The EMBO Journal vol.9 no.3 pp.641 - 651, 1990

# *In vitro* reconstitution of CDC25 regulated *S.cerevisiae* adenylyl cyclase and its kinetic properties

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Communicated by A.Levitzki

**The attenuated GTP regulation adenylyl cyclase (CDC35) lysates or membranes prepared from cells of a cdc25$^{ts}$ strain is enhanced 2.5- to 6-fold by mixing these lysates or membranes with lysates or membranes from a cdc35$^{ts}$ strain harboring wild-type CDC25. The kinetics of activation of the *Saccharomyces cerevisiae* adenylyl cyclase *in vitro* is first order, as is the activation of mammalian adenylyl cyclase. The rate of enzyme activation in the presence of non-hydrolysable analogs of GTP increases with the number of *CDC25* gene copies present in the cell. When GppNHp was used the rate of activation of the cyclase in a strain harboring a multicopy plasmid of *CDC25* was 7.0-fold higher than the rate in an isogenic strain with the *cdc25-2* mutation. The rate of adenylyl cyclase activation from a strain with a disrupted *CDC25* gene is 14.7-fold lower than the rate in an isogenic strain containing the *CDC25* gene on a multicopy plasmid. The reconstitution experiments described provide direct biochemical evidence for the role of the CDC25 protein in regulating the RAS dependent adenylyl cyclase in *S.cerevisiae*. The reconstitution experiments and the kinetic experiments may also provide a biochemical assay for the CDC25 protein and can form the basis for its characterization. In this study we also show that adenylyl cyclase activity in ras1ras2byc1 cells is found in the soluble fraction, whereas in wild-type strain it is found in the membrane fraction. Overexpression of the gene *CDC25* in the ras1ras2bcy1 strain relocalizes adenylyl cyclase activity to the membrane fraction. This finding suggests a biochemical link between CDC25 and CDC35 in the absence of RAS, in addition to its role in regulating RAS dependent adenylyl cyclase.**

*Key words:* adenylyl cyclase/*CDC25*/RAS/*S.cerevisiae*

## Introduction

*ras* is a family of protooncogenes which are highly conserved in evolution from yeast to man. Although *ras* genes are expressed in most mammalian tissues and a mutated form of *ras* is found in many human tumors as well as in tumor viruses (for reviews, see Barbacid, 1987; Lowy and Willumsen, 1986), their function in mammalian cells is unknown. The products of mammalian *ras* genes are small membrane associated proteins (mol. wt 21 kd) which, like heterotrimeric GTP binding proteins (G proteins), bind guanine nucleotides with high affinity and hydrolyze GTP. G proteins are activated by ligand bound hormone receptors which regulate the exchange reaction from GDP to GTP in a first order reaction. The active (GTP bound) G protein then activates intracellular effectors (Gilman, 1987). Mammalian *ras* proteins too are believed to mediate the transduction of an as yet unknown proliferative signal to an as yet unknown effector. A recently reported candidate for the *ras* effector in mammalian cells is the GTPase-activating protein (GAP) described by Trahey and McCormick (1987; for review, see McCormick, 1989).

In the yeast *Saccharomyces cerevisiae* which contains two *RAS* genes, the RAS effector is known to be the adenylyl cyclase (Toda *et al.*, 1985). Yeast cells that lack an active RAS protein do not produce cAMP and are not viable unless extremely active protein kinases are present in the cell. The adenylyl cyclase activity in membranes prepared from these mutated cells is not sensitive to GTP. The sensitivity to GTP and cell viability can be restored, however, if either yeast, human or viral *RAS* gene is introduced into the mutated cell (Clark *et al.*, 1985; Kataoka *et al.*, 1985a). The sensitivity of the adenylyl cyclase to GTP in membranes prepared from RAS deficient yeast cells is restored *in vitro* if purified RAS proteins are added to the membrane preparation (Broek *et al.*, 1985). On the other hand, yeast cells that contain an activated RAS mutant gene (*RAS2*$^{val19}$) show enhanced growth, do not accumulate storage carbohydrates and a diploid heterozygote carrying this mutation cannot sporulate in sporulation medium (Kataoka *et al.*, 1984). The *RAS2*$^{val19}$ mutation is analogous to the *ras*$^{val12}$ mutation in the mammalian *ras* gene that transforms cells in culture and is found in many tumors.

The external signal that activates the yeast RAS/cyclase pathway is glucose. When glucose is added to yeast derepressed cells the cAMP level rises 3- to 5-fold within 30 s (Purwin *et al.*, 1982; Eraso and Gancedo, 1985). Although the exact biochemical mechanism of this activity is unknown, the quick rise in cAMP concentration is totally dependent on the presence of an active *RAS* gene in the cell (Monyi *et al.*, 1988) and on the presence of another gene, *CDC25* (Munder and Kuntzel, 1989; Tanaka *et al.*, 1989; Abeliovich *et al.*, unpublished results). The *CDC25* gene was first identified by a temperature sensitive mutation that causes *S.cerevisiae* cells to arrest at the G1 stage of the cell cycle (Pringle and Hartwell, 1981). Cells that are mutated in the *CDC25* gene do not respond to glucose (Munder and Kuntzel, 1989; Tanaka *et al.*, 1989; Abeliovich *et al.*, unpublished), contain very low levels of cAMP (Camonis *et al.*, 1986; Broek *et al.*, 1987) and sporulate in rich medium (Shilo *et al.*, 1978). The phenotype of cells carrying a disruption or a temperature sensitive mutation in *CDC25* is suppressed by the presence of a very active component of the RAS/cyclase system such as a high copy number plasmid carrying a gene coding for a protein kinase (*TPK1* or *TPK2* or *TPK3*) or adenylyl cyclase (*CYR1*/*CDC35*) (Broek *et al.*, 1987). Furthermore, a mutated, permanently

