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The glucose-induced polyphosphoinositides turnover in *Saccharomyces cerevisiae* is not dependent on the CDC25-RAS mediated signal transduction pathway

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Recently the polyphosphoinositides (PI) turnover has been related to the control of growth and cell cycle also in *Saccharomyces cerevisiae*, and the *RAS2* and *RAS1* gene products have been shown to be involved in the stimulation of PI turnover in G0/G1 arrested yeast cells. Here we show that addition of glucose to previously glucose-starved cells, stimulates the PI turnover with fast kinetics also in yeast cells that were not arrested in the G0/G1 phase of the cell cycle. In addition PI turnover is equally stimulated in temperature sensitive *cdc25*-1 and *cdc25*-5 strains at restrictive temperature, as well as in *ras1,ras2*-ts strain, suggesting that PI turnover stimulation is not dependent on the *CDC25-RAS* mediated signal transduction pathway.

Polyphosphoinositide turnover; Phospholipid; *Ras* mutant; *Cdc25* mutant; *Saccharomyces cerevisiae*

1. INTRODUCTION

Growth and cell cycle progression in the budding yeast *Saccharomyces cerevisiae* appear to be regulated by the intracellular cAMP level [1]. In fact mutations that decrease the intracellular cAMP level bring about both a cell cycle arrest in the G0/G1 phase and a growth arrest, while mutant strains that show high unregulated cAMP levels fail to arrest the cell cycle during starvation conditions [1]. The cAMP level appears to be regulated throughout a signal transduction pathway, that operates in response to nutrients availability and involves the activity of the *CDC25, RAS1* and *RAS2* gene products [2,3].

However there are some indications that other intracellular second messengers play a role in yeast. For instance polyphosphoinositides (PI) turnover has been related to the control of growth and cell cycle by Kaibuchi et al. that showed that glucose stimulates PI turnover in G0/G1 arested cells [4] and by Uno et al. that showed that mutants unable to accumulate phosphatidylinositol 4,5-bisphosphate (PIP_2) are arrested in G1 as unbudded cells [5]. Besides the *RAS2* and *RAS1* gene products appear to be involved in the stimulation of PI turnover in G0/G1 arrested cells [4].

Since the *CDC25* gene product operates upstream to the RAS proteins [2,3], we studied the PI turnover in *cdc25*-1 and *cdc25*-5 mutants, and we report in this note that, under our experimental conditions, the glucose-induced stimulation of PI turnover does not appear to be dependent upon the CDC25 activity and that also it is not related to the G0/G1 arrest, but it seems to directly respond to glucose addition independently from the position of the cell cycle arrest. Moreover it does not appear to be related to the transient increase of intracellular cAMP.

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Abbreviations: ANS, 8-anilino,2-naphtalen sulphonic acid; Mes, 2-(*N*-morpholino) ethanesulphonic acid; PI, polyphosphoinositides; PIns, phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PIP_2, phosphaditylinositol 4,5-bisphosphate; YNB, yeast nitrogen base

2. MATERIALS AND METHODS

2.1. *Yeast strain, media and growth conditions*

Yeast strains A364A (*MAT*a, *ade1, ade2, ura1, his7, lys2, tyr1, gal1*) 321 (*MAT*a, *ade1, ade2, ura1, his7, lys2, tyr1, gal1, cdc25*-1), OL86 (*MATα, ade2, leu2, trp1, cdc25*-5) NA1 (*MATa/MATα, his3/his3, leu2/leu2, ade2/+, lys2/+, tyr1/+, met6/+, cdc25*-1/+), VNA1-1 (*MATa/MATα, his3/his3::pHIS3-RAS2*^{Val19}, *leu2/leu2, ade2/+, lys2/+, tyr1/+, met6/+, cdc25*-1/+) and TS1-6 (*MATα, ade2, can1*-100, *his3, leu2*-3,112, *lys1, ura3*-52, *ras1::URA3, ras2-ts1::SUP16*) were used in this work. Strains A364A and 321 were obtained from Yeast Genetic Stock Center, strains NA1 and VNA1-1 were constructed in our laboratory [6]. OL86 was obtained from M. Jacquet [7], TS1-6 was obtained from O. Fasano [8]. Strain X4004[pIND25-2] is a transformant of X4004-3A (*MATα, lys5, met2, ura3, trp1*) bearing a multicopy plasmid pIND25-2 that contains the whole ORF of *CDC25* gene cloned under the control of an inducible *GAL* promoter [9,10].

