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Activity-dependent Reversible Inactivation of the General Amino Acid Permease

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The general amino acid permease, Gap1p, of *Saccharomyces cerevisiae* transports all naturally occurring amino acids into yeast cells for use as a nitrogen source. Previous studies have shown that a nonubiquitinateable 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 *et al.*, 1998; Iraqui *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 *et al.*, 1999; Omura *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 *et al.*, 1970; Chen and Kaiser, 2002).

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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 *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 *et al.*, 2001; Soetens *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

Table 1. Strains

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 gap1Δ::LEU2 can1 leu2-3 ura3-52*	

All are isogenic with S288C. All are from this article unless noted.

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

MATERIALS AND METHODS

Strains, Plasmids, and Media

All of the yeast strains used in this study are of the S288C background that expresses high Gap1p activity in minimal ammonia medium (Table 1; Courchesne and Magasanik, 1983).

The integrated *GAP1* strains were constructed by ligating a 500-base pair 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 *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 (La Jolla, CA) on the above plasmids to introduce the lysine mutations before 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; Difco, Detroit, MI). 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 base pairs of the *ADH1* promoter and 800 base pairs of the *GAP1* 3′ UTR was amplified in four 50-μl reactions with AmpliTaq Gold (Perkin-Elmer Cetus, Norwalk, CT) and 0.1 mM $MnCl_2$. PCR products were transformed along with gapped pEC221 plasmid (lacking the *GAP1* ORF) into CKY701 (*bul1Δ bul2Δ gap1Δ ura3-52*), and gap-repaired plasmids were isolated by selection for Ura$^+$ transformants. Citrulline-resistant transformants were identified by replica plating onto SD with 4 mM citrulline at 30°C. Resistant clones were then tested for sensitivity to glycine by replica plating to SD with 1 mM 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 $\sim 10^{-3}$.

Amino Acid Uptake Assays

Strains were cultured to 4–8 × 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 *et al.*, 1997). The specific activity of glycine was ~112 mCi/mmol.

Amino Acid Accumulation Assays

GAP1^{K9R,K16R} (CKY893) was cultured at a concentration of 5 × 10^6 cells/ml in minimal SD medium and distributed into 1-ml 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 1 mM. The total accumulated radiolabeled amino acid was measured after 20 min.

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 300 mM Tris, pH 8, 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 *et al.*, 2002). Antibodies used were as follows: rabbit anti-Gap1p; rabbit anti-Pma1p (gift of S. Losko and R. Kolling, Dusseldorf, Germany); and horseradish peroxidase–coupled sheep anti-rabbit (Amersham Pharmacia, Piscataway, NJ).

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 *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 *mks1Δ* 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 *et al.*, 2002; Sekito *et al.*, 2002) and *mks1Δ* strains have elevated internal amino acid concentrations sufficient to cause Gap1p to be sorted to the vacuole in a wild-type cell (Figure 1; Chen and Kaiser, 2002;

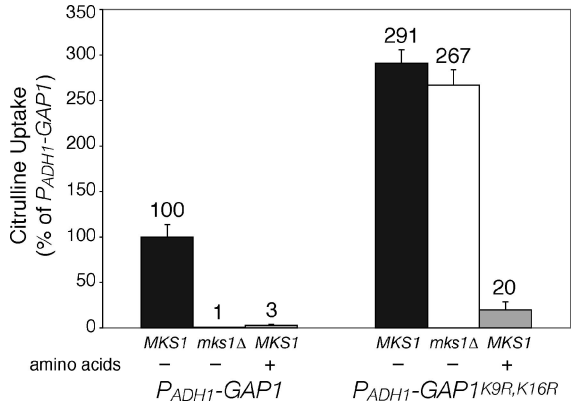

Figure 1. Gap1p that cannot be ubiquitinated bypasses amino acid–dependent sorting but can be inactivated by exogenous amino acids. P_{ADH1}-*GAP1* (CKY759), P_{ADH1}-*GAP1 mks1*Δ (CKY763), P_{ADH1}-*GAP1*^{K9R,K16R} (CKY890), or P_{ADH1}-*GAP1*^{K9R,K16R} *mks1*Δ (CKY891) were grown in minimal ammonia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

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 (Figure 2, A 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 1 h after amino acid addition Gap1p activity had decreased to <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, because they exhibit different sensitivities to the concentration of exogenous amino acids.

