the transfer of ubiquitin from the E2 to the substrate. This can occur simply by bringing the E2 into close proximity of the substrate, as is done by the RING type ubiquitin ligases, or by receiving the ubiquitin molecule from the E2 enzyme and transferring it to the substrate, the method of the HECT type ubiquitin ligases. Polyubiquitin chains can be formed on substrate molecules through the linkage of ubiquitin molecules by one of 7 lysines. Polyubiquitin chains connected via Lys48 or Lys63 are the most common. Interestingly, proteasome substrates commonly receive Lys48-linked chains, while vacuolar sorting appears to rely on Lys63-linked chains (Glickman and Ciechanover, 2002; Belgareh-Touzé et al., 2008).

The HECT type E3 ubiquitin ligase Rsp5p is an essential protein in yeast and participates in several aspects of protein trafficking, including endocytosis and direct sorting to the vacuole (Belgareh-Touzé et al., 2008). Rsp5p is required for both the endocytosis and direct vacuolar sorting of Gap1p (Hein et al., 1995; Helliwell et al., 2001). In addition to its C-terminal HECT catalytic domain, Rsp5p also contains WW protein-protein interaction domains that bind PY domains on the partner protein. These WW domains have been suggested as involved in substrate recognition, yet many Rsp5p substrates do not contain a PY domain. Several PY domain-contanining proteins have been suggested as adaptors for Rsp5p. The best characterized of these are the Bul (Binds Ubiqutin Ligase) proteins, Bul1p and Bul2p. Bul1p and Bul2p are partially-redundant nonessential proteins that each contain a PY domain. Bul1p was originally identified as an interactor of Rsp5p; Bul2p was identified due to its homology to Bul1p (Yashiroda et al., 1998; Yashiroda et al., 1996).

The contribution of Bul1p and Bul2p to Gap1p trafficking was discovered by a genetic screen that identified *BUL1* and *BUL2* as genes that when overexpressed conferred greater resistance to the toxic proline analog L-azetidine 2-carboxylic acid (ADCB) (Helliwell et al., 2001). An abundance of Bul1p or Bul2p causes a decrease in Gap1p activity, which correlates with an increase in poly-ubiquitination (Helliwell et al., 2001; Rubio-Texeira and Kaiser, 2006). A *bul1*Δ*bul2*Δ strain causes constitutive sorting of Gap1p to the plasma membrane in equivalent amounts to that observed in an *rsp5-1* mutant, suggesting that the three proteins function together (Helliwell et al., 2001). Endocytosis does not require Bul1/2p, as Gap1p is endocytosed even in a *bul1*Δ*bul2*Δ strain, yet endocytosis of Gap1p is faster when Bul1/2p are present, indicating that both

Bul-dependent and –independent endocytic pathways control Gap1p endocytosis (Risinger and Kaiser, 2008). Total ubiquitination of Gap1p can be blocked by mutation of two acceptor lysines on the N terminus of Gap1p, Lys9 and Lys16 (Soetens et al., 2001). A Gap1p^{K9R,K16R} mutant can not reach the vacuole, either by direct sorting or endocytosis and is therefore constitutively present at the plasma membrane. The E3 complex does not appear to preferentially ubiquitinate one lysine over the other for direct sorting, though Bul-independent, Rsp5p-dependent endocytosis occurs specifically on Lys16 (Risinger and Kaiser, 2008).

Golgi-endosome trafficking

Ubiquitinated cargo requires vesicular transport from the *trans*-Golgi to the vacuole. The cargo must be identified, via its ubquitin modification, and packaged into secretory vesicles bound for the endosome. Adaptor proteins are needed to bind ubiquitin, as well as the clathrin coat of vesicles to facilitate this process. The Golgi-localized, gamma-ear-containing, Arf- binding (GGA) proteins have been identified as participants in Golgi-endosome trafficking in both yeast and mammalian cells (Bonifacino, 2004; Pelham, 2004). Gga proteins have four domains that are conserved across species: a VHS (Vps27, Hrs, STAM) domain in the N terminus, a GAT (GGA and TOM) domain, a flexible hinge, and a C terminal γ-adaptin domain. The GAT domain is of particular interest, as not only does it bind the coat-recruiting GTPase Arf, but has also been shown to bind ubiquitin. Yeast Gga1p and Gga2p have been implicated in Gap1p trafficking from the *trans*-Golgi to the endosome. Mutation of the GAT domain impairs, but does not completely disrupt, Gap1p delivery to the vacuole (Scott et al., 2004). Thus,

while the GGA proteins may participate in Gap1p trafficking, they are not absolutely required.

Vesicles reaching the endosome require Pep12p for fusion (Becherer et al., 1996). Pep12p is a member of the syntaxin family and has a central role in all traffic entering and leaving the endosome. Mutation of Pep12p disrupts fusion of vesicles from the Golgi, plasma membrane, and vacuole (Gerrard et al., 2000). This indicates that not only is Pep12p a multifunctional syntaxin that can incorporate into several different SNARE complexes, but also that the late endosome is a central location for many trafficking events in the cell. Accordingly, in *pep12*Δ mutants CPY is secreted and Gap1p is inappropriately sorted to the plasma membrane (Becherer et al., 1996; Roberg et al., 1997b).

The screen for VPS genes identified a group of mutants, dubbed "Class C" mutants that appeared to have no vacuoles (Raymond et al., 1992). Four class C genes were identified that caused mutant cells to lack an identifiable vacuole: *VPS11/PEP5, VPS16, VPS18/PEP3,* and *VPS33* (Sato et al., 2000). These genes appeared to function in multiple trafficking steps, including Golgi-MVE, MVE-vacuole, and vacuole-MVE trafficking (Peterson and Emr, 2001). These four proteins comprise what is now known as a "Vps C core" complex and form the basis of two larger trafficking complexes known as CORVET and HOPS. Although the HOPS complex functions primarily in trafficking events at the vacuole, CORVET has several functions that center on the MVE (Nickerson et al., 2009). CORVET associates with the Rab5 homolog Vps21p to facilitate membrane fusion at the late endosome (Peplowska et al., 2007). Disruptions in CORVET not only cause endosomal/vacuolar dysfunction, but also disrupt the traffic of

cargo destined for the vacuole. Trafficking of Gap1p to the vacuole is disrupted in *vps18* mutants (Srivastava et al., 2000).

Multivesicular endosome formation

To facilitate the delivery of cargo to the vacuole, the endosome undergoes a deliberate inward budding process that results in the formation of the MVE (Saksena et al., 2007). The MVE then fuses with the vacuole, at which point the intralumenal vesicles (ILV) are exposed to vacuolar proteases and cargo is degraded. The formation of MVEs is the coordinated effort of the four arms of the ESCRT pathway, ESCRT complexes 0-4. Mutants in ESCRT were identified as class E VPS mutants, and loss of ESCRT function results in an aberrant, enlarged endosome referred to as the class E compartment (Raymond et al., 1992).

In yeast, internalization into MVEs requires ubiquitination of the cargo by Rsp5p. Membrane proteins that reach the endosomal membrane without ubiquitination are not internalized (Katzmann et al., 2004). Members of ESCRT-0, I, and II contain ubiquitin binding domains that facilitate the sequential transfer of the cargo. Ubiquitinated cargo is first recognized by Vps27-Hse1, also known as ESCRT-0. Vps27-Hse1 is recruited to the endosomal membrane by the lipid phosphatidylinositol 3-phosphate (PI-3P), which is enriched in the endosomal membrane and is recognized by the FYVE domain of Vps27. Vps27 binds the ubiquitinated cargo. This allows ESCRT-I to dock, followed by ESCRT-II. This sequence of events allows the cargo to cluster on the endosomal membrane in preparation for membrane invagination (Hurley and Emr, 2006). ESCRT-II can then act as a scaffold for ESCRT-III (Teis et al., 2010). ESCRT-III is believed to be

responsible for the initial formation of ILV. The ESCRT-III component Snf7p oligomerizes into a ring-like filament before being capped by Vps24. Finally, the Vps4 AAA-ATPase complex disassembles ESCRT-III so the process can repeat (Teis et al., 2008). An important step in this process is the recruitment of deubiquitinating enzymes (DUB) that remove the ubiquitin molecules or chains before internalization. Deubiquitination is important not only to maintain cellular levels of free ubiquitin, but also to serve as a means for cargo to escape the MVE membrane and be recycled to the *trans*-Golgi (Saksena et al., 2007).

Interestingly, a genome-wide screen for mutants that caused increased ADCB sensitivity (and by inference, overactive Gap1p) identified many of the ESCRT components. Apparently, disruption of MVE formation causes Gap1p to undergo recycling to the *trans*-Golgi and reach the plasma membrane (Rubio-Texeira and Kaiser, 2006). Similar results are observed for a mutation in Doa4p, the major deubiquitinating enzyme located to endosomes (Springael et al., 1999). When ubiquitin is limiting, Gap1p is unable to be poly-ubiquitinated and cannot enter MVEs.

Post-translational amino acid regulation of Gap1p

Gap1p was initially identified as a broad specificity amino acid transporter that is most active in poor nitrogen sources and least active in good nitrogen sources. Although this chapter has detailed how the transcription of Gap1p is regulated by nitrogen, transcriptional regulation of Gap1p only accounts for a small fraction of the total effect of high amino acids on Gap1p activity (Stanbrough and Magasanik, 1995). The following section describes current knowledge of the effects of amino acids on post-translational regulation of Gap1p.

Discrepancies between laboratory strains

The early characterization of Gap1p regulation was performed in the Belgian *S. cerevisiae* strain Σ1278b. The experiments of Grenson, Andre, and colleagues identified ammonium as a repressive nitrogen source that signals the internalization of Gap1p from the cell surface, a phenomenon they termed "ammonium inactivation" (Grenson, 1983; Hein et al., 1995). The work presented in this thesis, as well as previous work performed in the Kaiser laboratory, was performed in the S288C strain background. This strain displays high activity when ammonium is the sole nitrogen source (Courchesne and Magasanik, 1983). Therefore, ammonium inactivation is not observed in this strain. In studies of Gap1p regulated trafficking, experiments using Σ1278b compare cells grown in proline (a poor nitrogen source) to cells grown in ammonia, whereas experiments using S288C compare cells grown in proline or urea to cells grown in glutamine or glutamate. Although the response to nitrogen sources differs, many of the genes identified as effectors of Gap1p trafficking have equivalent function in both strains.

The specific reason for the differing effects of ammonium between strains is not clear, but has been ascribed to genomic differences between Σ1278b and S288C. In diploids generated from mating Σ1278b and S288C, Gap1p activity is low in ammonium, indicating that the S288C trait is recessive (Rytka, 1975). S288C may less efficiently convert ammonia into glutamate and glutamine, but the genetic basis for such a difference is unknown. Recently, Boone and colleagues systematically compared the genomes of Σ1278b and S288C by whole genome sequencing and deletion analysis (Dowell et al., 2010). The total sequence identity between the strains was approximately equal to the similarity between the genomes of two humans, with the majority of differences arising from small insertions, deletions, or SNPs. They found that 5% of genes essential to Σ1278b were non-essential to S288C and 1% of genes essential to S288C were non-essential to Σ1278b. A subset of genes essential only to Σ1278 was analyzed further by tetrad analysis of Σ1278b/S288C hybrid heterozygous diploid strains. They concluded that the lethality of a particular mutation required the presence of at least two but typically four or more unlinked modifiers specific to Σ1278b. Thus, in many cases, difference in phenotypes is due to multiple background-specific modifiers. If the difference in Gap1p trafficking in ammonium between Σ1278b and S288C is genetic in nature, it is unlikely to be isolated to the effect of one gene.

Amino acids regulate Gap1p trafficking

The contribution of a post-translational regulation step for Gap1p was made clear by the observation that cells grown in glutamate transcribed equivalent levels of *GAP1* mRNA to cells grown in urea, but growth on urea resulted in ~100 times more Gap1p

activity than growth on glutamate (Stanbrough and Magasanik, 1995; Roberg et al., 1997b). It was apparent that although Gap1p is transcriptionally regulated, Gap1p activity is primarily controlled at the protein level. To fully decouple the effects of transcription from any post-translational regulation of Gap1p, the *GAP1* gene was placed under the control of the non-nitrogen regulated *ADH1* promoter. This constitutively expressed Gap1p construct displayed high activity in urea or ammonia and low activity in glutamate or glutamine, similar to Gap1p expressed from the endogenous promoter (Chen and Kaiser, 2002). The decrease in activity was further attributed to a decrease in Gap1p sorting to the plasma membrane and increased sorting to the vacuole (Roberg et al., 1997b; Chen and Kaiser, 2002).

Further study of the constitutively promoted Gap1p construct revealed that the signal for vacuolar sorting of the permease was not connected to the quality of the nitrogen source, but rather the level of amino acids present. Amino acids that serve as poor nitrogen sources, such as glycine, histidine, or methionine, could nonetheless cause a decrease in Gap1p activity within two hours of addition. The same was true for several glutamate analogs that *S. cerevisiae* is unable to use as a nitrogen source. Notably, all the analogs tested that fit the class of L-amino acid were able to down-regulate Gap1p, while other amino acid derivatives such as *N*-methyl amino acids or amino acid amides were not (Chen and Kaiser, 2002).

Control of Gap1p trafficking is not limited to exogenous amino acids. Manipulation of amino acid biosynthetic pathways is equally effective in signaling Gap1p trafficking to the plasma membrane or sorting to the vacuole. Mutation of *GDH1* reduces glutamate synthesis as described above; in S288C a *gdh1* mutant reduces

intracellular glutamate by 11%. This results in a twofold increase in Gap1p activity. A *gln1* mutation, which decreases glutamine production, caused a nearly threefold increase in Gap1p activity. By contrast, mutation of *MKS1* increases intracellular glutamate by increasing production of α-ketoglutarate. Gap1p activity in an *mks1* mutant is less than one percent of that observed in a wild type strain (Chen and Kaiser, 2002).

Surprising results were observed when the effect of TOR signaling on Gap1p trafficking was measured. At the level of transcription, rapamycin treatment appears to mimic the effects of nitrogen starvation; the effects of NCR are released and pathways for poor nitrogen source utilization are activated. Indeed, rapamycin treatment induces transcription of Gap1p by activating Gln3p and Nil1p (Rohde et al., 2008). In nitrogen starvation conditions, Gap1p is highly active. Thus it was expected that rapamycin treatment would increase Gap1p trafficking to the plasma membrane. Instead, rapamycin treatment resulted in Gap1p sorting to the vacuole (Chen and Kaiser, 2002). Similarly, mutation of the TOR component Lst8p causes a substantial decrease in Gap1p activity and sorting of Gap1p to the vacuole (Roberg et al., 1997a; Chen and Kaiser, 2003). Rapamycin treatment causes intracellular amino acids to increase as much as threefold. Therefore regulation of Gap1p trafficking occurs through a separate mechanism that is independent of TOR but dependent on amino acid levels in the cell (Chen and Kaiser, 2003).

Regulated trafficking in response to amino acid substrates is not unique to Gap1p. The tryptophan specific permease Tat2p also undergoes regulated trafficking, albeit in a somewhat inverse fashion to Gap1p. Under conditions of nitrogen starvation, Tat2p is directly sorted to the vacuole, while in complete medium it is sorted to the plasma

membrane (Beck et al., 1999). Like Gap1p, Tat2p is poly-ubiquitinated by Rsp5p/Bul1p/Bul2p on as many as five lysines on its N terminus (Beck et al., 1999; Umebayashi and Nakano, 2003). While initially it was believed that Tat2p was regulated completely opposite Gap1p, further study found that sorting of Tat2p responds to three signals: starvation, in which it is sorted to the vacuole, low tryptophan, in which it is sorted to the plasma membrane, and high tryptophan, in which it is sorted to the vacuole (Umebayashi and Nakano, 2003). Because rapamycin treatment increases internal amino acid levels it is possible that Tat2p, like Gap1p, is responding to high amino acid levels upon rapamycin treatment.

Redistribution of Gap1p from the MVE

Direct sorting of Gap1p in high amino acids requires ubiquitination by Rsp5p/Bul1p/Bul2p; mutations in Bul1/2p or lysines 9 and 16 on Gap1p cause constitutive sorting to the plasma membrane (Helliwell et al., 2001; Soetens et al., 2001). Yet, increasing amino acid levels do not correspond to a substantial increase in the ubiquitination of Gap1p (Rubio-Texeira and Kaiser, 2006). Additionally, a significant amount of Gap1p associates with internal membranes even when grown in amino acid free media (Roberg et al., 1997b). This may partially be caused by the rate at which Gap1p navigates the early secretory pathway; nonetheless, amino acids do not appear to act on the rate of Gap1p ubiquitination, but an event downstream of ubiquitination. That event appears to be a recycling step from the MVE back to the Golgi. In cells shifted from glutamate media to urea media with cycloheximide, Gap1p activity increases rapidly, reaching a maximum within 15 minutes (Roberg et al., 1997b). Cells grown in

glutamine do not display this behavior, presumably because Gap1p synthesis is completely suppressed in glutamine and therefore any reactivation in urea would require new protein synthesis. The reactivation in glutamate is therefore due to redistribution of an existing intracellular pool of Gap1p (Figure 4).

The existence of a recycling step explains the apparent paradox that Gap1p is synthesized in glutamate or any amino acid other than glutamine, only to be directly sorted to the vacuole unused. Gap1p synthesis in glutamate is entirely dependent on Gln3p. When wild type cells grown in glutamate are shifted into urea, Gap1p activity increases rapidly and dramatically. By contrast, a *gln3*Δ strain requires over an hour to achieve the same levels of Gap1p activity (Risinger and Kaiser, unpublished data). Therefore, in cells where Gap1p is synthesized but not sorted to the plasma membrane, a pool of Gap1p is available on the MVE membrane to be mobilized if nutrient conditions change from abundant to limiting. Because recycling of Gap1p is much faster than induction of new synthesis and trafficking through the early secretory pathway, dual control of Gap1p transcription by Gln3p and Nil1p gives a competitive advantage, in that cells are able to quickly respond to shifting environmental conditions.

Although genetic data has suggested that amino acids are regulating the recycling of Gap1p from the MVE to the Golgi, the specific mechanism for this regulation has been unclear. In several mutant strains that cause abnormally high Gap1p activity, Gap1p is still regulated by amino acids. For example, mutations in the ESCRT pathway cause increased Gap1p activity in ammonium, presumably because the inability of Gap1p to enter the vacuole forces Gap1p to cycle backwards to the Golgi. However, these mutants have low activity in glutamate, comparable to a wild type strain. Similarly, *mks1*Δ is

epistatic to ESCRT mutants. In these cells, Gap1p is observed in an enlarged endosome, or class E compartment (Rubio-Texeira and Kaiser, 2006). Therefore, amino acids act downstream of ubiquitination, but upstream of internalization into MVEs. Amino acid regulation of Gap1p recycling will be further addressed in Chapter 2.

Genetic screens have been performed to identify additional genes involved in the trafficking of Gap1p. A class of mutants was identified that down-regulates Gap1p in ammonium. Because mutations in ubiquitination are epistatic to mutants of this class, we have concluded that these genes are required for Gap1p recycling from the vacuole. However, the function of these genes is still largely unknown. A screen for mutants lethal with *sec13-1* revealed that separately from its role in the COPII coat, Sec13p also functions in the trafficking of permeases, particularly Gap1p. One class of genes lethal with *sec13-1* included amino acid biosynthesis genes *SER2, HOM6*, and *THR4*. These genes caused synthetic lethality with *sec13-1* because both synthesis and uptake of amino acids were impaired. *LST8* was also isolated in this screen, as the combined effects of TOR disruption and *sec13-1* interfered with the trafficking of Gap1p to the plasma membrane in an amino acid auxotrophic strain. However, two other genes, *LST4* and *LST7,* were previously unknown. Although they are required for Gap1p trafficking to the plasma membrane, their role is not yet clear (Roberg et al., 1997a).

A later screen identified two GTPases, Gtr1p and Gtr2p, that when deleted cause a similar Gap1p phenotype to *lst4* and *lst7*. Gtr1p and Gtr2p are part of a larger complex known as GSE/EGO that is localized to endosomal membranes. Work in the Kaiser laboratory found that Gtr2p binds to the C terminal tail of Gap1p, suggesting that the GSE complex is involved in recruitment of Gap1p to recycling endosomes (Gao and

Kaiser, 2006). Because the activities of Gtr1p and Gtr2p are controlled by their nucleotide bound state, it was proposed that they act as part of a coat protein complex, similarly to Sar1p and Arf1p. Other studies have tied the GSE/EGO complex to TOR signaling. In yeast, the GSE/EGO complex is required for TOR induction of microautophagy (Dubouloz et al., 2005). Gtr1p and Gtr2p each have two homologs in mammalian cells. The Gtr1p homologs RagA and RagB form heterodimers with RagC and RagD, respectively. After amino acid stimulation, the Rags are required to localize mTOR to the endosomal membrane (Sancak et al., 2008). Because disruption of TOR causes Gap1p sorting to the vacuole due to high amino acid levels, the effect of GSE/EGO could be indirect. However, GSE/EGO may have multiple roles in Gap1p trafficking.

Initial studies of *LST4, LST7*, and the GSE/EGO complex could not distinguish the functions of these proteins based solely on steady-state Gap1p activity. However, in Chapter 4 data will be presented that suggest that all of these genes do not affect the same step in Gap1p trafficking.

Cell surface inactivation of Gap1p

We have also observed that Gap1p is also regulated by amino acids at the cell surface. The Gap1p^{K9R,K16R} allele is constitutively trafficked to the plasma membrane and is highly active in amino acid-free medium. However, addition of casamino acids, a rich amino acid mixture containing all amino acids except tryptophan, caused Gap1p^{K9R,K16R} to decrease in activity with a half time of approximately 10 minutes. Localization experiments confirmed that this decrease in activity occurred despite the

continued presence of Gap1p^{K9R,K16R} at the plasma membrane. Therefore the regulation of Gap1p^{K9R,K16R} was occurring at the level of activity, not expression or trafficking. Intriguingly, we observed that this inactivation was reversible; removal of amino acids allowed Gap1p to reactivate with a half time of approximately 20 minutes. Inactivation appears to be dependent on Gap1p activity (Risinger et al., 2006). The surface inactivation of Gap1p is discussed in depth in Appendix I.

Amino acid toxicity

Gap1p activity is tightly controlled by amino acids. The presence of any amino acid is sufficient to prevent Gap1p from performing its function at the plasma membrane. An increase in amino acids in every way we have tested has resulted in Gap1p sorting to the vacuole. Moreover, the difference between Gap1p activity in permissive to restrictive conditions is extreme – Gap1p is at least 100 times more active in amino acid free media than in rich media. Indeed, the level of Gap1p activity in nutrient rich conditions is comparable to a *gap1*Δ strain. However, the cell has the ability to rapidly up-regulate Gap1p in the event of nutrient shortage. If Gap1p activity is necessary to rescue cells from potential nitrogen starvation, the question remains: why regulate Gap1p at all?

We have made several observations that suggest a completely unregulated Gap1p can be deleterious to cells. Experiments using P_{ADH1}-$GAP1^{K9R,K16R}$ showed a puzzling phenomenon: these cells were inviable on minimal ammonium plates supplemented with 1mM glycine. When this strain was grown in liquid culture, a growth arrest was observed after the addition of glycine. Because these cells did not grow when transferred to a glycine-free plate, we concluded that glycine had a toxic effect on this strain. Further

experimentation showed that 18 of the 20 naturally occurring amino acids are toxic to some degree, as well as citrulline, a precursor to arginine. Alanine and phenylalanine did not cause any toxic effects. The toxicity of amino acids was most pronounced when amino acids were added individually. However, cells were viable when multiple toxic amino acids were added in combination (Risinger et al., 2006). This suggests that cells suffer toxic effects from an imbalance in the intracellular amino acid pool, although the mechanism of toxicity is still unclear.

Summary

Gap1p activity is tightly controlled by amino acids. Regulation at the levels of transcription, trafficking, and activity allow cells to utilize Gap1p only when facing starvation. In nutrient-replete conditions, cells can efficiently down-regulate Gap1p to protect cells from the toxic effects of amino acid overabundance. In the following chapters, I will describe new insights into the regulated trafficking of Gap1p gained through analysis of several Gap1p mutant alleles. To test the hypothesis that regulation of Gap1p by amino acids requires Gap1p activity, I isolated mutants of Gap1p with altered transport abilities for use as tools to establish a link between activity and regulation. First, I show that intracellular sorting and inactivation of Gap1p are substrate-specific and require Gap1p to undergo the full transport cycle. Then, examination of a constitutive yeast amino acid transporter, Hip1p, suggests that while substrate-specific down-regulation may be a general property of yeast permeases, intracellular sorting and cell surface inactivation may be specific to Gap1p.

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Figures

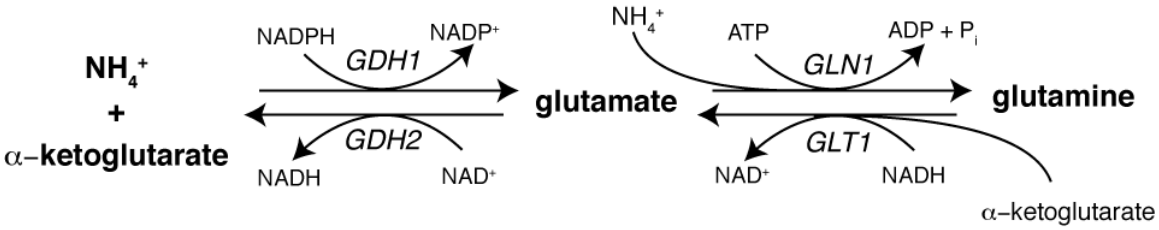
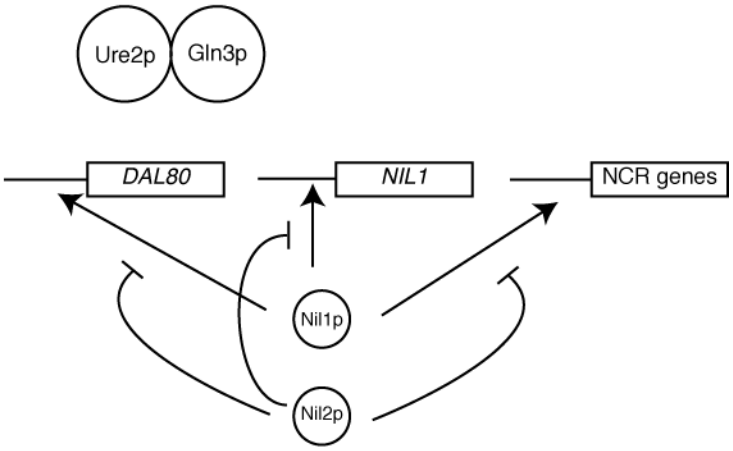


Figure 1. Core pathway for nitrogen metabolism in *S. cerevisiae*. Ammonium that is imported from the extracellular environment or generated from the metabolism of other nitrogenous compounds is combined with α-ketoglutarate, TCA cycle intermediate. Glutamine is generated by combinding ammonium with glutamate. Glutamine can donate an ammonium group to α-ketoglutarate to form two molecules of glutamate. The genes responsible for each step are shown in italics.

A. Glutamine



B. Proline

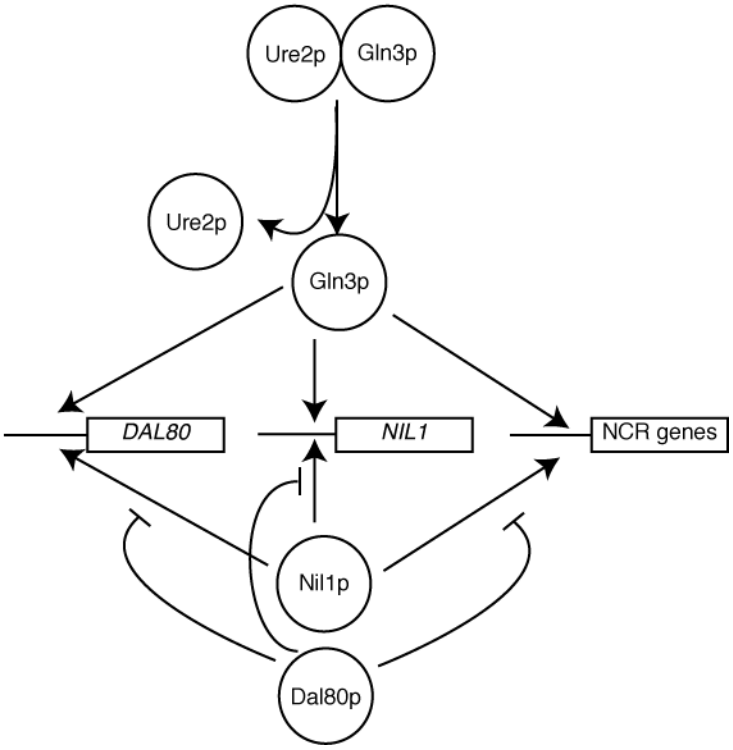


Figure 2. Four GATA transcription factors participate in NCR. (A) In glutamine, Gln3p is kept inactive through an interaction with Ure2p. Nil2p blocks the auto-activation of Nil1p. As a result, NCR target genes are not expressed. (B) In proline, Ure2p releases Gln3p, which enters the nucleus and activates transcription of *NIL1, DAL80*, and NCR target genes. Nil1p auto-activates its expression and also activates *DAL80* and NCR target genes. Dal80p antagonizes Nil1p activation of expression, which sets a limit to nitrogen-regulated gene expression.

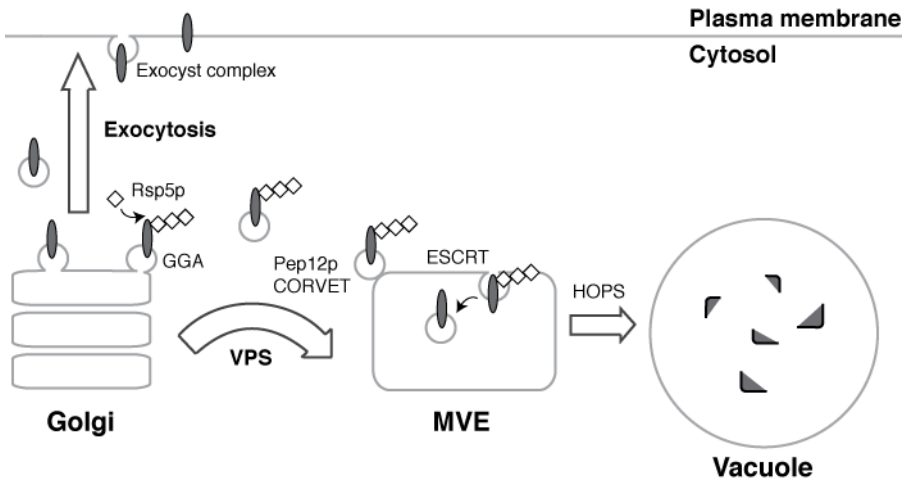


Figure 3. A schematic of trafficking pathways in *S. cerevisiae*. A membrane protein at the *trans*-Golgi can proceed to the plasma membrane (Exocytosis) or undergo vacuolar protein sorting (VPS). Each trafficking step is labeled by the protein or protein complex that facilitates it. Fusion of post-Golgi vesicles with the plasma membrane requires the exocyst complex. Proteins destined for the VPS pathway are first ubiquitinated (diamonds), here by Rsp5p, and may be recruited to vesicles by GGA. Tethering and fusion of vesicles to the MVE require Pep12p and CORVET, respectively. Proteins on the MVE membrane are incorporated into ILVs by ESCRT. Finally, delivery of MVE contents to the vacuole is facilitated by HOPS.

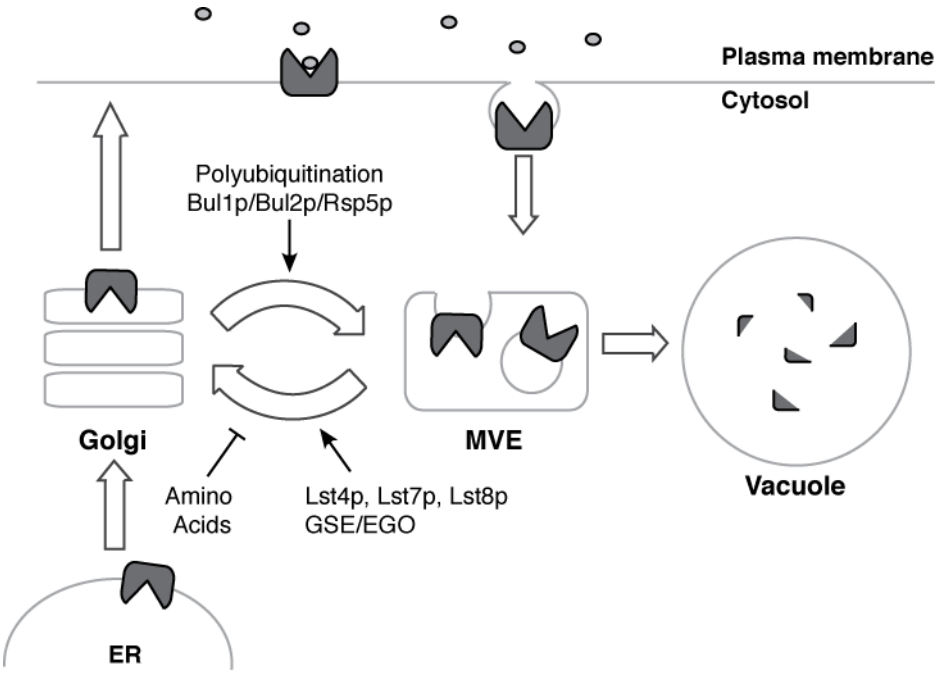


Figure 4. Trafficking of Gap1p through the late secretory pathway. At the trans-Golgi, Gap1p can be sorted to the plasma membrane or ubiquitinated and sorted to the MVE. When amino acids are abundant, Gap1p is internalized into MVEs and delivered to the vacuole. If amino acid levels drop, any Gap1p present on the MVE membrane can be redistributed to the trans-Golgi and sorted to the plasma membrane. Recycling requires several proteins whose functions are not fully understood.

Chapter 2

Transport Activity Dependent Intracellular Sorting of the Yeast General Amino Acid Permease

Abstract

Intracellular trafficking of the general amino acid permease, Gap1p, of *S. cerevisiae* is regulated by amino acid abundance. Gap1p is sorted to the plasma membrane when amino acids are scarce, whereas amino acid abundance causes Gap1p to be sorted from the *trans*-Golgi to the multivesicular endosome (MVE) and the vacuole. Here we test the hypothesis that Gap1p itself is the sensor of amino acid abundance by examining the trafficking of Gap1p mutants with altered substrate specificity and transport activity. We show that the trafficking behavior of substrate specificity mutant Gap1p^{A297V} does not respond to amino acids that are not transport substrates. Further, we have identified a catalytically inactive mutant that does not respond to complex amino acid mixtures and constitutively sorts Gap1p to the plasma membrane. Previously we showed that amino acids govern the propensity of Gap1p to recycle from the MVE to the plasma membrane. Here we propose that in the presence of substrate the steady-state conformation of Gap1p shifts to a state that is unable to be recycled from the MVE. These results indicate a parsimonious regulatory mechanism by which Gap1p senses its transport substrates to set an appropriate level of transporter activity at the cell surface.

Introduction

The yeast *S. cerevisiae* encodes 24 members of the amino acid-polyamines-organocation (APC) superfamily, which are responsible for transport of amino acids and other amines across the plasma membrane (Nelissen et al., 1997; Jack et al., 2000). APC proteins share a common topology with 12 trans-membrane domains (TMD) and cytosolic N and C termini. The physiological function of individual APC family members can be deduced from their mode of regulation. For example, the activity of several transporters, including *AGP1, BAP2, BAP3, GNP1, TAT1,* and *TAT2*, have been shown to be induced by amino acids through regulation of the SPS amino acid sensor complex (Didion et al., 1998; Iraqui et al., 1999; Forsberg et al., 2001). Because the transporters regulated by the SPS complex have relatively low capacity and are most active when amino acids are present in the growth medium, these transporters at thought to function opportunistically to transport specific amino acids for use in protein synthesis. By contrast, Gap1p is a high capacity transporter of all naturally occurring amino acids as well as many amino acid analogs, and is inactive when amino acids are present in the growth medium (Grenson et al., 1970; Chen and Kaiser, 2002). Gap1p is therefore thought to function as a high capacity scavenger of amino acids for use as a source of nitrogen.