active adenylyl cyclase (*CRI4*; De Vendittis *et al.*, 1986), or mutated RAS proteins *RAS2*$^{val19}$ (Broek *et al.*, 1987; Robinson *et al.*, 1987) and RAS$^{Ile-152}$ (Camonis and Jacquet, 1988) also suppresses the cdc25$^{ts}$ phenotype.

The adenylyl cyclase activity in membranes prepared from cdc25$^{ts}$ or cdc25 disrupted mutants shows reduced response to guanine nucleotides (Broek *et al.*, 1987; Daniel *et al.*, 1987; Robinson *et al.*, 1987). A recent study characterized point mutations in yeast and human *RAS* genes that are lethal to the yeast cells. The lethality can be suppressed by a high copy number of the *CDC25* gene plus a copy of the normal *RAS* gene. The normal *RAS* gene by itself could not rescue these cells, suggesting a strong physical interaction between the mutated RAS molecule and the CDC25 protein that titrated all the CDC25 protein molecules in the cell (Powers *et al.*, 1989).

Considering the phenotype of cdc25$^{ts}$ and cdc25 disrupted mutants and the genetic results described, together with the reduced GTP sensitivity of adenylyl cyclase activity measured in membranes prepared from cdc25$^{ts}$ and cdc25 disrupted cells, it appears that the *CDC25* gene product is a key component in the regulation of yeast adenylyl cyclase, acting upstream to RAS. It was proposed (Broek *et al.*, 1987; Daniel *et al.*, 1987; Robinson *et al.*, 1987) that the role of CDC25 is to activate RAS by facilitating the GDP/GTP exchange on the molecule in analogy to the regulation of GDP/GTP exchange on heterotrimeric G proteins by hormone bound receptors.

The pathway that leads to the activation of *ras* and its effector in mammalian cells is unknown, leaving the *S.cerevisiae* as the exclusive experimental system available to study the activation of RAS *in vivo*. Further clarification of the mechanism of CDC25/RAS in yeast may be of value in our understanding of *ras* activation in mammalian cells.

We have therefore performed biochemical experiments aimed at further elucidating the role of the *CDC25* gene product in the yeast RAS/cyclase system and to develop an *in vitro* assay for the *CDC25* gene product. We have used cell lysates and membranes in mixing experiments and examined whether the CDC25 protein is indeed a regulator of the yeast adenylyl cyclase system. We have also studied the kinetics of activation of yeast adenylyl cyclase and obtained kinetic evidence that this activation is dependent on the *CDC25* gene product which most probably regulates GDP/GTP exchange on RAS.

## Results

### *Mixing of cdc25$^{ts}$ membranes and lysates with cdc35$^{ts}$ membranes and lysates*

Strains that contain a mutated or disrupted *CDC25* gene show reduced adenylyl cyclase activity when assayed *in vitro* in the presence of $Mg^{2+}$, or $Mg^{2+}$ with GTP or its analogs. The activity in the presence of $Mn^{2+}$, which directly activates the enzyme itself, is less affected (Broek *et al.*, 1987; Daniel *et al.*, 1987; Robinson *et al.*, 1987). These findings suggested that the *CDC25* gene product is involved in the regulation of the adenylyl cyclase activity but does not affect the intrinsic activity of the enzyme itself. To further study the role of the *CDC25* gene product in the cyclase system we dissolved membranes prepared from a cdc25$^{ts}$ mutant (strain 352-1-5A2 which carries the *cdc25-2* mutation) and a cdc35$^{ts}$ mutant (strain 353; for description of strains see Table I) after each strain was shifted to restrictive temperature (36°C) for 2 h. We then assayed the adenylyl cyclase activity of each membrane preparation alone and of the two preparations mixed together. If indeed the CDC25 protein plays a role in the cyclase activation by GTP, one would expect the activity measured with $Mg^{2+}$ or $Mg^{2+}$ plus guanosine-5($\beta$,$\gamma$-imino)triphosphate (GppNHp) in the mixed system to be higher than the arithmetic average of the separate activities of the two strains involved. The activity of the reconstituted system in the presence of

