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Role of guanine nucleotides in the regulation of the Ras/cAMP pathway in *Saccharomyces cerevisiae*

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Abstract

The *CDC25* gene product is a guanine nucleotide exchange factor for Ras proteins in yeast. Recently it has been suggested that the intracellular levels of guanine nucleotides may influence the exchange reaction. To test this hypothesis we measured the levels of nucleotides in yeast cells under different growth conditions and the relative amount of Ras2-GTP. The intracellular GTP/GDP ratio was found to be very sensitive to growth conditions: the ratio is high, close to that of ATP/ADP during exponential growth, but it decreases rapidly before the beginning of stationary phase, and it drops further under starvation conditions. The addition of glucose to glucose-starved cells causes a fast increase of the GTP/GDP ratio. The relative amount of Ras2-GTP changes in a parallel way suggesting that there is a correlation with the cytosolic GTP/GDP ratio. In addition 'in vitro' mixed-nucleotide exchange experiments done on purified Ras2 protein demonstrated that the GTP and GDP concentrations influence the extent of Ras2-GTP loading giving further support to their possible regulatory role.

Keywords: Nutrient; Growth regulation; Guanine nucleotide exchange factor; CDC25; Ras; *Saccharomyces cerevisiae*

1. Introduction

In budding yeast the activity of adenylyl cyclase is regulated by two Ras proteins (Ras1 and Ras2) which activation state is positively regulated by guanine nucleotide exchange factor (GEF) and negatively by GAPs (GTPase activating proteins) [1]. The *CDC25* gene product constitutes the principal GEF of *Saccharomyces cerevisiae*, and genetic data suggest that it is one of the upstream elements of the signal transduction pathway that regulates adenylyl cyclase [2]. The *CDC25* gene product is a 180 kDa protein (p180Cdc25) membrane bound [3] that contains in its C-terminal region (aa 1256–1541) the domain responsible for guanine nucleotide exchange activity [4].

For the present is not clear which mechanism regulates the activity of the Cdc25/Ras/cAMP pathway in response to nutrient(s). For the glucose response it is required the presence of low-affinity glucose carriers, of hexokinase and of the *GGS1* gene product [5]. However a basal level of this pathway is required for growth independently from the carbon source, suggesting that this pathway is not limited to the glucose signaling but it generally 'senses' the presence of the carbon source(s) in a *CDC25* dependent man-

Abbreviations: GEF, guanine nucleotide exchange factor; YNB, yeast nitrogen base; GST, glutathione-*S*-transferase; RBD, Ras-binding domain

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ner [5,6]. In addition the Cdc25/Ras/cAMP pathway operates also during the normal cell cycle progression with a modulation of the critical cell sizes required both for budding and for mitosis [7]. Since p180Cdc25 is a large protein, it may contain one or more regulatory domains in the N-terminal region (aa 1–1000). Indeed this region contains a cyclin destruction box [8] and an SH3 domain that has been reported to interact with adenylyl cyclase [9]. Moreover either the deletion of the first 900 aa or the overexpression of N-terminal region did not produce phenotypes compatible with a postulated regulatory function, as observed, for instance, with the mammalian GEFs (Sos or Ras-GRF) [10,11]. The Cdc25 protein is continuously synthesized during growth and it is a labile protein [8]. Therefore a possible regulatory mechanism could be related to the amount of p180 Cdc25 in the cell. However it is clear that for a normal growth a GEF activity is required, but the amount or the type of GEF is not relevant, since heterologous GEFs work as well as Cdc25 protein [12,13].

Recently it has been shown that the catalytic domain of Ras-exchange factors operates generating a nucleotide-free Ras/exchange factor complex [14] that can be dissociated either by GTP or by GDP. Therefore the exchange reaction does not unequivocally promote the GDP to GTP exchange, but is a reversible cycle in which the relative concentration of GDP and GTP may be relevant for the process [15,16]. Starting from this observation it is clearly reasonable to put forward an alternative regulatory model, firstly hypothesized by Haney and Broach [15] which suggests a possible role played by the GTP and GDP concentrations inside the cytoplasm. According to this model the exchange factor would operate in a constitutive way, trying to put in equilibrium the Ras-GTP and Ras-GDP forms with the effective guanine nucleotides levels. In turn the GTP/GDP ratio would be greatly influenced by nutritional conditions, so to generate a signal, in the form of Ras-GTP that is related to the nutritional (and energetic) state of the cell.

