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Amino acid transport through the *Saccharomyces cerevisiae* Gap1 permease is controlled by the Ras/cAMP pathway

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Abstract

The general amino acid permease (Gap1p) of *Saccharomyces cerevisiae* is a broad range, low affinity permease that imports amino acids in cells growing on poor nitrogen sources. This permease also signals the presence of amino acids through the fermentable growth medium pathway allowing the cell to respond to new sources of nitrogen in the surrounding medium. Yeast with an activated Ras2/cAMP pathway show many phenotypes indicative of altered nitrogen uptake and metabolism; sensitivity to nitrogen starvation, low amino acid pools. We have shown that Gap1p activity is lowered in cells with an activated $RAS2^{val19}$ allele or elevated cAMP levels whereas cells with inactive *ras2* allele lose ammonia repression of Gap1p-mediated transport. This regulation is through a post-transcriptional mechanism; transcription of *GAP1* is not affected by cAMP level. A mechanism by which the Ras2/cAMP/PKA pathway controls the ubiquitin-dependent degradation of Gap1p is most consistent with the data.

Keywords

GAP1; general amino acid permease; Gap1p; Ras2/cAMP pathway; post-transcriptional control

INTRODUCTION

The mechanisms by which yeast cells detect, and respond to, available nutrients have been studied extensively. There are two major signaling pathways, the Ras/cAMP pathway and the fermentable growth medium pathway, which signal sugar and nitrogen source availability respectively and converge through the regulation of protein kinase A (PKA; Thevelein and de Winde, 1999). The subsequent activity of PKA substrates controls the cell's readiness for continued growth in adequate nutrient conditions or its transition to stationary phase in response to starvation (Broach and Deschenes, 1990).

Yeast contain a series of transport molecules responsible for the uptake of nitrogen sources from the medium (André, 1995) some of which also act as receptors signaling the availability of their substrate to the interior of the cell (Holsbeeks et al., 2004). In a useful oversimplification of the regulation mechanisms, one can classify growing cells as being either nitrogen-repressed or –derepressed (Magasanik and Kaiser, 2002). Nitrogen-repressed cells have a 'good' nitrogen source available, which can be imported through specific permeases (Mep1 and Mep2 – ammonia, Gnp1 – glutamine). Alternatively, there are permeases that are induced at the

transcriptional level by the presence of external substrates (Nielson et al., 2001) that can take up an amino acid either as a nitrogen source for growth or as an additional nutrient to supplement an auxotrophy. These can be either broad-range (e.g.Agp1) or group-specific (e.g.Bap2 – branched chain amino acids) permeases. In contrast, cells growing on a derepressing nitrogen source like proline induce the expression of the general amino acid permease gene (*GAP1)* as well as the proline specific permease (*PUT4)*. This allows the cell to take up a broad range of amino acids and related compounds as they become available in the medium to the nitrogen-limited cells (Magasanik and Kaiser, 2002).

Yeast cells employ multiple mechanisms to control Gap1 activity in response to different nitrogen sources. Transcription of *GAP1* and other nitrogen-repressible genes, is controlled by the GATA type transcription factors (Gln3p, Nil1p, Nil2p, Dal80p) in response to the presence of glutamine or asparagine (Stanbrough and Magasanik, 1995). In de-repressing media, the levels of Gap1p found in the membrane is also regulated by the control of permease trafficking to the vacuole by amino acids. Cells growing on glutamate synthesize Gap1p but the protein is sorted directly to the vacuole and never reaches the plasma membrane (Chen and Kaiser, 2002).

In nutrient conditions where mature permease does localize to the plasma membrane, Gap1p stability, and thus activity, is regulated in multiple ways. One important activation/inactivation mechanism involves the *NPR1* and *NPI1/RSP5* gene products (Vandenbol et al., 1990). In non-repressing conditions the action of these enzymes results in a steady state activity of the permease, however, once ammonia is added, Gap1p is internalized and degraded by a pathway involving ubiquitination (Springael and André, 1998; Springael et al., 2002). While the molecular mechanism controlling these processes is not understood, one component of the pathway may be Aua1p. *aua1* mutants have lost ammonia repression of Gap1p but show no other phenotypes (Sophianopoulou & Diallinas, 1993). Recently, an additional reversible inhibition of Gap1p activity in response to the transport of excess amino acid has been elucidated (Risinger et al., 2006). In this case, it appears that the permease remains within the plasma membrane in an inactive, but unmodified, form.

