The Cdc25 protein of *Saccharomyces cerevisiae* is required for normal glucose transport

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The essential *CDC25* gene product of *Saccharomyces cerevisiae* is the most upstream known component of the RAS/adenylate cyclase pathway. Cdc25 is a GTP-exchange protein involved in activating RAS in response to fermentable carbon sources. In this paper it is reported that the Cdc25 protein, in addition to its stimulatory role in the RAS/adenylate cyclase pathway, regulates glucose transport. Continuous culture studies and glucose uptake experiments showed that the *cdc25-1* and the *cdc25-5* temperature-sensitive mutants exhibit decreased glucose uptake activity at the restrictive temperature under both repressed and derepressed conditions as compared to the wild-type strain. Because the *cdc25-1* mutant is not impaired in its cAMP metabolism, it is concluded that this effect on glucose transport is independent of cAMP levels. Furthermore, it is shown that the decrease in glucose uptake activity is not due to a decrease in protein synthesis or to an arrest in the G1 phase of the cell cycle. In addition to a defect in glucose uptake, the *cdc25-5* mutant strain exhibited differences in glucose metabolism, probably due to the decreased cAMP level and hence decreased protein kinase A activity. Because the Cdc25 protein is localized at the membrane, these results indicate that Cdc25 is directly involved in glucose transport and may be in direct contact with the glucose transporters.

Keywords: *Saccharomyces cerevisiae*, *cdc25* mutants, glucose metabolism, glucose uptake, continuous culture

INTRODUCTION

The RAS/adenylate cyclase signal transduction pathway plays an important role in the regulation of metabolism and in cell cycle control in the yeast *Saccharomyces cerevisiae* (Broach, 1991; Thevelein, 1992, 1994). Addition of fermentable carbon sources to a yeast culture growing on a non-fermentable carbon source results in the activation of the RAS/adenylate cyclase pathway, which leads to a transient rise in the intracellular cAMP level (Thevelein, 1992). This in turn results in the liberation of the cAMP-dependent protein kinase A (PKA) (Toda *et al.*, 1987a,b) from the regulatory subunit (Toda *et al.*, 1987a) resulting in the activation of PKA. PKA in turn phosphorylates key enzymes of carbon metabolism, for example fructose-1,6-bisphosphatase (Rittenhouse *et al.*, 1987) and phos-

phofructokinase 2 (François *et al.*, 1984), resulting in a net increase in glycolytic activity versus gluconeogenic activity.

The most upstream known activator of the RAS/adenylate cyclase pathway is the Cdc25 protein (Broek *et al.*, 1987; Camonis *et al.*, 1986; Van Aelst *et al.*, 1991). Cdc25 is a guanine-nucleotide-exchange factor (GEF) replacing GDP bound to RAS protein by GTP, resulting in the activation of RAS. GTP-activated RAS in turn activates adenylate cyclase, which catalyses the conversion of ATP into cAMP, stimulating PKA activity. The nucleotide sequence of the *CDC25* gene codes for a large protein of 1588 or 1589 amino acid residues (Broek *et al.*, 1987; Camonis *et al.*, 1986). Gene disruption and deletion experiments have identified three functional domains of the Cdc25 protein. The amino-terminal half (the α -domain), containing an SH3 domain (Rodaway *et al.*, 1989), is required for efficient sporulation, gluconeogenic functions and glucose-induced cAMP signalling, the carboxy-terminal half (the β 1-domain), containing the

Abbreviations: cdc, cell division cycle; PKA, protein kinase A; G6P, glucose 6-phosphate; F1,6BP, fructose 1,6-bisphosphate; GEF, guanine-nucleotide-exchange factor.

guanine-exchange domain, is essential for viability. The last 38 residues of the carboxy terminus, the β 2-domain, are required for downregulating the cAMP signal transduction pathway (Munder *et al.*, 1988; Munder & Küntzel, 1989). *cdc25* disruption mutants and *cdc25* temperature-sensitive mutants have been used to elucidate the function of the Cdc25 protein. In this study we used the *cdc25-1* and the *cdc25-5* temperature-sensitive mutants, which have point mutations in the essential β 1-domain. The *cdc25-1* point mutation (Ala-1403 $\rightarrow$ Val) is situated within a strongly conserved box found in many GEFs, while the *cdc25-5* point mutation (Glu1-328 $\rightarrow$ Lys) is situated between two strongly conserved boxes (Boguski & McCormick, 1993). In the *cdc25-5* mutant this substitution probably results in a change in the α -helical structure (Petitjean *et al.*, 1990). This results in the loss of its function in cAMP metabolism after a shift from the permissive temperature to the restrictive temperature (Oehlen *et al.*, 1993; Van Aelst *et al.*, 1991). The substitution in the *cdc25-1* mutant is reported not to have an effect on the α -helical structure (Petitjean *et al.*, 1990) and cAMP metabolism is still normal in this mutant even at the restrictive temperature (Martegani *et al.*, 1986; Oehlen *et al.*, 1993; Van Aelst *et al.*, 1991). Both mutants arrest in the G1 phase and are impaired in carbon metabolism at the restrictive temperature (Oehlen *et al.*, 1993). This suggests that Cdc25 has another function in addition to the regulation of adenylate cyclase activity. Because no changes in glycolytic enzyme activities were observed at the restrictive temperature, it has been suggested that this reduction in carbon metabolism is located at the level of uptake (Oehlen *et al.*, 1993).

