Involvement of the *CDC25* gene product in the signal transmission pathway of the glucose-induced RAS-mediated cAMP signal in the yeast *Saccharomyces cerevisiae*

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Addition of glucose or related fermentable sugars to derepressed cells of the yeast *Saccharomyces cerevisiae* triggers a RAS-protein-mediated cAMP signal, which induces a protein phosphorylation cascade. Yeast strains without a functional *CDC25* gene were deficient in basal cAMP synthesis and in the glucose-induced cAMP signal. Addition of dinitrophenol, which in wild-type strains strongly stimulates *in vivo* cAMP synthesis by lowering intracellular pH, did not enhance the cAMP level. *cdc25* disruption mutants, in which the basal cAMP level was restored by the *RAS2*^{val19} oncogene or by disruption of the gene (*PDE2*) coding for the high-affinity phosphodiesterase, were still deficient in the glucose- and acidification-induced cAMP responses. These results indicate that the *CDC25* gene product is required not only for basal cAMP synthesis in yeast but also for specific activation of cAMP synthesis by the signal transmission pathway leading from glucose to adenylyl cyclase. They also show that intracellular acidification stimulates the pathway at or upstream of the *CDC25* protein. When shifted to the restrictive temperature, cells with the temperature sensitive *cdc25-5* mutation lost their cAMP content within a few minutes. After prolonged incubation at the restrictive temperature, cells with this mutation, and also those with the temperature sensitive *cdc25-1* mutation, arrested at the 'start' point (in G₁) of the cell cycle, and subsequently accumulated in the resting state G₀. In contrast with *cdc25-5* cells, however, the cAMP level did not decrease and normal glucose- and acidification-induced cAMP responses were observed when *cdc25-1* cells were shifted to the restrictive temperature. These results show that, in the original genetic background at least, growth arrest of *cdc25-1*-bearing cells at the restrictive temperature is not due to cAMP deficiency. Previous experiments have provided evidence for the presence of a glucose-repressible protein in the signalling pathway. Exponential-phase glucose-grown cells of a strain with overexpression of *CDC25* unexpectedly showed a glucose-induced cAMP signal. Control experiments, however, indicated that overexpression of *CDC25* caused a defect in glucose repression. Introduction of the *cat1* derepression mutation in the strain with overexpression of *CDC25* restored glucose repression and abolished the glucose-induced cAMP signal, indicating that the *CDC25* protein is not the glucose-repressible component of the signalling pathway.

Introduction

When glucose or related fermentable carbon sources are added to derepressed cells, stationary-phase cells or to ascospores of the yeast *Saccharomyces cerevisiae*, a cAMP signal is induced which triggers a protein phosphorylation cascade, similar to the hormone-induced cascades in mammalian cells (for a recent review, see Thevelein, 1988). The mechanism by which glucose triggers the cAMP signal is not well understood. It has been shown that transient plasma membrane depolarization, transient intracellular acidification and increased energy

supply are not triggers for the cAMP signal. Recent studies have provided evidence for the existence of a specific signal transmission pathway. The RAS proteins, which in yeast probably act in a way similar to the G_s proteins of mammalian adenylyl cyclase (Defeo-Jones *et al.*, 1983, 1985; Dhar *et al.*, 1984; Kataoka *et al.*, 1984, 1985; Toda *et al.*, 1985), have been implicated as signal transmitters in the pathway (Mbonyi *et al.*, 1988). The requirement of glucose metabolism for induction of the cAMP signal is very limited. Transport of glucose alone is unable to induce the cAMP signal, but the presence of a functional sugar kinase (hexokinase 1, hexokinase 2 or

glucokinase) in the only other requirement. Whether the kinase is needed for phosphorylation of the sugar, or for another reason, is not yet clear (Beullens *et al.*, 1988). Experiments with a mutant lacking the *SNF3* gene, which encodes the high-affinity glucose carrier, have shown that this carrier is not required for induction of the cAMP signal (Mbonyi & Thevelein, 1988). This implies that the putative mechanism of transport-associated phosphorylation of sugar is not involved in induction of the cAMP signal (Beullens & Thevelein, 1990).

