Volume 307, number 3, 249–252 FEBS 11349 August 1992


# *CDC25*-dependent induction of inositol 1,4,5-trisphosphate and diacylglycerol in *Saccharomyces cerevisiae* by nitrogen

Christof Schomerus and Hans Küntzel

*Max-Planck-Institut für experimentelle Medizin, Hermann-Rein-Str. 3, D-3400 Göttingen, Germany*

Received 27 April 1992; revised version received 16 June 1992

The addition of ammonium sulfate to starved yeast cells leads to a 3- to 4-fold rapid increase of the second messengers inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG), the products of phosphoinositide-specific phospholipase C (PI-PLC). This response is reduced by dissecting the RAS-activating Cdc25 protein, and is completely abolished by the *cdc25-1* mutation even at permissive temperature. Starved *cdc25-1* mutant cells have a strongly reduced $IP_3$ content, but an at least 10-fold increased DAG level compared to the isogenic wild-type strain. $NH_4$ does not stimulate cAMP synthesis, and glucose does not induce $IP_3$ and DAG. Our data suggest that the Cdc25 protein controls a nitrogen-specific signalling pathway involving the effector PI-PLC, in addition to the glucose-induced activation of adenylyl cyclase (AC).

*Saccharomyces cerevisiae*; Inositol 1,4,5-trisphosphate; Diacyl glycerol; CDC25; Nitrogen signalling; Phospholipase C

## 1. INTRODUCTION

The *Saccharomyces cerevisiae* cell division cycle protein Cdc25 is a multifunctional activator of the cAMP signalling chain. It catalyzes the GDP/GTP exchange on Ras proteins [1] and plays a critical role in the nutrient-dependent decision between growth, starvation arrest and entry into the meiotic pathway during the G1 period of the cell cycle [2–4]. There is also evidence for a cAMP-independent signalling pathway controlled by the Cdc25 protein: the *cdc25-1* mutant arrests at restrictive temperature although the cAMP level is not decreased [5], and the mutant is complemented by the cAMP-independent protein kinase Sch9 [6]. A likely candidate for an alternative and cAMP-independent growth-promoting effector is PI-PLC, an enzyme producing the second messengers $IP_3$ and DAG by cleaving phosphatidyl 4,5-bisphosphate (PIP2) [7]. Although PI-PLC isozymes have not yet been studied in yeast, the substrate $PIP_2$ has been shown to be required for growth of *S. cerevisiae* [8].

We have tested the possibility that PI-PLC may be involved in a cAMP-independent *CDC25*-dependent growth control pathway, by determining the products of PI-PLC-catalyzed cleavage of $PIP_2$, the second messengers $IP_3$ and DAG in wild type and *cdc25-1* mutant strains. We demonstrate that the addition of nitrogen to starved cells leads to a *CDC25*-dependent accumulation of the two metabolites, whereas the cAMP level is not increased under these conditions. Since glucose, the major carbon source known to induce a *CDC25*-dependent cAMP signal [9], does not stimulate $IP_3$ and DAG, we suggest that the Cdc25 protein controls two different nutrient-specific signalling pathways in starved cells.

*Correspondence address:* H. Küntzel, Max-Planck-Institut für experimentelle Medizin, Hermann-Rein-Str. 3, D-3400 Göttingen, Germany. Fax: (49) (551) 3899-388.