To gain further insight into the role of the *CDC25* gene product in the regulation of glycolytic flux, we have grown the *cdc25-1* and *cdc25-5* temperature-sensitive mutant strains in glucose-limited continuous cultures. In this way we were able to study physiological parameters such as O_2 consumption, CO_2 production and concentrations of glycolytic metabolites under well-defined physiological conditions. To investigate the role of the Cdc25 protein in regulating glucose uptake, glucose uptake experiments were performed on batch cultures at both the permissive and the restrictive temperatures.

It is demonstrated that inactivation of the Cdc25 protein lowers the glucose uptake rate. This effect is independent of cAMP levels and is not due to cell cycle arrest. Because Cdc25 is located at the plasma membrane (Gross *et al.*, 1992), it is suggested that Cdc25 is directly involved in glucose transport, probably via interaction with the glucose transporters.

METHODS

Strains and growth conditions. Strain JC530-4B and the construction of the isogenic temperature-sensitive strains AP124 and AP127 (*cdc25-1* and *cdc25-5*, respectively) were described by Petitjean *et al.* (1990). The strains were grown on minimal medium as described by Sierkstra *et al.* (1992), with the addition of 600 mg leucine l^{-1} , 200 mg histidine l^{-1} and 200 mg uracil l^{-1} . They were grown at a dilution rate of $0.1\ h^{-1}$ at $22\ ^\circ C$ under glucose limitation, with 10 g glucose l^{-1} in the feed, in a

Bioflo III fermenter (New Brunswick) with a 2 l working volume. The fermenter was connected to a computer and controlled by the advanced fermentation software (AFS) (New Brunswick). The airflow and stirrer speed were set on $2\ l\ min^{-1}$ and 500 r.p.m., respectively, and the O_2 tension in the medium was kept above 50% (v/v). The pH was automatically controlled at a value of 5.0 by the addition of 2 M NH_4OH . CO_2 production and O_2 consumption were measured on-line by connection of the headspace of the fermenter to a URAS3G carbon dioxide analyser and a MAGNOS4G oxygen analyser (Hartmann & Braun). For batch growth Yeast Nitrogen Base (YNB) (Sigma) was used, supplemented with the same amounts of leucine, histidine and uracil as indicated above and with either 2% (w/v) glucose or 2% (w/v) galactose as carbon source.

Glucose pulses. Fifty millilitres of glucose solution (2 M) was injected directly into the fermenter chamber resulting in a final concentration of glucose in the fermenter of 50–60 mM.

Samples. Samples for the determination of residual glucose, extracellular and intracellular metabolites and dry weight were taken from the fermenter as described previously (Sierkstra *et al.*, 1992). Extracts for metabolite determination were prepared as described previously (Sierkstra *et al.*, 1992). Residual glucose concentrations, and extra- and intracellular metabolites were determined according to Sierkstra *et al.* (1992), with the modification that all spectrophotometric assays were performed on a Hitachi U2000 spectrophotometer. Total cAMP was measured with the Amersham cAMP kit according to the manufacturer's instructions. Trehalose was measured essentially as described by Neves *et al.* (1991).

Calculation of glucose fluxes in the fermenter. Under glucose-limited conditions glucose flux was calculated by multiplication of the dilution rate by the difference between the glucose concentration in the feed and the residual glucose concentration in the fermenter divided by the dry weight concentration. After a glucose pulse the glucose flux was calculated by dividing the decrease in residual glucose concentration with time by the dry weight concentration. The glucose flux from the feed into the fermenter was added to this value.

Glucose uptake experiments. These were performed essentially as described by Walsh *et al.* (1994). Cells were cultured in batch at $22\ ^\circ C$ in YNB with 2% glucose or 2% galactose as carbon source. Cultured cells (80 ml) were harvested by filtration at $OD_{600}\ 0.7$, washed with 100 mM potassium phosphate buffer (pH 6.5) and resuspended in 4 ml of this buffer. Uptake experiments were performed in triplicate with $[U-^{14}C]$ glucose (Amersham) with glucose concentrations of 0.1–250 mM and a specific activity of $0.6\text{--}739.8\ kBq\ \mu mol^{-1}$. Samples of yeast cells (100 μl) were incubated with 100 μl twice-concentrated radio-labelled glucose for 5 s at $22\ ^\circ C$. Quenching, filtration and counting were essentially the same as described by Walsh *et al.* (1994). Uptake experiments at $37\ ^\circ C$ were performed with yeast cells which were harvested after a 90 min incubation at $37\ ^\circ C$. All data were analysed by computer-assisted nonlinear regression by using Enzfitter software. Standard errors were estimated by Enzfitter.