Here we show that the intracellular level of GTP and GDP is clearly related to the availability of nutrients (carbon source) and in starvation condition the intracellular level of GDP is the same (or even higher) than GTP. In addition we give evidence that a correlation exists between the cytosolic GTP/GDP ratio and the relative amount of Ras2-GTP. In addition 'in vitro' exchange experiments demonstrated that the relative concentration of GTP and GDP might be relevant for the nucleotide exchange reaction.

2. Materials and methods

2.1. Strains, plasmids, media, and yeast methods

The *S. cerevisiae* strains X4004–3A (*MAT*a, *lys5, met2, ura3, trp1*), KP-2 (*MAT*a, *leu2, his3, trp1, ade8, ura3, ras2::URA3*) (obtained by J. Thevelein, University of Leuven, Leuven, Belgium), W303-1A (*MAT*a, *leu2-3, 112 ura3-1, trp1-92, his3-11,15 ade2-1 can1-100 GAL SUC mal*) were used. Strain W303-1A was transformed with the plasmid YEp-*RAS2* [17]. Yeast cells were grown in batch at 30°C in minimal medium (yeast nitrogen base (YNB)-glucose) containing 0.67%(w/v) YNB without amino acids, supplemented with 50 mg/l of each required nutrient and 2% glucose. Solid media contained 2% (w/v) agar. Growth was monitored by counting the cell number/ml with a Coulter Counter ZBI (Coulter Electronics) after sonication and appropriate dilution with Isoton (Coulter Electronics). The fraction of budded cells (budding index, %) was determined by microscopic counting of a yeast cell suspension, previously sonicated and fixed in 10% formalin [6]. At least 400 cells were counted for each determination.

Glucose-starved cells were obtained by collecting cells growing in YNB containing 0.3% glucose in late exponential phase (approximately 10^7 cell/ml) by filtration and subsequent resuspension in MES buffer (25 mM *N*-morpholino-ethanesulfonic acid/KOH, pH 6) for at least 20 min. *Escherichia coli* strain SCS1 and the pGEX2T-RAS2 and pGEX2T-CDC25-509 plasmids were obtained by A. Parmeggiani [4].

2.2. Nucleotides extraction and determination

Yeast cells (approximately 10^8 cells) were rapidly collected by filtration on nitrocellulose filters (Sartorius, 5 cm diameter, 0.45 μ). The filters with the collected cells were immediately put in small plastic

dishes containing 2 ml of ice-cold 1 M formic acid, previously saturated with *n*-butanol. After 20–30 min the acid suspension was centrifuged, and the clear supernatant was collected, lyophilized and stored at −80°C. Just before the analysis the lyophilized pellet was resuspended in pure MilliQ water and nucleotides were separated and quantitated with HPLC (Waters W600) using a Partisil10-SAX column (Whatman) as described previously [18,19]. The instrument was calibrated with pure standard nucleotides (Sigma) ADP, ATP, GDP, GTP, CDP, CTP, UDP and UTP. Quantitative determinations were done using W486 spectrophotometer detector and Millennium 4.1 software (Waters Inst.).

2.3. Assay for Ras2-GTP 'in vivo'

Activated Ras2 (Ras2-GTP) was detected essentially as described by Taylor and Shalloway [20] using an assay that exploits the very high affinity of Ras-GTP for the Ras-binding domain of Raf-1 (RBD). Cells (3×10^8) were harvested either by centrifugation or by fast filtration on nitrocellulose filters and, after addition of ice-cold lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% Na deoxycholate, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM Na vanadate, 10 μg/ml leupeptin and 10 μg/ml aprotinin), the cells were disrupted with glass beads in a Fastprep instrument (Savant). Cleared supernatant (containing 200–400 μg proteins) was incubated with glutathione-*S*-transferase (GST)–RBD fusion protein pre-bound to glutathione–Sepharose for 1 h at 4°C. The GST–RBD fusion protein was prepared using the expression vector pGEX-RBD, which encodes aa 1–149 of Raf-1 fused to GST. This plasmid was kindly provided by A. Wittinghofer (Max-Planck Institute, Dortmund, Germany). The expression of GST–RBD fusion protein in *E. coli* was induced with 0.1 mM IPTG for 4–5 h at 30°C and the fusion protein was purified on glutathione–Sepharose beads. The beads were washed with PBS containing 1 mM EDTA and subsequently with PBS buffer (PBS 1×, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 0.5 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 0.1 mM Na-vanadate). Bound proteins were eluted with 2× SDS-sample buffer (100 mM Tris–HCl pH 6.8, 2% β mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), separated by SDS-PAGE, blotted to nitrocellulose, immunodecorated with anti-Ras2 polyclonal antibodies (Santa Cruz Biotechnology) and revealed with ECL Western blotting analysis system (Amersham Pharmacia Biotech). Total Ras2 protein was detected on cleared lysate (3–5 μg of total protein) by Western blotting using the same anti-Ras2 antibodies. The total protein concentration was estimated by DC protein assay (Bio-Rad) following the manufacturer's instructions. The bands were quantitated by densitometric analysis (NIH-Image software) and the fraction of Ras2-GTP was estimated taking in account the amounts of proteins loaded.

