Amino Acids Regulate the Transcription, Internal Sorting, and Intrinsic Activity of the General Amino Acid Permease *(GAP1)* in *S. cerevisiae*

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Submitted to the Department of Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Massachusetts Institute of Technology
June 2007

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Abstract

The high capacity general amino acid permease in *Saccharomyces cerevisiae* (*GAP1*) is regulated such that it actively imports amino acids into the cell from the extracellular medium only when internal amino acid levels are low. Regulation of Gap1p activity allows the cell to rapidly and reversibly modulate amino acid import according to the nitrogen requirements of the cell. I have explored three distinct modes of action by which amino acids repress *GAP1* activity: transcriptionally, by regulation of ubiquitin-mediated intracellular sorting, and by transport-dependent inactivation at the plasma membrane.

Transcriptional regulation of *GAP1* by two nutrient responsive GATA transcription factors, Nil1p and Gln3p, allows the cell to modulate expression of the permease in response to both amino acid quantity and nitrogen source quality. Any Gap1p that is expressed in the presence of elevated internal amino acids is sorted to the vacuole and degraded or stored in internal compartments from which the permease can be rapidly mobilized to the plasma membrane when amino acid levels become limiting. Redistribution of Gap1p from the plasma membrane to internal compartments upon an increase in internal amino acid levels involves three ubiquitin-mediated sorting steps that each require unique *cis*- and *trans*-acting factors. A constitutively expressed, non-ubiquitinateable form of Gap1p can also be downregulated by the addition of amino acids through reversible, transport-dependent inactivation of the permease at the plasma membrane.

Since amino acids are the primary source of nitrogen in the cell, upregulation of Gap1p activity allows the cell to rapidly import nitrogen-containing compounds when internal amino acid pools are limiting. Conversely, downregulation of Gap1p activity when sufficient intracellular amino acids have accumulated allows the cell to avoid toxicity that results from unrestricted amino acid uptake. Therefore, amino acid regulated transcription, sorting, and activity of Gap1p are important to maintain the proper balance of intracellular amino acid levels in diverse and rapidly changing nutritional environments.

Thesis supervisor: Chris A. Kaiser
Title: Professor and Department Head of Biology

Acknowledgements

I would like to thank my advisor, Chris Kaiser, for guidance, support, and encouragement during my graduate work.

I would also like to thank the members of my committee: Gerry Fink, Boris Magasanik, Daniel Finley, and Michael Laub.

I am grateful to members, past and present, of the Kaiser lab for making it a place that I've enjoyed coming to work everyday: Michelle Crotwell, Esther Chen, Darcy Morse, Rahel Siegenthaler, Natalie Cain, Marta Rubio, Andrea Vala, Lori Huberman, Max Juchheim, Eric Spear, Carolyn Sevier, Hongjing Qu, and Minggeng Gao. I would especially like to thank Esther Chen for imparting all of her Gap1p knowledge to me and for her encouragement in the early, murkier stages of my project and Carolyn Sevier for her immense scientific (and non-scientific) guidance, and friendship.

I would like to thank all the members of my family who have surrounded me with so much love and support: my sisters, Robin Heyen and Melissa Peterson, my grandparents, W.A. and Marlene Peterson, Linda and Tim Henning, Shane Heyen, Vicky and Jimmy Risinger, Rusty Risinger, and all of the Blackledge/Risinger family.
I especially thank my parents, Rodney and Rebecca Peterson for believing in me from such an early age and fostering my love of learning and science while keeping me grounded.

Finally, I would like to dedicate this thesis to my husband, Aaron Risinger, whose love and faith are the driving force in all that I do.

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Chapter 1

Introduction

NUTRIENT AVAILABILITY, TRANSPORT, AND SENSING

Nitrogen Catabolite Repression

Sufficient nutrient availability is an important requirement for the growth and development of all cells. One major nutritional requirement is the import of nitrogen-containing compounds into the cell and the subsequent production of nitrogen-containing macromolecules such as protein and DNA. Therefore, most organisms have established a system to provide a continuous supply of nitrogen while minimizing the energy required to maintain this supply.

Certain nitrogenous molecules such as ammonia, glutamate, or glutamine are the preferred nitrogen sources for yeast such as *Saccharomyces cerevisiae.* The presence of these preferred compounds represses synthesis of the enzymes required for catabolism of less preferred nitrogen sources through a process referred to as nitrogen catabolite repression (NCR) (Magasanik 1992). Conversely, when these preferred nitrogen sources are unavailable, NCR allows for the synthesis of enzymes that can catabolize molecules such as allantoin, purines, and proline to create the required nitrogen supply. In this way, the cell is able to monitor the available nitrogen supply to determine the optimum way to expend energy in the process of nitrogen metabolism.

Amino Acids

Monitoring amino acid availability and import is an important aspect of NCR as the amino nitrogen of glutamate is the source of 85% of the total cellular nitrogen in the yeast cell while the amide group of glutamine is the source of the remaining 15%

(Cooper 1982). The import of amino acids from the extracellular environment is regulated by the activity of several distinct amino acid permeases. Many of these amino acid permeases were first isolated as mutants possessing decreased susceptibility to toxic amino acid analogs or increased sensitivity to limiting extracellular concentrations of amino acids (Grenson, Mousset et al. 1966; Crabeel and Grenson 1970; Grenson, Hou et al. 1970; Lasko and Brandriss 1981; Grauslund, Didion et al. 1995; Isnard, Thomas et al. 1996; Zhu, Garrett et al. 1996). Additional amino acid permeases were identified through genomic analysis due to their similarity to previously identified amino acid permeases prior to directly demonstrating amino acid permease activity (Didion, Regenberg et al. 1998; Regenberg, Holmberg et al. 1998; Schreve, Sin et al. 1998). Sequence comparisons of the known amino acid permeases (which each consist of twelve trans-membrane domains and a cytosolic N and C-termini) combined with genomic analysis have determined that the amino acid permease family in S.cerevisiae is comprised of 24 members (Nelissen, Mordant et al. 1995; Gilstring and Ljungdahl 2000).

Amino Acid Permeases

The amino acid permeases in yeast can be distinguished both by their substrate preferences and their regulation. Many amino acid permeases in S.cerevisiae such as Hip1p, Can1p, Apl1p, and Lyp1p have a relatively high specificity for the import of one or a few amino acids that is unaffected by the addition of amino acids to the medium (Hoffmann 1985; Tanaka and Fink 1985; Sychrova and Chevallier 1993; Sychrova and Chevallier 1994). In contrast, the Agp1p, Gnp1p, Bap2p, and Bap3p permeases can transport a fairly broad subset of amino acids into the cell with a relatively low rate that is

increased upon addition of amino acids to the medium as a result of transcriptional induction of the permeases (Grauslund, Didion et al. 1995; Zhu, Garrett et al. 1996; Didion, Regenberg et al. 1998; Schreve, Sin et al. 1998; Iraqui, Vissers et al. 1999). The intracellular localization of the Tat2p high-affinity tryptophan permease is regulated by tryptophan levels such that it is present at the plasma membrane when tryptophan levels are low and sorted to the vacuole when tryptophan levels are elevated (Umebayashi and Nakano 2003). The General Amino Acid Permease (*GAP1*) is unique in that it has a high affinity for all naturally occurring amino acids as well as several amino acid analogs as long as cells are grown on a poor nitrogen source such as proline or urea (Wiame, Grenson et al. 1985). However, good nitrogen sources such as ammonia or glutamine have been shown to repress the ability of Gap1p to import amino acids from the medium (Jauniaux and Grenson 1990). This repression of Gap1p activity in preferred nitrogen sources has been described as a prime example of nitrogen catabolite repression (Grenson, Hou et al. 1970).

The SPS Amino Acid Sensor

In addition to the classes of amino acid permeases described above, there is an additional protein that shares significant homology with the other amino acid permeases although it fails to transport amino acids (Didion, Regenberg et al. 1998; Iraqui, Vissers et al. 1999). The role of this transporter homolog, Ssy1p, is to sense the level of amino acids in the extracellular medium, resulting in the transcriptional upregulation of several amino acid induced permeases as described above (Didion, Grausland et al. 1996; Didion, Regenberg et al. 1998; Jorgensen, Bruun et al. 1998; Iraqui, Vissers et al. 1999;

Klasson, Fink et al. 1999). When sufficient amino acid is present in the extracellular medium, Ssy1p, along with its associated peripheral membrane proteins Ptr3p and Ssy5p, allow proteolytic processing of the plasma membrane localized Stp1p transcription factor (Andreasson and Ljungdahl 2002). Once cleaved, the C-terminal domain of Stp1p is released from the plasma membrane and translocated into the nucleus where it acts together with the Uga35p/Dal81p transcription factor to express the amino acid inducible amino acid permeases including *AGP1* (Abdel-Sater, Iraqui et al. 2004).

Although Ssy1p is unable to transport amino acids, its structural similarity to amino acid permeases suggests that it is able to bind amino acids on either the intracellular and extracellular side of the plasma membrane depending on its conformational state and that these respective binding states may determine the equilibrium between signaling and non-signaling Ssy1p (Abramson, Smirnova et al. 2003; Gaber, Ottow et al. 2003; Huang, Lemieux et al. 2003; Poulsen, Wu et al. 2005). Indeed, it was recently demonstrated that the ability of Ssy1p to sense extracellular amino acids is attenuated by elevated intracellular concentrations of amino acids, suggesting that Ssy1p senses the difference in amino acid concentration across the membrane instead of the absolute extracellular concentration of amino acids (Abramson, Smirnova et al. 2003; Gaber, Ottow et al. 2003; Huang, Lemieux et al. 2003; Poulsen, Wu et al. 2005).

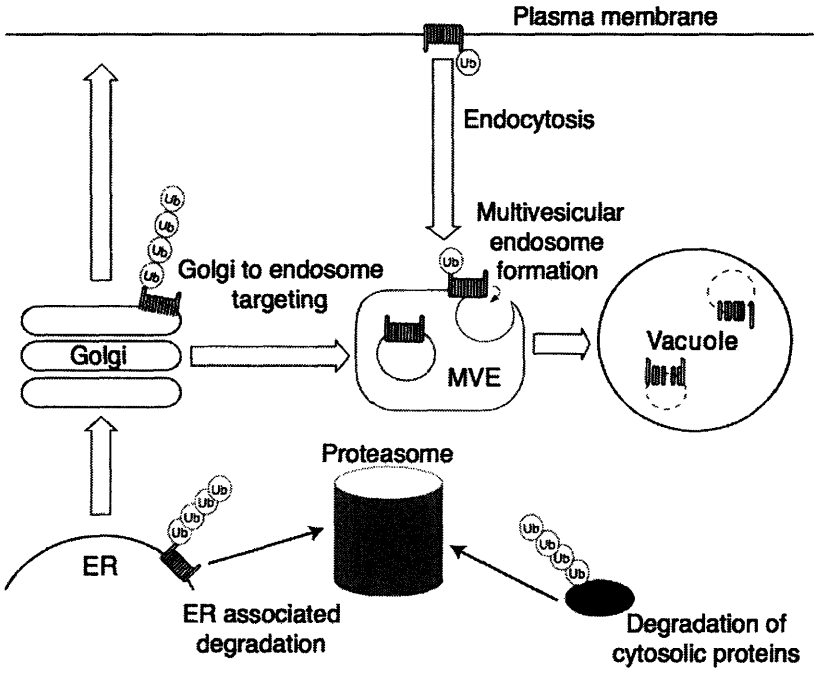
TOR

The TOR protein kinases are conserved in all eukaryotes to control cell growth in response to nutrient availability. Additionally, in mammals it has been demonstrated that growth factors such as insulin also promote signaling through TOR through stimulation

of PI3K and subsequent signaling through the AKT pathway (Chan 2004). When sufficient nutrients are present, TOR promotes the transcription of ribosomal subunits while also enhancing general protein synthesis through activation of the translational initiation factor eIF4E. However, when nutrients become limiting, TOR becomes inactive resulting in a general decrease in translation along with specific upregulation of several genes involved in nutrient transport (Dann and Thomas 2006). Although the exact mechanism by which nutrients regulate TOR function is unknown, it has recently been discovered that the hVps34 protein 3-kinase is involved in transducing amino acid and glucose signals to TOR in mammalian cells (Byfield, Murray et al. 2005; Nobukuni, Joaquin et al. 2005). Vps34p has been shown to localize to the endosome in *Saccharomyces cerevisiae* and its activity is required for proper trafficking from the endosomal compartment to the Golgi, suggesting a potentially interesting interrelationship between nutrient sensing and protein sorting (Burda, Padilla et al. 2002).

UBIQUITINATION

Ubiquitin is a highly conserved 76 amino acid protein that can be covalently bonded to lysine residues in a substrate protein. The posttranslational conjugation of ubiquitin to a protein is carried out by the sequential transfer of ubiquitin from an E1 ubiquitin activating enzyme to an E2 ubiquitin conjugating enzyme and finally through addition of ubiquitin to a lysine residue in the target protein by an E3 ubiquitin ligase. Proteins can either be modified by the addition of a single ubiquitin molecule, termed mono-ubiquitination, or a chain of ubiquitin molecules, termed poly-ubiquitination.



Ubiquitin regulates protein localization and degradation

Poly-ubiquitin chains are formed by the conjugation of ubiquitin to one of the seven lysine resides in another ubiquitin molecule. Covalent ubiquitin attachment to proteins directs several intracellular processes including degradation of cytosolic proteins by the proteasome, ER associated degradation, and several intracellular transport events such as endocytosis, entry into multivesicular endosomes, and Golgi to endosome targeting (reviewed in Hicke and Dunn 2003; Staub and Rotin 2006).

Ubiquitin-mediated protein sorting

As proteins travel through the secretory or endocytic pathways, cargo is selected, concentrated, and loaded into vesicles that are targeted to distinct cellular organelles. Selection of cargo often depends on intrinsic signals and/or modifications on the cargo protein. Covalent attachment of ubiquitin is one type of modification that can mediate the intracellular trafficking of transmembrane proteins. Indeed, recent studies have found that some protein transport machinery components contain ubiquitin-binding domains required for proper intracellular trafficking of ubiquitinated proteins (reviewed in Hicke, Schubert et al. 2005). Additionally, the finding that some proteins of the trafficking machinery are themselves modified and regulated by ubiquitin conjugation adds an additional layer of complexity to the role of ubiquitin in protein sorting (Stamenova, Dunn et al. 2004).

Endocytosis

Endocytosis of proteins involves the coordinated processes of cargo selection, coat formation, membrane invagination, and vesicle formation and requires a functional actin network (Kubler and Riezman 1993). The initial observations that ubiquitination plays a role in endocytosis came from studies of the Ste6p a-factor transporter and the Ste2p α–factor receptor. Ubiquitination of Ste6p was observed in endocytosis deficient mutants and required for the rapid turnover of Ste6p in the vacuole (Kolling and Hollenberg 1994). Soon afterward, it was determined that Ste2p is ubiquitinated upon binding to α–factor and that this ubiquitination was required for endocytosis and subsequent degradation of Ste2p in the vacuole (Hicke and Riezman 1996). Endocytosis

of Ste2p was found to be mediated by addition of a single ubiquitin molecule onto a lysine reside in the C-terminus of the receptor. Covalent attachment of an in-frame mono-ubiquitin tag to the C-terminus of the protein containing a mutation of the normally ubiquitinated lysine residue is also sufficient for internalization (Terrell, Shih et al. 1998).

Subsequent work has implicated mono-ubiquitination as an internalization signal for several receptors and transporters in yeast including constitutive endocytosis of the Pdr5p multidrug transporter, glucose induced endocytosis of the Gal2p galactose transporter, and metal induced endocytosis of the Smf1p manganese transporter (reviewed in Horak 2003). It has also been determined that mono-ubiquitination directs endocytosis of several plasma membrane receptors and transporters in mammalian cells including ion channels, receptor tyrosine kinases, T-cell receptors, and GPCRs (reviewed in Staub and Rotin 2006). Therefore, it appears that covalent attachment of mono-ubiquitin to proteins at the plasma membrane is a common theme for both constitutive and induced endocytosis.

Multivesicular endosome formation

Another protein trafficking step that is regulated by ubiquitination is entry of cargo into multivesicular endosomes en route to the vacuole. The yeast vacuole or mammalian lysosome is the common site of degradation of many endocytosed and biosynthetic membrane proteins. Before reaching the vacuole, these doomed proteins are delivered to endosomes where multi-protein complexes termed ESCRT-I, II, and III work in a coordinated fashion to concentrate the cargo into invaginations in the membrane that eventually bud off as vesicles inside of the endosome (reviewed in Hurley and Emr

2006). This unique organelle, referred to as a multivesicular endosome, fuses with the vacuole, delivering the invaginated cargo into the interior lumen of the vacuole where these proteins are degraded by vacuolar hydrolases. Proteins that are not selected for entry into these vesicles remain on the endosomal membrane and end up residing in the limiting membrane of the vacuole upon fusion.

Ubiquitination is often required for protein cargo to be recognized by the ESCRT complexes and ultimately enter the vacuolar lumen. Vps27p (sometimes referred to as ESCRT-0), Vps23p (a component of the ESCRT-I complex), and Vps36p (a component of the ESCRT-II complex) each contain ubiquitin-interacting motifs termed UIM, UEV, and NZF respectively (Katzmann, Babst et al. 2001; Bilodeau, Urbanowski et al. 2002; Alam, Sun et al. 2004). These motifs recognize mono-ubiquitin or short ubiquitin chains on the cytosolic domain of cargo proteins and allow the sequential transfer of cargo through the ESCRT pathway. In most cases, mutation of the ubiquitinated lysine residues in the cargo protein or deletion of a component of the ESCRT complex results in delivery of cargo to the limiting membrane instead of the lumen of the vacuole. At least one case of a non-ubiquitinated cargo being delivered to the vacuolar lumen through the ESCRT pathway has been demonstrated, although the mechanism of this ubiquitin-independent MVE sorting is unclear and does require Rsp5p ubiquitin ligase activity (Reggiori and Pelham 2001; McNatt, McKittrick et al. 2006; Oestreich, Aboian et al. 2006).

Defects in general multivesicular pathway function (including deletion of ESCRT components) result in formation of a so called class E compartment, which consists of an enlarged endosomal structure where cargo can accumulate (Raymond, Howald-Stevenson

et al. 1992). However, in certain ESCRT mutants, some cargo (including Gap1p) can be recycled from endosomes and redirected to the plasma membrane (Bugnicourt, Froissard et al. 2004; Rubio-Texeira and Kaiser 2006).

The GGAs

The GGA (Golgi-localizing, γ-adaptin ear domain homology, ADP-ribosylation factor ARF-binding) proteins are conserved adaptor proteins involved in protein sorting that have been shown to localize to the Golgi and bind ubiquitin in both yeast and mammalian cells (reviewed in Bonifacino 2004). Delayed vacuolar delivery and aberrant secretion of carboxypeptidase Y was observed in a *gga1*Δ *gga2*Δ double yeast mutant, first suggesting a role of the Gga proteins in internal sorting of cargo from the *trans*-Golgi to the vacuole (Hirst, Lui et al. 2000). The additional finding that the mammalian GGA proteins bind to and mediate sorting of the mannose 6-phosphate receptor between the *trans*-Golgi and the endosome supports their role in sorting at the *trans*-Golgi (Puertollano, Aguilar et al. 2001).

The ubiquitin-binding GAT-domain of the yeast Gga proteins has also been implicated in the intracellular sorting of Gap1p. In a *gga1*Δ *gga2*Δ double mutant, sorting of Gap1p to the vacuole upon glutamine addition is slowed but not inhibited (Scott, Bilodeau et al. 2004). Analysis of Gap1p vacuolar delivery in an endocytosis deficient *end3*Δ mutant suggests that the delayed vacuolar delivery of Gap1p is due at least in part to a defect in internal sorting of the permease that requires the ubiquitin-binding GAT domain of Gga2p, presumably at the *trans*-Golgi. However, Gga2p appears to play an additional role in the vacuolar delivery of Gap1p independently of this

GAT domain, presumably at the level of MVE formation (Scott, Bilodeau et al. 2004). The mammalian GGA proteins have also been implicated in MVE sorting as GGA3 depleted cells accumulate cargo endosomes and GGA3 has been shown to bind Tsg101, a component of the machinery required for ubiquitin-mediated MVE sorting (Puertollano and Bonifacino 2004).

REGLUATION OF GAP1

The presence of a general amino acid permease in *S.cerevisiae* was first identified when the initial uptake rate of several amino acids was found to increase when cells were shifted from ammonia to proline medium (Grenson, Hou et al. 1970). Therefore, it has been known from its discovery that Gap1p activity is regulated by nitrogen source. Eventually, it was found that Gap1p activity was affected by both transcriptional and post-translational levels of regulation (Stanbrough and Magasanik 1995).

Transcriptional Regulation

Gln3p and Nil1p

The GATA transcription factor Gln3p was originally isolated as a key regulator of nitrogen catabolite repression due to its role as the transcriptional inducer of the glutamine synthetase gene *GLN1* upon a shift from glutamine to glutamate medium (Mitchell and Magasanik 1984; Mitchell and Magasanik 1984). Similarly, it was later determined that Gln3p is the sole transcription factor required for expression of *GAP1* on glutamate and that this activity was repressed upon the addition of glutamine (Stanbrough and

Nitrogen source	amino acid quantity	Nitrogen source quality	*GAP1* transcription	Gap1p sorting	Gap1p activity
Urea	low	low	Gln3 & Nil1	PM	high
ammonia	low	high	Nil1	PM	high
glutamate	high	low	Gln3	internal	low
glutamine	high	high	off	(internal)	low
amino acid mixture	high	high	off	(internal)	low

Regulation of Gap1p in various nitrogen sources.
PM: plasma membrane, (internal): any expressed Gap1p would be found in internal compartments.

Magasanik 1996). However, it was also shown Gln3p was not the sole contributor toward *GAP1* expression since *GAP1* transcription was only decreased by 50% in *gln3* mutants grown in the poor nitrogen source urea. This finding led toward further analysis that suggested there was a second transcriptional activator of *GAP1* that contained the same characteristic GATA zinc finger sequence found in Gln3p.

Nil1p was subsequently identified through the screening of a yeast genetic library by hybridization and was confirmed to be responsible for the residual *GAP1* expression in a *gln3* mutant grown on urea (Stanbrough, Rowen et al. 1995). Additional mutational analysis confirmed that both Gln3p and Nil1p contribute toward *GAP1* expression in poor nitrogen sources such as on urea or proline medium; Gln3p-dependent transcription is specifically repressed by ammonia and Nil1p-dependent transcription is specifically repressed by glutamate. *GAP1* is not expressed by either transcription factor in glutamine medium where the intracellular concentration of both ammonia and glutamate is elevated (Stanbrough, Rowen et al. 1995).

Ure2p and TOR

Upstream regulators of Gln3p and Nil1p have also been identified. *URE2* was isolated as a mutant having the opposite phenotype of a *gln3* mutation; it was determined that *gln3* mutations were epistatic to *ure2* mutations suggesting that Ure2p is a negative regulator of Gln3p (Drillien, Aigle et al. 1973; Courchesne and Magasanik 1988). Indeed, it was found that Ure2p binds Gln3p and prevents its translocation into the cytoplasm when glutamine is present (Blinder, Coschigano et al. 1996; Beck and Hall 1999). There is conflicting data as to whether and to what extent Ure2p negatively

regulates Nil1p and no interaction between the two proteins has ever been detected (Coffman, Rai et al. 1996; Stanbrough and Magasanik 1996; Cunningham, Andhare et al. 2000).

Like Ure2p, The TOR complex has also been implicated as a negative regulator of Gln3p and Nil1p since addition of the TOR inhibitor Rapamycin results in nuclear localization of both transcription factors in the presence of repressive nitrogen sources (Beck and Hall 1999). The fact that TOR is a protein kinase, along with findings that the Ure2p-Gln3p interaction requires Gln3p to be phosphorylated and Rapamycin addition disrupts the Ure2p-Gln3p interaction strongly suggested that TOR-dependent phosphorylation dictates the localization of Gln3p (Beck and Hall 1999).

More recent data has demonstrated that the phosphorylation state of Gln3p does not always correlate with its affinity to Ure2p or ability to enter the nucleus, suggesting that the TOR complex may have a more indirect role in Gln3p nuclear localization (Cox, Kulkarni et al. 2004; Tate, Rai et al. 2005). Interestingly, another protein previously thought to be directly involved in regulating the translocation of Gln3p to the nucleus in a nitrogen source-dependent manner, the Npr1p kinase, was recently shown to have its affect as an indirect consequence of affecting ammonia uptake (Crespo, Helliwell et al. 2004; Tate, Rai et al. 2006). The fact that Npr1p is itself a target of TOR-dependent phosphorylation along with accumulating evidence that addition of rapamycin and actual nitrogen starvation elicit many differing effects on cellular physiology strongly suggest that the effect of TOR on Gln3p localization may be, at least partially, indirect (Schmidt, Beck et al. 1998).

Post-translational Regulation

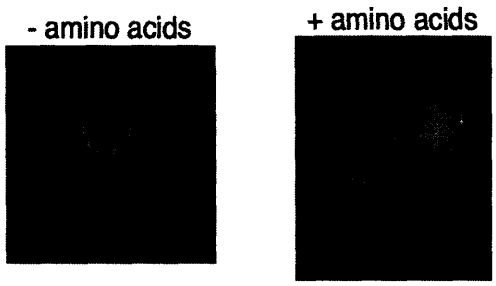
GAP1 is tightly controlled at the transcriptional level, however Gap1p activity does not always correlate with the level of mRNA or protein expression, indicating that Gap1p is also regulated post-translationally (Stanbrough and Magasanik 1995). This posttranslational regulation allows Gap1p to be present at the plasma membrane and actively transport amino acids into the cell when internal amino acid levels are low while rapidly redistributing to internal compartments when sufficient amino acids are available. It is worth noting that there are differences in this posttranslational regulation depending on which yeast genetic background is used.

Strain background

Strain differences in the regulation of Gap1p activity were first discovered when the D-amino acid sensitivity of different strains was explored; cells from the S288C background were sensitive to D-amino acid addition in minimal ammonia medium while cells from the ∑1278 Belgian strain background were resistant (Rytka 1975). Indeed, in ammonia medium, strains from the ∑1278 background have been shown to have low Gap1p activity while strains from the S288C background have high Gap1p activity. In our studies, we grow S288C derived cells in ammonia (where Gap1p activity is high) and add amino acids to the medium to determine the ability of Gap1p to be downregulated. However, I will also discuss findings from laboratories that add ammonia to minimal proline medium to measure ammonia-dependent Gap1p downregulation in the ∑1278 background.

Amino acids regulate Gap1p sorting

The ability of Gap1p to be posttranslationally regulated in a nutrient dependent manner came from the observation that cells grown in either urea or glutamate expressed *GAP1* at a similar level although the Gap1p activity in ammonia was 100-fold greater than in glutamate (Stanbrough and Magasanik 1995). To study the posttranslational regulation of Gap1p independently of the regulation of its expression, *GAP1* can be expressed under control of the nutrient insensitive *ADH1* promoter (Chen and Kaiser 2002). In the presence of elevated amino acid levels, any expressed Gap1p is degraded in the vacuole or retained in intracellular compartments from which it can be mobilized to the plasma membrane upon a decrease in internal amino acid levels (Roberg, Rowley et al. 1997; Chen and Kaiser 2002).



Gap1p localization. Gap1p-GFP is localized to the plasma membrane in the absence of amino acids and delivered to the vacuolar lumen in the presence of elevated amino acid levels

It was additionally shown that internal amino acid levels are the direct signal for this protein sorting decision as elevated levels of any amino acid result in redistribution of Gap1p from the plasma membrane to internal compartments regardless of whether the amino acid could be converted into a nitrogen source or whether the amino acid was exogenously added or internally produced (Chen and Kaiser 2002). Once amino acid levels are elevated, internal pools of Gap1p are unable to be delivered to the plasma

membrane and are eventually sent through the vacuolar sorting pathway to the vacuole where the permease is degraded (Roberg, Rowley et al. 1997; Rubio-Texeira and Kaiser 2006). This amino acid dependent posttranslational regulation allows for rapid changes in Gap1p activity according to its substrate availability; Gap1p activity can be increased or decreased by redistribution of the permease faster than protein could be expressed or turned over.

Amino acid production and ammonia inactivation

The finding that elevated internal amino acid levels are sufficient for intracellular retention and vacuolar sorting of Gap1p suggests that downregulation of Gap1p activity by the addition of ammonia to strains of the ∑1278 background may be indirect. Internal amino acids are produced by Gdh1p, which combines ammonia and α-ketoglutarate to produce glutamate, the precursor of other amino acids and nucleotides in the yeast cell (Grenson, Dubois et al. 1974). In glucose grown cells, production of α-ketoglutarate requires expression of four TCA cycle genes by the Rtg1p/Rtg3p transcription factor complex (Liu and Butow 1999). When glutamate is present at sufficient levels in the cell, Rtg1p and Rtg3p are sequestered in the cytoplasm by Mks1p, preventing synthesis of α-ketoglutarate (Liu and Butow 1999; Dilova, Aronova et al. 2004).

We have found mutations that allow unregulated α-ketoglutarate synthesis, such as *mks1Δ,* result in elevated internal amino acid levels and Gap1 sorting to the vacuole in minimal ammonia medium, setting a precedent for the hypothesis that the ability of ammonia to sort Gap1p to internal compartments in the ∑1278 background may be due,

at least in part, to an indirect effect of increased amino acid synthesis (Chen and Kaiser 2002).

TOR

The TOR complex has been implicated in the nutrient-dependent trafficking of both the Gap1p and Tat2p amino acid permeases. Initial analysis using the TOR inhibitor, rapamycin, suggested that Tat2p was sorted to the vacuole and degraded under conditions of nitrogen starvation. Since it was well documented that Gap1p is localized to the plasma membrane under nitrogen starvation, it was thought that these two permeases were differentially regulated.

Upon finding that loss-of-function mutations in *LST8* (a component of the TOR complex) resulted in constitutive vacuolar localization of Gap1p, the link between TOR function and Gap1p sorting was further explored. Although it had been assumed that addition of the TOR inhibitor rapamycin fully mimicked nitrogen starvation, it was determined that Gap1p was actually localized to the vacuole when cells were grown in the presence of a sublethal concentration of rapamycin.

This result is less puzzling when it is appreciated that addition of rapamycin and subsequent inhibition of TOR cause the cell to behave as if it is nitrogen starved even though nutrients are readily available. In this case, rapamycin treatment actually promotes an intracellular environment of nitrogen excess that is in contradiction with the direct effect of rapamycin on the inhibition of the TOR complex. The ensuing measurement of metabolic outputs when cells simultaneously perceive a state of nitrogen excess and starvation can be misleading. However, the fact that the high internal amino

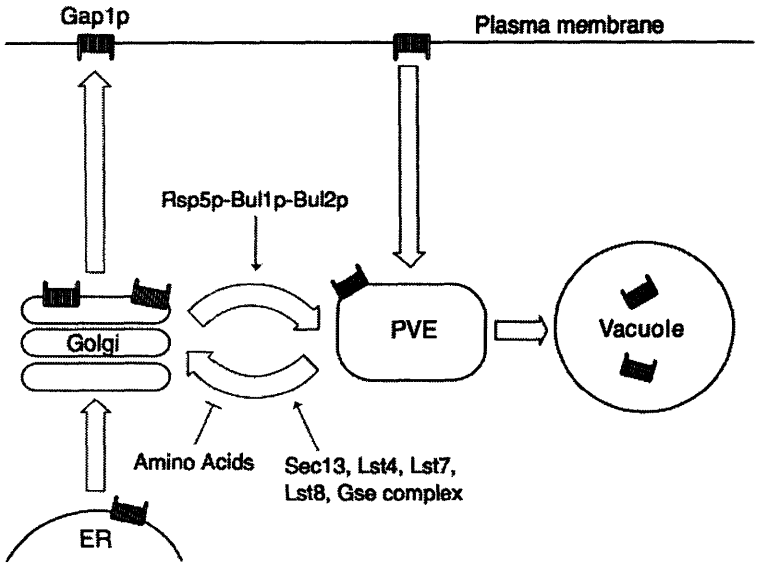
acid levels present in rapamycin-treated cells result in the sorting of Gap1p to the vacuole indicates that the amino acid signal is sensed and transmitted by a novel internal amino acid sensor independently of the TOR complex.