RESULTS

Glycolytic flux

The role of the Cdc25 protein in regulating glycolytic flux was examined in continuous cultures. In this way we were able to change one parameter at a time while all other parameters, such as pH, nutrient concentration and O_2

Fig. 1. CO₂ production rates of *S. cerevisiae* grown in glucose-limited continuous cultures at a dilution rate of 0.1 h⁻¹ at 22 °C. CO₂ production rates were determined on-line as described in Methods. Measurements were performed twice with similar results. ■, Wild-type strain JC530-4B; △, *cdc25-1* mutant strain AP124; ◇, *cdc25-5* mutant strain AP127. (a) Temperature shift from 22 °C to 37 °C; (b) addition of 50 mM glucose at 37 °C (the temperature was raised to 37 °C 90 min before glucose addition); (c) addition of 50 mM glucose at 22 °C.

tension, were kept constant. The *S. cerevisiae* mutant strains *cdc25-1* (AP124) and *cdc25-5* (AP127), and the isogenic wild-type strain JC530-4B were grown under glucose limitation at a dilution rate of 0.1 h⁻¹ at the permissive temperature of 22 °C. Under these conditions the CO₂ production rate of all three strains was approximately 3.5 mmol g⁻¹ h⁻¹ and the O₂ consumption rate was approximately 3.1 mmol g⁻¹ h⁻¹. Thus the respir-

ation coefficient (RQ) was about 1.1, indicating that under these conditions glucose metabolism was totally respiratory. This was supported by the observation that no ethanol, glycerol or acetate were detected in the culture medium (detection limits 0.05 g l⁻¹) (data not shown). Furthermore, the biomass yield under glucose limitation was about 0.45 g dry wt (g glucose)⁻¹ for all strains.

To investigate the effect of altered Cdc25 function on glucose metabolism, a temperature shift from 22 °C to 37 °C was performed. This resulted in a transient increase in CO₂ production rate and O₂ consumption rate for about 40 min in all strains (Fig. 1a). The CO₂ production rate reached a new steady state value of 3.8 mmol g⁻¹ h⁻¹ after about 60 min in the case of the *cdc25-1* mutant and wild-type strains and of 2.8 mmol g⁻¹ h⁻¹ for the *cdc25-5* mutant strain. The pattern of increase in O₂ consumption rates in all experiments was similar to that of CO₂ production rates, giving an almost constant RQ of about 1.1 (data not shown). This indicates that carbon metabolism remains respiratory under the conditions used. No differences in biomass concentrations between the three strains were observed under these conditions. Thus, under glucose-limited conditions at 37 °C only the *cdc25-5* mutant exhibited a different CO₂ production rate and O₂ consumption rate as compared to the wild-type strain. This may be due to a decrease in glucose uptake or glycolysis or to a routing of glucose metabolites in another direction, for example into reserve carbohydrates.

To establish the response of glucose metabolism to high glucose concentrations at the restrictive temperature, glucose pulses were given as shown in Fig. 1(b). These were given 90 min after the change in temperature from 22 °C to 37 °C, at which time the CO₂ production rates had reached a new steady value. In this way, the effects of high glucose concentrations on glycolytic flux in the *cdc25* mutant strains were not complicated by the initial temperature effects on carbon metabolism. The addition of about 50 mM glucose to the three strains resulted in an immediate increase in the CO₂ production rates (Fig. 1b). In the wild-type strain, it increased from 3.8 to 19.4 mmol g⁻¹ h⁻¹ (a 5.1-fold increase) whereas in the *cdc25-1* and *cdc25-5* mutant strains it increased from 3.9 to 9.8 mmol g⁻¹ h⁻¹ (a 2.5-fold increase) and from 3.0 to 9.2 mmol g⁻¹ h⁻¹ (a 3.0-fold increase), respectively. Thus the increase in CO₂ production rate in the *cdc25* mutant strains was less than half that in the wild-type strain. Furthermore, the pattern of increase in CO₂ production rate in the *cdc25* mutant strains differed from that in the wild-type strain. In the *cdc25* mutant strains a rapid increase in CO₂ production rate for 5 min was followed by a steady CO₂ production rate. The rapid increase in CO₂ production rate in the wild-type strain lasted about 10 min and was followed by a slower increase. After 150 min, the CO₂ production rate in the wild-type strain decreased due to the fact that all glucose had been consumed. Compared to the CO₂ production rates, O₂ consumption rates increased only slightly in the three strains (data not shown). Thus respiratory metabolism remained the same in the three strains, but the increase in fermentative metabolism was almost threefold higher in the wild-type