The yeast CDC25 protein forms a functional complex with the RAS-adenyl cyclase system (Broek *et al.*, 1987; Camonis *et al.*, 1986; Daniel, 1986; Daniel *et al.*, 1987; Martegani *et al.*, 1986a; Robinson *et al.*, 1987). In the absence of CDC25 or RAS proteins, the basal cAMP level in yeast cells is extremely low (Broek *et al.*, 1987). To differentiate between the alternatives that the *CDC25* gene product is merely needed to sustain basal cAMP synthesis and that it acts as a component of the signal transmission pathway leading from glucose to adenyl cyclase, we have investigated glucose-induced cAMP signalling in *cdc25* mutants in which the capacity for basal cAMP synthesis was restored by the presence of the *RAS2^{val19}* oncogene in the genome or on a plasmid. These strains always contained at least one additional wild-type *RAS* gene which allows for transmission of the glucose-induced cAMP signal, even in the presence of the *RAS2^{val19}* oncogene product (Mbonyi *et al.*, 1988). These experiments showed that the CDC25 protein functions as a component of the signalling pathway. Recent experiments by Munder & Küntzel (1989) also pointed to involvement of CDC25 in glucose-induced cAMP signalling.

In vivo cAMP synthesis in yeast is strongly stimulated by intracellular acidification (Caspani *et al.*, 1985; Purwin *et al.*, 1986; Thevelein *et al.*, 1987a). Previous evidence indicated that lowered intracellular pH does not act directly on adenyl cyclase, but rather on the RAS proteins or on other controlling elements situated upstream in the activation pathway of the enzyme (Mbonyi *et al.*, 1988). In this paper, we have investigated whether a functional CDC25 protein is required for the acidification-induced increase in cAMP. Experiments with glucose-repressed and derepressed wild-type cells, and with repression and derepression mutants, have shown that fermentable sugar is able to induce a cAMP signal only in derepressed cells and not in glucose-repressed cells. This has led to the hypothesis that the signalling pathway contains a glucose-repressible protein (Beullens *et al.*, 1988; Argüelles *et al.*, 1990; Mbonyi *et al.*, 1990). We have made use of strains with overexpression of CDC25 to determine whether CDC25 is the putative glucose-repressible protein of the pathway.

Temperature-sensitive *cdc25* mutants behave as cell

cycle mutants. They arrest at the restrictive temperature at a point called 'start' in the G₁ phase of the cell cycle and subsequently accumulate as round unbudded cells in the resting state G₀. The same is true for temperature-sensitive mutants in *CDC35*, the gene for adenyl cyclase (Boutelet *et al.*, 1985; Casperson *et al.*, 1985; Kataoka *et al.*, 1985). Whereas *cdc25* disruption mutants clearly have very low cAMP levels (Broek *et al.*, 1987), conflicting results have been published for cAMP levels in cells bearing temperature-sensitive alleles of *CDC25*, incubated at restrictive temperatures. Experiments with cells carrying the *cdc25-5* allele showed a rapid drop of the cAMP level upon shift to the restrictive temperature (Camonis *et al.*, 1986). On the other hand, in cells of the *cdc25-1* mutant the cAMP level did not significantly change (Martegani *et al.*, 1986b; Portillo & Mazon, 1986). These results were obtained by different authors using different methods for cAMP extraction and determination. Because of the proposed importance of the CDC25/RAS/adenyl cyclase pathway for progression over the 'start' point of the yeast cell cycle we have reinvestigated the possible difference between *cdc25-1* and *cdc25-5* and found it, at least in the original genetic background of the two strains, to be true.