As the Ras2/cAMP and FGM pathways control yeast cell growth in response to nutrients and permease molecules provide those nutrients, it is not surprising that specific links have been found between these pathways. Certain permeases have been shown to signal the presence of their substrate generating an intracellular signal and response Holsbeeks et al., 2004). For example, Gap1p has been shown to be involved in the regulation of PKA targets in response to the addition of amino acids to the medium (Donaton et al., 2003). As activated Ras2/cAMP pathway mutants show phenotypes suggestive of altered amino acid metabolism and transport, it is of interest to determine if there is feedback control of amino acid transport by Ras2/cAMP/PKA activities. Preliminary studies have suggested that cAMP levels affect amino acid transport (Amitrano et al., 1997; Saenz et al., 1997). In this paper we demonstrate that elevated cAMP levels decrease Gap1p activity and this control is exerted at a post-transcriptional step in Gap1 regulation. Also, that ammonia inactivation of Gap1p requires a functional Ras2p. We suggest that the Ras2/cAMP pathway is involved in regulating the ubiquitin-dependent degradation of Gap1p.

MATERIALS AND METHODS

Yeast strains and plasmids

The strains used in this study are listed in Table 1. The construction of strains HR125Δgap1 and TC41-1Δgap1 was as described previously (Schreve & Garrett, 1997). The plasmids pSE (promoter *GAP1-lacz)* and pPDE2 (phosphodiesterase 2 over-expression plasmid) were the kind gifts of Mike Stanbrough and Mike Wigler respectively (Stanbrough et al., 1995;Sass et

al., 1986). All DNA manipulations in yeast and bacteria were done using standard protocols (Sherman et al., 1986;Sambrook et al., 1989).

Growth conditions

Yeast strains were routinely grown in standard yeast media (Sherman et al., 1986; Wickerham, 1946). Minimal medium contained the following supplements: L-histidine (20mg/l), L-leucine (30mg/l), L-tryptophan (20mg/l), L-lysine (30mg/l), adenine (20mg/l) and uracil (20mg/l). For the determination of balanced growth rates on different nitrogen sources, the culture was repeatedly diluted into fresh media during mid-exponential phase and the doubling time determined from at least three growth phases. The amino acid acting as principal nitrogen source was added at 0.01% (w/v) and the supplements were added at 0.1x normal concentration.

Amino Acid Transport and β-galactosidase Assays

L-^{14}C-amino acid uptake was assayed by the method of Woodward and Cirillo (1977), except that cycloheximide was omitted from the buffer. Cells grown in minimal ammonia media were harvested during log phase (OD_{550} = 0.1 – 0.3; 0.058 – 0.174 mg dry weight/ml), washed with water and resuspended in 10mM potassium phthalate (pH = 5.5), 2% glucose to an OD_{550} of approximately 0.8. After incubation at 30°C for 30 minutes ^{14}C-labelled amino acid was added. Samples were withdrawn at 30 second intervals, filtered, and washed with 20 ml ice cold water. Filters were placed in vials and 5 ml liquid scintillation fluid was added. Radioactivity was determined on a Wallac LSC. β-galactosidase activity was determined as described previously (Miller, 1972; Schreve et al., 1998).

RESULTS

Yeast strains with elevated *RAS*/cAMP pathway activity show phenotypes that indicate altered nitrogen metabolism, including sensitivity to starvation and decreased vacuolar basic amino acid pools (Markwardt et al., 1995). The balanced growth rates of wild-type and activated *RAS2* mutants were measured in different amino acids as principal nitrogen source. This data (Table 2) shows that strains containing the *RAS2^{val19}* allele are differentially impaired for growth on different amino acids suggesting a defect in either transport or utilization of amino acids in these strains. Alteration of amino acid transport activity in *RAS2^{val19}* is also indicated by their hypersensitivity to growth in the presence of the amino acid analogs cycloleucine (125 μg/mL), γ-hydroxyglutamate (500 μg/mL) and norleucine (100 μg/mL) (data not shown). Strains in which *RAS*/cAMP pathway activity is decreased (*ras2*) grow at the same rate as their parental strain in these conditions (data not shown).