Fig. 2. Glucose consumption rates of *S. cerevisiae* grown in continuous cultures as described in Fig. 1. Glucose consumption rates were calculated from the residual glucose concentrations in the fermenter and the glucose concentration in the medium feed as described in Methods. Residual glucose concentrations were determined in triplicate and mean values were used which had an SE of <5%. ■, Wild-type strain JC530-4B; △, *cdc25-1* mutant strain AP124; ◇, *cdc25-5* mutant strain AP127. (a) Temperature shift from 22 °C to 37 °C; (b) addition of 50 mM glucose at 37 °C (the temperature was raised to 37 °C 90 min before glucose addition); (c) addition of 50 mM glucose at 22 °C.

than in the *cdc25-1* and *cdc25-5* mutant strains. A glucose pulse at 22 °C resulted in almost the same increases in CO₂ production rates in all three strains (Fig. 1c). However,

Fig. 3. Effect of glucose on concentrations of G6P (a) and F1,6BP (b) in *S. cerevisiae* grown in glucose-limited continuous cultures at 22 °C. The temperature was raised to 37 °C 90 min before the addition of 50 mM glucose. G6P and F1,6BP concentrations were measured as described in Methods. Values are means of two determinations and the SE was <15%. ■, Wild-type strain JC530-4B; △, *cdc25-1* mutant strain AP124; ◇, *cdc25-5* mutant strain AP127.

the increase in CO₂ production was less than at 37 °C due to the lower temperature. In addition, under these conditions O₂ consumption rates increased only slightly as compared to the CO₂ production rates (data not shown).

From these results it was concluded that the lower increase in CO₂ production rates after the glucose pulse at 37 °C in the *cdc25-1* and *cdc25-5* mutant strains as compared to the wild-type strain is caused by the altered functionality of the Cdc25 protein. To determine if this lower increase was due to a lower glucose uptake rate, residual glucose concentrations in the fermenter were measured and the glucose consumption rates of the cells calculated.

Glucose consumption

Glucose consumption rates following the temperature shift, and the glucose pulses at 37 °C and 22 °C are shown in Fig. 2. At 22 °C, glucose consumption rates were almost the same in the three strains. A switch to the restrictive temperature resulted in a small increase in

glucose consumption rates in all strains (Fig. 2a). Thus, the lower CO₂ production in the *cdc25-5* mutant strain at 37 °C was not due to decreased glucose consumption by the cells.

The glucose consumption rate after a glucose pulse at the restrictive temperature was higher in the wild-type strain as compared to the *cdc25-1* and *cdc25-5* mutant strains (Fig. 2b). The glucose consumption rate during the first 20 min after the pulse, calculated from residual glucose concentrations, was 9.20 mmol g⁻¹ h⁻¹ for the wild-type strain, and 6.07 and 7.48 mmol g⁻¹ h⁻¹ for the *cdc25-1* and *cdc25-5* mutant strains, respectively. It is noteworthy that the glucose consumption rate of the *cdc25-5* mutant strain was higher than of the *cdc25-1* strain, although this difference was not observed in the CO₂ production rates. In the wild-type strain, a further increase in the glucose consumption rate from 9.20 to 11.02 mmol g⁻¹ h⁻¹ was observed, followed by a decrease due to exhaustion of the glucose in the medium (Fig. 2b). This slow increase correlates with the CO₂ production data shown in Fig. 1b. The slow increase in glucose consumption rate after the initial increase was absent in the *cdc25* mutant strains, which is again in accordance with the CO₂ production data (Fig. 1b). In the *cdc25-5* mutant strain the glucose consumption rate decreased after 30 min, possibly due to the lowered glucose concentration. The difference in glucose consumption rates between the strains after a glucose pulse at 22 °C was much smaller than at 37 °C (Fig. 2c). These data demonstrate that the differences in fermentative metabolism (Fig. 1b) after a glucose pulse at 37 °C are partly due to a lower glucose consumption rate of the *cdc25-1* and *cdc25-5* mutant strains as compared to the wild-type strain. This could be caused by lower glycolytic activity or by lower glucose uptake activity. To discriminate between these two possibilities glycolytic intermediates were measured.

Concentrations of glucose metabolites, cAMP and ATP

Following the temperature shift to 37 °C cAMP levels were analysed to verify the difference between the two *cdc25* mutant strains. In both the wild-type and the *cdc25-1* mutant strain an increase in cAMP levels was observed, from 14.3 to 22.8 nmol g⁻¹ and from 16.1 to 25.7 nmol g⁻¹, respectively. In the *cdc25-5* mutant however, a decrease in cAMP levels was observed, from 16.3 to 11.1 nmol g⁻¹. This is in accordance with most data in the literature (Martegani *et al.*, 1986; Oehlen *et al.*, 1993; Van Aelst *et al.*, 1991), although Petitjean *et al.* (1990) observed no differences in cAMP levels in these strains.