2.4. Purification of Ras2 and Cdc25-509 proteins

The Ras2 and Cdc25-509aa protein were produced as GST fusion from *E. coli* cultures and purified by affinity chromatography on glutathione–Sepharose resin [4]. After purification the Cdc25-509aa protein was stored at −20°C in 25 mM Tris (pH 7,5), 25 mM NaCl, glycerol 50%; the Ras2 fusion protein was stored at −20°C in 25 mM Tris (pH 7,5), 25 mM NaCl, 1 μM GDP, 1 mM $MgCl_2$, glycerol 50%.

2.5. 'In vitro' nucleotide exchange assay

The activity of Cdc25-509aa exchange factor was measured by the nitrocellulose-binding assay [4,14]. Ras2 protein (0.5 μM) and [^{3}H]GTP (1 μM, specific activity 1000 Ci/mol) were incubated in buffer A (50 mM Tris pH 7.5, 1 mM $MgCl_2$, 100 mM NH_4Cl, BSA 0.5 mg/ml) at 30°C together with Cdc25-509aa (20 nM) or EDTA (3 mM). At different times aliquots were withdrawn and filtered on nitrocellulose filters. Filter-bound radioactivity was measured with a liquid scintillation counter (Tri-Carb 2100TR-Packard).

2.6. 'In vitro' loading of GDP and GTP on Ras2

Ras2 protein (0.5 μM) and Cdc25-509 protein (20 nM) were incubated in buffer A at 30°C with equimolar concentrations of [^{3}H]GDP and [γ-^{32}P]GTP (1 μM, 10 μM, 100 μM final concentration). Specific activities were approximately 800 Ci/mol for GTP and 2000 Ci/mol for GDP. For each experiment

the exact specific activity was determined by counting a sample of nucleotide mother solutions. After 2, 5 and 10 min, aliquots were filtered on nitrocellulose filters and ^{3}H and ^{32}P radioactivity was measured with a liquid scintillation counter. The ^{3}H and ^{32}P cpm were corrected for efficiency and converted in GDP/GTP ratio taking into account the specific activity used in each experiment.

2.7. Determination of GTPase and nucleotidase activities

[α-^{32}P]GTP (10 μM) was incubated in buffer A with Cdc25-509aa (20 nM) or Cdc25-509aa and Ras2 (0.5 μM) in buffer A at 30°C. At time 0 and after 10 min, 20 μl were withdrawn and mixed with an equal volume of pre-chilled trichloroacetic acid (10%). Samples were separated by TLC on F-PEI cellulose [21]. GTP and GDP spots were evidenced by autoradiography.

3. Results

3.1. Nucleotide levels during batch growth

The levels of nucleotides were initially determined during mid-exponential growth in glucose minimal medium (cell density 6.4×10^{6} per ml). In this condition we found approximately 12 nmol of ATP in 3.2×10^{8} cells that, considering the internal water, corresponds to a concentration of about 1 mM. The GTP peak was 1/5 of ATP (that is about 200 μM) and the ratio ATP/ADP and GTP/GDP around 4, in good agreement with previously reported data [18,19]. When the same determination was done in late exponential phase (2.4×10^{7} cells/ml), while the ATP/ADP ratio was slightly reduced (3.5), the GTP/GDP ratio was markedly reduced being between 1.5 and 2. By comparison other nucleotides were practically unaffected, for example the CTP/CDP ratio was around 7 in exponential growth and 6 in early stationary phase (Table 1). It is relevant to underline that the nucleotides ratios ATP/ADP and GTP/GDP were extremely sensitive to carbon source deprivation, in fact a simple fast washing of the cells with ice-cold water reduced the ATP/ADP and GTP/GDP ratio to 1, or less (Table 1).

