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Activation of the Ras/Cyclic AMP Pathway in the Yeast *Saccharomyces cerevisiae* Does Not Prevent G_1 Arrest in Response to Nitrogen Starvation

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Cells carrying mutations that activate the Ras/cyclic AMP (Ras/cAMP) pathway fail to accumulate in G_1 as unbudded cells and lose viability in response to nitrogen starvation. This observation has led to the idea that cells carrying this type of mutation are sensitive to nitrogen starvation because they are unable to appropriately arrest in G_1. In this study, we tested predictions made by this model. We found that cells with activating Ras/cAMP pathway mutations do not continue to divide after nitrogen starvation, show a normal decrease in steady state levels of START-specific transcripts, and are not rescued by removal of cAMP during nitrogen starvation. These findings are inconsistent with the idea that activation of the Ras/cAMP pathway prevents growth arrest in cells starved for nitrogen. Our finding that cells with an active Ras/cAMP pathway have dramatically reduced amino acid stores suggests an alternative model. We propose that cells at high cAMP levels are unable to store sufficient nutrients to allow return to the G_1 phase of the cell cycle when they are suddenly deprived of nitrogen. It is this inability to return to G_1, rather than a failure to arrest, which leaves cells at different points in the cell cycle following nitrogen starvation.

In *Saccharomyces cerevisiae*, cyclic AMP (cAMP) plays an important role in transmitting nutrient signals into the cell. Glucose levels in the growth medium are coupled to intracellular cAMP concentrations (10, 12). Furthermore, cAMP has been shown to regulate many different nutrient responses. For example, a number of metabolic processes associated with glucose depletion, such as glycogen storage and oxidative metabolism of ethanol, are inhibited by cAMP (5, 8, 21), while synthesis of ribosomal proteins is enhanced by cAMP (18). Genetic evidence indicates that cAMP production is also important in regulating cellular proliferation in response to nutrient signals. Mutations that block the production of cAMP block progression through a point in late G_1 called START, leaving cells arrested in G_1 in a manner that very closely resembles the cell cycle arrest produced by nutrient limitation (22, 26, 31). More recently, cAMP has been shown to be necessary for the transcription of two G_1 cyclins, encoded by *CLN1* and *CLN2* (16). The products of these genes have been shown to stimulate the $p34^{CDC28}$ protein kinase to promote passage through START. Taken together, these results indicate that cAMP plays an important role in both metabolic and cell cycle responses to nutrients.

While mutations that block cAMP production leave cells permanently arrested in G_1, mutations that activate the Ras/cAMP pathway, such as activating mutations in the *RAS2* gene and *bcy1* mutations that activate the cAMP-dependent protein kinase (cAPK), appear to produce the opposite phenotype. Normal cells arrest in G_1 as unbudded cells in response to nutrient depletion (25), but cells carrying activating mutations in the Ras/cAMP pathway fail to accumulate as G_1-arrested cells when nutrients are withdrawn (6, 17, 30). This is particularly well characterized with nitrogen starvation. Cells carrying activating Ras/cAMP mutations have been shown to rapidly lose viability when shifted to nitrogen starvation medium. This loss of viability is associated with an unusually high percentage of budded cells in the population of nitrogen-starved cells. Thus, an attractive and often-cited interpretation for these results is that the Ras/cAMP pathway is in some way involved in signaling nitrogen starvation and that cells with an activated Ras/cAMP pathway are unable to respond to the loss of nitrogen. These cells then are proposed to fail to arrest in G_1, becoming stranded throughout the cell cycle as they run out of resources (29).

Although this model fits well with other data indicating a role for cAMP in regulating the cell cycle in response to nutrients, it has not been carefully tested. In this paper, we describe experiments designed to determine whether activation of the Ras/cAMP pathway prevents G_1 arrest in response to nitrogen starvation. Our results are inconsistent with the idea that the loss of viability is caused by a failure to arrest growth. Rather, they favor a model in which cells with activating Ras/cAMP mutations are deficient in storing nutrients. These cells then remain budded when suddenly starved for nitrogen because they lack the resources to complete the cell cycle and return to G_1.