Much of the change in Gap1p activity in response to amino acid abundance is due to regulated alternative trafficking patterns of Gap1p in the secretory pathway. At the *trans*-Golgi, Gap1p can proceed directly to the plasma membrane, or it can be directly sorted to the multivesicular endosome (MVE) ultimately to be delivered to the vacuole via the vacuolar protein-sorting (VPS) pathway (Roberg et al., 1997; Rubio-Texeira and

Kaiser, 2006). Sorting to the MVE requires poly-ubiquitination on lysine 9 and/or 16 of Gap1p by the E3 ubiquitin ligase complex of Bul1p, Bul2p, and Rsp5p (Helliwell et al., 2001; Soetens et al., 2001). Deletion of the redundant *BUL1* and *BUL2* accessory proteins or mutation of both ubiquitin-acceptor lysines on Gap1p prevents ubiquitination of Gap1p and results in constitutive sorting of Gap1p to the plasma membrane. Such mutants that constitutively express Gap1p at the cell surface become hypersensitive to amino acid analogs as well as naturally occurring amino acids, probably because of aminoacyl tRNA mischarging brought about by imbalances in the intracellular concentration of amino acids (Risinger et al., 2006). We have used amino acid sensitivity to develop genetic screens for *GAP1* mutants that are resistant to some amino acids but remain sensitive to others as a means to find substrate selectivity mutants (Risinger et al., 2006).

In the absence of amino acids, such as in growth on ammonia as a nitrogen source, Gap1p that has been ubiquitinated and sorted to the MVE will recycle from the MVE to the plasma membrane where it is active for uptake of extracellular amino acids (Roberg et al., 1997; Rubio-Texeira and Kaiser, 2006). In the presence of amino acids this recycling of Gap1p from the MVE is blocked and Gap1p is efficiently sorted to the vacuole. We have tested a variety of compounds for their ability to cause Gap1p sorting to the vacuole and have found that all naturally occurring amino acids as well as amino acid analogs that carry both α-carboxylate and α-amino moieties are effective at signaling for Gap1p sorting, regardless of their quality as a source of nitrogen (Chen and Kaiser, 2002). Moreover, mutants that cause overproduction of intercellular amino acids such as *mks1*Δ and temperature sensitive alleles of *LST8,* which encodes a subunit of the

TORC1 complex, will also trigger Gap1p trafficking to the vacuole, despite the induction of many cellular responses associated with nitrogen starvation (Chen and Kaiser, 2002; Chen and Kaiser, 2003). Although the mechanism by which Gap1p recycling from the MVE to the plasma membrane is inhibited by amino acids is not understood, these pharmacological and genetic tests for the conditions required for sorting of Gap1p to the vacuole suggest that the sensor for amino acid signaling is not related to nitrogen availability per se, but instead the depends on direct recognition of the presence of amino acids themselves.

Because the broad variety of amino acids that are transport substrates for Gap1p largely coincides with the spectrum of compounds that can trigger Gap1p sorting to the vacuole we suspected that Gap1p itself might be the sensor for amino acids. Furthermore, characterization of the non-ubiquitinateable Gap1p^{K9R,K16R} allele revealed that complex mixtures of amino acids, such as Casamino acids, that are not toxic to cells, will reversibly inactivate Gap1p^{K9R,K16R} transport activity as the protein remains at the cell surface. Importantly, a mutant of Gap1p, which has a selective defect for transport of arginine, is not inactivated at the cell surface by arginine (Risinger et al., 2006). These findings establish a precedent for Gap1p transport activity acting as a sensor of amino acids, in this case to reversibly regulate transporter activity itself.

In this paper we apply a similar strategy using Gap1p mutants with altered specificity or transport activity to test the hypothesis that the sorting of Gap1p acts in an auto-regulatory fashion, in which active turnover of amino acid substrates in the transport cycle prevents recycling to the plasma membrane and leads to Gap1p sorting to the vacuole.

Materials and Methods

Strains, Plasmids, and Media

All of the yeast strains used in this study (Table 1) were constructed in the S288C genetic background. Wild type S288C strains exhibit relatively high Gap1p activity in ammonia medium, in contrast to strains in the Σ1278b genetic background, which exhibit low Gap1p activity when grown on ammonia (Stanbrough and Magasanik, 1995). Deletions of *CAR1* and *END3* were made by one-step gene replacement in CKY482. Disruption of *CAR1* was verified by loss of ability to utilize arginine as a sole nitrogen source. Disruption of *END3* was verified by PCR.

Plasmids used in this study are listed in Table 2. Plasmid pNC26 was constructed by amplifying the *GAP1* ORF with adjoining 580 bp of 3' sequence using oligos OES43 and OES44. The resulting fragment was digested with EcoRV and XhoI and ligated into pCD43 (Shaywitz et al., 1995), which was digested with EcoRI, blunted with Mung Bean nuclease, and digested again with XhoI. Plasmids containing $GAP1^{T106K}$ and $GAP1^{T106K}$-*GFP* were created using the QuikChange kit (Stratagene, La Jolla, CA). Other mutations were introduced into the plasmid borne *GAP1* gene by sub-cloning mutations from existing plasmids.

Strains were grown at 24˚C unless otherwise noted. Minimal ammonia medium (SD) is composed of Difco (Detroit, MI) yeast nitrogen base without amino acids or ammonium sulfate (YNB), 2% glucose, and 0.5% ammonium sulfate (adjusted to pH 4.0 with HCl). Nitrogen-free medium is SD medium without ammonium sulfate. Galactose medium (SGal) is composed of YNB, 2% raffinose, 0.25% galactose, and 0.5% ammonium sulfate (adjusted to pH 4.0 with HCl). Casamino acids (Difco) were added

from a 10% stock (pH 4) to SD or SGal at a final concentration of 0.025% or 0.25%. Basic amino acid mix (BAM) is comprised of arginine, citrulline, and lysine (Sigma, St. Louis, MO) diluted from a stock solution of 100 mM each (pH 4).

For measurement of Gap1p recycling, strains expressing P_{GAL10}-*GAP1* or P_{GAL10}-*GAP1-GFP* were cultured ~16 hours in SGal to induce Gap1p expression. 0.25% Casamino acids were added for one hour to sequester Gap1p in the MVE. Cells were then filtered and suspended in SD with 0.25% Casamino acids and cultured for 1 hour to arrest P_{GAL10}-*GAP1* transcription and allow for trafficking of all protein through the early secretory pathway. The culture was then split, filtered, washed in an equal volume of nitrogen-free medium, and resuspended in either SD or SD + BAM (1 mM each of arginine, citrulline, and lysine) and cultured for an additional 30 minutes.

Amino Acid Uptake Assays

Approximately 2 x 10^7 cells (one OD_{600} unit) of cells in exponential phase were washed in nitrogen-free medium by filtration on 0.45 µM nitrocellulose filters before amino acid uptake was measured as described previously (Roberg et al., 1997). [^{14}C]-labeled alanine, phenylalanine, threonine, lysine, arginine, and glutamate were used at a specific activity of 125 mCi/mmol.

For estimation of the K_M for glycine transport through Gap1p^{K9R,K16R}, uptake was measured for [^{14}C]-glycine concentrations ranging from 1 µM to 80 µM at a specific activity of approximately 10 µCi/µmol. Inhibition of glycine transport by citrulline was measured by suspending cells in nitrogen-free medium supplemented with citrulline at concentrations ranging from 10 nM to 1 mM. K_M and IC_{50} values were calculated using

BioDataFit (Chang Biosciences, Castro Valley, CA). K_I values were calculated by relating K_M and IC_{50} values (Cheng and Prusoff, 1973).

Fluorescence microscopy

Cells in exponential phase were harvested, suspended in PBS with 10mM NaN_3 and visualized via fluorescence microscopy. Images were captured with a Nikon E800 microscope (Melville, NY) equipped with a Hamamatsu digital camera (Bridgewater, NJ) with 1x1 binning. Image analysis was performed with Improvision OpenLabs 2.0 software (Lexington, MA).

Equilibrium Density Centrifugation and Antibodies

Yeast cellular membranes were fractionated by equilibrium density centrifugation on continuous 20-60% sucrose gradients containing EDTA or $MgCl_2$ as described previously (Kaiser et al., 2002). Golgi-containing fractions were identified by measurement of GDPase activity. Plasma membrane fractions were identified by the presence of Pma1p. Gap1p-GFP and Pma1p were detected in membrane fractions by immunoblotting and imaging with the LI-COR Odyssey Infrared Imaging System using the following antibodies: mouse anti-GFP (Covance, Princeton, NJ), mouse anti-Pma1 (40B7; Abcam, Cambridge, MA), and IRDye 800CW goat anti-mouse (LI-COR, Lincoln, NE). Quantitation was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/).

Immunoprecipitation and Western blotting of Gap1p.

Detection of ubiquitin conjugated $Gap1p^{HA}$ was performed as described previously (Helliwell et al. 2001) with modifications. Cells were grown overnight in SD medium to exponential phase. Amino acids were then added for two hours where

indicated. Approximately 4.0 x 10^8 cells were collected by filtration, washed in 10mM NaN_3 and suspended in lysis buffer (0.3M sorbitol, 10mM Tris pH 7.5, 100mM NaCl, 5mM $MgCl_2$) with 50mM NEM, 1mM PMSF, and Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN). Cells were lysed by vortexing with glass beads, 100µl of 1X sample buffer (80 mM Tris pH 6.8, 10% glycerol, 2% SDS) was added, and lysates were incubated at 37˚C for 30 minutes. Lysates were then diluted in 1mL of IP buffer (50mM Tris pH 7.5, 150mM NaCl, 1% Triton) with 10mM NEM and protease inhibitors and centrifuged at 16,000 x g at 4˚C. $Gap1p^{HA}$ was immunoprecipitated overnight at 4˚C with 10µl rat anti-HA (3F10; Roche), followed by incubation for 90 minutes at 4˚C after addition of 50µl of a 50% suspension of Protein G-Sepharose 4 fast flow (GE Healthcare). Beads were washed three times in IP buffer, followed by two washes in PBS, and solublized in 50µl 2X sample buffer with 2% BME at 37˚C. Immunoprecipitates were resolved by 8% SDS-PAGE. Antibodies used were rabbit anti-Gap1p, mouse anti-*myc* (9E10; Santa Cruz Biotechnology, Santa Cruz, CA), and HRP-coupled sheep anti-rabbit and sheep anti-mouse (GE Healthcare).

Results

Gap1p^{A297V} is defective for transport of basic amino acids

We previously isolated Gap1p^{A297V} as a substrate specificity mutant that did not transport citrulline or arginine, but was able to transport glycine (Risinger et al., 2006). We further tested Gap1p^{A297V} for uptake of alanine, phenylalanine, threonine, glutamate, and lysine to further characterize its catalytic defect. A strain expressing *GAP1^{A297V}* showed the same impairment of transport of citrulline, arginine, and lysine as a *gap1*Δ strain (Figure 1A). Gap1p^{A297V} allowed normal transport of all other amino acids tested, although uptake of phenylalanine was decreased to approximately 50% of uptake observed for wild type Gap1p. Thus the transport defect for Gap1p^{A297V} appeared largely to affect amino acids with basic side chains. Notably, cells either containing Gap1p^{A297V} or *gap1*Δ cells were nevertheless able to transport significant amounts of lysine, and arginine. Transport assays in *can1*Δ (Risinger et al., 2006) and *lyp1*Δ cells showed that this residual transport activity was due to the activities of the basic amino acid permease (Can1p) and the lysine specific permease (Lyp1p) (data not shown).

The Ala^{297} residue, mutated in Gap1p^{A297V}, lies in the middle of TMD 6 and thus could alter the amino acid binding pocket so as to exclude binding of basic residues. To test for a possible defect in binding of basic amino acids, we tested whether citrulline could act as a competitive inhibitor of glycine transport for Gap1p^{A297V}. Competition assays employed either *GAP1^{K9R,K16R}* or *GAP1^{K9R,K16R,A297V}* expressed in a *gap1*Δ *can1*Δ strain, which allowed for maximum levels of the Gap1p at the plasma membrane. We first calculated the apparent K_M of glycine transport and found that Gap1p^{K9R,K16R} and Gap1p^{K9R,K16R,A297V} have similar K_M values for glycine: 20.0 ± 5.3 μM and 12.7 ± 1.12

μM respectively (Figure 1B). Although there appeared to be an approximately two-fold difference between the V_{max} for glycine uptake for Gap1p^{K9R,K16R} and Gap1p^{K9R,K16R,A297V} (1587 pmol/min and 850.7 pmol/min, respectively), this difference is probably not due to a significance in the specific activity and can most easily be explained by differences in the abundance of Gap1p at the plasma membrane in the two strains. We then tested the ability of unlabeled citrulline to act as a competitor for uptake of [^{14}C]-glycine. Citrulline could inhibit glycine uptake through both Gap1p^{K9R,K16R} and Gap1p^{K9R,K16R,A297V} (Figure 1, C and D). Using the Cheng-Prusoff equation (1973), we calculated K_I values for citrulline inhibition to be ~36.6 μM for Gap1p^{K9R,K16R} and ~35.6 μM for Gap1p^{K9R,K16R,A297V}. In parallel experiments using arginine as an inhibitor we found that the K_I for arginine inhibition of [^{14}C]-glycine transport by Gap1p^{K9R,K16R,A297V} was the same as for Gap1p^{K9R,K16R} (data not shown). Based on the ability of basic amino acids to compete for transport of glycine, basic amino acids appear to bind to the active site of Gap1p^{A297V} with the same affinity as for wild type Gap1p. Thus, we conclude that the Gap1p^{A297V} is blocked at a step of the transport cycle that takes place after binding of a basic amino acid. Perhaps Gap1p^{A297V} cannot undergo the conformational changes necessary to complete a transport cycle when a basic amino acid occupies the active site.

Previously we showed that reversible inactivation of intrinsic Gap1p activity at the cell surface requires active transport through Gap1p (Risinger et al., 2006). We therefore tested various amino acids and combinations of amino acids for their ability to inactivate Gap1p^{K9R,K16R,A297V} at the cell surface (Figure 2). Cells expressing Gap1p^{K9R,K16R,A297V} were cultured in either a basic amino acid mix (hereafter referred to as BAM) consisting of arginine, citrulline, and lysine at 1 mM each, and Casamino acids

(CAS), a mixture containing all amino acids except tryptophan, at 0.25% (approximately 10 mM total amino acid concentration). As expected, Gap1p^{K9R,K16R,A297V} was not inactivated by BAM (Figure 2A), but was inactivated by CAS to the same degree as Gap1p^{K9R,K16R} (Figure 2B). The failure of Gap1p^{K9R,K16R,A297V} to be inactivated at the cell surface by basic amino acids provides independent evidence that the Ala^{297} to Val mutation specifically blocks completion of a transport cycle for basic amino acid substrates.

Sorting of Gap1p into the MVE requires direct recognition of transport substrates

Amino acid regulation of the distribution of Gap1p between the plasma membrane and the vacuole is primarily determined by the ability of amino acids to inhibit recycling of Gap1p from the MVE to the plasma membrane (Rubio-Texeira and Kaiser, 2006). To assess the effects of Gap1p mutations on the response to amino acids, we designed a redistribution assay to measure the rate of traffic of Gap1p from the MVE to the plasma membrane. The idea was to accumulate Gap1p in the MVE by expressing Gap1p in the presence of a large excess of CAS, and then to shut off Gap1p synthesis for a period of time sufficient to clear newly synthesized Gap1p from the ER and Golgi compartments and then to follow the extent of redistribution of Gap1p from the MVE to the plasma membrane after removal of amino acids. An appropriately regulated form of Gap1p was constructed by placing *GAP1* under control of the *GAL10* promoter (P_{GAL10}-*GAP1*). Synthesis of P_{GAL10}-*GAP1* was induced on galactose medium supplemented with CAS. Transcription of Gap1p was stopped by incubation of cells in glucose medium with CAS for 1 hour, a time sufficient for any newly synthesized Gap1p to transit the early compartments of the secretory pathway and reach the MVE. At this time, CAS was

removed, and cells were placed into glucose medium with no amino acids (SD) or with basic amino acids (SD + BAM). The redistribution assay was performed in a *car1*Δ strain, which lacks arginase thus inhibiting conversion of arginine to glutamate and other amino acids (Sumrada and Cooper, 1982). To determine a maximum level of Gap1p reactivation after CAS removal, we followed the increase in glycine uptake over the 30 minutes after removal of amino acids (SD) and found that activity increases approximately tenfold. Cells shifted into SD with BAM did not increase significantly, showing that BAM was sufficient to prevent wild type Gap1p form redistributing to the plasma membrane (Figure 3A). In contrast to wild type, Gap1p^{A297V} showed efficient redistribution to the plasma membrane in either SD or SD + BAM. (Figure 3B). This crucial result showed that Gap1p^{A297V}, which has a specific defect in the transport of basic amino acids, also fails to be regulated by basic amino acids at the stage of amino acid regulated sorting in the MVE.

As a control, we asked whether disruption of post-Golgi secretory vesicle trafficking machinery could disrupt the redistribution of Gap1p^{A297V}. Previously we showed that redistribution of Gap1p observed in cells shifted from glutamate to urea as a nitrogen source requires the exocyst complex and is blocked in temperature sensitive mutants such as *sec6-4* (Roberg et al., 1997). We anticipated that the redistribution of active Gap1p^{A297V} to the plasma membrane in the presence of BAM would require vesicle trafficking and thus should be blocked in *sec6-4* at the restrictive temperature. Strains expressing Gap1p or Gap1p^{A297V} were cultured in duplicate as described above and recycling was measured at both the permissive temperature of 24˚C and the restrictive temperature of 36˚C. Prior to removal of CAS from the glucose medium, one

culture of each strain was shifted to 36˚C for 10 minutes, and then filtered into pre-warmed SD or SD with BAM at 36˚C. The control performed on Gap1p expressing cells confirmed previous results; only cells at 24˚C in SD showed reactivation (Figure 3C). Gap1p^{A297V} reactivated in both SD and SD with BAM at 24˚C, as observed in Figure 3B. However, Gap1p^{A297V} was not reactivated in SD or SD with BAM at 36˚C (Figure 3D). By comparison, Gap1p^{K9R,K16R}, which is constitutively present at the plasma membrane but reversibly inactivated by amino acids, increases in activity to 80% of maximum within 30 minutes of amino acid washout (Figure 3E). Thus we conclude that Gap1p redistributes from the MVE to the plasma membrane, and that this recycling relies on the same machinery required for vesicular trafficking between the *trans*-Golgi and the plasma membrane.

To visualize the redistrubution of Gap1p, we localized GFP-tagged Gap1p by fluorescent microscopy during the redistribution assay (Figure 4A). Prior to removal of CAS, both Gap1p-GFP and Gap1p^{A297V}-GFP were primarily located the vacuole as well as associated organelles (probably corresponding to the MVE). After removal of CAS, cells were cultured for 30 minutes before imaging. When transferred into medium that did not contain amino acids (SD), both Gap1p-GFP and Gap1p^{A297V}-GFP were observed at the plasma membrane, although an appreciable amount was associated with the vacuole, probably corresponding to the pool of Gap1p already in the vacuole or irreversibly committed to transport to the vacuole at the time of amino acid removal. When transferred to SD with BAM, Gap1p-GFP did not redistribute to the plasma membrane, but Gap1p^{A297V}-GFP did, to about the same extent as in amino acid free medium. These localization studies confirm the results of the activity assays that

Gap1p^{A297V} does not respond to the presence of basic amino acids in redistributing from the MVE to the plasma membrane.

We were concerned about the possibility that the diminished response of Gap1p^{A297V} to basic amino acids could be due to an indirect effect on intracellular amino acid pools resulting from a decreased capacity of Gap1p^{A297V} mutants to take up basic amino acids. To control for this possibility, we assayed accumulation of radio-labeled basic amino acids over the 30 minutes after CAS removal and found the total amino acid uptake for the Gap1p^{A297V} mutant is similar as for wild type, 17.2 nmoles/OD_{600} and 20.1 nmoles/OD_{600}(data not shown). This equivalence of Gap1p mutant and wild type for basic amino acid uptake can be explained by the presence of *CAN1* and *LYP1* permeases that provide robust transport of arginine and lysine independently of Gap1p. Moreover, the contribution of wild type Gap1p to amino acid uptake should be negligible since both wild type and Gap1p^{A297V} mutant strains have very little Gap1p permease activity after growth on CAS.

To show that the insensitivity of Gap1p^{A297V} mutant to sorting by basic amino acids is due to an intrinsic property of the mutant protein and not some unanticipated effect of the mutant on cellular amino acid pools, we performed a *cis/trans*-test, in which redistribution of GFP-tagged Gap1p or Gap1p^{A297V} was followed in heterozygotes also expressing untagged Gap1p^{A297V} or Gap1p, respectively. We observed that Gap1p-GFP did not reappear at the plasma membrane in SD+BAM even in cells expressing Gap1p^{A297V}, whereas Gap1p^{A297V}-GFP was recycled to the plasma membrane in the presence of SD+BAM even in cells also expressing wild type Gap1p (Figure 4B). Formally these results show that the behavior of the Ala^{297} to Val mutant, which is to

allow trafficking to the plasma membrane even in the presence of BAM, acts in *cis* but not in *trans*. This result shows that the mutation exerts its effect on the mutated Gap1p protein and not on the physiology of the whole cell.

*A catalytically inactive mutant displays reduced sensitivity to amino acid sorting***.**

To extend the results with the substrate specificity mutant Gap1p^{A297V}, we wished to identify a catalytically inactive mutant of Gap1p that could not transport any amino acids with the expectation that such a mutant may not be regulated by any amino acids. The aim was to identify a Gap1p mutant that was specifically defective for amino acid transport, but that could fold normally as judged by the ability of the mutant protein to pass the ER quality control checkpoint and be trafficked to the late secrteory pathway. To find rare transport-defective mutants that were also properly folded, we took a targeted mutagenesis approach based on sequence similarity of Gap1p to prokaryotic transporter proteins for which structural information was available. A common feature of these structures is a short, unwound section in both TMD1 and TMD6. Substrate-bound forms of LeuT (Yamashita et al., 2005; Singh et al., 2008) and AdiC (Gao et al., 2010) show these regions as part of the amino acid binding site. In AdiC, Gly^{25}-Ser^{26}-Gly^{27} makes up the unwound section of TMD1, and initial characterizations of AdiC showed that a Ser^{26} to Lys substitution blocked antiport activity (Gao et al., 2009). This GSG motif in AdiC aligns with a conserved GTG motif in five amino acid transporters of *S. cerevisiae*, including Gap1p (Gly^{105}-Thr^{106}-Gly^{107}).

To determine if this GTG motif in Gap1p serves a similar function as the GSG motif of AdiC, we generated the analogous mutation in Gap1p, Thr^{106}Lys. We tested amino acid uptake abilities of Gap1p^{T106K} and found that it was unable to import any

amino acid we tested (Figure 5A), supporting the hypothesis that the GTG motif of Gap1p is involved in amino acid binding. Importantly, localization of Gap1p^{T106K}-GFP revealed that the protein was expressed and localized to the plasma membrane normally. For comparison we show a typical randomly generated mutant, Gap1p^{T21S,C397R}-GFP which is also inactive for uptake but is primarily located in the ER presumably because of a folding defect (Figure 5B). We tested a range of citrulline and glycine concentrations from 4μM up to 1mM to determine if the loss of amino acid uptake ability of Gap1p^{T106K} was due to an increase in K_M, but did not observe uptake of either citrulline or glycine even at the highest substrate concentrations (data not shown). However, we cannot rule out the possibility that Gap1p^{T106K} may have K_M for transport of some amino acids in the millimolar range.

Next we tested how sorting of Gap1p^{T106K} to the plasma membrane would respond to a complex mixture of amino acids. Because we were interested in assessing direct sorting to the vacuole, we used an *end3*Δ mutant to block endocytosis of Gap1p from the plasma membrane (Tang et al., 1997). In an *end3*Δ mutant any Gap1p present in the vacuole must have reached that location by direct sorting via the *trans*-Golgi and MVE, not by endocytosis from the plasma membrane. We expressed P_{ADH1}-*GAP1*-*GFP* or P_{ADH1}-*GAP1*^{T106K}-*GFP* in a *gap1*Δ *end3*Δ strain cultured in SD medium. CAS was then added to half of each culture at a final concentration of 0.025% (corresponding to a total amino acid concentration of 1– 2 mM), an amount in about ten-fold excess of the minimum concentration needed to signal direct sorting of Gap1p to the vacuole (Risinger et al., 2006). As expected, wild type Gap1p-GFP was located primarily at the plasma membrane when grown in SD, but was located primarily in the vacuole after 3 hours

growth in SD + 0.025% CAS (Figure 7A). A small amount of Gap1p-GFP was seen at the plasma membrane in some cells, presumably corresponding to Gap1p-GFP that was localized to the plasma membrane prior to the addition of CAS and was trapped there because of *end3*Δ. In contrast, Gap1p^{T106K} was located primarily in the plasma membrane when cells were grown in either SD or SD + 0.025% CAS (Figure 6A).

Because Gap1p^{T106K} is catalytically inactive we could not use activity assays to confirm the localization of this protein to the plasma membrane. Instead we used density gradients to fractionate plasma membrane from Golgi and MVE compartments. Membranes from cells expressing P_{ADH1}-*GAP1*-*GFP* or P_{ADH1}-*GAP1*^{T106K}-*GFP* grown in either SD or SD + 0.025% CAS were separated over a continuous 20-60% sucrose gradient in the presence of EDTA. Under these conditions, the plasma membrane, identified by the marker Pma1p, fractionates at a considerably higher density than intracellular membranes, including the Golgi (identified by GDPase activity), MVE and ER (Figure S1). After growth in SD, Gap1p-GFP was present both in the plasma membrane and in internal fractions in approximately equal amounts. However, after three hours growth in SD + 0.025% CAS, the majority of Gap1p-GFP fractionated in internal membranes, with only a small amount still seen at the plasma membrane (Figure 6B and S1A). The pool of Gap1p at the plasma membrane in CAS probably corresponds to Gap1p that was sorted to the plasma membrane prior to the addition of CAS and was prevented from reaching the vacuole by endocytosis due to the *end3*Δ mutation. Gap1p^{T106K}-GFP is primarily observed at the plasma membrane both in SD and SD with amino acids, with more than 70% co-fractionating with Pma1p (Figure 6C and S1B).

We considered the possibility that Gap1p^{T106K} is inefficiently sorted to the vacuole in CAS because lower levels of amino acid import than wild type cells. Therefore, we also performed fluorescence microscopy on Gap1p^{T106K}-GFP in an *end3*Δ strain also expressing wild type Gap1p (expressed constitutively from P_{ADH1}-*GAP1* promoter fusion). Even in the presence of wild type Gap1p, the majority of Gap1p^{T106K}-GFP was located at the plasma membrane (Figure S2). Taken together, these fluorescence microscopy and membrane fractionation experiments show that the catalytically inactive mutant Gap1p^{T106K} displays a reduced sensitivity to the amino acid signal for sorting to the vacuole.

Discussion

Through the use of mutants that alter the substrate specificity or activity of Gap1p we have shown that a given amino acid must be a transport substrate for Gap1p in order to signal for the inhibition of Gap1p recycling to the plasma membrane. Based on the observation that citrulline and arginine can competitively inhibit glycine uptake through Gap1p^{A297V}, the Ala^{297} to Val substitution blocks the translocation, not the binding, of basic amino acids. Therefore, binding of Gap1p to its substrate is not sufficient to signal sorting. Rather, a transport cycle is required. This sets the regulation of Gap1p apart from the many examples of plasma membrane proteins that are down regulated in response to the binding of their substrates, such as the manganese transporter Smf1p (Liu and Culotta, 1999; Jensen et al., 2009) and the uracil permease Fur4p (Seron et al., 1999; Blondel et al., 2004).

We were not able to perform similar mechanistic tests on Gap1p^{T106K}, as the complete lack of amino acid transport ability prevented measurement of amino acid binding by competition experiments. However, existing structural data offers clues to the defect of Gap1p^{T106K}. In the recently solved crystal structure of AdiC bound to arginine, Ser^{26} is shown forming a hydrogen bond with the α-carboxylate of arginine (Gao et al., 2010). As Ser^{26} of AdiC corresponds to Thr^{106} of Gap1p, it is likely that the Thr^{106} to Lys substitution disrupts amino acid binding, presumably preventing it from undergoing the transport cycle. It is possible that the K_M of amino acid binding to Gap1p^{T106K} is sufficiently large that our assays have not been able to measure it, which could explain why a small amount of vacuolar fluorescence is observed in Gap1p^{T106K}-GFP cells grown in CAS. Nonetheless, it appears that in the absence of amino acid binding or amino acid

translocation, Gap1p is unable to be retained at the MVE. Substrate transport and regulation of intracellular trafficking are therefore tightly linked.

The alternate access model for APC transporter function (Jardetzky, 1966), depicted in Figure 7, provides a useful scheme for thinking about how substrate transport and trafficking fate might be coupled. As Gap1p alternates between extracellular and cytoplasmic-facing states at the plasma membrane to facilitate transport, it may similarly alternate between exoplasmic and cytoplasmic facing states on the MVE membrane to signal for either recycling or retention, respectively. Although the kinetics of the transport cycle of Gap1p have not been determined, if isomerization of empty Gap1p from the cytoplasmic state to the exoplasmic state (CF to EF; step 4) is significantly slower than substrate translocation (EB to CB; step 2), an increase in amino acids on the exoplasmic face should lead to an abundance of the cytoplasmic oriented state (CF or CB) relative to the exoplasmic oriented state (EF or EB). This idea, combined with the observation that a mutant unable to perform step 2 is efficiently recycled from the MVE, suggests the following model: when Gap1p assumes the cytoplasmic oriented state, it is blocked from recycling, leading to its internalization into intralumenal vesicles (ILV) and delivery to the vacuole. Conversely, when amino acids are scarce, the majority of Gap1p present on the MVE membrane would be in the exoplasmic oriented state, which would be competent for recycling and sorting to the plasma membrane.

We can imagine two ways in which the conformational state of Gap1p could affect the propensity to recycle from the MVE membrane. The first is that the cytoplasmic oriented state could more efficiently interact with the ESCRT complex, increasing the efficiency of ILV formation. Because internalization into ILV requires the

recognition of ubiquitinated cargo (Urbanowski and Piper, 2001), the cytoplasmic state could make the ubiquitinated N-terminus of Gap1p more accessible to ESCRT. Gap1p in the EF or EB conformations would therefore be left on the MVE membrane until it is recycled to the Golgi by a passive process. Alternatively, the exoplasmically oriented state could more efficiently interact with the as yet undefined components necessary for recycling, leaving the cytoplasmically oriented state on the MVE membrane to be internalized into ILV by ESCRT. Although we did not observe a noticeable defect in ubiquitination of Gap1p^{A297V} or Gap1p^{T106K} in the presence of BAM or CAS respectively (Figure S3), we cannot rule out the former possibility, as ubiquitin recognition by ESCRT could also be affected by deubiquitination of Gap1p on the MVE membrane.

Because the intracellular sorting step governed by amino acid abundance takes place in the membrane of the MVE, Gap1p is likely sensing amino acids at that location. The signal for Gap1p retention may not be amino acid abundance per se, but rather a gradient of amino acids across the MVE membrane. This idea agrees with the observation that a signal of amino acid abundance causing Gap1p to be sorted to the vacuole can be produced by either exogenous addition of amino acids to the growth medium or regulatory mutant such as *mks1*Δ, which causes endogenous overproduction of amino acids (Chen and Kaiser, 2002). In either case we would expect that when amino acids are abundant, Gap1p fails to recycle from the MVE to the plasma membrane because of an amino acid gradient across the MVE membrane caused by amino acids concentrated in the lumen of the MVE.

Although it has not been directly demonstrated that the MVE, like the vacuole, is an acidic organelle capable of storing amino acids, indirect evidence such as the

capability of proteinase A with an acidic pH optimum to process pro-CPY in the MVE (Piper et al., 1995; Rieder et al., 1996), suggests that the MVE is acidic. Further, because import of amino acids into the vacuole occurs through the action of proton-driven amino acid transporters (Li and Kane, 2009), the acidity of the MVE may similarly facilitate storage of amino acids. Notably, basic amino acids, as we have used here to cause internal sorting of Gap1p, are highly represented in the vacuolar lumen (Messenguy et al., 1980), and may therefore be particularly effective in control of Gap1p sorting. We would therefore predict that disruption of the amino acid gradient would lead to mis-sorting of Gap1p to the plasma membrane even under conditions of amino acid abundance. Future work will explore possible mechanisms for establishment of such an amino acid gradient.

The model we propose echoes that of the dual function amino acid transporter/receptor, or "transceptor" (Hundal and Taylor, 2009). An example of transceptor function is that of Ssy1p, the transporter-like amino acid sensor component of the SPS complex. Although Ssy1p resembles APC transporters, it cannot translocate amino acids across the plasma membrane. Rather, binding of extracellular leucine to Ssy1p has been proposed to induce a conformational change that activates the SPS sensing response, leading to induction of permease gene expression. Because Ssy1p cannot shift from EB to CB (Figure 7, step 2), an increase in extracellular amino acids is thought to drive Ssy1p into an exoplasmically-oriented conformation by mass action. This model is supported by experiments in which overproduction of intracellular leucine inhibits the response of Ssy1p to extracellular leucine, suggesting that the intracellular leucine can drive Ssy1p in a cytoplasmically oriented, non-signaling conformation (Wu et al., 2006; Poulsen et al., 2008). Gap1p has also been proposed to function similarly as a

tranceptor at the plasma membrane to activate PKA signaling. Because PKA pathway activation requires substrate binding by Gap1p, but not the completion of a transport cycle, Gap1p is thought to assume a unique signaling conformation distinct from the four transport cycle conformations (Donaton et al., 2003; Van Zeebroeck et al., 2009).

We propose that the examples of Ssy1p and Gap1p represent two fundamentally different mechanisms by which a transporter-like protein can signal by switching its conformational state in response to a substrate concentration gradient, the "tranceptor" type and the "transport-coupled" type. In the case of Ssy1p, which cannot execute an amino acid translocation step, a high concentration of amino acids on the exoplasmic side of the membrane is thought to drive Ssy1p into an exoplasmically-oriented conformation, which signals amino acid abundance. In contrast, as we have shown here, intracellular sorting of Gap1p requires an amino acid translocation step in order to respond to the amino acid signal. It is therefore likely that Gap1p is driven into a cytoplasmically oriented conformation, which is the signal for amino acid abundance. For Ssy1p the conformational switch activates a signal transduction pathway, whereas a conformational switch in Gap1p regulates the intracellular sorting of Gap1p in the MVE. For both signaling mechanisms, it is of great interest to understand the nature of the different conformational states and how these states are recognized.