The nucleotide triphosphate/diphosphate ratio also changes continuously during a batch growth as shown in Fig. 1. The ratio increases rapidly at the beginning of exponential growth, it remains high during mid-exponential growth then decreases in the late growth phase. The GTP/GDP ratio was again the most sensitive and precedes by several hours the decrease of the ATP/ADP ratio. Then a

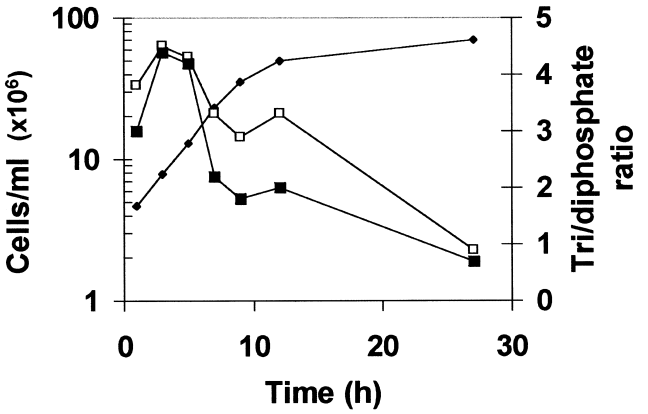

Fig. 1. Nucleotides triphosphate/diphosphate ratio during a batch growth. X4004 cells were grown in YNB-glucose minimal medium at 30°C. Approximately 10^{5} cells/ml were inoculated overnight in 1 l conical flasks containing 250 ml of medium. Growth was monitored by counting the cell number/ml and appropriate aliquots, containing about 10^{8} cells, were collected and processed for nucleotides extraction and analysis. (♦) Cell number/ml of culture; (□) ATP/ADP ratio; (■) GTP/GDP ratio.

Table 1
Ratio of nucleotide triphosphate/diphosphate in yeast cells

Cell density	6.4×10^{6}/ml	2.4×10^{7}/ml	Washed
Budding index (%)	72	44	n.d.
ATP/ADP	4.4 ± 0.2	3.2 ± 0.2	1.0 ± 0.1
GTP/GDP	3.9 ± 0.2	1.7 ± 0.2	0.9 ± 0.1
CTP/CDP	7.3 ± 0.4	6.5 ± 0.4	2.0 ± 0.3
UTP/UDP	9.5 ± 0.5	9.8 ± 0.8	1.9 ± 0.3

Nucleotides were extracted from X4004-3A yeast cells growing in YNB-glucose medium as described in Section 2 and separated by HPLC according to Freese et al. [19]. Extraction and assay were done in triplicate, and mean ± standard deviation is reported. A cell density of 6.4×10^{6}/ml corresponds to a mid-exponential growth, while a density of 2.4×10^{7}/ml corresponds to a late exponential growth.

'Washed' cells represent samples of exponentially growing cells that after the collection on nitrocellulose filter were rapidly washed with ice-cold distilled water, before the incubation in formic acid. The washing time was approximately 10 s.

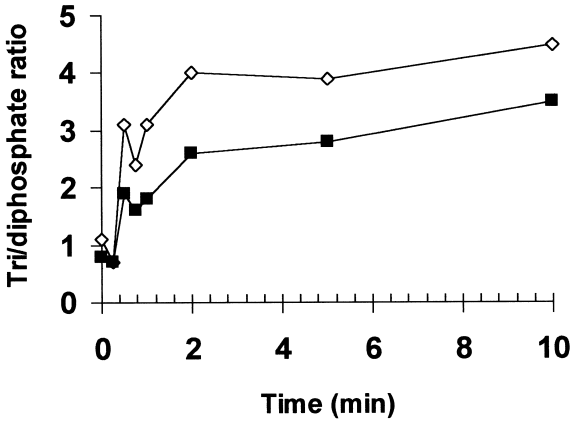

Fig. 2. Nucleotides triphosphate/diphosphate ratio after glucose addition to glucose-starved cells. X4004 cells were grown until late exponential phase in YNB containing 0.3% glucose. Cells were collected by filtration and resuspended in MES buffer (10^8 cells/ml) for 20 min, at 30°C. At time zero glucose (final concentration 100 mM) was added and appropriate samples were taken and processed for nucleotides extraction and analysis. (◇) ATP/ADP ratio; (■) GTP/GDP ratio.

slow decrease continues during the limiting growth condition that led to stationary phase.