In order to establish at which level the differences in glycolytic fluxes originated, the concentrations of glycolytic intermediates and ATP were measured. Upon temperature shift to 37 °C the glucose 6-phosphate (G6P) concentrations decreased from about 4.4 µmol g⁻¹ to about 2.0 µmol g⁻¹ in all three strains. However, no significant changes were found in the concentrations of fructose 1,6-bisphosphate (F1,6BP) (2.0 µmol g⁻¹), ATP (4.8 µmol g⁻¹) or the citric acid cycle intermediate α-

ketoglutarate (33 µmol g⁻¹). Because a discrepancy between CO₂ production and glucose consumption was observed in the *cdc25-5* mutant, trehalose levels were measured in the temperature shift experiment in order to establish if the 'lost' glucose had been routed in other directions, such as the production of reserve carbohydrates. A higher increase in trehalose levels was observed in the *cdc25-5* mutant (40 to 182 µmol g⁻¹) compared to the wild-type and the *cdc25-1* mutant (21 to 104 µmol g⁻¹ and 31 to 98 µmol g⁻¹, respectively). Although this difference cannot explain the large difference in CO₂ production, it indicates that at least some glucose metabolites are rerouted in the *cdc25-5* mutant.

A glucose pulse at 37 °C resulted in a transient increase in G6P in all three strains, although it was less marked in the *cdc25-1* strain (Fig. 3a). However, in the *cdc25-5* mutant the G6P concentration remained high, while in the *cdc25-1* mutant and wild-type strain it decreased rapidly to its initial value. The slower decrease in G6P concentration in the *cdc25-5* mutant after the glucose pulse at 37 °C suggests a downstream bottleneck in this mutant. A large transient increase in F1,6BP concentration, from 2.3 µmol g⁻¹ to 31.1 µmol g⁻¹, was observed in the wild-type strain (Fig. 3b). The increase in F1,6BP level was lower in the mutant strains, from about 2.3 µmol g⁻¹ to about 13.3 µmol g⁻¹ (Fig. 3b). The higher transient increase in F1,6BP concentration in the wild-type strain after the glucose pulse at 37 °C suggests that the initial glucose influx in this strain is higher than in the *cdc25* mutant strains. This observation is supported by the glucose consumption rate results presented in Fig. 2(b). An increase in α-ketoglutarate concentration was observed in all three strains 10 min after the glucose pulses, while the ATP levels remained constant except for a transient decrease immediately after the pulse (data not shown). The three strains showed no differences in G6P, ATP or α-ketoglutarate concentrations following addition of glucose at 22 °C (data not shown). Only the F1,6BP concentration exhibited a transient increase from about 3.0 µmol g⁻¹ to about 26.7 µmol g⁻¹ in all three strains.

These results suggest that the lower glucose metabolism in the mutant strains after the glucose pulse at 37 °C is at least partially located at the level of glucose uptake. Furthermore, they show that in the *cdc25-5* mutant glycolytic differences are present in addition to a glucose uptake defect. To demonstrate that the lower glucose consumption rate in the *cdc25* mutant strains was due to a lower glucose uptake activity, uptake experiments were performed.

Glucose uptake experiments

These were performed in batch cultures growing on either 2 % glucose or 2 % galactose. In this way we were able to investigate glucose uptake of glucose-repressed cells (grown on glucose) and glucose-derepressed cells (grown on galactose). Glucose uptake was measured over 5 s. During this time period glucose uptake is linear under all experimental conditions.

Fig. 4. Glucose transport of *S. cerevisiae* under repressed and derepressed conditions at 37 °C. Transport was assessed by measuring the amount of radiolabelled glucose taken up by the cells in 5 s, as described in Methods. Different concentrations of radiolabelled [U-¹⁴C]glucose were used. Cells growing at 22 °C were shifted to 37 °C 90 min before the glucose uptake experiments and results are presented as Eadie-Hofstee plots. ■, Wild-type strain JC530-4B; △, *cdc25-1* mutant strain AP124; ◇, *cdc25-5* mutant strain AP127. (a) Glucose-repressed cells grown on 2% glucose; (b) glucose-derepressed cells grown on 2% galactose.

Table 1. Glucose uptake characteristics of *S. cerevisiae* at 22 °C and 37 °C

The results are means of at least three determinations $\pm$ SEM.