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Tables

Table 1: Strains

Strain	Genotype	Source
CKY482	*MATα leu2-3 ura3-52 gap1::LEU2*	Kaiser strain collection
CKY701	*MATα leu2-3 ura3-52 gap1::LEU2 bul1::kanMX6 bul2::kanMX6*	Kaiser strain collection
CKY703	*MATa leu2-3 ura3-52 his3 gap1::LEU2*	Kaiser strain collection
CKY1025	*MATa leu2-3 ura3-52 gap1::LEU2 can1*	Kaiser strain collection
CKY1080	*MATa leu2 ura3-52 gap1::LEU2 car1::kanMX6 GAL2*	This study
CKY1081	*MATa ura3-52 gap1::LEU2 car1::kanMX6 sec6-4 GAL2*	This study
CKY1082	*MATα ura3-52 leu2 gap1::LEU2 end3::kanMX6*	This study
CKY1085	*MATa ura3-52 leu2,3 his3 gap1::LEU2 car1::KanMX*	This study

Table 2: Plasmids

Name	Description	Markers	Source
pAR1	P_{ADH1}-*HA-GAP1*^{K9R,K16R}	*CEN, URA3*	Risinger et al. (2006)
pAR13	P_{ADH1}-*GAP1-GFP*	*CEN, URA3*	Risinger et al. (2006)
pAR77	P_{ADH1}-*GAP1*^{K9R,K16R}	*CEN, URA3*	This study
pCK227	P_{ADH1}-*HA-GAP1*	*CEN, URA3*	Chen and Kaiser (2002)
pCK231	P_{CUP1}-*UB1-c-myc*	2μ, *HIS3*	Helliwell et al. (2001)
pEC221	P_{ADH1}-*GAP1*	*CEN, URA3*	Risinger et al. (2006)
pNC7	P_{ADH1}-*GAP1*^{A297V}	*CEN, URA3*	Risinger et al. (2006)
pNC9	P_{ADH1}-*GAP1*^{K9R,K16R,A297V}	*CEN, URA3*	This study
pNC15	P_{ADH1}-*HA-GAP1*^{A297V}	*CEN, URA3*	This study
pNC26	P_{GAL10}-*GAP1*	*CEN, URA3*	This study
pNC27	P_{GAL10}-*GAP1*^{A297V}	*CEN, URA3*	This study
pNC29	P_{GAL10}-*GAP1-GFP*	*CEN, URA3*	This study
pNC30	P_{GAL10}-*GAP1*^{A297V}-*GFP*	*CEN, URA3*	This study
pNC69	P_{GAL10}-*GAP1*^{K9R,K16R}	*CEN, URA3*	This study
pNC74	P_{ADH1}-*GAP1*^{T106K}	*CEN, URA3*	This study
pNC84	P_{ADH1}-*GAP1*^{T106K}-*GFP*	*CEN, URA3*	This study
pNC113	P_{ADH1}-*GAP1*^{T21S,C397R}-*GFP*	*CEN, URA3*	This study
pNC115	P_{ADH1}-*HA-GAP1*^{T106K}	*CEN, URA3*	This study
pNC119	P_{GAL10}-*GAP1-GFP*, P_{GAL1}-*GAP1*^{A297V}	*CEN, URA3*	This study
pNC120	P_{GAL10}-*GAP1*^{A297V}-*GFP*, P_{GAL1}-*GAP1*	*CEN, URA3*	This study

Figures

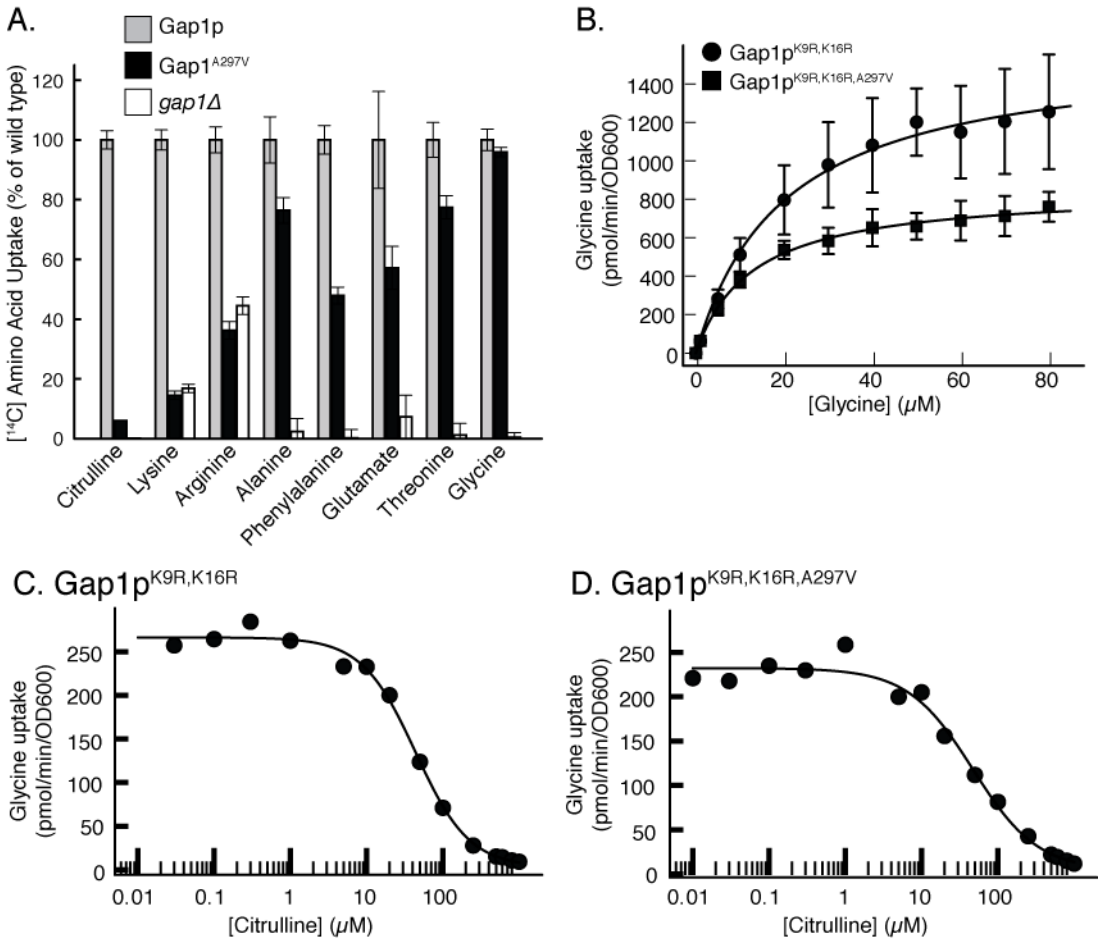

Figure 1. A Gap1p mutation, which specifically blocks the uptake of positively charged amino acids. **(A)** Initial rate of uptake for [^{14}C]-amino acids were determined for wild type *GAP1* (pEC221), *gap1*^{A297V} (pNC7), or empty vector (pRS316), expressed in a *gap1*Δ *bul1*Δ *bul2*Δ (CKY701) strain grown in SD medium. Mean uptake rates (n = 3) were normalized to the wild type rate for each amino acid. (B) Kinetic parameters for [^{14}C]-glycine uptake were determined for *gap1*Δ *can1* strains expressing P_{ADH1}-*GAP1*^{K9R,K16R} or P_{ADH1}-*GAP1*^{K9R,K16R,A297V}. The mean (± SEM; n = 3) initial velocity of uptake was measured as a function of [^{14}C]-glycine concentration. Fits to the Michaelis-Menten equation revealed similar K_M values: K_M = 20.0 ± 5.3 μM for Gap1p^{K9R,K16R} and K_M of 12.7 ± 1.1 μM for Gap1p^{K9R,K15R,A297V}. (C, D) Similarly Kinetic parameters for citrulline inhibition of [^{14}C]-glycine uptake were determined for Gap1p^{K9R,K16R} and Gap1p^{K9R,K15R,A297V}. Initial velocity of uptake was measured for 4 μM [^{14}C]-glycine in the presence of varying concentrations of unlabeled citrulline. Fits to a four parameter sigmoidal equation gave similar IC50 values: IC50 = 30.9 μM for Gap1p^{K9R,K16R} and IC50=27.0 μM for Gap1p^{K9R,K16R,A297V}.

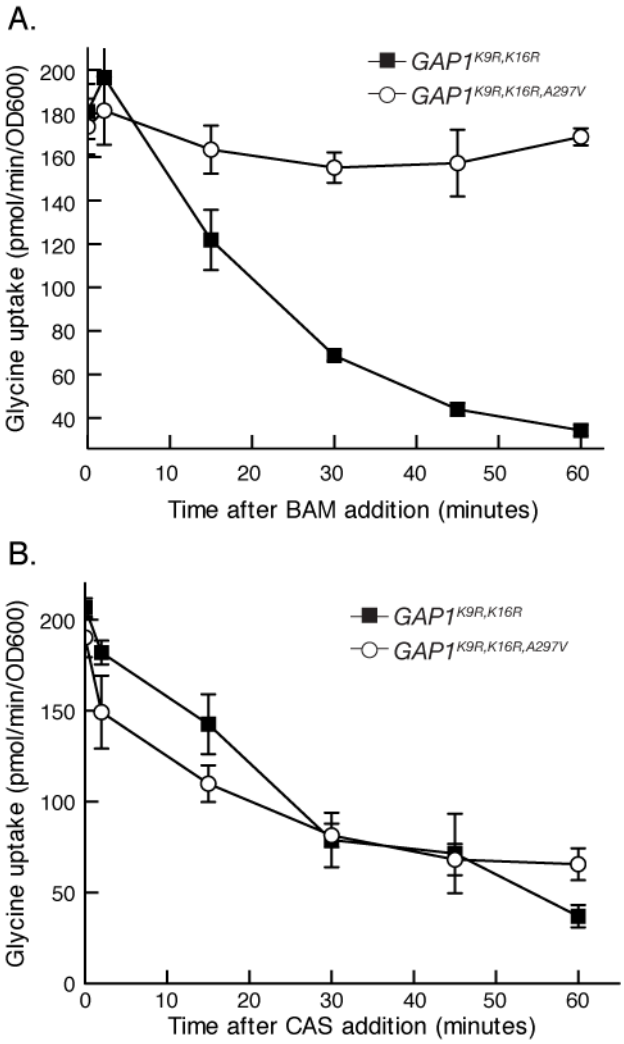

Figure 2. Inactivation of Gap1p at the plasma membrane requires transport of amino acid substrates. A *gap1*Δ strain (CKY482) expressing either $GAP1^{K9R,K16R}$ (pAR77) or $GAP1^{K9R,K16R,A297V}$ (pNC9) was cultured to exponential phase before addition of (A) BAM mix or (B) CAS mix. The mean (± SEM; n = 3) initial velocity of [^{14}C]-glycine showed that $Gap1p^{K9R,K16R,A297V}$ is inactivated by CAS but not by BAM.

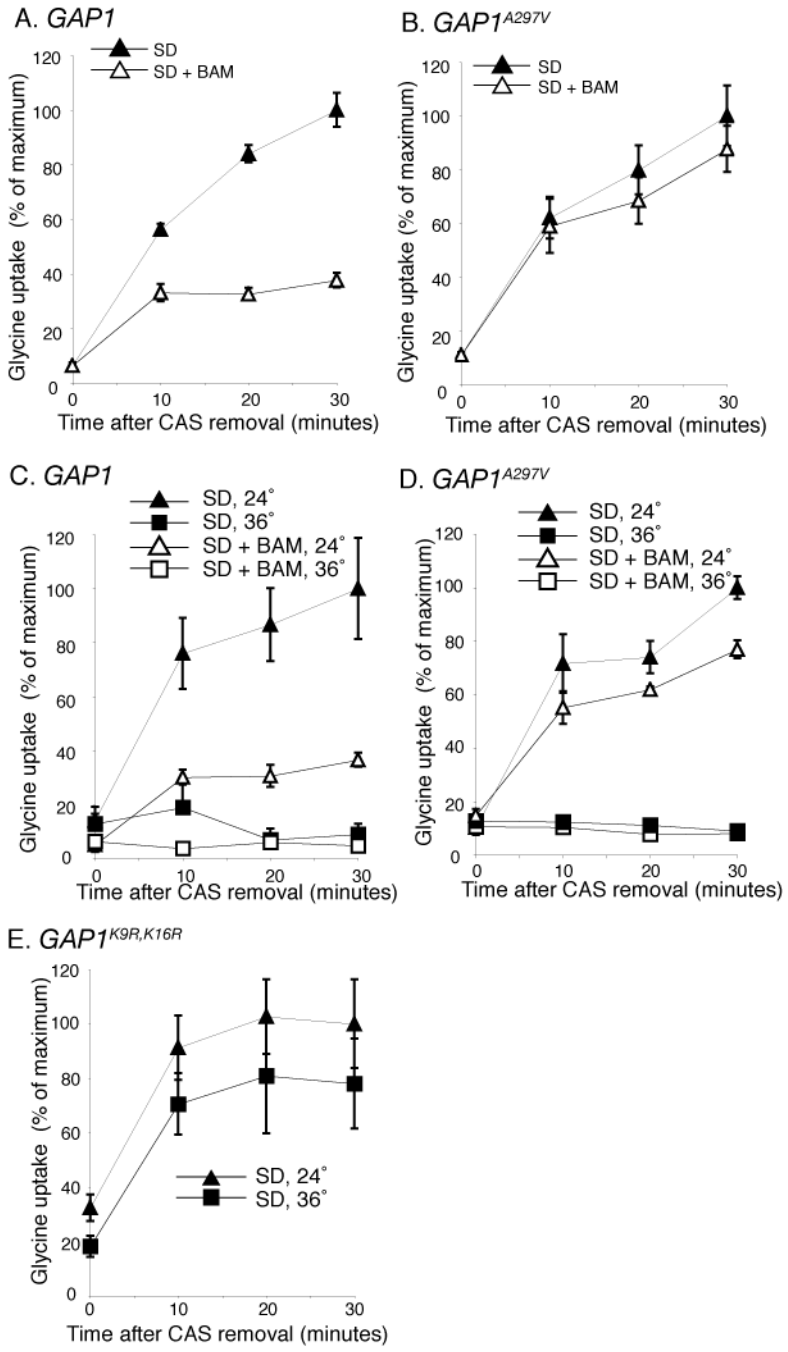
A. GAP1
SD
SD + BAM
Glycine uptake (% of maximum)
Time after CAS removal (minutes)
B. GAP1A297V
SD
SD + BAM
Glycine uptake (% of maximum)
Time after CAS removal (minutes)
C. GAP1
SD, 24°
SD, 36°
SD + BAM, 24°
SD + BAM, 36°
Glycine uptake (% of maximum)
Time after CAS removal (minutes)
D. GAP1A297V
SD, 24°
SD, 36°
SD + BAM, 24°
SD + BAM, 36°
Glycine uptake (% of maximum)
Time after CAS removal (minutes)
E. GAP1K9R,K16R
SD, 24°
SD, 36°
Glycine uptake (% of maximum)
Time after CAS removal (minutes)

Figure 3. Recycling of Gap1p^{A297V} to the plasma membrane is not blocked by BAM. Gap1p expressed from the *GAL10* promoter was accumulated in the MVE during growth on SGal medium + CAS. After shutoff of new Gap1p expression with glucose and removal of CAS, recycling of preexisting Gap1p to the plasma membrane was measured by initial rate of [^{14}C]-glycine uptake (± SEM; n =5). (A) Wild type Gap1p was recycled efficiently in the absence of CAS, but was blocked in the presence of BAM, whereas (B) Gap1p^{A297V} was recycled efficiently in the presence of BAM. (C-E) The recycling assay was performed in a *gap1*Δ *car1*Δ *sec6-4* strain expressing (C) P_{GAL10}-*GAP1* (pNC26), (D) P_{GAL10}-*GAP1*^{A297V} (pNC27), or (E) P_{GAL10}-*GAP1*^{K9R,K16R}. Where indicated the temperature sensitive *sec6-4* defect was imposed by shifting cells to 36˚ for 10 minutes while in SD + CAS before transfer into media pre-warmed to 36˚C. The mean initial rate of [^{14}C]-glycine uptake (± SEM; n =5) is shown.

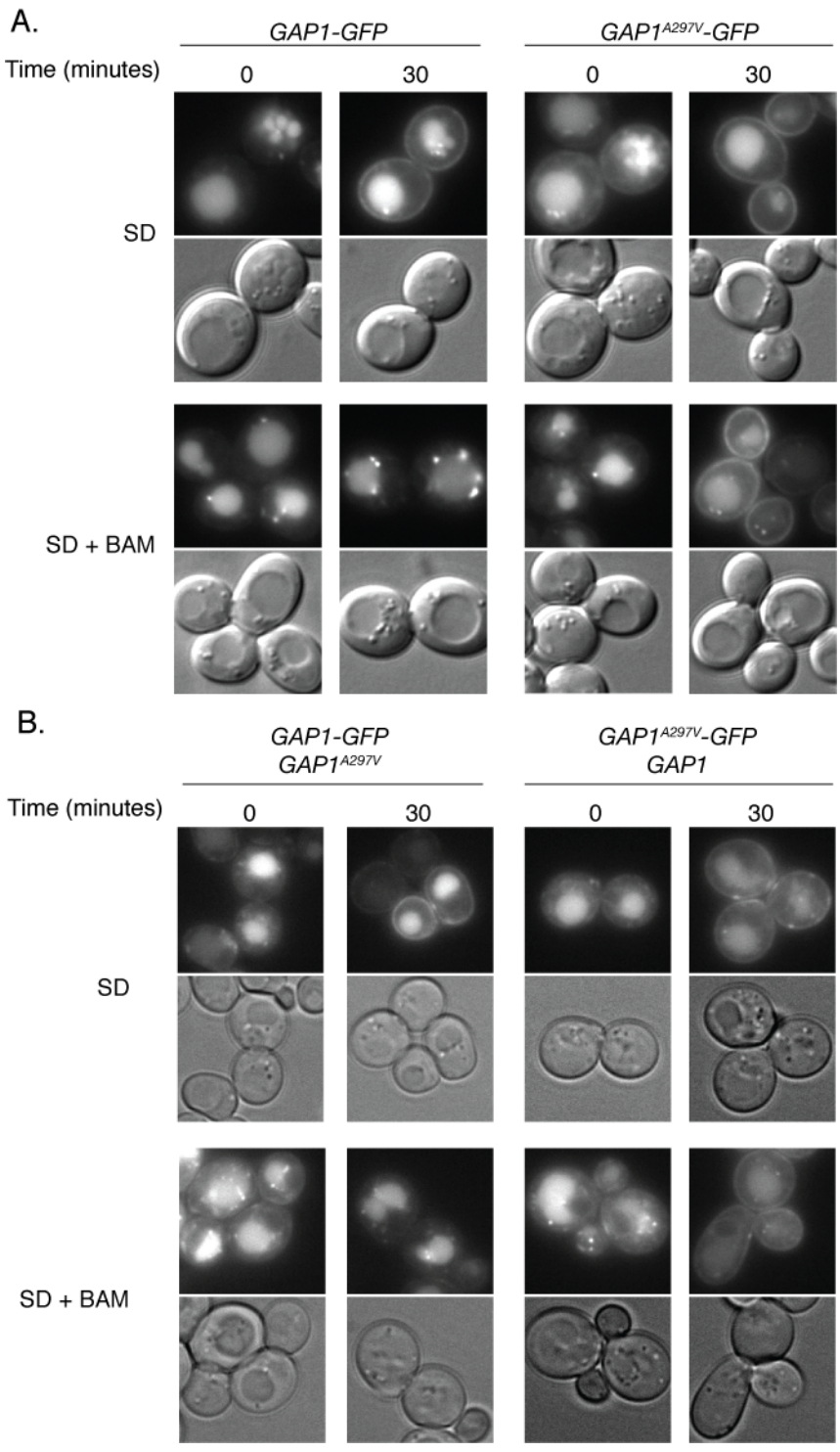
A.
GAP1-GFP
GAP1A297V-GFP
Time (minutes)
0
30
0
30
SD
SD + BAM
B.
GAP1-GFP
GAP1A297V
GAP1A297V-GFP
GAP1
Time (minutes)
0
30
0
30
SD
SD + BAM

Figure 4. Redistribution of Gap1p^{A297V} from the MVE to the plasma membrane in the presence of BAM. (A) *gap1*Δ *car1*Δ cells expressing P_{GAL10}-*GAP1*-*GFP* (pNC29) or P_{GAL10}-*GAP1*^{A297V}-*GFP* (pNC30) were cultured to early exponential phase in SGal medium + CAS before glucose shutoff, and CAS removal. (B) *gap1*Δ *car1*Δ cells expressing P_{GAL10}-*GAP1*-*GFP* with P_{GAL1}-*GAP1*^{A297V} (pNC119) or P_{GAL10}-*GAP1*^{A297V}-*GFP* with P_{GAL1}-*GAP1* (pNC120) were treated as in (A). (A and B) Samples were collected immediately prior to and 30 minutes post CAS removal. Gap1p-GFP was imaged by fluorescence microscopy.

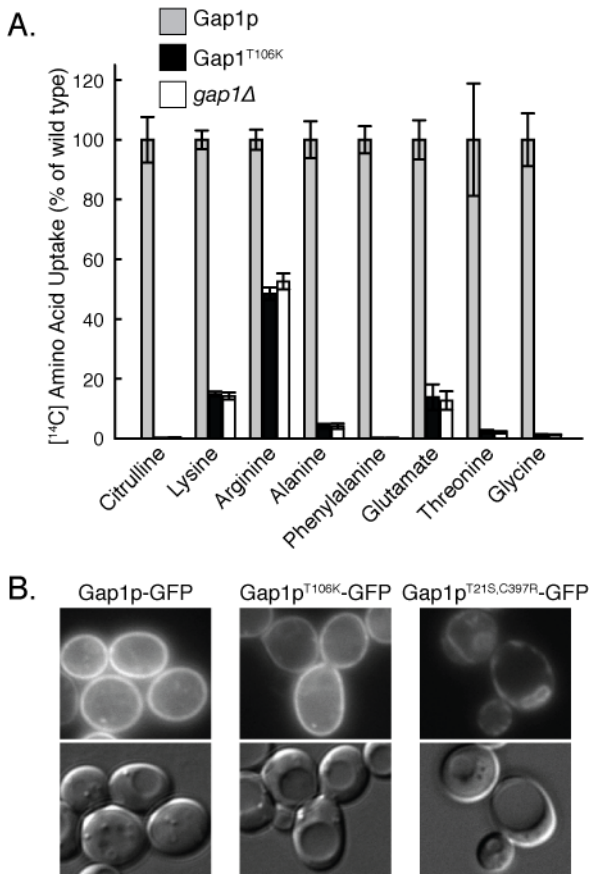

Figure 5. A mutation in Gap1p with a general defect in amino acid transport. (A) *ADH1*-promoted wild type *GAP1* (pEC221), Gap1^{T106K} (pNC74), or empty vector (pRS316) were expressed in a *gap1*Δ *bul1*Δ *bul2*Δ (CKY701) strain grown in SD medium. Initial rate of uptake was determined for the [^{14}C]-amino acids indicated. Mean uptake rates (n = 3) were normalized to the wild type rate for each amino acid. (B) P_{ADH1}-*GAP1*-*GFP* (pAR13), P_{ADH1}-*GAP1*^{T106K}-*GFP* (pNC84), or P_{ADH1}-*GAP1*^{T21S,C397R} were expressed in CKY701 cells grown in SD medium. Gap1p-GFP was imaged by fluorescence microscopy.

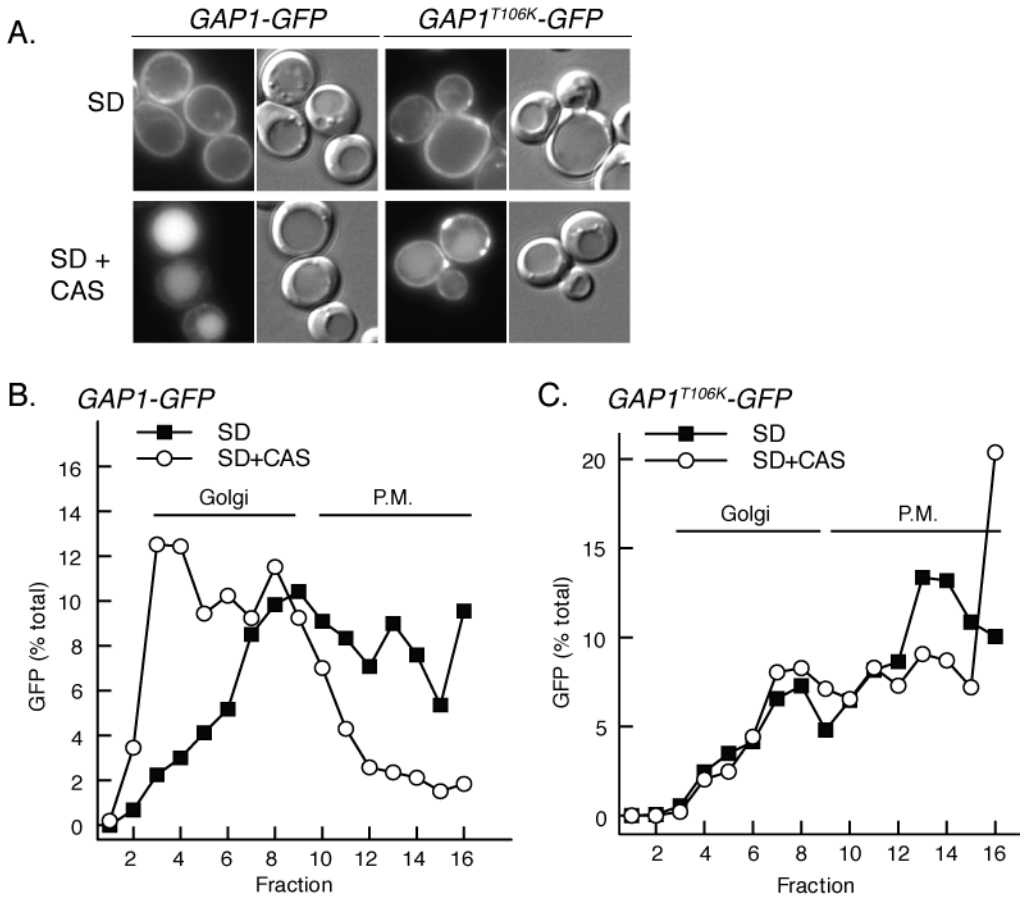

Figure 6. Sorting of Gap1p^{T106K}-GFP to the vacuole does not respond correctly to amino acid abundance. P_{ADH1}-*GAP1-GFP* or P_{ADH1}-*GAP1*^{T106K}-*GFP* were expressed in *gap1*Δ *end3*Δ cells and grown in SD. Where indicated, CAS was added for 3 hours. (A) Gap1p-GFP was imaged by fluorescence microscopy. (B, C) Quantitation of cellular distribution of Gap1p-GFP and Gap1pT106K-GFP measured by cell fractionation. Lysates were prepared and fractionated over a continuous 20 - 60% sucrose gradient with 10mM EDTA. Proteins were separated by SDS-PAGE and immunoblotted with anti-GFP or or anti-Pma1p. Golgi fractions were identified by GDPase activity. Gel images and GDPase activity graphs are shown in Supplemental Figure 1.

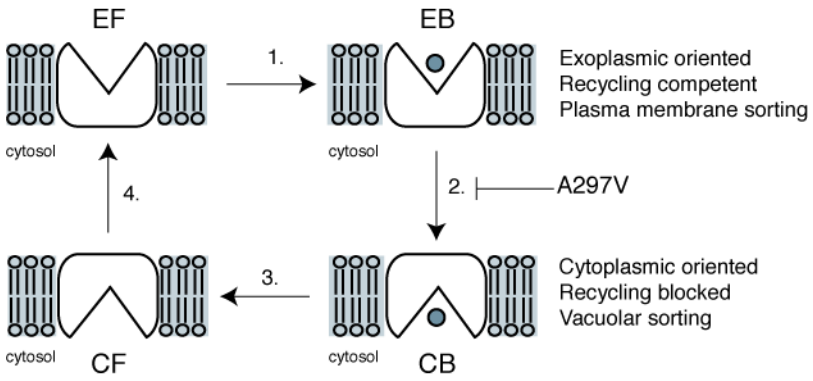

Figure 7. The alternate access model for substrate transport showing how the conformational state may switch in response to amino abundance. During the transport cycle, Gap1p assumes four different states: exoplasmically oriented free (EF), exoplasmically oriented bound (EB), cytoplasmically oriented bound (CB), and cytoplasmically oriented free (CF). In state EF or EB, Gap1p would be competent to recycle from the MVE to the plasma membrane. In CF or CB, Gap1p would be prevented from recycling and would enter ILVs destined for the vacuole. The Ala^{297} to Val mutation in Gap1p prevents the transition from EB to CB (step 2) for basic amino acids, preventing Gap1p from switching to cytoplasmically oriented thus preventing vacuolar trafficking of Gap1p in response to basic amino acids.

Supplementary Figures

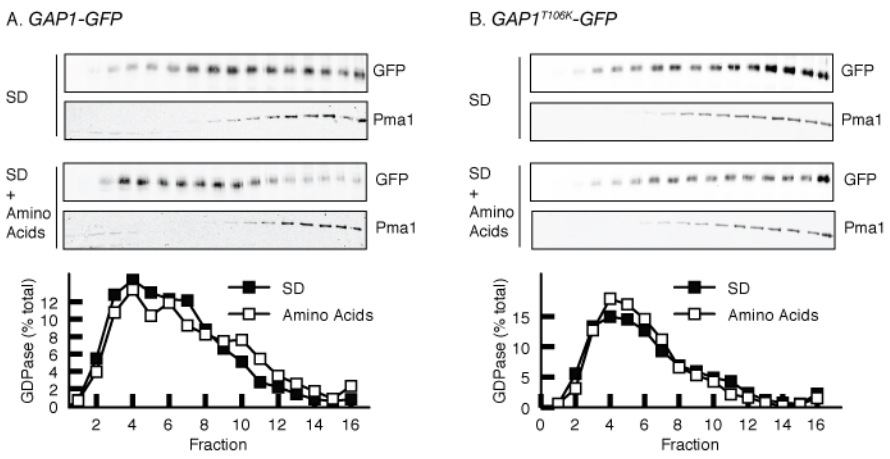

Figure S1. Equlibrium density sedimentation of Gap1p-GFP and Gap1p^{T106K}-GFP. Lysates were prepared and fractionated over a continuous 20 - 60% sucrose gradient with 10mM EDTA. Sixteen fractions were collected from the top of the gradient. Proteins were separated by SDS-PAGE and immunoblotted with monoclonal GFP antibody or monoclonal Pma1 antibody. Golgi fractions were identified by GDPase activity.

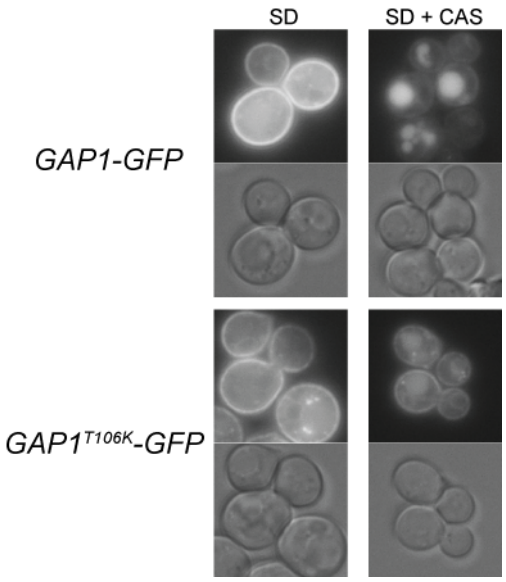

Figure S2. Sorting of Gap1p^{T106K}-GFP is less responsive to amino acids. *P_{ADH1}-GAP1-GFP* or *P_{ADH1}-GAP1^{T106K}-GFP* were expressed in *P_{ADH1}-GAP1 end3*Δ cells and grown in SD. Where indicated, CAS was added for 3 hours. Gap1p-GFP was imaged by fluorescence microscopy.

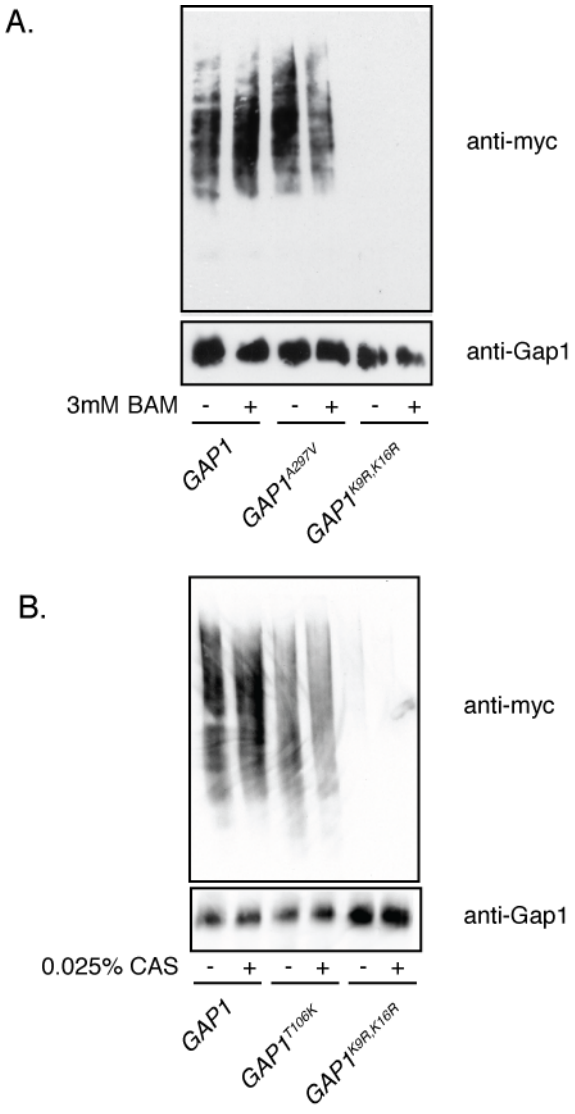

Figure S3. Gap1p^{A297V} and Gap1p^{T106K} are polyubiquitinated. HA-tagged *GAP1* constructs were transformed into (A) *gap1*Δ *car1*Δ (CKY1085) or (B) *gap1*Δ (CKY703) cells with P_{CUP1}-*UB1-myc* (pCK231) and cultured to early exponential phase in SD with 1μM $CuSO_4$. 3mM BAM or 0.025% casamino acids were added for 2 hours where indicated. Gap1p-containing plasmids used were pCK227 (P_{ADH1}-*HA-GAP1*), pAR1 (P_{ADH1}-*HA-GAP1*^{K9R,K16R}), pNC15 (P_{ADH1}-*HA-GAP1*^{A297V}), and pNC115 (P_{ADH1}-*HA-GAP1*^{T106K}). Gap1p was immunoprecipitated with rat anti-HA (3F10) antibody and immunoblotted with rabbit anti-Gap1p antibody. Ubiquitin conjugates were detected by anti-myc (9E10) antibody.

Chapter 3

Post-translational Regulation of the Yeast Histidine Permease

Abstract

Post-translational regulation of Gap1p occurs at the level of the individual protein, as Gap1p is directly influenced by amino acids. It was possible, then that other permeases could be subject to the same regulation when separated from transcriptional control. Alternatively, Gap1p could be the only permease that is regulated in this extensive way. Here we show that the histidine specific transporter Hip1p is repressed specifically by histidine addition. To determine the extent of this regulation, we adapted assays used to study Gap1p regulation for a constitutively expressed Hip1p construct. We found that Hip1p was not reversibly inactivated at the cell surface, nor was Hip1p competent to recycle from internal compartments after direction to the vacuolar protein sorting (VPS) pathway. Histidine-stimulated endocytosis is likely the main post-translational method of regulation that controls Hip1p activity. Gap1p therefore has a unique role in the cell to be rapidly mobilized in starvation conditions and strongly repressed in nutrient rich conditions.

Introduction

Saccharomyces cerevisiae expresses 24 amino acid transporters that are members of the amino acids/polyamines/organocation (APC) superfamily. The majority of these are high affinity, low capacity transporters that provide the cell with amino acids for protein synthesis. Transporters of this class are either constitutively expressed, or are induced by amino acid activation of the SPS amino acid sensor (Iraqui et al., 1999; Forsberg and Ljungdahl, 2001). By contrast, the general amino acid permease Gap1p activity is induced when amino acids are limiting, but is down-regulated when amino acid levels are high (Grenson et al., 1970; Chen and Kaiser, 2002). Downregulation of Gap1p in amino acid abundant conditions serves a crucial role in cell viability, as unregulated amino acid uptake is toxic (Risinger et al., 2006).

We have shown that Gap1p is specifically regulated by substrates and have suggested that amino acids induce a conformational change in Gap1p that favors vacuolar sorting over plasma membrane sorting (Chapter 2). Because this regulation is apparently occurring at the level of the individual protein, we wondered if this regulation extends to other transporters besides Gap1p. In pursuit of this question, we focused on the histidine specific permease Hip1p (Tanaka and Fink, 1985). Gap1p and Hip1p show strong sequence similarity, despite their differences in substrate specificity (Figure 1). Our first goal was to use this similarity to generate Gap1p-Hip1p chimeras, in which the N and C termini of Gap1p were fused to the membrane-spanning region of Hip1p. While we were not able to construct functional chimeras, results of control experiments suggested that Hip1p might be regulated specifically by histidine.