MATERIALS AND METHODS

Strains and media. The strains used are listed in Table 1. Cells were grown either in rich medium (YEPD), containing 1% yeast extract, 2% Bacto-peptone, and 2% glucose, or in synthetic medium, containing 6.7 g of yeast nitrogen base (Difco) per liter, 2% glucose, and appropriate factors to support auxotrophic strains. For experiments involving nitrogen limitation, the synthetic medium was made with yeast nitrogen base without amino acids or ammonium sulfate (Difco). When necessary, cells were transferred from one medium to another by centrifugation in a Beckman J6 centrifuge for 5 min at 3,000 rpm.

Preparation of RNA and Northern (RNA) blots. RNA was prepared by the method of Ellwood and Craig (9). The RNA samples (15 μg per lane) were separated by formaldehyde-agarose gel electrophoresis and transferred to a GeneScreen Plus membrane according to the manufacturer's instructions (New England Nuclear). To ensure uniform loading and transfer of RNA, ethidium bromide was added to the samples prior to loading the gel. Following blotting,

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TABLE 1. Yeast strains used in this study

Strain	Description
TW38	*MAT***a** *leu2-3 leu2-112 trp1-1 his3-532 his4*
TC41-1	Isogenic to TW38 except Δ*cyr1*; *MAT***a** *leu2-3 leu2-112 trp1-1 his3-532 his4 cyr1*::*URA3 cam*
GC118	Isogenic to TW38 except *bcy1*; *MAT***a** *leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 bcy1-1*
SP1	*MAT***a** *his3 leu2 ura3 trp1 ade8 can1*
TK161-R2V	*MAT***a** *his3 leu2 ura3 trp1 ade8 can1 RAS2*$^{Val-19}$

ethidium-stained rRNA was visualized on the blots by UV illumination and photographed. Only blots with even rRNA loading and transfer were used. Blots were probed with a 2.5-kb *Hin*dIII fragment from *HO* and a 1-kb *Sac*I-*Xho*I fragment from *CLN2*. Probes were radiolabeled with ^{32}P by the random primer method to a specific activity of 10^9 cpm/μg. Radioactive signals were quantified with a PhosphorImager SI and ImageQuaNT software (Molecular Dynamics, Sunnyvale, Calif.).

Measurement of amino acid pools. Cultures were grown at 30°C in YEPD medium. Exponentially growing cells were harvested at an A_{600} of 0.4, and stationary-phase cells were collected 1 day after reaching maximum density. Amino acid pools were measured as described by Oshumi et al. (24). Briefly, cytoplasmic amino acid pools were released by treatment of the cells with 100 mM $CuCl_2$ and collected. The vacuolar amino acid pools were subsequently extracted by boiling the treated cells in distilled water. The derivatized amino acids were analyzed by high-pressure liquid chromatography (HPLC) at the Biotechnology Analytical and Synthetic Facility, Cornell University. Values for 19 of the 20 physiological amino acids were obtained for both the vacuolar and cytoplasmic samples: tyrosine levels could not be quantitated because tyrosine eluted with the peak of a derivatizing reagent. The arginine, histidine, and lysine vacuolar pools were totaled to give the vacuolar amino acid value.

RESULTS

Cell division after nitrogen starvation. If cAMP prevents cells from arresting at START in response to nitrogen starvation, then we would expect cells with activated Ras/cAMP pathways to continue to divide for a time after transfer to nitrogen starvation medium. To test this, we transferred *bcy1* mutant cells to nitrogen starvation plates, incubated these plates for 24 h, and then used a microscope to determine whether the cells had continued dividing to form microcolonies (Fig. 1). Cells carrying a *bcy1* mutation did not go on to divide any more than the wild-type control cells. With both the mutant and wild-type control strains, most of the colonies consisted of one or two cells.

Cells carrying a *RAS2*$^{Val-19}$ mutation also showed no tendency to continue to divide after transfer to nitrogen starvation medium, although the wild-type control in this strain background tended to continue through two or three rounds of division under these conditions.

We obtained similar results with a strain (TC41-1) in which we can manipulate cAMP levels. This strain has a deletion of the adenylyl cyclase gene, *CYR1*, and cannot produce cAMP but remains viable if exogenous cAMP is supplied in the medium. To test whether cells held at constant cAMP levels are unable to arrest growth in nitrogen starvation medium, we plated TC41-1 cells onto nitrogen starvation plates containing 2 mM cAMP and examined the number of cells in each colony after 24 h as described above. These cells also divided no more than the control wild-type cells, forming mostly colonies of one or two cells.