Methods

Yeast strains. *Saccharomyces cerevisiae* strains used in this study are shown in Table 1. Plasmid pL25S-P [a derivative of YRP7 (*TRP1*) which contains the *CDC25* gene] was kindly provided by M. Jacquet (Orsay, France) and introduced into strains SP1, JT2000 and JT2010 to give, respectively, strains JT4101, JT4200 and JT4300 (Table 1). Plasmid YEp (*RAS2^{val19}* + *LEU2*) was isolated from strain TT1A-5, kindly provided by M. Wigler (Cold Spring Harbor) and introduced into strain SP1 to give strain JT4100. Strain JT1821 was obtained from a cross between strain TK161-R2V and strain TT1A-2 after selection of the diploid for plasmid (*LEU2*) loss. Heat shock sensitivity, basal trehalase activity and trehalose levels in the newly constructed strains were consistent with previous results reported in the literature (Broek *et al.*, 1987). Strain JT5011 (*cdc25⁻ pde2*) was obtained from a cross between strains JT5001 (*pde2*) and T139-5A-6A (*cdc25::HIS3*)/YEp (*RAS2^{val19}*) after selection of the diploid for plasmid (*LEU2*) loss. Strains JT2000 (*cat1*) and JT2010 (*CAT1*) were obtained from a cross between HRX.42-13C (*cat1*), kindly provided by K.-D. Entian (Tübingen, FRG) and DBY747 (wild-type).

Culture and heat shock conditions. The cells were grown to a density of about 8 mg (wet weight) ml⁻¹ in a gyrotatory incubator (200 r.p.m.) at 22 °C (temperature-sensitive strains), 37 °C (temperature-sensitive strains containing plasmids) or 30 °C (all other strains). Composition of the growth media was as follows. YPG: 3% (v/v) glycerol, 2% (w/v) peptone (Merck 7224) and 1% (w/v) yeast extract. YPD: 2% (w/v) glucose, 2% (w/v) peptone and 1% (w/v) yeast extract. YPGal: 2% (w/v) galactose, 2% (w/v) peptone and 1% (w/v) yeast extract. Strains in which the plasmid is not required for growth on rich media were cultured on minimal media (Sherman *et al.*, 1985) lacking the nutrient specified by the selectable marker on the plasmid.

Heat shock was performed by replica-plating cell patches to plates (YPD or minimal glucose medium without leucine) that had been preheated at 55 °C. The plates were incubated for 30, 60, 90 or 120 min

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source (and reference)
A364A	<i>MATa ade1 ade2 ura1 his7 lys2 tyr1 gal1</i>	Yeast Genetic Stock Center
SP1	<i>MATa his3 leu2 ura3 trp1 ade8 can1</i>	M. Wigler (Toda <i>et al.</i> , 1985)
TK161-R2V	<i>MATa his3 leu2 ura3 trp1 ade8 can1 RAS2^{val19}</i>	M. Wigler (Toda <i>et al.</i> , 1985)
T139-5A-6A [*] /YEp(CDC25)	<i>MATa his3 leu2 ura3 trp1 ade8 cdc25::HIS3 pCDC25 (LEU2)</i>	M. Wigler (Broek <i>et al.</i> , 1987)
T139-5A-6A [*] /pC1 ²⁵⁻⁸	<i>MATa his3 leu2 ura3 trp1 ade8 cdc25::HIS3 pTPK1 (LEU2)</i>	M. Wigler (Broek <i>et al.</i> , 1987)
T139-5A-6A [*] /YEp(RAS2 ^{val19})	<i>MATa his3 leu2 ura3 trp1 ade8 cdc25::HIS3 pRAS2^{val19} (LEU2)</i>	M. Wigler (Broek <i>et al.</i> , 1987)
JT1821	<i>MATa his3 leu2 ura3 trp1 ade8 can1 cdc25::HIS3 RAS2^{val19}</i>	This work
JT4100	<i>MATa his3 leu2 ura3 trp1 ade8 can1 pRAS2^{val19} (LEU2)</i>	This work
JT5001*	<i>MATa his3 leu2 ura3 trp1 ade8 pde2::URA3</i>	This work
JT5011	<i>MATa his3 leu2 ura3 trp1 ade8 cdc25::HIS3 pde2::URA3</i>	This work
t.s. 321	<i>MATa cdc25-1 ade1 ade2 ura1 his7 lys2 tyr1 gal1</i>	Yeast Genetic Stock Center
OL86	<i>MATa cdc25-5 ade2 leu2 trp1</i>	M. Jacquet (Camonis <i>et al.</i> , 1986)
OL97-1-11B pL25	<i>MATa cdc25-5 leu2 ura3 his⁻ pH61 (CDC25 + URA3)</i>	M. Jacquet (Camonis <i>et al.</i> , 1986)
DBY747	<i>MATa his3-Δ1 leu2-3 leu2-112 ura3-52 trp1-289_a</i>	Yeast Genetic Stock Center
HRX.42-13C	<i>MATa cat1-42 trp1 ade1 his4 MAL2-8^c MAL3 SUC3</i>	K.-D. Entian
JT2000	<i>MATa cat1-42 ura3 trp1 his3</i>	This work
JT2010	<i>MATa CAT1 ura3 trp1</i>	This work
JT4101	<i>MATa his3 leu2 ura3 trp1 ade8 can1 pL25S-P (CDC25 + TRP1)</i>	This work
JT4200	<i>MATa cat1-42 ura3 trp1 his3 pL25S-P (CDC25 + TRP1)</i>	This work
JT4300	<i>MATa CAT1 ura3 trp1 pL25S-P (CDC25 + TRP1)</i>	This work