In this chapter, we explore post-translational effects of histidine on Hip1p. We find that, although histidine likely stimulates endocytosis of Hip1p from the cell surface,

we see no evidence of the additional regulatory steps that we have previously described for Gap1p. Therefore it appears that post-translational regulation of Gap1p is unique and does not apply to other yeast transporters.

Materials and methods

Strains, plasmids, and media

Strains used in this study were NCY33 (*gap1::LEU2 hip1::KanMX6, leu2-3,112 ura3-52 GAL2*) and NCY47 (*gap1::LEU2 hip1::KanMX6 sec6-4 ura3-52 GAL2*). Both are derived from the S288C background, in which Gap1p activity is high in ammonia medium without amino acids (Stanbrough and Magasanik, 1995).

Plasmids used in this study were pEC221, P_{ADH1}-*GAP1* in pRS316 (Risinger et al., 2006), pNC45, Hip1p^{3myc} (Kuehn et al., 1996) fused behind the *ADH1* promoter in pRS316, pNC49, P_{ADH1}-*HIP1* in pRS316, pNC51, P_{ADH1}-*HIP1-GFP* in RS316, and pNC59, P_{GAL10}-*HIP1* in pRS316.

Strains were grown at 24˚C unless otherwise noted. Minimal ammonia medium (SD) is composed of Difco (Detroit, MI) yeast nitrogen base without amino acids or ammonium sulfate (YNB), 2% glucose, and 0.5% ammonium sulfate (adjusted to pH 4.0 with HCl). Nitrogen-free medium is SD medium without ammonium sulfate. Galactose medium (SGal) is composed of YNB, 2% raffinose, 0.25% galactose, and 0.5% ammonium sulfate (at pH 4 as for SD). Basic amino acid mix (BAM) is comprised of arginine, citrulline, and lysine (Sigma, St. Louis, MO) diluted from a stock solution of 100 mM each (pH 4). Histidine (Sigma) was added from a 100mM stock solution (pH 4).

Amino acid uptake assays

Strains were cultured to early exponential phase, 4-8 X 10^6 cells/ml. Approximately 2 X 10^7 cells were harvested and washed with nitrogen-free medium on 0.45µm nitrocellulose filters. [^{14}C] histidine (GE Healthcare, Piscataway, NJ) was

adjusted to a specific activity of 64.8 mCi/mmol before use. Uptake of [^{14}C] citrulline or [^{14}C] histidine was performed as described previously (Roberg et al., 1997).

Fluorescence microscopy

Cells in exponential phase were harvested, suspended in Tris pH 8.0 with 0.5% sodium azide and visualized via fluorescence microscopy. Images were captured with a Nikon E800 microscope (Melville, NY) equipped with a Hamamatsu digital camera (Bridgewater, NJ). Image analysis was performed with Improvision OpenLabs 2.0 software (Lexington, MA).

Results

Hip1p is down regulated specifically by histidine

In order to examine post-translational regulation of Hip1p independently of any transcriptional control, we followed the precedent established for Gap1p, placing Hip1p under control of the *ADH1* promoter, which allows for robust and consistent protein expression under a variety of media conditions (Chen and Kaiser, 2002; Risinger et al., 2006; Risinger and Kaiser, 2008). *HIP1* and $HIP1^{3myc}$ (Kuehn et al., 1996) were fused to the *ADH1* promoter and transformed into *gap1*Δ*hip1*Δ cells where they could be assayed as the sole histidine transporter in the cell. We found that histidine uptake of Hip1p and $Hip1p^{3myc}$ were equivalent, indicating that the 3myc tag in Hip1p does not compromise its function (data not shown).

We considered the possibility that Hip1p could be regulated specifically by histidine, or by a general increase in amino acid levels. To determine if Hip1p is at all regulated post-translationally, P_{ADH1}-$HIP1^{3myc}$ was grown in three different media: SD (no amino acids), SD with histidine, and SD with a basic amino acid mix (BAM), as used in Chapter 2. If, like Gap1p, Hip1p is regulated only by substrates, we expected that Hip1p activity would be lower in histidine media than either SD or SD with BAM. Indeed, we observed that in histidine media, Hip1p activity was approximately 25% of that observed in SD or SD with BAM. As a control, we cultured *gap1*Δ*hip1*Δ cells expressing Gap1p under the same conditions. As expected, in both SD with histidine and SD with BAM, Gap1p activity was approximately 10% of that observed in SD (Figure 2).

The decrease in Hip1p activity observed in SD with histidine could indicate that Hip1p activity is regulated by histidine. Alternatively, cold histidine could be

contaminating the uptake assay and acting as a competitive inhibitor of labeled histidine uptake. Although cells are washed thoroughly in nitrogen free media prior to addition of labeled amino acid, Hip1p is a high affinity transporter, and the wash step may not be sufficient to remove all bound histidine. To confirm that Hip1p is indeed downregulated, two steps were taken. The amount of histidine added to the media was reduced to 100μM and a time course was performed to measure the effect of histidine on Hip1p activity over 90 minutes after addition (Figure 3). As in Figure 2, we compared the response of Hip1p in histidine medium to that of Gap1p under the same conditions. After addition of histidine, Hip1p activity decreased consistently over 90 minutes to less than 40% of the starting amount. This result confirms that Hip1p is in fact regulated and is not simply inhibited by cold histidine; if cold histidine were simply inhibiting uptake of labeled histidine, we would expect Hip1p activity in histidine medium to be less than activity in SD, but constant over the time course. However, Gap1p activity decreased to 10% of the starting amount in histidine, suggesting that Gap1p is regulated more strongly by histidine than Hip1p.

Hip1p accumulates in the vacuole after histidine addition

P_{ADH1}-*HIP1* was fused to GFP and localization in histidine medium was followed by fluorescence microscopy. Hip1p-GFP was mostly observed at the plasma membrane in SD, although in some cells, fluorescent puncta near the plasma membrane were observed. After addition of histidine, fluorescence begins to appear in the vacuole after 60 minutes and is considerably brighter after 3 hours, indicating that Hip1p is sorted to the vacuole after histidine treatment (Figure 4). In contrast to our observations of Gap1p,

after three hours a significant amount of Hip1p remains at the plasma membrane. This agrees with the observation that Hip1p activity does not decrease to the extent that Gap1p activity does in amino acid media. Thus, the regulation of Hip1p is not as strong as the regulation of Gap1p.

Hip1p is not reversibly inactivated at the cell surface

Gap1p is regulated post-translationally by amino acids in three distinct ways: internal sorting (Chen and Kaiser, 2002; Rubio-Texeira and Kaiser, 2006), endocytosis (Risinger and Kaiser, 2008), and reversible inactivation (Risinger et al., 2006). Substrate stimulated endocytosis has been reported for other yeast permeases, including Mup1p and Lyp1p (Lin et al., 2008). Therefore we thought it likely that histidine stimulates endocytosis of Hip1p. However, we wondered whether the other regulatory steps thought unique to Gap1p, surface inactivation and recycling from internal compartments, also applied to Hip1p.

We first asked whether Hip1p is reversibly inactivated at the cell surface prior to internalization. P_{ADH1}-*HIP1* was expressed in a *sec6-4* strain background. Under these conditions, Golgi to plasma membrane trafficking is blocked at the restrictive temperature of 36˚C (Novick and Schekman, 1983; Walworth and Novick, 1987). Previously, we showed that amino acid inactivation of Gap1p^{K9R,K16R} at the plasma membrane was reversible at the restrictive temperature in a *sec6-4* mutant (Risinger et al., 2006). Duplicate cultures were grown in SD with histidine at 24˚C. One culture was shifted to 36˚C, and cells were shifted into SD at either 24˚C or 36˚C. At 24˚C, Hip1p activity increased approximately threefold over 90 minutes, while at 36˚C, Hip1p activity

did not increase, but rather decreased by 50% over the same time period (Figure 5). Therefore, in contrast to Gap1p, which can be reactivated at the cell surface, reactivation of Hip1p after histidine removal requires delivery of newly synthesized protein to the plasma membrane.

Hip1p does not recycle from internal compartments

We then asked if, like Gap1p, Hip1p recycles from internal compartments when shifted from histidine media to media lacking histidine. In Chapter 2, I described an assay to measure Gap1p redistribution from the MVE to the plasma membrane. Briefly, P_{GAL10}-*GAP1* is induced in galactose medium containing a rich amino acid mixture. After addition of glucose for one hour to halt new synthesis and direct all protein to the MVE, the amino acids are removed. We observed that under these conditions, Gap1p activity sharply increases over 30 minutes. We used this general framework to design an assay to ask whether a pre-existing pool of Hip1p could be sorted to the plasma membrane after histidine removal. Synthesis of P_{GAL10}-*HIP1* was induced in histidine media, followed by growth in glucose medium with histidine for one hour. Cells were then shifted into SD without histidine (SD). As a postive control, we allowed a duplicate culture to remain in galactose medium with histidine before transferring in to galactose medium without histidine (SGal). In SD, Hip1p activity did not increase significantly over 60 minutes, while in SGal, Hip1p activity increased nearly fourfold (Figure 6). Because Hip1p synthesis continues in SGal, this increase in activity is likely due to delivery of newly synthesized protein to the plasma membrane. This indicates that when

Hip1p is directed to the MVE, it is committed for degradation in the vacuole and unlike Gap1p, cannot redistribute to the plasma membrane if conditions change.

Discussion

Here we have shown that Hip1p is down regulated at the plasma membrane in response to histidine. This down-regulation is most likely stimulated endocytosis, as Hip1p-GFP accumulated in the vacuole after histidine addition and reactivation of Hip1p after histidine removal was dependent on delivery of newly synthesized protein to the plasma membrane. We also observed that any Hip1p directed to the VPS pathway was irreversibly committed to degradation after internalization. This is in contrast to Gap1p, which can be recycled from the MVE membrane prior to internalization into intralumenal vesicles (ILV). The experiments presented here do not eliminate the possibility that Hip1p can be directly sorted from the Golgi to the vacuole, but prevented from recycling. This question could be addressed by localization of Hip1p-GFP in an *end3*Δ strain treated with histidine. As we demonstrated in Chapter 2, *end3*Δ effectively traps Gap1p at the plasma membrane, but amino acid stimulation results in newly synthesized Gap1p being sorted directly to the vacuole from the Golgi. Similarly, if histidine stimulates direct sorting of Hip1p to the vacuole, in *end3*Δ cells Hip1p-GFP would be observed both at the plasma membrane and the vacuole.

Future studies of histidine-induced regulation of Hip1p could also explore the potential role of ubiquitin. Both endocytosis and internal sorting of Gap1p require ubiquitination on two acceptor lysines in the N terminus, Lys9 and Lys16 (Helliwell et al., 2001; Soetens et al., 2001). The N terminus of Hip1p contains three lysines similarly located to the ubiquitin acceptor lysines in Gap1p: Lys7, Lys8, and Lys19. We did not examine the ubiquitination state of Hip1p, but it is possible that Hip1p trafficking could be controlled by ubiquitination. This has been observed for other permeases besides Gap1p. For example, Bul1p/Bul2p/Rsp5p have been shown to control the trafficking of

the tryptophan permease Tat2p (Abe and Iida, 2003). In addition, the proline-specific permease Put4p is down regulated when Bul1p is overexpressed (Helliwell et al., 2001).

Apart from the similar position of lysines, the N termini of Gap1p and Hip1p are sufficiently different that a chimeric protein made of the N terminus of Gap1p fused to the transmembrane domains and C terminus of Hip1p is misfolded and retained in the ER (data not shown). This misfolding occurred when the junction site was placed immediately before the predicted start of transmembrane domain (TMD) 1 and when the junction site was placed after TMD1, suggesting that the misfolding was not due to interruption of the TMD segment. Perhaps the N terminus of Gap1p adopts a radically different conformation than the N termini of other transporters, and therefore in that context disrupts normal amino acid transport. However, experiments to determine the secondary structure of the cytosolic N-terminal region of Gap1p were inconclusive (Eluère and Kaiser, unpublished data).

Taken together, these results suggest that yeast cells have developed different methods of regulation for the various permeases based on their usage. The specific permeases, such as Hip1p and others, are permitted to function in all but the most extreme environmental conditions, as their lower expression levels and transport capacity make it unlikely that amino acids will be taken up in toxic quantities. Even in high substrate levels, the cellular response is subtle – in very high levels of histidine, a population of Hip1p continued to be present and active at the plasma membrane. This is in contrast to our observations of Gap1p, in which an influx of amino acids can repress Gap1p to levels comparable with a *gap1*Δ. This is clearly because the high capacity of Gap1p allows cells to take up toxic quantities of substrate when Gap1p is unregulated,

and it is therefore advantageous for cells to exert complete inhibition of Gap1p activity. At the same time, in starvation conditions, the high capacity and broad specificity of Gap1p change from a liability to an asset. Gap1p is a more efficient scavenger for nitrogen sources than a low-capacity, narrowly specific transporter. The exact differences between Gap1p and the specific yeast transporters that allow for this additional regulation of Gap1p will be the focus of future study.

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Figures

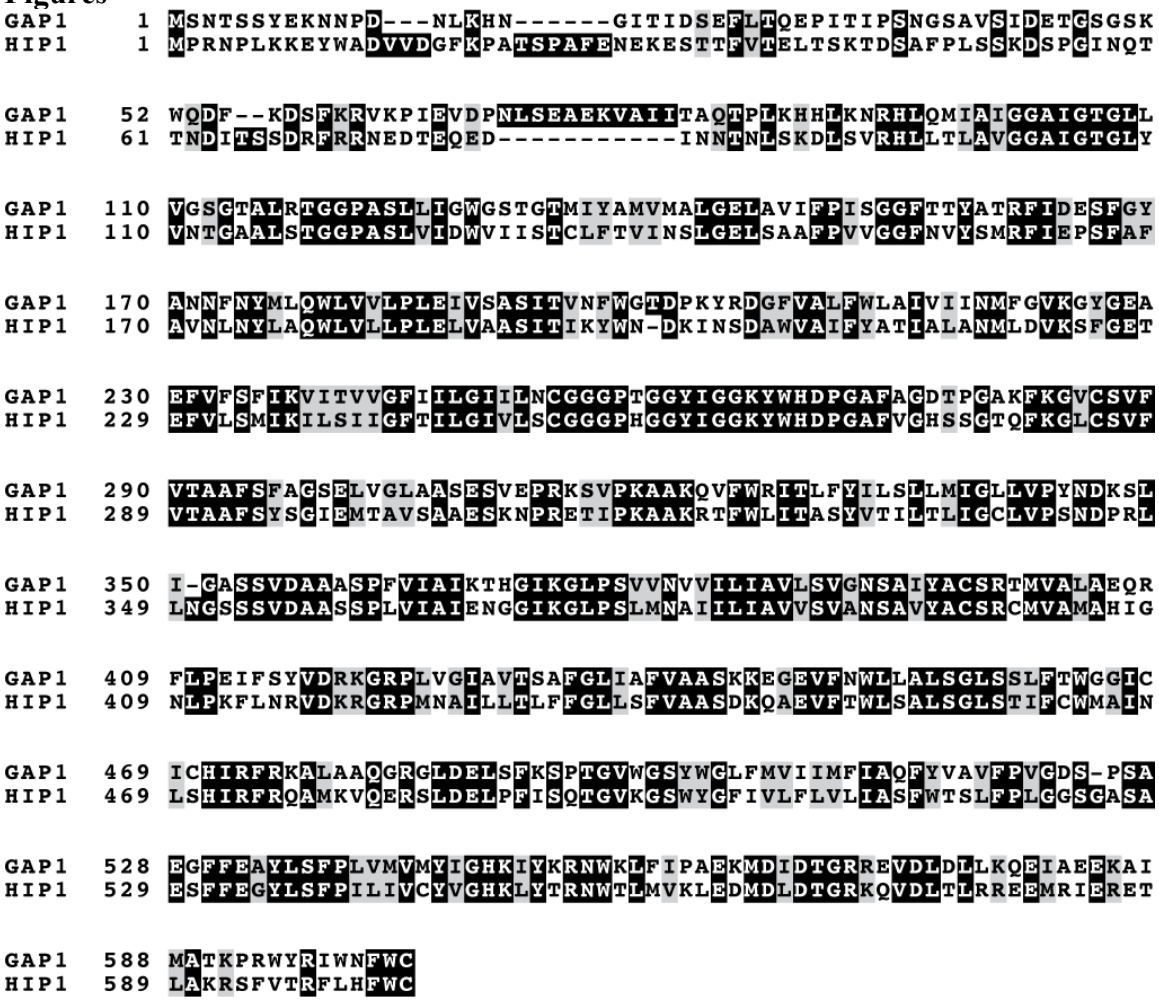

Figure 1. Alignment of Gap1p and Hip1p protein sequences. Alignment was performed by ClustalW and processed by BOXSHADE 3.21.

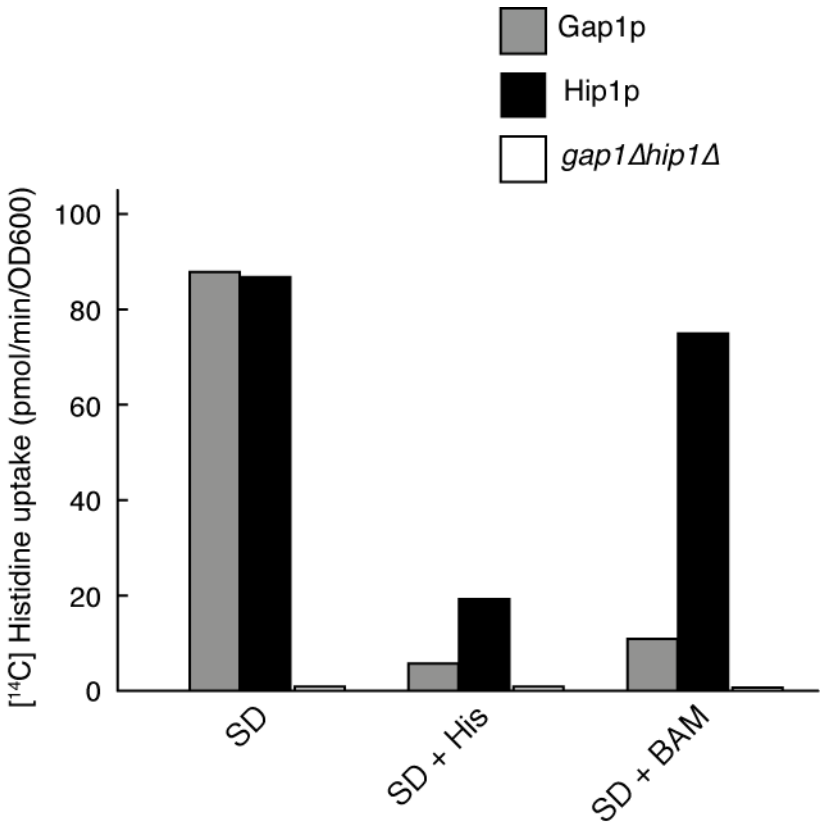

Figure 2. Hip1p is specifically down-regulated by histidine. *gap1*Δ*hip1*Δ (NCY33) cells expressing P_{ADH1}-*GAP1* (pEC221), P_{ADH1}-$HIP1^{myc}$ (pNC45), or empty vector (pRS316) were cultured to early exponential phase in SD medium. Where indicated, 1mM histidine or 1mM BAM were added to the culture for 3 hours before measuring initial uptake of [^{14}C] histidine.

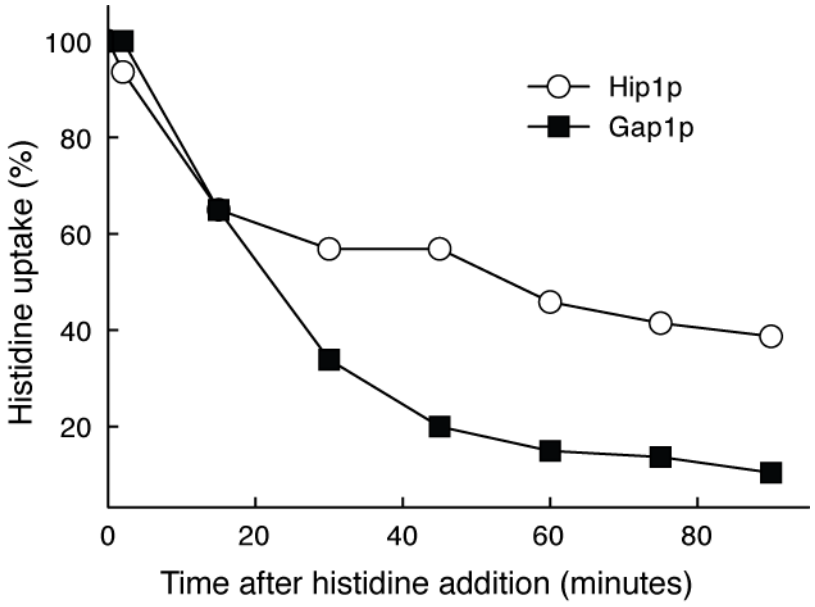

Figure 3. Hip1p activity decreases after growth in histidine. *gap1*Δ*hip1*Δ (NCY33) cells expressing P_{ADH1}-*HIP1* (pNC49) or P_{ADH1}-*GAP1* (pEC221) were cultured to early exponential phase. Initial rate of [^{14}C]-histidine uptake was measured at the indicated times after addition of 100μM unlabeled histidine to the culture. Values were normalized to the amount of uptake at t=0.

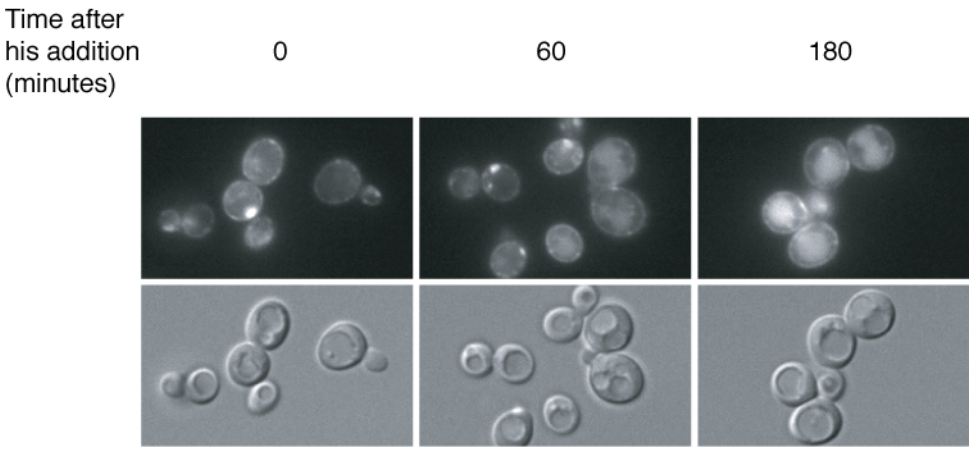

Figure 4. Hip1p accumulates in the vacuole after histidine addition. *gap1*Δ*hip1*Δ (NCY33) cells expressing P_{ADH1}-*HIP1-GFP* (pNC51) were cultured to early exponential phase in SD medium. 100μM histidine was added, and cells were harvested at the indicated times. GFP was visualized by fluorescence microscopy.

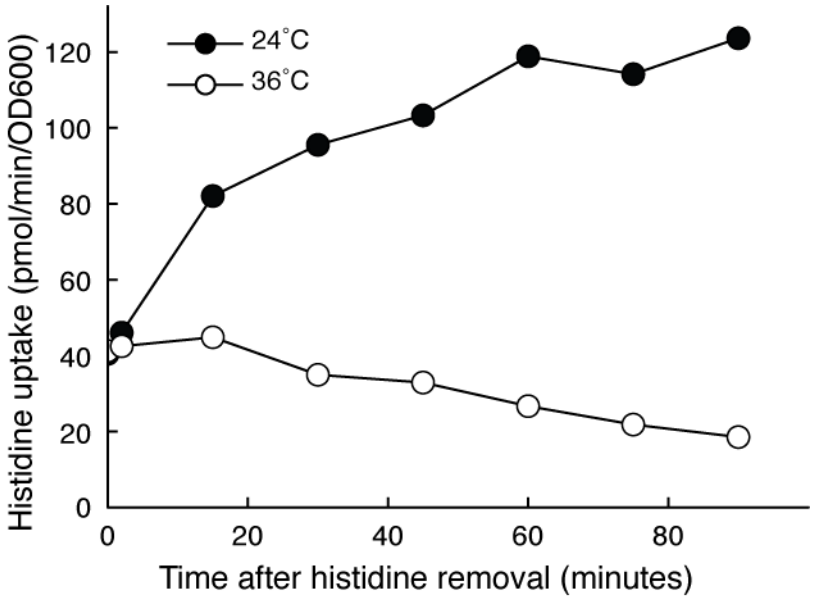

Figure 5. Hip1p does not undergo reversible inactivation at the cell surface. *gap1*Δ*hip1*Δ*sec6-4* (NCY47) cells expressing P_{ADH1}-*HIP1* (pNC49) were cultured to early exponential phase in SD with 100μM histidine, then washed and transferred into SD medium without histidine. Where indicated, cells were shifted to 36˚C for ten minutes before transfer into SD medium pre-warmed to 36˚C. [^{14}C] histidine uptake was measured at the indicated times after histidine removal.

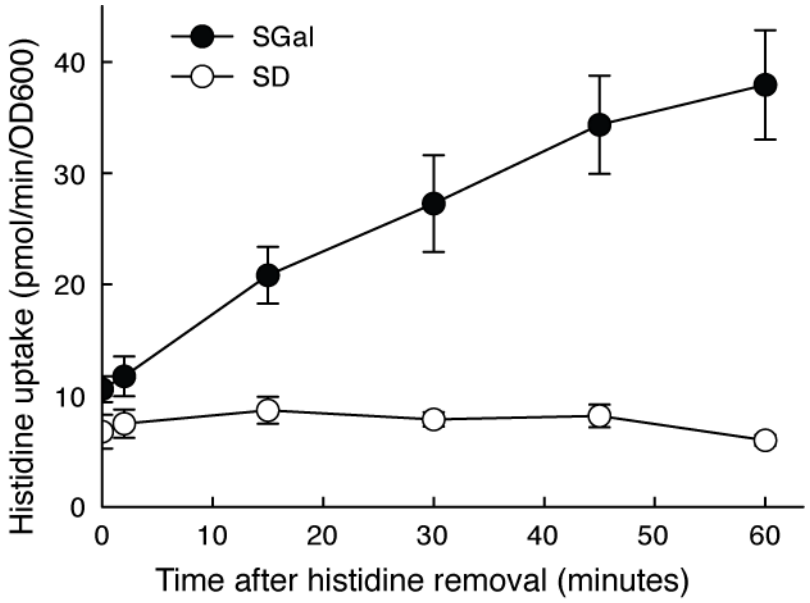

Figure 6. Hip1p is not recycled from internal compartments. *gap1*Δ*hip1*Δ (NCY33) cells expressing P^{GAL10}-*HIP1* (pNC59) were cultured to early exponential phase in SGal medium with 100μM histidine. The experimental culture (SD) was transferred into SD with histidine for one hour, then transferred into SD without histidine. A control culture (SGal) was transferred into SGal without histidine. [^{14}C] histidine uptake was measured at the indicated times after histidine removal.

Chapter 4

Ubiquitin-mediated *Trans*-regulation of C-terminal Gap1p Mutants

Abstract

The C terminus of Gap1p is required for sorting to the vacuole. Mutations in the C terminus of Gap1p cause Gap1p to bypass direct sorting to the multivesicular endosome (MVE) and proceed to the plasma membrane. Here we describe the isolation of a C-terminal mutant, Gap1p^{E583G}, based on its hyperactivity in a strain overproducing amino acids. It was previously shown that a different mutation at this position, Glu^{583}Asp, blocks polyubiquitination and causes increased trafficking to the plasma membrane. Gap1p^{E583G} and Gap1p^{E583D} have elevated activity in several mutant strains that prevent Gap1p sorting to the plasma membrane in ammonium medium. Yet in one of these sorting mutants, *lst4*Δ, Gap1p^{E583G} and Gap1p^{E583D} are *trans*-regulated by wild type Gap1p. We show that this *trans*-regulation is ubiquitin-dependent, but does not result from increased ubiquitination. Further, we show that polyubiquitinated Gap1p is not observed at the plasma membrane, suggesting that deubiquitination may be a key step in Gap1p sorting. Although the function of Lst4p is not known, the results here suggest that it could have a distinct role in regulation of Gap1p trafficking.

Introduction

The trafficking of Gap1p through the late secretory pathway is regulated by amino acids and requires many *trans*-acting factors. Several genome-wide screens in our laboratory have identified mutants that alter Gap1p trafficking (Roberg et al., 1997a; Gao and Kaiser, 2006; Rubio-Texeira and Kaiser, 2006). These Gap1p sorting mutants can be placed into two classes. The first are mutants that increase Gap1p sorting to the plasma membrane. Members of this class include the double mutant *bul1*Δ*bul2*Δ, which blocks internal polyubiquitination of Gap1p at the Golgi and thus prevents direct sorting of Gap1p to the multivesicular endosome (MVE) (Helliwell et al., 2001; Risinger and Kaiser, 2008). Other mutants in this class are those that decrease internal amino acid pools, such as mutants of the glutamate dehydrogenase *GDH1* or the glutamine synthetase *GLN1*, which decrease intracellular pools of glutamate and glutamine respectively, mimicking the effects of nitrogen starvation (Chen and Kaiser, 2002).

A second class of mutants decreases Gap1p activity by preventing its trafficking to the plasma membrane. *MKS1* encodes a negative regulator of glutamate synthesis; *mks1*Δ mutant cells have elevated amino acid levels and in this background, Gap1p is sorted to the vacuole even when extracellular amino acids are low (Liu et al., 2003; Chen and Kaiser, 2002). *GTR1* and *GTR2* encode two GTPases that are members of the GSE/EGO complex that assembles on endosomal membranes (Gao and Kaiser, 2006; Dubouloz et al., 2005). The C terminus of Gap1p has been shown to interact with Gtr2p, suggesting that Gtr1p and Gtr2p may be responsible for loading Gap1p into recycling vesicles (Gao and Kaiser, 2006). *LST4* was identified in a screen for mutants that are synthetic lethal with *sec13-1*. Further studies indicated that *lst4* mutants cause low

Gap1p activity in all nutrient conditions (Roberg et al., 1997a). The function of Lst4p is poorly understood, as its sequence has no identifiable functional motifs, and there are no known homologs in higher organisms. However, the mutant phenotype of *lst4* can be suppressed by a *bul1*Δ*bul2*Δ mutant, placing Lst4p downstream of ubiquitination (Helliwell et al., 2001). Because of this, and because the *lst4* phenotype mimics that of GSE complex mutants and *mks1*Δ, Lst4p has been predicted to function in Gap1p recycling from the endosome to the Golgi.

In this work, we present evidence that the function of Lst4p can be separated from that of the GSE complex and amino acid homeostasis. A C terminal mutant of Gap1p that bypasses the endosome is *trans*-regulated by wild type Gap1p only in *lst4*Δ mutants. We show that this effect requires ubiquitination, but is not due to increased ubiquitination of the mutant Gap1p protein. Although the role of Lst4p is still poorly understood, the data presented here indicates that Lst4p may have a unique function in Gap1p trafficking.

Materials and Methods

Strains, plasmids, and media

All of the yeast strains used in this study (Table 1) were constructed in the S288C genetic background. Wild type S288C strains exhibit relatively high Gap1p activity in ammonia medium, in contrast to strains in the Σ1278b genetic background, which exhibit low Gap1p activity when grown on ammonia (Stanbrough and Magasanik, 1995).

Plasmids used in this study are listed in Table 2. All alleles of *GAP1* were fused to the *ADH1* promoter for constitutive expression. *UBI-myc* was fused to the *CUP1* promoter for overexpression in $CuSO_4$.

Strains were grown at 24˚C unless otherwise noted. Minimal ammonia medium (SD) is composed of Difco yeast nitrogen base without amino acids or ammonium sulfate, 2% glucose, and 0.5% ammonium sulfate (adjusted to pH 4.0 with HCl). Nitrogen-free medium is SD medium without ammonium sulfate. For induction of P_{CUP1}*-UBI-myc,* 1μM $CuSO_4$ was added for ~16 hours.

Screen for Gap1p hyperactive mutants

GAP1 mutations were generated by mutagenic PCR using pEC221 (P_{ADH1}*- GAP1*) as a template and methods described previously (Sevier and Kaiser, 2006) with modifications. A fragment including the entire *GAP1* ORF as well as 500 base pairs of the *ADH1* promoter and 800 base pairs of the *GAP1* 3' UTR was amplified in four 50 μl reactions with AmpliTaq Gold (Perkin-Elmer Cetus, Norwalk, CT) and 0.3 mM $MnCl_2$. PCR products were transformed along with gapped pEC221 plasmid (lacking the *GAP1* ORF) into ARY117 (*gap1*Δ*, mks1*Δ*, ura3-52*), and gap-repaired plasmids were isolated by selection for Ura^+ transformants. Hyperactive mutants were identified by replica

plating to SD supplemented with 10mg/L L-azetidine 2-carboxylic acid (ADCB). ADCB sensitivity was confirmed by streaking the original Ura^+ colony on a fresh SD + ADCB plate. Plasmids were isolated from ADCB-sensitive colonies, re-transformed into ARY117, and re-tested for ADCB sensitivity.

Amino acid uptake assays

Strains were cultured to early exponential phase, 4-8 X 10^6 cells/ml. Approximately 2 X 10^7 cells were harvested and washed with nitrogen-free medium on 0.45µm nitrocellulose filters. Uptake of [^{14}C] citrulline was performed as described (Roberg et al., 1997b).

Immunoprecipitation of ubiquitin conjugates

Detection of ubiquitin conjugates was performed as described previously (Helliwell et al., 2001) with modifications. Cell pellets were resuspended in equal volume lysis buffer (0.3M sorbitol, 10mM HEPES pH 7.5, 10mM NaN_3) with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN), 1mM PMSF, and 50mM NEM. Cells were lysed by vortexing with glass beads at 4˚C. 100µl of sample buffer (2% SDS, 10% glycerol, 80mM Tris pH 6.8) was added and lysates were incubated at 37˚C for 30 minutes. 1mL of IP buffer (50mM Tris pH 7.5, 150mM NaCl, 1% Triton) was added with protease inhibitors and 10mM NEM. Lysates were incubated on ice for 10 minutes before insoluble material was removed by centrifugation at 12,000 X g. Immunoprecipitation was performed overnight by incubation with anti-HA antibody (3F10 rat anti-HA, Roche Diagnostics), followed by 2 hours at 4˚C with 50 µl of 50%

Protein G-Sepharose 4 fast flow (Amersham Pharmacia Biotech). Beads were washed three times with IP buffer and two times with PBS. Immunoprecipitates were solubilized with sample buffer for 1 hour. Proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes by a 90 minute semi-dry transfer. Antibodies used were rabbit anti-Gap1, mouse anti-myc (9E10, Covance), HRP-coupled sheep anti-rabbit and HRP-coupled sheep anti-mouse (Amersham Pharmacia Biotech).

Equilibrium density centrifugation

Fractionation of membranes was performed as described (Kaiser et al., 2002) with modifications. After centrifugation, eight fractions were collected from the top of the gradients and concentrated by TCA precipitation. The pellets were resuspended in sample buffer before pooling every two fractions. The fractions were then diluted in IP buffer and immunoprecipitation was carried out as described (Risinger and Kaiser, 2008). Antibodies used were rabbit anti-Gap1, mouse anti-myc (9E10, Covance), rabbit anti-Pma1, HRP-coupled sheep anti-rabbit, and HRP-coupled sheep anti-mouse (Amersham Pharmacia Biotech).