Addition of glucose to glucose-starved cell generates fast signaling responses, like the fast cAMP increase and stimulation of phospholipids turnover [22,23]. In this condition the nucleotide triphosphate/diphosphate ratio responds very fast to glucose addition (Fig. 2). In glucose-starved cells the ATP/ADP and GTP/GDP ratios were very low (less that one). A fast increase was observed after 30 s, while ratios comparable with that observed during exponential growth were reached within 5 min.

Therefore we can conclude that both ATP/ADP and more interestingly GTP/GDP ratios are largely influenced by nutritional condition and therefore they could take part in a signaling mechanism relating carbon sources availability with growth and cell cycle progression.

3.2. *Loading equilibrium of GDP and GTP on Ras2 'in vitro'*

To mimic an in vivo situation, in which the nucleotide exchange of Ras protein occurs in the presence of a relative high amount of GDP (as occurs in late stationary phase or in sugar starvation), we performed 'in vitro' exchange experiments.

We measured the amount of Ras2-GDP and Ras2-GTP generated by exchange catalyzed by Cdc25 protein in the presence of equimolar amounts of labeled GDP and GTP. In the presence of only GTP all the Ras2 protein was converted in Ras2-GTP within 10 min in our experimental conditions (Ras2 100 nM, Cdc25 20 nM, [^{3}H]GTP 1 μM) (not shown).

The kinetics was similar under condition of mixed nucleotides, however a significant amount of Ras2 resulted loaded with [^{3}H]GDP (an example is shown in Fig. 3). The relative amount of Ras2-GDP was influenced by the nucleotide concentration and with 0.1 mM GDP and GTP 50% of Ras2 protein resulted loaded with [^{3}H]GDP. Under these conditions the ratio of Ras2-GTP/Ras2-GDP resulting from catalyzed exchange equals the free GTP/GDP ratio, while at low concentrations the GTP loading is favored (Fig. 4), in agreement with the lower K_m reported for GTP exchange in comparison with GDP reaction [15].

Interestingly the spontaneous exchange induced by EDTA favors, at all the tested concentrations, the loading with GTP (the Ras2-GTP/Ras2-GDP ratio was around 5 at all the tested nucleotides concentrations) suggesting that this exchange operates through a different mechanism from the catalyzed one.

Under our experimental conditions the amount of

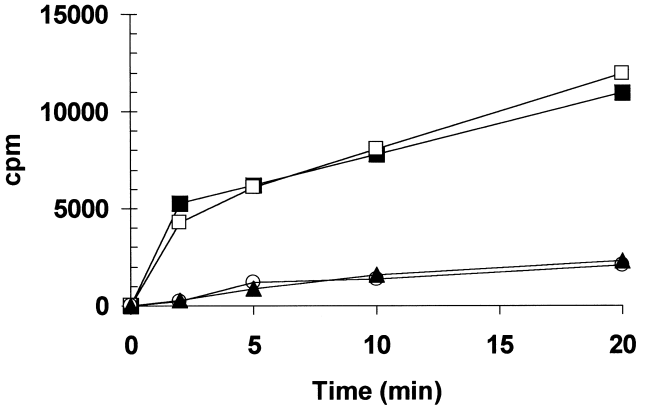

Fig. 3. Cdc25-stimulated exchange reaction of Ras2-GDP in the presence of 100 μM ^{3}H-GDP and 100 μM ^{32}P-GTP. Ras2-GDP protein (0.5 μM) was incubated with 100 μM [^{3}H]GDP (specific activity 2000 Ci/mol) and 100 μM [γ-^{32}P]GTP (specific activity 800 Ci/mol), at 30°C in buffer A in presence of Cdc25-509 protein (20 nM), 20 μl aliquots were filtered on nitrocellulose filters (4) and counted. (■) Ras2-^{3}H-GDP cpm; (□) Ras2-^{32}P-GTP cpm with Cdc25-509. (○) Ras2-^{3}H-GDP cpm; (▲) Ras2-^{32}P-GTP cpm without exchange factor.