The pleiotropic amino acid-related phenotypes of *RAS2^{val19}* strains suggest a general defect in amino acid transport and/or metabolism. We assayed general amino acid permease levels in yeast with normal, elevated and attenuated *RAS*/cAMP pathway activity in two strain backgrounds. The assays were performed in both repressing (ammonia) and non-repressing (proline) media. In all conditions tested, cells with an overactive *RAS*/cAMP pathway (TR161-R2V, Y1020) showed lower Gap1 transport as measured by uptake of the Gap1 permease-specific substrate, citrulline than the parental strain (Figure 1). In contrast, *ras2* mutants (T3–24C, Y420) showed normal or slightly elevated Gap1-mediated transport on proline media and lost ammonia repression of the transporter in ammonia-grown cells The two strains studied showed different levels of ammonia repression characteristic of S288C-derived (SP1, partial repression) and Σ1278B-derived (Y294, full repression) strains and in both cases the Gap1 transport levels of ammonia-grown *ras2* cells equaled that of proline-grown parental strain. Thus we have demonstrated that mutant *RAS2* alleles affect Gap1 activity in two ways; a decrease in amino acid transport in *RAS2^{val19}* cells that is not specific to nitrogen source and a loss of ammonia repression in *ras2* cells.

In order to determine if the decreased Gap1 activity in *RAS2*^{val19} cells is the result of elevated cAMP levels we assayed ^{14}C-citrulline uptake in cells engineered to be sensitive to external cAMP levels (Russell et al., 1993). The results in Figure 2 clearly demonstrate that Gap1 activity is decreased with increasing cAMP levels in a cAMP-sensitive strain whereas Gap1 activity is not affected in the isogenic wild-type strain. The observed effect of the *RAS*/cAMP pathway on amino acid transport appears to be confined to regulation of Gap1 activity, we tested three specific amino acid transporters and found no evidence that they are regulated in a similar manner. The transport of amino acids that are good nitrogen sources like glutamate and glutamine is unaffected by the *RAS2*^{val19} allele (Table 3). Similarly, uptake of glutamate and glutamine are not affected by external cAMP concentration in the cAMP-sensitive strain (Table 3). Transport of leucine, a poor nitrogen source, is only slightly reduced in the *RAS2*^{val19} mutant. Further evidence that the decrease in Gap1 activity is the result of elevated cAMP levels comes from the finding that overexpression of Pde2 (phosphodiesterase that degrades cAMP) on a multicopy plasmid restores Gap1 activity in TR161-R2V to wildtype levels (SP1 = 2.83 +/− 0.28 nmoles ^{14}C-citrulline/min^{-1}/OD$_{600}$, TR161-R2V = 1.39 +/− 0.31 nmoles ^{14}C-citrulline/min^{-1}/OD$_{600}$, TR161-R2Vp*PDE2* = 3.39 +/− 0.68 nmoles ^{14}C-citrulline/min^{-1}/OD$_{600}$).

The transport activity of Gap1 has been shown to be regulated in multiple ways both transcriptional and post-transcriptional (Stanbrough and Magasanik, 1995; Springael et al., 2002; Risinger et al., 2006). In order to determine if transcription of *GAP1* is affected by cAMP levels we transformed the expression construct pSE (*GAP1* promoter fused to *lacz*) into the cAMP-dependent strain TC41. As shown in Figure 3, while rising cAMP levels caused Gap1 transport activity of cells grown in 5mM cAMP to fall to 20% of that if 0.5mM cAMP grown cells, β-galactosidase levels remained constant. Thus cAMP is not affecting transcription of the *GAP1* gene and the loss of activity must be the result of alteration of the activity of a post-transcriptional control mechanism, e.g. permease localization or turnover.