Results

A C-terminal mutation in Gap1p prevents Golgi-endosome trafficking

We were interested in isolating mutant alleles of Gap1p that were hyperactive in high amino acid conditions, with the goal of identifying regions of Gap1p that are required for responding to amino acids. We generated random Gap1p mutant alleles by error-prone PCR using P_{ADH1}-*GAP1* as a template. These mutants were transformed into a *gap1*Δ*mks1*Δ strain, which has high levels of intracellular glutamate and glutamine due to increased expression of α-ketoglutarate through the RTG pathway (Dilova et al., 2002; Liu et al., 2003). An *mks1*Δ mutation had been previously shown to increase Gap1p sorting to the vacuole (Chen and Kaiser, 2002). Transformants were screened for sensitivity to 10mg/L ADCB, a concentration that is tolerated by strains expressing wild type Gap1p, but lethal to strains expressing the non-ubiquitinated $Gap1p^{K9R,K16R}$. Of approximately 10,000 colonies screened, one mutant allele, $Gap1p^{1\text{-}1}$, was isolated that displayed a reproducible, plasmid-dependent ADCB sensitive phenotype. This result was confirmed by measurement of citrulline uptake, which was approximately 60 times higher than that observed for wild type Gap1p (Figure 1). $Gap1p^{1\text{-}1}$ had five point mutations: D54G, D68G, N92I, A99S, and E583G. The first four all mapped to the cytosolic N terminus of Gap1p while the E583G mutation mapped to the cytosolic C terminus.

Glu^{583} has been previously implicated in proper Gap1p sorting. The $GAP1^{pgr}$ allele, which was found to resist ammonia-induced endocytosis in Σ1278b, was identified as a glutamate to lysine mutation at position 583 (Grenson and Acheroy, 1982; Hein et al., 1995). More recently, $Gap1p^{E583D}$ was isolated in a random screen of mutant Gap1p alleles with increased sensitivity to glycine toxicity (Risinger and Kaiser, 2008).

Therefore we thought it possible that the E583G mutation may be sufficient to confer the ADCB sensitivity and citrulline uptake phenotypes of the Gap1p$^{1-1}$ mutant. Citrulline uptake of Gap1p^{E583G} was indeed elevated in similar fashion to Gap1p$^{1-1}$. It also displayed high activity in *lst4*Δ and *gtr1*Δ strains. Lst4p and the GSE complex are thought to act downstream of ubiquitination, as ubiquitination mutants suppress the low Gap1p activity phenotype of *lst4*Δ and *gtr1*Δ mutants (Helliwell et al., 2001; Gao and Kaiser, 2006). Therefore Gap1p^{E583G} appears to be deficient in Golgi-endosome trafficking.

*Trans-regulation of Gap1p E583 mutants in lst4*Δ

Initial tests of Gap1p^{E583G} were performed in a *gap1*Δ background where each mutant was the sole copy of Gap1p. We wondered if it were possible for Gap1p^{E583G} to interact with wild-type Gap1p and if so, whether such an interaction could restore Golgi-to-MVE of Gap1p^{E583G}. To test this, we expressed Gap1p^{E583G} and the previously characterized Gap1p^{E583D} in either *gap1*Δ*lst4*Δ cells or *lst4*Δ cells. Because wild type Gap1p is mostly internal in *lst4* mutants, we predicted that any activity we observed in *lst4*Δ cells expressing either E583 mutant would be due to the mutant Gap1p allele. Both Gap1p^{E583G} and Gap1p^{E583D} showed a 50% decrease in inactivity when co-expressed with wild type Gap1p (Figure 2). Therefore wild type Gap1p can at least partially complement the sorting defect of C terminal Gap1p mutants.

Trans-regulation of E583 mutants by wild type could occur through a physical interaction between the two. Presumably the mutants are only partially regulated because they interact either with themselves or with wild type, and mutant-mutant interactions would result in no down-regulation. If Gap1p oligomerizes as it is sorted through the late

secretory pathway, perhaps an oligomer containing both wild type and mutant Gap1p would be sorted in a wild type manner. Wild type Gap1p could then carry mutant Gap1p along with it to the vacuole, even if the mutant does not contain the proper sorting signal to be sorted on its own. Alternatively, the wild type and mutant Gap1p could interact briefly to allow for ubiquitination of the mutant, or recognition of ubiquitin attachment.

In order to distinguish between these two possibilities, we expressed the non-ubiquitinated *GAP1*^{K9R,K16R} allele in *gap1*Δ*lst4*Δ or *lst4*Δ cells. If the *trans*-regulation we observe is due to an oligomerization event during trafficking, we expected that Gap1p^{K9R,K16R} could be sorted with wild type Gap1p to the vacuole, despite its inability to be polyubiquitinated. However, in contrast to the 50% reduction in activity we observed with Gap1p^{E583G} and Gap1p^{E583D}, we observed no effect on the activity of Gap1p^{K9R,K16R} by the addition of endogenous Gap1p. From this we concluded that the ability to be ubiquitinated was necessary for *trans*-regulation of Gap1p mutants. Because Gap1p^{K9R,K16R} has no ubiquitin acceptor lysines, it continues to bypass the endosome and is sorted directly to the plasma membrane.

Trans-regulation is not caused by increased ubiquitination

Previous studies of Gap1p^{E583D} showed that it does not receive polyubiquitin chains (Risinger and Kaiser, 2008). Thus, our initial hypothesis for *trans*-regulation of E583 mutants by wild type was that the wild type Gap1p allowed for increased ubiquitination of the mutant. As both the N and C termini of Gap1p appear to be required for proper sorting, wild type Gap1p could provide the necessary C terminal sequence to the E583 mutants during ubiquitination by Bul1p/Bul2p/Rsp5p. To examine levels of

ubiquitinated Gap1p in both a *gap1*Δ*lst4*Δ strain and a *lst4*Δ strain, we expressed HA-tagged *GAP1, GAP1*^{E583G}*, GAP1*^{E583D}*,* and *GAP1*^{K9R,K16R} along with myc-tagged ubiquitin. HA tagged Gap1p was immunoprecipitated and ubiquitinated species were detected by immunoblotting for ectopically overexpressed myc-ubiquitin (Figure 4). Surprisingly, ubiquitinated species were observed for both Gap1p^{HA,E583G} and Gap1p^{HA,E583D} in *gap1*Δ*lst4*Δ cells, although at much lower levels than for Gap1p^{HA}. Further, levels of ubiquitination did not increase in *lst4*Δ cells; ubiquitination appeared to decrease in all lanes, likely because the untagged endogenous Gap1p was also ubiquitinated with the myc-tagged ubiquitin, yet was not collected by immunoprecipitation. Nonetheless, the results do not indicate that the *trans*-regulation of the E583 mutants in *lst4*Δ is due to an increase in ubiquitination.

We then considered the possibility that in *lst4*Δ cells, C terminal mutants are ubiquitinated, but are inefficiently detected by recognition machinery and escape Golgi-endosome vesicular trafficking. In this case, it was possible that polyubiquitinated species could be detected at the plasma membrane. To test this, we expressed *GAP1*^{HA} or *GAP1*^{HA,E583G} along with myc-tagged ubiquitin in *gap1*Δ*pep4*Δ*doa4*Δ cells. Deletion of the vacuolar protease Pep4p and the deubiquitinating enzyme Doa4p stabilizes ubiquitinated proteins by preventing removal of the ubiquitin chain and degradation in the vacuole. Although this strain background contains wild type Lst4p, we observed robust ubiquitination of Gap1p^{HA} and Gap1p^{HA,E583G}. Cells were first fractionated over continuous 20-60% sucrose density gradients with EDTA, which effectively separates the denser plasma membrane from less dense internal membranes. Each gradient was concentrated into four fractions before immunoprecipitating Gap1p and immunoblotting

for Gap1p, myc-ubiquitin, and the plasma membrane marker Pma1p (Figure 5). The majority of ubiquitinated species in both $Gap1p^{HA}$ and $Gap1p^{HA,E583G}$ cells did not co-fractionate with Pma1p, suggesting that polyubiquitinated mutant Gap1p protein is not sorted to the plasma membrane.

*Partial suppression of C terminal mutants requires lst4*Δ

The specific role of Lst4p in Gap1p sorting is poorly understood. It has typically been classified with other mutants that cause low Gap1p activity in SD, and is presumed to be required for endosome-Golgi trafficking. The *trans*-regulation of E583 Gap1p mutants is an additional phenotype that could be used to classify *trans* Gap1p sorting mutants. By testing other Gap1p sorting mutants for the ability to partially down-regulate $Gap1p^{E583G}$ and $Gap1p^{E583D}$, the mutants could potentially be placed into classes, which could lead to deduction of common functions. We performed citrulline uptake assays on *mks1*Δ, *gtr1*Δ, and *gtr2*Δ strains both with and without the endogenous *GAP1*. As shown in Figure 1, these mutants have low Gap1p activity, although they have no effect on the E583 mutants. Strikingly, we observed no significant difference in citrulline uptake of either $Gap1p^{E583G}$ or $Gap1p^{E583D}$ in any of the mutant strains with the endogenous *GAP1* (Figure 6). Therefore, the *trans*-regulation of E583 mutants cannot be observed in all Gap1p sorting mutant strains, but is specific to *lst4*. Lst4p then must have a role in Gap1p sorting that is distinct from Mks1p and the GSE complex.

Discussion

Both the cytosolic N and C termini of Gap1p are required for vacuolar sorting. The ubiquitin acceptor lysines are located at the N terminus, and mutations in the C terminus allow Gap1p to bypass the MVE even when the N terminal lysines are intact. In particular, Glu^{583} is critical for vacuolar sorting – three different mutations at this position cause sorting defects. The C terminus is required for efficient ubiquitination of the N terminus. Our laboratory recently reported that $Gap1p^{E583D}$ was not polyubiquitinated at all in a *pep4*Δ*doa4*Δ strain background (Risinger and Kaiser, 2008). We were therefore surprised when, in the same strain, $Gap1p^{E583G}$ was polyubiquitinated at similar levels to Gap1p. However, the *pep4*Δ and *doa4*Δ mutations have a stabilizing effect on ubiquitinated species. Ubiquitinated $Gap1p^{E583G}$ may accumulate in *pep4*Δ*doa4*Δ to a greater extent than in a wild type strain background. Nonetheless, both E583 mutants exhibited the same low level of polyubiquitination in *gap1*Δ*lst4*Δ, although this amount of ubiquitination did not correspond to a significant decrease in Gap1p activity as measured by citrulline uptake. The majority of $Gap1p^{E583G}$ and $Gap1p^{E583D}$ may avoid polyubiquitination and be sorted directly to the plasma membrane.

Both of the E583 mutants we tested were *trans*-regulated in *lst4*Δ, suggesting that Gap1p may form oligomers. Many transporters have been shown to assemble in oligomeric structures, although each amino acid binding channel is distinct. For example, the arginine/agmatine antiporter AdiC of *E. coli* was crystallized as a dimer via interactions between the last two transmembrane segments that were not part of the pore (Gao et al., 2009). It has also been shown that neurotransmitter transporters require oligomerization for proper trafficking through the early secretory pathway (Farhan et al., 2006). Interaction between wild type Gap1p and E583 mutants is only observed in *lst4*Δ.

The function of Lst4p must then be distinct from the functions of the other mutants tested, Mks1p and Gtr1/2p, although all four ultimately cause low Gap1p activity. Lst4p may be spatially separate from the other mutants we tested as well. Mks1p increases internal amino acid levels and we have shown that amino acids specifically inhibit endosome-Golgi trafficking (Chen and Kaiser, 2002; Rubio-Texeira and Kaiser, 2006). The GSE complex has been localized to endosomal membranes, although its role there is not yet clear (Gao and Kaiser, 2006; Dubouloz et al., 2005). Because *trans*-regulation is not observed in any of these mutants, wild type and mutant Gap1p must interact prior to trafficking to the endosome in order for *trans*-regulation to occur.

Additionally, ubiquitination is necessary for *trans*-regulation, as the completely non-ubiquitinated Gap1p^{K9R,K16R} is not regulated at all by wild type Gap1p. This allowed us to hypothesize that a *trans*-interaction between the C terminus of wild type Gap1p and the N terminus of an E583 mutant allows for increased ubiquitination of the E583 mutant, leading to vacuolar sorting. However, no significant increase in ubiquitination was observed, indicating that the interaction does not allow for more ubiquitination of the mutant. Interaction must therefore occur after the ubiquitination event, but before Golgi-endosome trafficking. Possibly *lst4*Δ does not increase Golgi-to-MVE trafficking, but rather traps Gap1p in a pre-endosomal compartment, and through oligomerization, E583 mutants are sequestered with wild type Gap1p.

Although Gap1p can be polyubiquitinated regardless of nutrient conditions, polyubiquitinated Gap1p is not observed at the plasma membrane. This indicates a role for deubiquitination in trafficking of Gap1p to the plasma membrane. *S. cerevisiae* contains 16 deubiquitinating enzymes (DUB) (Amerik et al., 2000). Several of them

could be acting on Gap1p at different stages of post-Golgi trafficking. A genome-wide analysis of genetic interactions has implicated the DUB Ubp15p in Gap1p trafficking (Costanzo et al., 2010), and preliminary work in our laboratory has shown that *ubp15*Δ strains have lower Gap1p activity and increased Gap1p ubiquitination (Eluère and Kaiser, unpublished data).

Our screen was designed with the goal of isolating mutants that are constitutively recycled from the endosome, and thus insensitive to amino acid regulation. The sole mutant identified by this screen, Gap1p^{E583G}, was determined not to be a constitutively recycled mutant, but rather a mutant that could not reach the endosome from the Golgi. The inability to isolate the desired mutant may have been due to the inherent inefficiency of the screen. True mutants were difficult to isolate due to the nature of the screen – screening for a death phenotype rather than selecting a growth phenotype. Additionally, the screen had a high level of background. Both wild type *GAP1* and *gap1*Δ cells were resistant to ADCB. Therefore, both wild type and null mutants were enriched in the selection. As a result, truly ADCB-sensitive colonies were difficult to identify among the large number of colonies growing on the screening plates.

Because the aim of the screen is to identify mutants with high activity, one potential solution is to increase the rate of lethality of the mutant colonies. This could be achieved through two steps: decreasing the amount of mutagenesis while simultaneously increasing the amount of ADCB to a level where only the null mutation could survive. Citrulline uptake assays would be needed to distinguish the wild type-like mutants from the hyperactive mutants. By taking this approach, it may be possible to isolate mutants that were otherwise missed.

We also considered the possibility that the screen as designed may not allow us to isolate the specific mutant we were seeking. This screen relied on a fundamental assumption that an amino acid insensitive mutant would be hyperactive when amino acid levels are high. We hypothesized that Gap1p may contain a sorting signal that was separate and distinct from residues required for amino acid transport, and that mutation of that signal would increase trafficking to the plasma membrane without affecting the catalytic activity of Gap1p. Because we did not identify such a mutant, we considered instead the alternative hypothesis that transport activity and regulated sorting are so tightly linked they cannot be separated. In this case, an amino acid insensitive mutant would not be hyperactive at the plasma membrane, but non-functional. This idea directly led to the work that is presented in Chapter 2.

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Tables

Table 1: Yeast strains used in this study

Strain	Genotype	Source
ARY117	*gap1::KanMX6 mks1::KanMX6 ura3-52*	This study
CKY482	*gap1::LEU2 leu2-3,112 ura3-52*	Kaiser Strain Collection
CKY695	*lst4::KanMX6 ura3-52*	Kaiser Strain Collection
CKY702	*gap1::LEU2 lst4::KanMX6 leu2-3,112 ura3-52*	Kaiser Strain Collection
CKY776	*mks1::KanMX6 ura3-52*	Kaiser Strain Collection
CKY1031	*gap1::LEU2 pep4::LEU2 doa4::KanMX6 leu2-3,112 ura3-52*	Kaiser Strain Collection
ESY25	*gtr1::KanMX6 ura3-52*	This study
ESY25	*gtr2::KanMX6 ura3-52*	This study
MGY9	*gap1::LEU2 gtr2::KanMX6 leu2-3,112 ura3-52*	This study
MGY13	*gap1::LEU2 gtr1::KanMX6 leu2-3,112 ura3-52*	This study
NCY73	*gap1::NatMX4 lst4::KanMX6 leu2-3,112 ura3-52*	This study
NCY74	*lst4::KanMX6 leu2-3,112 ura3-52*	This study

Table 2: Plasmids

Name	Description	Markers	Source
pAR1	P_{ADH1}-*GAP1*^{K9R,K16R,HA}	*CEN, URA3*	Risinger et al. 2006
pAR77	P_{ADH1}-*GAP1*^{K9R,K16R}	*CEN, URA3*	This study
pAR101	P_{ADH1}-*GAP1*^{E583D}	*CEN, URA3*	This study
pAR103	P_{ADH1}-*GAP1*^{HA,E583D}	*CEN, URA3*	This study
pCK227	P_{ADH1}-*GAP1*^{HA}	*CEN, URA3*	Chen and Kaiser 2002
pCK231	P_{CUP1}-*UBI-c-myc*	2μ, *HIS3*	Helliwell et al. 2001
pEC221	P_{ADH1}-*GAP1*	*CEN, URA3*	Risinger et al. 2006
pNC11	P_{ADH1}-*GAP1*^{E583G}	*CEN, URA3*	This study
pNC12	P_{ADH1}-*GAP1*^{HA,E583G}	*CEN, URA3*	This study
pSL3	P_{CUP1}-*UBI-c-myc*	2μ, *LEU2*	This study

Figures

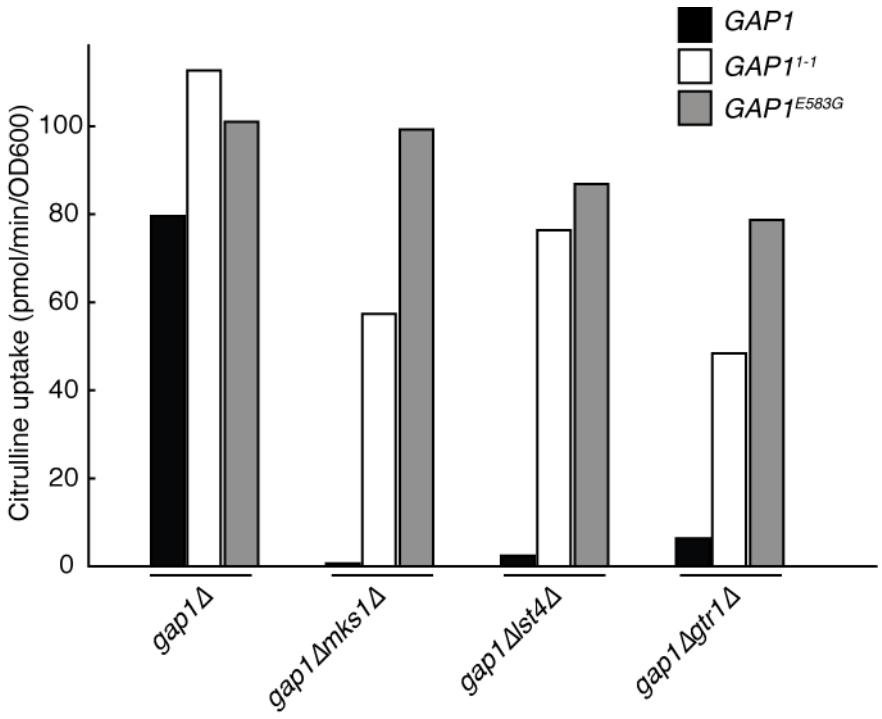

Figure 1. A C-terminal mutation in Gap1p causes high activity in three mutant strains that typically have low Gap1p activity. *gap1*Δ (CKY482), *gap1*Δ*mks1*Δ (ARY117), *gap1*Δ*lst4*Δ (CKY702), and *gap1*Δ*gtr1*Δ (NCY20) cells were transformed with P_{ADH1}-*GAP1* (pEC221), P_{ADH1}-$GAP1^{1\text{-}1}$, or P_{ADH1}-$GAP1^{E583G}$ (pNC11). Cells were grown to early exponential phase and initial rate of [14C] citrulline uptake was measured.

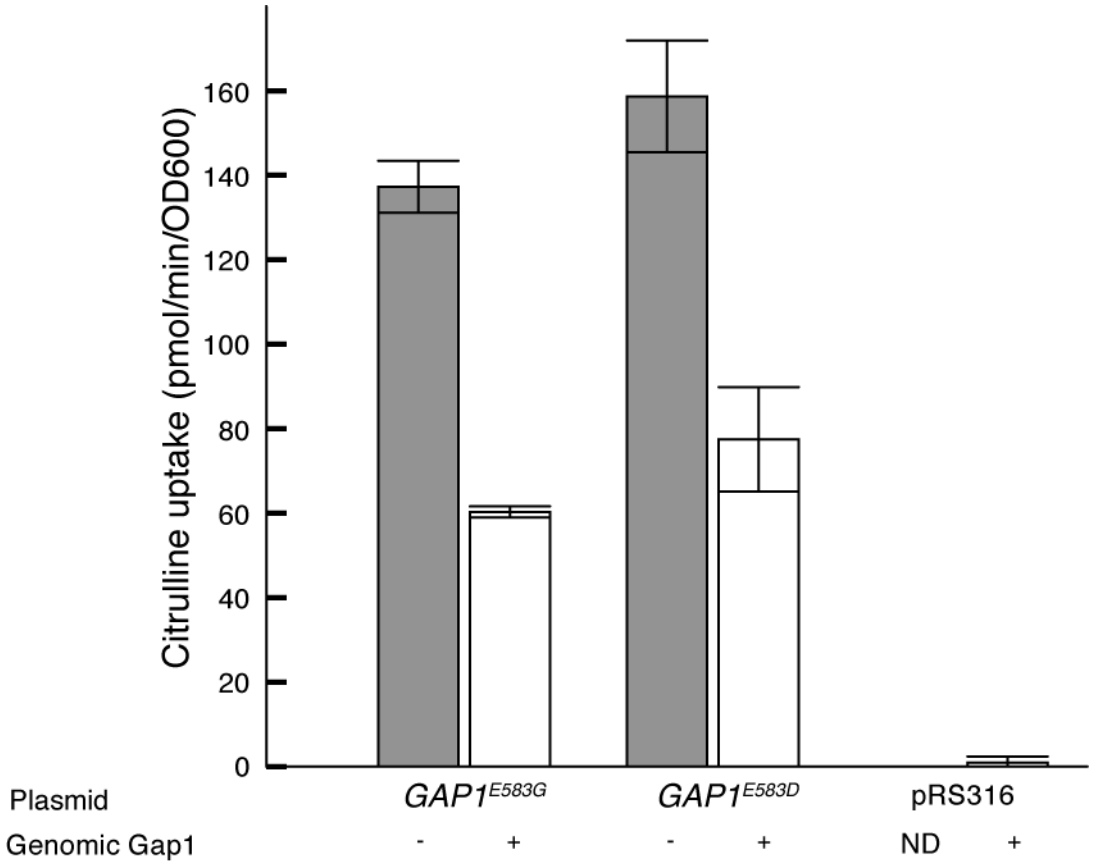

Figure 2. Wild type Gap1p partially suppresses C-terminal mutant phenotypes in a *lst4*Δ strain. CKY702 (*gap1*Δ*lst4*Δ) was transformed with pNC11 (P_{ADH1}-$GAP1^{E583G}$) or pAR101 (P_{ADH1}-$GAP1^{E583D}$) and CKY695 (*lst4*Δ) was transformed with pNC11, pAR101, or pRS316 (empty vector). Cells were grown to early exponential phase in SD and initial uptake of [^{14}C]-citrulline was measured. Error bars represent 1 S.E.M. for three independent measurements. N.D. - not done.

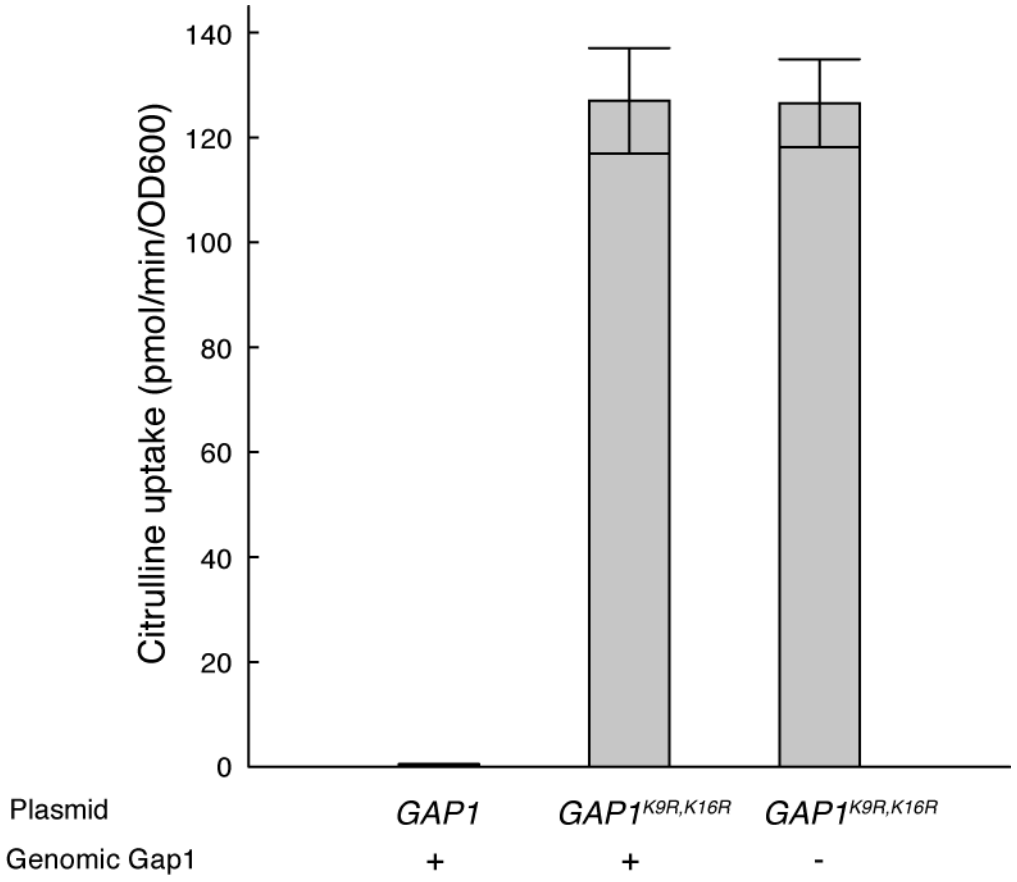

Figure 3. Wild type Gap1p has no effect on Gap1p^{K9R,K16R} in a *lst4*Δ strain. CKY695 (*lst4*Δ) was transformed with pEC221 (P_{ADH1}-*GAP1*) or pAR77 (P_{ADH1}-*GAP1*^{K9R,K16R}) and CKY702 (*gap1*Δ*lst4*Δ) was transformed with pAR77. Cells were grown to early exponential phase in SD. Initial uptake of [^{14}C] citrulline was measured. Error bars represent 1 S.E.M for three independent measurements.

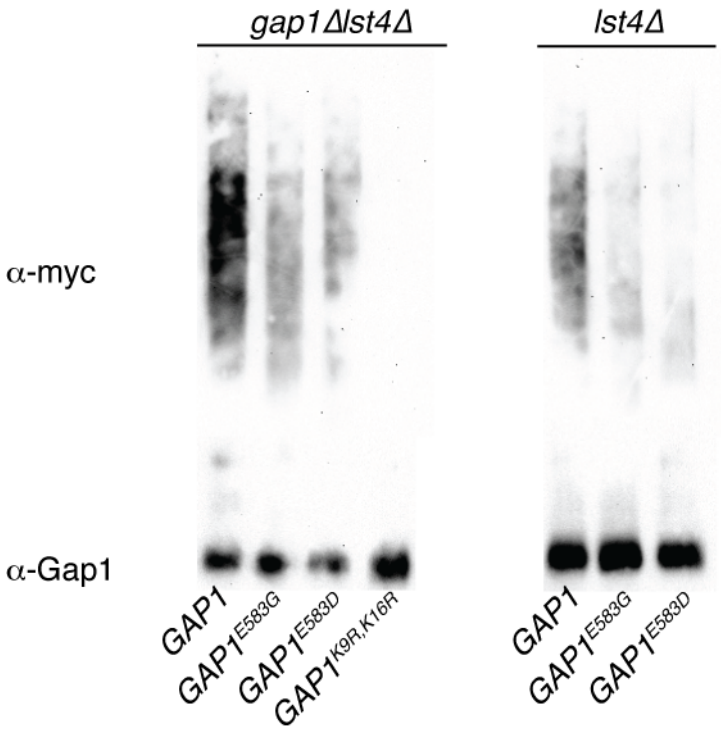

Figure 4. Suppression of C terminal mutants by wild type Gap1p in *lst4*Δ strains is not due to increased ubiquitination. *gap1*Δ*lst4*Δ (NCY73) or *lst4*Δ (NCY74) strains were transformed with P_{ADH1}-$GAP1^{HA}$ (pCK227), P_{ADH1}-$GAP1^{HA,E583G}$ (pNC12), P_{ADH1}-$GAP1^{HA,E583D}$ (pAR103), or P_{ADH1}-$GAP1^{K9R,K16R,HA}$ (pAR1), along with P_{CUP1}-*myc*-*UBI* (pSL3). Cells were grown to exponential phase in SD medium with 1μM $CuSO_4$. Lysates were immunoprecipitated with 3F10 rat anti-HA and separated by SDS-PAGE. Gap1p was detected by rabbit antiserum and myc was detected with 9E10 monoclonal antibody.

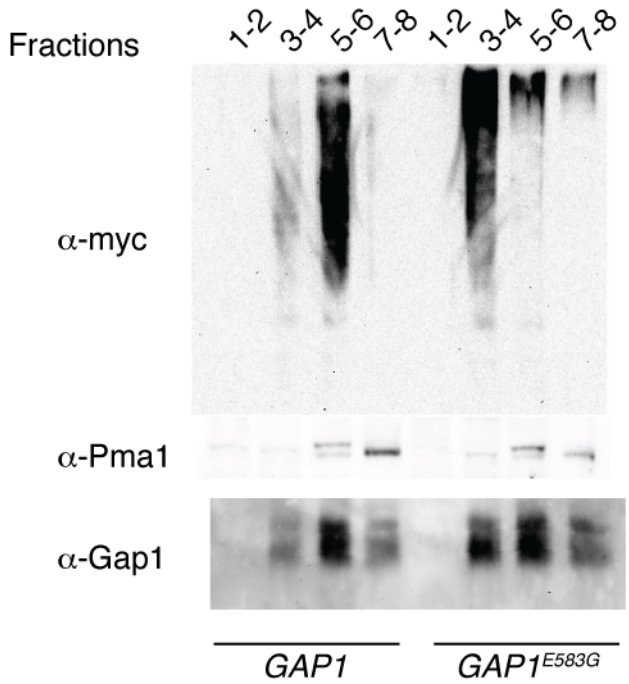

Figure 5. The majority of polyubiquitinated Gap1p is observed in internal membranes. CKY1031 (*pep4*Δ*doa4*Δ*gap1*Δ) was transformed with pCK231 (P_{CUP1}-*myc*-*UBI*) and either pCK227 (P_{ADH1}-$GAP1^{HA}$) or pNC12 (P_{ADH1}-$GAP1^{HA,E583G}$). Cells were grown in SD with 1μM $CuSO_4$ and fractionated over a 20-60% continuous sucrose gradient with 10mM EDTA. Eight fractions were collected from the top of each gradient. Fractions were TCA precipitated and every two were pooled before immunoprecipitation with 3F10 anti-rat antibody. Immunoprecipitated proteins were detected with rabbit anti-Gap1, rabbit anti-Pma1, and 9E10 mouse anti-myc antibodies.

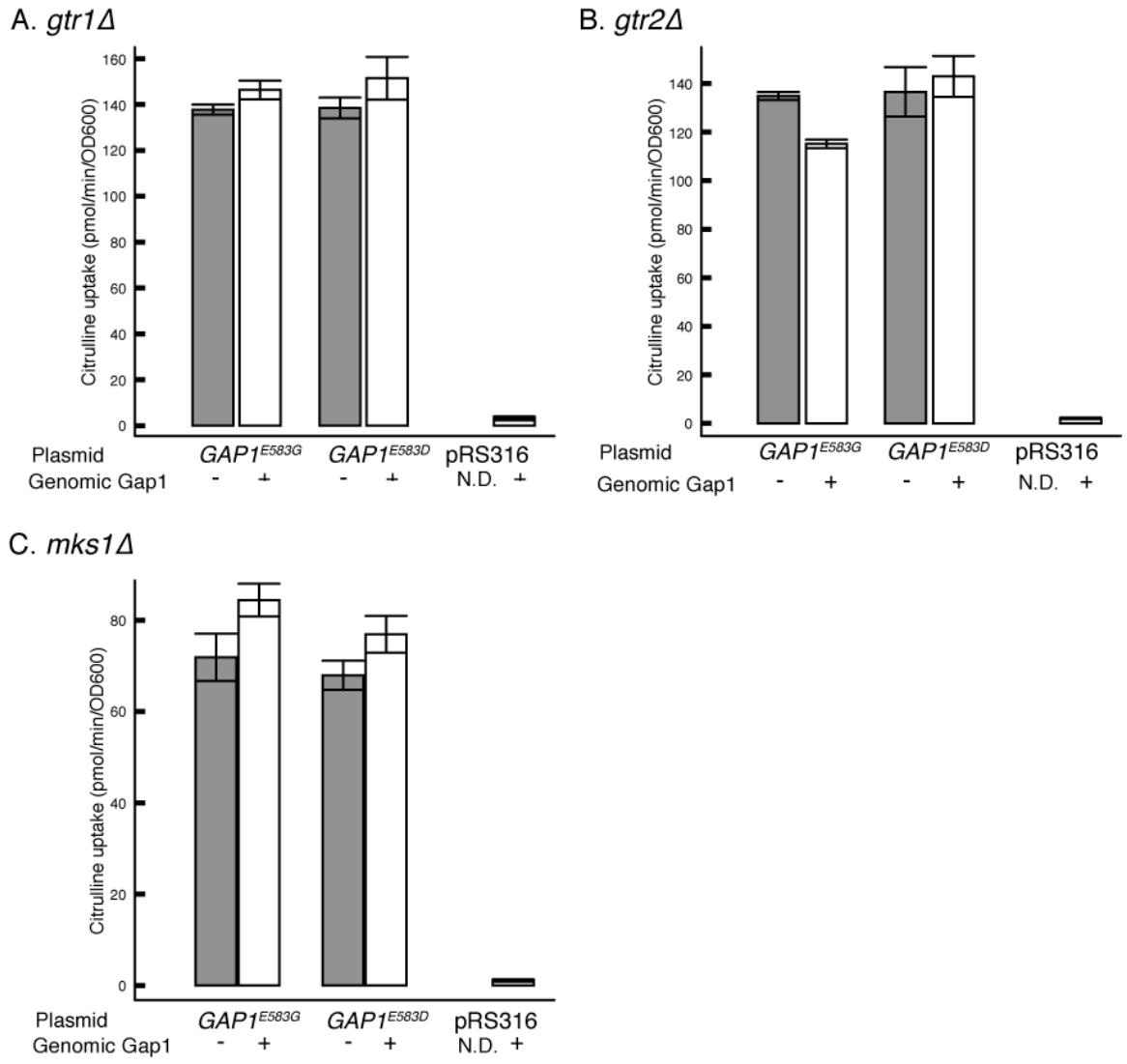

Figure 6. C terminal mutants are not affected by wild type Gap1p in GSE complex mutants or *mks1*Δ. As described in Figure 2, $GAP1^{E583G}$ or $GAP1^{E583D}$ were expressed in *gtr1*Δ, *gtr2*Δ, or *mks1*Δ strains with or without the endogenous *GAP1*. Cells were grown to early exponential phase in SD and initial uptake of [^{14}C]-citrulline was measured. Error bars represent 1 S.E.M. for three independent measurements. N.D. - not done.

Chapter 5

Prospectus

Summary

Down-regulation of Gap1p in response to abundant nutrient conditions has been known for many years. Understanding of Gap1p regulation was greatly expanded by the findings that individual substrates are sufficient to regulate Gap1p post-translationally and that they do so by preventing the redistribution of Gap1p from internal compartments to the plasma membrane (Chen and Kaiser, 2002; Rubio-Texeira and Kaiser, 2006). Yet the mechanism of amino acid down-regulation of Gap1p remained poorly understood. The work presented here provides strong evidence that signaling to Gap1p in response to rising amino acid levels is provided by Gap1p itself. Although transporter-like proteins have been previously implicated in *trans*-signaling (Wu et al., 2006; Dietvorst et al., 2010), the sensing function of Gap1p is distinct, in that the target of Gap1p signaling is Gap1p itself. Although we found that the closest homolog to Gap1p in *S. cerevisiae*, Hip1p, is not regulated to the same extent as Gap1p, this mechanism of regulation may nonetheless be true for other transporter proteins throughout evolution.