**Table I.** Yeast strains

| Strain | Genotype | Source of reference | Short name |
|---|---|---|---|
| AM18-5C | MATα,*cyr1-1* | (Matsumoto *et al.*, 1982) | cyr1 |
| T27-10D | MATα,*his3,leu2,can1,bcy1,ura3,ras1* :: *HIS3,ras2* :: *LEU2* | (Toda *et al.*, 1985) | ras1ras2bcy1 |
| T27-10D-1 | MATα,*his3,leu2,can1,bcy1,ura3,ras1* :: *HIS3, ras2* :: *LEU2*, with plasmid pFL1-6[CDC25, 2 μ, URA3] | This work | ras1ras2bcy1[CDC25, 2 μ] |
| T27-10D-2 | MAT?,*his3,leu2,can1,bcy1,ura3,ras1* :: *HIS3, ras2* :: *LEU2*, with plasmid YCp50-2[CDC25, URA3] | This work | ras1ras2bcy1[CDC25, CEN] |
| T27-10D-3 | MATα,*his3,leu2,can1,bcy1,ura3,ras1* :: *HIS3, ras2* :: *LEU2*, with plasmid YCp50[URA3] | This work | ras1ras2bcy1[CEN] |
| T27-10D-4 | MATα,*his3,leu2,can1,bcy1,ura3,ras1* :: *HIS3, ras2* :: *LEU2*, with plasmid pFL1-8[TPK1, 2 μ, URA3] | This work | ras1ras2bcy1[TPK1, 2 μ] |
| 352-1-5A2 | MATα,*ade5,cdc25-2,his7,lys2,met10,trp1-289,ura3-52* | Segregant progeny of 352 (Hartwell *et al.*, 1973; Shilo *et al.*, 1978) | cdc25$^{ts}$ |
| 352-1-5A2-1 | MATα,*ade5,cdc25-2,his7,lys2,met10,trp1-289,ura3-52*, with plasmid YCp50-2[CDC25, URA3] | (Daniel *et al.*, 1987) | cdc25$^{ts}$[CDC25, CEN] |
| 352-1-5A2-2 | MATα,*ade5,cdc25-2,his7,lys2,met10,trp1-289,ura3-52*, with plasmid pFL1-6[CDC25, 2 μ, URA3] | (Daniel *et al.*, 1987) | cdc25$^{ts}$[CDC25, 2 μ] |
| 353 | MAT**a**,*ade2,arg,cdc35-1,his7-1,leu,lys2-2,trp,tyr1-2,ura1* | (Shilo *et al.*, 1978) | cdc35$^{ts}$ |
| TT1A-3 | MATα,*his3,leu2,ura3,trp1,ade8,cdc25* :: *URA3*, with plasmid pTPK1[TPK1, 2 μ, LEU2] | (Broek *et al.*, 1987) | cdc25Δ[TPK1, 2 μ] |
| TT1A-4 | MATα,*his3,leu2,ura3,trp1,ade8,cdc25* :: *URA3*, with plasmid pCDC25[CDC25, 2 μ, LEU2] | (Broek *et al.*, 1987) | cdc25Δ[CDC25, 2 μ] |
| A364A | MAT**a**,*ade1,ade2,ura1,lys2-2,his7-1,tyr1-2,gal1-4* | (Shilo *et al.*, 1978) | wild-type |

$Mn^{2+}$, however, is expected to equal the arithmetic average of the separate activities.

Figure 1 shows that the activity of the mixed system in the presence of $Mg^{2+}$ is 1.3-fold higher and with $Mg^{2+}$/GppNHp 2.3-fold higher than the calculated arithmetic average. The activity in the presence of $Mn^{2+}$, however, is very close to the expected average. The $Mg^{2}$/GppNHp dependent activity reaches 46% of the activity measured in A364A wild-type strain (Figure 1) showing significant reconstitution efficiency.

During the course of our studies we found that in strains which are mutated in one of the genes coding for the components of the adenylyl cyclase system, the other components are affected too. For example, in mutants of the *RAS* or the *CDC25* genes, where theoretically the cyclase molecule itself (coded by the *CDC35/CYR1* gene) is intact, we measured $Mn^{2+}$ induced cyclase activity in the soluble fraction of the cell whereas in wild-type strains most of the cyclase activity is found in membranes (see below). These results encouraged us to perform the $cdc25^{ts}$/$cdc35^{ts}$ reconstitution experiments using whole cell lysates rather than the membrane fraction alone. Using lysates, we could improve our reconstitution (Figure 2) and the $Mg^{2+}$ dependent cyclase activity of the reconstituted system in this case was 1.9-fold higher than the calculated arithmetic average while the $Mg^{2+}$/GppNHp dependent activity was between 5- to 6-fold higher. In this experiment the $Mg^{2+}$/GppNHp dependent activity reached 21% of the maximal activity measured in lysate of wild-type strain (Figure 2, see Discussion). It should be noted that the activity of the adenylyl cyclase in our $cdc35^{ts}$ mutant is high in the presence of $Mn^{2+}$. This phenomenon was also observed by Casperson *et al.* (1985) and Sy and Tamai (1986) working with the *cdc35-1* allele which is the same allele as in our strain 353. Sy and Tamai (1986) suggested that this allele of *CDC35* is defective at a site involved in the interaction of the cyclase with its regulatory molecules while the active site remains intact.