GTP hydrolysis caused either by spontaneous GTPase activity of Ras2 or by non-specific nucleotidase activities was negligible.

3.3. Determination of Ras2-GTP 'in vivo'

To investigate whether the cytosolic GTP/GDP ratio modulates the GTP/GDP ratio of the Ras2-bound guanine nucleotides, we used an assay based on the high affinity interaction of Ras-GTP with the Ras binding domain (RBD) of Raf1 [20]. Yeast cells were collected during a batch growth on YNB-glucose at different cell densities corresponding to an early, mid and late exponential growth. Ras2-GTP was then isolated from the total lysates using a GST–RBD fusion protein coupled with glutathione–Sepharose resin and detected by Western blotting; an aliquot of total lysate was also used to evaluate the amount of total Ras2 protein (Fig. 5A,B). The relative intensity of the bands was estimated by densitometric analysis and shown in Fig. 5C. The specificity of antibodies and their ability to detect the small amounts of Ras2-GTP bound to GST–RBD were tested using yeast strains with a deletion of *RAS2* gene (KP-2), or overexpressing

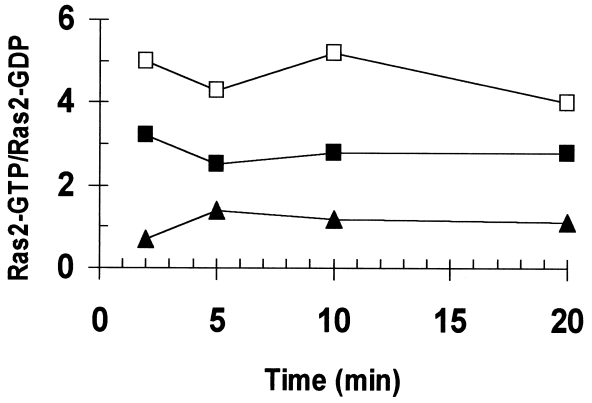

Fig. 4. Ras2-GTP/Ras2-GDP ratio during Cdc25-stimulated exchange reactions in the presence of equimolar concentrations of GDP and GTP. Ras2-GDP protein (0.5 μM) and Cdc25-509 protein (20 nM) were incubated with [γ-^{32}P]GTP (specific activity 800 Ci/mol) and [^{3}H]GDP (specific activity 2000 Ci/mol) present in concentrations of 1 μM (□); 10 μM (■) and 100 μM (▲). 20 μl aliquots were filtered and counted. The Ras2-GTP/Ras2-GDP ratio was determined by correcting the ^{3}H and ^{32}P counts for efficiency and taking in account the exact specific activity of the labeled nucleotides. The exchange experiments were done in triplicate, the standard deviation was less than 10%.

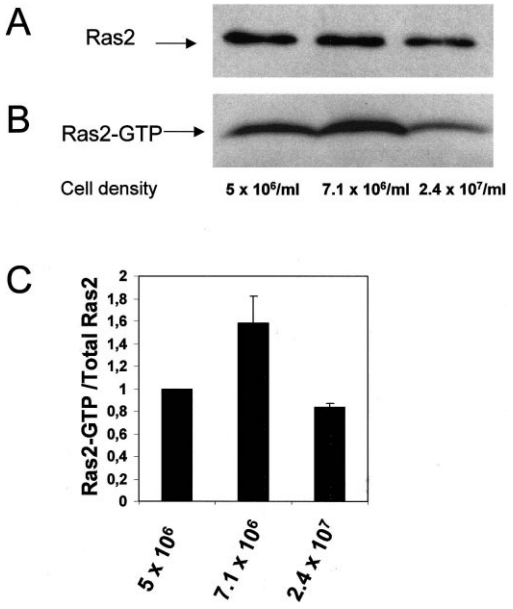

Fig. 5. Activation of Ras2 as a function of the cell density. X4004 cells were grown in YNB containing 2% glucose. Cells were collected by centrifugation. Lysates (200 μg of total protein) were subjected to affinity precipitation with GST–RBD pre-bound to glutathione–Sepharose beads. Ras2 proteins were detected by immunoblotting with anti Ras2 polyclonal antibodies. A cell density of 5×10^6 corresponds to an early exponential growth, a density of 7.1×10^6 corresponds to a mid-exponential growth, while a density of 2.4×10^7 corresponds to a late exponential growth. (A) 2 μg of total cell lysate (total Ras2 level). (B) Bound proteins eluted with SDS-PAGE sample buffer (level of Ras2-GTP). (C) Quantitative analysis of Ras-GTP levels. The ratio Ras2-GTP/Total Ras2 was calculated by densitometry and normalized to the value determined for the early exponential growth, set as 1. Values are reported as the mean of two independent experiments.