It is likely that the trafficking of Tat2p to the vacuole upon addition of rapamycin is also an indirect effect of increased internal amino acid levels. Studies of Tat2p localization in media with varying concentrations of tryptophan have demonstrated that Tat2p is delivered to the plasma membrane in low tryptophan and to the vacuole in high tryptophan. Therefore, it appears that rapamycin and nitrogen starvation have opposing roles in the intracellular nutrient-dependent trafficking of both Gap1p and Tat2p.

Recycling from internal compartments

Our lab has identified several proteins required for the redistribution of internal pools of Gap1p to the plasma membrane when internal amino acid levels are lowered. *LST4, LST7,* and *LST8* were first identified in a screen for mutants that are synthetically lethal with *sec13-1*, an allele of the essential COPII coat that specifically blocks delivery of Gap1p to the plasma membrane at the permissive temperature (Roberg, Bickel et al. 1997). It was later determined that Lst8p is part of the TOR complex and that impaired delivery of Gap1p to the plasma membrane in *lst8* mutants was a result of derepression of amino acid biosynthesis leading to increased internal amino acid levels (Chen and Kaiser 2003). The exact role *SEC13, LST4,* and *LST7* play in delivery of Gap1p to the plasma membrane is currently unclear.

Another screen to isolate mutants defective in Gap1p sorting to the plasma membrane in low amino acid concentrations was carried out using the toxic amino acid



Internal sorting of Gap1p. Gap1p is translocated in the ER and sorted through the secretory pathway to the *trans*-Golgi. In the *trans*-Golgi, Gap1p is either delivered to the plasma membrane or polyubiquitinated by the Rsp5p-Bul1p-Bul2p ubiquitin ligase complex and sorted to the prevacuolar endosome (PVE). Once in the PVE, Gap1p is either delivered to the vacuole and degraded via entry into multivesicular endosomes or recycled back to the *trans*-Golgi. This recycling step is inhibited by elevated internal amino acid levels.

analog L-azetidine-2-carboxylic acid (ADCB). Two genes identified as ADCB resistant were *GTR1* and *GTR2*, two GTPases that were found to be in a complex required for delivery of Gap1p to the plasma membrane (Gao and Kaiser 2006). Additional proteins in this GSE complex, Gse1p, Gse2p, and Ltv1p were isolated from the original screen and by mass spectrometry. The GSE complex is a 600KD complex that is localized to the endosomal compartment and is required for sorting of Gap1p to the plasma membrane from internal compartments. Although the exact mechanism of GSE-dependent delivery of Gap1p to the plasma membrane is unclear, the finding that Gtr2p binds directly to the C-terminal tail of Gap1p suggests that the GSE complex may be part of a cargo specific coat that facilitates recycling of Gap1p from the endosome to the plasma membrane, presumably through the *trans*-Golgi.

A role for ubiquitin in Gap1p sorting

Complete redistribution of Gap1p from the plasma membrane to internal compartments requires both endocytosis of plasma membrane localized Gap1p as well as internal retention of any newly synthesized Gap1p; both of these sorting events appear to require ubiquitination of Gap1p. The role of ubiquitin in Gap1p trafficking was first suggested when mutations in either the Rsp5p ubiquitin ligase or the Doa4p deubiquitinase were found to suppress delivery of Gap1p to internal compartments (Grenson 1983; Hein, Springael et al. 1995; Springael, Galan et al. 1999). The inability of Gap1p to be sorted in a *doa4* mutant is an indirect consequence of the low levels of free ubiquitin in this strain; overexpression of ubiquitin abolishes this Gap1p sorting defect (Springael, Galan et al. 1999). It was determined that Gap1p was directly ubiquitinated on lysines 9 and 16 by the Rsp5p-Bul1p-Bul2p ubiquitin ligase complex and that this ubiquitination was required for delivery of both endocytosed and newly synthesized Gap1p to the vacuole (Springael and Andre 1998; Helliwell, Losko et al. 2001; Soetens, De Craene et al. 2001).

SUMMARY

In the following chapters, I describe three ways in which Gap1p is regulated in response to elevated levels of amino acid substrate. *GAP1* is transcribed by two nutrient responsive GATA transcription factors, Nil1p and Gln3p. In chapter two, I show that Nil1p is repressed by increased levels of any intracellular amino acid regardless of whether it can be used as a cellular nitrogen source. In contrast, Gln3p is only repressed by amino acids, such as glutamine, that are also excellent nitrogen sources. Dual regulation of *GAP1* transcription in response to nitrogen source quality and amino acid quantity allows the cell to titrate the expression of *GAP1* according to each of these variables.

Once Gap1p is expressed, it is sorted to the plasma membrane when internal amino acid levels are low and redirected to the vacuole and degraded when amino acid levels are elevated. Delivery of Gap1p to the vacuole requires ubiquitination on two N-terminal, cytosolic lysine residues. In chapter three, I characterize three independent ubiquitin-mediated sorting steps involved in delivery of Gap1p to the vacuole. I found that unique *cis* and *trans*-acting elements are required for each sorting event, demonstrating an underappreciated level of specificity in ubiquitin-mediated protein sorting.

We were surprised to uncover an additional level of Gap1p regulation when an ubiquitin deficient form of *GAP1* was expressed under the amino acid insensitive *ADH1* promoter. In chapter four, I describe this novel type of permease regulation: reversible and transport-dependent inactivation at the plasma membrane. Finally, we determine that downregulation of Gap1p activity is required to avoid toxicity that results from

unrestricted amino acid uptake. This multifaceted regulation of Gap1p activity in response to its amino acid substrate allows the cell to rapidly upregulate amino acid transport when intracellular pools of amino acids become limiting, while avoiding amino acid toxicity.

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Chapter 2

Two GATA Transcription Factors Allow the Yeast Gap1p Permease to Respond to Changing Nutritional Conditions

Abstract

The activity of the general amino acid permease in *S. cerevisiae,* Gap1p, is tightly regulated by amino acids through intracellular sorting as well as transport dependent inactivation at the plasma membrane. Here, we show that the two GATA type transcription factors responsible for *GAP1* transcription also respond to unique physiological amino acid inputs: Nil1p/Gat1p activity is repressed by elevated levels of any amino acid, whereas Gln3p is repressed specifically by glutamine. Upon further exploration of the amino acid specificity of Nil1p and Gln3p repression, we found both a molecular basis and physiological rational for this dual regulation. Ure2p negatively regulates both transcription factors in glutamine, while a parallel TOR-dependent pathway is involved in the specific general amino acid repression of Nil1p activity. The amino acid-dependent repression of Nil1p activity provides the cell yet a third mechanism to down-regulate Gap1p activity when its substrate is abundant. In contrast, Gln3p allows the cell to express *GAP1* in amino acid-containing poor nitrogen sources, allowing rapid mobilization of the permease to the cell surface when amino acid levels become limiting.

Introduction

All cells must be able to regulate nutritional uptake systems and metabolic pathways in response to their environment, but it is particularly important for free-living microbes to respond rapidly to a wide variety of nutritional conditions. Yeast cells monitor carbon and nitrogen containing nutrients using both extracellular and intracellular nutrient sensors to make appropriate adjustments in the activity of nutrient uptake and utilization pathways (Iraqui, Vissers et al. 1999; Forsberg and Ljungdahl 2001). The general amino acid permease encoded by the *GAP1* gene, is a high capacity transporter of all naturally occurring amino acids for use as a nitrogen source. Gap1p is regulated both transcriptionally and post-translationally as a function of nitrogen availability (Grenson, Hou et al. 1970; Courchesne and Magasanik 1983; Magasanik and Kaiser 2002).

GAP1 transcription is positively controlled by two GATA transcription factors: Gln3p, which is repressed on glutamine or ammonia medium, and Nil1p/Gat1p, which is repressed on glutamate or glutamine medium (Daugherty, Rai et al. 1993; Stanbrough, Rowen et al. 1995; Crespo, Powers et al. 2002). Therefore, *GAP1* is expressed largely by Nil1p in ammonia medium and by Gln3p in glutamate medium. On poor nitrogen sources, such as urea or proline, both transcription factors contribute to *GAP1* transcription, while in glutamine medium *GAP1* is not transcribed (Magasanik and Kaiser 2002).

Gln3p-dependent transcription is repressed by a physical interaction with Ure2p that sequesters the Gln3p transcription factor in the cytoplasm when intracellular glutamine levels are elevated (Courchesne and Magasanik 1988; Beck, Schmidt et al.

1999). Although Gln3p activity is also repressed in ammonia media, this repression is likely an indirect effect of increased glutamine synthesis as it is alleviated upon addition of the glutamine synthetase inhibitor L-methionine sulfoximine (MSX) to minimal ammonia media (Crespo, Powers et al. 2002). A negative effect of Ure2p on Nil1p activity is suggested by the finding that over-expression of Ure2p decreases Nil1p activity in either proline or ammonia medium (Cunningham, Rai et al. 2000). However, is unclear to what extent Ure2p is involved in the normal inhibition of Nil1p activity in repressive nitrogen sources.

Gap1p permease activity is also regulated in response to nitrogen source by sorting in the *trans*-Golgi and endosomal compartments. Gap1p is an integral membrane protein that is transported through the secretory pathway to the *trans*-Golgi where it can either be delivered to the plasma membrane or sent to the vacuole for degradation. This sorting decision is sensitive to the presence of amino acids; when amino acids are absent from the medium, Gap1p is sorted to the plasma membrane where it actively transports amino acids into the cell (Stanbrough and Magasanik 1995; Roberg, Rowley et al. 1997; Chen and Kaiser 2002). When any amino acid, including glutamate, is present in the growth medium, Gap1p is diverted to the vacuole through the vacuolar-sorting pathway (Chen and Kaiser 2002; Nikko, Marini et al. 2003; Rubio-Texeira and Kaiser 2006). Even when amino acids are absent from the growth medium, large intracellular pools of Gap1p are present allowing Gap1p to be rapidly redistributed to the cell surface in response to changing nutritional conditions (Roberg, Rowley et al. 1997).

Mutations that affect Gap1p sorting can be grouped into three general classes (Figure 1). Class I mutations prevent transport of Gap1p from the *trans*-Golgi to the pre-

vacuolar endosome (PVE) and include the Rsp5p-Bul1p-Bul2p ubiquitin ligase complex (Hein, Springael et al. 1995; Springael and Andre 1998; Helliwell, Losko et al. 2001). Ubiquitin ligase mutants, as well as a *cis*-acting mutation in *GAP1* in which the N-terminal ubiquitinated lysine residues are mutated to arginines *($GAP1^{K9R,K16R}$)* result in plasma membrane localization of the internally stored pools of Gap1p and consequently produce constitutive high levels of Gap1p activity (Helliwell, Losko et al. 2001; Soetens, De Craene et al. 2001). Class II mutations cause constitutive sorting of Gap1p to the vacuole regardless of nitrogen source and as a consequence exhibit very low Gap1p activity (Roberg, Rowley et al. 1997). When class I and class II mutations are combined Gap1p activity is high, implying that in class I mutants Gap1p never enters the compartment where class II mutants can influence sorting. The most likely site of action of the class II mutants is in the retrieval of Gap1p from the PVE to the *trans*-Golgi (Helliwell, Losko et al. 2001; Gao and Kaiser 2006). Finally, class III mutants have an indirect effect on Gap1p sorting by altering the intracellular concentration of amino acids. Class III mutants that have increased internal amino acid levels result in Gap1p sorting to the vacuole while those that have decreased internal amino acid levels result in Gap1p sorting to the plasma membrane (Chen and Kaiser 2002).

Among the first class III mutants isolated were alleles of *LST8,* an essential component of the TOR complex that negatively regulates both *GLN3* and *RTG1/3* transcription factors through distinct, genetically separable mechanisms (Roberg, Bickel et al. 1997; Chen and Kaiser 2003). The *RTG1/3* transcription factors are involved in the transcription of TCA cycle enzymes responsible for α-ketoglutarate synthesis (Dilova, Chen et al. 2002). Therefore the derepression of *RTG1/3* in *lst8* mutants results in high

internal amino acid levels that are also observed when the direct negative regulator of *RTG1/3*, *MKS1*, is mutated (Liu, Sekito et al. 2001; Chen and Kaiser 2003). The TOR pathway is required for processes such as cell growth and the initiation of translation and is inhibited upon addition of the immunosuppressant rapamycin. Inhibition of the TOR complex by rapamycin results in pleiotropic effects upon the cell that include transcriptional induction of several nitrogen-regulated genes including *GAP1, NIL1, GLN3,* and *URE2* and redistribution of Nil1p and Gln3p to the nucleus (Beck and Hall 1999; Cardenas, Cutler et al. 1999). Although treatment of yeast cells with rapamycin produces many of the same effects as nitrogen starvation, the level of nutrients such as amino acids are abnormally high in rapamycin treated cells since many biosynthetic pathways are derepressed. As a result, Gap1p is sorted to the vacuole in *lst8* mutants or upon rapamycin addition although it is present at the plasma membrane in nitrogen starved cells (Chen and Kaiser 2003). It has also been demonstrated that rapamycin addition and nitrogen starvation are additive with regard to Gln3p nuclear localization and not equivalent with regard to Gln3p phosphorylation, further suggesting that the two treatments are not redundant (Tate, Rai et al. 2005).

In this paper we show that Nil1p-dependent *GAP1* transcription is repressed by elevated internal amino acid levels in a manner similar to Gap1p sorting to the vacuole. We also show that Gln3p-dependent *GAP1* transcription in the presence of amino acids is required for a rapid mobilization of Gap1p to the plasma membrane when cells are shifted from conditions of amino acid excess to starvation, demonstrating a physiological role for Gln3p-dependent *GAP1* transcription under conditions where the permease is not active.

Materials and Methods

Strains, plasmids, and media

All of the yeast strains used in this study (listed in Table 1) are in the S288C background, which has high Gap1p activity in minimal ammonia media (SD) (Courchesne and Magasanik 1983). Gene disruptions of *GLN3, NIL1, GAP1, MKS1, LST4, CAR1, URE2*, and *SSY1* were constructed by homologous recombination with *kanMX4 or natMX4* gene replacement cassettes (Wach, Brachat et al. 1994; Goldstein and McCusker 1999). These strains were subsequently crossed to obtain double and triple mutants.

The transcriptional reporter pMS29 consists of a P_{GAP1}-*lacZ* fusion at codon 53 of *GAP1* carried on an *URA3-CEN* vector (Stanbrough and Magasanik 1995).

Minimal medium contains Difco yeast nitrogen base without amino acids and without ammonium sulfate, 2% glucose, and a nitrogen source: 0.5% ammonium sulfate (SD), 0.2% urea, 0.1% glutamate, or 0.1% glutamine and adjusted to a pH of 4 with either NaOH or HCl. Amino acid stocks were made at 40-200 mM (depending on solubility) in SD medium at pH 4.0, filter sterilized, and stored at 4° C. For rapamycin treatment, a sublethal dose is considered to be the concentration at which there is a 2- to 4-fold inhibition in the growth of the culture as measured by the final OD_{600} value at the time of the assay. This concentration is 12 ng/ml in ammonia medium, 15 ng/ml in glutamate medium, and 25 ng/ml in glutamine medium. The sublethal rapamycin concentrations used in these experiments are higher than those used in previous studies since *gln3*Δ strains, which are used in this study, are partially resistant to rapamycin (Cardenas, Cutler et al. 1999; Chen and Kaiser 2003).

β-galactosidase reporter expression

β-galactosidase activity was measured by the permeabilized cell method (Adams, Gottschling et al. 1996). Two independent transformants were cultured at 30° C in indicated medium for 16-22 hours to a cell density of 2-8 x 10^6 cells/ml and assayed in duplicate. Each experiment was performed at least two times with similar results. For nitrogen starvation conditions, cells were washed and cultured at 30°C in nitrogen free medium for four hours prior to assay. Where amino acid or rapamycin addition is noted, cells were cultured in the presence of these compounds for the entire growth period.

Amino acid uptake assays

Strains were cultured to 4-8x10^6 cells/ml in minimal medium containing 0.1% glutamate or 1mM leucine as a sole nitrogen source, washed with nitrogen-free medium by filtration on a 0.45-mm nitrocellulose filter, and suspended in minimal urea media at a concentration of 4x10^6 cells/ml. At the indicated time, cells were again washed with nitrogen-free medium by filtration on a 0.45-mm nitrocellulose filter, and amino acid uptake assays were performed as described previously (Roberg, Rowley et al. 1997).

Results

Amino acids down-regulate Nil1p-dependent *GAP1* transcription

Gln3p-dependent transcription of *GAP1* is repressed by glutamine, whereas Nil1p-dependent transcription of *GAP1* is repressed by glutamate or glutamine (Stanbrough, Rowen et al. 1995; Crespo, Powers et al. 2002). To determine whether amino acids other than glutamate and glutamine could also repress Nil1p-dependent *GAP1* transcription, we compared P_{GAP1}-*lacZ* reporter expression driven by Nil1p alone (in a *gln3*Δ strain) or Gln3p alone (in a *nil1*Δ strain) to a wild-type strain. As reported previously, both Gln3p and Nil1p contribute toward *GAP1* transcription on urea medium (Table 2). Gln3p-dependent *GAP1* transcription was repressed by the excellent nitrogen sources ammonia and glutamine, while poorer nitrogen sources such as glutamate or leucine did not repress Gln3p activity (Table 2). In contrast, Nil1p-dependent *GAP1* transcription was repressed when amino acids such as glutamate or leucine were used as a sole nitrogen source (Table 2). We also determined that addition of any single amino acid at a concentration of 3 mM to minimal ammonia medium resulted in a significant decrease in Nil1p-dependent *GAP1* expression (Table 3). In contrast, the addition of the imino acid proline caused only a slight decrease in Nil1p-dependent *GAP1* transcription (Table 3). These results indicate that, like intracellular sorting of Gap1p, Nil1p activity is repressed by amino, but not imino acids (Chen and Kaiser 2002).

To determine whether amino acids directly repress Nil1p-dependent *GAP1* transcription independently of their ability to be converted into other nitrogen containing compounds, we utilized a *car1*Δ strain that is deficient in the first step of arginine catabolism and therefore unable to utilize arginine as a source of nitrogen (Figure 2A)

(Sumrada and Cooper 1982). We determined that *CAR1* was not required for repression of Nil1p-dependent *GAP1* transcription in response to arginine addition (Figure 2B), indicating that Nil1p-dependent *GAP1* transcription can be inhibited by arginine independently of whether it is broken down or used as a source of nitrogen. These findings indicate that Nil1p activity is regulated in response to an amino acid signal including, but not limited to, glutamate.

Nil1p responds to intracellularly synthesized amino acids

In mutants such as *mks1*Δ, which cause increased synthesis of intracellular amino acids, Gap1p is sorted to the vacuole even when amino acids are not present in the growth medium (Chen and Kaiser 2002). We found that the *mks1*Δ mutant also results in repression of Nil1p-dependent *GAP1* transcription in amino acid free minimal medium, suggesting that elevated internal amino acid levels are sufficient to repress Nil1p activity (Figure 3A). When *mks1*Δ mutants were cultured in nitrogen free medium, the repression of *GAP1* transcription was largely alleviated (Figure 3B). Therefore, repression of Nil1p-dependent *GAP1* transcription in *mks1*Δ was an indirect effect of high intracellular amino acid levels since the observed repression in *mks1*Δ depended on the cell's ability to synthesize high internal amino acid pools from a nitrogen source provided in the medium. Other mutants, such as *lst4*Δ, that cause Gap1p sorting to the vacuole without altering internal amino acid levels did not affect Nil1p-dependent transcription (Figure 3A).

To test the effect of TOR complex inhibition on Nil1p activity, we assayed Nil1p-dependent *GAP1* transcription in *lst8* mutants or upon addition of the TOR inhibitor rapamycin. We determined that several mutant alleles of *LST8* caused repression of

Nil1p-dependent *GAP1* transcription in minimal ammonia medium (Figure 3C). This effect could be attributed to loss of TOR complex function in *lst8* mutants resulting in activation of the *RTG1/3* transcription factors leading to unrestricted synthesis of the amino acid precursor α-ketoglutarate and elevated internal amino acid levels (Roberg, Bickel et al. 1997; Chen and Kaiser 2003). Addition of a sub-lethal dose of the TOR inhibitor rapamycin also results in increased internal amino acid levels that should be sufficient to repress Nil1p-dependent *GAP1* transcription (Chen and Kaiser 2003). However, when cells were cultured in sublethal rapamycin concentrations we observed a derepression of Nil1p-dependent *GAP1* transcription in spite of the elevated internal amino acid levels (Figure 3C). This finding is consistent with the fact that Nil1p is rapidly translocated to the nucleus upon the addition of rapamycin (Beck and Hall 1999). Intriguingly, rapamycin addition to *lst8* mutants caused only a slight derepression of Nil1p activity, suggesting that the repression of Nil1p in *lst8* mutants can occur independently of TOR. Therefore, inhibition of the TOR complex has two opposing effects on Nil1p-dependent *GAP1* transcription: an increase in internal amino acid levels and resulting decrease in Nil1p activity in *lst8* mutants and a more direct alleviation of repression observed upon rapamycin treatment.

URE2 negatively regulates *NIL1* activity

The *URE2* gene product negatively regulates Gln3p by sequestering the transcription factor in the cytoplasm when glutamine is abundant (Blinder, Coschigano et al. 1996; Beck and Hall 1999). To better understand the role *URE2* plays in regulating Nil1p activity, we determined the effect of *ure2*Δ on Nil1p-dependent *GAP1* transcription

in ammonia medium, where Nil1p is active, or in medium containing glutamate or glutamine as the sole nitrogen source, where Nil1p is repressed. In all three media *ure2*Δ partially derepressed Nil1p-dependent *GAP1* transcription, indicating that Ure2p is a negative regulator of Nil1p (Figure 4A). To ensure that the effect of *URE2* on Nil1p activity was not an indirect result of altered amino acid import in the *ure2*Δ strain, we determined that Nil1p-dependent *GAP1* transcription remained high in ammonia-grown *ure2*Δ strains when the internal amino acid concentration was elevated by *mks1*Δ (Figure 4A).

Although Nil1p activity in ammonia, glutamate, or glutamine was derepressed by loss of *URE2*, we noticed that Nil1p activity in an *ure2*Δ strain was still significantly repressed by glutamate (Figure 4A). This finding suggested that other negative regulators of Nil1p activity may exist. Since we also observed derepression of Nil1p activity when the TOR inhibitor rapamycin was added to cells (Figure 3C, 4A), we postulated that TOR either acts as a positive upstream regulator of *URE2,* or that *URE2* and *TOR* operate in parallel to repress Nil1p activity. We found that Nil1p-dependent *GAP1* transcription in medium containing glutamate or glutamine as a sole nitrogen source was derepressed to a greater extent by the addition of a sublethal dose of rapamycin to *ure2*Δ strains than with either *ure2*Δ or rapamycin addition alone (Figure 4B). Interestingly, we also found that Nil1p activity in a *ure2*Δ mutant was most strongly repressed by glutamate while Nil1p activity in rapamycin-treated cells was most strongly repressed by glutamine, suggesting that Ure2p responds to glutamine while TOR responds to the general amino acid signal (Figure 4B). The additive effect of *ure2*Δ and rapamycin on Nil1p derepression as well as the differential strength of derepression

elicited by *ure2*Δ and rapamycin in glutamate versus glutamine medium suggest that Ure2p and the TOR complex act in parallel to inhibit Nil1p in repressive, amino acid containing nitrogen sources.

Nil1p regulation by amino acids does not require the amino acid sensor Ssy1p or the Gap1p permease itself

The *SSY1* gene product is a member of the amino acid permease family that is responsible for inducing transcription of a subset of amino acid permeases, most prominently *AGP1*, in response to amino acids (Didion, Regenberg et al. 1998; Iraqui, Vissers et al. 1999; Klasson, Fink et al. 1999). *SSY1* has also been implicated in transcriptional repression of *GAP1* in the presence of glutamate (Klasson, Fink et al. 1999), but other studies suggest that this could be the result of an indirect effect of *SSY1* on amino acid import (Kodama, Omura et al. 2002). To determine whether *SSY1* is directly required for Nil1p repression by amino acids, we took advantage of a subset of amino acids (e.g. asparagine and arginine), that can enter the cell independently of the *GAP1* and *AGP1* permeases (Iraqui, Vissers et al. 1999). We determined that Nil1p-dependent *GAP1* transcription was repressed by these amino acids regardless of the presence of *SSY1*, demonstrating that Nil1p repression by exogenous amino acids does not require *SSY1* (Figure 5A). Moreover, we found that the elevated intracellular amino acids in *mks1*Δ inhibited Nil1p activity independently of *SSY1*; the diminution in Nil1p activation of *GAP1* transcription caused by *mks1*Δ was unaltered by *ssy1*Δ (Figure 5A).

It has also been suggested that the Gap1p permease itself can sense amino acids and transduce this signal to activate protein kinase A targets in the cell (Donaton,

Holsbeeks et al. 2003). We determined that, even in a *gap1*Δ strain, Nil1p activity was repressed by amino acids that are imported into the cell through other permeases (Figure 5B), indicating that the amino acid-dependent repression of Nil1p activity does not require Gap1p as a sensor. These findings indicate that the reported amino acid sensors Ssy1p and Gap1p are not responsible for the Nil1p-dependent down-regulation of *GAP1* in response to amino acids.

Gln3p-dependent *GAP1* expression is required for rapid activation of Gap1p in amino acid free medium

When yeast cells are grown on an amino acid such as glutamate, Nil1p-dependent *GAP1* transcription is repressed while Gln3p continues to give rise to a pool of intracellular Gap1p that is not delivered to the plasma membrane. A possible reason for this seemingly futile expression of nonfunctional Gap1p could be to produce internal stores of Gap1p under amino acid rich conditions in preparation for rapid mobilization to the plasma membrane upon a shift to conditions of amino acid limitation. To test this hypothesis, we evaluated the contribution of Gln3p to the rate at which Gap1p activity increased in response to a shift from glutamate to urea as the sole nitrogen source. A wild type strain grown on glutamate had very low Gap1p activity that increased five-fold after 10 minutes in amino acid free (urea) medium (Figure 6A). In contrast, a *gln3*Δ strain required over an hour to achieve a similar increase in activity after a shift from glutamate to urea medium (Figure 6A). Nevertheless, *gln3*Δ strains were eventually able to achieve the high Gap1p activity levels seen for the wild type strain, indicating that

*gln3*Δ cells do not posses a general defect in steady state Gap1p activity in amino acid free medium.

We found that growth in a variety of different amino acids allowed Gln3p-dependent expression of *GAP1*, but resulted in very low permease activity because Gap1p was sorted to the vacuole (data not shown). Figure 6B shows the increase in Gap1p activity for wild type and *gln3*Δ strains shifted from leucine to urea as a sole nitrogen source. Again, we observed that Gap1p activity in the wild type strain rapidly increased upon a shift to amino acid free medium and that this effect was markedly delayed in a *gln3*Δ mutant. Together these results show that Gln3p-dependent *GAP1* transcription in the presence of amino acids allows the synthesis of intracellular pools of Gap1p that can be rapidly mobilized to the plasma membrane when amino acid levels become limiting and provide a rational for the dual regulation of *GAP1* transcription.

Discussion

It is well established that Nil1p and Gln3p are two transcriptional activators responsible for mediating nitrogen-responsive gene expression including the transcription of *GAP1*. Nil1p and Gln3p possess nearly identical zinc finger domains and are each required for the full expression of most nitrogen sensitive genes due to overlapping sites of recognition (Stanbrough, Rowen et al. 1995; Stanbrough and Magasanik 1996). However, Nil1p and Gln3p respond differently to nutrient availability (Daugherty, Rai et al. 1993; Stanbrough, Rowen et al. 1995; Crespo, Powers et al. 2002). Previously, it was believed that Nil1p is repressed specifically by glutamate while Gln3p is repressed specifically by glutamine; the mechanism and rational for this dual regulation was poorly understood. Here we show that all amino acids, even those that are poor nitrogen sources, can repress Nil1p activity while Gln3p continues to transcribe *GAP1* in preparation for worsening nutritional conditions.

Upon analysis of *trans*-acting mutants, we found that the combination of rapamycin treatment and *ure2Δ* have additive effects on the derepression of Nil1p activity in repressive nitrogen sources, indicating that Ure2p and TOR contribute toward repression of Nil1p through parallel pathways. Additionally, we observed that, in rapamycin treated cells, Ure2p responds primarily to glutamine while, in *ure2Δ* mutants, TOR-dependent repression responds to the general amino acid signal (Figure 4B). This finding allows us to simplify our view of Ure2p as a negative regulator of both Nil1p and Gln3p in the presence of glutamine, while another less well-understood TOR-mediated pathway regulates the specific repression of Nil1p by amino acids.

The repression of Nil1p-dependent *GAP1* transcription by amino acids closely corresponds to the amino acid dependent regulation of Gap1p internal sorting. Thus, the likely role of Nil1p repression by amino acids is to conserve the energy of *GAP1* expression when the permease would not be directed to the plasma membrane. In contrast, Gln3p continues to activate transcription of *GAP1* in the presence of amino acids (as long as the quality of the nitrogen source is relatively low) in preparation for worsening nutritional conditions. Indeed, we have found that the ability of yeast cells to rapidly induce Gap1p activity upon a shift from amino acid containing medium to minimal urea medium is dependent on Gln3p-dependent *GAP1* transcription. Gln3p dependent transcription is shut off only under conditions that would give high intracellular levels of glutamine such as during growth on glutamine, ammonia, or the complex mixture of amino acids present in rich medium. Thus the physiological inputs for Nil1p and Gln3p differ as follows: Nil1p as well as the machinery that controls intracellular sorting of Gap1p respond to the *quantity* of intracellular amino acids regardless nitrogen source quality, whereas Gln3p specifically responds to intracellular glutamine levels and thus appears to be a sensor of nitrogen *quality*.

Nil1p dependent transcription and intracellular sorting of Gap1p appear to have evolved as feedback mechanisms to down regulate amino acid uptake through Gap1p, thus preventing excessive uptake of amino acids when they are abundant in the extracellular medium, which has been shown to be toxic (Risinger, Cain et al. 2006). The finding that Nil1p-dependent *GAP1* transcription and sorting of Gap1p to the plasma membrane are each repressed by an amino acid signal suggests that these two pathways may share a common mechanism of amino acid sensing. An advantage of having two

distinct outputs of a single amino acid sensing pathway is the ability to differentiate components involved in the upstream sensing and processing of the amino acid signal from those that act specifically in either the transcription or sorting branches of the pathways. Further mutational analysis of each of these processes may give insight as to whether a single amino acid sensor is indeed responsible for both levels of *GAP1* regulation and the identity of that sensor.