We also attempted to reconstitute guanyl nucleotide dependent cyclase by mixing membranes or lysates from $cdc25^{ts}$ with membranes or lysates prepared from a strain which harbors the *cyr1* mutation in the *CDC35/CYR1* gene (strain AM18-5C; Matsumoto *et al.*, 1982). In this experiment, the reconstituted system showed no significant improvement in activity over the calculated arithmetic average (data not shown). To study the reason for this unexpected result we performed a control experiment in which we used the cyr1 mutant as a partner for reconstitution with the strain T27-10D harboring a disruption of its *RAS* genes and the *bcy1* mutation. Although this reconstitu-

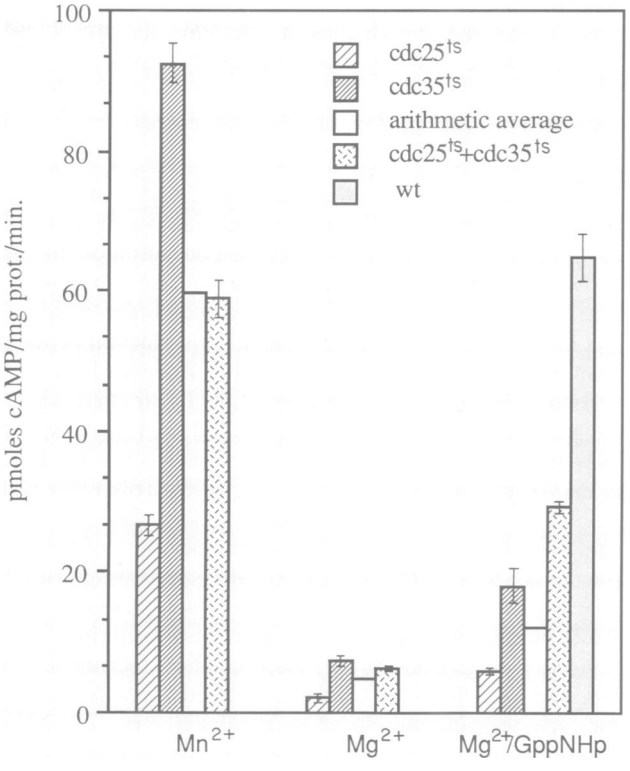



**Fig. 1.** Reconstitution of guanyl nucleotide sensitive cyclase by fusion of membranes from $cdc25^{ts}$ and $cdc35^{ts}$. Adenylyl cyclase activity was measured in membrane preparations of strains 352-1-5A2 ($cdc25^{ts}$) and 353 ($cdc35^{ts}$). Membranes were prepared after each strain was incubated for 2 h at the restrictive temperature. Equal quantities from each membrane preparation were mixed and treated as described in Materials and methods. Both preparations and the mixed sample were assayed in the presence of either 2.5 mM $Mn^{2+}$, 10 mM $Mg^{2+}$, or 10 mM $Mg^{2+}$ plus 75 $\mu$M GppNHp. ▨, activity in the $cdc25^{ts}$ strain, ▨, activity in the $cdc35^{ts}$ strain, □, calculated arithmetic average of the two, ▩, activity measured in the mixed membranes and ▩, activity measured in membranes of wild-type strain A364A in the presence of 10 mM $Mg^{2+}$ plus 75 $\mu$M GppNHp. The experiment depicted is a representative of four independent experiments; it was performed in triplicate and the results shown are the mean ± standard deviation.

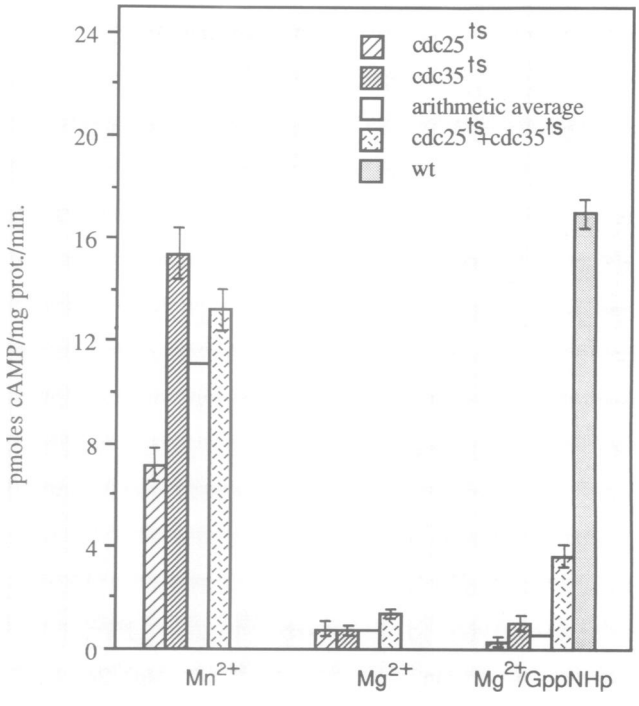



**Fig. 2.** Reconstitution of guanyl nucleotide sensitive cyclase by mixing lysates of $cdc25^{ts}$ and $cdc35^{ts}$. Adenylyl cyclase activity was measured in whole cell lysates of strains 352-1-5A2 ($cdc25^{ts}$) and 353 ($cdc35^{ts}$). The whole cell lysate was treated as described for the membranes in the legend to Figure 1. The assay conditions are also as described in the legend to Figure 1. ▨, activity of lysates of the 352-1-5A2 strain, ▨, activity of the 353 strain, □, calculated arithmetic average of the two, ▩, activity of the mixed membranes and ▩, activity of lysates of wild-type strain A364A in the presence of $Mg^{2+}$/GppNHp.