Ras2p (W303 [Yep-Ras2]) (results not shown). All experiments were done in triplicate and as shown in Fig. 5 the Ras2-GTP level was higher in mid-exponential growth compared to early and late exponential growth, in good agreement with the cytosolic GTP/GDP (see Fig. 1).

By a careful evaluation of the densitometric data, taking in account the amounts of total lysate used for purification of Ras2-GTP and for determination of total Ras2, the initial Ras2-GTP/total Ras2 ratio (set to one in Fig. 5C) was estimated to be about 0.015 (i.e. Ras2-GTP was 1.5%). This value is very low in comparison with the Ras-GTP levels found in mammalian cells but it is comparable with the pre-

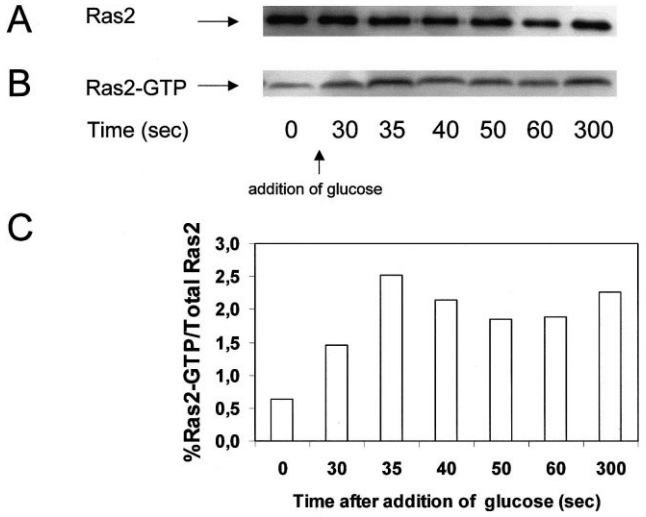

Fig. 6. Activation of Ras2 after addition of 100 mM glucose to glucose-starved cells. X4004 cells were grown till late exponential phase in YNB containing 2% glucose. Cells were collected by centrifugation and resuspended in MES buffer for about 20 min. At time zero glucose was added and appropriate samples were taken by filtration. Lysates (200 μg of total protein) were subjected to affinity precipitation with GST–RBD pre-bound to glutathione–Sepharose. Ras2 proteins were detected by immunoblotting with anti Ras2 polyclonal antibodies. (A) 4 μg of total cell lysate (total Ras2 level). (B) Bound proteins eluted with SDS-PAGE sample buffer (level of Ras2-GTP). (C) Quantitative analysis of Ras2-GTP levels. The ratios of Ras2-GTP/Total Ras2 were determined by densitometric analysis.

viously reported data for yeast [17] using a ^{32}P-orthophosphate labeling.

In a second sets of experiments we evaluated the amount of Ras2-GTP after addition of 100 mM glucose to glucose-starved cells, in the same conditions used for the experiments reported in Fig. 2.

The Ras2-GTP level was quite low in glucose-starved cells (between 0.005 and 0.01, i.e. 0.5–1% in different experiments), while a 2–3 fold increase was observed within 30–40 s. after glucose addition, and a plateau is reached and maintained for at least 5 min. An example is reported in Fig. 6.

4. Discussion

The Cdc25 protein is an exchange factor for Ras and its activity is essential for normal growth of yeast cells. However it is still unclear if the activity of Cdc25 protein is regulated and how the Cdc25/Ras/cAMP pathway transduces signals originated by nutrients. The presence of regulatory regions in Cdc25 proteins was never clearly demonstrated and several reports showed that also heterologous GEF activities could substitute *CDC25* function in yeast cells [12,13]. In addition it has been shown that the GEF-catalyzed reaction is a reversible cycle and not a unidirectional GDP/GTP exchange reaction [14–16], therefore the nucleotide loaded on Ras could be GTP or GDP depending from the availability of the nucleotides.