The ultimate question is why the cell employs two homologous transcription factors, Nil1p and Gln3p, to integrate multiple nutritional inputs into the expression of a single gene, *GAP1*. We propose that when internal amino acids are scarce, the high-capacity, low-specificity *GAP1* permease acts as an efficient scavenger of amino acids from the medium. When cells are shifted to conditions where external amino acid levels are abundant, Gap1p would continue to import amino acids until the internal amino acid concentration rose to a level that could trigger a yet unidentified internal amino acid sensor or sensors. The amino acid sensor in turn would cause *GAP1* transcription to be down-regulated through repression of Nil1p while newly synthesized Gap1p protein would be sorted to the vacuole and preexisting permease cleared from the plasma membrane by endocytosis. Gln3p would continue to drive transcription of *GAP1* in the presence of amino acids to produce a pool of intracellular Gap1p that could be rapidly mobilized to the plasma membrane should extracellular amino acid levels fall. While Gap1p activity decreases as a result of elevated internal amino acid levels, the cell could continue to take advantage of available amino acids in the medium through the *SSY1*-dependent induction of relatively low-affinity or low-capacity permeases that would allow for a more controlled uptake of external amino acids. In this manner the yeast cell

is able to adjust the rate of amino acid uptake in response to both the quantity of amino acids available in the medium and the quality of the nitrogen source.

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Table 1. Strains (all are isogenic with S288c). All are from this paper

Strain	Genotype
CKY772	*MAT***a** *ura3-52*
CKY878	*MAT***a** *gln3Δ::kanMX6 mks1Δ::kanMX6 ura3-52*
CKY879	*MAT*α *gln3Δ::kanMX6 lst4Δ::kanMX6 ura3-52*
CKY880	*MAT***a** *gln3Δ::kanMX6 lst8-1 ura3-52*
CKY881	*MAT***a** *gln3Δ::kanMX6 lst8-6 ura3-52*
CKY882	*MAT***a** *gln3Δ::kanMX6 lst8-7 ura3-52*
CKY883	*MAT***a** *gln3Δ::kanMX6 lst8-15 ura3-52*
CKY884	*MAT*α *gln3Δ::kanMX6 gap1Δ::LEU2 ura3-52*
CKY886	*MAT***a** *gln3Δ::kanMX6 ure2Δ::kanMX6 ura3-52*
CKY887	*MAT***a** *gln3Δ::kanMX6 ssy1Δ::kanMX6 ura3-52*
CKY888	*MAT***a** *nil1Δ::kanMX6 ura3-52*
CKY889	*MAT*α *gln3Δ::kanMX6 mks1Δ::kanMX6 ssy1Δ::kanMX6 ura3-52*
CKY894	*MAT***a** *gln3Δ::kanMX6 ura3-52*
CKY898	*MAT***a** *gln3Δ::natMX car1Δ::kanMX ura3-52*
CKY1036	*MAT***a** *gln3Δ::natMX nil1Δ::kanMX ura3-52*

Table 2. Amino acids down-regulate Nil1p-dependent *GAP1* transcription

Nitrogen Source	*GAP1* transcription (β–galactosidase units)	Gln3p-dependent *GAP1* transcription (β-galactosidase units)	Nil1p-dependent *GAP1* transcription (β-galactosidase units)
Urea	106	187	95
Ammonia	176	14	232
Glutamine	6	3	6
Glutamate	211	150	4
Leucine	224	203	9

β-galactosidase assays were performed for wildtype (CKY772), *gln3*Δ (CKY894), or *nil1*Δ (CKY888) with a P_{GAP1}-*LacZ* reporter construct (pCK211) after growth in the indicated sole nitrogen source for 16 hours.

Table 3. Amino acids repress Nil1p-dependent *GAP1* transcription

Amino acid	P_{GAP1} - *LacZ*
None (SD)	1.00
Asparagine	0.01
Glutamine	0.02
Aspartate	0.03
Glutamate	0.03
Leucine	0.03
Methionine	0.05
Lysine	0.05
Phenylalanine	0.10
Serine	0.11
Threonine	0.12
Arginine	0.13
Alanine	0.14
Isoleucine	0.14
Tryptophan	0.28
Tyrosine	0.33
Glycine	0.37
Histidine	0.42
Proline	0.80

β-galactosidase assays were performed for *gln3*Δ (CKY894) with a P_{GAP1}-*LacZ* reporter construct (pCK211) after growth in minimal ammonia media (SD) with 3 mM of the indicated amino acid for 16 hours.

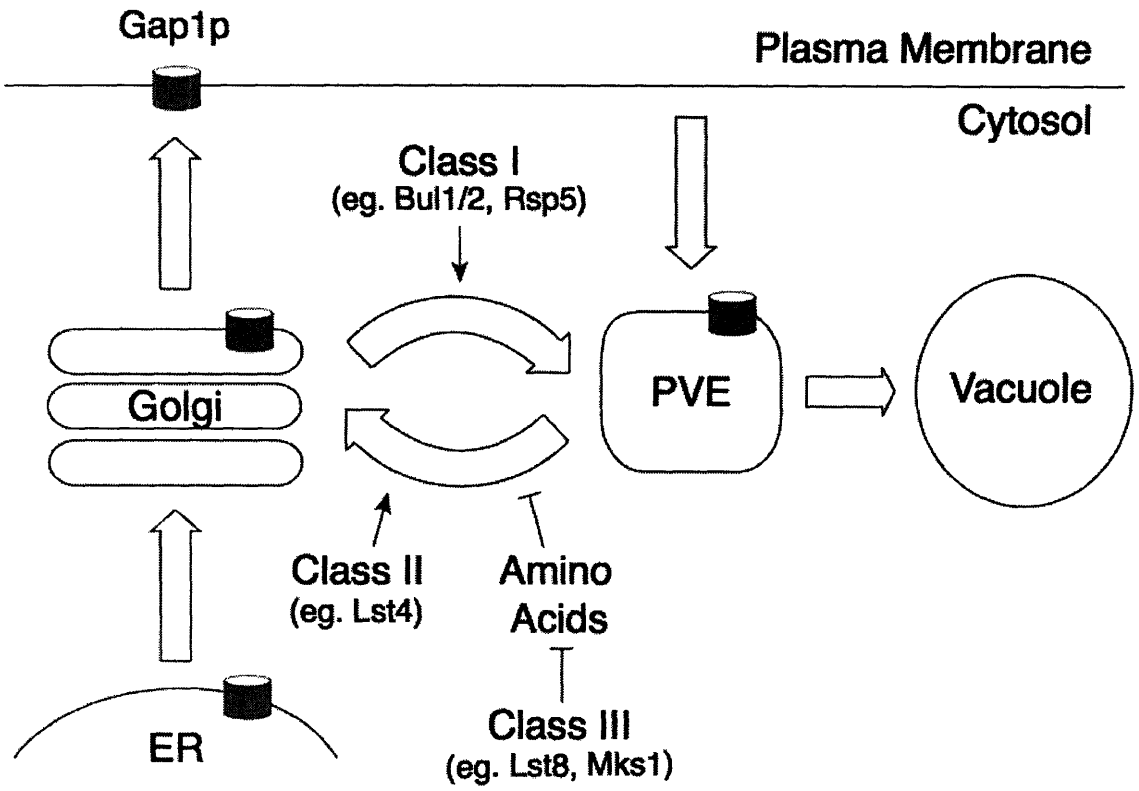


Figure 1. Genetic control of Gap1p sorting. Class I mutants are defective in transport of Gap1p from the *trans*-Golgi to the PVE and include components of the E3-ubiquitin ligase complex. Class II mutants are defective in retrieval of Gap1p from the pre-vacuolar endosome (PVE) to the *trans*-Golgi. Class III mutants alter internal amino acid levels and affect Gap1p sorting indirectly as a result of these altered amino acid levels.

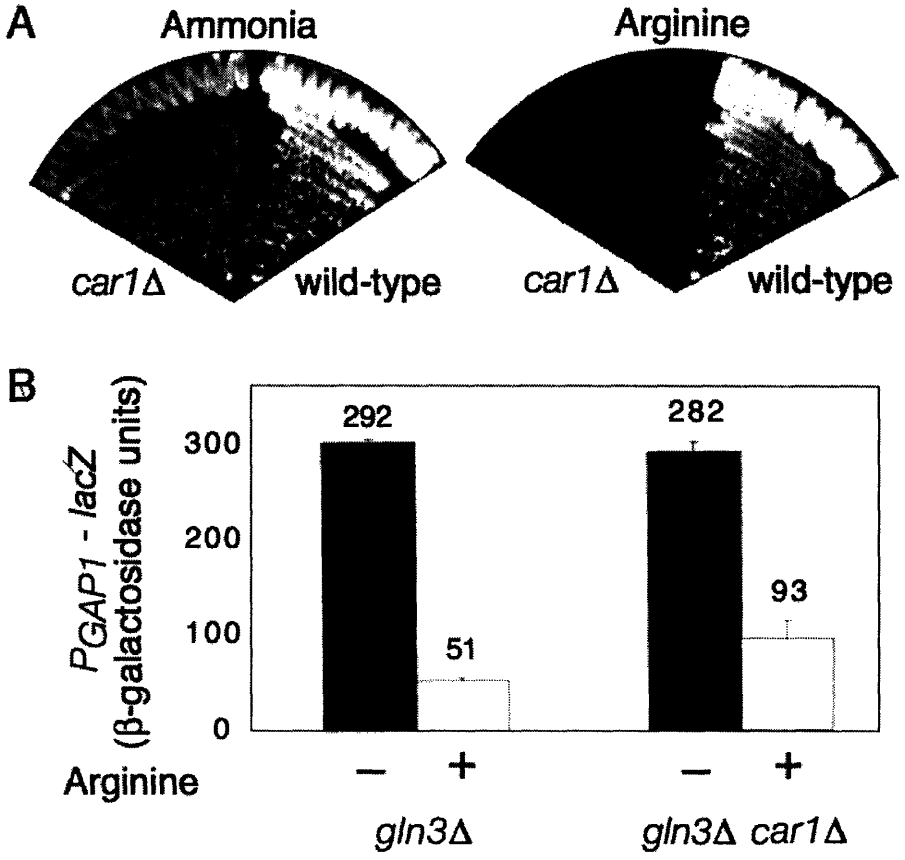


Figure 2. Amino acid metabolism is not required for repression of Nil1p-dependent *GAP1* transcription. (A) *gln3*Δ (CKY894) or *gln3*Δ *car1*Δ (CKY898) with a P_{GAP1}-*lacZ* reporter (pMS29) were streaked onto SD plates or minimal plates containing arginine as a sole nitrogen source and incubated at 30° C. (B) β-galactosidase assays were performed for *gln3*Δ (CKY894) or *gln3*Δ *car1*Δ (CKY898) with a P_{GAP1}-*lacZ* reporter (pMS29) in minimal ammonia medium alone or with 3 mM arginine.

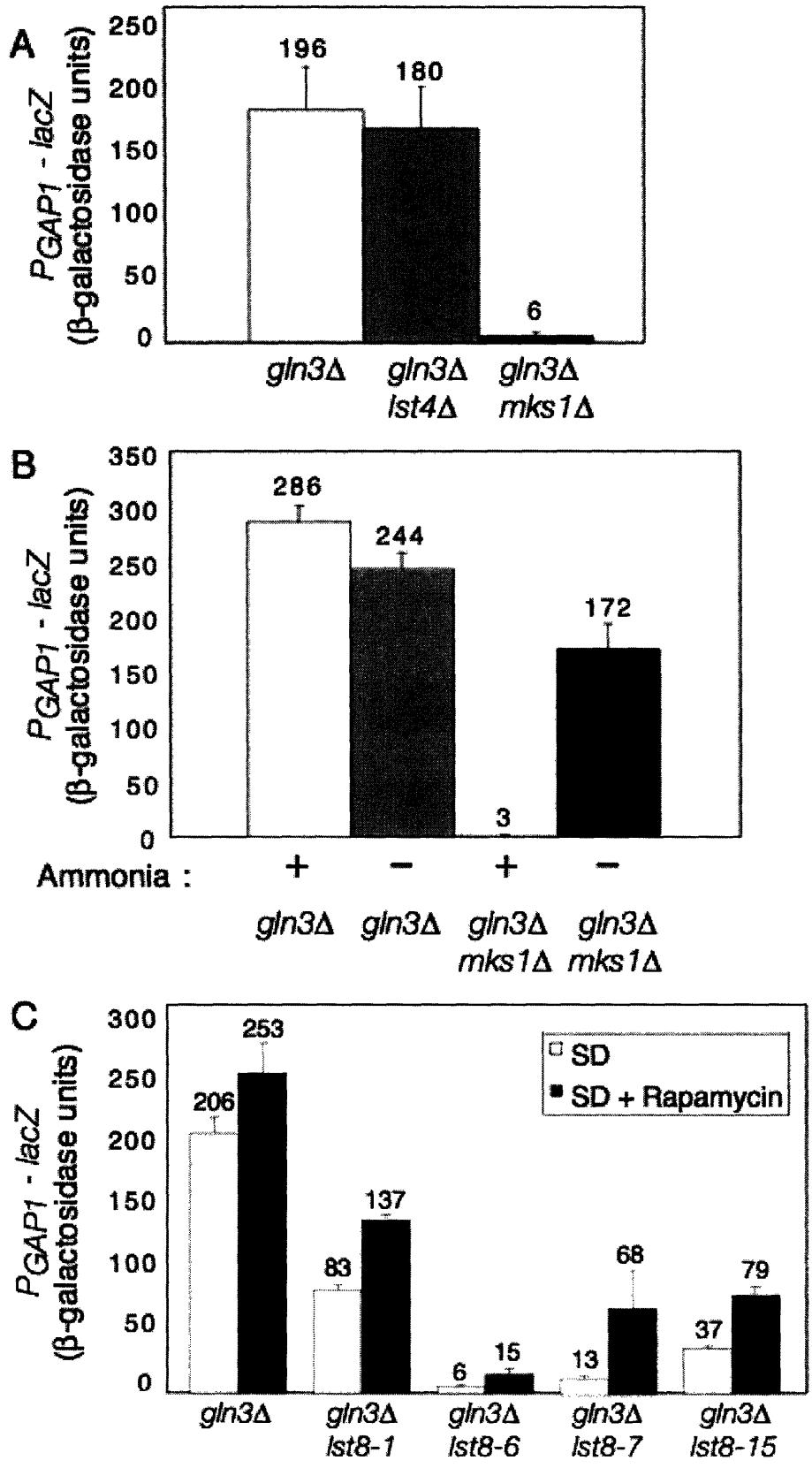

A
P_{GAP1} - lacZ
(β-galactosidase units)
250
200
150
100
50
0
196
180
6
gln3Δ
gln3Δ lst4Δ
gln3Δ mks1Δ
B
P_{GAP1} - lacZ
(β-galactosidase units)
350
300
250
200
150
100
50
0
286
244
3
172
Ammonia :
+
−
+
−
gln3Δ
gln3Δ
gln3Δ mks1Δ
gln3Δ mks1Δ
C
P_{GAP1} - lacZ
(β-galactosidase units)
300
250
200
150
100
50
0
SD
SD + Rapamycin
206
253
83
137
6
15
13
68
37
79
gln3Δ
gln3Δ lst8-1
gln3Δ lst8-6
gln3Δ lst8-7
gln3Δ lst8-15

Figure 3. **Nil1p-dependent *GAP1* transcription is repressed by high internal amino acid levels.** (A) β-galactosidase assays were performed for *gln3D* (CKY894), *gln3*Δ *lst4*Δ (CKY879), or *gln3*Δ *mks1*Δ (CKY878) with a P_{GAP1}-*lacZ* reporter (pMS29) in SD medium. (B) β-galactosidase assays were performed as in (A). Cells were washed and cultured in either ammonia medium (ammonia +) or nitrogen free medium (ammonia –) for four hours prior to assay. (C) β-galactosidase assays were performed for *gln3*Δ (CKY894), *gln3*Δ *lst8-1* (CKY880), *gln3*Δ *lst8-6* (CKY881), *gln3*Δ *lst8-7* (CKY882), or *gln3*Δ *lst8-15* (CKY883) with a P_{GAP1}-*lacZ* reporter (pMS29) in SD medium. Where rapamycin treatment is indicated, a sublethal dose of rapamycin was present during the full growth period.

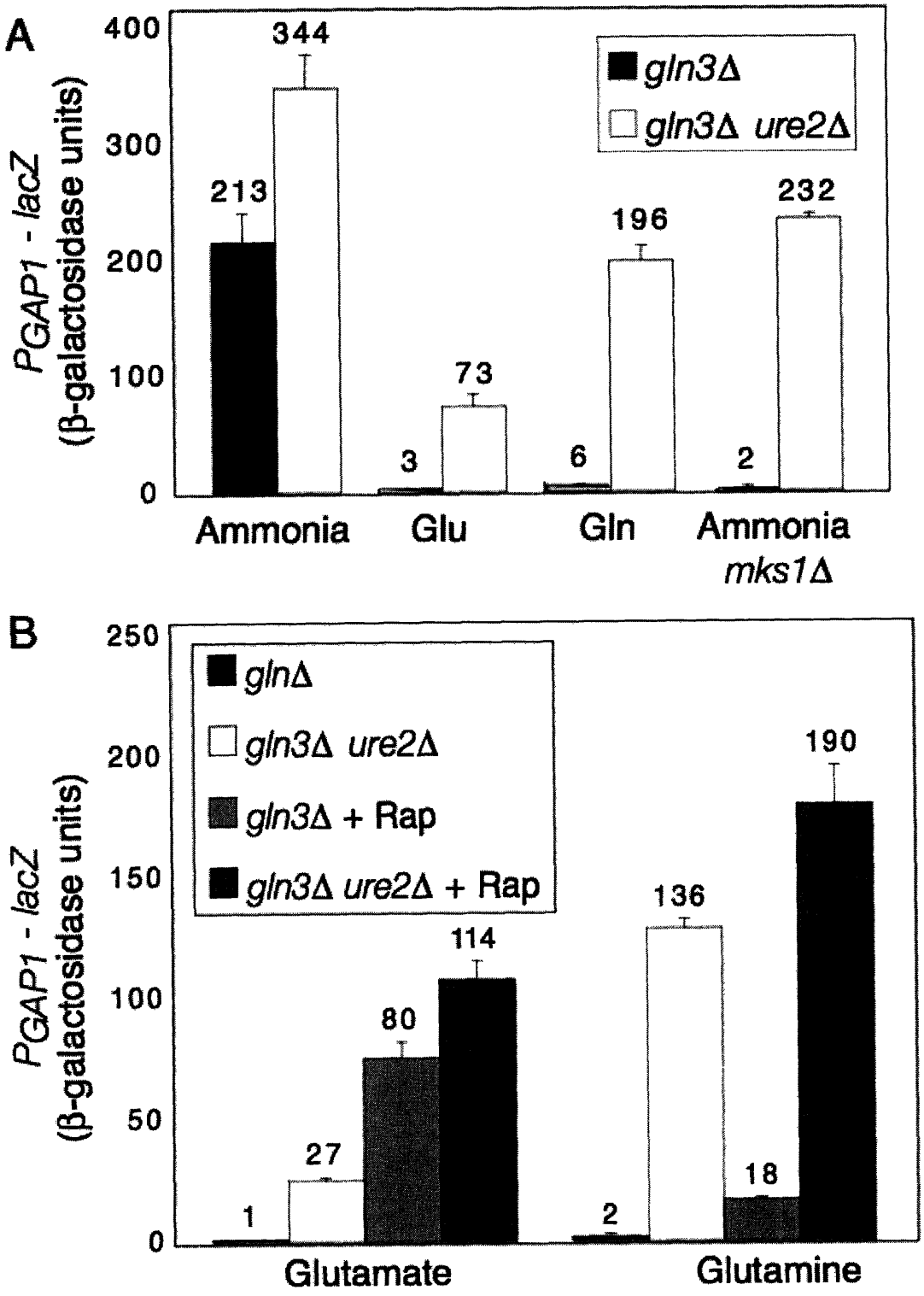

A
400
300
200
100
0
PGAP1 - lacZ
(β-galactosidase units)
344
213
73
3
6
196
2
232
gln3Δ
gln3Δ ure2Δ
Ammonia
Glu
Gln
Ammonia
mks1Δ
B
250
200
150
100
50
0
PGAP1 - lacZ
(β-galactosidase units)
glnΔ
gln3Δ ure2Δ
gln3Δ + Rap
gln3Δ ure2Δ + Rap
1
27
80
114
2
136
18
190
Glutamate
Glutamine

Figure 4. *URE2* and the *TOR* pathway independently control Nil1p-dependent *GAP1* transcription. (A) and (B) β-galactosidase assays were performed for *gln3*Δ (CKY894) or *gln3*Δ *ure2*Δ (CKY886) with a P_{GAP1}-*lacZ* reporter (pMS29) in medium containing ammonia, glutamate, or glutamine as the nitrogen source. Where rapamycin treatment is indicated, a sublethal dose of rapamycin was present during the entire growth period.

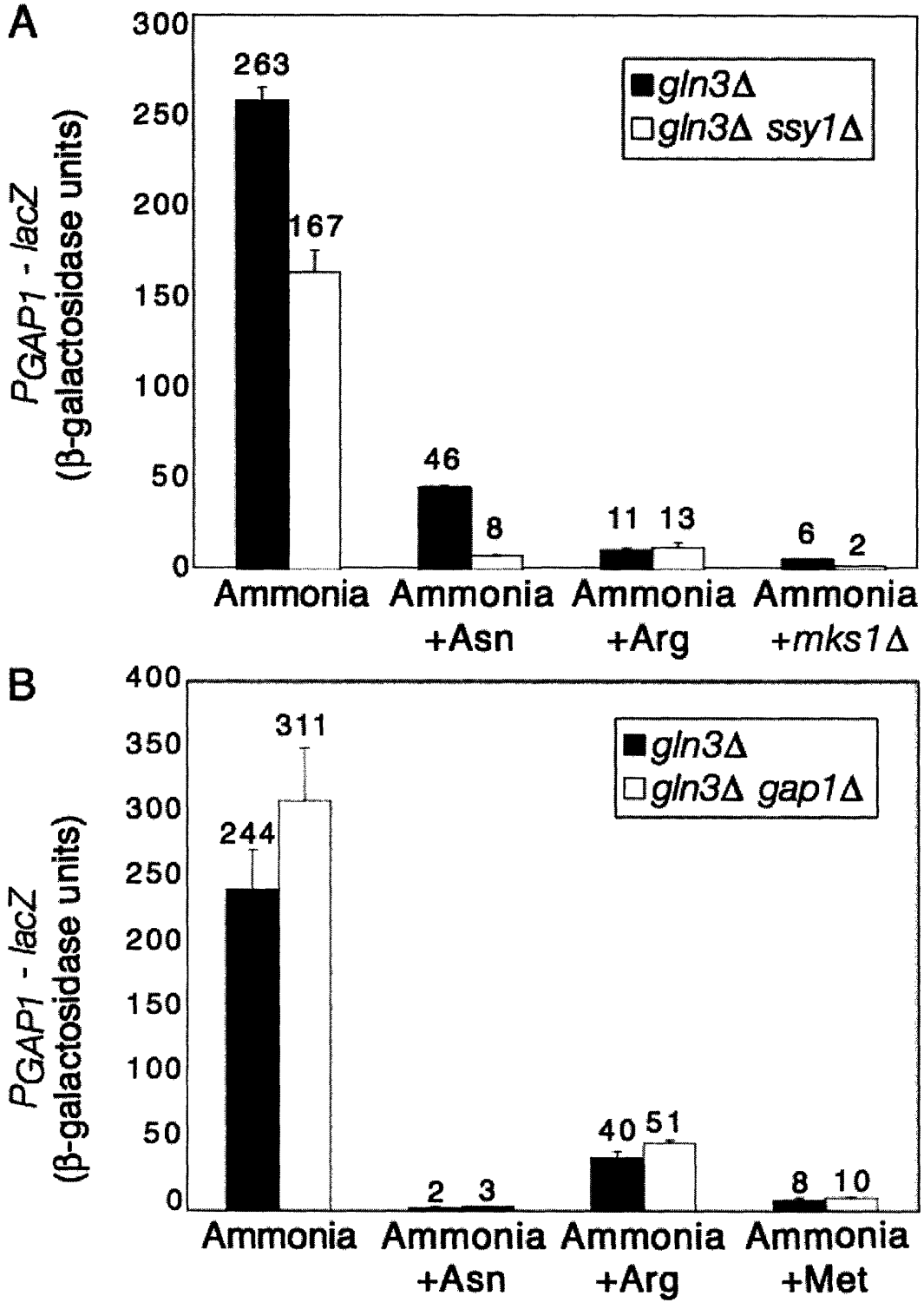

A
300
250
200
150
100
50
0
PGAP1 - lacZ
(β-galactosidase units)
263
167
46
8
11
13
6
2
gln3Δ
gln3Δ ssy1Δ
Ammonia
Ammonia +Asn
Ammonia +Arg
Ammonia +mks1Δ
B
400
350
300
250
200
150
100
50
0
PGAP1 - lacZ
(β-galactosidase units)
311
244
2
3
40
51
8
10
gln3Δ
gln3Δ gap1Δ
Ammonia
Ammonia +Asn
Ammonia +Arg
Ammonia +Met

Figure 5. The amino acid sensor *SSY1* and the Gap1p permease are not required for the repression of Nil1p-dependent *GAP1* transcription in response to amino acids. (A) β-galactosidase assays were performed for *gln3*Δ (CKY894), *gln3*Δ *ssy1*Δ (CKY887), *mks1*Δ *gln3*Δ (CKY878), or *mks*1Δ *gln3*Δ *ssy1*Δ (CKY889) with a P_{GAP1}-*lacZ* reporter (pMS29) in SD alone or SD + 3 mM of the indicated amino acid. (B) β-galactosidase assays were performed as in (A) for *gln3*Δ (CKY894), or *gln3*Δ *gap1*Δ (CKY884).

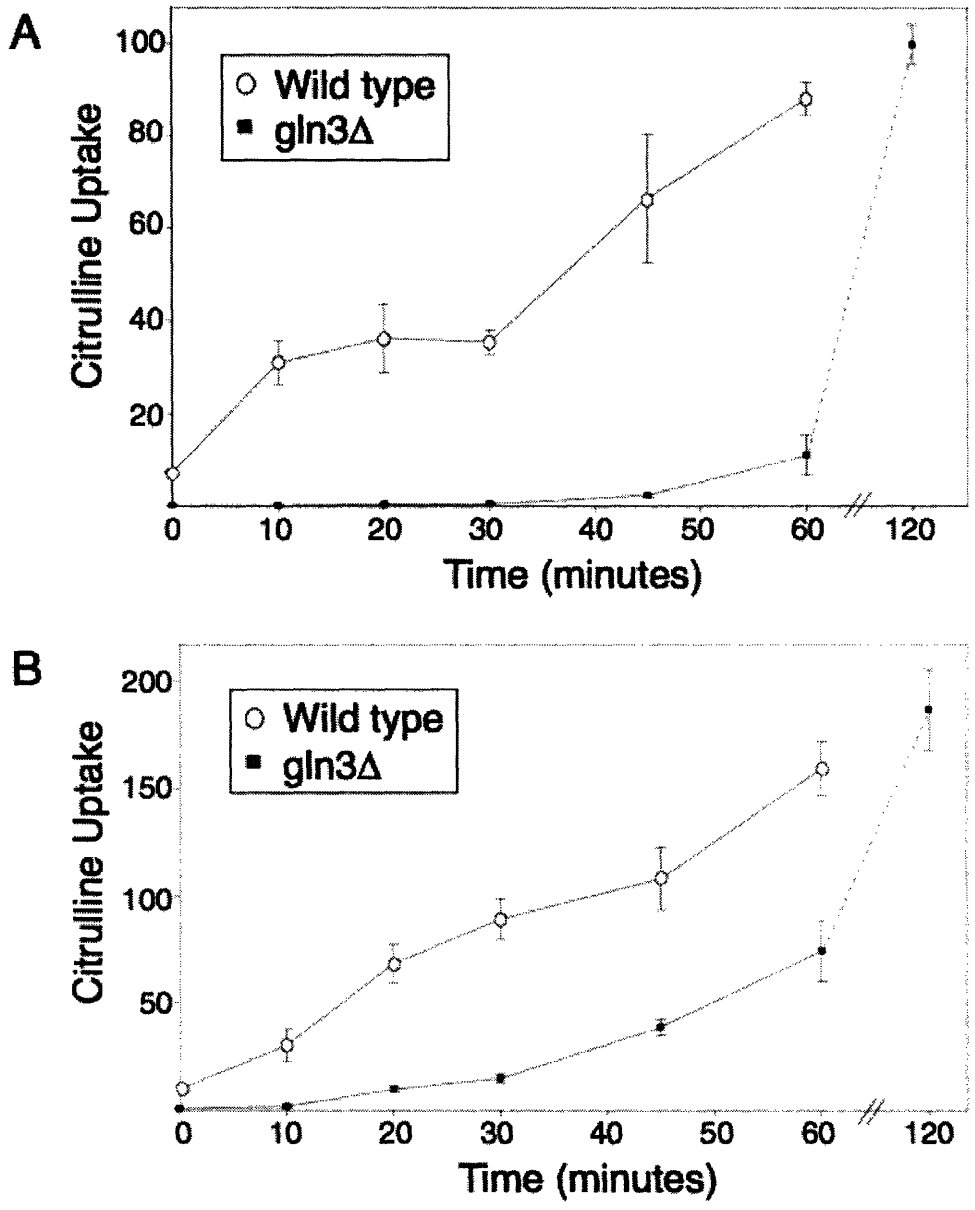
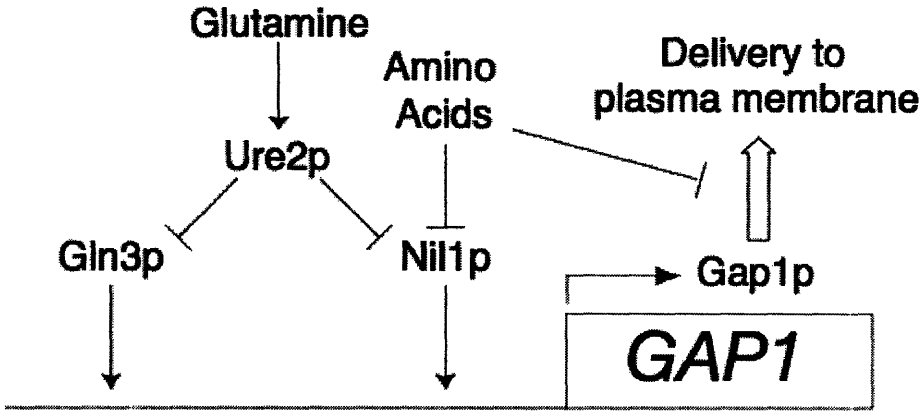

A
Citrulline Uptake
100
80
60
40
20
Wild type
gln3Δ
0
10
20
30
40
50
60
120
Time (minutes)
B
Citrulline Uptake
200
150
100
50
Wild type
gln3Δ
0
10
20
30
40
50
60
120
Time (minutes)

Figure 6. Gln3p-dependent *GAP1* expression in the presence of amino acids is required for rapid mobilization of Gap1p upon a shift to amino acid free medium. (A) Citrulline uptake assays were performed for wild type (CKY772) or *gln3*Δ (CKY894) at the indicated time after shift from minimal medium with glutamate as the nitrogen source to minimal medium with urea as the nitrogen source. (B) Citrulline uptake assays were performed as in (A) for a shift from minimal medium with leucine as the nitrogen source to minimal medium with urea as the nitrogen source.