* Obtained from a cross between SP1 and a *pde1 pde2* strain (DJ23-3C, provided by M. Wigler).

at 55 °C, cooled on ice and then transferred to a 30 °C incubator. Growth was scored after 2 d.

Incubation conditions and cAMP determinations. For cAMP measurements, cells were incubated at a density of 30 mg (wet weight) ml⁻¹ in a reciprocating water-bath at a temperature of 22 °C, 30 °C or 37 °C as indicated in the figure legends. Incubation was carried out in 25 mM-MES/KOH buffer (pH 6) for 10 min or 20 min (in the case of 37 °C) before addition of 100 mM-glucose or 2 mM-2,4-dinitrophenol (DNP). For temperature shift experiments, cells were incubated in YPD, YPG or 25 mM-MES/KOH buffer (pH 6) for 1 h at 22 °C and then rapidly (1–2 min) shifted to 37 °C. cAMP was determined as described previously (Thevelein *et al.*, 1987a). Glucose-induced cAMP responses were routinely followed for 3 min, while DNP-induced responses were followed for 5 min.

Cell transformation and DNA isolation. Transformation of *E. coli* cells was performed by standard methods (Maniatis *et al.*, 1982). Transformation of yeast cells was done by the lithium acetate method (Ito *et al.*, 1983). Yeast DNA was prepared essentially as described by Struhl *et al.* (1979). Plasmid DNA was isolated from *E. coli* (HB101) by the alkali lysis method (Maniatis *et al.*, 1982).

In vivo ³¹P-NMR spectroscopy. High-resolution ³¹P-NMR spectra of living cells were obtained on a Bruker AM-400 WB NMR spectrometer operating in the Fourier transform mode at 161.9 MHz. The spectra were accumulated at 25 °C for 3 min (312 accumulations) after a 10 min pre-incubation period (for temperature equilibration). The polyphosphate peak was used for calibration (–22.45 p.p.m.). Cells (4 g wet wt) were suspended in 25 mM-MES/KOH buffer (pH 6) (total volume 13 ml) in 20 mm NMR tubes. The cells were continuously bubbled with O₂ (or N₂ in some control experiments) using two glass capillaries. No substrate was added except in control experiments with mutants having high protein kinase activity (which are known to have low endogenous reserves). They were given 3% glycerol after the normal incubation period and after a second pre-incubation period of 10 min, a new spectrum was accumulated.

Preparation of crude extracts and enzyme assays. Crude enzyme extracts for measurement of invertase and trehalase activity were prepared as described previously (Thevelein & Beullens, 1985). Invertase activity was assayed by the procedure of Goldstein & Lampen (1975) as modified by Celenza & Carlson (1984). Trehalase activity was determined as described before (Thevelein *et al.*, 1983), and trehalose levels were determined by the anthrone method (Ashwell, 1957). Total protein content was determined by the Lowry method.

Reproducibility of results. All experiments were repeated at least twice with consistent results. Representative results are shown.