## 2. MATERIALS AND METHODS

### 2.1. *Strains and growth conditions*

The *CDC25* wild-type strain CS14 (*MATα ura3 his3*, pCS2) was obtained by introducing the multicopy plasmid pCS2 into strain HK1b [4]. pCS2 contains in addition to *URA3* the *HIS3* gene as a 1.6 kb *Bam*HI fragment in the respective site of pFL1 (YEp24). The *cdc25* mutant strains CS15 (*MATα cdc25-d4::HIS3 ura3 his3*, pFL1) and CS27 (*MATα cdc25-1 ura3*, pFL1) were obtained by introducing plasmid pFL1 into strains KM28 [4] and KL1b [9]. In strain CS28 (*MATα cdc25-1 ura3*, p25/1) the *cdc25-1* mutation is complemented by the multicopy *CDC25* gene [10,11]. Strains were propagated at 30°C (CS14, CS15 and CS28) or 25°C (CS27) in minimal medium (MM) containing 2% glucose and 6.8 g/l Difco N base without amino acids. Nitrogen starvation medium (MM-N) contained Difco N base without ammonium sulfate and amino acids, and glucose starvation medium (MM-C) lacked the carbon source. Solid media contained 2% agar. For nutrient induction experiments cells were grown in liquid MM to mid-log phase, transferred to starvation medium by centrifugation and resuspension in MM-N or MM-C at a density of 4 OD (600 nm), and incubated for 16 h at 160 rpm. Cells were then resuspended in 0.25 vols. of 10 mM MES (pH 6), 0.1 mM EDTA, and incubated for 2 h before adding nutrients. All steps were performed at temperatures optimal for growth (25°C or 30°C). Before and after adding ammonium sulfate or glucose to final concentrations of 30 mM or 25 mM, respectively, samples were taken at indicated times for 3 parallel determinations of each metabolite, and for protein determination. Microscopical examination and plating experiments have indicated an arrest as unbudded viable cells.

### 2.2. *Determination of $IP_3$ and DAG*

For measuring $IP_3$ 0.3 ml aliquots of cellular suspensions were mixed with equal vol. of 10% (v/v) $HClO_4$ and 0.3 g glass beads (diameter 0.5 mm), and cells were broken by vortexing at 4°C for 4

min at 30 s intervals. After centrifugation for 10 min at 2,000 × g, 400 μl aliquots of the supernatants were mixed with 100 μl of 10 mM EDTA (pH 7) and neutralized with a 1:1 (v/v) mixture of Freon and tri-*n*-octylamine as described [12]. The $IP_3$ content was determined by using the Amersham $IP_3$ radioligand assay system.

DAG was determined by quantitative conversion to [$^{32}$P]phosphatidic acid (PA) under mixed micellar conditions, using *E. coli* DAG kinase (Calbiochem). Lipids were extracted according to Preiss et al. [13] and resuspended by bath sonication in an assay buffer essentially as described [14]. Reactions were initiated in a final volume of 90 μl by adding 10 μl of 5 mM [$\gamma$-$^{32}$P]ATP (spec. act. 1 μCi/50 nmol) in 100 mM imidazole, 1 mM diethylenetriaminepentaacetic acid (pH 6.6). After incubation at 25°C for 30 min the reaction was stopped by the addition of 20 μl 1% $HClO_4$ and 450 μl methanol/chloroform (2:1). Samples were vigorously vortexed and incubated for 10 min at room temperature. Phases were broken by adding 150 μl 1% $HClO_4$ and 150 μl $CHCl_3$. The lower organic phase was washed twice with 750 μl 1% $HClO_4$, evaporated to dryness and resuspended in 30 μl chloroform/methanol (95:5). 20 μl aliquots were analyzed by thin layer chromatography [13]. Total cellular and soluble protein was determined according to the Lowry method.

## 3. RESULTS

We have used a bovine adrenocortical $IP_3$-binding protein to determine the $IP_3$ content in crude cellular lysates by a radioligand competition assay. This procedure should give reliable data, since the binding protein is highly specific for $IP_3$ [15]. Crude lysates of exponentially grown *CDC25* cells (CS14) were found to contain 6 ± 2 pmol $IP_3$ per mg total cellular protein. This level slightly decreases to 3 ± 1 pmol/mg protein during starvation and increases up to 5-fold within a few minutes upon addition of ammonium sulfate, as shown in Fig. 1. The induction response is reduced in the mutant strain CS15 containing a centrally dissected *CDC25* gene (allele d4), and is completely abolished in the *cdc25-1* mutant strain CS27 even at permissive temperature (25°C). The $IP_3$ content of the latter strain drops to background level during the starvation period. Fig. 1B demonstrates that the addition of glucose to starved wild-type cells leads to a slow decrease of $IP_3$. Similarly, the initial $IP_3$ levels decreased or remained constant in the two *cdc25* mutant strains CS15 and CS27 during glucose treatment (data not shown).