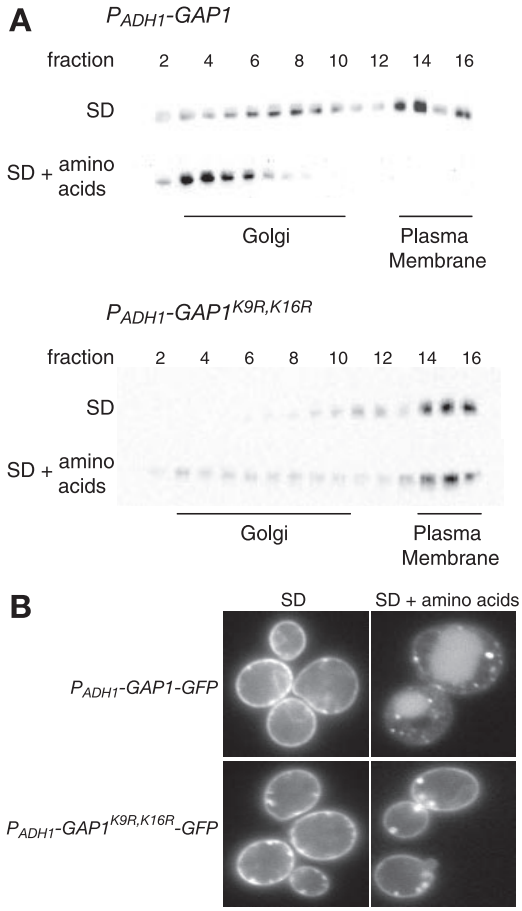

Figure 2. Inactive Gap1p^{K9R,K16R} remains in the plasma membrane in the presence of amino acids. (A) Membranes from P_{ADH1}-*GAP1* (CKY759) or P_{ADH1}-*GAP1*^{K9R,K16R} (CKY890) cell extracts were fractionated on 20–60% sucrose density gradients containing EDTA. Fractions were collected from the 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.

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 *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

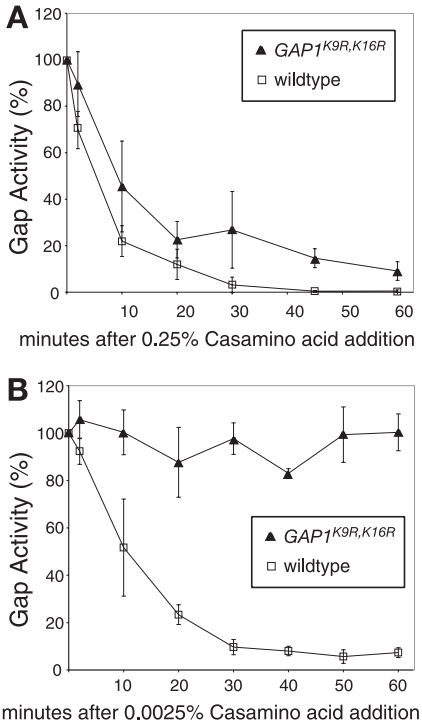

Figure 3. Gap1p sorting and inactivation occur in response to distinct amino acid concentrations. Wild type (CKY443) or *GAP1*^{K9R,K16R} (CKY1024) were grown to exponential phase in SD medium. Gap1p activity was measured by assaying the initial rate of [^{14}C]citrulline uptake at the indicated time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

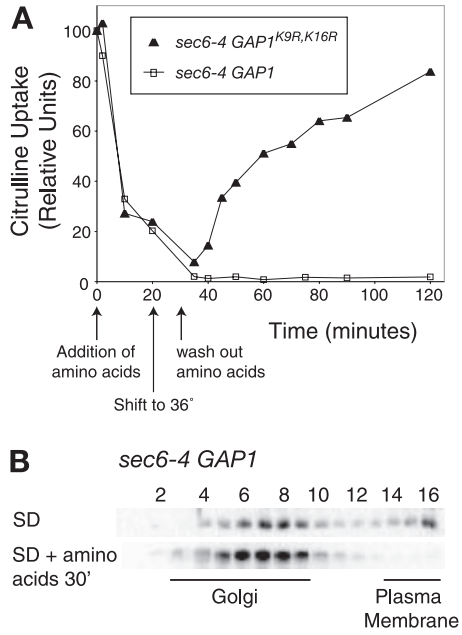

Figure 4. Amino acid import through Gap1p^{K9R,K16R} is rapidly and reversibly inactivated upon amino acid treatment. (A) *sec6-4 GAP1*^{K9R,K16R} (CKY895) or *sec6-4* (CKY517) were grown in minimal SD medium at 24°C. Casamino acids were added to cells for 20 min after which cells were shifted to 36°C for 10 min. 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 min 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.

most of Gap1p had been removed from the plasma membrane), cells were shifted to 36°C for 10 min, washed, and resuspended in prewarmed 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 because 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 nonubiquitinated form of Gap1p was not internalized (Figure 2, A 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, whereas 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 because 3 mM 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, because less than 1% of Gap1p^{K9R,K16R}-expressing cells were able to form colonies on medium lacking amino acids after incubation in 3 mM glycine for 22 h (Table 2).