Cells were grown in minimal media, 0.67% Yeast Nitrogen Base (Difco), supplemented with 50 mg/l of each required nutrient and 2% glucose (YNB-glucose) or galactose (YNB-galactose). Cultures were

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grown in conical flasks, shaken in a water bath at 24°C. Growth was monitored by counting the number of cells per ml with a Coulter Counter [6]. The fraction of budded cells was determined by microscopic counting of at least 400 cells, fixed in 4% formalin and mildly sonicated [6].

2.2. *Labelling extraction and separation of phospholipids*

Yeast cell exponentially growing in YNB-glucose containing 50 μM [^{3}H]inositol (Amersham) (1 μCi/ml), were harvested, washed with sterile water and resuspended in 0.1 M 2-(*N*-morpholino)ethanesulphonic acid (Mes)/Tris, pH 6.5 (Mes buffer) at a density of about 10^8 cells/ml. Cells were incubated for 1 h and then 60 μCi/ml of carrier free [^{32}P]orthophosphate (Amersham) were added. After an additional 1 h incubation yeast cells were stimulated by addition of 25 mM glucose (final concentration). This incubation in Mes buffer was done either at 24°C and at 36°C for *ts* strains.

Incubations were stopped by addition of cold trichloroacetic acid (10% final concentration) and phospholipids were extracted according to Roldan and Harrison [11]. Phospholipids were separated on TLC plates of silica gel 60, 0.25 mm thick (Merck) pretreated with 1% potassium ossalate, activated at 110°C for 15 min, and developed with chloroform/methanol/acetic acid/acetone/water (43:13:12:15:8 v/v). The phospholipids were evidentiated by spraying with 1 mM 8-anilino,2-naphtalen sulphonic acid (ANS) in water. The fluorescent spots of phospholipids were identified with the aid of appropriate standards, and the plates were autoradiographed. Radioactive spots were scraped and counted by liquid scintillation, using a two-channel Packard-Prias beta-counter settled for ^{3}H and ^{32}P determination. The data were corrected for efficiency, spilling of 5% ^{32}P counts in the ^{3}H channel and background subtraction.

3. RESULTS AND DISCUSSION

3.1. *Glucose stimulation of PI turnover in yeast*

Kaibuchi et al. [4] have shown that when yeast cells previously arrested in G0/G1 phase by carbon source starvation are stimulated by addition of glucose the PI turnover increases. However the response appears to be rather slow, the maximum of phosphate incorporation being observed after 2 h of stimulation.

The increased PI turnover may be related either to the cell cycle arrest, or to a metabolic response to the glucose addition. To discriminate between these two possibilities we performed similar experiments but in yeast cells that were not previously arrested in the G0/G1 phase.

Yeast cells exponentially growing in minimal glucose media containing [^{3}H]inositol were rapidly harvested by filtration and resuspended in glucose-free buffer for 2 h in the presence of [^{32}P]orthophosphate. During this short period cells are deprived of glucose, but they fail to arrest in G1/G0 unbudded phase of the cell cycle and probably they remain freezed in the various phases of the cell cycle (Fig. 1). At the end of this period one half of the culture was stimulated by addition of 25 mM glucose, while the other half was used as the untreated control. At different times the cellular phospholipids were extracted, separated and the ^{3}H- and ^{32}P-radioactivity present in phosphoinositides (phosphatidylinositol (PIns), phosphatidylinositol monophosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2)) was determined. As shown in Fig. 2 the phosphoinositides,

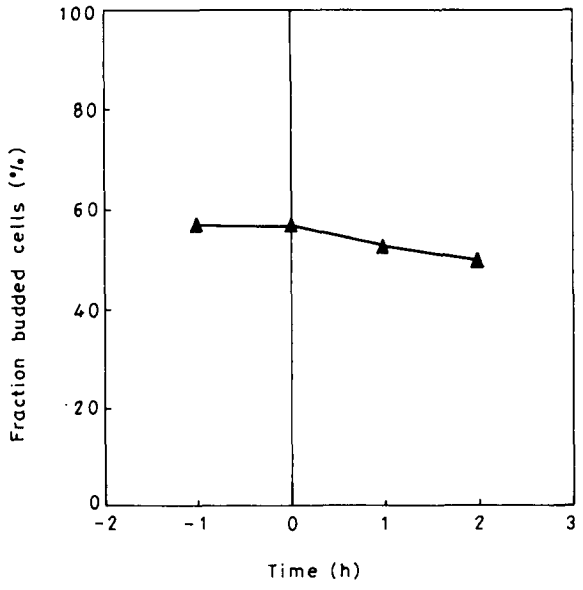

Fig. 1. Fraction of budded cells during the glucose starvation period. Yeast cells of strain A364A exponentially growing in YNB-glucose medium, were collected by filtration and resuspended in Mes/Tris buffer at time zero.