DISCUSSION

Many of the phenotypes of yeast mutants with an activated Ras2/cAMP pathway are not fully explained by the current model of carbon and nitrogen growth control. It has been shown that amino acid availability is signaled by Gap1p through the fermentable growth pathway to activate protein kinase A resulting in trehalase activation and storage carbohydrate breakdown (Donaton et al., 2003). But many questions remain. Why are activated *RAS* strains sensitive to nitrogen starvation and unable to accumulate vacuolar amino acids (Markwardt et al., 1995)? Why are they unable to grow on many amino acids as nitrogen source (Table 2) and hypersensitive to some amino acid analogs (data not shown). These phenotypes suggest that amino acid transport and/or metabolism is altered in *RAS2*^{val19} strains. They raise the possibility that the Ras2/cAMP/PKA pathway not only assimilates signals of nutrient availability but also provides feedback controls on the acquisition of further nutrients for growth.

There are multiple transporters for most amino acids and they vary in their regulation (André, 1995). The experiments presented here indicate that elevated RAS pathway activity (Fig. 1) or elevated cAMP levels (Fig. 2) decrease the activity of the general amino acid permease and thus impair the ability of the cell to take up a wide range of amino acid substrates. The effect on citrulline uptake is the most marked as Gap1 is the only transporter of this substrate, however a decrease in leucine uptake was also observed consistent with loss of the contribution of Gap1 to uptake of this amino acid. We found no evidence of cAMP control of uptake of the good nitrogen sources glutamate and glutamine.

Previous studies on the effect of cAMP levels on amino acid transport have reached different conclusions. Saenz and colleagues found decreased levels of leucine transport observed in *RAS2*^{val19} mutants and concluded these were the result of changes in other permeases (S1 –Bap2p, S2 –Agp1p) and that Gap1p transport was not affected (Saenz et al., 1997). The strains used in that experiment were designated as *gap1* mutants because they showed no detectable citrulline transport. However, the initial leucine uptake they observe is three times higher in proline-grown wild-type cells than those grown in ammonia. The difference is leucine uptake is not present in their activated *RAS* strain indicating that an ammonia-sensitive activity has been lost. This pattern of regulation is typical of an activity subject to ammonia repression like Gap1, not permeases regulated by amino acid induction like Bap2 (S1). While the lack of citrulline transport in their strain does indicate the loss of Gap1 activty, it is also possible that this strain contains a mutant Gap1p permease with an altered active site that does not recognize citrulline but still transports leucine.

Our studies indicate that elevated Ras2p activity or high cAMP levels result in lowered Gap1p permease activity and this could explain the sensitivity to nitrogen starvation observed in these strains. Previous results have have also demonstrated that yeast general amino acid permease is sensitive to cAMP levels (Amitrano et al., 1997). However, in those studies the level of ^{14}C-citrulline uptake increased as cells were grown in 0.25–1.0mM cAMP, but higher levels of exogeneous cAMP were not tested. The low Gap1 activity at low cAMP levels is not consistent with the finding that *ras2*mutants, which have only partially active Ras pathway have elevated Gap1 levels on all media tested (Fig. 1). Nor with the low Gap1p activity in *RAS2*^{val19} mutants we observed (Fig. 1) It is possible that the decreased Gap1p activity at low cAMP observed by Amitano et al (1997) is related to decreased viability and slow growth rate in these conditions and these workers did not assay cAMP concentrations high enough (>1mM) to see the effect on Gap1p activity. Finally, we do not believe that the loss of Gap1 activity we observe at high cAMP is directly correlated with cell viability because, while Gap1 activity decreased steadily from 0.5–5.0mM cAMP, the cells grew at essentially the same rate with no loss of viability from 0.5–2.0mM cAMP.