On the other hand, both the TC41-1 cells held at constant cAMP and the *bcy1* strain lost viability during nitrogen starvation, as expected. After 48 h in nitrogen starvation medium, fewer than 50% of the TC41-1 cells held at a constant cAMP level were able to form colonies when plated onto fresh YEPD medium, and only 10% of the *bcy1* mutants remained viable. In contrast, at least 90% of the wild-type control cells remained able to form colonies following 48 h of nitrogen starvation.

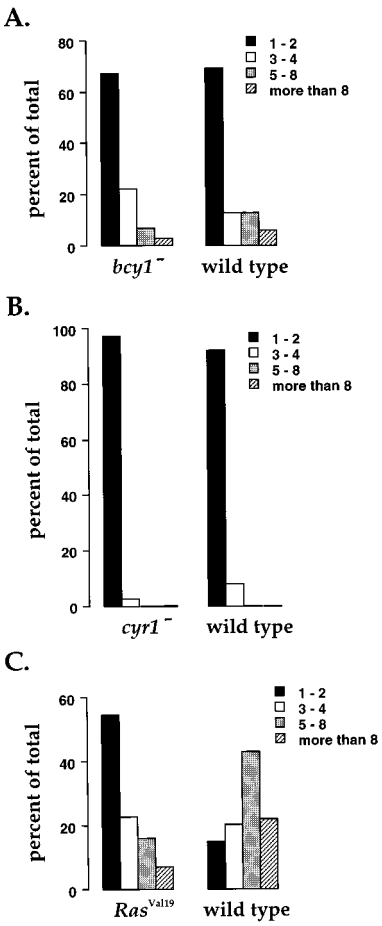

FIG. 1. Cells with activated Ras/cAMP pathways do not continue to divide after nitrogen starvation. Cells were grown in YEPD to mid-log phase (1 OD_{660}) and diluted 1:10 in synthetic medium without a nitrogen source, and 50-μl samples were spread onto plates containing synthetic medium without any nitrogen source. The cells were incubated for 24 h at 30°C, and the plates were examined microscopically to determine how many cells were in each microcolony. For each strain, six groups of 100 microcolonies were counted; results are presented as the average. (A) *bcy1* (GC118) and the otherwise isogenic wild-type (TW38) cells. (B) Wild-type cells (TW38) or otherwise isogenic cells carrying a deletion of *CYR1* (TC41-1). The TC41-1 cells were grown as described above except that 2 mM cAMP was added to the liquid medium and to the nitrogen starvation plates. (C) *RAS2*$^{Val-19}$ (TK161-R2V) and the otherwise isogenic wild-type strain SP1.

Changes in G_1 transcripts in response to nitrogen starvation. In order to further test whether cells carrying activating Ras/cAMP mutations are unable to arrest in G_1, we examined the abundance of START-specific transcripts in these cells immediately following nitrogen starvation. Normal cells arrest growth in G_1 in response to nitrogen starvation, decreasing the levels of these mRNAs. If the mutant cells are continuing to pass from G_1 into S following nitrogen starvation, then we should see greater levels of START-specific transcripts persisting in the mutant populations as they continue to pass START. In this experiment, we transferred log-phase cultures into nitrogen starvation medium and collected samples for RNA preparation at intervals (Fig. 2). As expected (1, 34), in the wild-type control strains (SP1 and TW38), nitrogen starvation caused a marked decrease in the levels of the mRNAs encoded by *HO* and *CLN2*. The levels of these messages also fell by at