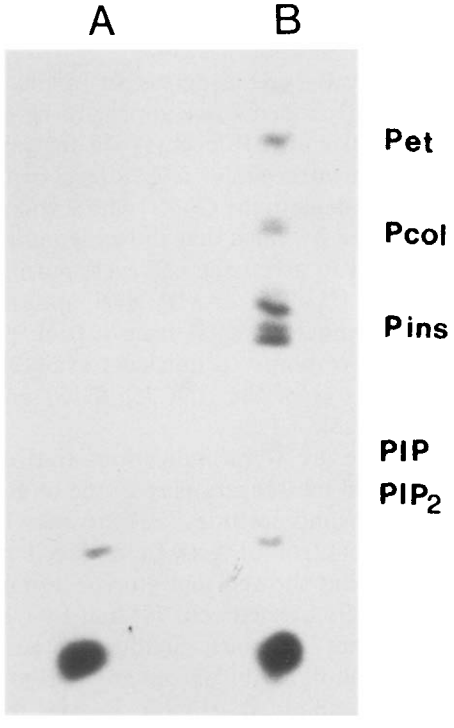

Fig. 2. Autoradiography of silica-gel TLC plates of labelled phospholipids extracted from unstimulated cells (A) and from cells stimulated for 15 min with 25 mM glucose (B). Yeast cells of A364A strain were labelled at 24°C as indicated in section 2 for 1 h with [^{32}P]orthophosphate in Mes/Tris buffer. After glucose stimulation (15 min) phospholipids were extracted and separated on silica-gel TLC. Appropriate standards were added and revealed after fluorescent staining with ANS. PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol phosphate; PIns, phosphatidylinositol; Pcol, phosphatidylcholine; Pet phosphatidylethanolamine. The 3 closely migrating PIns radioactive bands may reflect a different lipid composition.

in particular phosphatidylinositol (PIns), appear to be preferentially phosphorylated, in comparison with the other more abundant cellular phospholipids (phosphatidylcholine, phosphatidylethanolamine etc.), indicating a strong stimulation of their turnover rate. In Fig. 3 is reported the stimulation of turnover, calculated as ratio between the values of $^{32}P/^{3}H$ for stimulated/unstimulated cells. The response was very fast and a steady state situation was reached after 15–20 min.

As reported previously also by Kaibuchi et al. [4] PIns is the more responding molecular species. Since the yeast cells were not specifically arrested in G0 or G1 this stimulation of PI turnover does not appear to be dependent upon a cell cycle response, but it is related only to a specific metabolic signal elicited by glucose. Moreover the response we have observed was much faster than that observed by Kaibuchi et al. and it resembles the increase of intracellular cAMP observed in the same experimental conditions [12].

3.2. *Effects of* RAS2^{Val19} *and* cdc25-*ts mutations*

It has been recently shown that the increase of cAMP elicited by glucose addition to yeast cells is mediated by a signal transduction pathway and that the products of

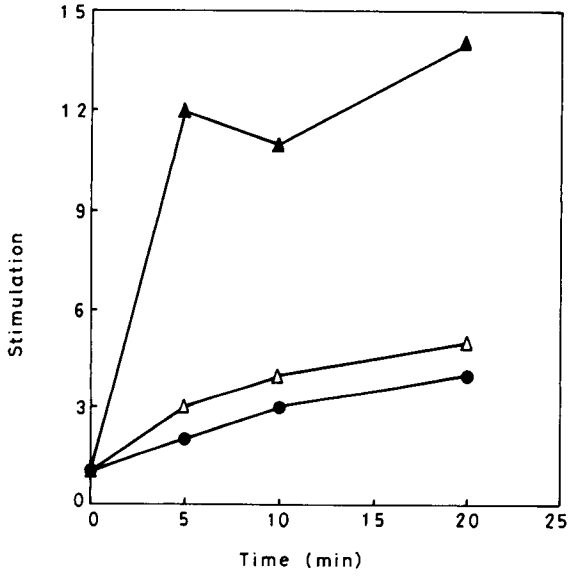

Fig. 3. Kinetics of PI turnover stimulation after glucose addition to A364A yeast cells. Yeast cells were labelled with [^{3}H]inositol during growth in YNB-glucose medium. Exponentially growing cells were rapidly harvested and resuspended in Mes/Tris buffer for 1 h. Then cells were labelled with [^{32}P]orthophosphate and incubated in Mes buffer for one additional hour. Cells were stimulated by addition of 25 mM glucose (final concentration), and at different times after glucose addition, phospholipids were extracted, separated on TLC plates, and the ^{3}H- and ^{32}P-radioactivity present in PIns, PIP and PIP_2 was measured. The stimulation of PI turnover was measured as ratio between the $^{32}P/^{3}H$ value of stimulated cells and $^{32}P/^{3}H$ value of unstimulated cells. (▲) PIns; (△) PIP; (•) PIP_2. The experiments were repeated 3 times and the difference among them was less than 15%.