General amino acid permease is controlled by multiple regulation mechanisms both transcriptional and post-transcriptional. Gene expression studies of a *GAP1-lacZ* expression construct indicate that there is no repression of transcription in high cAMP conditions (Fig. 3). Compared with 0.5mM cAMP-grown cells, cells grown in 5mM cAMP have lost 80% of their Gap1p activity and yet have slightly higher *GAP1*-driven β-galactosidase levels. Thus, the Ras2/cAMP pathway must effect one of the post-transcriptional control mechanisms; permease localization, activation or turnover. As the loss of Gap1p activity in *RAS2*^{val19} mutants occurs in all media tested and *ras2* mutants lose ammonia repression it is most likely that it is the ubiquitination and subsequent turnover of the permease that is being affected. We suggest the following model. In normal cells, growing on adequate nitrogen and carbon sources, the Ras2/cAMP/PKA pathway signals ‘growth’ which includes depression of Gap1p as the permease is not needed when cells have a good nitrogen source. Conversely when cells are starving, Gap1 is up-regulated to allow uptake of any nitrogen sources that become available. Thus, in *RAS2*^{val19} mutants the cells are unable to respond to poor nitrogen conditions and lose Gap1p activity. This is achieved by stimulating the ubiquitin-dependent turnover of the permease in a nitrogen-insensitive manner. *ras2* mutants maintain high Gap1p levels even in ammonia because the turnover of Gap1p is inhibited in these strains even when ammonia is present in the medium. Thus, the observed loss of ammonia repression in both strains.

In this study we have demonstrated the regulation of Gap1p permease in response to the Ras2/cAMP/PKA levels. Elevated Ras2p or cAMP levels resulted in a decrease in Gap1 transport. Loss of Ras2p or low cAMP resulted in elevated Gap1p activity in ammonia-grown cells. This effect on Gap1 regulation occurs at a post-transcriptional step in the pathway of Gap1p

maturation. Studies are underway to further test the model that cAMP levels affect the ubiquitin-dependent turnover of Gap1p in response to the addition of ammonia or amino acids.