Results and Discussion

Stimulation of cAMP synthesis by glucose and by intracellular acidification

Addition of 100 mM-glucose or 2 mM-dinitrophenol (DNP) to wild-type yeast cells grown on non-fermentable carbon sources triggers a rapid increase in the cAMP level (Fig. 1). With glucose, the cAMP increase is always transient so that it takes the form of a cAMP signal. A yeast strain with a disrupted *CDC25* gene in which viability was restored by a multicopy plasmid carrying the *TPK1* gene has a very low cAMP level (Broek *et al.*, 1987) and did not show a glucose- or DNP-induced cAMP increase (results not shown). To investigate whether the *CDC25* protein truly acts as a component of the signalling pathway, or whether it is merely required

Fig. 1. Glucose-induced cAMP signal (a) and DNP-induced cAMP increase (b) at 22 °C (●) and 37 °C (○) in derepressed cells of the wild-type strain A364A.

for basal cAMP synthesis, we measured the cAMP responses in strains lacking the *CDC25* gene but able to synthesize cAMP: *cdc25::HIS3 pRAS2^{val19}* and *cdc25::HIS3 RAS2^{val19}*. The *RAS2^{val19}* oncogene product does not depend on the *CDC25* protein for its activity, and restores cAMP synthesis in strains lacking *CDC25* (Fig. 2). The cAMP level was higher in the *cdc25::HIS3 pRAS2^{val19}* strain than in the *cdc25::HIS3 RAS2^{val19}* strain, probably owing to the higher copy number of *RAS2^{val19}* in the former strain, caused by the presence of the multicopy YEp vector. The *RAS2^{val19}* oncogene product itself is unable to transmit the cAMP signal. The strains however still contain *RAS1* (and also *RAS2* in the *cdc25::HIS3 pRAS2^{val19}* strain), which is able to transmit the cAMP signal in the presence of *RAS2^{val19}* (Mbonyi *et al.*, 1988). Addition of glucose to

Fig. 2. cAMP level in derepressed cells of strain T139-5A-6A **/YEp RAS2^{val19}* (*cdc25 pRAS2^{val19}*) after addition of glucose (●) and DNP (○), and in cells of strain JT1821 (*cdc25 RAS2^{val19}*) after addition of glucose (▲) and DNP (△).

Fig. 3. Glucose-induced cAMP signal (●, ordinate on left scale) and DNP-induced cAMP increase (○, ordinate on right scale) in derepressed cells of the control strain JT4100 (wild-type strain SP1 with a plasmid containing the *RAS2^{val19}* oncogene).

cells with a disrupted *CDC25* gene did not induce a typical cAMP signal. Neither did DNP cause a cAMP increase comparable to that in wild-type cells (Fig. 2). In the strain containing *pRAS2^{val19}* some residual effect on the cAMP level was observed, the reason for which is not known. Control experiments with wild-type cells containing the *RAS2^{val19}* oncogene in the genome (Mbonyi *et al.*, 1988) or on a plasmid (Fig. 3) showed that the latter is not responsible, e.g. through increased feedback-inhibition (Nikawa *et al.*, 1987; Mbonyi *et al.*, 1990), for the absence of the cAMP responses.

Disruption of both *RAS* genes in yeast is lethal, except for instance in strains lacking both the low-affinity (*PDE1*-encoded) and the high-affinity (*PDE2*-encoded) phosphodiesterases. In these strains, the basal cAMP level is also restored (Nikawa *et al.*, 1987). In this paper we show that lethality due to *CDC25* disruption can be rescued by disruption of the gene encoding high-affinity

phosphodiesterase. Comparison of the glucose- and DNP-induced cAMP increases in the *cdc25 pde2* strain, in which the basal cAMP level is also restored, and the control strain *CDC25 pde2* confirmed the importance of CDC25 for induction of the cAMP responses (results not shown). These results show that the CDC25 protein is required for transmission of the glucose-induced cAMP signal. Since the DNP effect also largely disappears when CDC25 is inactivated, intracellular acidification apparently activates the signal transmission pathway at the CDC25 protein or at some point upstream of CDC25. This point, however, must be located downstream of glucose phosphorylation, since yeast mutants lacking sugar kinase activity are only deficient in the glucose-induced cAMP signal, and not in the DNP-induced cAMP increase (Beullens *et al.*, 1988).