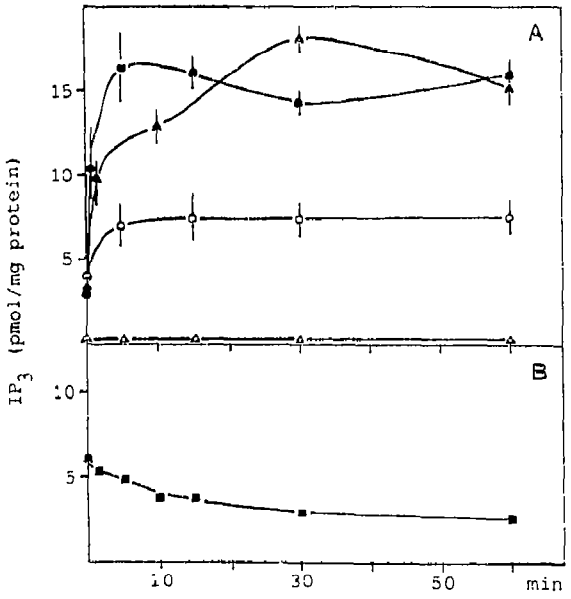


Fig. 1. $IP_3$ content in starved wild type CS14 (●,▲) and *cdc25* mutant strains CS15 (○) and CS27 (△) induced by (A) ammonium sulfate and (B) glucose (CS14, ■).

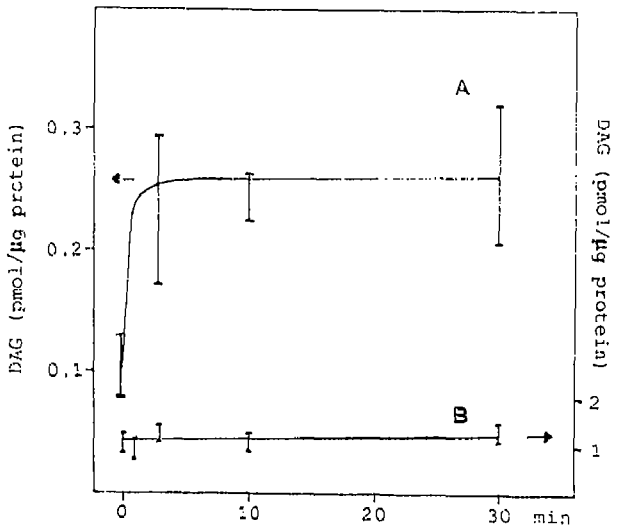


Fig. 2. DAG content in starved wild-type CS14 (A) and *cdc25* mutant strains CS15 (B) induced by ammonium sulfate.

The amount of DAG extractable from whole CS14 cells (150 or 100 ± 10 pmol per mg total cellular protein in growing or starved cells, respectively) exceeds that of extractable $IP_3$ by approx. a factor of 30. Fig. 2A demonstrates a rapid increase of DAG upon addition of ammonium to starved CS14 cells, persisting for at least 30 min at the elevated level. Furthermore, both *cdc25* mutations abolish the nitrogen effect, since the DAG levels remain constant in strains CS15 and CS27 after addition of nitrogen (Figs. 2B and 3A). While the basal DAG level remains fairly constant in the mutant strain CS15 during starvation, a dramatic increase up to 12 ± 1 pmol/μg is observed in the *cdc25-1* mutant strain CS27 after growth and starvation at permissive temperature (see Fig. 3A). This starvation-induced DAG accumulation is prevented by introducing the wild-type *CDC25* gene into the *cdc25-1* mutant, since the DAG level in the starved strain CS28 (0.2 pmol/μg protein) is only slightly higher than in CS14. Glucose has only a minor transient effect on DAG (Fig. 3B), and ammonium sulfate does not induce a transient cAMP signal like glucose (data not shown).