Future Directions

Amino acid sensing

Amino acids regulate Gap1p sorting whether they are imported from the extracellular environment or are produced in high amounts by the cell. Yet I have shown that amino acids imported from outside the cell must be recognized by Gap1p inside the cell to allow vacuolar sorting to occur. Proper orientation of Gap1p in the membrane dictates that the extracellular face is open to the lumen of the MVE. Taken together, these findings allow us to make predictions about the intracellular location of amino acids that are sensed by Gap1p. Amino acid regulation may require an amino acid gradient

across the endosomal membrane. Therefore, cellular control of amino acid pools is an important focus of future study.

The rapid response of Gap1p located at the endosomal membrane suggests that amino acids imported from the extracellular environment are concentrated in the MVE. How might this concentration occur? Our current hypothesis is that the lumen of the MVE is similar to that of the vacuole, in that excess amino acids are concentrated there for storage. Amino acid levels in the vacuole are controlled by a diverse group of transporters that are distantly related to plasma membrane amino acid permeases (Li and Kane, 2009). Because most of these transporters are specific to classes of amino acids, we predict that vacuolar transporter mutants would cause inappropriate Gap1p sorting in certain amino acids due to an inability to concentrate them in the MVE/vacuole. We can imagine that wild type Gap1p in certain vacuolar transporter mutants would display a phenotype similar to Gap1p^{A297V} – selectively amino acid insensitive. Because basic and neutral amino acids are most concentrated in the vacuole (Messenguy et al., 1980; Klionsky et al., 1990), our model predicts that these amino acids will be most effective at directing Gap1p sorting. As acidic amino acids are not abundant in the vacuole, recycling of Gap1p may be more efficient when cells are shifted from casamino acids to aspartate, than from casamino acids into arginine.

Transport of amino acids and other metabolites across the vacuolar membrane requires a proton gradient. Accordingly, the vacuolar lumen is more acidic (pH 6) than the cytosol (Graham et al., 2003). The vacuolar-ATPase complex maintains the proton gradient across the vacuolar membrane. It is made up of two subcomplexes, V_1 and V_0, which are composed of eight and five subunits respectively (Nishi and Forgac, 2002). In

the aforementioned deletion collection screen, five components of the V-ATPase, as well as three assembly factors, were found to cause increased Gap1p activity when deleted (Table 1; Rubio-Texeira and Kaiser, unpublished data). We hypothesized that the loss of the V-ATPase would cause a decrease in endosomal amino acid pools due to neutralization of the MVE lumen, resulting in increased Gap1p recycling to the plasma membrane. We therefore expected that these *vma* mutants would not properly sort Gap1p to the vacuole in response to rising amino acid levels. However, in *vma10*Δ cells cultured in amino acids, Gap1p-GFP was localized to the vacuolar membrane (Figure 1). The V-ATPase has been implicated in membrane fusion at the vacuole (Peters et al., 2001). Therefore, it appears that like mutants of the ESCRT complex, V-ATPase mutants increase Gap1p sorting to the plasma membrane in amino acid-free media because Gap1p is unable to enter the vacuole. When amino acids are present, however, Gap1p is prevented from either entering the vacuolar lumen or recycling to the Golgi.

Because Gap1p sorting in response to amino acids is determined at the endosomal membrane, demonstration that the amino acid content of the MVE is a factor in the propensity of Gap1p to avoid vacuolar sorting is important to verify the conformational switch model of Gap1p sorting. If mutants can be identified that render wild type Gap1p insensitive to increasing amino acids, this would also provide a useful tool to examine *trans*-acting Gap1p sorting mutants whose functions are still ill defined. We would expect that such mutants would be epistatic to any Gap1p sorting mutant that acts indirectly by raising amino acid levels, for example *mks1*Δ. These mutants may thus provide a valuable tool to expand our understanding of Gap1p trafficking.

Transport mechanism of Gap1p

Because the structure of Gap1p has not been solved, information about its catalytic mechanism has thus far been inferred from genetic manipulation. Here I described the identification of two classes of Gap1p catalytic mutants. The first class was defined by an inability to transport basic amino acids. Although much of the work presented here focused on characterization of Gap1p^{A297V}, I confirmed that all five citrulline-resistant, glycine-sensitive mutants were also deficient in uptake of lysine and arginine. Citrulline and arginine were chosen for the screen based on their extreme toxicity to *bul1*Δ*bul2*Δ strains expressing P_{ADH1}-*GAP1,* as well as their difference in size (Risinger et al., 2006). That all the mutants isolated had the exact same phenotype was unexpected, but suggests that choosing different amino acid pairs could result in the isolation of different selective mutants. Threonine, cysteine, lysine, and isoleucine were found to be similarly toxic to citrulline and glycine, making them the most suitable for plate-based assaying. While a selection for lysine-resistant colonies would likely result in the isolation of similar mutants to those described here, one could imagine that selection for threonine or isoleucine-resistant colonies could result in different classes of selective mutants.

We were surprised that although Gap1p^{A297V} cannot transport citrulline or arginine, it nonetheless retains the ability to bind them. Ala297 is in the 6th transmembrane domain (TMD) of Gap1p. In the recently solved structure of arginine-bound AdiC, residues in the 6th TMD interacted with the substrate molecule via hydrogen bonds to the α-amino group of arginine (Gao et al., 2010). The fact that A297V specifically blocks basic amino acid uptake, and that another mutation at this position, A297P, blocks all amino acid uptake (Appendix II), suggests either that the 6th TMD of

Gap1p interacts with the R group of the substrate molecule, or that mutation of Ala297 affects the ability of residues in other TMDs to interact with the substrate R group. For example, A297V may not be able to complete the transport cycle with citrulline or arginine because the mutation shifts the position of a basic residue in Gap1p that causes charge repulsion with the substrate molecule. In efforts to determine if the A297V mutation could be compensated for by second site mutations, I performed a pilot screen of random mutants generated by error-prone PCR with $GAP1^{A297V}$ as a template and screened for citrulline sensitivity. However, the strain background I used contained the *gap1::LEU2* disruption made by Magasanik and colleagues that contains the latter half of the endogenous *GAP1* gene (Stanbrough and Magasanik, 1995). As a result, the isolates from the pilot screen contained plasmids that had recombined with the *GAP1* locus to reverse the A297V mutation. This could be corrected very simply in the future by using a complete gene disruption of *GAP1*, as is employed by the S288C deletion collection (Brachmann et al., 1998).

I also described the isolation of a catalytically inactive mutant, $Gap1p^{T106K}$, based on sequence similarity of yeast transporters to the *E. coli* APC protein AdiC (Gao et al., 2009; Gao et al., 2010). The GSG motif that makes up the unwound section in TMD 1 of AdiC corresponds to a conserved GTG motif in at least five yeast transporters, including Gap1p. In contrast to the strong conservation of this section in TMD1, the unwound section of TMD 6, GVESA in AdiC, is less similar among yeast transporters although the center glutamate residue (Glu300 in Gap1p) is conserved. Gao et al. (2009) suggested that this residue changes protonation states during the transport cycle due to alternate exposure to the acidic environment of the stomach and the less acidic intracellular

environment. If so, the corresponding residue in Gap1p could be responsible for proton symport. I generated a Glu300Lys *GAP1* allele and found that although this mutant had extremely low citrulline and glycine uptake, this was due to the fact that the mutant was misfolded and retained in the ER (Figure 2). Thus it is more likely that Glu300 is required for maintaining the structural integrity of Gap1p and may not participate in substrate recognition or transport. Notably, in a later structure of AdiC, it was observed that Glu 208 is in fact not exposed to the periplasm, and therefore exists in a non-protonated state throughout the transport cycle (Gao et al., 2010).

Conformational states of Gap1p

In Chapter 2, we proposed that amino acids induce a conformational change in Gap1p that prevents its recycling from the MVE and thus leads to degradation in the vacuole. Further, the selective mutant Gap1p^{A297V} and the inactive mutant Gap1p^{T106K} were insensitive to amino acids because they lack the ability to make the necessary conformational shift. If conformational state is the determining factor in Gap1p regulation, not all "inactive" mutants may be sorted in the same way. For instance, an inactive mutant locked in an inward facing state would presumably be sorted to the vacuole even in the absence of amino acids. Alternatively, some mutations may block amino acid uptake without affecting the ability of the protein to assume either conformational state.

Concurrently with the directed mutagenesis I described to isolate Gap1p^{T106K}, I screened for catalytically inactive mutants by a random mutagenesis approach similar to the screen that identified selective mutants. In this screen, untagged Gap1p and Gap1p-

GFP mutants were screened for citrulline and glycine resistance. Although the majority of mutants were not expressed or unable to exit the ER and reach the plasma membrane, two mutants able to reach the plasma membrane were isolated, Gap1p^{W179R} and Gap1p^{S388P}. These mutations are in TMD3 and TMD8 respectively. TMD3 and TMD8 have also been suggested to participate in amino acid binding (Singh et al., 2008; Gao et al., 2010). Similarly to Gap1p^{T106K}, both Gap1p^{W179R} and Gap1p^{S388P} were deficient in uptake of all amino acids tested. To determine if these mutants were sorted to the vacuole by amino acids, I localized them by fluorescence microscopy in an *end3*Δ strain, as described in Chapter 2. Interestingly, these mutants displayed an intermediate phenotype, in which protein was observed both at the plasma membrane and in the vacuole, suggesting that response to amino acids was reduced, but not eliminated (Figure 3). The reason why these mutants have little measurable catalytic activity, yet retain some ability to respond to amino acid levels is still unclear. Activity based assays such as those described here are not informative for these mutants, making it difficult to identify their specific defect as was done for Gap1p^{A297V}. A more direct method for measuring conformational state needs to be employed.

We have not yet developed an experiment to test if amino acids induce a conformational change in Gap1p, in no small part due to the difficulty of performing biochemical assays with Gap1p. Like all multispanning membrane proteins, Gap1p is extremely hydrophobic, making it difficult to purify it in its native folded state. Expression of eukaryotic transporter proteins in heterologous systems has proved challenging (Tate et al., 2003), as has solublization of proteins after extraction (Talvenheimo and Rudnick, 1980). Thus, purification of Gap1p from yeast offers the

best chance to obtain functional protein. Efforts are underway in our laboratory to isolate Gap1p in proteoliposomes, with the goal of performing in vitro experiments (O'Malley, Cain, and Kaiser, unpublished data).

Another possibility for determining the conformational state of Gap1p could use cell surface labeling of Gap1p to determine the accessibility of the amino acid binding pocket. One commonly used technique involves labeling cysteine residues with thiol-modifying reagents, the idea being that cysteine residue positioned in or near the amino acid binding pocket will be accessible to modification when the protein is outward-facing, but not accessible when inward-facing. A biotinylated methanethiosulfonate (MTS) derivative, MTSEA-biotin, was used to demonstrate a conformational change in the first extracellular loop of Ste2p (Hauser et al., 2007). Wild type Gap1p has six cysteine residues, although control experiments I performed to determine if any are accessible to MTSEA biotinylation were inconclusive. Cysteine modification of cell surface proteins is often inefficient in yeast due to the cysteine-rich cell wall. It was reported that citrulline uptake of an S388C mutant of Gap1p is inhibited by treatment with non-biotinylated MTSEA (Van Zeebroeck et al., 2009). Nevertheless, I could not verify that this mutant was labeled with MTSEA biotin. Because the previous study never directly verified that S388C was modified, it is possible that the effect on citrulline uptake by MTSEA treatment was indirect. Another possibility is that while MTSEA can access S388C, MTSEA-biotin cannot, suggesting that another thiol-modifying reagent could be used. Kaback and colleagues have used [^{14}C]-labeled N-ethylmaleimide (NEM) to identify residues necessary for LacY activity (Kaback et al., 2007). Similar experiments performed with Gap1p may therefore be more successful.

An alternative to modifying Gap1p with thiol-modifying reagents would be to design a conformational probe that resembles a Gap1p substrate. Such a probe could be crosslinked to Gap1p after interacting with the amino acid binding site. The advantage of this approach is that it targets the amino acid binding pocket, whereas cysteine modification may not occur directly where amino acids bind. Analogs of leucine and methionine that contain a UV-activated diazirine motif have been used to crosslink interacting proteins in mammalian cells (Suchanek et al., 2005), and are now commercially available. However, a method to detect crosslinked Gap1p is needed. Transporter proteins such as Gap1p are presumed to bind one substrate molecule at a time, and the addition of one amino acid is unlikely to cause a significant gel mobility shift. Thus, further modification to photo-leu or photo-met would be necessary. The simplest option would be to radiolabel the probe, for example with [^{14}C], and detect crosslinked species by autoradiography. Alternatively, the probe could be modified with a tag, such as biotin. However, the tag would have to be attached to the R group, as I found by performing competition experiments with various glutamate analogs that modifications to the α-amine or α-carboxylate groups prevent Gap1p from binding the analog.

Response to Gap1p conformation

If amino acids signal a conformational change in Gap1p, the next question is which cellular processes are affected by this change. A conformational change in Gap1p could affect its interactions with the recycling machinery. In its inward facing conformation, Gap1p might not be recruited into recycling vesicles, whereas in the

outward-facing state, recruitment into vesicles might be enhanced. As the process of Gap1p recycling from the MVE is not fully understood, this hypothesis is difficult to test. It has been shown previously, and I have confirmed with the recycling assay described in Chapter 3, that Lst4p and Gtr1p are required for Gap1p recycling from the MVE (Gao and Kaiser, 2006; Rubio-Texeira and Kaiser, 2006). However, the role of Lst4p is not yet understood, and the data presented in Chapter 4 suggests that it may not be acting at the MVE. The role of the GSE complex in Gap1p sorting also appears to be complex and it may have multiple functions. Because it has been implicated in TOR signaling, mutations in the GSE complex may affect Gap1p sorting indirectly by altering cellular amino acid levels (Dubouloz et al., 2005; Binda et al., 2009). Thus, further study of the mechanism of Gap1p recycling is needed to determine if this step could be affected by Gap1p conformation.

Alternatively, the conformational state of Gap1p may affect the rate of incorporation into intralumenal vesicles (ILV) by the ESCRT complex. In support of this idea is the observation that many of the ESCRT components were identified in a screen of the deletion collection for mutants with increased Gap1p activity. In SD (amino acid free) medium, a block in MVE formation causes increased recycling of Gap1p to the plasma membrane (Rubio-Texeira and Kaiser, 2006). In this scenario, one might imagine that recycling is a passive process that is much less efficient than ESCRT-mediated internalization. The prediction, then, is that high amino acids would have no effect on Gap1p sorting in an ESCRT mutant. This is not what we observe – in amino acids, ESCRT mutants cause Gap1p to be retained in enlarged endosomal structures collectively called the class E compartment (Rubio-Texeira and Kaiser, 2006). Therefore, either

recycling is not a constitutive process, or another step occurs between delivery to the MVE membrane and internalization by ESCRT that may be controlled by the conformation of Gap1p.

Deubiquitination is thought to be required for recycling of Gap1p to the plasma membrane because although Gap1p is ubiquitinated regardless of amino acid levels, poly-ubiquitinated Gap1p is not observed at the plasma membrane. Deubiquitination could therefore be the regulated step between recycling and internalization into MVEs by ESCRT. We could imagine that the conformational state of Gap1p could affect recruitment of the deubiquitinating enyme (DUB), or could affect the accessibility of the ubiquitin chains on the N-terminus of Gap1p. The deubiquitinating enzyme Ubp15p was recently identified as required for Gap1p trafficking (Costanzo et al., 2010), and recent work has shown that Ubp15p controls Gap1p ubiquitination levels. Ubp15p is also required for Gap1p recycling from the MVE, as measured in the assay described in Chapter 2 (Eluère and Kaiser, unpublished data). Future work will determine if amino acids regulate the deubiquitination of Gap1p by Ubp15p. It has been shown that Ubp15p interacts directly with Gap1p (Eluère and Kaiser, unpublished data). This interaction might only when Gap1p is in the recycling competent conformational state. This could explain why in ESCRT mutant strains, Gap1p is still retained internally in amino acids – Gap1p would presumably be in the recycling-blocked conformation, preventing deubiquitination by Ubp15p.

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Tables

Table 1: V-ATPase mutants that cause high Gap1p activity

Gene	Description*
VMA1	Catalytic Subunit A of the V_1 subcomplex
VMA6	Subunit d of the V_0 subcomplex
VMA7	Subunit F of the V_1 subcomplex
VMA10	Subunit G of the V_1 subcomplex
VMA11	Subunit c' of the V_0 subcomplex
VPH2/VMA12	ER localized assembly factor required for V-ATPase function
VMA21	ER localized assembly factor required for V-ATPase function
VMA22	ER localized assembly factor required for V-ATPase function

*Descriptions obtained from the *Saccharomyces* Genome Database (SGD)

Figures

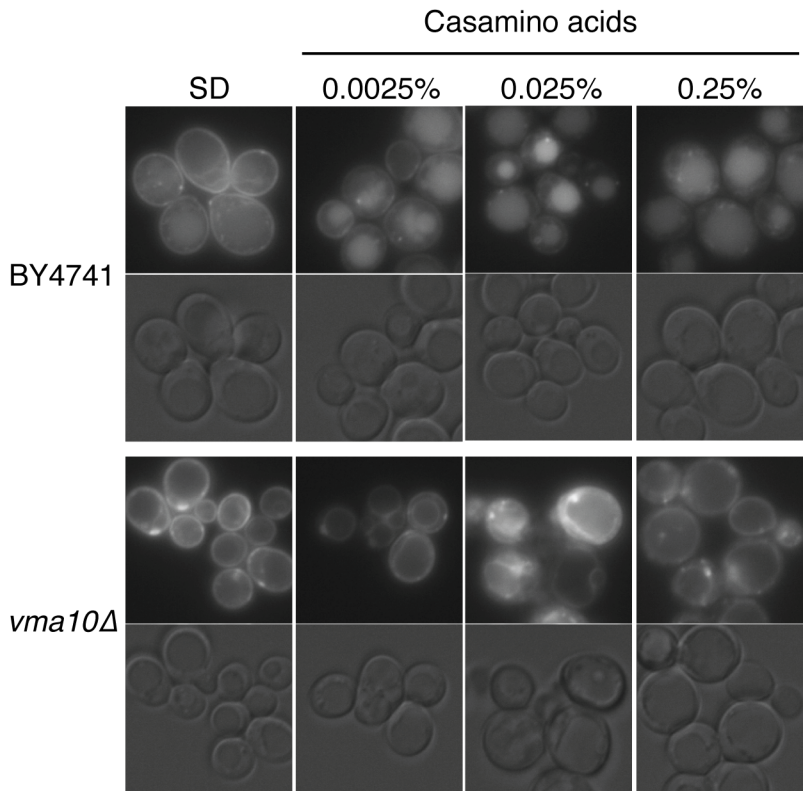

Figure 1. A V-ATPase mutant accumulates Gap1p on endosomal or vacuolar membranes in the presence of amino acids. Wild type (BY4741) or *vma10*Δ cells expressing P_{ADH1}-*GAP1*-*GFP* were cultured to early exponential phase in SD medium. Where indicated, casamino acids were added for two hours. Cells were imaged by fluorescence microscopy.

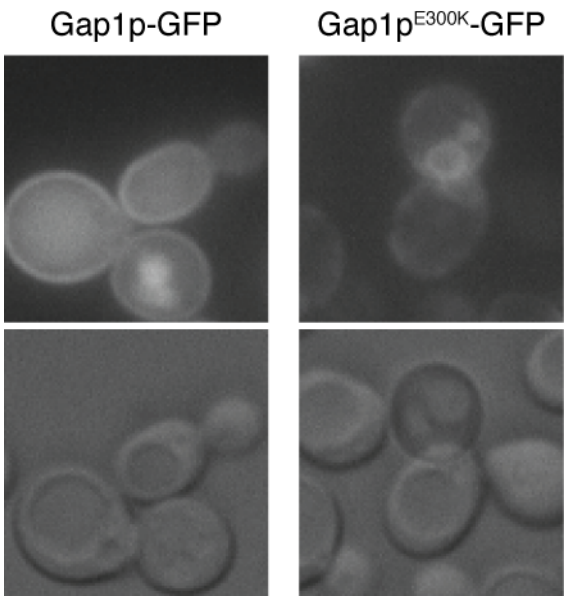

Figure 2. Gap1p^{E300K} is retained in the ER. P_{ADH1}-*GAP1* (ECY739) cells expressing *GAP1-GFP* (pAR13) or *GAP1*^{E300K}-*GFP* (pNC86) were cultured to early exponential phase in SD medium. Cells were imaged by fluorescence microscopy.

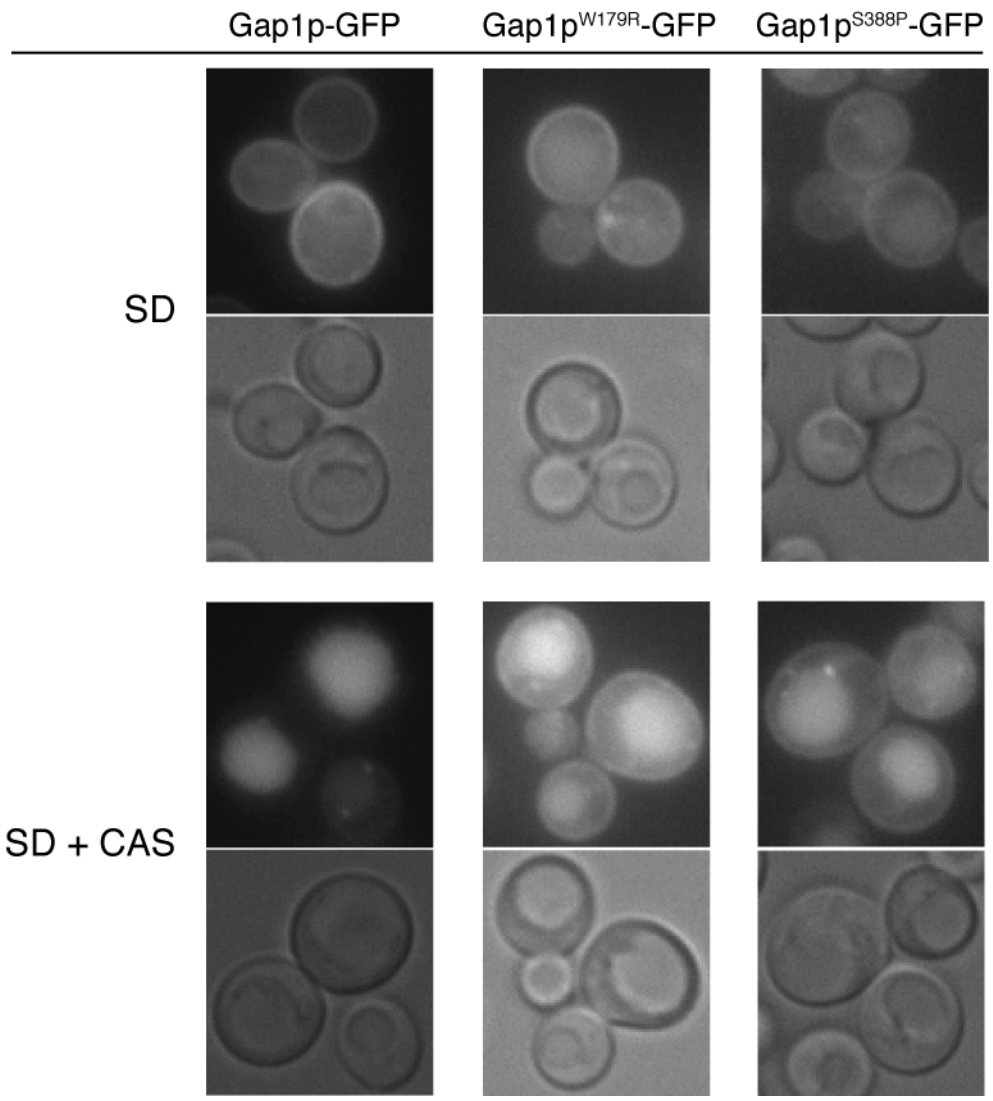

Figure 3. Two catalytically inactive mutants display an intermediate sorting phenotype. *gap1*Δ*end3*Δ (CKY1082) cells expressing P_{ADH1}-*GAP1*-*GFP* (pAR13), P_{ADH1}-*GAP1*^{W179R}-*GFP* (pNC19), or P_{ADH1}-*GAP1*^{S388P}-*GFP* (pNC67) were grown to early exponential phase in SD and cultured for an additional three hours in SD alone or SD with 0.025% casamino acids. Cells were imaged by fluorescence microscopy.

Appendix I

Activity-Dependent Reversible Inactivation of the General Amino Acid Permease

Preface

This appendix was previously published as: April L. Risinger, Natalie E. Cain, Esther J. Chen, and Chris A. Kaiser. Activity-dependent reversible inactivation of the general amino acid permease. Mol. Biol. Cell. 2006 Oct;17(10):4411-4419.

This work describes the screen to identify selective Gap1p mutants. The initial characterizations are shown in Figure 9.

Abstract

The general amino acid permease, Gap1p, of *S. cerevisiae* transports all naturally occurring amino acids into yeast cells for use as a nitrogen source. Previous studies have shown that a non-ubiquitinateable form of the permease, Gap1p^{K9R,K16R}, is constitutively localized to the plasma membrane. Here, we report that amino acid transport activity of Gap1p^{K9R,K16R} can be rapidly and reversibly inactivated at the plasma membrane by the presence of amino acid mixtures. Surprisingly, we also find that addition of most single amino acids is lethal to Gap1p^{K9R,K16R} expressing cells, whereas mixtures of amino acids are less toxic. This toxicity appears to be the consequence of uptake of unusually large quantities of a single amino acid. Exploiting this toxicity, we isolated *gap1* alleles deficient in transport of a subset of amino acids. Using these mutations, we show that Gap1p inactivation at the plasma membrane does not depend on the presence of either extracellular or intracellular amino acids, but does require active amino acid transport by Gap1p. Together, our findings uncover a new mechanism for inhibition of permease activity in response to elevated amino acid levels and provide a physiological explanation for the stringent regulation of Gap1p activity in response to amino acids.

Introduction

The yeast *Saccharomyces cerevisiae* imports amino acids from the surrounding medium through a set of amino acid permeases (Sophianopoulou and Diallinas 1995). The activity of many amino acid permeases is regulated by the quantity of available amino acids as well as the quality of the nitrogen source. Genes encoding several amino acid permeases, such as *AGP1*, *GNP1, BAP2, BAP3, DIP5, TAT1,* and *TAT2* are transcriptionally induced by the presence of extracellular amino acids (Didion, Regenberg et al. 1998; Iraqui, Vissers et al. 1999). This transcriptional induction requires the extracellular SPS amino acid sensor that proteolytically activates the transcription factor Stp1p in the presence of amino acids (Forsberg and Ljungdahl 2001; Andreasson and Ljungdahl 2002). There is also evidence that the Tat2p and Bap2p permeases are regulated post-translationally such that these permeases are only present at the plasma membrane when their substrate amino acids are available for import from the extracellular medium (Beck, Schmidt et al. 1999; Omura, Kodama et al. 2001).
The subset of permeases induced by elevated amino acid levels include both specific and general amino acid transporters, but all have a relatively low capacity for transport. In contrast, the activity of the general amino acid permease (*GAP1*), which is responsible for the high capacity transport of all naturally occurring amino acids for use as a nitrogen source, is repressed by amino acids both transcriptionally and posttranscriptionally through a sorting process in the late secretory pathway (Grenson, Hou et al. 1970; Chen and Kaiser 2002).

GAP1 is transcribed by two GATA transcription factors: Gln3p, which is repressed in glutamine or ammonia medium and Nil1p, which is repressed by elevated levels of glutamate or any other amino acid (Stanbrough, Rowen et al. 1995; Magasanik

and Kaiser 2002) (Risinger and Kaiser, unpublished data). Therefore, the *GAP1* gene product is produced when the nitrogen source in the growth medium is poor, or when total intracellular amino acid levels are low.

Gap1p is an integral membrane protein that is transported through the secretory pathway to the *trans*-Golgi. At the *trans*-Golgi, Gap1p is either delivered to the plasma membrane, where it can import amino acids from the medium, or sent to the vacuole for degradation. Poly-ubiquitination of Gap1p by the Rsp5p-Bul1p-Bul2p ubiquitin ligase complex at the *trans*-Golgi is required for targeting of the permease to the prevacuolar endosome; mutation of the ubiquitin ligase machinery (*bul1Δ bul2Δ*) or the ubiquitinated lysine residues of Gap1p ($GAP1^{K9R,K16R}$) results in constitutive plasma membrane localization of Gap1p (Helliwell, Losko et al. 2001; Soetens, De Craene et al. 2001). When Gap1p is ubiquitinated and sorted to the prevacuolar endosome, it can recycle back to the *trans*-Golgi for another attempt to reach the plasma membrane; it is this recycling step that is blocked by the presence of amino acids (Chen and Kaiser 2002; Rubio-Texeira and Kaiser 2006). Therefore, elevated internal amino acid levels cause any expressed Gap1p to be sorted to the vacuole, resulting in low amino acid transport through Gap1p (Stanbrough and Magasanik 1995; Chen and Kaiser 2002). However, when internal amino acid levels are scarce, Gap1p is able to reach the plasma membrane where it can scavenge any available amino acids in the medium through high affinity transport.

We were interested in finding a physiological rationale for Gap1p repression in response to elevated internal amino acid levels given that the activity of most other amino acid permeases is induced by amino acids. Surprisingly, when we explored the

physiological consequences of disrupting Gap1p regulation in response to amino acids, we discovered a novel mechanism of amino acid-dependent repression of Gap1p activity: rapid and reversible inactivation of amino acid transport through the permease at the plasma membrane. Interestingly, we also found that exposure of cells expressing Gap1p^{K9R,K16R} to any one of a number of individual amino acids caused a rapid cessation of growth and a loss of viability despite the ability of the permease to be inactivated at the plasma membrane.

Materials and Methods

Strains, plasmids, and media

All of the yeast strains used in this study are of the S288C background that expresses high Gap1p activity in minimal ammonia medium (Courchesne and Magasanik 1983).

The integrated *GAP1* strains were constructed by ligating a 500 bp fragment containing 5' *GAP1* sequences (-1149 to –747 upstream of ATG) followed by the *kanMX6* cassette in front of either the wild-type *GAP1* or *ADH1* promoter and the *GAP1* ORF (tagged version from pPL257 (Ljungdahl, Gimeno et al. 1992)) in pRS316. The plasmid was cleaved in the 5' sequence with *EagI* and within the *GAP1* ORF with *Bsu36I* and transformed into either wild-type or *sec6-4* strains where the fragment homologously recombined with the endogenous *GAP1* allele, replacing it with the new version and inserting the *kanMX6* gene upstream of the promoter. Lysine mutants were constructed in a similar manner by performing Stratagene Quick-Change site directed mutagenesis on the above plasmids to introduce the lysine mutations prior to homologous recombination. Plasmids used in this study were pAR70, *GAP1* under its own promoter in a *URA3-CEN* vector; pAR73, $GAP1^{K9R,K16R}$ under its own promoter in a *URA3-CEN* vector; pEC221, *ADH1* promoted *GAP1* in a *URA3-CEN* vector; pAR1, *ADH1* promoted $GAP1^{K9R,K16R\text{-}HA}$ in a *URA3-CEN* vector; pAR13, *ADH1* promoted *GAP1-GFP* in a *URA3-CEN* vector; pAR14, *ADH1* promoted $GAP1^{K9R,K16R\text{-}GFP}$ in a *URA3-CEN* vector; pNC3, *ADH1* promoted $gap1^{V363G}$ in a *URA3-CEN* vector; pNC4, *ADH1* promoted $gap1^{L185V}$ in a *URA3-CEN* vector; pNC5, *ADH1* promoted $gap1^{A497V}$ in a *URA3-CEN* vector; pNC6, *ADH1* promoted $gap1^{A365V,T590A}$ in a *URA3-CEN* vector; pNC7, *ADH1* promoted $gap1^{A297V}$ in a *URA3-CEN* vector; and pNC8, *ADH1* promoted $GAP1^{K9R,K16R,A297V\text{-}HA}$ in a

URA3-CEN vector.

Minimal (SD) medium is composed of Difco yeast nitrogen base without amino acids and without ammonium sulfate, 2% glucose, 0.5% ammonium sulfate (adjusted to pH 4.0 with HCl). Individual amino acid stocks were made at 40-200 mM in SD medium at pH 4.0, filter sterilized, and stored at 4°C. Casamino acid medium contains SD with Casamino acids (Difco) added from a 10% stock (pH 4.0) to a final concentration of 0.25% or 0.0025%.

Screen for Gap1p transport mutants

GAP1 mutations were generated by mutagenic PCR using pEC221 (P_{ADH1} *GAP1)* as a template and methods described previously (Sevier and Kaiser 2006) with modifications. A fragment including the entire *GAP1* ORF as well as 500 bp of the *ADH1* promoter and 800 bp of the *GAP1* 3' UTR was amplified in four 50 µl reactions with AmpliTaq Gold (Perkin Elmer) and 0.1mM $MnCl_2$. PCR products were transformed along with gapped pEC221 plasmid (lacking the *GAP1* ORF) into CKY701 (*bul1Δ bul2Δ gap1Δ ura3-52*) and gap repaired plasmids were isolated by selection for Ura+ transformants. Citrulline resistant transformants were identified by replica plating onto SD with 4 mM citrulline at 30°C. Resistant clones were then tested for sensitivity to glycine by replica plating to SD with 1mM glycine. Plasmids were isolated from citrulline resistant, glycine sensitive colonies, retransformed into CKY701, and retested for citrulline and glycine sensitivity. Plasmids conferring resistance to citrulline and sensitivity to glycine arose at a frequency of about 10^{-3}.

Amino acid uptake assays

Strains were cultured to 4-8 x 10^6 cells/ml, subjected to indicated treatment, and washed with nitrogen-free medium by filtration on a 0.45-µm nitrocellulose filter before amino acid uptake assays were performed as described previously (Roberg, Rowley et al. 1997). The specific activity of glycine was about 112 mCi/mmol.

Amino acid accumulation assays

GAP1^{K9R,K16R} (CKY893) was cultured at a concentration of 5x10^6 cells/ml in minimal SD medium and distributed into 1ml aliquots. [^{14}C]-glycine, [^{14}C]-lysine, [^{14}C]-threonine, or [^{14}C]-lysine were added either alone or in combination with the other three unlabeled amino acids to a final concentration of 1mM. The total accumulated radiolabeled amino acid was measured after 20 minutes.

Fluorescence Microscopy

Strains expressing P_{ADH1}-*GAP1*-*GFP* or P_{ADH1}-*GAP1*^{K9R,K16R}-*GFP* were cultured overnight in minimal SD medium to exponential phase at 24°C. Cells were harvested, resuspended in 300mM Tris pH8 with 1.5% NaN_3 and visualized using a fluorescence microscope. Images were captured with a Nikon E800 microscope (Melville, NY) equipped with a Hamamatsu digital camera (Bridgewater, NJ). Image analysis was performed using Improvision OpenLabs 2.0 software (Lexington, MA).

Equilibrium Density Centrifugation and Antibodies

Yeast membranes were fractionated by equilibrium density centrifugation on continuous 20-60% sucrose gradients containing EDTA as described (Kaiser, Chen et al.

2002). Antibodies used were: rabbit anti-Gap1p; rabbit anti-Pma1p (gift of S.Losko and R.Kolling, Dusseldorf, Germany); and horseradish peroxidase-coupled sheep anti rabbit (Amersham Pharmacia).