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Figure 1A

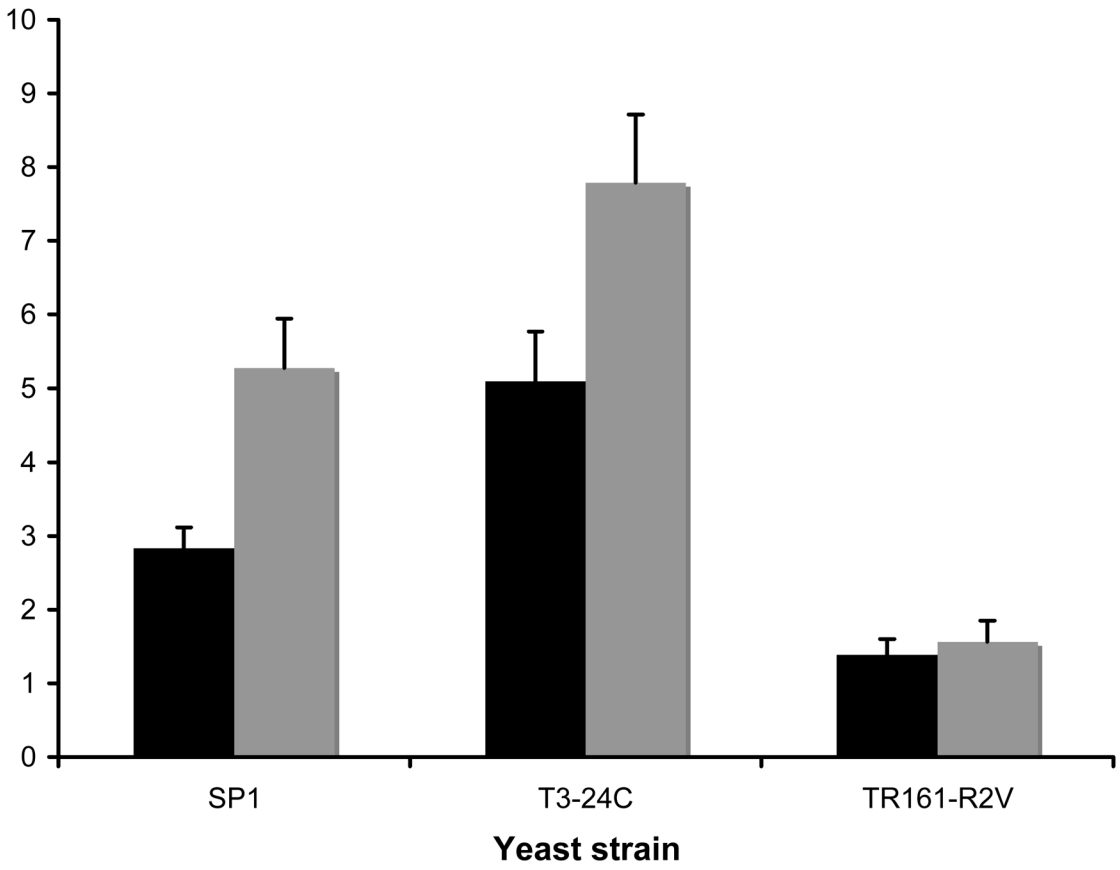

Figure 1B

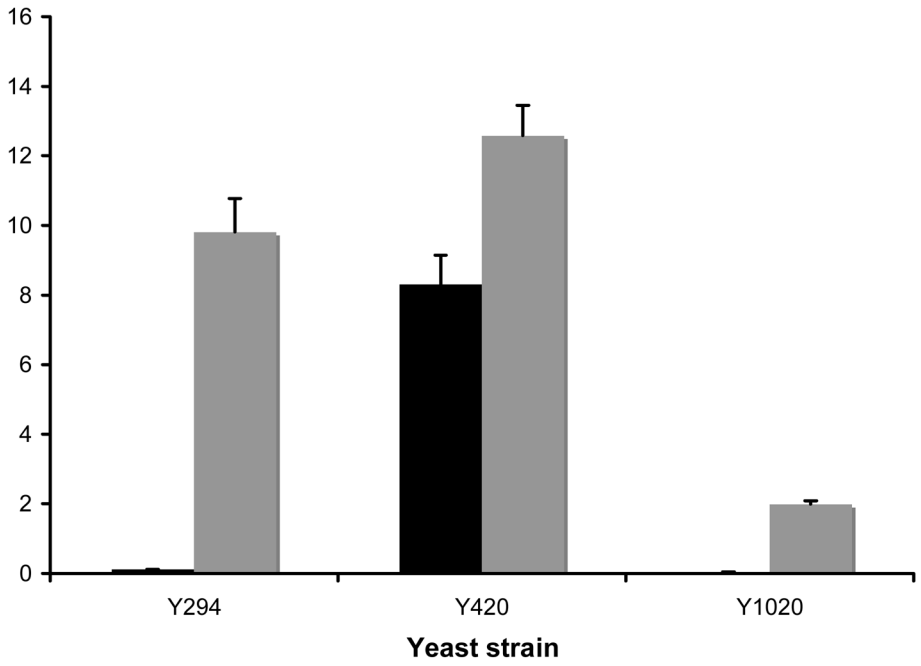

Figure 1. General amino acid permease activity in yeast *RAS2* mutants

Strains were grown in either minimal ammonia (black bars) or minimal proline (grey bars) medium and assayed for ^{14}C-citrulline uptake (2.0mM) as described in the methods. A. S288C-derived strains. B. Σ1278B-derived strains. Gap1p activity is measured as nmoles ^{14}C-citrulline/min^{-1}/OD550^{-1}.Results are the average of 4–10 independent assays, error bars denote the standard error.