## 4. DISCUSSION

The two major nutrient sources glucose (carbon) and ammonium (nitrogen) elicit specific responses in starved yeast: glucose induces a transient cAMP signal [9,16],

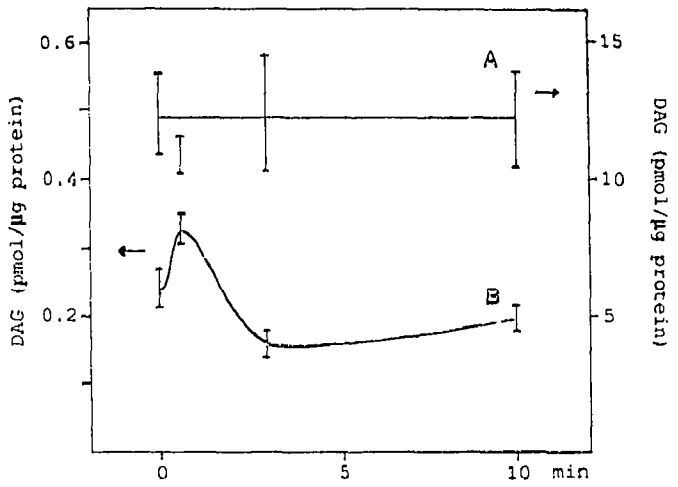


Fig. 3. DAG content in (A) starved CS27 cells induced by ammonium sulfate, and in (B) starved CS14 cells induced by glucose.

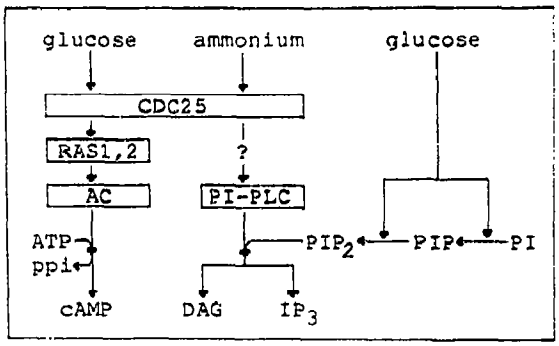


Fig. 4. *CDC25*-controlled nutrient signalling pathways in starved yeast cells.

and ammonium stimulates a sustained increase of DAG + $IP_3$, but not vice versa. While the receptor-coupled formation of DAG in mammalian cells is sometimes sustained for at least 30 min [17], the $IP_3$ response is usually transient, like the cAMP response [7]. Why the nitrogen-induced $IP_3$ level remains constant up to 60 min in starved yeast cells is difficult to explain, since virtually nothing is known about the metabolism of inositol phosphates in this organism. A sustained induction of radiolabelled inositol 1-phosphate and $IP_3$ was also observed after adding glucose to starved yeast cells prelabelled for 24 h with tritiated inositol [18], a finding difficult to reconcile with our data (no accumulation of total cellular $IP_3$ after adding glucose, see Fig. 1B). The observed nutrient induction effects clearly depend on a functional Cdc25 protein, which is required to activate Ras proteins by catalyzing the exchange of GDP by GTP on Ras [1]. It is interesting to note that two thermosensitive mutant alleles having different effects on signalling pathways map within the catalytic domain [19]. While the *cdc25-5* mutation leads to a decrease of cAMP [2,3], the *cdc25-1* mutation has little effect on cAMP levels [5] and glucose-induced cAMP signalling [9] at restrictive temperature, but completely prevents the nitrogen-induced induction of DAG and $IP_3$ even at permissive temperature. This may point to an interaction of the catalytic domain with two different small G proteins activating either adenylyl cyclase (Ras) or PI-PLC (to be identified). The apparent 10- to 15-fold excess of nitrogen-inducible DAG over $IP_3$ points to the activation of other phospholipases in addition to PI-PLC. On the other hand, the Cdc25 protein appears to be required to suppress the formation of DAG from other lipid sources during starvation, since starved *cdc25-1* cells have a strongly increased DAG content, but a very low $IP_3$ level, in comparison to starved wild-type cells. It remains to be shown whether this high DAG level results from an increased hydrolysis of major phospholipids like phosphatidylcholine, or from an elevated phosphatidic acid (PA) phosphatase activity [20]. Fig. 4 summarizes some features of nutrient signalling in quiescent yeast cells. It is obvious from our data that the Cdc25 protein controls a novel signalling pathway in addition to the previously reported glucose-dependent cAMP induction [9], namely the nitrogen-induced accumulation of $IP_3$ and DAG, the products of PI-PLC catalyzed $PIP_2$ hydrolysis. The two major nutrients show a remarkable specificity, since nitrogen does not induce cAMP, and glucose does not induce $IP_3$ and DAG. The previously reported glucose-stimulated turnover of phosphoinositides [18] does not require the Cdc25 and Ras functions [21] but may act in concert with the Cdc25-dependent ammonium-induced activation of PI-PLC to provide enough substrate for this enzyme during mitogenesis ($G_0$–$G_1$ transition). Our data are compatible with the idea that the Cdc25 protein is involved in a nutrient-dependent activation of a GTP-binding protein, which in turn might activate PI-PLC, in analogy to receptor-coupled G proteins activating PI-PLC during hormone response of mammalian cells [22]. Indeed, the Cdc25 protein physically interacts not only with Ras proteins, the activators of adenylyl cyclase [23], but also with other small G proteins not involved in the cAMP pathway (unpublished data).