It has been suggested that the CDC25 protein is a specific stimulator of GDP/GTP exchange on the RAS proteins (Broek *et al.*, 1987; Camonis *et al.*, 1986; Daniel, 1986; Daniel *et al.*, 1987; Marshall *et al.*, 1987). Addition of fermentable sugar to derepressed yeast cells probably activates the CDC25 protein, in turn causing a sudden stimulation of GDP/GTP exchange on the RAS proteins. This activates adenyl cyclase, which increases the cAMP level. The rapid decrease after the initial increase might be caused by feedback inhibition by cAMP-dependent protein kinase on one of the components of the signal transmission pathway (Nikawa *et al.*, 1987; Mbonyi *et al.*, 1990).

cAMP levels in the temperature-sensitive mutants *cdc25-1* and *cdc25-5*

Complementation experiments and analysis of the progeny of a cross of the *cdc25-1* and *cdc25-5* containing strains confirmed that the two genes truly represent alleles (results not shown). The temperature-sensitive mutant *cdc25-5* showed a glucose- and DNP-induced cAMP increase at the permissive but not at the restrictive temperature (37 °C) (Fig. 4a). The absence of DNP effect at the restrictive temperature was not due to absence of intracellular acidification as measured by *in vivo* NMR (results not shown). In contrast, the *cdc25-1* mutant showed normal cAMP increases at both the permissive (22 °C) and the restrictive temperature (37 °C) (Fig. 4b). In agreement with the results obtained by Camonis *et al.* (1986) we found that the cellular cAMP content in *cdc25-5* mutants dropped precipitously when the cells were shifted to 37 °C. This was observed both in MES buffer and in rich medium containing either a fermentable or a non-fermentable carbon source (Fig. 5). As opposed to *cdc25-5*-bearing cells, the cAMP level in the *cdc25-1* mutant did not drop, or even increased, when the cells were shifted to the restrictive temperature

Fig. 4. cAMP level in the temperature-sensitive strains OL86 (*cdc25-5*) (a) and t.s.321 (*cdc25-1*) (b) after addition of glucose (filled symbols) and DNP (open symbols) at the permissive temperature of 22 °C (circles) and at the restrictive temperature of 37 °C (triangles).

(37 °C) (Fig. 5). This is in agreement with results obtained previously by Martegani *et al.* (1986b) and Portillo & Mazon (1986). An increased cAMP level upon shift to high temperature is generally observed in wild-type strains (unpublished results). The experiments with *cdc25-1* and *cdc25-5* were carried out under exactly the same experimental conditions. Hence, our data confirm the discrepancy between the previous results obtained independently by different groups with the *cdc25-1* and *cdc25-5* mutants. It cannot be excluded however that the different genetic background in the strains also plays a role, as suggested by recent work from the group of Tatchell (Petitjean *et al.*, 1990) in which the nucleotides modified in the *cdc25-1* and *cdc25-5* gene were identified, and in which it was claimed that both *cdc25-1* and *cdc25-5*, when present in the same genetic background, resulted

Fig. 5. cAMP level in the temperature-sensitive strains OL86 (*cdc25-5*) (●) and t.s.321 (*cdc25-1*) (○) during incubation at the permissive temperature (22 °C) for 60 min and subsequent shift to the restrictive temperature (37 °C). The experiments were carried out in MES buffer (a), YPD (b) and YPG (c).

in a drop of the cAMP level at the restrictive temperature. Unfortunately, the cAMP level in the original *cdc25-1* strain (t.s. 321) was not investigated. Our results with the original *cdc25-1* strain demonstrate that in yeast cells cAMP depletion is not required for arrest at the 'start' point of the cell cycle and for subsequent accumulation in the resting state G_0 .

Is CDC25 the glucose-repressible component of the signal transmission pathway?

Glucose-repressed cells of wild-type strains do not show a glucose-induced cAMP signal, but they show a DNP-induced cAMP increase when they are provided with an

Fig. 6. cAMP level after addition at the permissive temperature (22 °C) of glucose (●) and DNP (○) to glucose-repressed cells of strain OL86 (*cdc25-5*).