CDC25, RAS1 and *RAS2* genes are elements of this pathway [13].

Thus we measured the stimulation of PI turnover in *ras* mutants and in *cdc25* mutants. The results are shown in Table I and it can be observed that mutants bearing temperature-sensitive mutations that inactivate the pathway, like *cdc25*-1, *cdc25*-5 and *ras1,ras2*-ts, continue to respond to glucose addition at restrictive temperature with a stimulation of PI turnover. A decreased response was observed however for TS1-6 strain (*ras1, ras2*-ts) at 24°C, but since we have not tested an isogenic *RAS1, ras2*-ts strain, it is not possible to relate this decrement to the *RAS1* gene disruption. On the contrary a mutant bearing an activated *RAS2*^{Val19} allele [4] showed an increased response to glucose addition, in fact the stimulation is about threefold as compared with an isogenic wild-type. These latter results are in agreement with the data of Kaibuchi et al. [4], and suggest that *RAS2* may be involved in the stimulation of PI turnover.

On the other hand the data obtained on temperature-sensitive mutants suggest that the *CDC25* gene product that is postulated to be a mediator of glucose response for adenylate cyclase activation [2,3,7], is not involved in glucose-mediated PI stimulation, and also that the stimulus appears to be RAS-independent, as indicated by the data observed with *ras1,ras2*-ts strain. The two *cdc25* alleles used show a difference for cAMP metabolism at restrictive temperature. In fact while the *cdc25*-1 mutant still responds to glucose addition with an increase of intracellular cAMP ([14], Thevelein, personal communication), the *cdc25*-5 mutants do not accumulate cAMP [7], in addition the level of intracellular cAMP remains high in *cdc25*-1 [15], while it

Table I

Stimulation of PI turnover in different yeast mutants

Strain	Relevant genotype	Stimulation of PI[a] turnover	
		24°C	36°C
A364A	wild-type	12 ± 2	11 ± 4
321	*cdc25*-1	14 ± 2	9 ± 3
OL86	*cdc25*-5	10 ± 3	12 ± 2
NA1	wild-type	10 ± 2	nd
VNA1-1	*RAS2*^{Val19}	32 ± 3	nd
TS1-6	*ras1,ras2*-ts	5 ± 1	9 ± 2
X4004[pIND25-2][b]		9 ± 1	nd

Yeast cells were labelled with [^{3}H]inositol during growth in YNB-glucose medium. Exponentially growing cells were rapidly harvested and resuspended in Mes/Tris buffer for 1 h. Then cells were labelled with [^{32}P]orthophosphate and incubated in Mes/Tris for one additional hour at the indicated temperature (24° or 36°C). Cells were stimulated by addition of 25 mM glucose (final concentration), and after 20 min, phospholipids were extracted and separated on TLC plates. The PIns spot was identified by the use of appropriate standard, scraped and counted by liquid scintillation. (a) Calculated as ($^{32}P/^{3}H$) of PIns stimulated/ ($^{32}P/^{3}H$) of PIns unstimulated after 20 min from glucose addition (mean ± SD of at least 3 independent experiments). (b) This strain was grown in YNB-galactose to induce the overexpression of *CDC25* gene that is cloned under the control of an inducible *GAL* promoter [10].

rapidly drops in *cdc25*-5 [7]. Since the stimulation of PI turnover at restrictive temperature is similar in strains bearing the two alleles, we can conclude that this stimulation is clearly not dependent on the transient increase of cAMP that occurs in wild type strains. The overexpression of CDC25 product does not markedly stimulate the PI turnover as well as it does not change significatively the intracellular cAMP level ([12], Frascotti et al., manuscript in preparation). Since G-proteins, different from RAS proteins, are present in yeast [16,17], it can be tentatively suggested that another G-protein may be responsible for the PI turnover in yeast, while RAS1 and RAS2 proteins work normally only on the adenylate cyclase pathway. An 'activating' *RAS* mutation, like $RAS2^{Val19}$ which may alter the target specificity of the protein, could originate the observed hyperstimulatory effect.

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