## REFERENCES

[1] Jones, S., Vignais, M.L. and Broach, J.R. (1991) Mol. Cell. Biol. 11, 2641–2646.
[2] Broek, D., Toda, T., Michaeli, T., Levin, L., Birchmeier, C., Zoller, M., Powers, S. and Wigler, M. (1987) Cell 48, 789–799.
[3] Camonis, J.H., Kalékine, M., Gondré, H.G., Boy-Marcotte, E. and Jacquet, M. (1986) EMBO J. 5, 375–380.
[4] Munder, T., Mink, M. and Küntzel, H. (1988) Mol. Gen. Genet. 214, 271–277.
[5] Martegani, E., Baroni, M. and Wanoni, M. (1986) Exp. Cell Res. 162, 544–548.
[6] Toda, T., Cameron, S., Sass, P., Zoller, M. and Wigler, M. (1988) Genes Dev. 2, 517–527.
[7] Majerus, P.W., Ross, T.S., Cunningham, T.W., Caldwell, K.K., Jefferson, A.B. and Bansal, V.S. (1990) Cell 63, 459–465.

[8] Uno, I., Fukami, K., Kato, H., Takenawa, T. and Ishikawa, T. (1988) Nature 333, 188–190.
[9] Munder, T. and Küntzel, H. (1988) FEBS Lett. 242, 341–345.
[10] Liszlewicz, J., Godany, A., Förster, H.H. and Küntzel, H. (1987) J. Biol. Chem. 262, 2549–2553.
[11] Schomerus, C., Munder, T. and Küntzel, H. (1990) Mol. Gen. Genet. 223, 426–432.
[12] Sharpes, E.S. and McCarl, R.L. (1982) Anal. Biochem. 124, 421–424.
[13] Preiss, J., Loomis, C.R., Bishop, W.R., Stein, R., Niedel, J.E. and Bell, R.M. (1986) J. Biol. Chem. 261, 8597–8600.
[14] Walsh, J.P. and Bell, R.M. (1986) J. Biol. Chem. 261, 6239–6247.
[15] Palmer, S., Hughes, K.T., Lee, D.Y. and Wakelam, M.J.O. (1989) Cell. Signalling 1, 147–156.
[16] Van Aelst, L., Jans, A.W.H. and Thevelein, J.M. (1991) J. Gen. Microbiol. 137, 341–349.
[17] Boeckino, S.B., Blackmore, P.F. and Exton, J.H. (1985) J. Biol. Chem. 260, 14201–14207.
[18] Kaibuchi, K., Miyajima, A., Arai, K. and Matsumoto, K. (1986) Proc. Natl. Acad. Sci. USA 83, 8172–8176.
[19] Petitjean, A., Hilger, F. and Tatchell, K. (1990) Genetics 124, 797–806.
[20] Carman, G.M. and Henry, S.A. (1989) Annu. Rev. Biochem. 58, 635–669.
[21] Frascotti, G., Baroni, D. and Martegani, E. (1990) FEBS Lett. 274, 19–22.
[22] Pouyssegur, J. and Seuwen, K. (1992) Annu. Rev. Physiol. 54, 195–210.
[23] Munder, T. and Fürst, P. (1992) Mol. Cell. Biol. 12, 2091–2099.