Results

Amino acids can inactivate Gap1p at the plasma membrane

To determine the physiological consequences of unregulated Gap1p activity, we expressed from the constitutive *ADH1* promoter a mutant of *GAP1* lacking ubiquitin acceptor sites (P_{ADH1}-$GAP1^{K9R,K16R}$), which is constitutively delivered to the plasma membrane (Soetens, De Craene et al. 2001; Chen and Kaiser 2002). As a control to show that this mutant no longer responded to high intracellular levels of amino acids we found Gap1p-dependent citrulline uptake activity of P_{ADH1}-$GAP1^{K9R,K16R}$ remained high in an *mks1*Δ strain (Figure 1). *MKS1* is a negative regulator of the Rtg1/3 transcription factors that are responsible for synthesis of α-ketoglutarate, an amino acid precursor (Dilova, Chen et al. 2002; Sekito, Liu et al. 2002) and *mks1Δ* strains have elevated internal amino acid concentrations sufficient to cause Gap1p to be sorted to the vacuole in a wild type cell (Figure 1) (Chen and Kaiser 2002; Rubio-Texeira and Kaiser 2006). The finding that the localization and activity of $Gap1p^{K9R,K16R}$ was not perturbed by elevated internal amino acid levels supports the hypothesis that Gap1p must first be ubiquitinated before sorting can be regulated by amino acids levels.

In contrast to the situation when internal amino acid levels were raised by an *mks1Δ* mutation, when a rich mixture of amino acids (0.25% Casamino acids) was added exogenously to a strain expressing P_{ADH1}-$GAP1^{K9R,K16R}$, amino acid import through $Gap1p^{K9R,K16R}$ was very low (Figure 1). We found $Gap1p^{K9R,K16R}$ localized to the plasma membrane both by fractionation and fluorescence microscopy in the presence of Casamino acids (Figures 2A and B), suggesting that exogenously added amino acids could inactivate amino acid import through Gap1p that resided at the plasma membrane. To explore this possibility further, we followed the change in $Gap1p^{K9R,K16R}$ activity with

time after the addition of 0.25% Casamino acids to the medium. Immediately after amino acid addition, amino acid import through Gap1p^{K9R,K16R} remained high, indicating that exogenously added Casamino acids were not simply blocking [^{14}C]-citrulline uptake by competitive inhibition (Figure 3A). The rate of [^{14}C]-citrulline uptake through Gap1p^{K9R,K16R} decreased with time, such that one hour after amino acid addition Gap1p activity had decreased to less than 10% the starting activity (Figure 3A). We conclude that the presence of extracellular amino acids was sufficient to inactivate amino acid transport through plasma membrane localized Gap1p, uncovering a new and distinct mechanism of amino acid dependent down-regulation of Gap1p activity.

Amino acids differentially affect Gap1p sorting and inactivation

To further characterize the relationship between different types of amino acid dependent regulation of Gap1p, we compared the relative responses of intracellular sorting of Gap1p and inactivation at the plasma membrane to different concentrations of amino acids. We found that a relatively low concentration of amino acids (0.0025% Casamino acids) was unable to inactivate Gap1p^{K9R,K16R} at the plasma membrane, but was sufficient to cause wild-type Gap1p to be sorted to the vacuole (Figure 3B). However, higher concentrations of amino acids (such as 0.25% Casamino acids) could cause inactivation of Gap1p^{K9R,K16R} at the plasma membrane and sorting of wild-type Gap1p to the vacuole (Figure 3A). Therefore, it appears that the intracellular sorting of Gap1p and inactivation of the permease at the plasma membrane depend on distinct mechanisms for sensing amino acid abundance since they exhibit different sensitivities to the concentration of exogenous amino acids.

Amino acid dependent inactivation of Gap1p is reversible

To determine whether the inactivation of Gap1p was reversible, we utilized a temperature sensitive *sec6-4* strain that blocks delivery of newly synthesized protein to the plasma membrane at its restrictive temperature (Novick, Field et al. 1980; Walworth and Novick 1987). When amino acids were added to *sec6-4* strains expressing wild-type Gap1p, we observed a rapid decrease in Gap1p activity (Figure 4A) that corresponded to a loss of the permease from the plasma membrane due to ubiquitin mediated endocytosis (Figure 4B). Twenty minutes after amino acid addition (a time when most of Gap1p had been removed from the plasma membrane) cells were shifted to 36°C for 10 minutes, washed, and resuspended in pre-warmed amino acid free medium at 36°C. We found that the temperature shift was sufficient to inhibit delivery of newly synthesized protein to the plasma membrane since no increase in Gap1p activity was observed after transfer to amino acid free medium (Figure 4A). When amino acids were added to *sec6-4* strains expressing Gap1pK9R,K16R, we also observed a rapid decrease in Gap1p activity (Figure 4A) even though this non-ubiquitinated form of Gap1p was not internalized (Figures 2A and B). Most importantly, the activity of Gap1pK9R,K16R regenerated after amino acids were washed from the medium even at the restrictive temperature for *sec6-4*, a condition that blocks Gap1p delivery to the plasma membrane by exocytosis (Figure 4A). The conclusion from these experiments is that inactivation of Gap1pK9R,K16R is reversible in the sense that permease that has been inactivated in the plasma membrane by the addition of exogenous amino acids will recover almost full activity once amino acids are withdrawn from the medium.

Unregulated uptake of individual amino acids rapidly inhibits yeast cell growth

As we have shown, when a complex mixture of amino acids was added to cells expressing Gap1p^{K9R,K16R} , the permease becomes inactive at the plasma membrane while cell growth remains normal. In contrast, addition of individual amino acids at a high concentration greatly inhibited growth of cells expressing constitutive forms of Gap1p. For example, strains bearing either *cis*-acting (*GAP1*^{K9R,K16R}) or *trans*-acting (*bul1Δ bul2Δ*) mutations that affect Gap1p ubiquitination were unable to grow in the presence of 1 mM glycine (Figure 5A). This inhibitory effect was not specific to glycine as 3mM addition of any amino acid except alanine or phenylalanine greatly inhibited the growth of *GAP1*^{K9R,K16R} (Table 2). Amino acid addition both inhibited cell growth and was cytotoxic, since less than one percent of Gap1p^{K9R,K16R} expressing cells were able to form colonies on medium lacking amino acids after incubation in 3 mM glycine for 22 hours (Table 2).

Complete growth inhibition of Gap1p^{K9R,K16R} expressing cells occurred within 30 minutes after addition of 1 mM glycine (Figure 5B). Interestingly, wild type strains showed a slight inhibition of growth thirty minutes after glycine addition, but then recovered to a normal growth rate at about the same time that it took for Gap1p activity to be downregulated by amino acids (Figure 6A and B). A *gap1Δ* strain showed no effect on growth after treatment with glycine, indicating that the transient growth inhibition of wild type strains was due to amino acid import through Gap1p (Figure 6C). Therefore, it appears that the ability of wild-type Gap1p to be efficiently ubiquitinated prevents wildtype cells from fully succumbing to amino acid induced toxicity (Chen and Kaiser

2002). From our findings, we determined that the regulation of Gap1p sorting in response to amino acids is required to protect cells from uptake of amino acids to lethal levels.

Amino acid mixtures relieve amino acid dependent toxicity

Before amino acid addition, Gap1p activity is approximately three-fold higher in Gap1p^{K9R,K16R} expressing strains than in wild type (Figure 1), suggesting that the susceptibility of *GAP1*^{K9R,K16R} to individual amino acid addition was due to the elevated initial rate of import in this strain due to the increased levels of Gap1p present at the plasma membrane. Indeed, we were able to abolish the glycine sensitivity of *GAP1*^{K9R,K16R} by pre-treatment with alanine (which is not toxic) for a period sufficient to cause Gap1p activity to decrease to wild type levels (Figures 7A and B). This finding supports the hypothesis that the sensitivity of *GAP1*^{K9R,K16R} to amino acids results from an increased initial rate of Gap1p activity, allowing accumulation of the amino acid to toxic levels before the permease can be fully inactivated.

We can envision three mechanisms by which amino acid import could be lethal to cells with high levels of Gap1p activity: (1) the flux of amino acids through Gap1p and corresponding proton flux could be toxic, (2) a high concentration of total intracellular amino acids could be toxic, or (3) the excess of a single intracellular amino acid could be toxic. We found that adding a mixture of the four most toxic amino acids (glycine, lysine, threonine, and citrulline) to *GAP1*^{K9R,K16R} greatly improved growth when compared to the addition of the amino acids individually even though equivalent levels of total amino acid were imported (Figure 8). Moreover, adding even more complex mixtures of amino acids (such as Casamino acids lacking the non-toxic amino acids alanine and phenylalanine) to

GAP1^{K9R,K16R} showed no toxic effect at all. Adding complex mixtures of amino acids to strains transcribing *GAP1*^{K9R,K16R} from the amino acid insensitive *ADH1* promoter also resulted in wild type growth, indicating that the viability of *GAP1*^{K9R,K16R} in amino acid mixtures was not due to a transcriptional effect (data not shown). The finding that mixtures of amino acids are less toxic than individual amino acids suggests that the amino acid-induced toxicity of *GAP1*^{K9R,K16R} is a consequence of a gross excess intracellular level of a single amino acid that may alter the balance of amino acid pools in the cell.

Amino acid transport is required for Gap1p inactivation

We took advantage of Gap1p sensitivity to individual amino acids in ubiquitination deficient strains to isolate *GAP1* mutations defective for the transport of specific amino acids. After mutagenesis, plasmid borne P_{ADH1}-*GAP1* was transformed into a *bul1Δbul2Δgap1Δ* strain and screened for mutants that were resistant to 4 mM citrulline, a concentration that is toxic to cells expressing wildtype Gap1p in a *bul1Δbul2Δ* background. Citrulline resistant clones were then counter screened for sensitivity to 1mM glycine, to eliminate mutants that had lost Gap1p activity altogether. Five *GAP1* alleles that conferred resistance to citrulline, but sensitivity to glycine were isolated. Four mutants contained single, but unique point mutations: Gap1p^{L185V}, Gap1p^{A297V}, Gap1p^{V363G}, and Gap1p^{A497V}, while one mutant contained two unique mutations: Gap1p^{A365V,T590A}. It was determined that each of these mutants retained the ability to transport [^{14}C]-glycine although ability to import [^{14}C]-citrulline was impaired to various extents (Figure 9A).

One of these mutants, Gap1p^{A297V}, which was deficient in citrulline uptake, was used to investigate whether active transport of amino acids through Gap1p was required for permease inactivation. To monitor inactivation independently of sorting, we introduced the Gap1p^{A297V} mutation into non-ubiquitinateable Gap1p^{K9R,K16R} to make Gap1p^{K9R,K16R,A297V}. We also utilized the amino acid arginine due to the structural similarity to citrulline, ability to enter yeast cells independently of Gap1p through the Can1 permease, and non-toxicity to Gap1p^{K9R,K16R} at low concentrations. Wild type cells import [^{14}C]-arginine through Gap1p as well as the arginine specific permease Can1 (Grenson, Mousset et al. 1966; Whelan, Gocke et al. 1979). Indeed, we found that although *gap1Δ* or *can1* mutants import arginine, the double *gap1Δcan1* mutant could not (Figure 9B). Similarly, when Gap1p$^{K9R,K16R,\ A297V}$ was the sole form of Gap1p expressed in a *can1* mutant, the strain was not able to import [^{14}C]-arginine, indicating that Gap1p$^{K9R,K16R,\ A297V}$ was defective for arginine import (Figure 9B). When we measured the ability of arginine to inactivate [^{14}C]-glycine import, we found Gap1p^{K9R,K16R,A297V} activity was unaffected by arginine while Gap1p^{K9R,K16R} activity dropped to less than five percent after an hour of arginine addition (Figure 9C). Since arginine is imported into both strains through the Can1 permease, the inability of Gap1p$^{K9R,K16R,\ A297V}$ to be inactivated upon arginine addition indicates that elevated external or internal arginine levels are insufficient to inactive the permease. Therefore, the amino acid dependent inactivation of Gap1p at the plasma membrane must require active transport of amino acids through the permease.

Discussion

The type and abundance of the available nitrogen source has been shown to govern the activity of Gap1p permease by regulating both transcription of the *GAP1* gene and intracellular sorting of the Gap1p protein (Stanbrough and Magasanik 1995; Stanbrough, Rowen et al. 1995; Chen and Kaiser 2002). Here we describe a third mode of regulation; activity dependent, reversible inactivation of permease activity at the plasma membrane. The key experiment that demonstrates inactivation is based a mutant form of Gap1p ($Gap1p^{K9R,K16R}$) that is constitutively expressed and trafficked to the plasma membrane. When amino acids are added to cells expressing $Gap1p^{K9R,K16R}$ the protein loses transport activity while remaining located in the plasma membrane. This inactivation is distinct from the well documented competitive inhibition of Gap1p transport of one amino acid by a different amino acid (Woodward and Cirillo 1977; Woodward and Kornberg 1981). Competitive inhibition occurs essentially instantaneously but requires the presence of the competing amino acid during the assay, whereas the inactivation we observe occurs with a half-time of about 20 minutes and can be assayed in the absence of a competing amino acid.

Two additional observations provide further insight into the mechanism of inactivation. Amino acids do not appear to inactivate the permease irreversibly, since after withdrawal of exogenous amino acids $Gap1p^{K9R,K16R}$ located in the plasma membrane regains activity with a half time of 20 minutes. Moreover, by evaluating a mutant of Gap1p that is defective for transport of arginine but not glycine we found that inactivation requires active amino acid transport through the Gap1p permease. From measurements of initial amino acid import rates and the half-time required for inactivation we estimate that an individual Gap1p permease is able to transport about

5,000 amino acid molecules before being inactivated. Together these results imply that inactivation involves some kind of reversible modification or conformation that occurs as part of the catalytic cycle.

It has previously been shown that Gap1p is de-phosphorylated upon glutamine addition to low-phosphate urea medium or upon ammonia addition to proline medium (Stanbrough and Magasanik 1995; De Craene, Soetens et al. 2001). We however failed to observe any change in permease mobility associated with the amino acid dependent inactivation or reactivation of the Gap1p^{K9R,K16R} by SDS-PAGE, suggesting that protein phosphorylation may not be involved in this process (data not shown). Additionally, it has been speculated that the manganese transporter, Smf1p, adopts a conformational change when bound to metal that influences trafficking of the permease since transport deficient Smf1p mutant proteins are unable to be redirected to the plasma membrane from internal compartments upon metal starvation (Liu and Culotta 1999). It is possible that a similar conformational change occurs to Gap1p upon amino acid transport that would alter the structure of the pore to impair further amino acid import. Additional mutational analysis and biochemical characterization of the Gap1p permease will be required to elucidate the exact mechanism of permease inactivation at the plasma membrane.

Interestingly, a similar type of substrate-induced inactivation may be involved in regulation of GLAST, a neuronal sodium-dependent glutamate transporter. GLAST is highly expressed in glial cells where it takes up extracellular glutamate and thus attenuates glutamate signaling between surrounding neurons (Gonzalez and Robinson 2004). Conditions that cause low GLAST-dependent glutamate import cause abnormally

high extracellular glutamate levels leading to neuronal cell death via excitotoxicity (Huguenard 2003). Preincubation of glial cells with glutamate or other transportable agonists can lead to a marked decrease in GLAST activity if sodium is present during the preincubation period, indicating that inactivation requires active glutamate transport (Gonzalez and Ortega 2000). It is further suggested that glutamate affects GLAST activity by altering the affinity and not the level of permease present at the plasma membrane as changes in the Km but not Vmax of GLAST activity are observed after glutamate preincubation (Gonzalez and Ortega 2000). Just as amino acids can regulate Gap1p activity by a variety of different mechanisms, glutamate not only inactivates GLAST permease in the plasma membrane, but also may influence the transcription and intracellular trafficking of GLAST (Lopez-Bayghen, Espinoza-Rojo et al. 2003; Gonzalez and Robinson 2004). The ability to study substrate induced, transport dependent permease inactivation in a genetically tractable yeast system should allow studies to elucidate a general understanding of the process of permease inactivation at the plasma membrane.

Our work with Gap1p^{K9R,K16R} also led to the surprising observation that cells expressing this hyperactive form of Gap1p are sensitive to addition of amino acids to the growth medium. Because these cells are more sensitive to individual amino acids than mixtures of amino acids we deduce that toxicity results from alterations in internal amino acid pools brought about by rapid uptake and internal accumulation of a single amino acid before the permease can be inactivated.

We considered the possibility that an overabundance of one amino acid may indirectly cause amino acid starvation by feedback regulation of amino acid biosynthetic

pathways. To test this idea we used *GCN4* as a reporter for amino acid starvation (Hinnebusch 2005). Previous studies showed that elevated internal levels of one amino acid can induce starvation for other amino acids; this starvation results in elevated *GCN4* expression (Niederberger, Miozzari et al. 1981; Hinnebusch 1984). However, we saw no increase in *GCN4* expression upon the addition of individual amino acids to $GAP1^{K9R,K16R}$, suggesting that the growth inhibition seen under these conditions is not a consequence of amino acid starvation (data not shown). As a control we found that 3-aminotriazole (a competitive inhibitor of histidine biosynthesis) induced *GCN4* expression in $GAP1^{K9R,K16R}$.

Another possibility we considered was that highly skewed internal amino acid pools could lead to frequent amino acid misincorporation into proteins. To test this idea we pre-treated $GAP1^{K9R,K16R}$ with cycloheximide to block all new protein synthesis prior to amino acid addition. Indeed, we found a three to five-fold increase in viability of $Gap1p^{K9R,K16R}$ expressing cells treated with cycloheximide before lysine addition (data not shown). Although this finding suggests protein synthesis may play a role in the sensitivity of cells to individual amino acid addition, it is important to note that approximately 90% of cells succumbed to toxicity even with cycloheximide treatment, indicating that the cause of toxicity may be more complex. We also used Hsp104 as a reporter for global protein misfolding (Sanchez, Taulien et al. 1992; Trotter, Kao et al. 2002). When single amino acids were added to $GAP1^{K9R,K16R}$, we failed to observe an increase in Hsp104 expression similar to that seen for misincorporated amino acid analogs such as L-azetidine-2-carboxylic acid (data not shown). Taken together these data suggest that further experimentation will need to be performed to determine whether

tRNA synthetase mischarging and protein misfolding play a role in amino acid induced toxicity.

Intracellular sorting of Gap1p appears to have evolved as a feedback mechanism to adjust Gap1p activity according to the quantity of amino acids in the cytoplasm. We propose that when internal amino acids are scarce, the high-affinity, low-specificity Gap1p permease acts as an efficient scavenger of amino acids in the extracellular environment. When cells encounter conditions of elevated external amino acids, transport of amino acids by Gap1p causes both inactivation of the permease at the plasma membrane and triggers sorting of newly synthesized Gap1p protein to the vacuole. Both effects act to limit the uptake of potentially toxic quantities of extracellular amino acids. Meanwhile, the cell can continue to take advantage of the nutrient rich situation without the threat of toxicity through the *SSY1*-dependent induction of low-affinity permeases that allow for a more controlled uptake of amino acids when they are readily available in the external medium. In this manner the cell can take full advantage of conditions ranging from amino acid shortage to abundance.

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Tables

Table 1. Strains (all are isogenic with S288C). All are from this paper unless noted

Strain	Genotype	Source
CKY482	MATa *gap1Δ::LEU2 leu2-3 ura3-52*	Kaiser strain collection
CKY443	MATa prototroph	Kaiser strain collection
CKY517	MATa *sec6-4*	Kaiser strain collection
CKY701	MATα *bul1Δ::kanMX6 bul2Δ::kanMX6 gap1Δ::LEU2 leu2-3 ura3-52*	Kaiser strain collection
CKY759	MATα P_{ADH1}-*GAP1-HA::kanMX6*	Kaiser strain collection
CKY763	MATα *mks1Δ::kanMX6* P_{ADH1}-*GAP1-HA::kanMX6*	Kaiser strain collection
CKY890	MATa P_{ADH1}-$GAP1^{K9R,K16R}$-*HA::kanMX6*	
CKY891	MATa *mks1Δ::kanMX6* P_{ADH1}-$GAP1^{K9R,K16R}$-*HA::kanMX6*	
CKY893	MATa $GAP1^{K9R,K16R}$-*HA::kanMX6*	
CKY833	MATa P_{ADH1}-*GAP1::kanMX6*	
CKY885	MATa P_{ADH1}-$GAP1^{K9R,K16R}$*::kanMX6*	
CKY895	MATa *sec6-4* $GAP1^{K9R,K16R}$*::kanMX6*	
CKY1024	MATa $GAP1^{K9R,K16R}$*::kanMX6*	
CKY1025	MATa *gap1D::LEU2 can1 leu2-3 ura3-52*	

Table 2. Most amino acids are toxic to *GAP1*^{K9R,K16R}

Amino Acid	Growth (% of SD alone)	Viability (% of SD alone)
None (SD)	1.00	1.00
Citrulline	0.01	0.01
Glycine	0.01	0.01
Lysine	0.01	0.01
Cysteine	0.01	0.01
Threonine	0.01	0.01
Isoleucine	0.02	0.01
Glutamate	0.02	0.04
Histidine	0.03	0.01
Tryptophan	0.04	0.04
Aspartate	0.06	0.25
Arginine	0.08	0.25
Proline	0.09	0.70
Valine	0.13	0.10
Methionine	0.16	0.70
Glutamine	0.18	0.70
Tyrosine	0.20	0.35
Serine	0.20	0.25
Leucine	0.34	0.35
Asparagine	0.35	0.70
Phenylalanine	0.80	0.80
Alanine	1.09	1.00

GAP1^{K9R,K16R} (CKY893) was grown to exponential phase in minimal ammonia media (SD) at 30° and 5x10^6 cells were cultured in SD with 3mM of the indicated amino acid. After 22 hours, growth was measured by optical density and viability was determined by counting the number of colonies formed by plating 10,000 cells onto minimal ammonia media without amino acids.

Figures

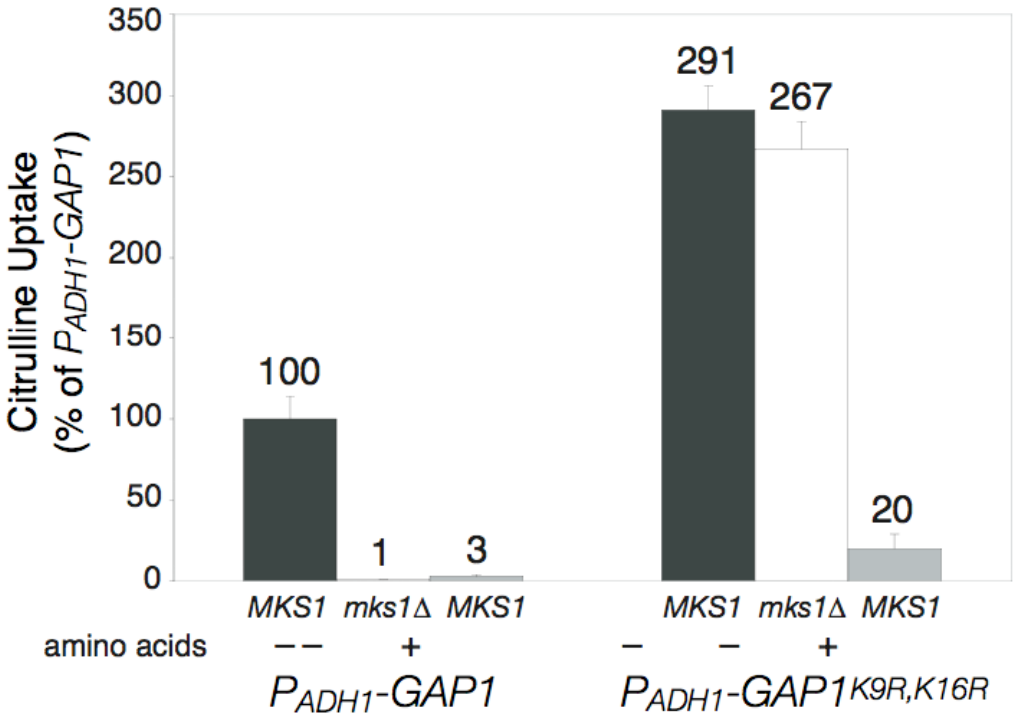


Figure 1. Gap1p that can not be ubiquitinated bypasses amino acid dependent sorting but can be inactivated by exogenous amino acids. *PADH1-GAP1* (CKY759), P_{ADH1}-*GAP1mks1Δ* (CKY763), P_{ADH1}-$GAP1^{K9R,K16R}$ (CKY890), or P_{ADH1}-$GAP1^{K9R,K16R}$ *mks1Δ* (CKY891) were grown in minimal ammonia medium (SD) or SD with the 0.25% Casamino acids. Gap1p activity was measured by assaying exponentially growing cells for the initial rate of [^{14}C]-citrulline uptake. (Three independent measurements were averaged).

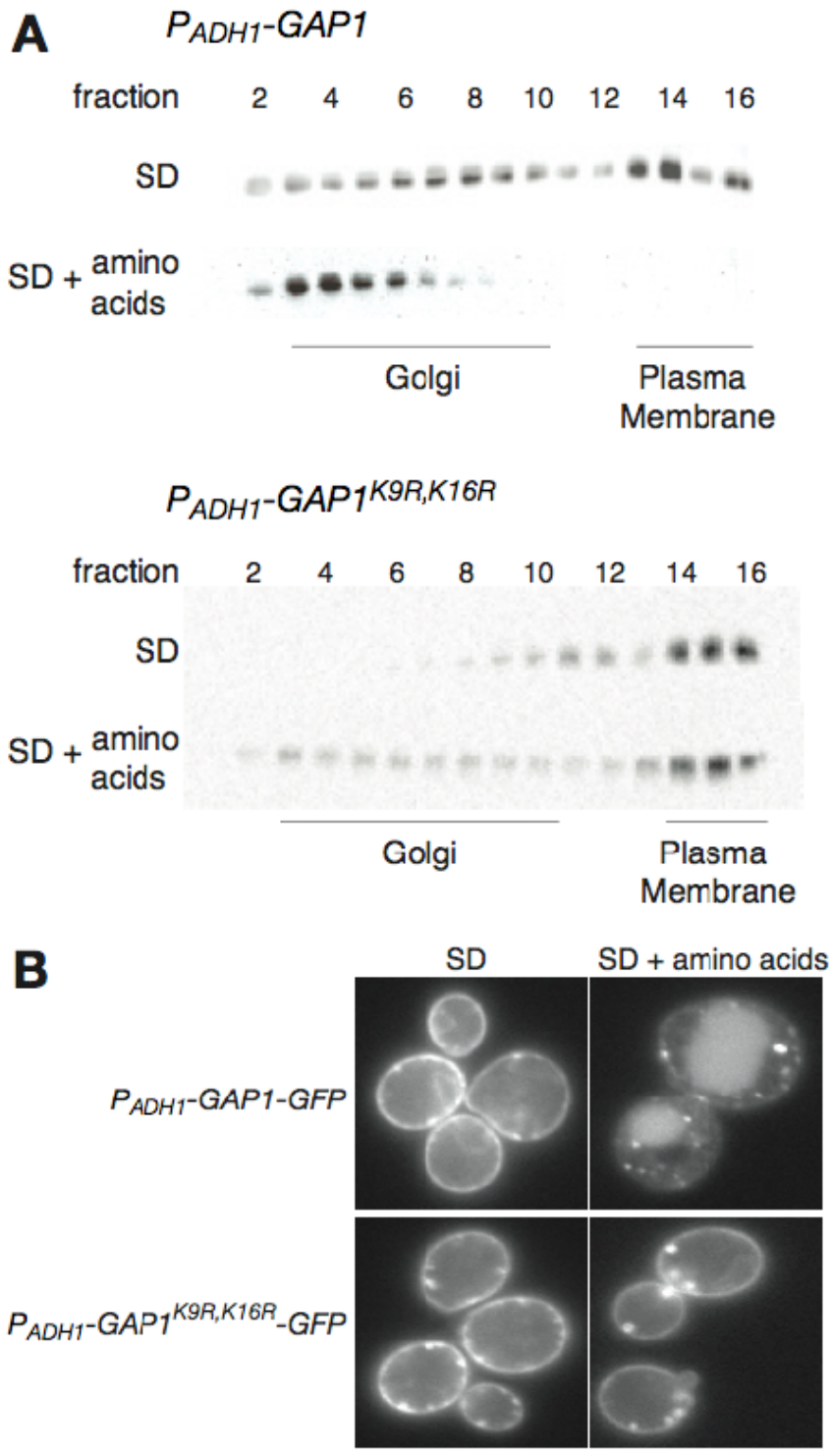
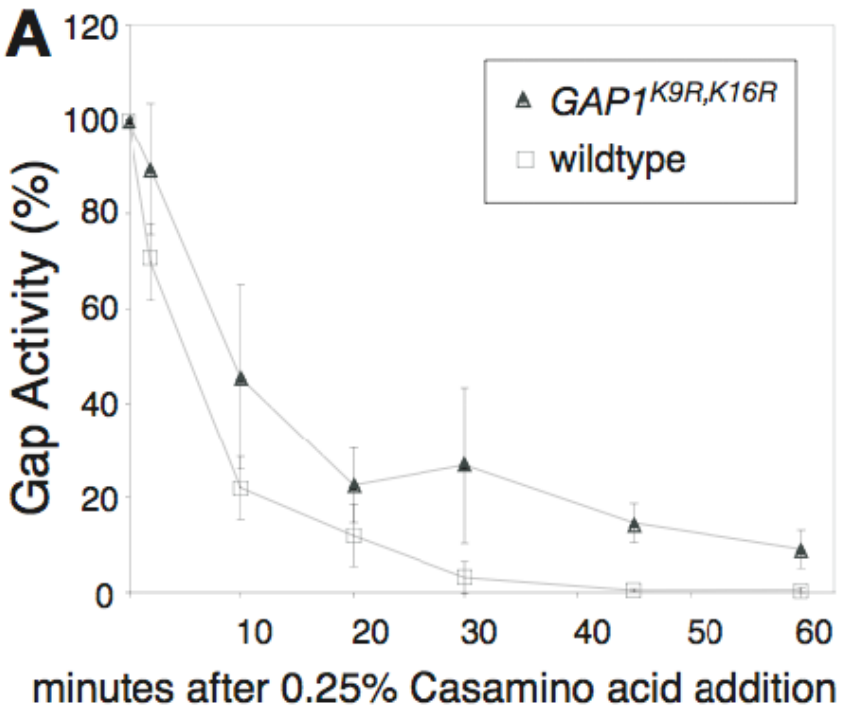

A
P_{ADH1}-GAP1
fraction 2 4 6 8 10 12 14 16
SD
SD + amino acids
Golgi
Plasma Membrane
P_{ADH1}-GAP1^{K9R,K16R}
fraction 2 4 6 8 10 12 14 16
SD
SD + amino acids
Golgi
Plasma Membrane
B
SD
SD + amino acids
P_{ADH1}-GAP1-GFP
P_{ADH1}-GAP1^{K9R,K16R}-GFP

Figure 2. Inactive Gap1p^{K9R,K16R} remains in the plasma membrane in the presence of amino acids. (A) Membranes from P_{ADH1}-*GAP1* (CKY759) or P_{ADH1}-*GAP1K9R,K16R* (CKY890) cell extracts were fractionated on 20-60% sucrose density gradients containing EDTA. Fractions were collected from the top of the gradients, proteins were separated by SDS/PAGE, and gradient fractions were immunoblotted with Gap1p antiserum. (B) *gap1Δ* strains (CKY482) were transformed with P_{ADH1}-*GAP1-GFP* (pAR13) or P_{ADH1}-$GAP1_{K9R,K16R}$-*GFP* (pAR14) and cultured in minimal SD medium alone or with 0.25% Casamino acids. GFP was imaged by epifluorescence microscopy.


A
120
100
80
60
40
20
0
Gap Activity (%)
GAP1K9R,K16R
wildtype
10
20
30
40
50
60
minutes after 0.25% Casamino acid addition

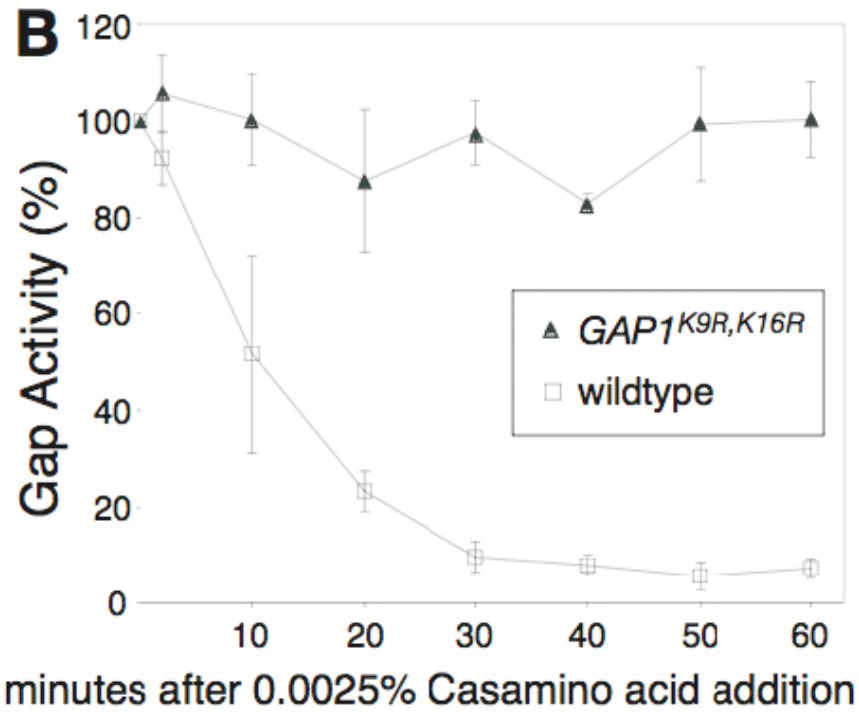

B
120
100
80
60
40
20
0
Gap Activity (%)
GAP1K9R,K16R
wildtype
10
20
30
40
50
60
minutes after 0.0025% Casamino acid addition

Figure 3. **Gap1p sorting and inactivation occur in response to distinct amino acid concentrations.** Wild type (CKY443) or $GAP1^{K9R,K16R}$ (CKY1024) were grown to exponential phase in SD medium. Gap1p activity was measured by assaying the initial rate of [^{14}C]-citrulline uptake at the indicated time after the addition of (A) a high (0.25%) or (B) a low (0.0025%) concentration of Casamino acids. (Three independent measurements were averaged).

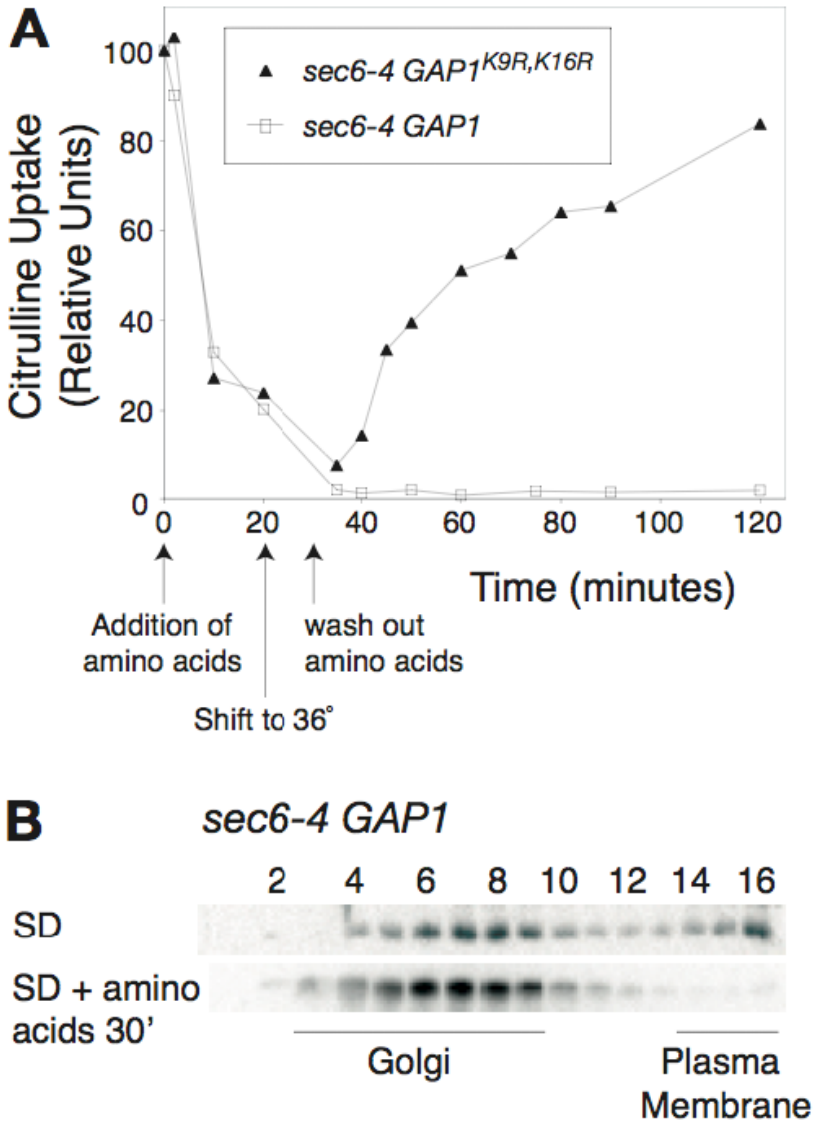

A
sec6-4 GAP1K9R,K16R
sec6-4 GAP1
Citrulline Uptake
(Relative Units)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (minutes)
Addition of
amino acids
wash out
amino acids
Shift to 36°
B
sec6-4 GAP1
2
4
6
8
10
12
14
16
SD
SD + amino
acids 30'
Golgi
Plasma
Membrane

Figure 4. Amino acid import through Gap1p^{K9R,K16R} is rapidly and reversibly inactivated upon amino acid treatment. (A) *sec6-4 GAP1*^{K9R,K16R} (CKY895) or *sec6-4* (CKY517) were grown in minimal SD medium at 24°C. Casamino acids were added to cells for 20 minutes after which cells were shifted to 36°C for 10 minutes. Cells were then washed and transferred to SD medium at 36°C. Gap1p activity was measured as the initial rate of [^{14}C]-Citrulline uptake at the indicated time after Casamino acid addition. (B) *sec6-4* (CKY517) was grown in minimal SD medium at 24°C. Lysates were prepared from cultures in SD or 30 minutes after the addition of 0.25% Casamino acids and fractionated on a 20-60% sucrose density gradient with EDTA. Fractions were collected from the top of the gradients, proteins were separated by SDS/PAGE, and gradient fractions were subjected to immunoblotting with Gap1p antiserum.