A



B

Nitrogen source	amino acid quantity	Nitrogen source quality	*GAP1* transcription	Gap1p sorting	Gap1p activity
Urea	low	low	Gln3 & Nil1	PM	high
ammonia	low	high	Nil1	PM	high
glutamate	high	low	Gln3	internal	low*
glutamine	high	high	off	(internal)	low
amino acid mixture	high	high	off	(internal)	low

*rapidly activated upon shift to amino acid free medium

Figure 7. Regulation of *GAP1* by two transcription factors and by intracellular sorting govern permease activity under a variety of growth conditions. (A) Transcription of *GAP1* is activated by Nil1p and Gln3p. Gln3p activity is repressed in rich nitrogen sources, such as glutamine; whereas Nil1p is inactivated under conditions of amino acid abundance. Ure2p is involved in repression of both Nil1p and Gln3p in glutamine. Sorting of Gap1p to the plasma membrane is regulated similarly to Nil1p and down-regulates Gap1p activity when any amino acid is present at high concentration. (B) The regulatory network of *GAP1* transcription allows cells to adjust Gap1p activity in response to both the quantity of amino acids and quality of nitrogen source. When cells are grown on a non-amino acid nitrogen source, Gap1p is transcribed by Nil1p and permease activity is high. When cells are grown on an amino acid that is a relatively poor nitrogen source such as glutamate or leucine, Gap1p activity is low, but intracellular Gap1p expressed by Gln3p can be rapidly activated upon transfer to amino acid free medium. When cells are grown in a high quality amino acid nitrogen source, such as glutamine or the mixture of amino acids present in rich medium, *GAP1* is not transcribed.

Chapter 3

The Ubiquitin Code of Gap1p Trafficking

Abstract

The high capacity general amino acid permease, Gap1p, in *S.cerevisiae* is redistributed between internal compartments and the plasma membrane upon alterations in amino acid availability. When internal amino acid levels are low, Gap1p is localized to the plasma membrane where it imports available amino acids from the medium. When sufficient amino acids are imported, Gap1p at the plasma membrane is endocytosed and newly synthesized Gap1p is delivered to the vacuole; both sorting steps require Gap1p ubiquitination. Although it has been suggested that identical *trans*-acting factors and Gap1p ubiquitin acceptor sites are involved in both processes, we clearly defined three unique ubiquitin-mediated sorting steps required for complete delivery of Gap1p to the vacuole upon amino acid addition. Our finding that distinct ubiquitin-mediated sorting steps employ unique *trans*-acting factors, ubiquitination sites on Gap1p, and types of ubiquitination demonstrates a previously unrecognized level of specificity in ubiquitin-mediated protein sorting.

Introduction

The movement of transmembrane proteins through the secretory pathway to a specific cellular or extracellular destination often requires specific targeting signals in the substrate protein (Harter and Wieland 1996). One signal utilized by transmembrane proteins to direct their intracellular distribution between organelles is ubiquitination. Ubiquitin is a highly conserved 76 amino acid peptide that can be covalently attached to lysine residues in a target protein through a series of coordinated enzymatic reactions (Pickart and Eddins 2004). Ubiquitin molecules can also be linked to one another through the covalent attachment of one ubiquitin molecule to an exposed lysine residue in another to form ubiquitin chains (Hoppe 2005). Although there are seven lysine residues in a single ubiquitin polypeptide, lysine 48 and lysine 63 are the most frequently used to form polyubiquitin chains (Peng, Schwartz et al. 2003). Therefore, proteins can be monoubiquitinated (one ubiquitin molecule on a single lysine residue), multiubiquitinated (one ubiquitin molecule on several separate lysine residues), or polyubiquitinated (several ubiquitin molecules in a chain on a single lysine residue).

Ubiquitin modification of transmembrane proteins has been shown to regulate several distinct intracellular processes including ER-associated degradation, (McCracken and Brodsky 2005), sorting in the *trans*-Golgi (Helliwell, Losko et al. 2001), endocytosis from the plasma membrane (Hicke and Riezman 1996), and entry into the multivesicular endosome (MVE) (Katzmann, Babst et al. 2001). Therefore, the ubiquitination status of a transmembrane protein can greatly impact its intracellular distribution, degradation, and resulting activity.

The expression, localization, and activity of the high capacity general amino acid permease in *S.cerevisiae*, Gap1p, is regulated by amino acids such that it transports available amino acids from the surrounding medium into the cell only when internal amino acid levels are low (Stanbrough, Rowen et al. 1995; Chen and Kaiser 2002; Risinger, Cain et al. 2006). Previous studies have shown that ubiquitination of Gap1p on two N-terminal lysine residues (9 and 16) is required for redistribution of the permease from the plasma membrane to internal compartments (Soetens, De Craene et al. 2001). This dynamic, ubiquitin-mediated regulation of Gap1p allows the cell to upregulate amino acid import rapidly when internal amino acid levels become limiting, while avoiding excess amino acid import which can be lethal to cells expressing a non-ubiquitinateable form of Gap1p (Roberg, Rowley et al. 1997; Risinger, Cain et al. 2006).

It is clear that ubiquitin-mediated sorting of Gap1p to the vacuole can occur independently of the ability of Gap1p to be endocytosed by what we will henceforth refer to as "direct sorting" of the permease to the vacuole (De Craene, Soetens et al. 2001; Helliwell, Losko et al. 2001). After newly synthesized Gap1p reaches the *trans*-Golgi, the permease can be poly-ubiquitinated on either lysine 9 or 16 by the Rsp5p-Bul1p-Bul2p ubiquitin ligase complex (Helliwell, Losko et al. 2001; Soetens, De Craene et al. 2001). At the *trans*-Golgi, an ubiquitin-dependent sorting decision is made; non-ubiquitinated Gap1p is sorted to the plasma membrane while poly-ubiquitinated Gap1p is sorted to the multi-vesicular endosome. Once Gap1p reaches the multi-vesicular endosome, the permease can either enter the vacuolar lumen and be degraded or recycle back to the *trans*-Golgi for another attempt at reaching the cell surface; this recycling step is inhibited by the presence of elevated internal amino acid levels or mutations such as

*lst4*Δ (Chen and Kaiser 2002; Rubio-Texeira and Kaiser 2006). This dynamic, ubiquitin-dependent recycling of Gap1p between the multi-vesicular endosome and the *trans*-Golgi allows for rapid redistribution of the permease upon changing nutritional conditions (Roberg, Rowley et al. 1997).

It appears that ubiquitination also plays a role in the endocytosis of Gap1p given the requirement of either one of the two ubiquitin acceptor lysine residues in the rapid loss of Gap1p activity at the plasma membrane upon ammonia addition in the Σ1278 strain background (Soetens, De Craene et al. 2001). Although it has been determined that direct sorting of Gap1p is independent of endocytosis, it is unclear what role ubiquitin-mediated endocytosis plays in delivery of Gap1p to the vacuole independently of direct sorting since all known mutants that affect Gap1p trafficking have been shown to affect direct sorting of the permease. With no known mutant that differentially affects direct sorting and endocytosis of Gap1p, it has been suggested that identical *cis* and *trans*-acting factors are required for both steps of ubiquitin-mediated Gap1p trafficking. Another possibility is that Gap1p endocytosis is a constitutive, ubiquitin-independent process and that the ubiquitin-mediated direct sorting of the permease dictates whether endocytosed Gap1p is redelivered to the plasma membrane or sent to the vacuole for degradation.

The isolation and characterization of mutants that affect the intracellular distribution of Gap1p has relied almost exclusively on the hypothesis that Gap1p activity is high when the permease is localized at the plasma membrane. However, our recent finding that Gap1p can be rapidly and reversibly inactivated at the plasma membrane prompted us to reevaluate this assumption (Risinger, Cain et al. 2006). Additionally, the effect of amino acid addition to mutants defective in Gap1p ubiquitination has been

previously unstudied as the addition of any single amino acid (other than alanine or phenylalanine) is toxic to mutants defective in ubiquitin-mediated sorting of the permease. Our finding that this toxicity is due to an internal amino acid imbalance and that addition of rich amino acid mixtures (such as Casamino acids) are not toxic has allowed us to observe previously unrecognized differences between *bul1/2*Δ and *GAP1*^{K9R,K16R} mutants (Risinger, Cain et al. 2006).

In this study, we clearly define the *cis*- and *trans*-acting factors for three unique ubiquitin-mediated sorting steps involved in delivery of Gap1p to the vacuole: 1) direct sorting from the *trans*-Golgi to the multivesicular endosome involving Rsp5p-Bul1/2p-dependent polyubiquitination of Gap1p on either lysine 9 or 16, 2) endocytosis involving Rsp5p-Bul1/2p-dependent monoubiquitination of Gap1p on lysine 9, and 3) endocytosis involving Rsp5p-dependent, Bul1/2p-independent monoubiquitination of Gap1p on lysine 16.

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).

Plasmids used in this study were pCK137, *GAP1*$^{-HA}$ in pRS316; pAR51, *GAP1*$^{K9R-HA}$ in pRS316; pAR58, *GAP1*$^{K16R-HA}$ in pRS316; pSH55, *GAP1*$^{K9R,K16R-HA}$ in pRS316; pCK231, *CUP1* promoted *UBI-c-myc* in pRS423; pCK232, *CUP1* promoted *UBI* in pRS423; pAR70, *GAP1* in pRS316; pAR71, *GAP1*^{K9R} in pRS316; pAR72, *GAP1*^{K16R} in pRS316; pAR73, *GAP1*^{K9R,K16R} in pRS316; pEC221, *ADH1* promoted *GAP1* in pRS316; pAR13, *ADH1* promoted *GAP1-GFP* in pRS316; pAR14, *ADH1* promoted *GAP1*^{K9R,K16R}-*GFP* in pRS316; pAR32, *ADH1* promoted *GAP1*^{K9R}-*GFP* in pRS316; pAR33, *ADH1* promoted *GAP1*^{K16R}-*GFP* in pRS316; pAR92, *CUP1* promoted *UBI*^{K63R}-*c-myc* in pRS316; pAR107, *CUP1* promoted *UBI-c-myc* in pRS316; pAR90, *CUP1* promoted *UBI* in pRS316; pAR91, *CUP1* promoted *UBI*^{K63R} in pRS316; pAR88, *GAP1*^{E583D} in pRS316; pAR18, *GAP1*$^{E583D-HA}$ in pRS316; pAR95, *GAP1*$^{K9R,\ E583D}$ in pRS316; pAR89, *GAP1*^{K16R,E583D} in pRS316; pAR96, *GAP1*^{K9R,K16R,E583D} in pRS316; pAR93, *GAP1*^{K16R,E583D}-*GFP* in pRS316; pAR41, *ADH1* promoted *GAP1*^{E583D}-*GFP* in pRS316. All *GAP1* constructs expressed from the wildtype *GAP1* promoter unless otherwise indicated. The levels of *GAP1* expressed from the *ADH1* promoter are roughly equivalent to the levels of *GAP1* expressed from the wildtype *GAP1* promoter in minimal ammonia medium (Chen and Kaiser 2002).

Strains are grown at 24° in minimal (SD) medium unless otherwise noted. SD medium is composed of Difco yeast nitrogen base without amino acids and without ammonium sulfate, 2% glucose, and 0.5% ammonium sulfate (adjusted to pH 4.0 with HCl). Casamino acid medium contains SD with Casamino acids (Difco) added from a 10% stock (pH 4.0) to a final concentration of 0.25%.

Screen for Gap1p ubiquitination 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 600 bp of the *GAP1* 3' UTR was amplified in four 50 µl reactions with AmpliTaq Gold (Perkin Elmer) and 0.3mM $MnCl_2$. PCR products were transformed along with gapped pEC221 plasmid (lacking the *GAP1* ORF) into CKY482 (*gap1Δ ura3-52*) and gap repaired plasmids were isolated by selection for Ura^+ transformants. Glycine sensitive transformants were identified by replica plating onto SD with 1 mM glycine at 30° C. Plasmids were isolated from glycine sensitive colonies, retransformed into CKY482, and retested for glycine sensitivity. Plasmids conferring sensitivity to glycine arose at a frequency of about 10^{-3}.

Immunoprecipitation and immunoblotting of Gap1p

For the detection of Gap1 protein levels and mono-ubiquitination, cultures were grown in SD medium with or without the addition of 0.25% Casamino acids at indicated temperature to early exponential phase. $4x10^7$ cells were collected and lysed in 50mM Tris pH 7.5 with 1mM EDTA pH 8 and protease inhibitors by glass bead lysis. Proteins

were solubilized by the addition of 4x sample buffer, resolved by 8% SDS-PAGE, and detected by immunoblot using rabbit anti-Gap1p antibody and HRP-coupled sheep anti-rabbit serum (Amersham Pharmacia).

For the detection of Gap1p poly-ubiquitination, Gap1p^{-HA} was immunoprecipitated and then detected by immunoblotting following an adaptation of the protocol described by Laney and Hochstrasser (Laney and Hochstrasser 2002). A *pep4*Δ *doa4*Δ strain expressing the indicated *GAP1*$^{-HA}$ allele was also transformed with the indicated *CUP1* promoted *UBI* allele and cultured in SD medium with 1μM $CuSO_4$ to exponential phase. $2x10^8$ cells were collected on 0.45-μm nitrocellulose filters, suspended in 200 μl SDS buffer (1% SDS; 45 mM Na-HEPES, pH 7.5; and 50 mM NEM containing protease inhibitors), and lysed with glass beads. Lysates were diluted in 700 μl of Triton buffer (1% Triton X-100; 150 mM NaCl; 50 mM Na-HEPES, pH 7.5; 5 mM Na-EDTA; and 10 mM NEM with protease inhibitors) and centrifuged at 4°C, 14,000*g*. Immunoprecipitation was carried out by overnight incubation at 4°C with 10 μl of rat anti-HA [3F10] (Roche, Indianapolis, IN), followed by a two hour incubation at 4°C upon addition of 60 μl of a 50% suspension of protein G-Sepharose 4 fast flow (Amersham Pharmacia Biotech, Piscataway, NJ). The beads were washed five times with 1% Triton in PBS and immunoprecipitates were solubilized by incubation in sample buffer for 1 h at 37°C and resolved by 8% SDS-PAGE. Antibodies used were Rabbit anti-Gap1p; mouse anti-*myc* [9E10] (Santa Cruz Biotechnology, Santa Cruz, CA); HRP-coupled sheep anti-rabbit serum (Amersham Pharmacia); and HRP-coupled sheep anti-mouse serum (Amersham Pharmacia).

Fluorescence Microscopy

GAP1-GFP expressing cells were cultured overnight in SD medium with or without the addition of 0.25% Casamino acids to exponential phase at 24°C. For latrunculin A treatment, $1x10^7$ cells were centrifuged into 50µl of SD medium and treated with 40µM latrunculin A (in EtOH) for 20 minutes prior to Casamino acid addition. For cycloheximide chases, 50µg/ml cycloheximide was added to cultures for 20 minutes prior to Casamino acid addition. 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).

Amino acid uptake assays

Strains were cultured to 4-8 x 10^6 cells/ml 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).

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). For detection of poly-ubiquitinated Gap1p, 50mM NEM was added to the lysis buffer. Antibodies used were: rabbit anti-Gap1p and horseradish peroxidase-coupled sheep anti rabbit (Amersham Pharmacia). GDPase assays and Pma1 immunoblotting

were performed on fractions to ensure proper separation of internal and plasma membrane fractions.

Internal mono-ubiquitination assay

Strains containing the *sec6-4* temperature sensitive mutation were grown at the permissive temperature of 24°C in SD medium with 3mM glutamine to repress *GAP1* expression. In exponential phase, cells were collected on 0.45-μm nitrocellulose filters, washed, and resuspended in SD medium pre-warmed to either 24°C or 36°C. $2x10^7$ cells were harvested at the indicated time after medium shift and subjected to lysis and Gap1p immunoblotting.

Results

Bul1/2p-independent monoubiquitination on lysine 16 is sufficient for delivery of Gap1p to the vacuole.

In agreement with previous studies (Helliwell, Losko et al. 2001; Soetens, De Craene et al. 2001), we found that Bul1/2p-dependent poly-ubiquitination of Gap1p required the presence of either lysine 9 or 16 of Gap1p (Figure 1A). We also reproduced the previously published but poorly understood result that, although Bul1p and Bul2p are required for Gap1p polyubiquitination, Gap1p retains the ability to be mono-ubiquitinated in an Rsp5p-dependent manner in the absence of Bul1p and Bul2p (Helliwell, Losko et al. 2001)(Figure 1B&C). We found that the Rsp5p-dependent, Bul1/2p-independent mono-ubiquitination of Gap1p was specifically due to modification of lysine 16 as this residual monoubiquitination was abolished upon expression of $GAP1^{K16R}$ in a *bul1/2*Δ mutant (Figure 1B).

It has been suggested that both biosynthetic and endocytic delivery of Gap1p to the vacuole require either Bul1p or Bul2p as well as either lysine 9 or 16 of Gap1p (Helliwell, Losko et al. 2001; Soetens, De Craene et al. 2001). We were surprised then to observe accumulation of Gap1p in the vacuolar lumen in a *bul1/2*Δ mutant when cells were grown in the presence of rich amino acid mixtures (Figure 2). In contrast, the non-ubiquitinated $Gap1p^{K9R,K16R}$ mutant failed to accumulate in the vacuole upon amino acid treatment (Figure 2). Together, these findings demonstrate the presence of a Bul1/2p-independent ubiquitin-mediated sorting step involved in delivery of Gap1p to the vacuole that requires one or both of the ubiquitin acceptor lysines in Gap1p. We speculated that Bul1/2p-independent delivery of Gap1p to the vacuole required lysine 16 of Gap1p since

this residue is required for the residual monoubiquitination of the permease observed in a *bul1/2*Δ mutant (Figure 1B). Indeed, we found that expression of $GAP1^{K16R}$ in a *bul1/2*Δ mutant completely abolished amino acid-dependent delivery of Gap1p to the vacuole (Figure 3B).

Gap1p is endocytosed by both Bul1/2p-dependent and independent pathways

To test whether the Bul1/2p-independent, lysine 16-dependent delivery of Gap1p to the vacuole was due to endocytosis or direct sorting, we utilized latrunculin A, a drug that binds actin monomers and effectively inhibits endocytosis in yeast (Ayscough, Stryker et al. 1997). Latrunculin A inhibited the ability of Gap1p to be endocytosed and delivered to the vacuolar lumen upon amino acid addition when cells were pre-treated with cycloheximide to inhibit delivery of newly synthesized Gap1p to the vacuole (Figure 3A). In contrast, Gap1p accumulated in vacuolar pools when amino acids were added to latrunculin A-treated cells without cycloheximide pretreatment, supporting the model that newly-synthesized Gap1p can reach the vacuole through a direct biosynthetic pathway independently of endocytosis (Figure 3B). Latrunculin A also inhibited delivery of Gap1p to the vacuole in the *bul1/2*Δ mutant, demonstrating that Bul1/2p-independent delivery of the permease to the vacuole was due to endocytosis and solidifying the role of Bul1/2p in biosynthetic delivery of Gap1p to the vacuole (Figure 3B). Therefore, Gap1p can reach the vacuole either by endocytosis (mediated by Rsp5p-dependent, Bul1/2p-independent monoubiquitination on lysine 16) or direct sorting (mediated by Rsp5p-Bul1/2p-dependent polyubiquitination on lysine 9 or 16).

To further explore the role of ubiquitination in Gap1p endocytosis, we monitored the rate of amino acid dependent Gap1p internalization following cycloheximide treatment. We were able to compare the rate of Gap1p endocytosis in different mutant backgrounds independently their effect on permease recycling given that amino acids inhibit delivery of any internalized Gap1p back out to the plasma membrane (Chen and Kaiser 2002; Rubio-Texeira and Kaiser 2006). We observed a slight delay in Gap1p^{K16R} internalization compared to wildtype Gap1p, indicating that lysine 16 contributed toward, but was not the sole means of Gap1p endocytosis (Figure 4). Gap1p endocytosis was also noticeably slowed in a *bul1/2*Δ mutant expressing wildtype *GAP1* (Figure 4) and completely inhibited when expressing *GAP1*^{K16R} (Figure 3B), suggesting the existence of two redundant pathways for Gap1p endocytosis. In contrast, we found that Gap1p^{K9R} was endocytosed at a rate indistinguishable from the wildtype permease, demonstrating that lysine 9 is not required for either ubiquitin-mediated endocytosis event when lysine 16 is present (data not shown). These results indicate that Gap1p can be endocytosed by either one of two ubiquitin-mediated pathways: one requiring Bul1/2p-dependent ubiquitination on lysine 9 or 16 of Gap1 and another requiring Bul1/2p-independent ubiquitination specifically on lysine 16.

Bul1/2-independent monoubiquitination occurs at the plasma membrane

The finding that Bul1/2p-dependent polyubiquitination directs sorting of Gap1p to the vacuole independently of endocytosis implies that the addition of polyubiquitin chains to Gap1p occurs prior to delivery of Gap1p to the plasma membrane. To further investigate the localization of Bul1/2p-independent mono-ubiquitin addition, we utilized

a *sec6-4* temperature sensitive mutation that inhibits delivery of protein to the plasma membrane at the restrictive temperature of 36°C (Novick, Field et al. 1980; Walworth and Novick 1987). We grew a *sec6-4 bul1/2Δ* mutant at the permissive temperature of 24°C in SD medium containing 3mM glutamine to repress *GAP1* transcription. In early log phase, cells were filtered, washed, and resuspended in SD medium to induce *GAP1* expression at either 24°C or 36°C. We found that Bul1/2p-independent mono-ubiquitination of Gap1p specifically occurred at the plasma membrane as we failed to observe any ubiquitination of Gap1p when Gap1p was unable to reach the plasma membrane at the restrictive temperature of 36°C (Figure 5A&B). As a control, we determined that Bul1/2p-independent mono-ubiquitination of Gap1p was not inhibited at 36°C in an otherwise wild-type *bul1/2Δ* strain, indicating that this modification could occur at high temperatures when Gap1p was able to reach the plasma membrane (Figure 5C).

Bul1/2-independent endocytosis is enhanced by amino acid addition

Gap1p is able to receive Bul1/2p-independent monoubiquitination in the absence of any exogenous amino acid addition (Figure 1). Indeed, we found that steady state levels of monoubiquitinated Gap1p in a *bul1/2Δ* cell were indistinguishable whether or not amino acids were present in the growth medium (Figure 6A). However, we did find that the rate of Bul1/2p-independent endocytosis was increased by in the addition of amino acids (Figure 6B). Since amino acids prevent redelivery of endocytosed Gap1p to the plasma membrane, we determined the effect of amino acids on the rate of Bul1/2p-independent endocytosis in the presence of a *lst4Δ* mutant that blocks this redelivery

independently of amino acid levels (Chen and Kaiser 2002; Rubio-Texeira and Kaiser 2006). We were unable to determine the effect of amino acid addition on the rate of Bul1/2p-dependent endocytosis independently of redelivery as Gap1p is constitutively internally localized in *lst4*Δ strains when Bul1p and Bul2p are expressed (Helliwell, Losko et al. 2001).

A single mutation in the C-terminal tail of Gap1p abolishes polyubiquitination and internal sorting of the permease

We isolated additional ubiquitination mutants in *GAP1* utilizing our previous observation that addition of most single amino acids is toxic to cells expressing ubiquitination deficient mutants of Gap1p (Risinger, Cain et al. 2006). We found that a *GAP1* mutant possessing a single mutation in the C-terminal cytosolic tail, Gap1p^{E583D}, was unable to receive Bul1/2p-dependent poly-ubiquitin chains on either of the N-terminal ubiquitin acceptor lysines in Gap1p (Figure 7A). Mutants that are deficient in direct sorting of Gap1p to the vacuole allow Gap1p to be delivered to the plasma membrane in the presence of a *lst4*Δ mutant, which is impaired in the recycling of Gap1p from the multivesicular endosome to the *trans*-Golgi (Rubio-Texeira and Kaiser 2006). We found that Gap1p^{E583D} was able to be sorted to the plasma membrane in a *lst4*Δ mutant, indicating that this mutant is impaired in direct sorting of the permease from the *trans*-Golgi to the multivesicular endosome (Figure 7B). The identification of a C-terminal *cis*-acting mutation in *GAP1* that abolished Bul1/2p-dependent polyubiquitination and direct sorting while leaving the N-terminal ubiquitin-acceptor

lysine residues in tact suggests that sequences in both the cytosolic N- and C-terminus of Gap1p cooperate in Bul1/2p-dependent ubiquitination.

Monoubiquitination is sufficient for both Bul1/2p-dependent and independent endocytosis

Although the Gap1p^{E583D} mutant was unable to receive Bul1/2p-dependent polyubiquitination, we determined that it retained the ability to be monoubiquitinated (Figure 7C). We found that the residual mono-ubiquitination on Gap1p^{E583D} consisted of both Bul1/2p-independent mono-ubiquitination on lysine 16 as well as Bul1/2p-dependent mono-ubiquitination on lysine 9 (and possibly lysine 16 as well, although this could not be determined directly) (Figure 7C). Bul1/2p-dependent mono-ubiquitination on lysine 9 of Gap1p^{K16R,E583D} was found primarily in plasma membrane containing fractions, suggesting that this modification was likely involved in mediating Bul1/2p-dependent endocytosis (Figure 7D). Indeed, we found that the rate of Gap1p^{K16R,E583D} endocytosis after cycloheximide treatment was indistinguishable from Gap1p^{K16R}, indicating that Bul1/2p-dependent mono-ubiquitination on lysine 9 was sufficient to direct endocytosis of the Gap1p^{E583D} mutant (compare Figures 4 & 7E).

To further support our assertion that Gap1^{E583D} was defective in Bul1/2p-dependent direct sorting of the permease we showed that the mutant permease was able to reach the vacuole in the presence of amino acids and that this delivery was abolished upon addition of the endocytosis inhibitor latrunculin A (Figure 7F). Therefore, we have isolated a novel *GAP1* mutant, *GAP1*^{E583D}, which is impaired for Bul1/2p-dependent

polyubiquitination and direct sorting although both Bul1/2p-dependent and Bul1/2p-independent monoubiquitination and endocytosis occur unaffected.

Lysine 63-linked ubiquitin chains are not required for direct sorting of Gap1p

Overexpression of a mutant form of ubiquitin where lysine 63 is replaced by arginine (Ub^{K63R}) has been shown to retard sorting of the permease to the vacuole and impair the formation of polyubiquitin chains upon ammonia addition to strains of the Σ1278 background (Springael, Galan et al. 1999). Therefore, it has been suggested that Bul1/2p-dependent polyubiquitination of Gap1p and subsequent internal sorting and/or endocytosis require lysine 63-linked ubiquitin chains. To further explore the role of lysine 63-linked ubiquitin chains in Gap1p trafficking, we measured Gap1p ubiquitination, activity, and intracellular sorting in strains expressing the Ub^{K63R} mutant as the sole form of ubiquitin in the cell (Spence, Sadis et al. 1995). To our surprise, we observed increased polyubiquitination of Gap1p in cells expressing the Ub^{K63R} mutant when compared to an isogenic strain expressing wildtype ubiquitin (Figure 8A). To determine whether these ubiquitin chains lacking lysine 63 were sufficient for internal sorting of Gap1p, we compared Gap1p activity in strains expressing either the wildtype or the Ub^{K63R} allele of ubiquitin. We found that the activity of strains expressing Ub^{K63R} was elevated compared to strains expressing wildtype ubiquitin, suggesting that, in spite of hyper-polyubiquitination, more Gap1p was delivered to the plasma membrane when lysine 63-linked polyubiquitin chains could not be synthesized (Figure 8B).

There are two known classes of mutants that result in increased delivery of Gap1p to the plasma membrane. The first class includes ubiquitination mutants such as *bul1/2Δ*

or *GAP1*^{K9R,K16R} that result in increased delivery of Gap1p to the plasma membrane because the permease is unable to be internally trafficked from the *trans*-Golgi to the endosome (Helliwell, Losko et al. 2001; Soetens, De Craene et al. 2001). The second class includes *vps27*Δ and other components of the vacuolar sorting pathway that allow Gap1p to be properly sorted from the *trans*-Golgi to the endosome but are unable to incorporate the permease into multivesicular endosomes (MVEs). In these mutants, Gap1p is unable to reach the vacuolar lumen and is instead rerouted to the plasma membrane in a *lst4* and amino acid-dependent manner, resulting in increased levels of Gap1p at the plasma membrane (Rubio-Texeira and Kaiser 2006).

We determined that the high Gap1p activity in a *Ub*^{K63R} expressing strain was dramatically decreased by introduction of a *lst4*Δ mutation (Figure 8B), suggesting that ubiquitin chains lacking lysine 63 were sufficient to allow sorting of Gap1p from the *trans*-Golgi to the endosome, but defective for incorporation of Gap1p into MVEs. Indeed, we found that when *Ub*^{K63R} was the sole form of ubiquitin expressed in the cell, Gap1p accumulated in the limiting membrane of the vacuole when amino acids or a *lst4*Δ mutation prevented recycling from the endosome to the plasma membrane, resembling the Gap1p sorting defect observed in *vps27*Δ (Figure 8C). In contrast to the increased levels of polyubiquitinated Gap1p observed in *Ub*^{K63R} expressing strains, we determined that levels of high molecular weight ubiquitinated species were decreased in whole cell lysates when compared to an isogenic wildtype strain (Figure 8D). We found that the addition of copper to the medium, which increased expression of *Ub*^{K63R} from the copper inducible *CUP1* promoter, not only increased levels of these high molecular weight

ubiquitinated species, but also allowed some Gap1p to be delivered to the vacuolar lumen upon amino acid addition (Figure 8D&E).

Discussion

Although ubiquitination was initially described as a signal to target cytosolic proteins to the 26S proteasome for degradation, there have been an increasing number of ubiquitin-mediated cellular processes identified in recent years. Several membrane transporters in both yeast and mammalian cells have been shown to require ubiquitin conjugation for constitutive or induced endocytic and biosynthetic delivery to the vacuole. For many of these substrates, work has been carried out comparing the activity and/or localization of the wildtype ubiquitinated protein with non-ubiquitinated substrates and in-frame ubiquitin fusions to non-ubiquitinated substrates (Terrell, Shih et al. 1998; Reggiori and Pelham 2001; Katzmann, Sarkar et al. 2004). While this type of analysis has elucidated many important aspects of ubiquitin-mediated protein sorting, the all or none view of protein ubiquitination overlooks the potential for contextual differences in ubiquitinated residues and discounts the possibility that ubiquitination of a single substrate may be involved in multiple cellular processes.

Our ability to separate by mutation the ubiquitin-mediated trafficking steps required for Gap1p trafficking to the vacuole has overturned some long-held views concerning Gap1p trafficking and ubiquitin-mediated sorting in general. Although Gap1p is polyubiquitinated in a Bul1/2p-dependent manner, the permease retains the ability to be monoubiquitinated by Rsp5p in a *bul1/2Δ* mutant (Helliwell, Losko et al. 2001)(Figure 1). It was assumed that this residual Rsp5p-dependent monoubiquitination on Gap1p indicated that monoubiquitination was not sufficient for sorting of Gap1p at the *trans*-Golgi. Our finding that Bul1/2p-independent ubiquitination occurs exclusively at the plasma membrane to direct endocytosis revealed that Gap1p is unable to be

monoubiquitinated by Rsp5p in the *trans*-Golgi independently of the Bul1/2p proteins (Figure 5).