tion experiment has been performed successfully before (Toda *et al.*, 1985), in our hands this reconstitution did not yield the hoped for and previously reported results. However, when we mixed whole cell lysates of the cdc35$^{ts}$ mutant with whole cell lysates prepared from the ras1ras2-bcy1 strain we observed a 2.9-fold increase in the activity of the mixed system over the calculated arithmetic average in the presence of $Mg^{2+}$, and a 4.4-fold increase with $Mg^{2+}$ /GppNHp (Figure 3). The activity induced by $Mn^{2+}$ is very close to the calculated arithmetic average, as expected. The reconstitution system in this case showed a $Mg^{2+}$/GppNHp dependent activity which is 20% of the activity in the wild-type strain (Figure 3) and very similar to that obtained with the mixed lysates of cdc25$^{ts}$ and cdc35$^{ts}$. A mixing experiment was also performed with the membrane preparations of cdc35$^{ts}$ and ras1ras2bcy1 and showed a 1.5-fold increase over calculated average with $Mg^{2+}$ and 2.0-fold with $Mg^{2+}$/GppNHp (data not shown). Thus mixing membranes or lysates preparations from cdc35$^{ts}$ (strain 353) with either ras1ras2bcy1 or cdc25$^{ts}$ (strain 352-1-5A2) yields similar results (compare Figure 2 with Figure 3), confirming that both the RAS and the CDC25 proteins are regulatory elements of the adenylyl cyclase. We believe that the *cyr1* mutation in the cyclase affects, in an as yet unresolved fashion, the activity of other components of the cyclase system which renders reconstitution studies extremely difficult. The lesion caused by the *cdc35-1* mutation seems to be milder and probably keeps regulatory molecules such as RAS and CDC25 intact.

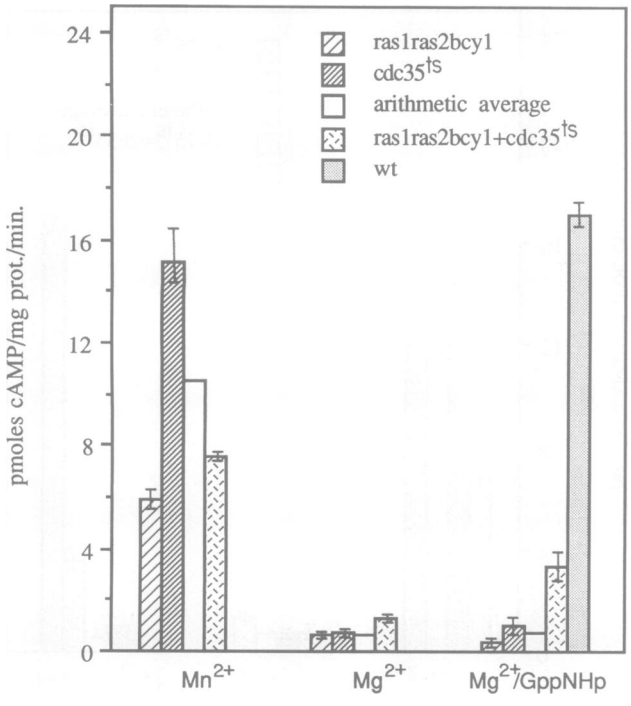



**Fig. 3.** Reconstitution of guanyl nucleotide sensitive cyclase by mixing lysates of cdc35$^{ts}$ and ras1ras2bcy1. Adenylyl cyclase activity was measured in whole cell lysates of strains 353 (cdc35$^{ts}$) and T27-10D (ras1ras2bcy1). Experimental details are as described in the legend to Figure 1 except that the T2-10D strain was grown at 30°C. ▨, activity of the 353 strain, ▩, activity of the T27-10D strain, □, calculated arithmetic average of the two ▨, activity in the mixed membranes and ▩, activity of wild-type strain A364A in the presence of $Mg^{2+}$/GppNHp.

### *CDC25 regulates the rate constant of adenylyl cyclase activation by $Mg^{2+}$ and $Mg^{2+}$ plus GTP analogs*

In order to study further the role of the *CDC25* gene product in the regulation of the adenylyl cyclase activity, we analyzed the kinetics of adenylyl cyclase activation *in vitro*, in five strains: 352-1-5A2, 352-1-5A2-1, 352-1-5A2-2, TT1A-3 and TT1A-4 (Table I). The first three strains harbor the temperature sensitive *cdc25-2* mutation, the same plus a centromeric (single copy) plasmid carrying the normal *CDC25* gene and the same carrying a multicopy plasmid with the normal *CDC25* gene respectively. Strains TT1A-3 and TT1A-4 contain a disruption in their genomic *CDC25* gene. Strain TT1A-3 carries the gene *TPK1* on a multicopy plasmid and TT1A-4 carries the *CDC25* gene on a multicopy plasmid (Broek *et al.*, 1987).