Complete growth inhibition of Gap1p^{K9R,K16R} expressing cells occurred within 30 min after addition of 1 mM glycine (Figure 5B). Interestingly, wild-type strains showed a slight inhibition of growth 30 min after glycine addition, but then recovered to a normal growth rate at about the same time that it took for Gap1p activity to be down-regulated by amino acids (Figure 6, A 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.

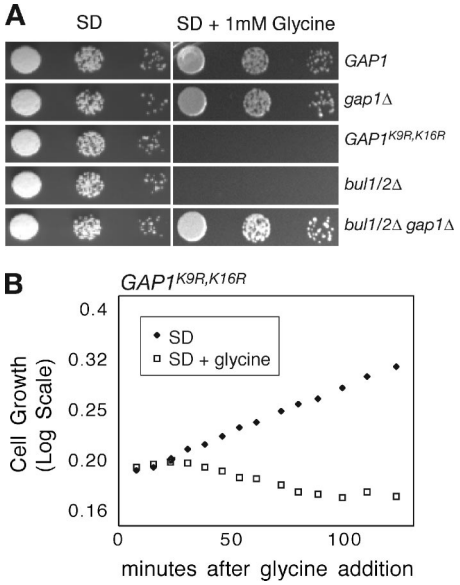

Figure 5. Excess amino acid addition is toxic to cells deficient in Gap1p ubiquitination. (A) *gap1*Δ (CKY482) expressing wild-type Gap1p (pAR70), Gap1p^{K9R,K16R} (pAR73), or pRS316 and *gap1*Δ *bul*Δ *bul2*Δ (CKY701) expressing wild-type Gap1p (pAR70) or pRS316 were serially diluted onto SD or SD + 1 mM glycine plates and incubated at 30°C. (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 (□), and an equal volume of SD was added to the other (♦). Cells were allowed to grow at 30°C, and growth was measured by optical density for 2 h after addition.

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°C, and 5×10^6 cells were cultured in SD with 3 mM of the indicated amino acid. After 22 h, 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.

Amino Acid Mixtures Relieve Amino Acid–dependent Toxicity

Before amino acid addition, Gap1p activity is approximately threefold 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 pretreatment with alanine (which is not toxic) for a period sufficient to cause Gap1p activity to decrease to wild-type levels (Figures 7, A 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 with 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 nontoxic 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 (unpublished data). 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 wild-type Gap1p in a *bul1*Δ *bul2*Δ background. Citrulline-resistant clones were then counterscreened for sensitivity to 1 mM 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}, and 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 indepen-