When Gap1p reaches the *trans*-Golgi, it is either processively polyubiquitinated by the Rsp5p-Bul1p-Bul2p ubiquitin ligase complex and sorted to the vacuole or escapes ubiquitin modification entirely and is delivered to the plasma membrane by default. In contrast, Rsp5p-Bul1p-Bul2p carry out monoubiquitination of Gap1p at the plasma membrane. The polyubiquitination of Gap1p in the *trans*-Golgi versus monoubiquitination of Gap1p at the plasma membrane by the Bul1p-Bul2p-Rsp5p ubiquitin ligase complex is reminiscent of the processive versus distributive polyubiquitination of substrates by the E3 ubiquitin ligase APC during cell cycle progression. Similarly to the ability of Rsp5p-Bul1p-Bul2p to mono or polyubiquitinate Gap1p, APC binding to substrate can result in either mono or polyubiquitination of the substrate protein depending on sequences that stabilize substrate interaction with APC (Prakash, Johnson et al. 2005). Although the processivity of ubiquitination in the case of APC dictates the order in which substrates are polyubiquitinated and thus degraded by the proteasome, it illustrates a precedent for substrate specific processivity of E3 ubiquitin ligases and suggests that the environment of Gap1p in the *trans*-Golgi may allow for a more processive interaction with the Rsp5p than at the plasma membrane.

The fact that endocytosis of Gap1p to the vacuole had not been observed in a *bul1/2*Δ mutant prior to our work also led to the faulty assumption that polyubiquitination was a prerequisite for Gap1p endocytosis as well as direct sorting (Soetens, De Craene et al. 2001). Identification of an allele of *GAP1*, $GAP1^{E583D}$, that failed to receive poly-ubiquitination but retained the ability to be endocytosed by both Bul1/2p-dependent and

Bul1/2p-independent pathways demonstrated that Gap1p endocytosis is not dependent or even enhanced by polyubiquitination (Figure 7). This finding reconciles Gap1p endocytosis with the rest of the ubiquitin-mediated endocytosis literature since it has been previously demonstrated that monoubiquitination is sufficient for endocytosis of many other plasma membrane proteins (Staub and Rotin 2006).

Another misconception we have uncovered with regard to ubiquitin-mediated sorting is the role of lysine 63-linked chains in the sorting of Gap1p. Since it has been suggested that lysine 63-linked ubiquitin chains are required for delivery of Gap1p from the *trans*-Golgi to the vacuole (Springael, Galan et al. 1999), we were surprised to find accumulation of Gap1p in the limiting membrane or lumen of the vacuole in Ub^{K63R} expressing strains (Figure 8C). A *GAP1* mutant that is truly impaired for internal sorting, $GAP1^{E583D}$, is delivered directly from the *trans*-Golgi to the plasma membrane, resulting in elevated activity even in the presence of a *lst4*Δ mutation (Figure 7). Therefore, the strongest indication that lysine 63-linked ubiquitin chains are not required for internal sorting from the *trans*-Golgi to the vacuole comes from the finding that Gap1p is localized to internal compartments in Ub^{K63R} expressing strains upon introduction of a *lst4*Δ mutation (Figure 8B&C).

Although we show lysine 63-linked polyubiquitin chains are not required for Gap1p polyubiquitination and internal sorting, the lysine 63 residue of ubiquitin may play a role in proper Gap1p trafficking to the vacuole as evidenced by accumulation of Gap1p on the vacuolar membrane in the Ub^{K63R} mutant. We are skeptical that lysine 63-linked ubiquitin chains on Gap1p are required for entry into multivesicular endosomes, as it has been shown that monoubiquitination of cargo is sufficient for this sorting step (Reggiori

and Pelham 2001; Urbanowski and Piper 2001; Katzmann, Sarkar et al. 2004). Also, since polyubiquitinated cargo (including Gap1p) can enter the vacuole in a *doa4*Δ mutant overexpressing ubiquitin, it also seems unlikely that deubiquitination of non-lysine 63-linked polyubiquitin chains by Doa4p is required for entry of Gap1p into multivesicular endosomes (Dupre and Haguenauer-Tsapis 2001; Rubio-Texeira and Kaiser 2006). Finally, lysine 63 is not required for recognition of substrate ubiquitin by the MVE machinery since an in frame fusion of Ub^{K63R} to otherwise nonubiquitinated cargo is sufficient to allow sorting into the vacuolar lumen (Urbanowski and Piper 2001).

One possibility is that, although the non-lysine 63-linked chains are sufficient for internal sorting, the Doa4p deubiquitinating enzyme may be unable to deubiquitinate cargo modified with these chains once they are delivered to the prevacuolar endosome. Although it has been demonstrated that Doa4p-dependent deubiquitination is not required for entry of polyubiquitinated proteins into the vacuolar lumen, a failure to deubiquitinate cargo decreases free ubiquitin pools in the cell, resulting in general defects in ubiquitin-mediated processes including multivesicular endosome formation (Swaminathan, Amerik et al. 1999; Dupre and Haguenauer-Tsapis 2001). We showed induction of high levels of Ub^{K63R} expressed under the copper inducible *CUP1* promoter increased the vacuolar delivery of Gap1p when it was the sole form of ubiquitin present in the cell, suggesting that the lysine 63 residue on ubiquitin is not absolutely required for vacuolar delivery (Figure 8E). Our finding that lysine 63-linked polyubiquitin chains are not required for direct sorting to Gap1p suggests that some or all of the Gap1p sorting defects observed in Ub^{K63R} mutants in previous work and in this study may be due to indirect effects of

decreased intracellular ubiquitin pools and/or impaired multivesicular body formation when Ub^{K63R} is the sole or primary form of ubiquitin expressed in the cell.

Although several ubiquitin-mediated sorting steps are required for redistribution of Gap1p from the plasma membrane to the vacuole in response to amino acid levels, it does not appear that covalent attachment of ubiquitin is the regulated step for any of these pathways. It has been previously shown that the level of Bul1/2p-dependent Gap1p poly-ubiquitination is not dramatically affected by an increase in internal amino acid levels, but that amino acids regulate trafficking of Gap1p by inhibiting redelivery of the permease to the plasma membrane once ubiquitin-mediated direct sorting has occurred (Rubio-Texeira and Kaiser 2006)(Figure 9). Similarly, in the current study we show that amino acids appear to regulate Bul1/2p-dependent endocytosis downstream of the ubiquitination event (Figure 6). In general, it appears that neither ubiquitination nor elevated amino acid levels are sufficient for redistribution of Gap1p. Instead, ubiquitin conjugation allows Gap1p to be modified in such a way to allow redistribution of the permease when amino acid levels are elevated.

There is evidence of other cases where protein ubiquitination has been shown to contain contextual information depending on the ubiquitinated residues of the substrate protein as well as the presence and linkage of ubiquitin chains. One elegant example of ubiquitin specificity is the differential regulation of the proliferating cell nuclear antigen (PCNA) by monoubiquitination, lysine 63-linked polyubiquitination, or sumolation to coordinate lesion bypass and recombinational repair during DNA synthesis with the extent and duration of DNA damage in the cell (Prakash, Johnson et al. 2005). Additionally, extensive studies of specificity in substrate phosphorylation during cellular

signaling have previously demonstrated the contextual importance of post-translational protein modification. Our ability to mutationally separate three distinct ubiquitin-mediated routes for delivery of Gap1p to the vacuole upon amino acid addition serves as an example of the underappreciated intricacy and specificity involved in ubiquitin-mediated protein sorting.

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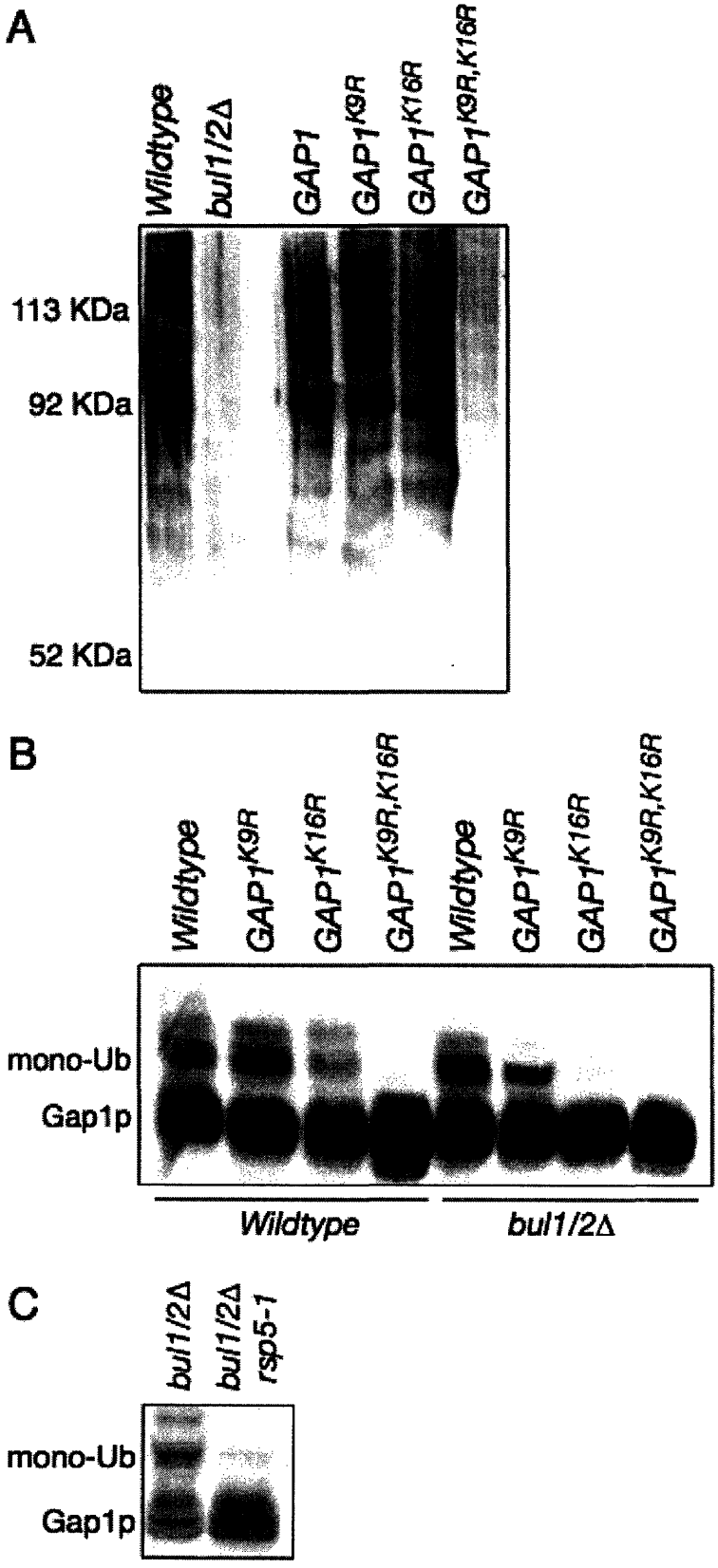

A
Wildtype
bul1/2Δ
GAP1
GAP1K9R
GAP1K16R
GAP1K9R,K16R
113 KDa
92 KDa
52 KDa
B
Wildtype
GAP1K9R
GAP1K16R
GAP1K9R,K16R
Wildtype
GAP1K9R
GAP1K16R
GAP1K9R,K16R
mono-Ub
Gap1p
Wildtype
bul1/2Δ
C
bul1/2Δ
bul1/2Δ rsp5-1
mono-Ub
Gap1p

Figure 1. **Bul1/2p-dependent polyubiquitination of Gap1p occurs on either lysine 9 or 16 whereas Bul1/2p-independent mono-ubiquitination occurs specifically on lysine 16.** (A) *pep4Δ doa4Δ gap1Δ* (CKY1031) or *bul1Δ bul2Δ pep4Δ doa4Δ gap1Δ* (CKY1032) expressing P_{cup1}-*myc-UBI* (pCK231) along with $GAP1^{-HA}$ (pCK137), $GAP1^{K9R-HA}$ (pAR51), $GAP1^{K16R-HA}$ (pAR58), or $GAP1^{K9R,K16R-HA}$ (pSH55) were grown in SD medium + 1μM $CuSO_4$. Gap1p was immunoprecipitated with rat anti-HA (3F10) and separated by SDS/PAGE. Gap1p and ubiquitin were detected by immunoblotting with anti-myc (9E10) or Gap1p antiserum respectively. Levels of unmodified Gap1 were equivalent in all lanes. (B) Lysates were prepared from *gap1Δ* (CKY482) or *bul1Δ bul2Δ gap1Δ* (CKY701) expressing *GAP1* (pAR70), $GAP1^{K9R}$ (pAR71), $GAP1^{K16R}$ (pAR72), or $GAP1^{K9R,K16R}$ (pAR73) and immunoblotted with Gap1p antiserum. (C) Lysates were prepared from *bul1Δ bul2Δ* (CKY698), or *bul1Δ bul2Δ rsp5-1* (CKY714) grown at 37°C and immunoblotted with Gap1p antiserum.

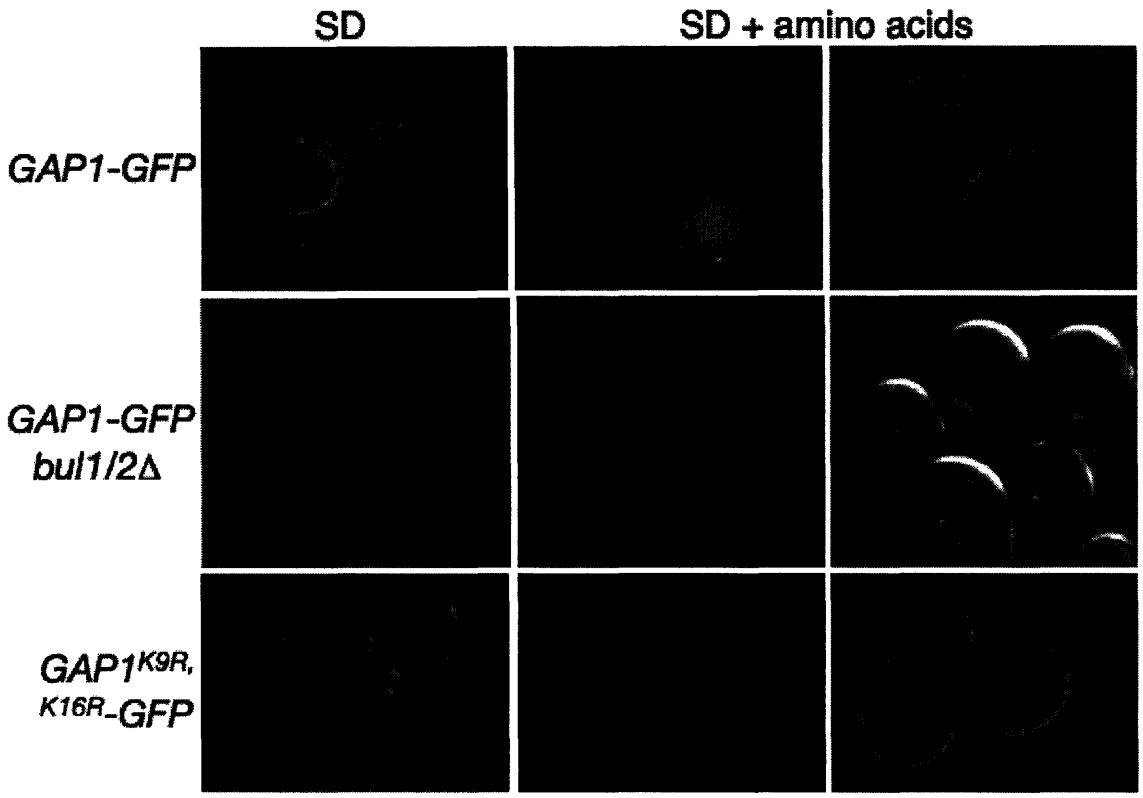


Figure 2. **Monoubiquitination of Gap1p is sufficient for delivery to the vacuole.** *gap1*Δ (CKY482) or *bul1*Δ *bul2*Δ *gap1*Δ (CKY701) expressing P_{ADH1}-*GAP1-GFP* (pAR13) or P_{ADH1}-$GAP1^{K9R,K16R}$-*GFP* (pAR14) were grown in SD medium with or without addition of 0.25% Casamino acids and visualized by fluorescence microscopy.

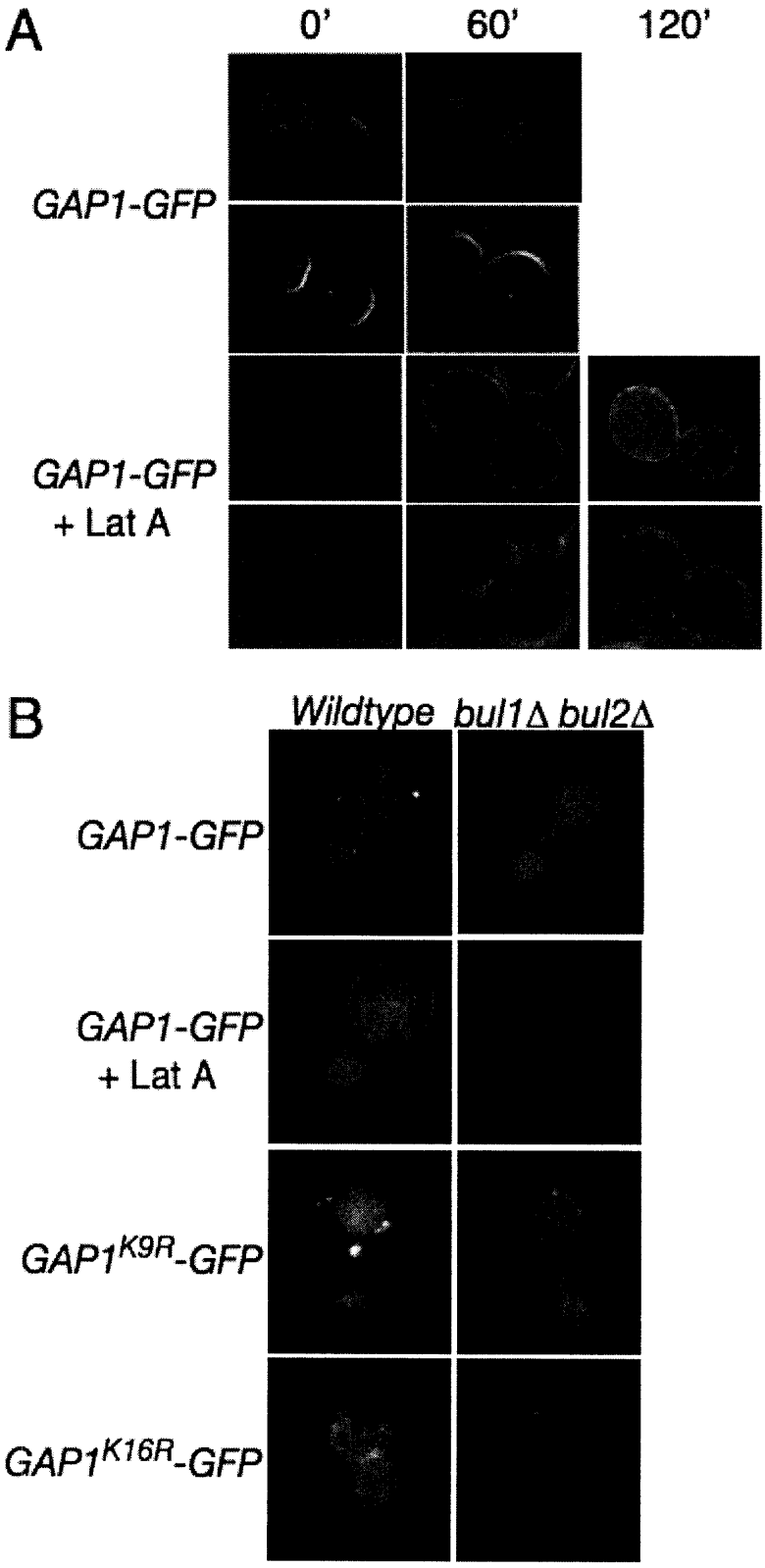

A
0'
60'
120'
GAP1-GFP
GAP1-GFP
+ Lat A
B
Wildtype
bul1Δ bul2Δ
GAP1-GFP
GAP1-GFP
+ Lat A
GAP1K9R-GFP
GAP1K16R-GFP

Figure 3. **Monoubiquitination of Gap1p is necessary and sufficient for endocytosis.** (A) *gap1*Δ (CKY482) expressing P_{ADH1}-*GAP1-GFP* (pAR13) was treated with 50μg/ml cycloheximide for 20 minutes before addition of 0.25% Casamino acids. At the indicated time after amino acid addition, cells were visualized by fluorescence microscopy. (B) *gap1*Δ (CKY482) or *bul1*Δ *bul2*Δ *gap1*Δ (CKY701) expressing P_{ADH1}-*GAP1-GFP* (pAR13), P_{ADH1}-*GAP1*^{K9R}-*GFP* (pAR32), or P_{ADH1}-*GAP1*^{K16R}-*GFP* (pAR33) were grown in SD medium with 0.25% Casamino acids and visualized by fluorescence microscopy. Where latrunculin A treatment is indicated, cells were incubated in the presence of 40μM latrunculin A for 20 minutes prior to amino acid addition.

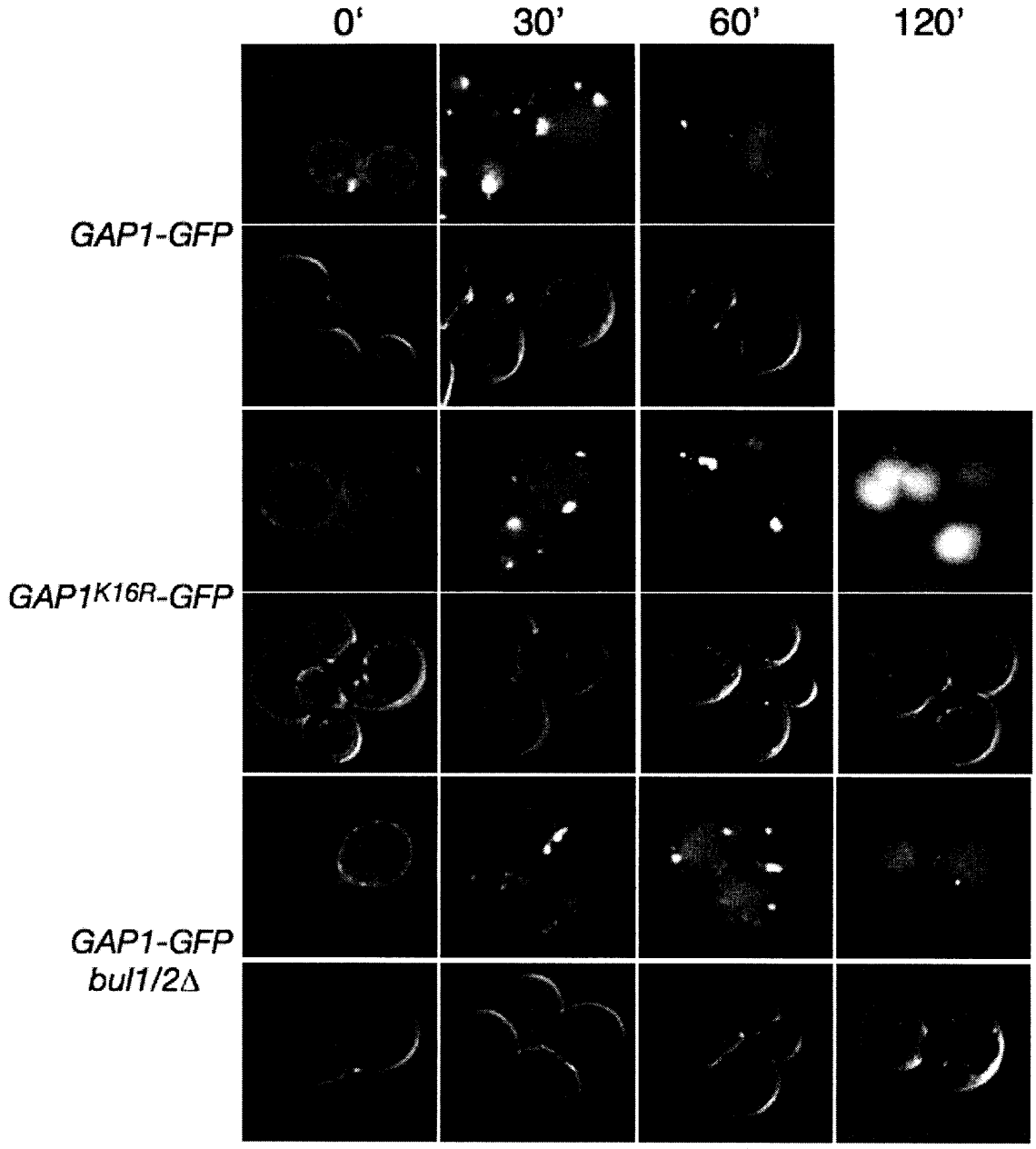

0'
30'
60'
120'
GAP1-GFP
GAP1K16R-GFP
GAP1-GFP
bul1/2Δ

Figure 4. **Bul1/2p-dependent and independent pathways for ubiquitin-mediated endocytosis of Gap1p.** *gap1*Δ (CKY482) or *gap1*Δ *bul1*Δ *bul2*Δ (CKY701) expressing P_{ADH1}-*GAP1-GFP* (pAR13) or P_{ADH1}-$GAP1^{K16R}$-*GFP* (pAR33) were grown in SD medium. Cells were treated with 50μg/ml cycloheximide for 20 minutes before addition of 0.25% Casamino acids. At the indicated time after amino acid addition, cells were visualized by fluorescence microscopy.

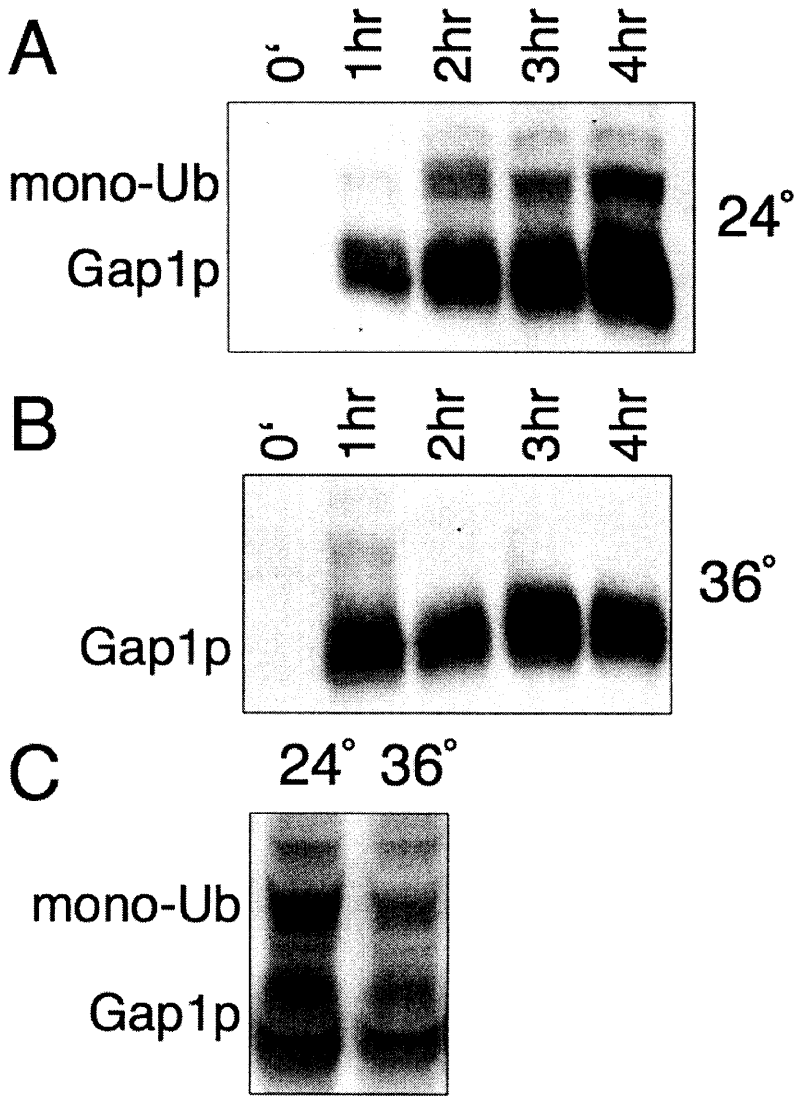


Figure 5. **Bul1/2p-independent ubiquitination does not occur prior to delivery of Gap1p to the plasma membrane.** *sec6-4 bul1Δ bul2Δ* (CKY1033) was grown in SD medium with 3mM glutamine at 24°C. In early log phase, cells were filtered, washed, and resuspended in SD medium at (A) 24°C or (B) 36°C. Lysates were prepared at the indicated time after media and temperature shift and immunoblotted with Gap1p antiserum. (C) *bul1Δ bul2Δ* (CKY698) was grown in SD medium at 24°C or 36°C. Lysates were immunoblotted with Gap1p antiserum.

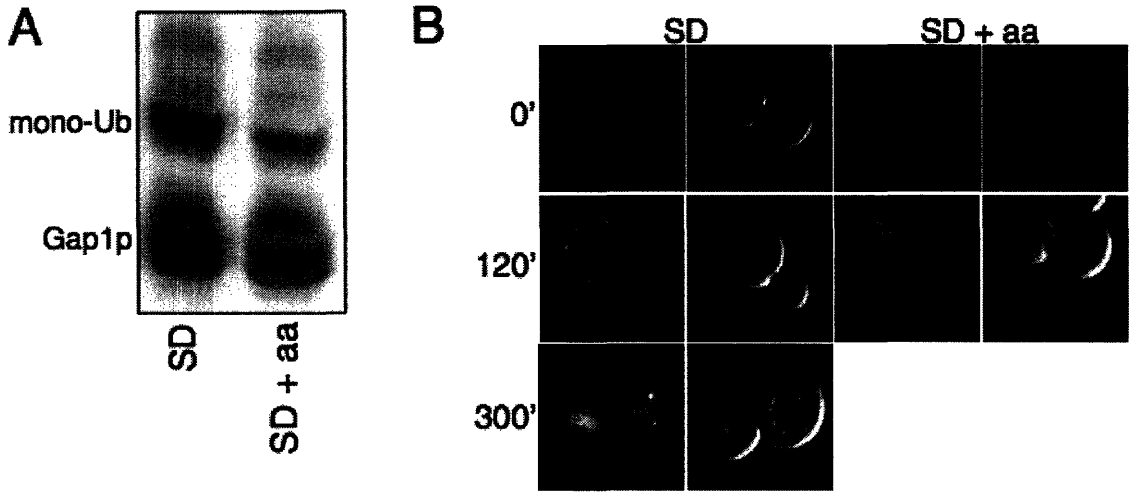


Figure 6. **The rate of Bul1/2p-independent endocytosis is enhanced by amino acid addition.** (A) Lysates were prepared from *bul1Δ bul2Δ* (CKY698) grown in SD medium with or without the addition of 0.25% Casamino acids and immunoblotted with Gap1p antiserum. (B) *bul1Δ bul2Δ lst4Δ GAP1-GFP* (CKY1034) cells were treated with 50μg/ml cycloheximide for 20 minutes, split, and then treated with or without 0.25% Casamino acids. At the indicated time after amino acid addition, cells were visualized by fluorescence microscopy.