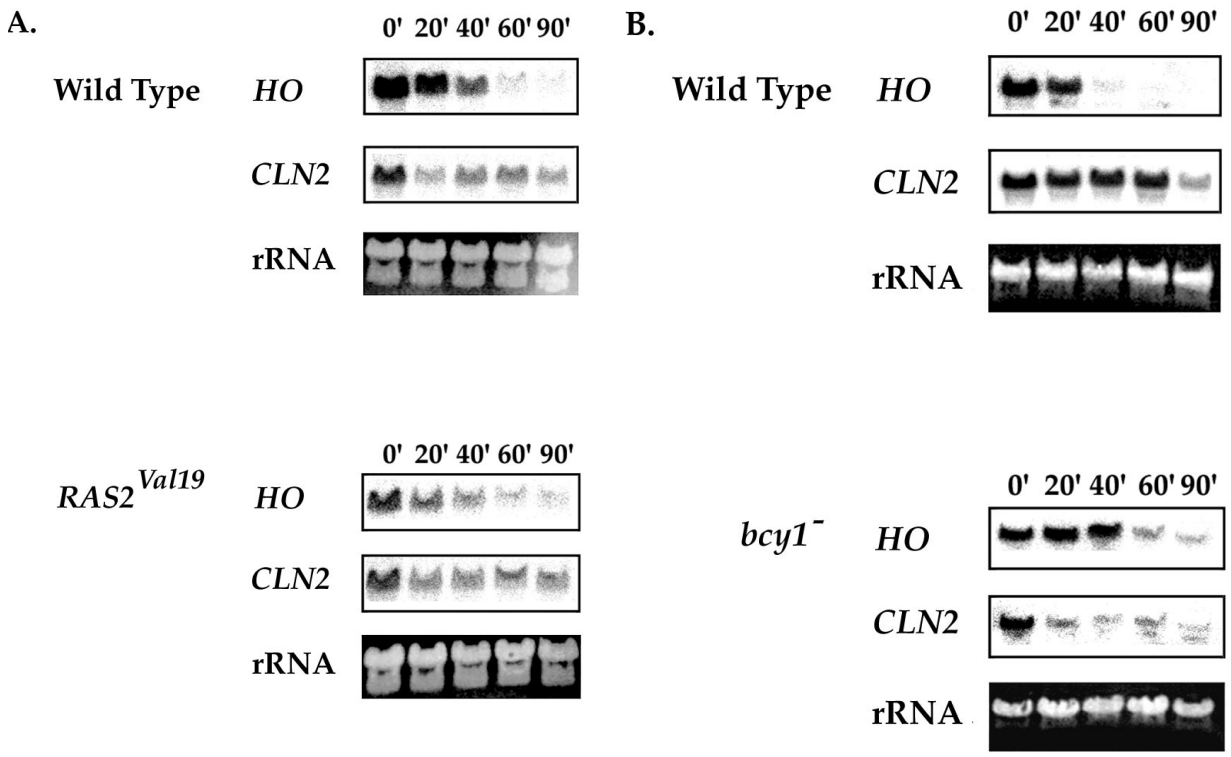

FIG. 2. Cells with activated Ras/cAMP pathways do not express G_1-specific mRNAs after nitrogen starvation. Cells were grown to mid-log phase (1 OD_{660}) in YEPD, spun down, and resuspended in medium lacking a nitrogen source. Samples (10 OD units) were removed at the indicated times for RNA preparation and Northern blotting with either a *CLN2* or *HO* probe. The strains tested include (A) TK161-R2V (*RAS2*$^{Val-19}$) and SP1 (wild-type isogenic to TK161-R2V) and (B) TC41-1 (*cyr1*Δ) and TW38 (wild type isogenic to TC41). The TC41-1 cells were grown as described for the other strains except that 2 mM cAMP was present in the medium at all times.

least as much in either *cyr1* mutant cells held at a constant cAMP level (TC41-1) or cells carrying an activating *RAS2*$^{Val-19}$ mutation (TK161-R2V). Thus, expression of START-specific transcripts was not elevated following nitrogen starvation in the cells with activating Ras/cAMP mutations. Although it is possible that the fall in G_1-specific transcripts in the cells that are unable to turn off the Ras/cAMP pathway is related to a loss in viability rather than to a normal shut-off of START, in either case, our data indicate that the cells are not continuing to pass out of G_1 into S.

Effect of cAMP on viability before and during nitrogen starvation. If cells lose viability during nitrogen starvation because they cannot lower cAMP levels to allow cell cycle arrest, then we should be able to preserve viability if we remove cAMP from the cells when we transfer them to nitrogen starvation medium. To test this, we grew TC41-1 cells at 2 mM cAMP and then shifted the cells to nitrogen starvation liquid medium containing various concentrations of cAMP. The cells were then incubated for 48 h and plated onto fresh YEPD-cAMP medium to measure viability (Fig. 3). Although removing cAMP from *cyr1* mutant cells is known to cause G_1 arrest (20), removing cAMP as the cells were transferred to nitrogen starvation medium did not prevent the loss in viability. Indeed, viability was slightly decreased in the culture that had no cAMP during nitrogen starvation.

These results show that cells carrying activating Ras/cAMP mutations do not continue to pass START following nitrogen starvation and that growth arrest is not sufficient to rescue the cells from the effects of nitrogen starvation. Instead, the results are more consistent with the idea that cells with activating mutations in the Ras/cAMP pathway are in some way unprepared for the stress of nitrogen starvation.