The kinetics of yeast adenylyl cyclase activation by $Mg^{2+}$, $Mg^{2+}$/GppNHp and by $Mn^{2+}$ was measured on lysates prepared from the five strains. Cells of strains 352-1-5A2, 352-1-5A2-1 and 352-1-5A2-2 were grown at 23°C, which is the permissive temperature of the cdc25$^{ts}$ strain, while cells of strains TT1A-3 and TT1A-4 were grown at 30°C.

We found, that the kinetics of activation (Figures 4 and 5, Table III) is first order and fits the function:

$$cAMP_t = k_{cat}[E_{max}]t + \frac{k_{cat}[E_{max}]}{k_{obs}}[\exp(-k_{obs}t) - 1] \quad (1)$$

where $cAMP_t$ is the absolute quantity of cAMP measured at any given time $t$, $k_{cat}$ is the turnover number of adenylyl cyclase, $[E_{max}]$ is the total enzyme concentration in the system and $k_{obs}$ the pseudo-first-order rate constant of activation. The term $[E_{max}]k_{cat}$ is actually the maximal specific activity ($V_{max}$) of the system. This function predicts a lag time in cAMP production which is followed by a time dependent linear curve. The slope of the linear curve yields the maximal specific activity ($k_{cat}[E_{max}] = V_{max}$) and the intercept on the time axis is $1/k_{obs}$, which is the lag time in cAMP production (Hanski *et al.*, 1979; Tolkovsky and Levitzki, 1978). When the activation of the cyclase is studied using non-hydrolysable GTP analogs, the value of the $k_{obs}$ (or the lag time) directly measures the rate constant of the guanyl nucleotide exchange reaction which takes place on the G protein, although it is not a direct measurement (for review, see Levitzki, 1988). Each kinetic experiment was repeated 3–5 times and all the experimental points (18–30 for each constant) were used to calculate the rate constants (Table II). Typical experiments are depicted in Figure 4 and Figure 5.

The rate constants ($k_{obs}$) of the activation reaction, when assayed in the presence of $Mg^{2+}$ or $Mg^{2+}$/GppNHp, are different in the various strains assayed and are higher when more gene copies of *CDC25* are present (Figures 4B, 4C, 5B, 5C, 5D, 5E and Table III). The $k_{obs}$ for 352-1-5A2-2, harboring the gene *CDC25* on a multicopy plasmid, is 4.5-fold higher than that of 352-1-5A2 (cdc25$^{ts}$) when assayed with $Mg^{2+}$ and 7.0-fold higher when assayed with $Mg^{2+}$/GppNHp. The $k_{obs}$ for TT1A-3, harboring a disruption in *CDC25* is 5.5-fold lower in the presence of $Mg^{2+}$ and 14.7-fold lower with $Mg^{2+}$/GppNHp than the equivalent rate constants measured in the isogenic strain TT1A-4, that contains the gene *CDC25* on a multicopy

plasmid. The rate constants of the activation in the presence of $Mn^{2+}$ is less affected by the number of active *CDC25* genes present. The $Mn^{2+}$ dependent adenylyl cyclase activity in the $cdc25^{ts}$ strain has a $k_{obs}$ which is 2.6 ± 0.9-fold lower than the $k_{obs}$ of the isogenic strain harboring the multicopy plasmid with *CDC25* (Figure 4A, Table II). The