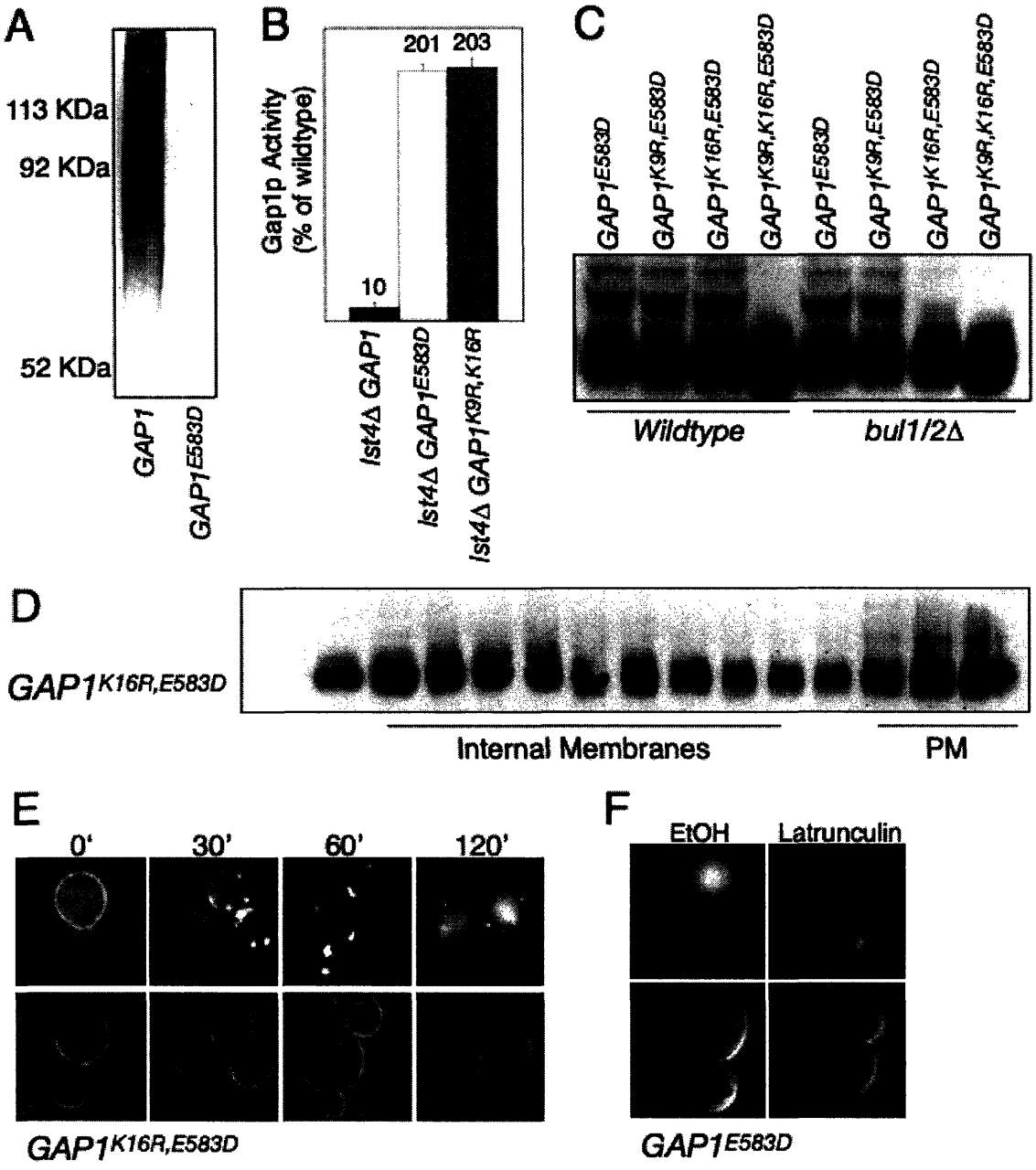

A
113 KDa
92 KDa
52 KDa
GAP1
GAP1E583D
B
Gap1p Activity (% of wildtype)
201
203
10
lst4Δ GAP1
lst4Δ GAP1E583D
lst4Δ GAP1K9R,K16R
C
GAP1E583D
GAP1K9R,E583D
GAP1K16R,E583D
GAP1K9R,K16R,E583D
GAP1E583D
GAP1K9R,E583D
GAP1K16R,E583D
GAP1K9R,K16R,E583D
Wildtype
bul1/2Δ
D
GAP1K16R,E583D
Internal Membranes
PM
E
0'
30'
60'
120'
GAP1K16R,E583D
F
EtOH
Latrunculin
GAP1E583D

Figure 7. **A cis acting mutant that blocks Gap1p polyubiquitination.** (A) *pep4*Δ *doa4*Δ *gap1*Δ (CKY1031) expressing P_{cup1}-*myc-UBI* (pCK231) and either $GAP1^{-HA}$ (pCK137) or $GAP1^{E583D-HA}$ (pAR18) was grown in SD medium + 1μM CuSO4. Gap1p was immunoprecipitated with rat anti-HA (3F10) and separated by SDS/PAGE. Gap1p and ubiquitin were detected by immunoblotting with anti-myc (9E10) or Gap1p antiserum respectively. Levels of unmodified Gap1 were equivalent in all lanes. (B) The Gap1p activity of *lst4*Δ *gap1*Δ (CKY702) expressing *GAP1* (pAR70), $GAP1^{E583D}$ (pAR88), or $GAP1^{K9R,K16R}$ (pAR73) was measured by assaying the initial rate of [^{14}C]-citrulline uptake. (C) Lysates from *gap1*Δ (CKY482) or *bul1*Δ *bul2*Δ *gap1*Δ (CKY701) expressing $GAP1^{E583D}$ (pAR88), $GAP1^{K9R,E583D}$ (pAR95), $GAP1^{K16R,E583D}$ (pAR89), or $GAP1^{K9R,K16R,E583D}$ (pAR96) were immunoblotted with Gap1p antiserum. (D) Membranes from *gap1*Δ (CKY482) expressing $GAP1^{K16R,E583D}$ (pAR89) were fractionated on 20-60% sucrose density gradients containing EDTA. Fractions were collected, proteins were separated by SDS/PAGE, and Gap1p was detected by immunoblotting with Gap1p antiserum. (E) *gap1*Δ (CKY482) expressing P_{ADH1}-$GAP1^{K16R,E583D}$-*GFP* (pAR93) was treated for 20 minutes with 50μg/ml cycloheximide, followed by addition of 0.25% Casamino acids. At the indicated time after amino acid addition, cells were visualized by fluorescence microscopy. (F) *gap1*Δ (CKY482) expressing P_{ADH1}-$GAP1^{E583D}$-*GFP* (pAR41) was treated with either 40μM latrunculin A or vehicle (EtOH) for 20 minutes before addition of 0.25% Casamino acids. Five hours after amino acid addition cells were visualized by fluorescence microscopy.

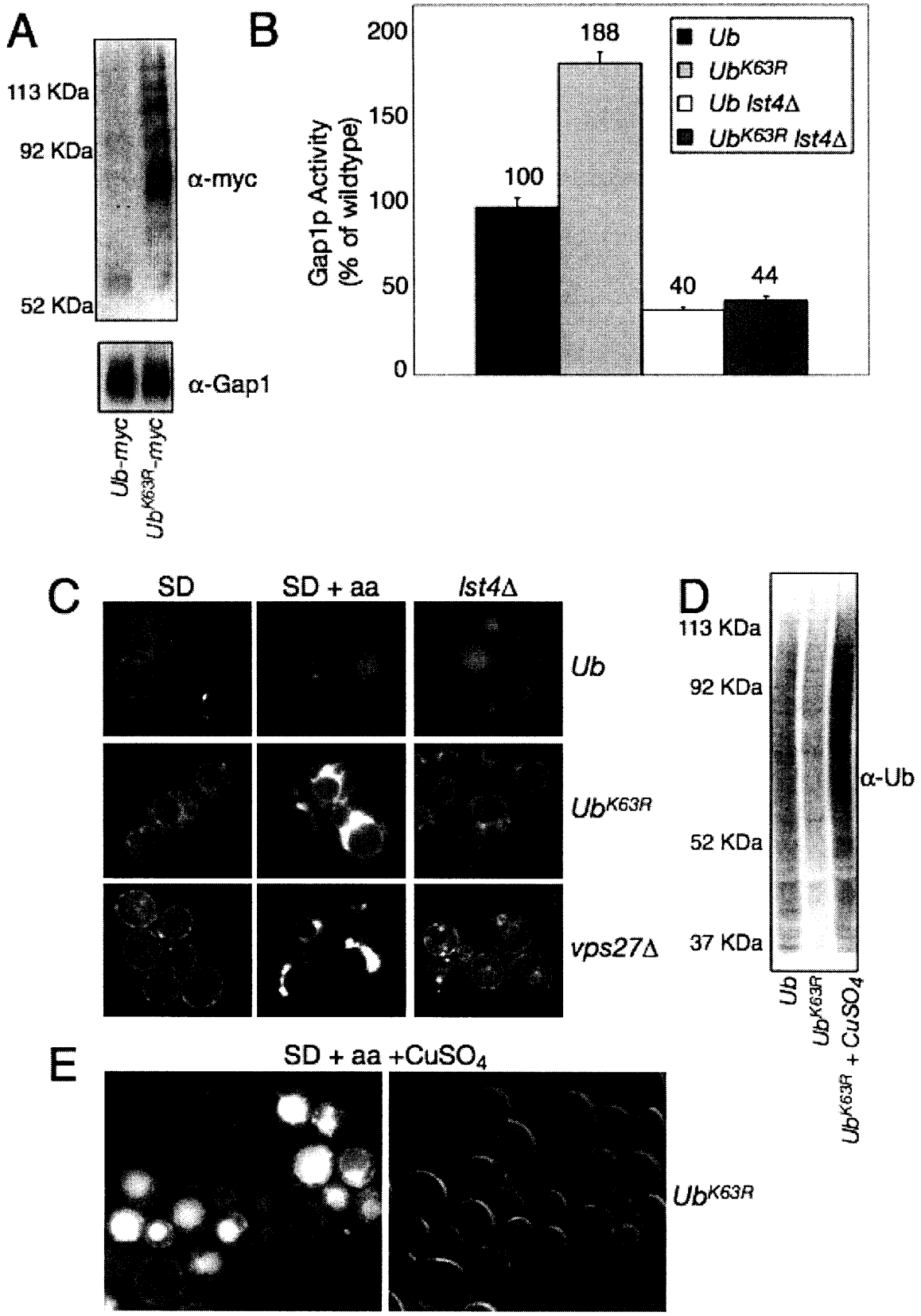
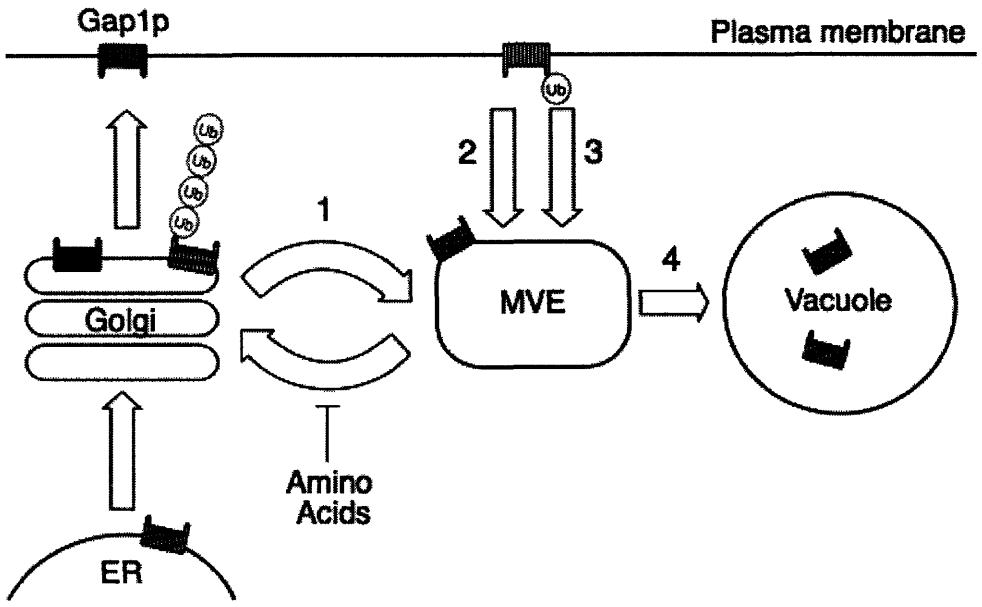

A
113 KDa
92 KDa
52 KDa
α-myc
α-Gap1
Ub-myc
Ub^K63R-myc
B
Gap1p Activity (% of wildtype)
200
150
100
50
0
188
100
40
44
Ub
Ub^K63R
Ub lst4Δ
Ub^K63R lst4Δ
C
SD
SD + aa
lst4Δ
Ub
Ub^K63R
vps27Δ
D
113 KDa
92 KDa
52 KDa
37 KDa
α-Ub
Ub
Ub^K63R
Ub^K63R + CuSO4
E
SD + aa +CuSO4
Ub^K63R

Figure 8. **Lysine 63-linked ubiquitin chains are not directly required for internal sorting of Gap1p.** Gap1p was immunoprecipitated from *ubi1 ubi2 ubi3 Δub-2 ubi4 ADH-GAP1^{HA}* (ARY175) expressing the *Ubi* tail (pUB100) and either *P_{cup1}-myc-UBI* (pCK231) or *P_{cup1}-myc-UBI^{K63R}* (pAR113) with rat anti-HA (3F10) and separated by SDS/PAGE. Gap1p and ubiquitin were detected by immunoblotting with anti-myc (9E10) or Gap1p antiserum respectively. Levels of unmodified Gap1 were equivalent in all lanes. (B) The Gap1p activity of *ubi1 ubi2 ubi3 Δub-2 ubi4* expressing the *Ubi* tail and either *P_{cup1}-UBI* (SUB280) or *P_{cup1}-UBI^{K63R}* (SUB430) and *ubi1 ubi2 ubi3 Δub-2 ubi4 lst4Δ* expressing the *Ubi* tail and either *P_{cup1}-UBI* (ARY180) or *P_{cup1}-UBI^{K63R}* (ARY181) was measured by assaying the initial rate of [^{14}C]-citrulline uptake. (C) *ubi1 ubi2 ubi3 Δub-2 ubi4* expressing the *Ubi* tail, *ADH-GAP1-GFP*, and either *P_{cup1}-UBI* (SUB280 + pAR13) or *P_{cup1}-UBI^{K63R}* (SUB430 + pAR13) and *ubi1 ubi2 ubi3 Δub-2 ubi4 lst4Δ* expressing the *Ubi* tail, *ADH-GAP1-GFP,* and either *P_{cup1}-UBI* (ARY180 + pAR13) or *P_{cup1}-UBI^{K63R}* (ARY181 + pAR13), and *vsp27Δ* (CKY837), and *vps27Δ lst4Δ* (CKY846), each expressing *ADH-GAP1-GFP* (pAR13) were grown in SD medium with or without the addition of 0.25% Casamino acids and visualized by fluorescence microscopy. (D) Lysates were prepared from *ubi1 ubi2 ubi3 Δub-2 ubi4* expressing the *Ubi* tail, *ADH-GAP1-GFP*, and *P_{cup1}-UBI* (SUB280 + pAR13) or *P_{cup1}-UBI^{K63R}* (SUB430 + pAR13) grown in SD medium with or without the addition of 200μM $CuSO_4$ and immunoblotted with PG47 anti-ubiquitin antiserum. (E) *ubi1 ubi2 ubi3 Δub-2 ubi4* expressing the *Ubi* tail, *ADH-GAP1-GFP*, and *P_{cup1}-UBI^{K63R}* (SUB430 + pAR13) was grown in SD medium with 0.25% Casamino acids and 200μM $CuSO_4$ and visualized by fluorescence microscopy.



	Process	Rsp5p dependent	Bul1/2p dependent	Ubiquitinated lysine(s)	Type of ubiquitination
1	Golgi to MVE	+	+	9 or 16	Poly
2	Endocytosis	+	+	9 or 16	Mono
3	Endocytosis	+	–	16	Mono
4	MVE sorting		–	9 or 16	Mono

Figure 9. **Model of Gap1p sorting.** Gap1p is sorted to the prevacuolar endosome (PVE) by four distinct ubiquitin-dependent mechanisms. (1) Direct sorting of Gap1p to the vacuole as a result of Bul1/2p-dependent poly-ubiquitination on either lysine 9 or 16, which is blocked in *bul1/2Δ*, $GAP1^{E8583D}$, $GAP1^{K9R,K16R}$, or upon cycloheximide treatment. (2) Endocytosis of Gap1p as a result of Bul1/2p-dependent mono-

ubiquitination on lysine 9 or 16, which is blocked in *bul1/2Δ*, $GAP1^{K9R,K16R}$, or upon latrunculin A treatment. (3) Endocytosis of Gap1p as a result of Bul1/2p-independent mono-ubiquitination on lysine16, which is blocked in $GAP1^{K16R}$ or upon latrunculin A treatment. Therefore, Gap1p trafficking to the vacuole is completely blocked in $GAP1^{K9R,K16R}$, *bul1/2Δ* $GAP1^{K16R}$, *bul1/2Δ* with latrunculin A treatment, or $GAP1^{E8583D}$ with latrunculin A treatment. (4) Entry of Gap1p into multivesicular bodies and subsequent fusion to the vacuole, which is able to occur in the absence of lysine 9 or 16 or ability of Gap1p to receive polyubiquitin chains. It is unclear whether Rsp5p is required for entry into multivesicular bodies as Gap1p is unable to be delivered to the multivesicular endosome in the absence of Rsp5p.

Chapter 4

Activity Dependent Reversible Inactivation of the General Amino Acid Permease

Preface

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

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 post-transcriptionally 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 (*bul1*Δ *bul2*Δ) 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.

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 $GAP1^{K9R,K16R}$-*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 (*bul1*Δ *bul2*Δ *gap1*Δ *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 *mks1D* 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 *mks1*Δ 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 *mks1*Δ 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 Gap1p^{K9R,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 Gap1p^{K9R,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 Gap1p^{K9R,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 (*bul1*Δ *bul2*Δ) 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 *gap1*Δ 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 wild-type 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 *bul1*Δ *bul2*Δ *gap1*Δ strain and screened for mutants that were resistant to 4 mM citrulline, a concentration that is toxic to cells expressing wildtype Gap1p in a *bul1*Δ *bul2*Δ 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 *gap1*Δ or *can1* mutants import arginine, the double *gap1*Δ *can1* 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 K_m but not V_{max} 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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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.

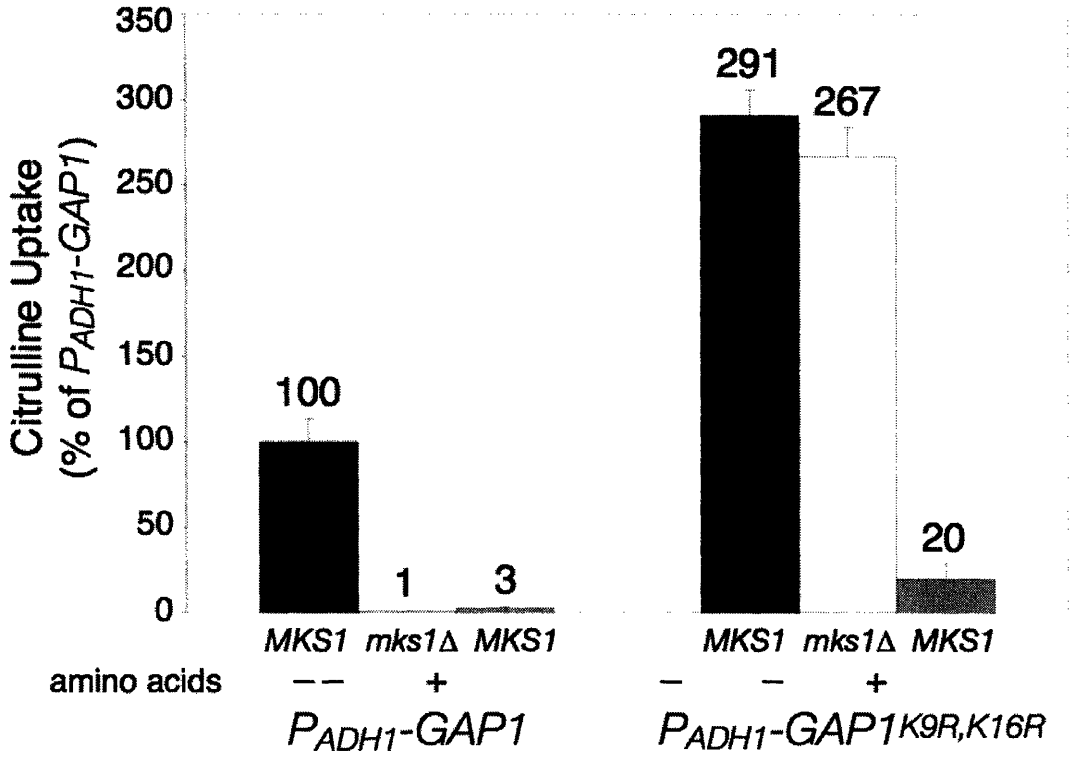
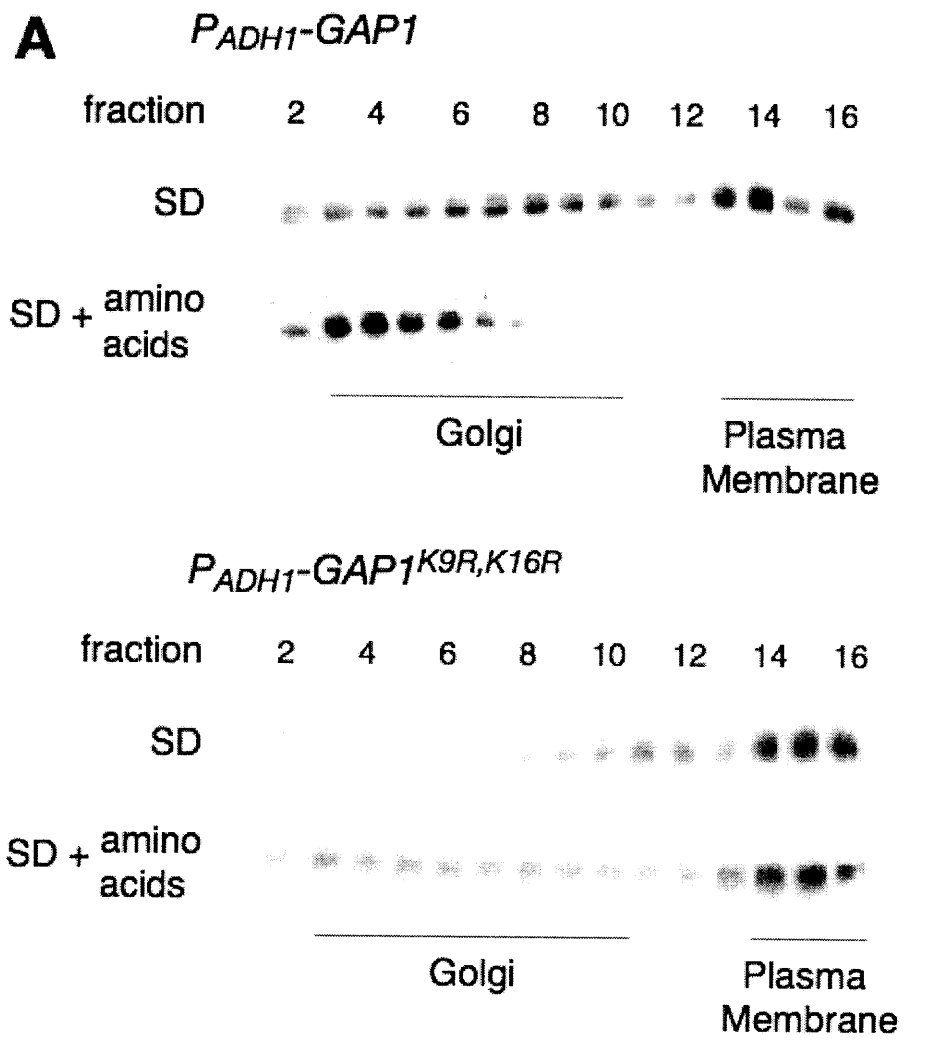


Figure 1. **Gap1p that can not be ubiquitinated bypasses amino acid dependent sorting but can be inactivated by exogenous amino acids.** *P_{ADH1}-GAP1* (CKY759), *P_{ADH1}-GAP1 mks1*Δ (CKY763), *P_{ADH1}-GAP1^{K9R,K16R}* (CKY890), or *P_{ADH1}-GAP1^{K9R,K16R} mks1*Δ (CKY891) were grown in minimal ammonia 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

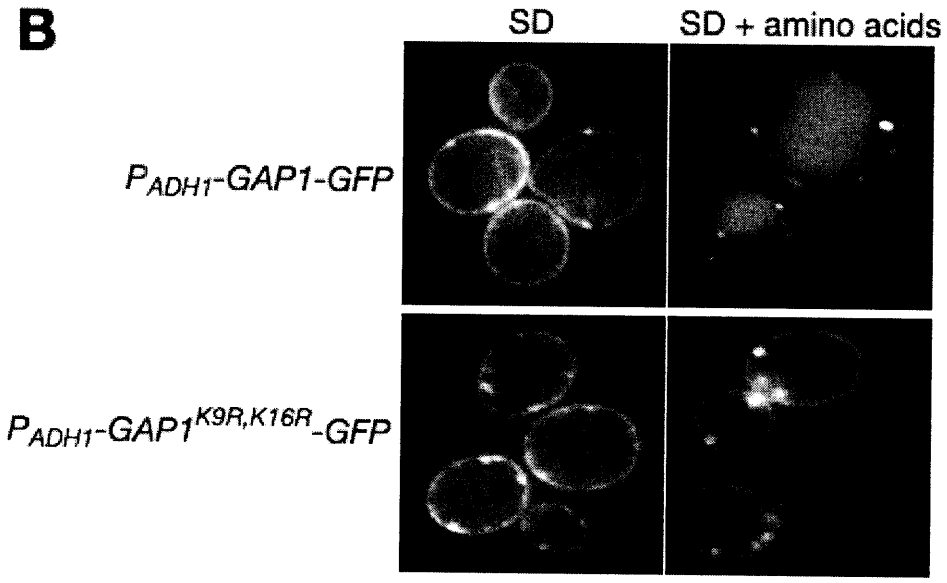
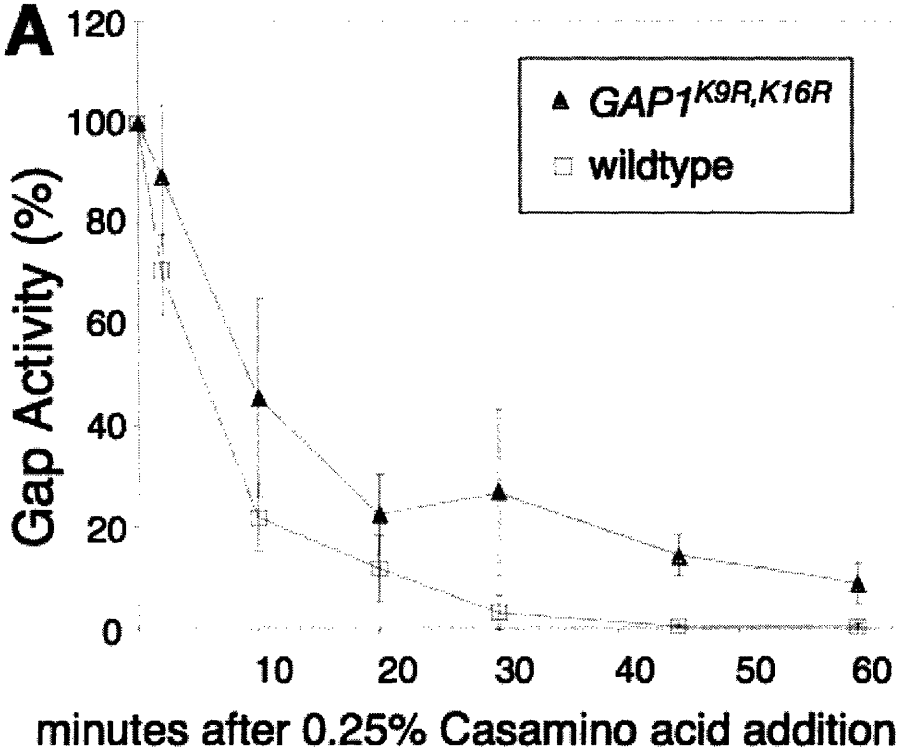

B
SD
SD + amino acids
P_{ADH1}-GAP1-GFP
P_{ADH1}-$GAP1^{K9R,K16R}$-GFP

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}-*GAP1*^{K9R,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) *gap1*Δ 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

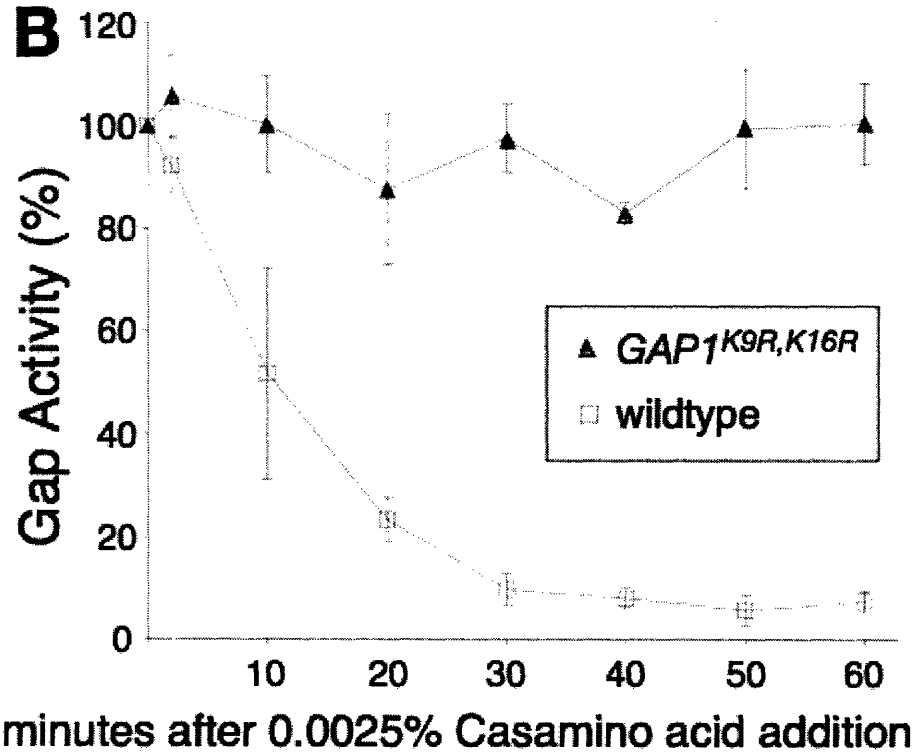

B
120
100
80
60
40
20
0
Gap Activity (%)
GAP1K9R,K16R
wildtype
10
20
30
40
50
60
minutes after 0.0025% Casamino acid addition

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).