Strain	22 °C*		37 °C†	
	$V_{\max}$ (mmol h ⁻¹ g ⁻¹)	K_m (mM)	$V_{\max}$ (mmol h ⁻¹ g ⁻¹)	K_m (mM)
(a) Glucose-repressed cells (grown on 2% glucose)				
JC530-4B (wild-type)	7.07 $\pm$ 0.24	24.0 $\pm$ 4.1	13.54 $\pm$ 0.54	49.1 $\pm$ 5.8
JC530-4B + cyc‡	ND	ND	12.37 $\pm$ 0.15	40.1 $\pm$ 1.6
AP124 (<i>cdc25-1</i>)	4.39 $\pm$ 0.20	27.8 $\pm$ 4.3	4.57 $\pm$ 0.24	36.4 $\pm$ 5.8
AP127 (<i>cdc25-5</i>)	5.17 $\pm$ 0.17	20.8 $\pm$ 2.5	6.51 $\pm$ 0.37	27.7 $\pm$ 5.1
(b) Glucose-derepressed cells (grown on 2% galactose)				
JC530-4B (wild-type)	3.45 $\pm$ 0.06	2.5 $\pm$ 0.2	6.90 $\pm$ 0.11	3.2 $\pm$ 0.3
AP124 (<i>cdc25-1</i>)	2.07 $\pm$ 0.12	2.6 $\pm$ 0.9	3.07 $\pm$ 0.17	7.2 $\pm$ 1.6
AP127 (<i>cdc25-5</i>)	2.46 $\pm$ 0.04	2.7 $\pm$ 0.3	3.33 $\pm$ 0.08	3.8 $\pm$ 0.5

ND, Not determined.

* Cells were grown as described in Methods.

† Cells were grown at 22 °C as described in Methods then shifted to 37 °C for 90 min.

‡ Cycloheximide (0.1 μ g ml⁻¹) was added at the time that the cells were shifted from 22 °C to 37 °C.

The results of the glucose uptake experiments under repressed and derepressed conditions at 37 °C are shown as Eadie-Hofstee plots in Fig. 4(a) and 4(b), respectively. Under both conditions a linear relation between the points was observed with the difference between repressed and derepressed states visible as differences in slopes of the lines. Furthermore it is clear that under both conditions the apparent $V_{\max}$ values of the *cdc25* mutant strains are lower than in the wild-type strain. From Fig. 4 the apparent K_m and $V_{\max}$ values were calculated and are presented in Table 1, together with the apparent $V_{\max}$ and K_m values obtained under the same conditions at 22 °C. At 22 °C no differences in apparent K_m values were observed between the strains under both growth conditions. The apparent $V_{\max}$ value for glucose transport at 22 °C was however about 1.5-fold higher in the wild-type strains as compared to the *cdc25* mutant strains under repressed and derepressed conditions. This indicates that at 22 °C glucose transport was affected by both *cdc25* point

mutations. At 37 °C the apparent K_m of the wild-type strain under repressed conditions was higher than that of the mutant strains. Under derepressed conditions this was not observed, but under these conditions the apparent K_m value of the *cdc25-1* mutant was about twofold higher as compared to the wild-type and the *cdc25-5* strain. At 37 °C the apparent $V_{\max}$ value for glucose transport was about 2- to 3-fold higher in the wild-type strain as compared to the *cdc25* mutant strains under both repressed and derepressed conditions. Furthermore, under all conditions the apparent $V_{\max}$ values of the *cdc25-1* mutant strain were lower than in the *cdc25-5* mutant strain, indicating allele-specific differences. From these results it is clear that inactivation of the Cdc25 protein results in a lowered glucose uptake rate. The effects were also observed at the permissive temperature, although were less marked. That these differences at 22 °C were not observed after the glucose pulses to the fermenter cultures is probably due to other downstream glycolytic reactions

Table 2. Glucose uptake of *S. cerevisiae* after a shift to 37 °C for 90 min and subsequent shift back to 22 °C

Uptake rates (expressed in mmol h⁻¹ g⁻¹) were measured at a final [U-¹⁴C]glucose concentration of 100 mM for 5 s. The results are means of three determinations ± SEM.

Strain	Before shift to 37 °C	After shift to 37 °C		
		Time (min) at 22 °C:		
		15	60	60 + cyc*
JC530-4B (wild-type)	5.19 ± 0.01	5.09 ± 0.00	4.95 ± 0.02	3.57 ± 0.02
AP124 (<i>cdc25-1</i>)	3.23 ± 0.00	2.04 ± 0.02	2.90 ± 0.17	0.85 ± 0.01
AP127 (<i>cdc25-5</i>)	4.26 ± 0.03	2.10 ± 0.01	4.51 ± 0.00	1.50 ± 0.04

* Cycloheximide (10 µg ml⁻¹) was added at the time the cells were shifted from 37 °C back to 22 °C.

which are rate-limiting at this low temperature. This conclusion is supported by the fact that at 22 °C G6P levels are higher than at 37 °C.