Storage of amino acids in *RAS2*$^{Val-19}$ cells. The experimental result supporting the hypothesis that Ras/cAMP activating mutants are defective in cell cycle arrest is the observation that these mutants are not found uniformly arrested in G_1 after nitrogen starvation, while almost 100% of wild-type cells arrest as unbudded cells under the same conditions. However, another model can explain this observation. In this model, cells carrying activating Ras/cAMP pathway mutations would fail to store nutrients. These cells would then be especially vulnerable

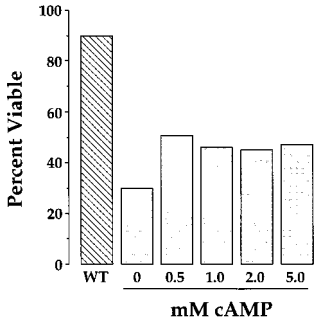

FIG. 3. Removal of cAMP during nitrogen starvation does not rescue Ras/cAMP pathway mutants from viability loss. Cells carrying a deletion in *CYR1* (TC41-1) were grown in YEPD at 2 mM cAMP to mid-log phase (1 OD_{660}) and transferred to nitrogen starvation medium containing the indicated concentration of cAMP. The cells were incubated in the nitrogen starvation medium at 30°C for 48 h, and then samples were plated onto YEPD plates with 1 mM cAMP to allow surviving cells to form colonies. Viability is expressed as the percentage of cells able to form colonies after 24 h. Control wild-type (WT) cells were treated in parallel except that cAMP was not added to the medium for these cells. Results are presented as the average.

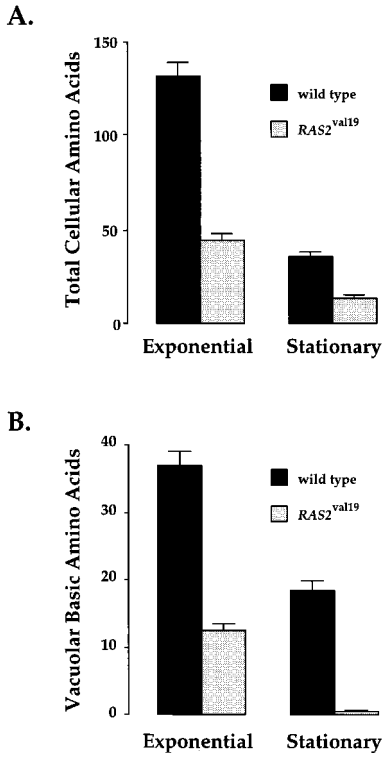

FIG. 4. *RAS2*$^{Val-19}$ cells have reduced amino acid reserves. Wild-type cells (SP1) and cells carrying a *RAS2*$^{Val-19}$ mutation (TK161-R2V) were grown in YEPD medium and harvested at mid-log phase (A_{600} of 0.4) or stationary phase (1 day after reaching the maximum A_{600}). Amino acid pools were measured as described in the text. Values are expressed as the averages of duplicate samples; error bars reflect the range of values. Basic amino acids are arginine, lysine, and histidine. All values are reported as micromoles per gram (wet weight) of cells. (A) Total cellular amino acids; (B) vacuolar basic amino acids.

to nitrogen starvation and might be unable to progress further through the cell cycle when suddenly deprived of nitrogen. This model accounts for the budded cells in the starved population by proposing that rather than failing to arrest at START, some of the cells fail to return to START.

Activation of the Ras/cAMP is known to prevent glycogen storage (11, 13). To determine whether these mutations also inhibit nitrogen storage, we examined amino acid storage levels in cells carrying a *RAS2*$^{Val-19}$ mutation. In this experiment, we separated cytoplasmic from vacuolar amino acids by $CuCl_2$ extraction of the cytoplasmic stores followed by separation of both vacuolar and cytoplasmic amino acids by HPLC. Amino acid stores were measured in both log-phase and stationary-phase cells. In all cases, the levels of stored amino acids were lower in the *RAS2*$^{Val-19}$ mutant strain than in the isogenic wild-type control (Fig. 4A). In particular, storage of the basic amino acids arginine, histidine, and lysine was almost undetectable in the vacuoles of stationary-phase *RAS2*$^{Val-19}$ cells (Fig. 4B). Thus, activation of the Ras/cAMP pathway inhibits storage of amino acids as well as carbohydrates.