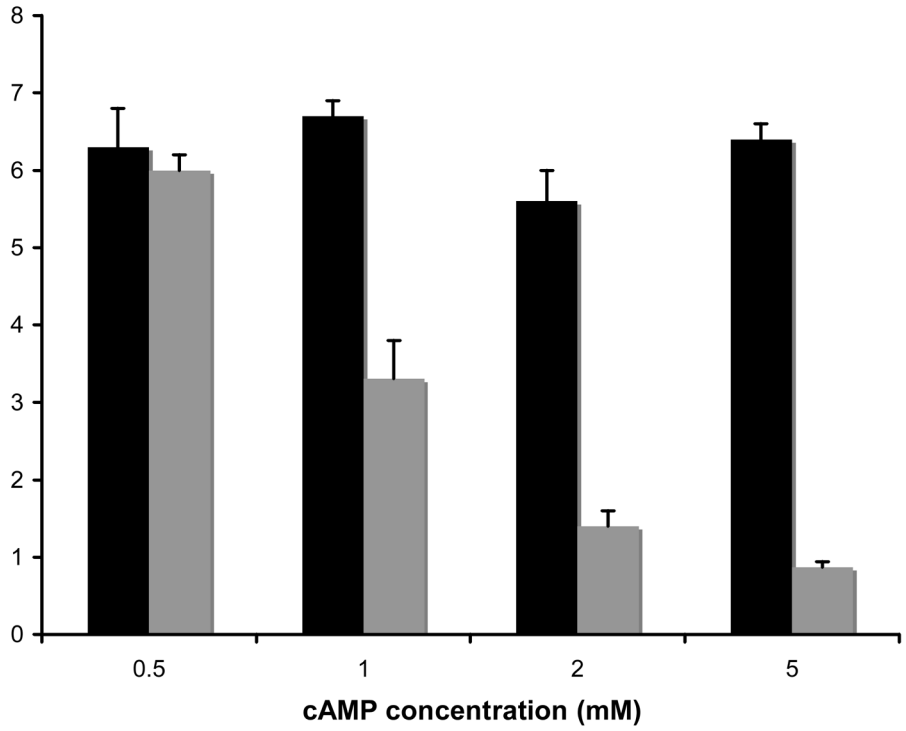

Figure 2. The effect of external cAMP concentration on Gap1p activity in a *cyr1* cAMP-sensitive strain

Strains were grown in minimal ammonia medium containing the cAMP concentration given and assayed as described for Fig. 1. Black bars – HR125 (wild-type), grey bars –TC41-1 (*cyr1* mutant). Results are the average of three independent assays.

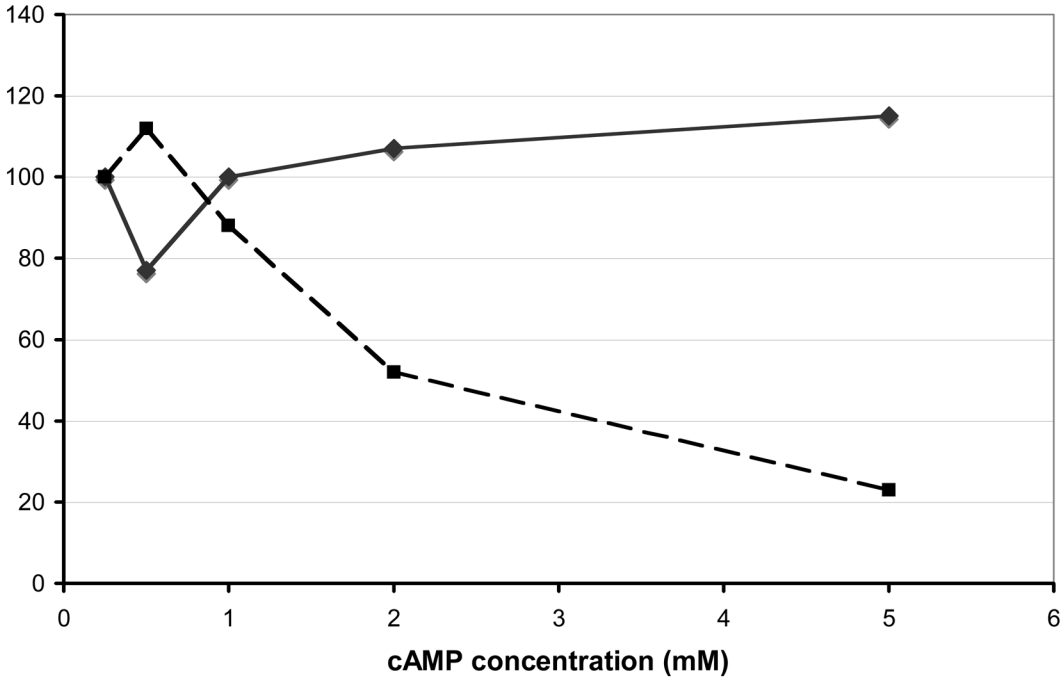

Figure 3. The control of Gap1p activity by cAMP is not through the regulation of transcription