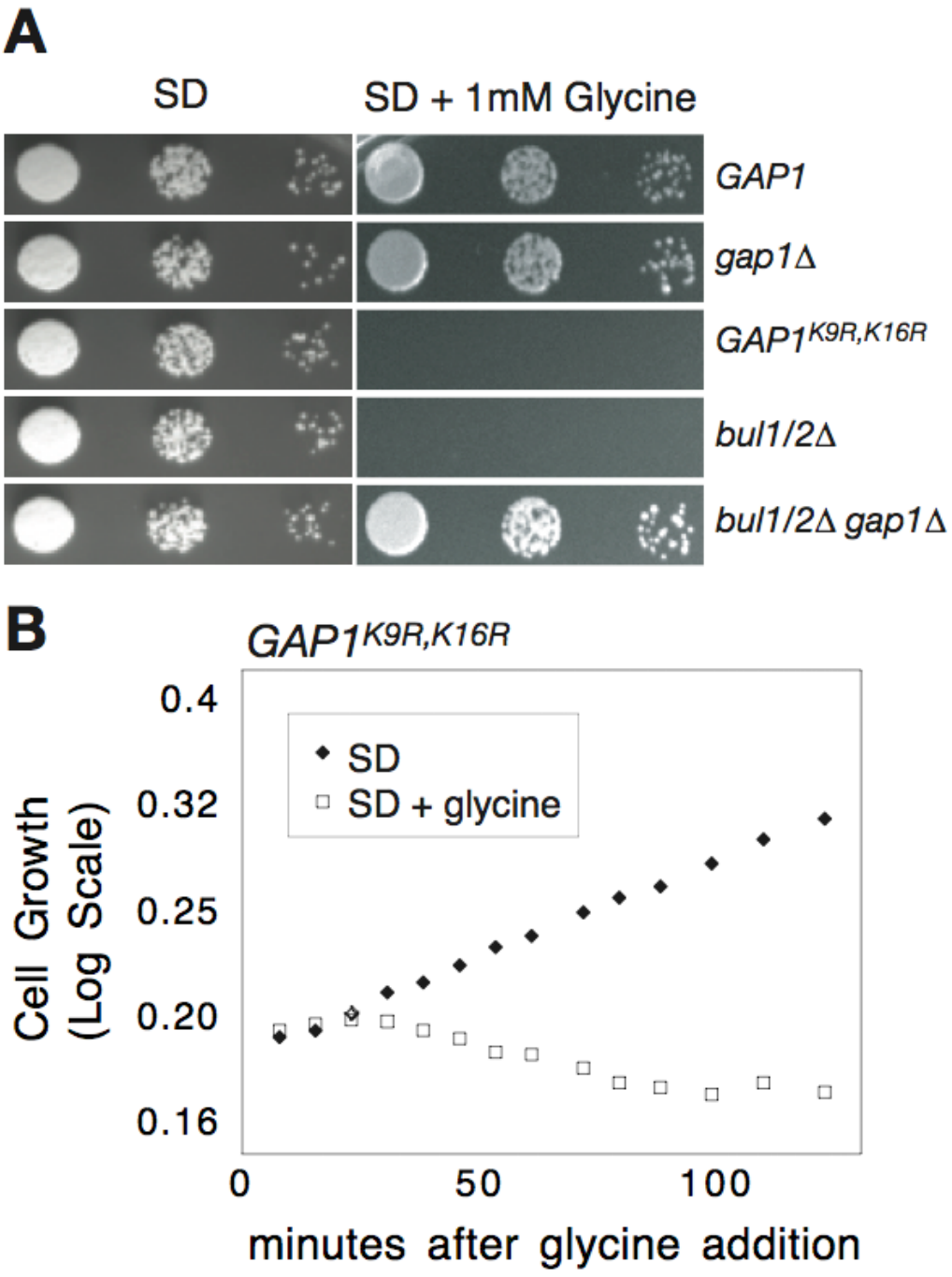

A
SD
SD + 1mM Glycine
GAP1
gap1Δ
GAP1K9R,K16R
bul1/2Δ
bul1/2Δ gap1Δ
B
GAP1K9R,K16R
0.4
0.32
0.25
0.20
0.16
SD
SD + glycine
Cell Growth
(Log Scale)
0
50
100
minutes after glycine addition

Figure 5. Excess amino acid addition is toxic to cells deficient in Gap1p ubiquitination. (A) *gap1Δ* (CKY482) expressing wildtype Gap1p (pAR70), Gap1p^{K9R,K16R} (pAR73), or pRS316 and *gap1ΔbulΔbul2Δ* (CKY701) expressing wild type Gap1p (pAR70) or pRS316 were serially diluted onto SD or SD + 1 mM glycine plates and incubated at 30°. (B) *GAP1*^{K9R,K16R} (CKY1024) was grown in SD at 30°C to early exponential phase. The culture was split and 1 mM glycine was added to one (open squares) while an equal volume of SD was added to the other (filled diamonds). Cells were allowed to grow at 30°C and growth was measured by optical density for two hours following addition.

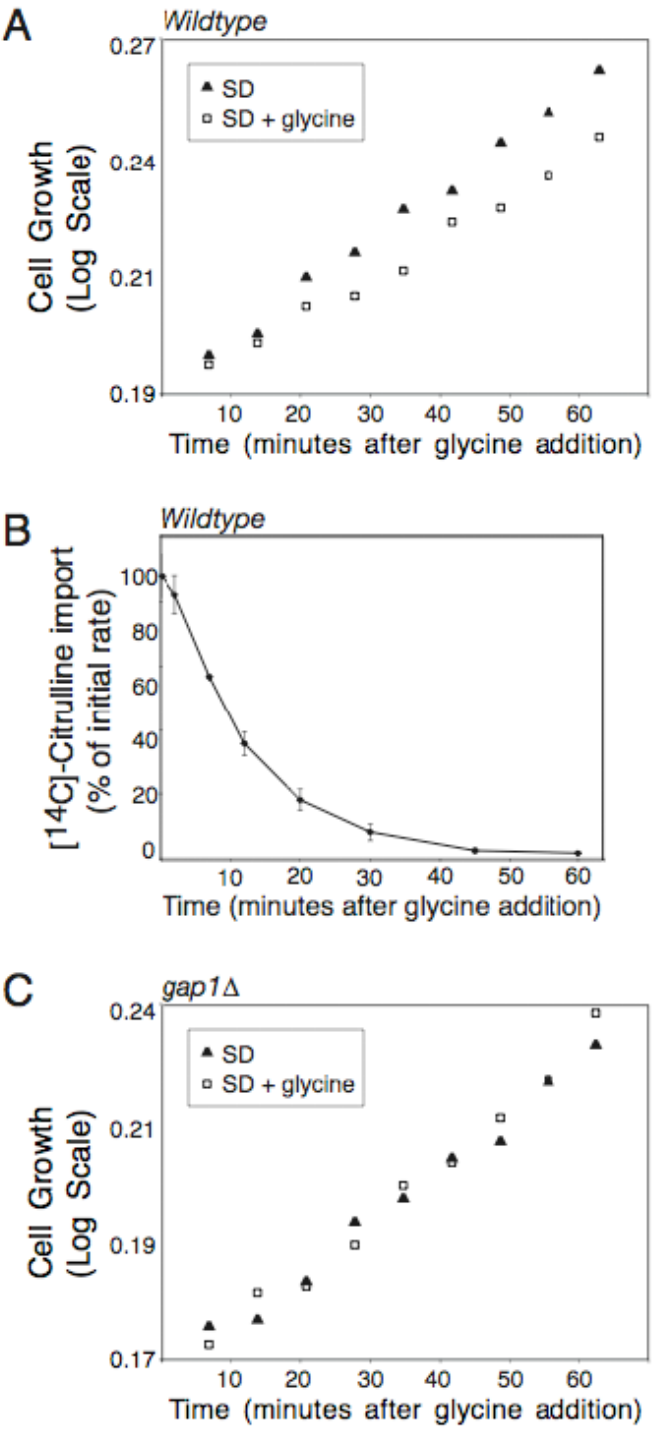

A
Wildtype
SD
SD + glycine
Cell Growth (Log Scale)
0.27
0.24
0.21
0.19
10 20 30 40 50 60
Time (minutes after glycine addition)
B
Wildtype
[14C]-Citrulline import (% of initial rate)
100
80
60
40
20
0
10 20 30 40 50 60
Time (minutes after glycine addition)
C
gap1Δ
SD
SD + glycine
Cell Growth (Log Scale)
0.24
0.21
0.19
0.17
10 20 30 40 50 60
Time (minutes after glycine addition)

Figure 6. Amino acid toxicity is physiological and Gap1p-dependent. (A) Wild type (CKY443) was grown in SD at 30°C. At early exponential phase, the culture was split and 1 mM glycine was added to one (open squares) while an equal volume of SD was added to the other (filled triangles). Cells were allowed to grow at 30°C and growth was measured by optical density. (B) 1 mM glycine was added to a wild-type strain (CKY443) and Gap1p activity was measured by [^{14}C]-Citrulline uptake at the indicated time after glycine addition. (Three independent measurements were averaged). (C) Performed as in (A) with *gap1Δ* (CKY482).

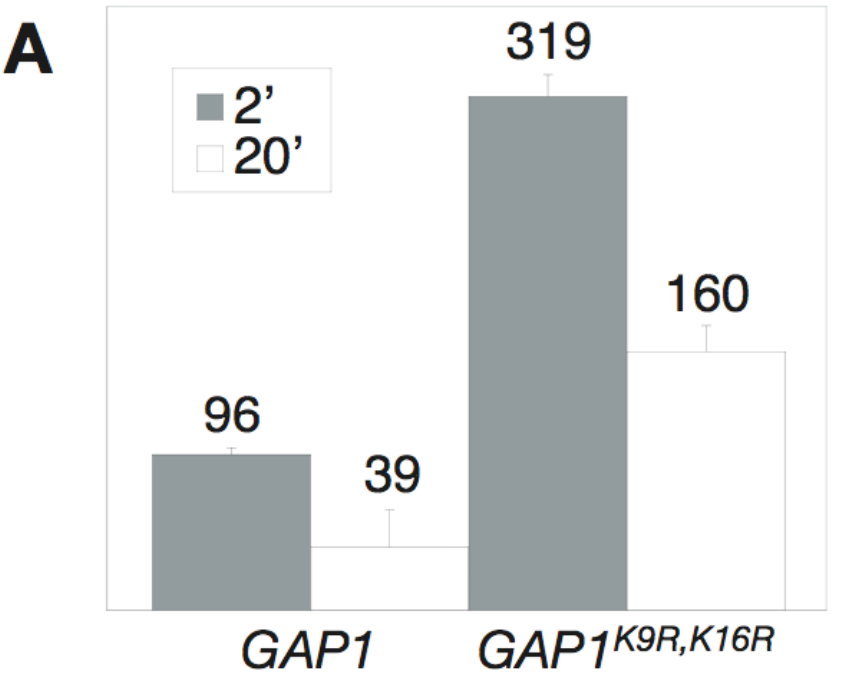
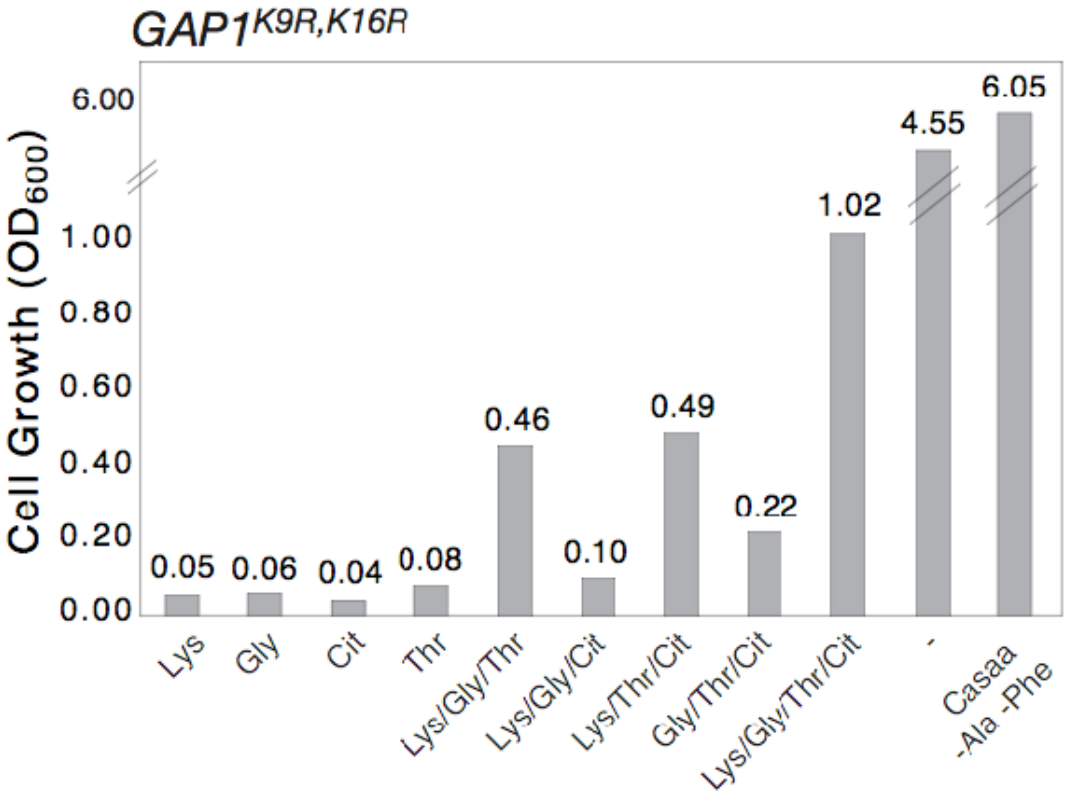

A
2'
20'
319
160
96
39
GAP1
GAP1K9R,K16R
B
GAP1
GAP1K9R,K16R
2'
20'

Figure 7. **Amino acid toxicity is a result of elevated amino acid import in Gap1p$^{K9R, K16R}$.** (A) Wildtype (CKY443) or *Gap1*^{K9R,K16R} (CKY1024) were grown to exponential phase in SD medium at 25°C. Gap1p activity was measured by assaying the initial rate of [14C]-citrulline uptake either immediately (0') or twenty minutes (20') after 1mM alanine addition. (B) Wild type (CKY443) or *Gap1*^{K9R,K16R} (CKY1024) were grown to exponential phase in SD medium at 25°C and 1mM alanine was added to cells. Cells were filtered, washed, and resuspended in SD + 1mM glycine either immediately or twenty minutes after alanine addition. Cells were then incubated in SD with 1mM glycine for thirty minutes, filtered, washed, and plated in serial dilutions onto minimal SD plates and grown at 24°C for three days to determine viability.



amino acid imported (nmol)	Lys	Gly	Cit	Thr	Lys/Gly/Thr/Cit	Casaa -Ala -Phe
Lys	56.7					23.7
Gly		89.6				15.6
Cit			89.2			21.7
Thr				142.1		38.7
total	56.7	89.6	89.2	142.1		99.7

Figure 8. Amino acid mixtures relieve amino acid dependent toxicity. (A) *GAP1*^{K9R,K16R} (CKY893) was grown to exponential phase in SD medium at 30°C and 5x10^6 cells were cultured in SD medium with 3 mM total of the indicated amino acid combination or SD + 0.25% Casamino acids (-ala, -phe). The optical density of the cultures was measured after 22 hours at 30°C (all had an initial OD600 value of 0.05). The amount of a given individual [^{14}C]-labeled amino acid that accumulated in the cell during the first twenty minutes after amino acid addition was determined.

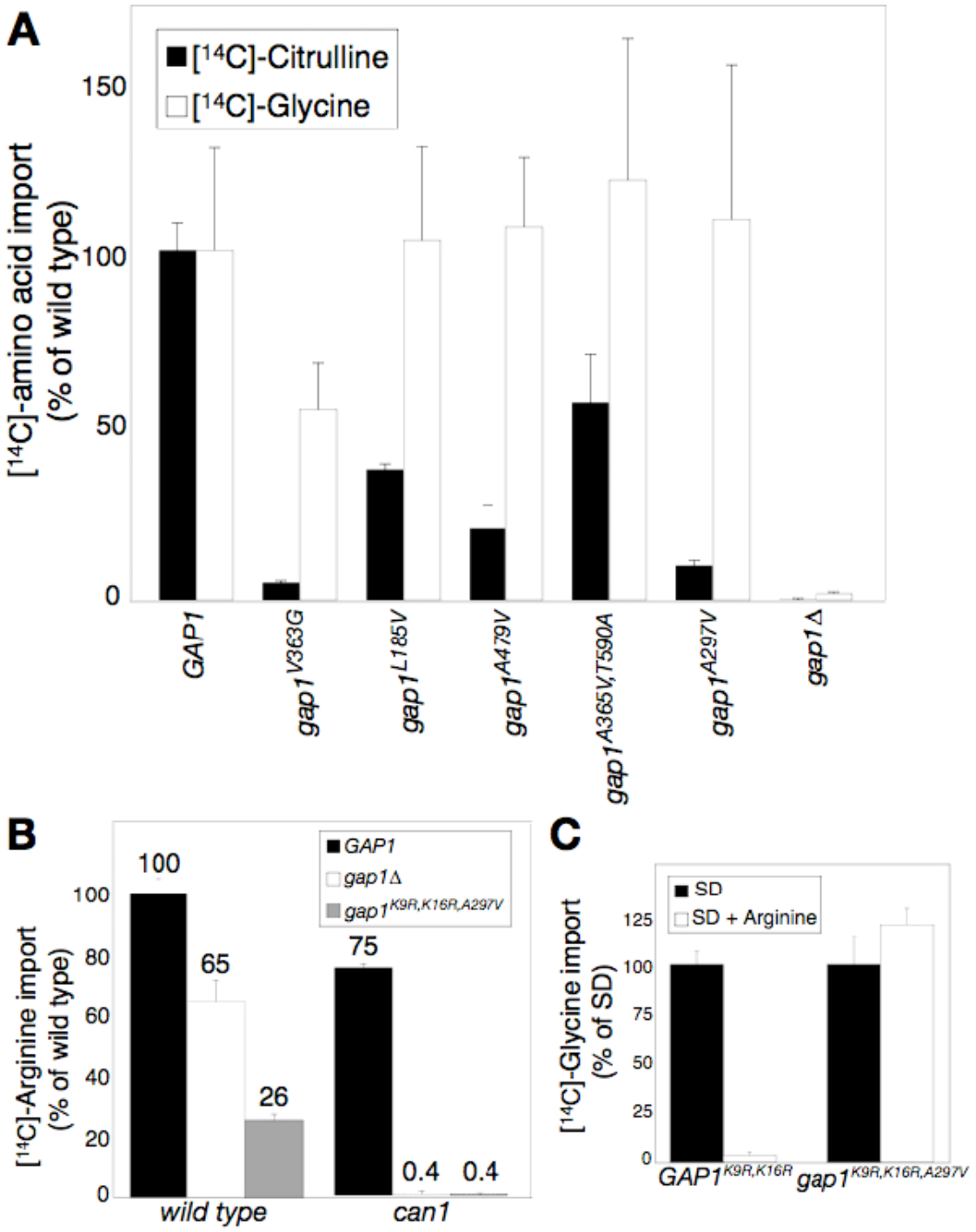

A
[14C]-Citrulline
[14C]-Glycine
[14C]-amino acid import (% of wild type)
150
100
50
0
GAP1
gap1V363G
gap1L185V
gap1A479V
gap1A365V,T590A
gap1A297V
gap1Δ
B
GAP1
gap1Δ
gap1K9R,K16R,A297V
[14C]-Arginine import (% of wild type)
100
80
60
40
20
0
100
65
26
75
0.4
0.4
wild type
can1
C
SD
SD + Arginine
[14C]-Glycine import (% of SD)
125
100
75
50
25
0
GAP1K9R,K16R
gap1K9R,K16R,A297V

Figure 9. **Inactivation of Gap1p requires active amino acid transport through the permease.** (A) The initial rate of [^{14}C]-citrulline or [^{14}C]-glycine uptake was determined for *gap1Δbul1Δbul2Δ* (CKY701) expressing wild type *GAP1* (pEC221), the indicated mutant *GAP1* allele (pNC3-7), or vector alone (pRS316) in SD medium. (B) The initial rate of [14C]-arginine uptake was determined for *gap1Δ* (CKY482) or *gap1Δcan1* (CKY1025) expressing wild type *GAP1* (pEC221), *GAP1*^{K9R,K16R,A297V} (pNC8), or vector alone (pRS316) in SD medium. (C) *gap1Δ* (CKY482) expressing *GAP1*^{K9R,K16R} (pAR1) or *GAP1*^{K9R,K16R,A297V} (pNC8) were grown to exponential phase in SD medium. Gap1p activity was measured by assaying the initial rate of [^{14}C]-glycine uptake at the indicated time after the addition of 1mM arginine. (Three independent measurements were averag

Appendix II

Identification and characterization of a catalytically inactive Gap1p mutant, Gap1p^{A297P}

Summary

I identified a point mutation in Gap1p, $Ala^{297}Val$, which selectively blocked the uptake of basic amino acids, but allowed uptake of all others (Risinger et al., 2006). Ala^{297} is predicted to be near the center of transmembrane domain (TMD) 6, which is implicated in amino acid binding in several recently crystallized bacterial amino acid transporters, including LeuT (Singh et al., 2008; Yamashita et al., 2005), AdiC (Gao et al., 2009; Gao et al., 2010), and ApcT (Shaffer et al., 2009). I therefore concluded that Ala^{297} is accessible in the amino acid binding pocket. To identify Gap1p mutants with altered transport abilities, I made additional mutations at position 297 and examined their transport ability profiles.

Six different mutants were made: A297P, A297D, A297S, A297F, A297I, and A297G. Citrulline and glycine uptakes of these mutants were measured and values were compared to wild type Gap1p or a *gap1*Δ strain (Table 1). A297D, A297F, and A297I most closely resembled the A297V phenotype, low citrulline uptake and high glyine uptake. A297S and A297G appeared most like wild type, with high uptake of both amino acids. A297P did not take up either amino acid. Because I was seeking a catalytically inactive mutant, I chose to focus on this allele.

Further examination of $Gap1p^{A297P}$ showed that uptake of any amino acid I tested was comparable to the *gap1*Δ allele, suggesting that this mutant was indeed catalytically inactive (Figure 1A). I generated a $Gap1p^{A297P}$-GFP construct to verify that this mutant is expressed and can reach the plasma membrane. In *bul1*Δ*bul2*Δ cells, where polyubiquitination is disrupted, $Gap1p^{A297P}$-GFP is mostly seen at the cell surface, although in some cells, additional fluorescence was observed in the ER (Figure 1B). Because proline can be very disruptive to α-helical structures (Chou and Fasman, 1978),

it may be that the insertion of a proline residue in place of alanine causes some Gap1p^{A297P} molecules to misfold, leading to retention in the ER (Ellgaard et al., 1999). Nonetheless, I confirmed by cell fractionation experiments that a significant amount of Gap1p^{A297P} was indeed present at the plasma membrane in *bul1*Δ*bul2*Δ cells (data not shown).

As described in Chapter 3, internal sorting of Gap1p^{A297P} was tested in *end3*Δ cells. Briefly, cells were cultured to early exponential phase and 0.025% casamino acids were added to half of each culture for 3 hours. When cells were imaged by fluorescence microscopy, the majority of Gap1p^{A297P}-GFP appeared at the plasma membrane in amino acids (Figure 2A). To confirm this, I performed equlibrium density sedimentation experiments as described in Chapter 3. When Gap1p^{A297P}-GFP was fractionated in gradients containing EDTA, where the plasma membrane is separated from all internal membranes, I observed that in both SD and SD with CAS, the majority of protein was in the internal fractions, although a significant amount was also at the plasma membrane (Figures B and D). This appeared to disagree with the microscopy images, where no significant vacuolar fluorescence was seen.

Because Gap1p^{A297P} could also accumulate in the ER (Figure 1B), we considered the possibility that the population of Gap1p^{A297P}-GFP we observed in internal fractions could be protein in the ER, not the vacuole. This was confirmed by repeating the fractionation over sucrose gradients with magnesium chloride and without EDTA. Under these conditions, ribosomes remain associated with ER membranes, causing the ER to co-fractionate with the denser plasma membrane fractions and separate from the less dense Golgi and vacuolar fractions (Kaiser et al., 2002). In gradients containing magnesium,

Gap1p^{A297P}-GFP was observed mainly in the denser fractions, and less in the Golgi-containing fractions (Figures C and E), in contrast to fractionation with EDTA, suggesting that the majority of Gap1p^{A297P}-GFP is indeed in the ER.

Here I have shown a catalytically inactive mutant, Gap1p^{A297P}, is catalytically inactive at the plasma membrane. This mutant appears to be insensitive to amino acid-regulated sorting, although a portion of the expressed protein is misfolded and subject to ER quality control.

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Tables

Table 1: Citrulline and glycine uptake of Ala^{297} mutants

GAP1 allele	Citrulline uptake (pmol/min)	Glycine uptake (pmol/min)
Wild type	149.5	128.9
*gap1*Δ	0.25	1.33
A297P	0.84	1.40
A297D	12.1	147.4
A297S	57.5	142.6
A297F	0.50	59.25
A297I	1.51	97.35
A297G	261.7	138.5

Figures

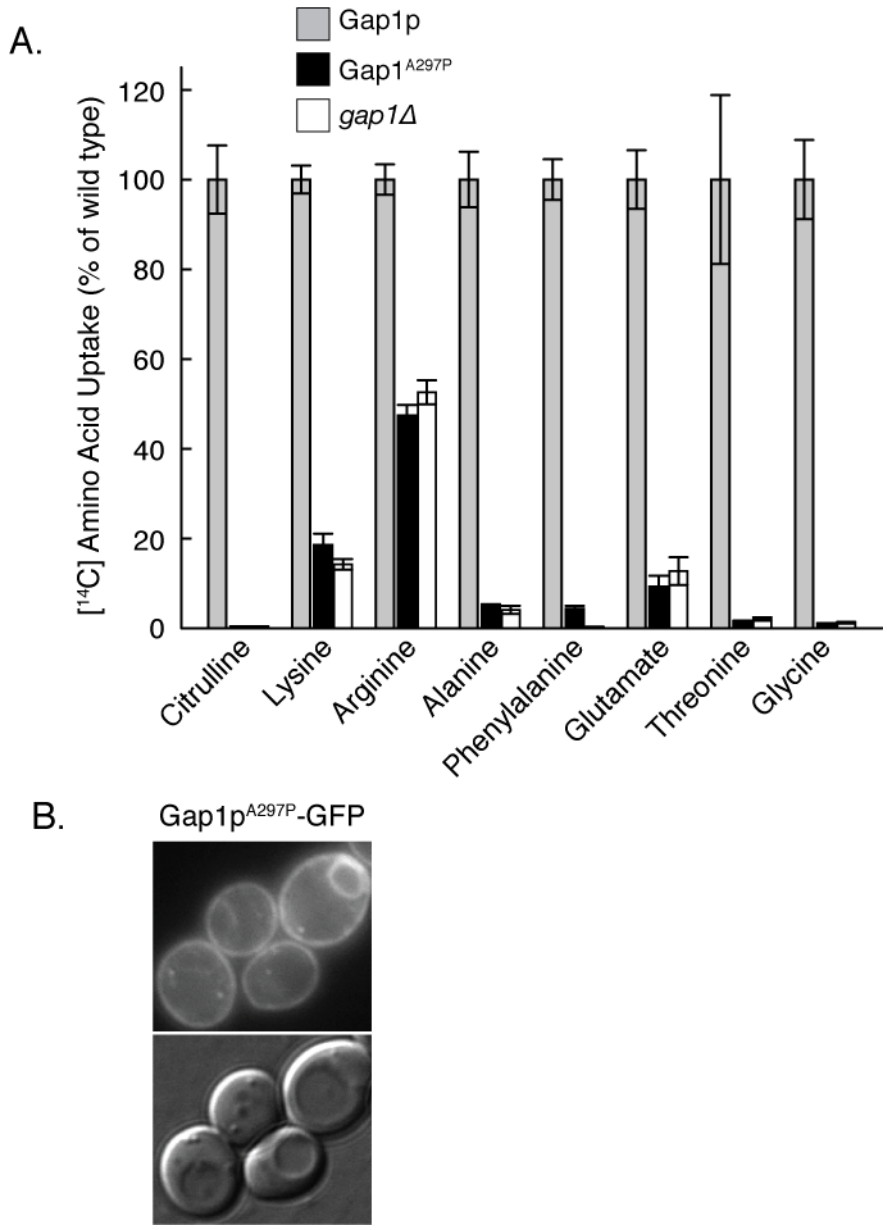

Figure 1. Gap1p^{A297P} does not uptake any amino acid, but can reach the plasma membrane. (A*) bul1*Δ*bul2*Δ (CKY701) cells expressing P_{ADH1}-*GAP1* (pEC221), empty vector (pRS316), or P_{ADH1}-$GAP1^{A297P}$ (pNC55) were cultured to early exponential phase before uptake of the indicated amino acids was measured. Values were normalized to the wild type uptake for each amino acid. (B) *bul1*Δ*bul2*Δ (CKY701) cells expressing P_{ADH1}-$GAP1^{A297P}$-*GFP* were cultured to early exponential phase and imaged by fluorescence microscopy.

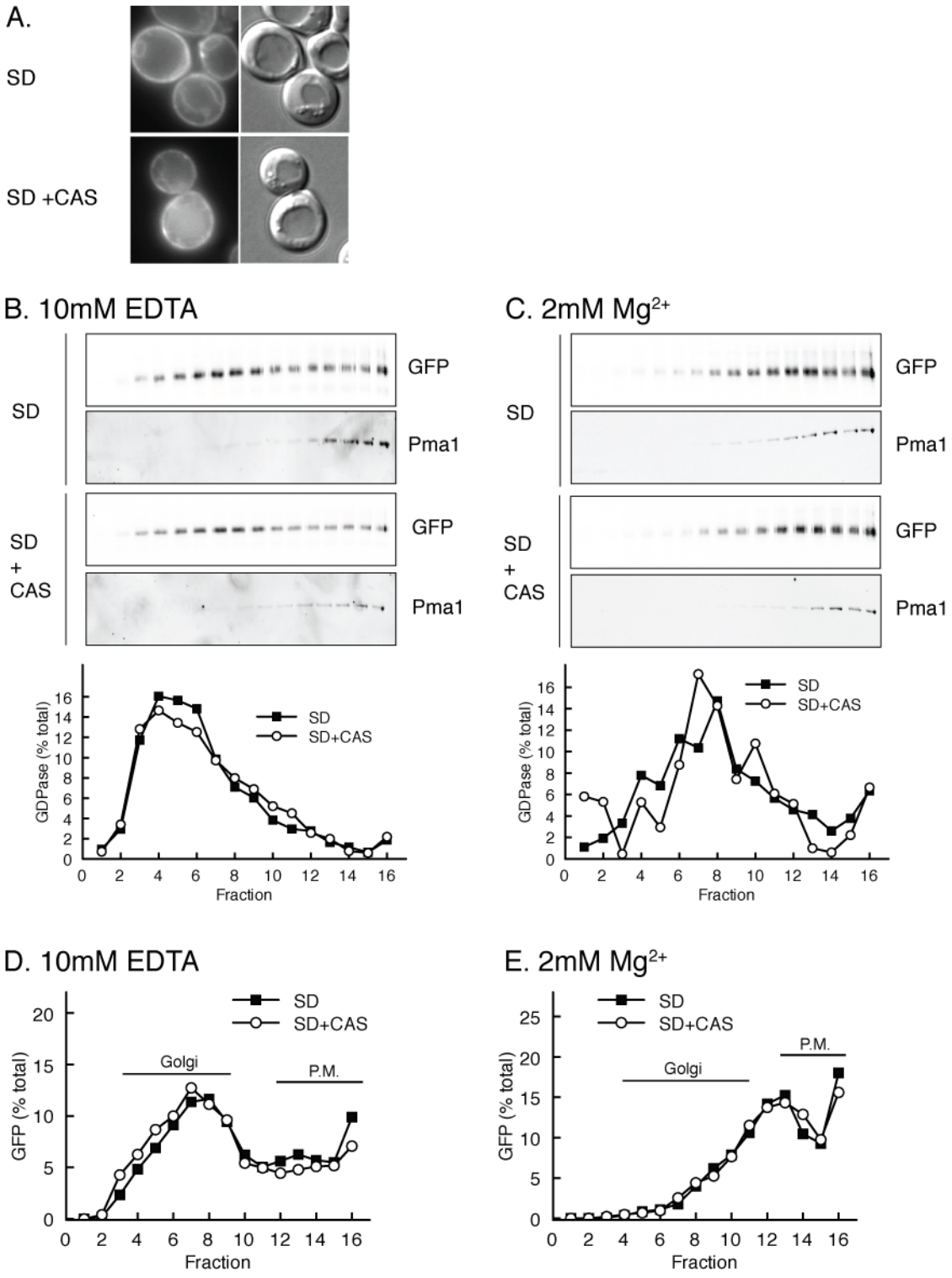
A.
SD
SD +CAS
B. 10mM EDTA
SD
SD + CAS
GFP
Pma1
GDPase (% total)
Fraction
SD
SD+CAS
C. 2mM Mg2+
D. 10mM EDTA
GFP (% total)
Golgi
P.M.
E. 2mM Mg2+

Figure 2. Gap1p^{A297P} is amino acid insensitive, but has an ER exit delay. *gap1*Δ*end3*Δ cells expressing P_{ADH1}-*GAP1*^{A297P}-*GFP* were cultured to early exponential phase in SD medium. 0.025% casamino acids (CAS) were added for three hours to half of each culture. (A) Cells were resuspended in PBS with 10mM sodium azide and imaged using fluorescence microscopy. (B-E) Cells were fractionated over continuous 20-60% sucrose density gradients with 10mM EDTA (B,D) or 2mM $MgCl_2$ (C,E). Sixteen fractions were collected from the top of the gradient. (B,C) Fractions were immunoblotted for Gap1p (mouse anti-GFP) or Pma1p (mouse anti-Pma1p), and Golgi fractions were identified by measuring GDPase activity. (D,E) GFP immunoblots in B and C were quantitated using ImageJ software.