DISCUSSION

When wild-type yeast cells deplete the nutrients in their environment, they undergo many well-described metabolic changes that presumably prepare them for a period of harsh conditions and allow them to remain viable over time (33). These cells become thermotolerant, rapidly accumulate glycogen and trehalose, and, if diploid, sporulate. In addition, they arrest proliferative growth and accumulate as unbudded cells. The best-studied signal transduction pathway related to nutrients in *S. cerevisiae* is the Ras/cAMP pathway. It is apparent that the fall in cAMP level that occurs as cells deplete the glucose in their environment (12, 27) triggers many of these processes.

Constitutive activation of cAPK has been widely thought to block arrest in G_1. The observation that mutations which activate cAPK produce cells that fail to accumulate in G_1 upon nitrogen starvation seems at first to confirm this assumption, suggesting a model in which an active cAPK causes cells to ignore signals that would normally cause them to arrest in G_1. As attractive as this model might be, nitrogen does not appear to affect intracellular cAMP levels (4, 15). Additionally, recent work by Wen et al. (32) indicates that G_1 arrest per se is not required for entry into a stable resting state. It is therefore worthwhile to test specific predictions of this model.

Our results do not support a model in which activation of the Ras/cAMP pathway leaves cells unable to arrest in G_1. We find that upon nitrogen starvation, these mutants do not continue to proliferate. Moreover, these mutants show a normal decrease in mRNAs associated with passing from G_1 into the S phase of the cell cycle. In addition, we demonstrate that allowing cells to arrest by removing cAMP at the time of nitrogen starvation does not rescue the cells from loss of viability.

The decreased amino acid reserves in cells carrying a *RAS2*$^{Val-19}$ mutation suggest an alternative model in which cells with an active cAPK fail to maintain adequate nutrient reserves. When cells face an environmental challenge such as nitrogen starvation, their remaining nutrient stores are insufficient to carry them back to G_1, and they become stranded at various points in the cell cycle as a mixed population of budded and unbudded cells.

It is likely that many of the pleiotropic effects of cAMP in *S. cerevisiae* also contribute to the loss of viability following nitrogen starvation. Not only do these cells have low amino acid pools, they also have low storage levels of carbohydrates (13, 21). While the supply of amino acids is reduced in these cells, activated cAPK continues to promote transcription of ribosomal genes through the *RAP1* gene product (18) and also prevents the mitochondrial stringent response, in which transcription of mitochondrial genes is normally inhibited in response to nitrogen starvation (23). Additionally, activated cAPK inhibits expression of genes known to protect cells from stress, including *UBI4*, *CTT1*, and *SSA3* (2, 3, 7, 28). Finally, cAMP is known to inhibit the process(es) by which cells enter a "resting state" in which they become resistant to heat stress and cell wall degradation (4). In light of this combination of effects, it is perhaps not surprising that these cells succumb fairly rapidly.

The decrease in amino acid pools may also explain the finding that cells carrying a *RAS2*$^{Val-19}$ mutation more readily form pseudohyphae in response to nitrogen starvation than wild-type cells (14). It is possible that pseudohyphal growth is triggered by falling amino acid pools. The *RAS2*$^{Val-19}$ mutation would then promote pseudohyphal growth by virtue of its effect on nitrogen stores. This is consistent with the finding that a mutation in *SHR3*, which also promotes pseudohyphal growth, is known to decrease amino acid transport into the cell (19).

Ras/cAMP can be thought of as a modulator of a wide range of processes involved in energy production and growth. The apparent failure of the activating Ras/cAMP mutants to arrest in G_1 after nitrogen starvation fits very nicely into a simplistic view in which cells unable to make cAMP cannot enter the cell cycle, while those that produce too much cAMP are unable to

leave it. As convenient as such a model might be, it appears to be only half right. Although *S. cerevisiae* cells that do not make cAMP are indeed prevented from passing from G_1 into S, we propose that the nitrogen sensitivity phenotype seen with mutants carrying an activated Ras/cAMP pathway can be explained in terms of metabolic effects rather than a defect in control of the cell cycle. In our model, activation of cAPK stimulates cells to pledge all of their resources to metabolic growth; this lack of reserves leaves them vulnerable to stressful conditions.

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