To check that the observed differences were not due to an arrest in G1 in the *cdc25* mutant strains, the protein synthesis inhibitor cycloheximide was added to the wild-type strain at a concentration of 0.1 µg ml⁻¹. This inhibitor was added at the time the cells were shifted from 22 °C to 37 °C. The addition caused an arrest in the G1 phase of the cell cycle but had only a minor effect on the apparent $V_{\max}$ of glucose uptake, indicating that cell cycle arrest is not the cause of the lowered glucose uptake in the *cdc25* mutant strains. Complete inhibition of protein synthesis in the wild-type strain by addition of 10 µg ml⁻¹ cycloheximide during the shift to 37 °C for 90 min resulted in a decrease in apparent $V_{\max}$ to 10.49 mmol h⁻¹ g⁻¹. Thus the glucose transporters have a relatively long half-life, indicating that the decrease in glucose transport in the mutant strains cannot be due to a decrease in expression of the glucose transporters. Therefore, the most likely explanation for the decreased glucose uptake in the *cdc25* mutants is that Cdc25 itself is directly involved in the regulation of glucose uptake. To examine whether, following a shift to 37 °C for 90 min and subsequent shift back to 22 °C, protein synthesis is necessary in the *cdc25* mutant strains for glucose uptake activity to return to original levels, samples were taken after 15 and 60 min and glucose uptake activity measured using 100 mM glucose (Table 2). In addition, a sample of the cells was treated with cycloheximide at the time of the shift back to 22 °C and glucose uptake activity measured after 60 min incubation (Table 2). Fifteen minutes after the shift back to 22 °C glucose uptake was decreased in the *cdc25* mutant strains, but not in the wild-type strain. After 60 min incubation at 22 °C, glucose uptake activity in the mutant strains had almost returned to the original levels. Cycloheximide addition prevented this return of normal glucose uptake activities. Although cycloheximide also affected glucose uptake in the wild-type, these results indicate that protein synthesis is necessary to regain normal glucose uptake activity in the *cdc25* mutant strains after the shift to 37 °C. At this time we are not able to

distinguish whether new glucose transporters have to be synthesized or new Cdc25 protein.

All the data presented show that the decreased glycolytic flux at the restrictive temperature in the *cdc25* mutant strains is at least partly due to decreased glucose uptake, and that this is not due to a decrease in protein synthesis or to G1 arrest. Since in the *cdc25-1* mutant cAMP levels are still normal at the restrictive temperature, the observed effects are independent of PKA activity.

DISCUSSION

To investigate the role of the Cdc25 protein in regulating carbon metabolism we have grown two temperature-sensitive *cdc25* mutant strains, *cdc25-1* and *cdc25-5*, and the isogenic wild-type under glucose-limited conditions in continuous cultures.

In the *cdc25-5* mutant, after a glucose pulse at the restrictive temperature metabolic differences compared to the wild-type strain were observed. The glucose consumption rate and CO₂ production rate were lower in the *cdc25-5* mutant as compared to the wild-type strain. Furthermore, under this condition G6P levels were higher in the *cdc25-5* mutant as compared to the other strains. It is likely that due to the impaired cAMP metabolism in the *cdc25-5* mutant, glycolysis is not activated upon glucose addition, resulting in higher G6P levels and a change in glucose metabolism. Also under glucose-limited conditions at the restrictive temperature, the *cdc25-5* mutant exhibited a change in carbon metabolism, which was manifested by the lowered CO₂ production rate. Under this condition no difference in glucose uptake rate or concentrations of glycolytic intermediates were observed, but a greater increase in trehalose concentrations was observed in the *cdc25-5* mutant as compared to the other strains. Although the increase in trehalose concentration cannot solely account for the lower CO₂ production rate, it suggests that at least some glucose is routed in directions other than glycolysis.

In the *cdc25-1* mutant, the glucose consumption rate was

also lower as compared to the wild-type strain after the glucose pulse at the restrictive temperature. In this mutant, no differences in concentrations of glycolytic intermediates such as G6P were observed as compared to the wild-type strain. This indicates that in this mutant glucose uptake but not glucose metabolism was affected. These data are in accordance with those reported by Oehlen *et al.* (1993) who suggested that metabolism in these mutants is affected in the first steps of glycolysis or at the level of uptake. It should be noted that the pattern of CO₂ production rate and glucose consumption rate differed between the wild-type strain and the *cdc25* mutant strains after a glucose pulse at the restrictive temperature. Possibly, the regulation of glucose uptake in the *cdc25* mutant strains is affected in such a way that they cannot respond properly to changing glucose concentrations.