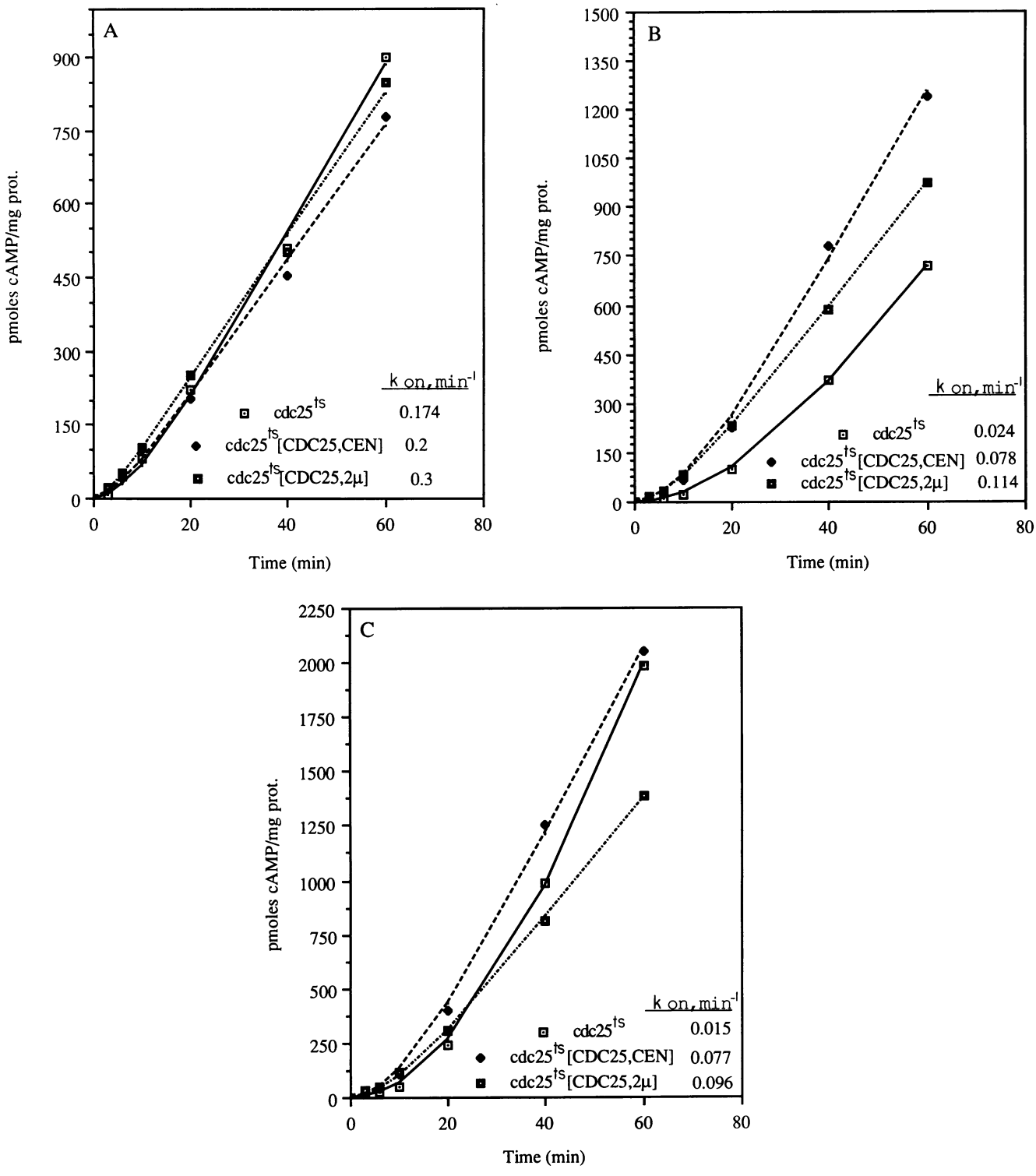



**Fig. 4.** Kinetics of cAMP formation in lysates of $cdc25^{ts}$, $cdc25^{ts}$[CDC25, CEN] and $cdc25^{ts}$[CDC25, 2 μ]. The kinetics of the activation of adenylyl cyclase was measured as described in Materials and methods. Experiments were performed either in the presence of 2.5 mM $Mn^{2+}$ $(acetate)_2$ (**A**), 10 mM $Mg^{2+}$ $(acetate)_2$ (**B**) or 10 mM $Mg^{2+}$ $(acetate)_2$ plus 100 μM GppNHp (**C**). The kinetics were measured in lysates prepared from three strains: 352-1-5A (⊡——), 353-1-5A-1 (◆ – – – –) and 352-1-5A-2 (□·····). Lines represent computer calculated curves according to kinetic parameters that best fit the experimental results, while symbols represent actual experimental results. This kinetic experiments were reproduced between three and five times and the results of all 3–5 experiments were pooled to compute the kinetic parameters with their standard errors of the mean (Table II). Thus each experimental time point was reproduced 3–4 times. A typical experiment is depicted in the Figure. Reproducibility can be judged by examination of Table II.

$k_{obs}$ in strain TT1A-3, in which the *CDC25* gene is disrupted, is 3.6 ± 1.35-fold lower than the $k_{obs}$ in TT1A-4 (Figure 5A, Table II).