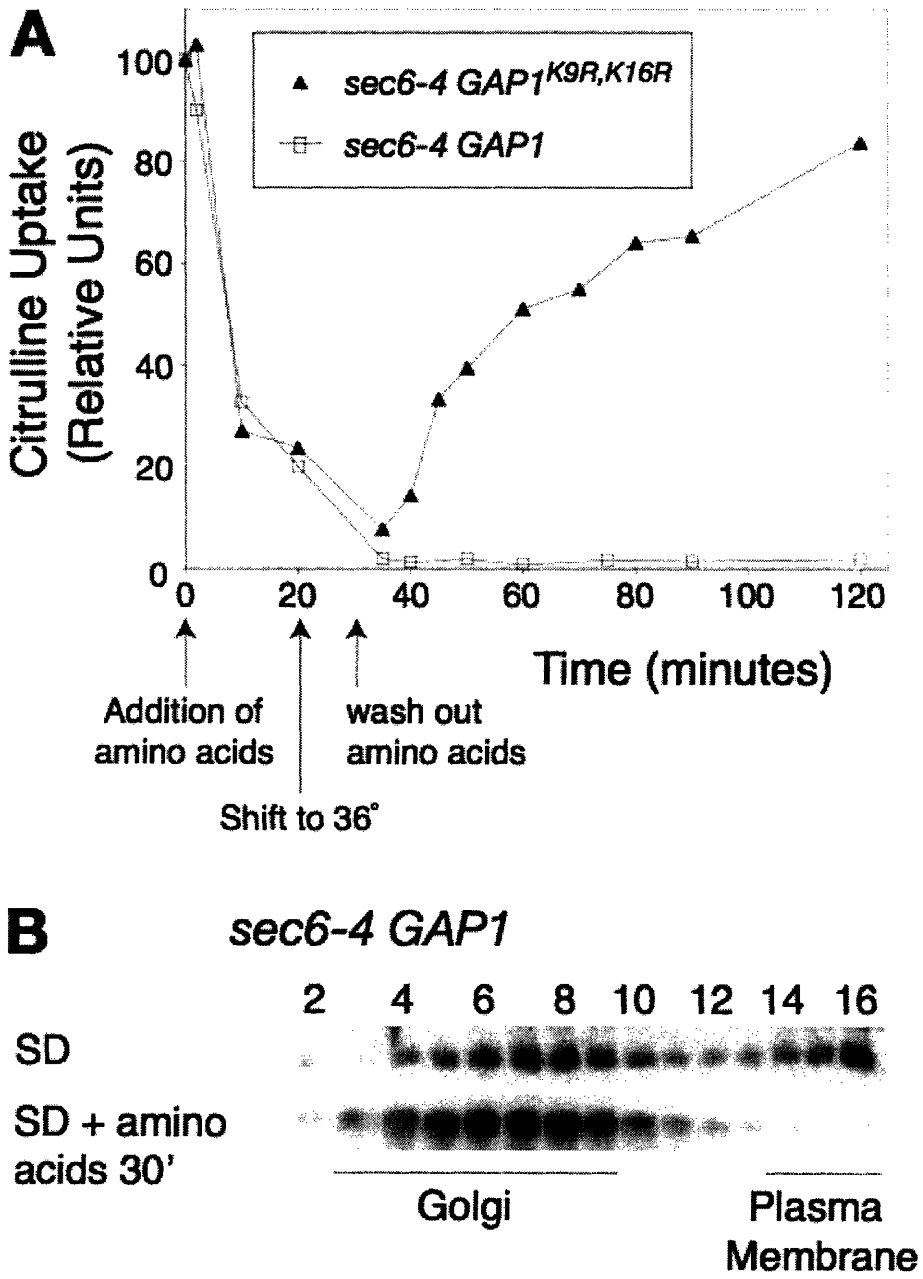

A
sec6-4 GAP1K9R,K16R
sec6-4 GAP1
Citrulline Uptake (Relative Units)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (minutes)
Addition of amino acids
wash out amino acids
Shift to 36°
B
sec6-4 GAP1
2
4
6
8
10
12
14
16
SD
SD + amino acids 30'
Golgi
Plasma Membrane

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.

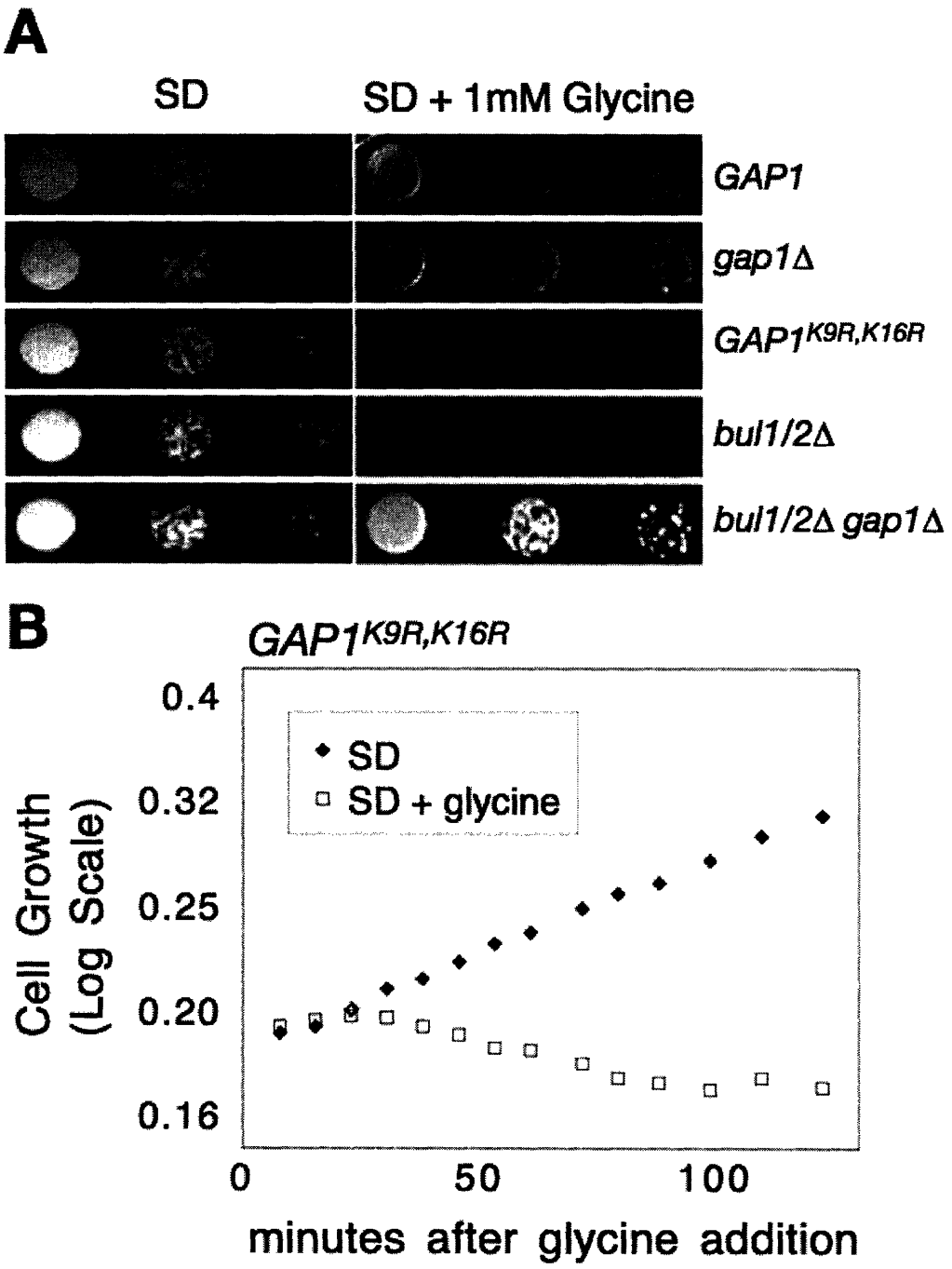

A
SD
SD + 1mM Glycine
GAP1
gap1Δ
GAP1K9R,K16R
bul1/2Δ
bul1/2Δ gap1Δ
B
GAP1K9R,K16R
0.4
0.32
0.25
0.20
0.16
SD
SD + glycine
Cell Growth
(Log Scale)
0
50
100
minutes after glycine addition

Figure 5. **Excess amino acid addition is toxic to cells deficient in Gap1p ubiquitination.** (A) *gap1*Δ (CKY482) expressing wildtype Gap1p (pAR70), Gap1p^{K9R,K16R} (pAR73), or pRS316 and *gap1*Δ *bul*Δ *bul2*Δ (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.

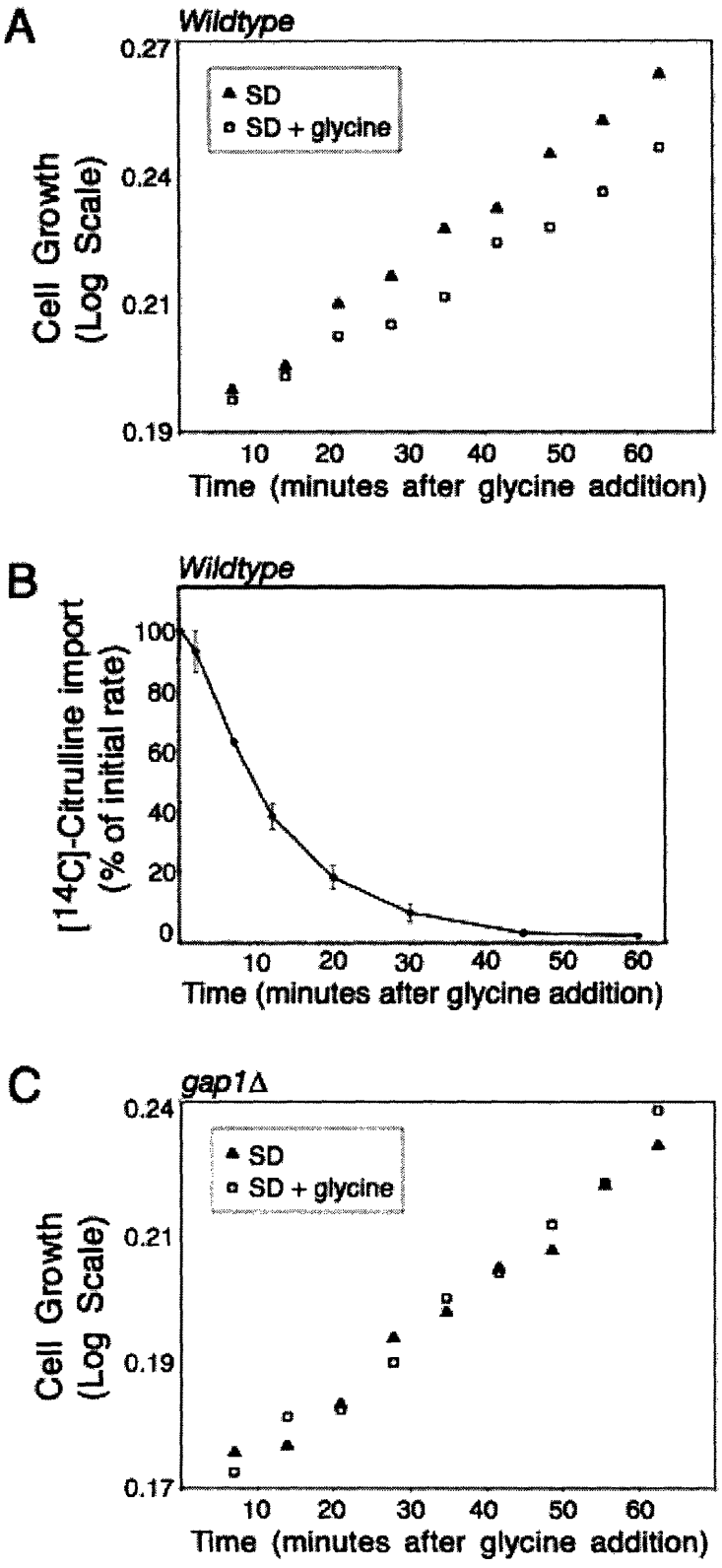

A
Wildtype
SD
SD + glycine
Cell Growth (Log Scale)
0.27
0.24
0.21
0.19
10 20 30 40 50 60
Time (minutes after glycine addition)
B
Wildtype
[14C]-Citrulline import (% of initial rate)
100
80
60
40
20
0
10 20 30 40 50 60
Time (minutes after glycine addition)
C
gap1Δ
SD
SD + glycine
Cell Growth (Log Scale)
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 *gap1*Δ (CKY482).

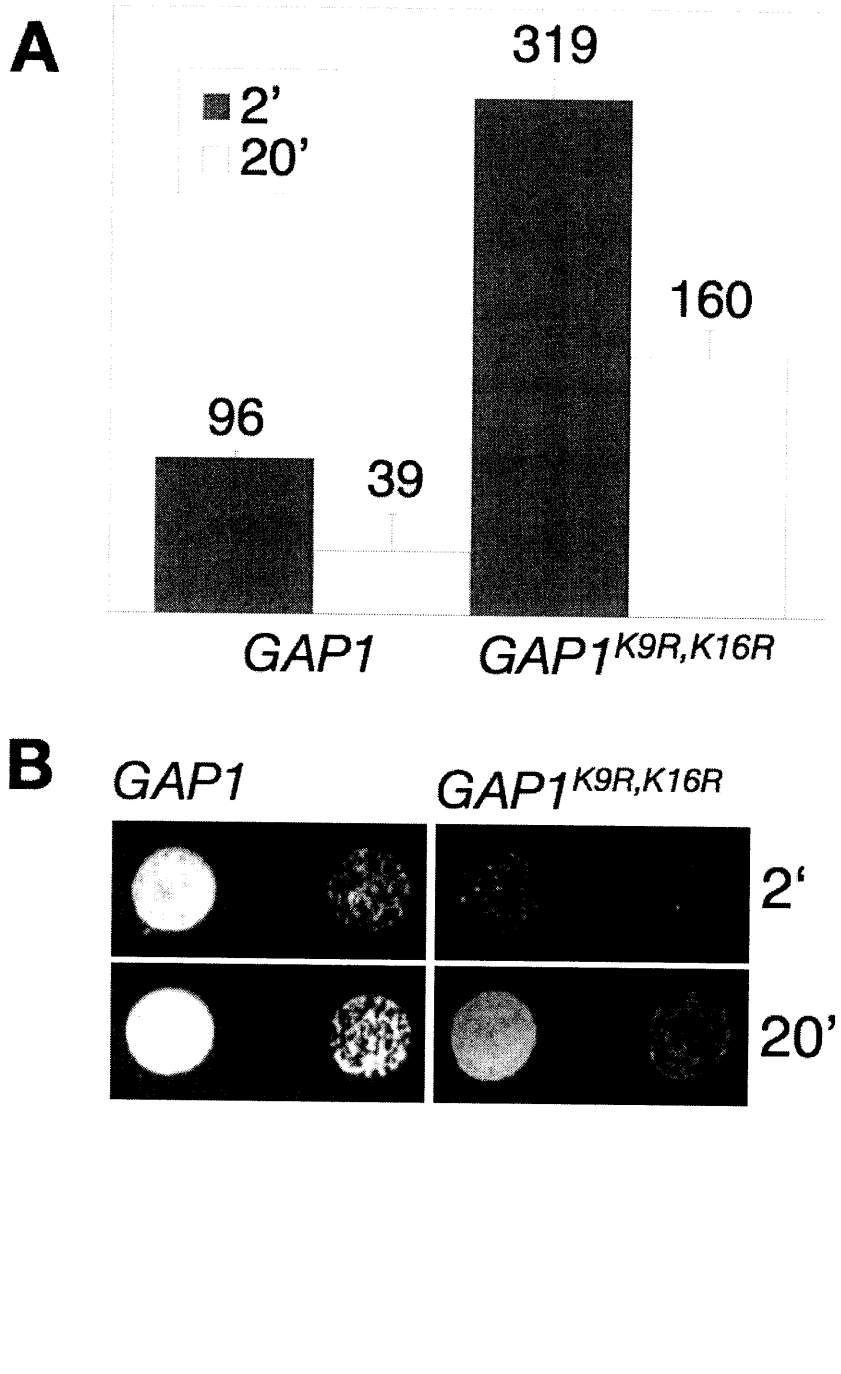
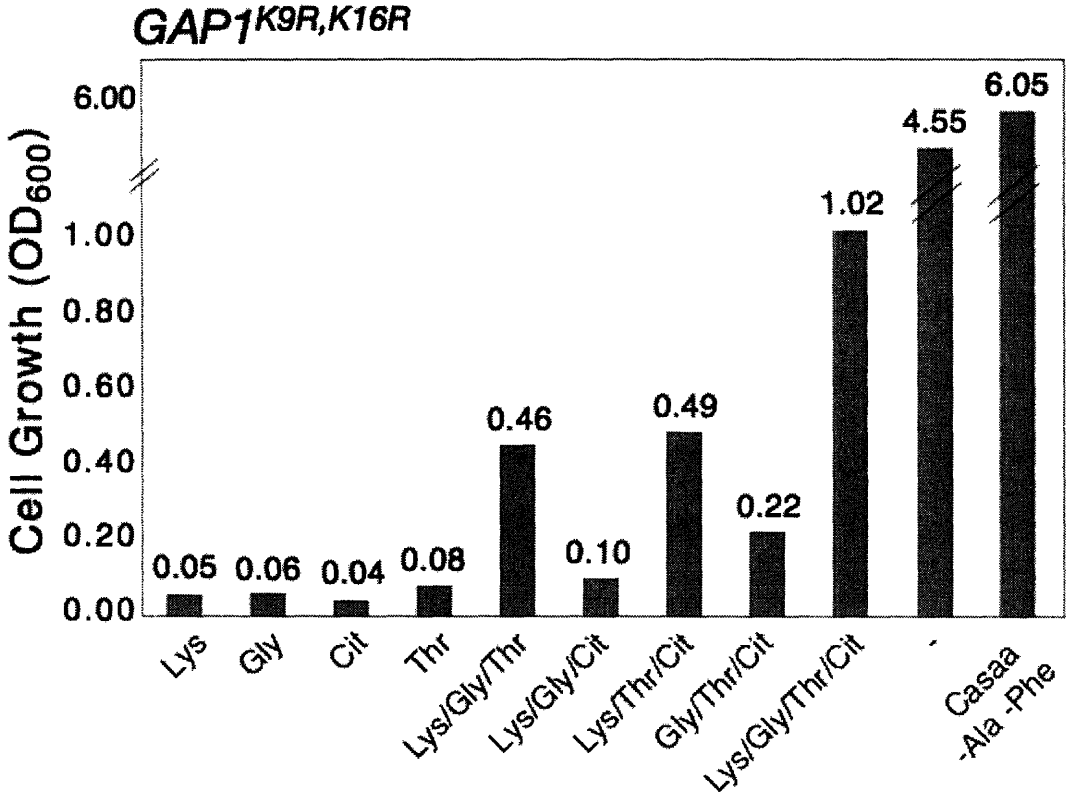

A
319
2'
20'
160
96
39
GAP1
GAP1K9R,K16R
B
GAP1
GAP1K9R,K16R
2'
20'

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 [^{14}C]-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	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 OD_{600} 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.

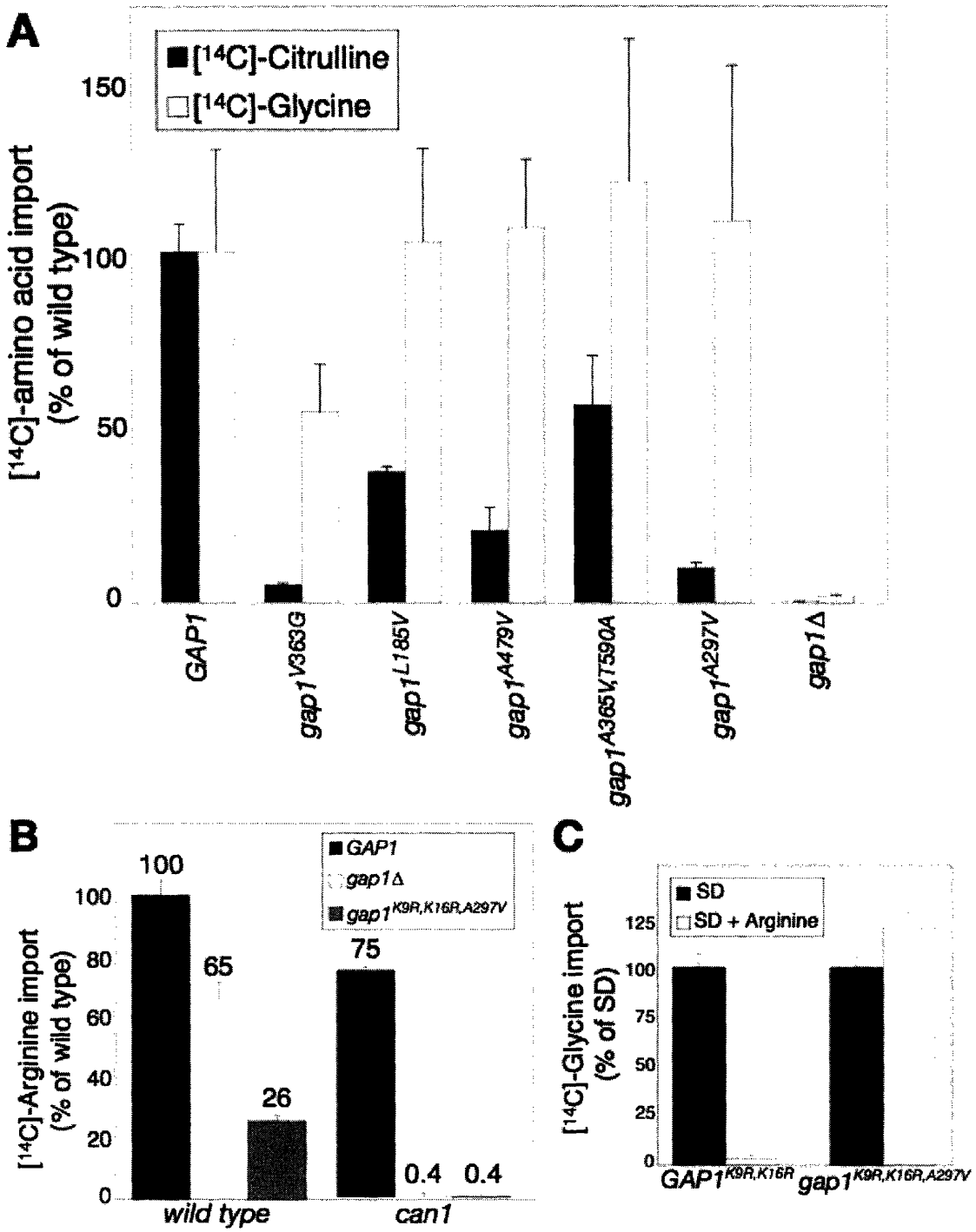


Figure 9. **Inactivation of Gap1p requires active amino acid transport through the permease.** (A) The initial rate of [^{14}C]-citrulline or [^{14}C]-glycine uptake was determined for *gap1Δ bul1Δ bul2Δ* (CKY701) expressing wild type *GAP1* (pEC221), the indicated

mutant *GAP1* allele (pNC3-7), or vector alone (pRS316) in SD medium. (B) The initial rate of [^{14}C]-arginine uptake was determined for *gap1*Δ (CKY482) or *gap1*Δ *can1* (CKY1025) expressing wild type *GAP1* (pEC221), $GAP1^{K9R,K16R,A297V}$ (pNC8), or vector alone (pRS316) in SD medium. (C) *gap1*Δ (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 averaged).

Chapter 5

Prospectus

GAP1 TRANSCRIPTION

In chapter 2, I discussed how two separate GATA transcription factors, Gln3p and Nil1p, regulate *GAP1* transcription in response to nitrogen source quality as well as amino acid quantity respectively. Interestingly, the same effectors, Ure2p and TOR, appear to be required for repression of both Nil1p and Gln3p in response to these distinct nutrient signals. To understand how different nutrients regulate separate transcription factors through identical effectors, I constructed and analyzed chimeric fusion proteins of the two transcription factors. This chimeric analysis takes advantage of the fact that both Nil1p and Gln3p poses similar GATA DNA binding domains near the middle of the protein with divergent N- and C-termini. Modular analysis of Gln3p function has determined that the region directly surrounding the GATA domain includes the sequences required for import and export of Gln3p from the nucleus. Additionally, the N-terminal region of Gln3p has been shown to be required for direct binding to Ure2p while the C-terminus is involved in TOR binding (Carvalho and Zheng 2003).

Using sequence alignment of the GATA domains and nuclear import and export sequences along with predictions for regions of low complexity secondary structure surrounding the GATA region, I constructed functional chimeric full-length and truncated transcription factors. The fusion and truncation constructs that were sufficient to express *GAP1* in a *gln3*Δ *nil1*Δ background in minimal urea medium are depicted in Figure 1. I explored the nutrient regulation of these chimeric transcription factors by measuring β-galactosidase levels in *nil1*Δ *gln3*Δ strains expressing a single chimera and the P_{GAP1}-*LacZ* (pCK211) reporter construct in various media. As expected, the wildtype Gln3p transcription factor actively transcribed *GAP1* when poor nitrogen sources such as

urea or glutamate were present as the sole nitrogen source and was repressed by excellent nitrogen sources such as ammonia or glutamine. Additionally, the wildtype Nil1p transcription factor actively transcribed *GAP1* when urea or ammonia were present as the sole nitrogen source and was repressed by the presence of either glutamate or glutamine (Figure 2).

Replacing the C-terminus of Gln3p with Nil1p resulted in a chimeric transcription factor, *GLN3/NIL1*, which actively transcribed *GAP1* in urea, glutamate, and ammonia but was repressed to some degree by glutamine. Replacing the N-terminus of Gln3p with Nil1p resulted in a chimeric transcription factor, *NIL1/GLN3*, which expressed high levels of *GAP1* in all media, including glutamine. Therefore, simply replacing either the N- or C-terminus of Gln3p with Nil1p is insufficient to confer repression of Gln3p-dependent *GAP1* expression in glutamate.

Since Nil1p-dependent repression of *GAP1* expression in glutamate could not be conferred to Gln3p by swapping domains, I was curious whether it would be possible to identify the amino acid responsive domain of Nil1p by making truncations of the wildtype protein. I found that truncation of the C-terminus of Nil1p resulted in a transcription factor that was competent for expression of *GAP1* in urea and repressed by glutamate, suggesting that the C-terminus of Nil1p is dispensable for amino acid-dependent repression of Nil1p activity (Figure 3). Additionally, I found that replacing the GATA domain of this truncated form of Nil1p with the GATA domain of Gln3p did not alter the repression of Nil1p by glutamate, further suggesting that the N-terminal domain of Nil1p is sufficient to confer amino acid-dependent repression of *GAP1* transcription.

In Chapter two, I showed that both Ure2p and the TOR complex contributed toward amino acid-dependent repression of Nil1p. I was interested in whether Ure2p, TOR, or both proteins were involved in amino acid-dependent repression of truncated forms of Nil1p. Surprisingly, I found that neither Rapamycin addition nor loss of *URE2* was sufficient to allow activation of truncated Nil1p. Upon further analysis, I determined that a truncated form of Gln3p was also unable to transcribe *GAP1* in an *ure2Δ* mutant in the presence of glutamine. This finding suggests that amino acids may be able to repress the activity of Nil1p and Gln3p independently of Ure2p and TOR function.

There are at least three distinct ways by which amino acids could regulate the activity of the truncated GATA transcription factors independently of the known Ure2p and TOR effectors. One possibility is that the truncated transcription factors are present in an inactive form in the nucleus. If this were the case, it would suggest that, although loss of Ure2p is sufficient for translocation to the nucleus, the truncated transcription factors can be inactivated in the nucleus by glutamate and/or glutamine. Another possibility is that the truncated transcription factors are excluded from the nucleus, a phenotype that has yet to be observed in a *ure2Δ* mutant. Finally, it is possible that the truncated transcription factors are unstable in the presence of amino acids such as glutamate and glutamine. In this scenario, the Ure2p and TOR effectors would have a negligible effect on *GAP1* transcription in these nitrogen sources since the transcription factors would not be present in the cell. Distinguishing between these three possibilities will require tagging of the wildtype, chimeric, and truncated transcription factors to detect their levels and localization in various nitrogen sources in wildtype and *ure2Δ* backgrounds.

References

Carvalho, J. and X. F. Zheng (2003). "Domains of Gln3p interacting with karyopherins, Ure2p, and the target of rapamycin protein." J Biol Chem **278**(19): 16878-86.

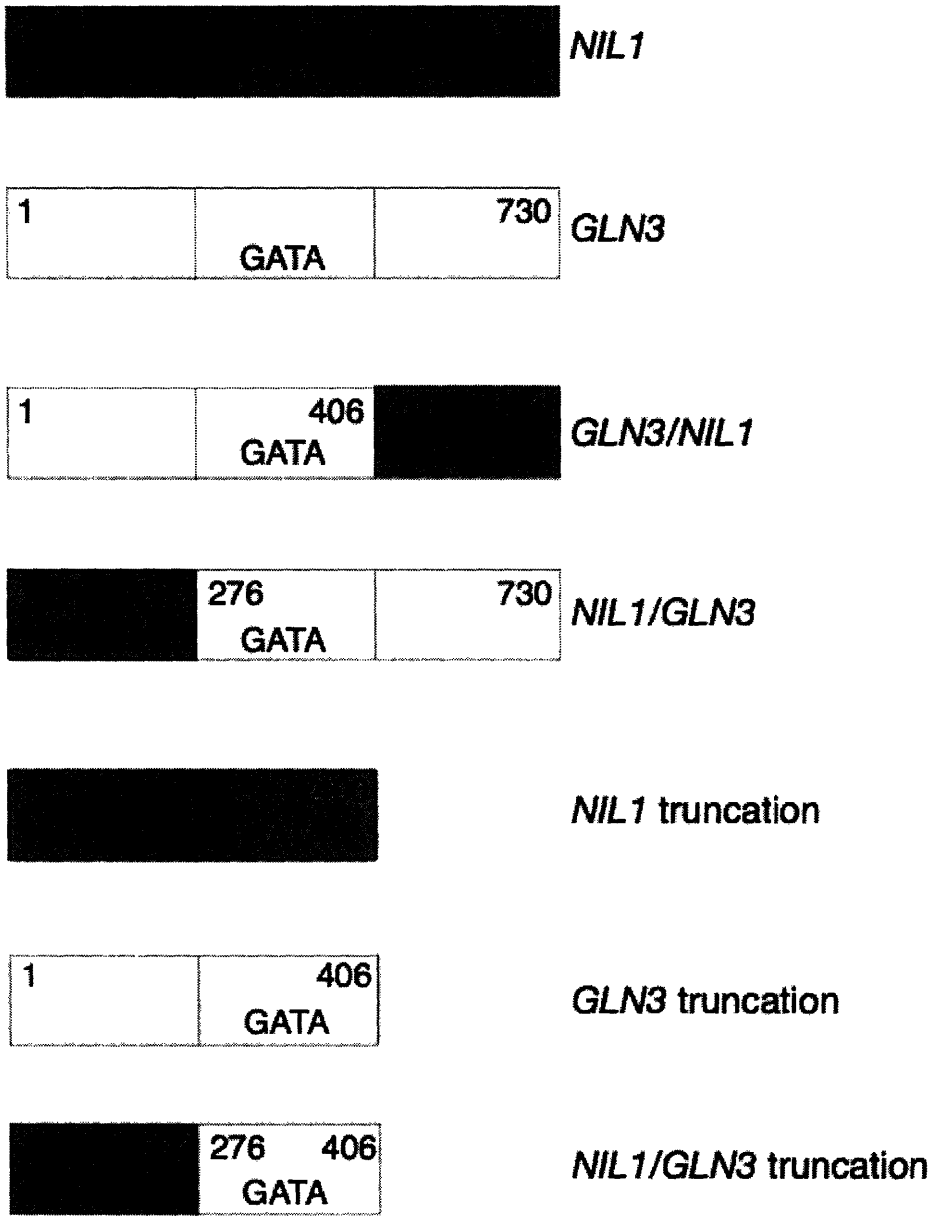


Figure 1. ***NIL1* and *GLN3* chimeras and truncations.** Regions of *NIL1* are in grey, regions of *GLN3* are in white. Numbers indicate amino acid residues of *NIL1* or *GLN3* present in the chimera or truncation.