Fig. 7. Glucose-induced cAMP signal at 22 °C (●) and 37 °C (○) and DNP-induced cAMP increase at 22 °C (▲) and 37 °C (△) in cells of strain OL97-1-11B pL25 (*cdc25-5 pCDC25*) grown in YPD (at 37 °C) and harvested in exponential phase.

adequate ATP level (Beullens *et al.*, 1988; Argüelles *et al.*, 1990). Glucose-repressed cells have a very low ATP level when suspended in buffer (Thevelein *et al.*, 1987b). The absence of both cAMP responses was observed with glucose-repressed cells of the *cdc25-5* strain (OL86) incubated at the permissive temperature in buffer (Fig. 6). However, exponential-phase glucose-grown cells of the *cdc25-5* mutant, containing a multi-copy plasmid with the *CDC25* gene, clearly showed a glucose- and DNP-induced cAMP increase (Fig. 7). This could indicate that the *CDC25* protein is the putative glucose-repressible component of the signalling pathway. To check whether the cells with overexpression of *CDC25* were still repressed we measured invertase activity and the level of mitochondrial respiration. For the latter, we investigated, by *in vivo* ³¹P-NMR, the ATP level and the intracellular pH in cells suspended in MES buffer and

Table 2. Invertase activity of cells grown in YPD and harvested in exponential phase or grown in YPD until the glucose in the medium was exhausted ('derepressed cells')

Strain	Relevant genotype	Invertase activity [nmol min ⁻¹ (mg protein) ⁻¹]	
		Exponential-phase glucose-grown cells	Derepressed cells
A364A	Wild-type	25.8	528
t.s.321	<i>cdc25-1</i>	45.9	1007
OL86	<i>cdc25-5</i>	12.0	169.5
OL97-1-11B pL25	<i>cdc25-5 pCDC25</i>	1151	1755

bubbled with oxygen (den Hollander *et al.*, 1981; Thevelein *et al.*, 1987b). Unexpectedly, overexpression of CDC25 abolished glucose repression as measured by the level of invertase activity (Table 2) and mitochondrial respiration (Fig. 8, Table 3). To solve this problem we made use of the *cat1* mutation, which prevents derepression. Because they lack respiration, *cat1* mutants are unable to grow on non-fermentable carbon sources. The mutation, however, does not affect derepression of invertase activity, because of the presence

of a suppressor allele in the genetic background of the *cat1* strains (Entian, 1986; Entian & Zimmerman, 1982; Zimmerman *et al.*, 1977). As is also the case for the *cat3* derepression mutant, *cat1* cells do not show a glucose-induced cAMP signal, nor do they show a DNP-induced cAMP increase when simply suspended in buffer (Argüelles *et al.*, 1990). Transformation of a *cat1* strain with the pCDC25 plasmid did not result in suppression of the deficiency in derepression of mitochondrial functions, and the transformed strain did not grow on non-fermentable carbon sources (results not shown). Investigation of glucose-grown cells of the *cat1 pCDC25* strain by *in vivo* ³¹P-NMR showed that the cells were repressed, compared to cells of a *CAT1 pCDC25* strain (Fig. 8,

Fig. 8. ³¹P-NMR spectra of yeast cells grown on YPD and harvested in exponential phase. The cells were incubated in MES buffer at 25 °C and bubbled with oxygen. (a) OL86 (*cdc25-5*); (b) OL97-1-11B pL25 (*cdc25-5 pCDC25*); (c) JT4200 (*cat1 pCDC25*); (d) JT4300 (*CAT1 pCDC25*). Pi, intracellular phosphate peak; Pi_{cyt}, cytoplasmic phosphate peak; Pi_{vac}, vacuolar phosphate peak. ATP_α, ATP_β, ATP_γ; α, β, γ phosphate groups of ATP.