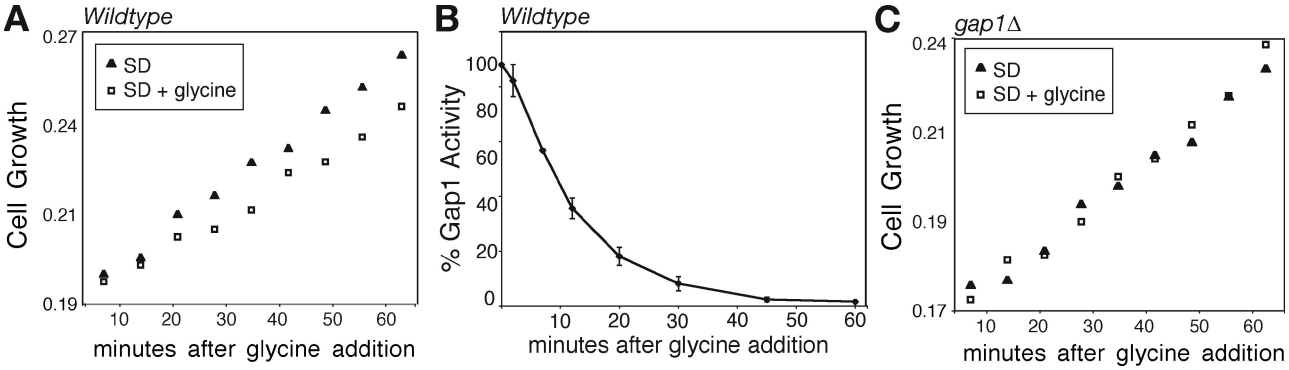

Figure 6. Amino acid toxicity is physiological and Gap1p-dependent. (A) Wild type (CKY443) was grown in SD at 30°C. At early exponential phase, the culture was split, and 1 mM glycine was added to one (□), and an equal volume of SD was added to the other (▲). Cells were allowed to grow at 30°C, and growth was measured by optical density. (B) Glycine, 1 mM, 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).

dently of sorting, we introduced the Gap1p^{A297V} mutation into nonubiquitinateable 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 nontoxicity 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 *et al.*, 1966; Whelan *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, whereas Gap1p^{K9R,K16R} activity dropped to less than 5% after an hour of arginine addition (Figure 9C). Because 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.

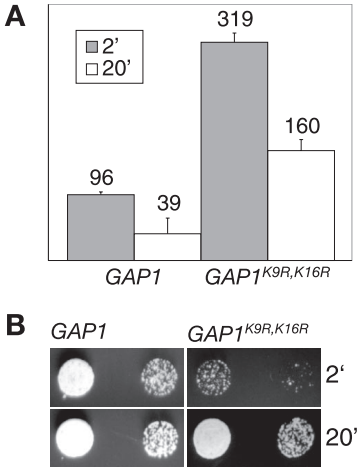

Figure 7. Amino acid toxicity is a result of elevated amino acid import in Gap1p$^{K9R,\ K16R}$. (A) Wild type (CKY443) or *GAP1*^{K9R,K16R} (CKY1024) were grown to exponential phase in SD medium at 25°C. Gap1p activity was measured by assaying the initial rate of [^{14}C]citrulline uptake either immediately (2′) or 20 min (20′) after 1 mM alanine addition. (B) Wild type (CKY443) or *GAP1*^{K9R,K16R} (CKY1024) were grown to exponential phase in SD medium at 25°C, and 1 mM alanine was added to cells. Cells were filtered, washed, and resuspended in SD + 1 mM glycine either immediately or 20 min after alanine addition. Cells were then incubated in SD with 1 mM glycine for 30 min, filtered, washed, and plated in serial dilutions onto minimal SD plates and grown at 24°C for 3 d to determine viability.

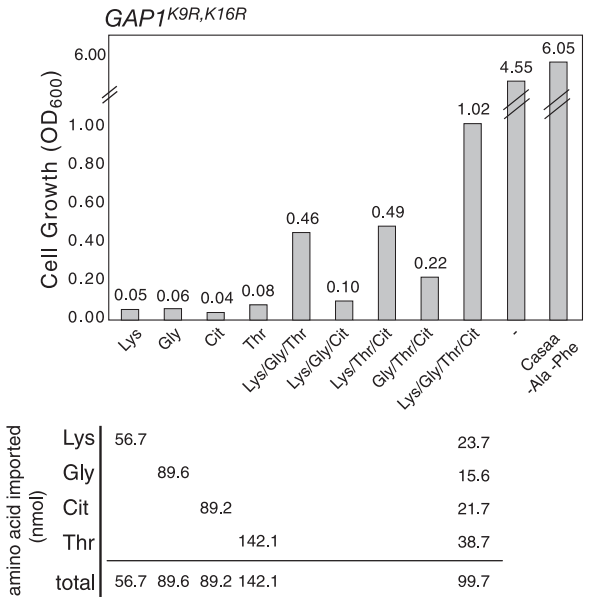

Figure 8. Amino acid mixtures relieve amino acid–dependent toxicity. (A) *GAP1*^{K9R,K16R} (CKY893) was grown to exponential phase in SD medium at 30°C, and 5 × 10^6 cells were cultured in SD medium with 3 mM total of the indicated amino acid combination or SD + 0.25% Casamino acids (−ala, −phe). The optical density of the cultures was measured after 22 h 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 20 min after amino acid addition was determined.