Glucose uptake experiments were performed to establish how glucose transport was affected in the strains. It must be emphasized that the glucose uptake system in *S. cerevisiae* is very complex, consisting of about 14 putative glucose transporters, of which at least 7 are transporters (Reifenberger *et al.*, 1995). The expression of these transporters is strongly regulated by environmental conditions (Özcan & Johnston, 1995). Thus, in the glucose uptake experiments not one transporter was being studied, but a large number of transporters, together resulting in average apparent K_m and V_{max} values of the transporters present under those particular conditions. The glucose uptake experiments with batch cultures revealed that at 37 °C the *cdc25-1* and *cdc25-5* mutant strains have a lower apparent V_{max} for glucose transport as compared to the wild-type strain under both repressed and derepressed conditions. Under both conditions, the *cdc25-1* mutant strain has an even lower apparent V_{max} than the *cdc25-5* mutant strain, which is in agreement with the lower glucose consumption rate in the *cdc25-1* mutant strain after the glucose pulses in the fermenter at 37 °C. The results of the glucose uptake experiments clearly indicate that the lower glucose consumption rate in the *cdc25* mutant strains after a glucose pulse at 37 °C is a result of a decreased apparent V_{max} in the mutant strains as compared to the wild-type strain. Addition of the protein synthesis inhibitor cycloheximide (0.1 µg ml⁻¹) to the wild-type strain resulted in G1 arrest, but had only a minor effect on glucose uptake activity. It is therefore concluded that the decrease in glucose uptake activity in the *cdc25* mutant strains is a direct result of a change in Cdc25 activity and is not due to an arrest in G1. Also, a complete inhibition of protein synthesis did not result in a decrease in glucose uptake in the wild-type strain to the same extent as in the *cdc25* mutant strains. This indicates that a possible change in expression of the *HXT* genes cannot be the only reason for the decreased glucose uptake activity in the *cdc25* mutant strains, due to the relatively long half-life of the transporters. It is therefore expected that the decreased glucose uptake activity in the mutants is due to a direct effect of Cdc25 on glucose transport. After a shift to the restrictive temperature, protein synthesis was necessary to regain the normal uptake levels in the *cdc25* mutants. However, it is not clear

if this is due to a decreased level of glucose transporters, or to the fact that the mutant Cdc25 protein cannot refold properly. At this time we are not able to resolve this question owing to lack of antibodies against the different glucose transporters and Cdc25. If the decrease in glucose transport activity is due to a breakdown of transporters, this is specific for glucose transporters, because no changes in the activities of other glycolytic enzymes, such as hexokinases, have been observed in these mutants (Oehlen *et al.*, 1993). If this is the case then Cdc25 may have a stabilizing effect on the glucose transporters.

Although less marked, the apparent V_{max} values of the glucose uptake system of the *cdc25-1* mutant strain and, to a lesser extent, the *cdc25-5* mutant strain were also lower at the permissive temperature compared with the wild-type strain. These differences between the strains were not observed in the fermenter studies, probably owing to the low temperature at which downstream glucose metabolism is limiting but glucose uptake is not. This is in agreement with the higher G6P levels observed at 22 °C as compared to 37 °C.

The results indicate that the Cdc25 protein is involved in glucose transport, in addition to its role in cAMP control and cell cycle regulation. In the *cdc25-1* mutant strain only glucose uptake is affected, while in the *cdc25-5* mutant strain metabolism is also affected. The decrease in glucose uptake activity in the *cdc25* mutant strains is not due to a decreased protein synthesis rate or G1 arrest in these strains. In the *cdc25-1* mutant strain the cAMP levels are still normal at the restrictive temperature. Therefore it is concluded that the decrease in the glucose uptake activity is independent of the cAMP levels and hence PKA activity. Thus Cdc25 is involved in glucose uptake, cell cycle progression and cAMP metabolism, but these processes seem to be independently regulated by Cdc25. It has been shown that in a *cdc35* (encoding adenylate cyclase) temperature-sensitive mutant with normal cAMP levels at the restrictive temperature, glucose uptake is decreased (Oehlen *et al.*, 1994), suggesting that Cdc25 and Cdc35 themselves are required for normal glucose uptake. Gross *et al.* (1992) have demonstrated that the Cdc25 protein is attached to the membrane cytoskeleton and is associated with the RAS proteins and adenylate cyclase. The RAS proteins are also attached to the membrane, via a farnesyl and a palmitoyl chain. Therefore the total complex of Cdc25/RAS/adenylate cyclase is situated at the plasma membrane and is thus in close vicinity, perhaps in direct contact, with the glucose transporter molecules or hexokinases. This increases the probability that the Cdc25/RAS/adenylate cyclase complex has a direct effect on glucose uptake and vice versa. The presence of multiple glucose transporters in *S. cerevisiae* explains why no other upstream activators of the Cdc25/RAS/adenylate cyclase pathway have been found. Also, in higher eukaryotes many GEF-like proteins are activated by receptors located in the plasma membrane. So, in an analogous situation, the glucose transporters in *S. cerevisiae* may also function as glucose sensors regulating the Cdc25/RAS/adenylate cyclase pathway.

ACKNOWLEDGEMENTS

We wish to thank Professor J. M. Thevelein for provision of the yeast strains.

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Received 7 February 1996; revised 19 February 1996; accepted 20 February 1996.