Table 3. ATP level and intracellular pH, as measured by *in vivo* ³¹P-NMR, in glucose-grown cells of strains with and without overexpression of CDC25

The cells were grown on YPD, harvested in exponential phase, suspended in MES buffer and bubbled with oxygen

Strain	Relevant genotype	ATP level*	Intracellular pH†
OL86 (22 °C)	<i>cdc25-5</i>	Low	6.0
OL97-1-11B pL25	<i>cdc25-5 pCDC25</i>	High	7.1
SP1	<i>CDC25</i>	Low	6.1
JT4101	<i>CDC25 pCDC25</i>	Intermediate	6.9
JT2000	<i>cat1</i>	Low	6.5
JT4200	<i>cat1 pCDC25</i>	Low	6.4
JT2010	<i>CAT1</i>	Low	6.0
JT4300	<i>CAT1 pCDC25</i>	High	7.1

* *In vivo* NMR is not well suited for accurate measurement of cellular ATP levels. The difference between the ATP level in repressed cells and derepressed cells however is very clear (Fig. 8). A high ATP level under these experimental conditions is typical for derepressed cells. Because they have respiration they can synthesize ATP from internal carbon sources. Repressed cells can only use fermentation to synthesize ATP and since there is no glucose in the medium, the ATP level of repressed cells under these conditions is low.

† A low intracellular pH under these experimental conditions is typical for repressed cells because they lack ATP. Derepressed cells have a high intracellular pH because they can use their ATP to pump protons out of the cell.

Fig. 9. cAMP level after addition of glucose (●) and DNP (○) to glucose-repressed cells of strain JT4200 (*cat1 pCDC25*).

Table 3). Addition of glucose or DNP to cells of the *cat1* strain with overexpression of CDC25 failed to produce cAMP increases (Fig. 9). This indicates that CDC25 is not the putative glucose-repressible component of the signal transmission pathway leading to activation of adenyl cyclase. Control experiments showed that overexpression of CDC25 in other wild-type strains (JT2010 and SP1) also resulted in the abolition of glucose repression, and the presence of glucose- and DNP-induced responses (results not shown).

Two possible explanations can be given for the abolition of glucose repression by overexpression of CDC25. Either overexpression of CDC25 interferes with the activity of a component needed for normal glucose repression (i.e. glucose repression is disrupted) or CDC25 protein acts as a positive stimulator of derepression, with the consequence that overexpression of CDC25 causes derepression under conditions in which the cells are normally repressed. In this respect, it might be recalled that in wild-type cells the level of *CDC25* transcript is very low (Camonis *et al.*, 1986; Martegani *et al.*, 1986a). Strain OL86 (*cdc25-5*) had a relatively low invertase activity under derepressed conditions (Table 2), which is apparently due to the genetic background. As can be seen in Table 2, overexpression of CDC25 resulted in a much higher activity of invertase. This result suggests that CDC25 protein acts as a positive stimulator of derepression and not as an artificial inhibitor of glucose repression.

It is important to note that the glucose-activation pathway of the CDC25/RAS/adenyl cyclase complex is dispensable for basal cAMP synthesis. The latter is shown by the fact that the glucose-activation pathway is glucose-repressible, and that it depends on sugar kinase activity, since mutants without sugar kinase activity display a normal basal cAMP level (Beullens *et al.*, 1988). Fermentable sugar might be considered an unusual trigger for a signal transmission pathway. In mammalian cells the best-understood signal transmission pathways are triggered by hormones and growth factors (Berridge, 1987; Gilman, 1987). In large multicellular organisms,

hormones and growth factors are prime regulators of cell metabolism and proliferation. In micro-organisms, on the other hand, nutrients are the prime regulators of metabolism and proliferation. In yeast, in particular, fermentable sugar is the preferred carbon substrate, and it should not surprise us if yeast cells contain a sophisticated system for sensing and responding to the level of fermentable sugar in their environment. In addition to fermentable sugar, however, there may exist other pathways for specific activation of the CDC25/RAS/adenyl cyclase complex. It must also be stressed that glucose-induced activation of cAMP synthesis through the RAS pathway is dispensable for growth on glucose and hence for progression over the 'start' point of the yeast cell cycle. The glucose-activation pathway is shut off as soon as the cells are repressed, and therefore it functions only during the transition from the derepressed state to the repressed state. If glucose acts as a stimulator of progression over the 'start' point of the yeast cell cycle, it is not through the glucose-activation pathway of the CDC25/RAS/adenyl cyclase complex.

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