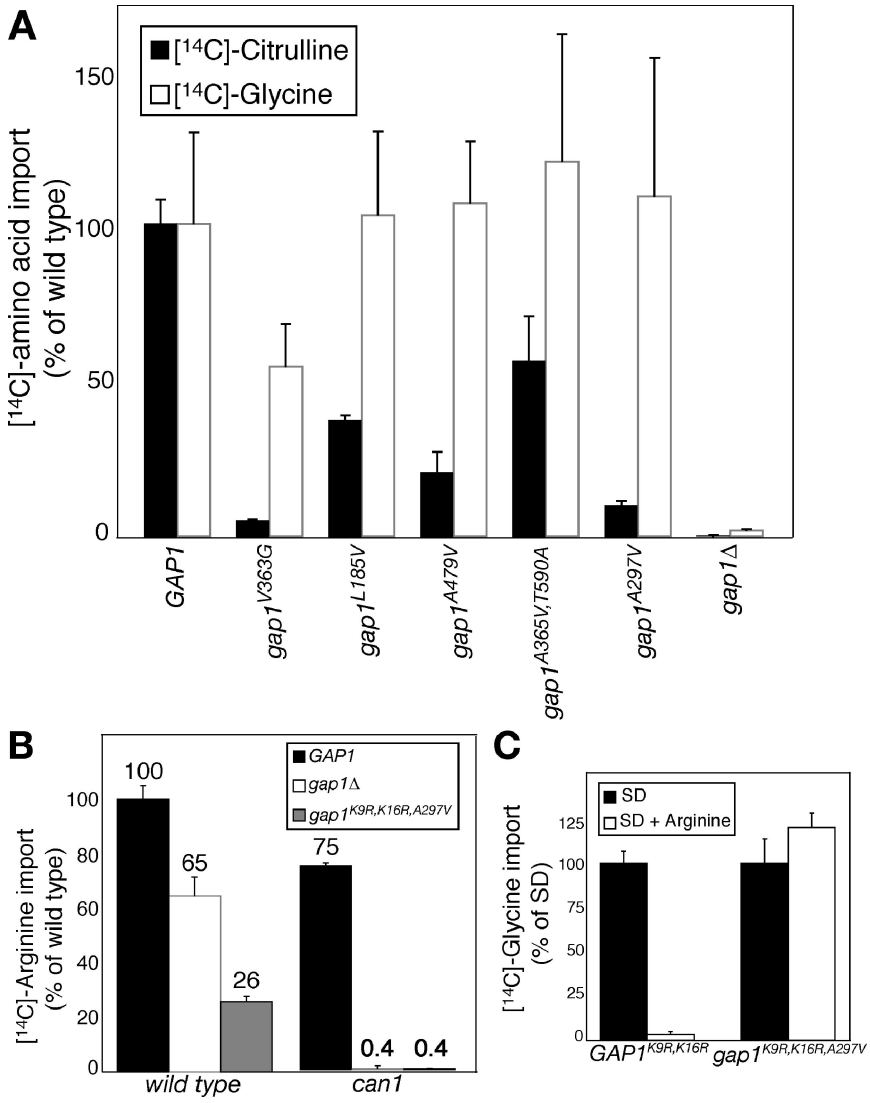

Figure 9. Inactivation of Gap1p requires active amino acid transport through the permease. (A) The initial rate of [^{14}C]citrulline or [^{14}C]glycine uptake was determined for *gap1*Δ *bul1*Δ *bul2*Δ (CKY701) expressing wild-type *GAP1* (pEC221), the indicated mutant *GAP1* allele (pNC3–7), or vector 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 1 h after the addition of 1 mM arginine. Three independent measurements were averaged.

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 *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 on 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 ~20 min 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, because after withdrawal of exogenous amino acids Gap1p^{K9R,K16R} located in the plasma membrane regains activity with a half-time of 20 min. 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 ~5000 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 *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 (unpublished data). Additionally, it has been speculated that the manganese transporter, Smf1p, adopts a conformational change when bound to metal that influences trafficking of the permease, because 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 *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 *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 (unpublished data). 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 pretreated *GAP1^{K9R,K16R}* with cycloheximide to block all new protein synthesis before amino acid addition. Indeed, we found a three- to fivefold increase in viability of Gap1p^{K9R,K16R} expressing cells treated with cycloheximide before lysine addition (unpublished data). 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 ~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 *et al.*, 1992; Trotter *et al.*, 2002). When single amino acids were added to *GAP1^{K9R,K16R}*, we failed to observe a increase in Hsp104 expression similar to that seen for misincorporated amino acid analogs such as L-azetidine-2-carboxylic acid (unpublished data). Taken together these data suggest that tRNA synthetase mischarging and protein misfolding may 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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