TC41-1 cells containing the expression plasmid pSE (promoter of *GAP1* fused to the *E. coli lacz* gene) was grown in ammonia medium containing the cAMP levels given. β-galactosidase levels were assayed by the method of Miller (1972). Gap1p activity (filled squares) and β-galactosidase levels (filled triangles, dashed line) are given as a percentage of the level obtained in 0.5mM cAMP where 100% Gap1p activity = 6.2 nmoles ^{14}C-citrulline/min^{-1}/OD$_{550}^{-1}$ and 100% β-galactosidase = 750 Units.

Table 1

Yeast Strains

Strain	Genotype	Reference
SP1	*MATa ura3–52 leu2–3,112 his3Δ1 trp1-289 ade8 can1.*	Toda et al, 1985.
T3-24C	*MATα ura3–52 leu2–3,112 his3Δ1 trp1–289 ade8 can1, ras2::URA3*	Toda et al, 1985.
TK161-R2V	*MATa ura3–52 leu2–3,112 his3Δ1 trp1–289, ade8 can1 RAS2^{val19}*	Toda et al, 1985.
Y294	*MATa ura3–52 leu2–3,112 his3Δ1 trp1–289*	Fedor-Chaiken et al, 1990.
Y420	*MATα ura3–52 leu2–3,112 his3Δ1 trp1–289 Ras2::GAL10-RAS2^{val19}**	Fedor-Chaiken et al, 1990.
Y1020	*MATa ura3–52 leu2–3,112 his3Δ1 trp1–289 RAS2^{val19}-URA3*	Fedor-Chaiken et al, 1990.
HR125	*MATa ura3–52 leu2–3,112 his3–532 his4*	Russell et al, 1993.
TC41-1	*MATa ura3–52 leu2–3,112 his3–532 his4 Cyr1::URA3 cam*	Russell et al, 1993.

* Y420 is a *ras2* mutant when grown in glucose-containing media as the *GAL10-RAS2^{val19}* construct is not induced.

Table 2

Growth rates of parental and *RAS2* strains on various amino acids as principal nitrogen source

	Balanced growth rate (h^{-1}) of			
	SP1 strains		Y294 strains	
Nitrogen source	***RAS2***	***RAS2^{Val19}***	***RAS2***	***RAS2^{Val19}***
–[a]	6.5	7.5	6.0	10.5
Ammonia	2.5	5.5	3.25	7.25
Alanine	2.5	NG[b]	NG	NG
Arginine	2.25	3.5	NG	NG
Aspartic acid	2.0	4.5	3.0	NG
Glutamate	3.75	NG	3.25	7.5
Glutamine	3.0	NG	3.25	NG
Proline	3.25	6.0	3.25	7.5
Serine	3.25	NG	NG	NG
Urea	3.5	5.5	3.5	7.0

[a] No additional amino acid added. The amino acids added to supplement the auxotrophic requirements of the cells sustain this growth rate.

[b] NG = no growth above background.

Table 3

The effect of an activated *RAS2* allele or elevated cAMP levels on glutamate, glutamine and leucine transport.

Strain	Amino acid uptake(nmoles ^{14C}-amino acid/min^{-1}/OD_{550})		
	Glutamine	Glutamate	Leucine
SP1	8.41 +/− 0.07	3.63 +/− 0.18	5.30 +/− 0.16
TR161-R2V	9.30 +/− 0.05	3.07 +/− 0.32	4.55 +/−0.21
TC41Δgap 0.5mM cAMP	8.57 +/− 0.43	4.53 +/− 0.21	ND
TC41Δgap 2.0mM cAMP	8.67 +/− 0.34	4.23 +/− 0.30	ND

Cells were grown on minimal ammonia medium and assayed as described in methods. All assays were performed at least three times.

ND – not determined