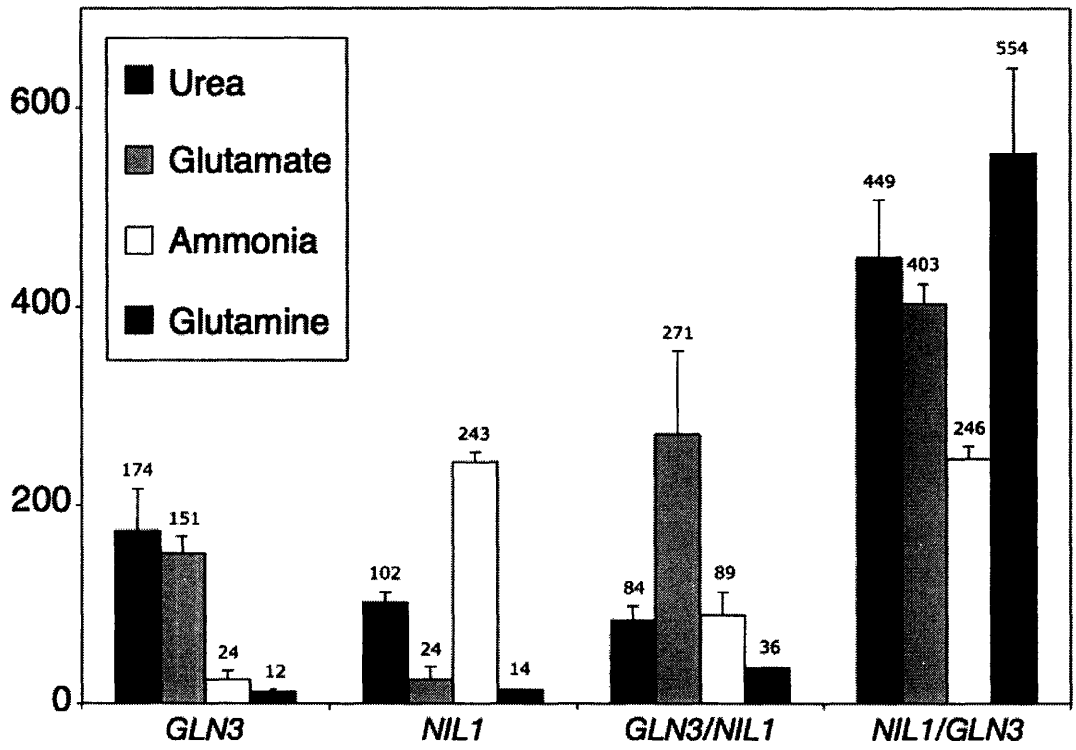


Figure 2. **Activity of chimeric transcription factors in different nitrogen sources.** β-galactosidase assays were performed for *nil1*Δ *gln3*Δ (CKY1036) expressing *NIL1*, *GLN3*, or chimeric fusions of the transcription factors and a P_{GAP1}-*lacZ* reporter (pMS29) in minimal medium with urea, ammonia, glutamate, or glutamine as a sole nitrogen source.

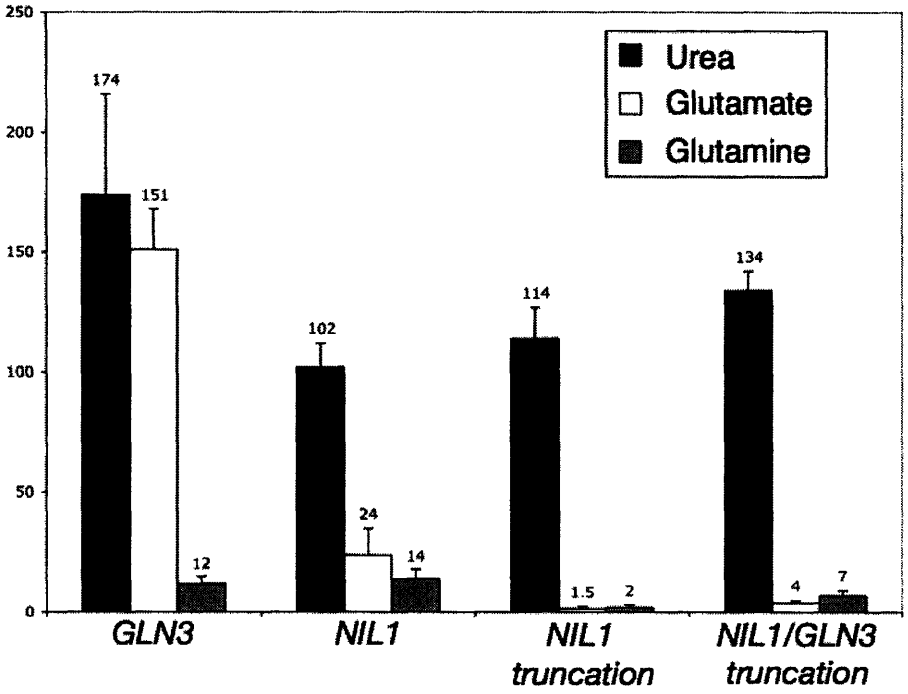


Figure 3. **The N-terminus of Nil1p is sufficient for amino acid-dependent repression of *GAP1* expression.** β-galactosidase assays were performed for *nil1Δ gln3Δ* (CKY1036) expressing *NIL1*, *GLN3*, the *NIL1* truncation, or the *NIL1/GLN3* truncation and a P_{GAP1}-*lacZ* reporter (pMS29) in minimal medium with urea, glutamate, or glutamine as a sole nitrogen source.

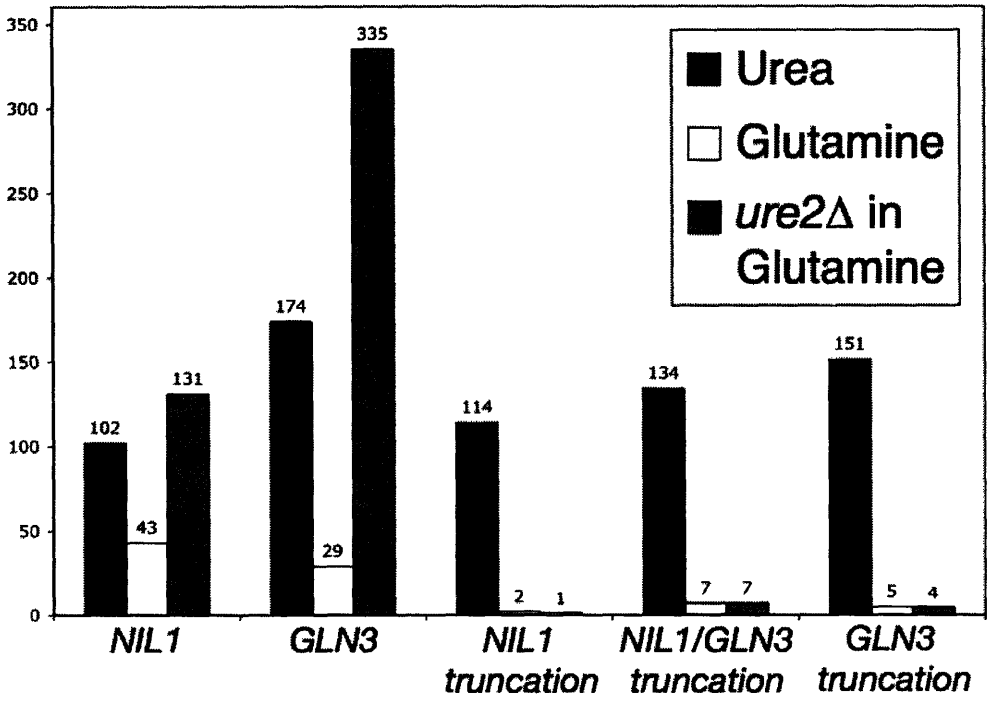


Figure 4. **C-terminal truncations of GATA transcription factors are unable to be derepressed in a *ure2*Δ mutant.** β-galactosidase assays were performed for *nil1*Δ *gln3*Δ (CKY1036) or *nil1*Δ *gln3*Δ *ure2*Δ expressing *NIL1*, *GLN3*, or truncations of the transcription factors and a P_{GAP1}-*lacZ* reporter (pMS29) in minimal medium with urea or glutamine as a sole nitrogen source.

GAP1 SORTING

I performed a screen for mutants in *GAP1* that resulted in missorting of the permease by taking advantage of our finding that sorting deficient alleles of Gap1p are sensitive to addition of glycine to the medium (Risinger, Cain et al. 2006). *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 600 bp of the *GAP1* 3' UTR was amplified in four 50 µl reactions with AmpliTaq Gold (Perkin Elmer) and 0.3mM $MnCl_2$. PCR products were transformed along with gapped pEC221 plasmid (lacking the *GAP1* ORF) into CKY482 (*gap1Δ ura3-52*) and gap repaired plasmids were isolated by selection for Ura^+ transformants. Glycine sensitive transformants were identified by replica plating onto SD with 1 mM glycine at 30° C. Plasmids were isolated from glycine sensitive colonies, retransformed into CKY482, and retested for glycine sensitivity. Plasmids conferring sensitivity to glycine arose at a frequency of about 10^{-3}.

I isolated six glycine sensitive mutants, three of which all contained mutations in the N-terminal cytosolic tail of Gap1p and three of which contained mutations in the C-terminal cytosolic tail of Gap1p (Figure 1). The mutations isolated in the screen include: $GAP1^{S2G}$, $GAP1^{I32K}$, $GAP1^{D23G}$, $GAP1^{E580D}$, $GAP1^{L77S,E580G}$, and $GAP1^{E583D}$. During my analysis of these mutants, I included two additional mutants that have a documented defect in sorting, $GAP1^{L76A,L77A}$ and $GAP1^{E583K}$ (Grenson 1983; Hein and Andre 1997). I compared the activity and ubiquitination state of each of these mutants to $GAP1^{K9R,K16R}$, a mutant lacking the ubiquitinated lysine residues, which is constitutively delivered to the

plasma membrane resulting in elevated ^{14C}-Citrulline import (Soetens, De Craene et al. 2001).

I found that each of the glycine sensitive mutants isolated in the screen as well as the three mutants described previously had an elevated rate of ^{14C}-Citrulline uptake in an otherwise wildtype background (Figure 2). I also measured ^{14C}-Citrulline import of these mutants in a *gtr1*Δ strain, which blocks recycling of Gap1p from the prevacuolar endosome at a step indistinguishable from amino acid addition; this mutation severely limits delivery of Gap1p to the plasma membrane when the permease is able to be ubiquitinated and sorted from the *trans*-Golgi to the prevacuolar endosome. Most of the glycine sensitive mutants retained the ability to import ^{14C}-Citrulline in a *gtr1*Δ background, suggesting that the mutants were defective in the ubiquitin-mediated internal sorting of Gap1p from the *trans*-Golgi to the prevacuolar endosome (Figure 3).

One possible explanation for the sorting defect observed in these mutants is that, like $GAP1^{K9R,K16R}$, they are unable to receive Bul1/2p-dependent polyubiquitin chains. However, I determined that all of the missorted Gap1p mutants (with the exception of $GAP1^{K9R,K16R}$ and the $GAP1^{E583D}$ mutant described in chapter 4) retained the ability to receive poly-ubiquitin chains (Figure 4). This finding is the first indication that polyubiquitin chains are not sufficient for sorting Gap1p in the *trans*-Golgi.

Isolation of *cis*-acting mutations in both the N- and C-terminus of *GAP1* that are defective in the internal sorting of the polyubiquitinated permease indicates that *trans*-acting factors involved in this sorting step recognize residues in both of these regions of Gap1p in addition to the polyubiquitin chains. It also suggests that the N- and C-terminal cytosolic tails cooperate in the sorting of Gap1p, possibly through a direct interaction. It

will be interesting to further characterize the sorting defect of these ubiquitinated but non-sorted mutants and to identify the *trans*-acting factors involved in this sorting step downstream of ubiquitination.

The inability of $GAP1^{E583D}$ to receive polyubiquitination indicates that residues on the C-terminus of Gap1p are required for ubiquitination of the two lysine residues on the N-terminus of the permease. This finding further supports our hypothesis that the two cytosolic tails work together to coordinate ubiquitination as well as internal sorting of ubiquitinated Gap1p. Finally, it is interesting that the mutation of a single residue to two different amino acids results in either a defect for polyubiquitination or internal sorting of a polyubiquitinated protein for $GAP1^{E583D}$ or $GAP1^{E583K}$ respectively. The fact that the same residues on Gap1p are involved in both polyubiquitination and in sorting downstream of the polyubiquitination event suggest that these two processes may be more tightly linked than previously thought. Further studies will need to be done to demonstrate the cooperativity between the two cytosolic tails of Gap1p and how they interact with both the known Rsp5p-Bul1p-Bul2p ubiquitin ligase machinery and the sorting machinery that directs delivery of the permease from the *trans*-Golgi to the prevacuolar endosome.

I determined that each of the mutants isolated in the screen received Bul1/2p-independent monoubiquitination on lysine 16 that was sufficient to direct Bul1/2p-independent endocytosis and vacuolar delivery of the mutant (Figure 5 and data not shown). It will be interesting to determine whether these mutants are capable of Bul1/2p-dependent endocytosis. This can be easily accomplished by mutating lysine 16, the residue required for Bul1/2p-independent endocytosis, in the context of the mutants we

pulled out of the screen. If the mutants do not affect endocytosis through the Bul1/2-dependent pathway, it would indicate that the machinery that directs sorting of Gap1p in the *trans*-Golgi is different from the machinery that directs endocytosis. However, if the same mutation in *GAP1* impairs both Bul1/2p-dependent sorting steps, it would strengthen the argument for a cooperative interaction between the ubiquitination and sorting machinery.

References

Grenson, M. (1983). "Inactivation-reactivation process and repression of permease formation regulate several ammonia-sensitive permeases in the yeast Saccharomyces cerevisiae." Eur J Biochem 133(1): 135-9.

Hein, C. and B. Andre (1997). "A C-terminal di-leucine motif and nearby sequences are required for NH4(+)-induced inactivation and degradation of the general amino acid permease, Gap1p, of Saccharomyces cerevisiae." Mol Microbiol 24(3): 607-16.

Risinger, A. L., N. E. Cain, et al. (2006). "Activity-dependent Reversible Inactivation of the General Amino Acid Permease." Mol Biol Cell.

Sevier, C. S. and C. A. Kaiser (2006). "Disulfide Transfer between Two Conserved Cysteine Pairs Imparts Selectivity to Protein Oxidation by Ero1." Mol Biol Cell.

Soetens, O., J. O. De Craene, et al. (2001). "Ubiquitin is required for sorting to the vacuole of the yeast general amino acid permease, Gap1." J Biol Chem 276(47): 43949-57.

1 MSNTSSYEKNNPDNLKHNGITIDSEFLTQEPITIPSNGSAVSIDETGSGS

51 KWQDFKDSFKRVKPIEVDPNLSEAEKVAIITAQTPLKHHLKNRHLQMIAI

101 GGAIGTGLLVGSGTALRTGGPASLLIGWGSTGTMIYAMVMALGELAVIFP

151 ISGGFTTYATRFIDESFGYANNFNYMLQWLVVLPLEIVSASITVNFWGTD

201 PKYRDGFVALFWLAIVIINMFGVKGYGEAEFVFSFIKVITVVGFIILGII

251 LNCGGGPTGGYIGGKYWHDPGAFAGDTPGAKFKGVCSVFVTAAFSFAGSE

301 LVGLAASESVEPRKSVPKAAKQVFWRITLFYILSLLMIGLLVPYNDKSLI

351 GASSVDAAASPFVIAIKTHGIKGLPSVVNVVILIAVLSVGNSAIYACSRT

401 MVALAEQRFLPEIFSYVDRKGRPLVGIAVTSAFGLIAFVAASKKEGEVFN

451 WLLALSGLSSLFTWGGICICHIRFRKALAAQGRGLDELSFKSPTGVWGSY

501 WGLFMVIIMFIAQFYVAVFPVGDSPSAEGFFEAYLSFPLVMVMYIGHKIY

551 KRNWKLFIPAEKMDIDTGRREVDLDLLKQLIAEEKAIMATKPRWYRIWNF

601 WC

Figure 1. ***GAP1*** **protein sequence.** Transmembrane domains are underlined. Ubiquitinated lysine residues are in bold. Mutations that result in glycine sensitivity when mutated are highlighted.

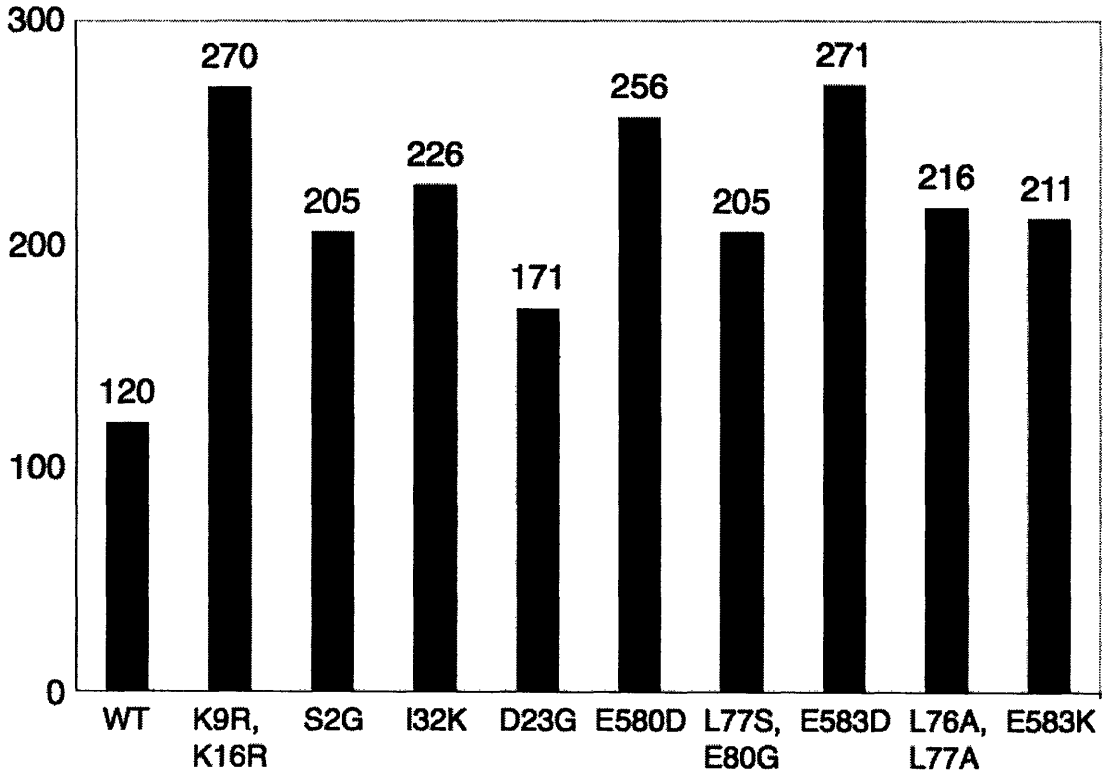


Figure 2. **Gap1p activity of glycine sensitive mutants in a wildtype background.** *gap1*Δ (CKY715) strains expressing the indicated *GAP1* allele were grown in minimal SD medium. Gap1p activity was measured by assaying exponentially growing cells for the initial rate of [^{14}C]-citrulline uptake.

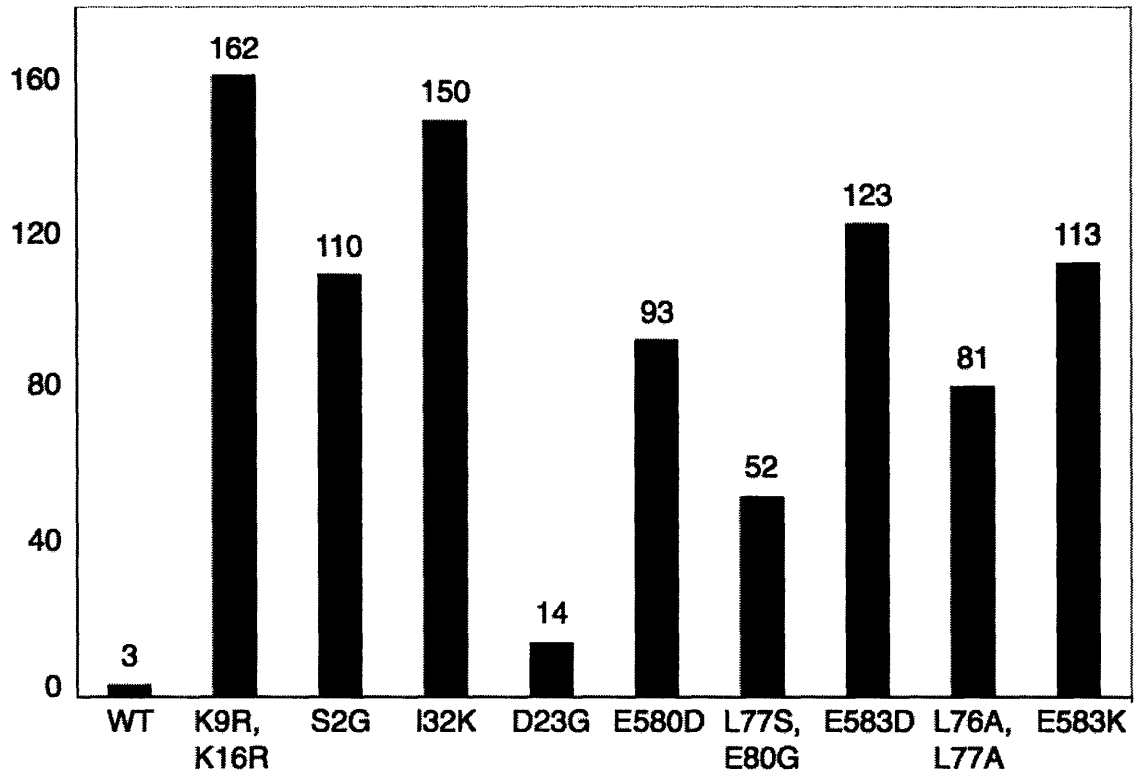


Figure 3. **Gap1p activity of glycine sensitive mutants in a *gtr1*Δ background.** *gap1*Δ *gtr1*Δ strains expressing the indicated *GAP1* allele were grown in minimal SD medium. Gap1p activity was measured by assaying exponentially growing cells for the initial rate of [^{14}C]-citrulline uptake.

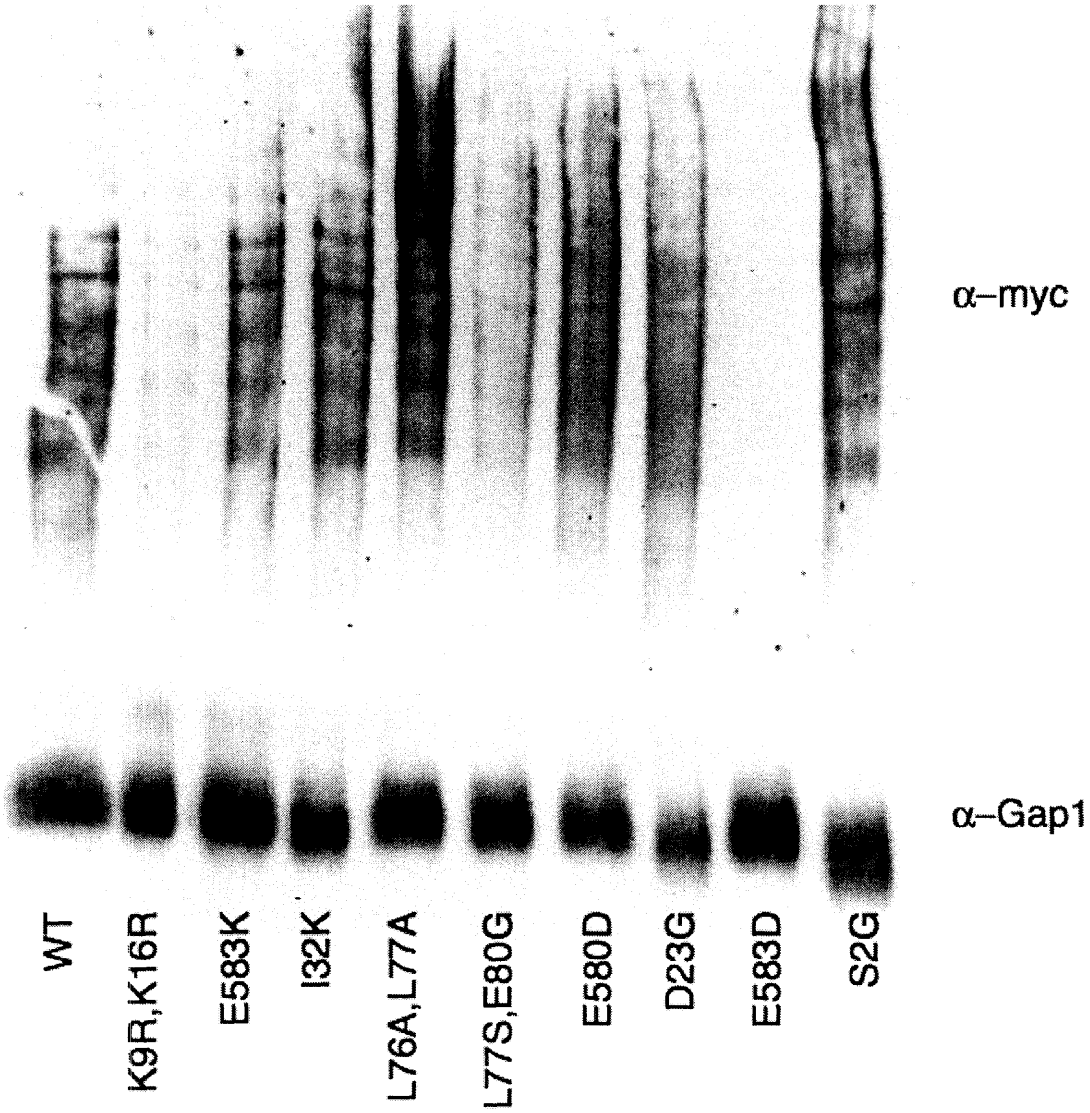


Figure 4. **Polyubiquitination of glycine sensitive mutants.** *pep4Δ doa4Δ gap1Δ* (CKY1031) strains expressing P_{cup1}-*myc-UBI* (pCK231) along with the indicated *HA* tagged *GAP1* allele were grown in minimal SD medium + 1μM $CuSO_4$ at 24°C. Immunoprecipitation of lysates was performed with 3F10 Rat-anti-HA. Immunoprecipitated proteins were separated by SDS/PAGE, and immunoblotted with 9E10 anti-myc or Gap1p antiserum.

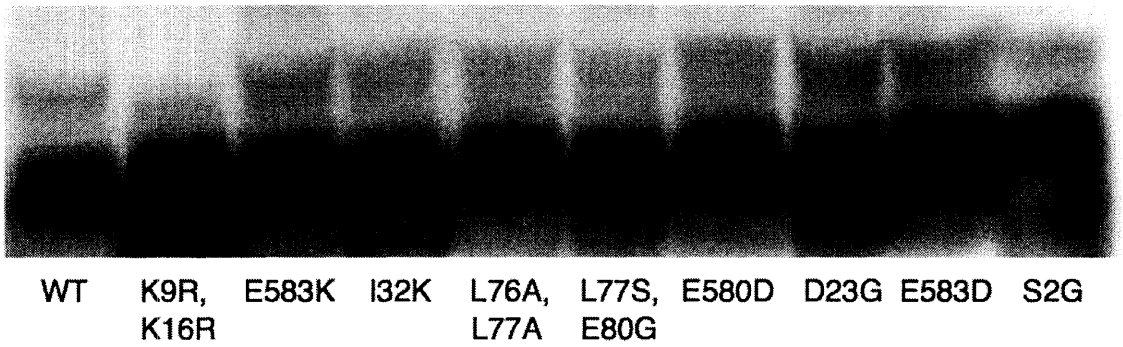


Figure 5. **Bul1/2p-independent monoubiquitination of glycine sensitive mutants.** Lysates were prepared from *gap1*Δ *bul1*Δ *bul2*Δ (CKY701) expressing the indicated *GAP1* allele grown at 24˚C in minimal SD medium and immunoblotted with Gap1p antiserum.

NUTRIENT SENSING

In this thesis, I have described multiple cellular processes including *GAP1* transcription and intracellular sorting that are affected by internal amino acid levels. However, we do not yet understand how the amino acid signal is transduced to affect these events. We have shown that the only known direct amino acid sensor in *S. cerevisiae*, Ssy1p, is not required for the transcriptional or post-translational regulation of *GAP1* in response to amino acids, indicating that an additional unidentified amino acid sensor exists in the cell.

One current hypothesis is that the Gap1p protein itself can serve as a sensor of intracellular amino acids. According to this theory, the ability of Gap1p to bind to its substrate amino acid in the cytosol could affect its conformation, which may alter the interaction of the permease with sorting machinery. If this is the case, we should be able to isolate mutants in the *GAP1* gene that are impaired in amino acid-dependent delivery of the permease to the vacuole. One approach we have taken to isolate a sorting-deficient *GAP1* allele is to screen for mutants that result in delivery of Gap1p to the plasma membrane in the presence of the elevated internal amino acid levels in a *mks1*Δ mutant. Although this screen has isolated several alleles of *GAP1* that are deficient in the ubiquitin-mediated sorting of Gap1p from the *trans*-Golgi to the endosome, it failed to pull out alleles that are insensitive to the amino acid dependent recycling of Gap1p from the endosome to the *trans*-Golgi.

One reason that Gap1p is an attractive candidate as an amino acid sensor is that it directly interacts with amino acids during their transport into the cell. It will be interesting to determine whether the ability of Gap1p to bind and transport amino acids is

required for the amino acid dependent retrieval of Gap1p from the endosome to the *trans*-Golgi. If active Gap1p transport is not required for amino acid dependent Gap1p trafficking, Gap1p may serve as an amino acid sensor through a mechanism uncoupled to transport or another protein may provide this function.

Regardless of whether Gap1p serves as an amino acid sensor with regard to permease trafficking, our finding that amino acids repress Nil1p-dependent *GAP1* expression in a *gap1*Δ mutant indicate that an unidentified amino acid sensor exists in the cell. Screens to isolate mutants deficient in amino acid dependent Gap1p sorting or repression of Nil1p activity could potentially identify this sensor as well as other proteins required for the transmission of the amino acid signal.