Haney and Broach [15] firstly suggested an alternative regulatory model where the exchange factor operates in a constitutive way, transducing a signal that is dependent upon the relative concentration of GTP and GDP present in the cytoplasm. Unfortunately no clear data relating the changes in concentration of GTP and GDP with the growth conditions were available in literature.

An obvious implication of this model is that the GTP/GDP ratio should be greatly influenced by nutritional conditions and in turn this ratio will determine the loading equilibrium of Ras-GTP/Ras-GDP and subsequently the amount of activation of adenylyl cyclase.

Here we showed that the GTP/GDP ratio 'in vivo' is clearly modulated by growth and nutritional conditions. The GTP/GDP ratio is high during exponential growth and decreases well before the entering in the stationary phase, in addition it is very sensitive to carbon sources deprivation and rises quickly after readdition of glucose to glucose-deprived cultures.

These data are in agreement and support the proposed model, although it cannot be excluded that other factors could cooperate to modulate the Cdc25/Ras pathway in response to nutrients. For example the activity of Ira1 and Ira2 proteins might change or could be regulated by the level of Cdc25 protein itself, as proposed by Kaplon and Jacquet [8].

The 'in vitro' exchange experiments, performed with 'mixed' nucleotides gave further support to the model. At a concentration of 0.1 mM the loading of GTP or GDP appear to be equivalent, at least in a catalyzed exchange, while at low concentrations a preference for GTP was evident, in agreement with the K_m for GTP and GDP reported for the overall exchange reaction [15].

Surprisingly in a spontaneous exchange reaction, induced by EDTA, a strong preference for GTP occurred at all the tested concentrations. This finding suggests that the two processes, i.e. the catalyzed one and that induced by EDTA, occurs via a different molecular mechanism as suggested also by Lenzen et al. [16] and by the recent resolution of the structure of the GEF domain of h-Sos1 [24].

Additional evidences for the proposed regulatory role of GTP/GDP ratio come from the direct determination of Ras2-GTP levels 'in vivo' under different growth conditions. The pull-down assay we used appeared to be enough sensitive to evidence changes in the steady state levels of Ras2-GTP without overexpression of Ras2 protein. For example during a batch growth experiment we showed a variation of Ras2-GTP in agreement with the changes in GTP/GDP ratio measured in a parallel growth curve. The level of Ras2-GTP was higher during mid-exponential phase of growth and decreased as the cells approached stationary phase. A quantitative analysis, although not very accurate, gives a value of Ras2-GTP in the range of 1–2% of total Ras2 protein. This value is very low in comparison with the Ras-GTP fraction measured in mammalian cells, but is in good agreement with the literature data reported for yeast [17]. This fact could be related to a very high activity of yeast GAPs (Ira1 and Ira2) or to a situation of limited Ras2 activation due for example to the presence of a limiting number of supramolecular complexes involved in the 'signal transduction' events.

Here we show also a fast increase of Ras2-GTP after addition of glucose to glucose-starved cells. For technical reasons our first determination was about 30 s after glucose addition and it would be of interest to improve the method in order to allow a fast time-course determination. Also in this case the increase of Ras2-GTP paralleled the increase of GTP/GDP ratio, but while the latter continues to increase for several minutes and reaches an higher value, the Ras2-GTP level reaches a steady state level after less than 1 min. Again this different behavior could be explained as a consequence of GAPs activity, that might damper the initial signal. In a previous paper of Colombo et al. [17], a marked increase of GTP/GDP bound to Ras2 in yeast cells was observed only after acidification induced by dinitrophenol, while a very limited increase was determined after glucose addition. However these data were obtained in yeast strains overexpressing Ras2p, since the method they used, based on 'in vivo' labeling with ^{32}P-phosphate, was not enough sensitive to measure the GTP/GDP ratio on Ras2 protein in wild type cells. Likely the pull-down assay we used is more sensitive, allowing a better and more accurate determination of the small amounts of Ras2-GTP present in wild type cells.

In conclusion our data strongly support the hypothesis that guanine nucleotide pools regulate the Ras pathway in yeast. However we are aware that additional regulatory mechanism could operate on this system. For example, since Cdc25p is subjected to a fast turnover [8], the amount of the exchange factor available could change under different growth condition and this could regulate also the activity of the pathway, or Cdc25 protein could interact with other protein(s) that might modulate its activity.

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