### *CDC25 molecule stabilizes a putative CDC25–RAS–CDC35 complex*

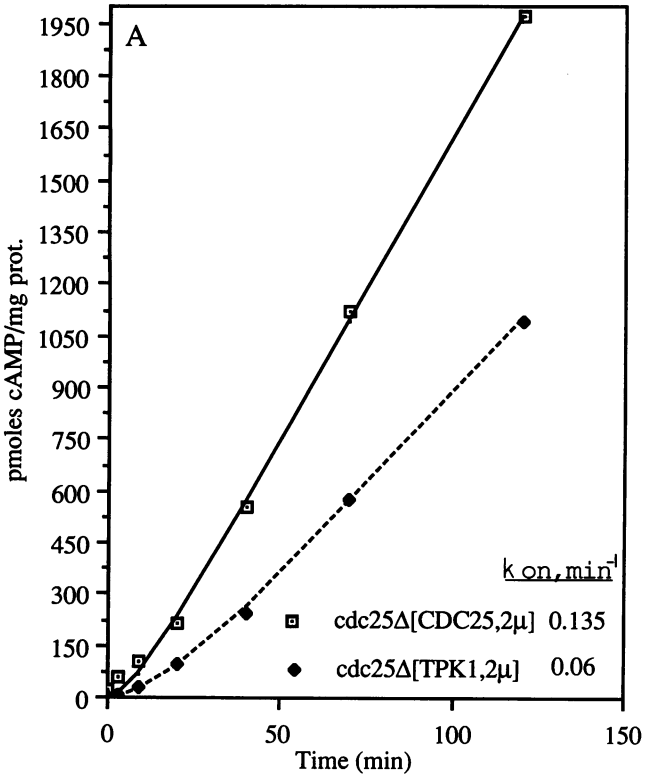
During our reconstitution experiments with the ras1ras2bcy1 strain (Figure 3) we noted that only 9.3% of the total adenylyl cyclase activity measured was recovered in the membrane fraction. The soluble, 100 000 *g* (30 min, 4°C) supernatant, contained >90% of the cyclase activity (Table III). We hypothesized that the absence of RAS proteins from the membrane affects the association of the CDC35 protein with the plasma membrane. We introduced into this strain the plasmid pFL1-6, which is a multicopy plasmid carrying the gene *CDC25* (Daniel and Simchen, 1986) and measured the distribution of cyclase activity in the transformants: 77.7% of the $Mn^{2+}$ dependent adenylyl cyclase activity was found to localize to the membrane fraction and only 22.3% was retained in the soluble fraction (Table III). In this transformed strain (ras1ras2bcy2[CDC25, 2 μ]) a very low $Mg^{2+}$ dependent activity (6 pmol cAMP/mg protein/min in the membrane preparation) was also measured. The distribution of the adenylyl cyclase activity between the membranes and the soluble fraction observed in the ras1ras2bcy1[CDC25, 2 μ] is similar to the distribution detected in wild-type strain (Table III). In order to ensure that the relocalization of the cyclase activity to the membrane is indeed a result of overexpression of the *CDC25* gene, we transformed the ras1ras2bcy1 strain with three other plasmids, YCp50, YCp50-2 and pFL1-8 (see Materials and methods and Table II) and measured the distribution of cyclase activity in the transformants. In all the transformants the major fraction of the activity was identified in the soluble fraction of the cell extract (100 000 *g* supernatant), as in the parental ras1ras2bcy1 cells.

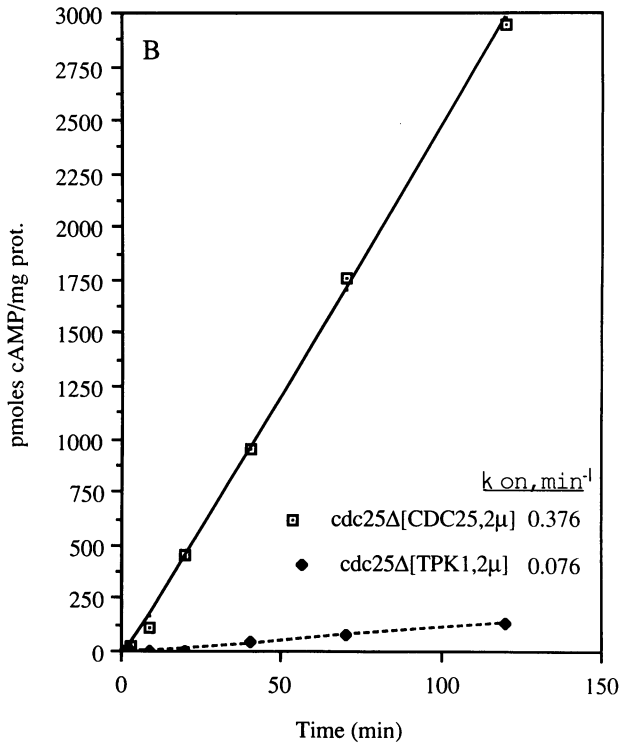
In order to test whether changes in the distribution of adenylyl cyclase activity measured *in vitro* reflect changes in the cells *in vivo*, we measured intracellular cAMP levels, generation time and the tendency for plasmid loss in the ras1ras2bcy1 strain and the transformed strains. Cells of the ras1ras2bcy1[CDC25, 2 μ] strain contain much higher intracellular levels of cAMP (3000 ± 250 fmol cAMP/$10^7$ cells) as compared to the parental ras1ras2bcy1 strain (25 ± 10 fmol cAMP/$10^7$ cells). Even wild-type cells contain lower levels of cAMP (750 ± 43 fmol cAMP/$10^7$ cells) than ras1ras2bcy1[CDC25, 2 μ]. Also, the generation time of the ras1ras2bcy1[CDC25, 2 μ] (2.7 h) is significantly shorter than the generation time of ras1ras2bcy1 (5.4 h). Furthermore, only a small fraction of the ras1ras2bcy1-[CDC25, 2 μ] cells (0.5%) loses the pFL1-6 plasmid when grown for 18 h on non-selective, YEPD media, while in the other transformants >50% of the cells lose their plasmid under the same conditions. It seems therefore that harboring a multicopy plasmid carrying the *CDC25* gene is beneficial to the yeast cells even though no RAS molecules are present.

## Discussion

### *cdc25$^{ts}$/cdc35$^{ts}$ reconstitution experiments*

Previous reports (Broek *et al.*, 1987; Daniel *et al.*, 1987; Robinson *et al.*, 1987) that linked the gene *CDC25* to the RAS/adenylyl cyclase system of *S.cerevisiae* led us to analyze in more detail the involvement of the *CDC25* gene product in this system. The biochemical complementation of the defect in GTP regulation of adenylyl cyclase in membranes of the cdc25$^{ts}$ strain by fusing them with membranes of the cdc35$^{ts}$ strain (which contains the normal



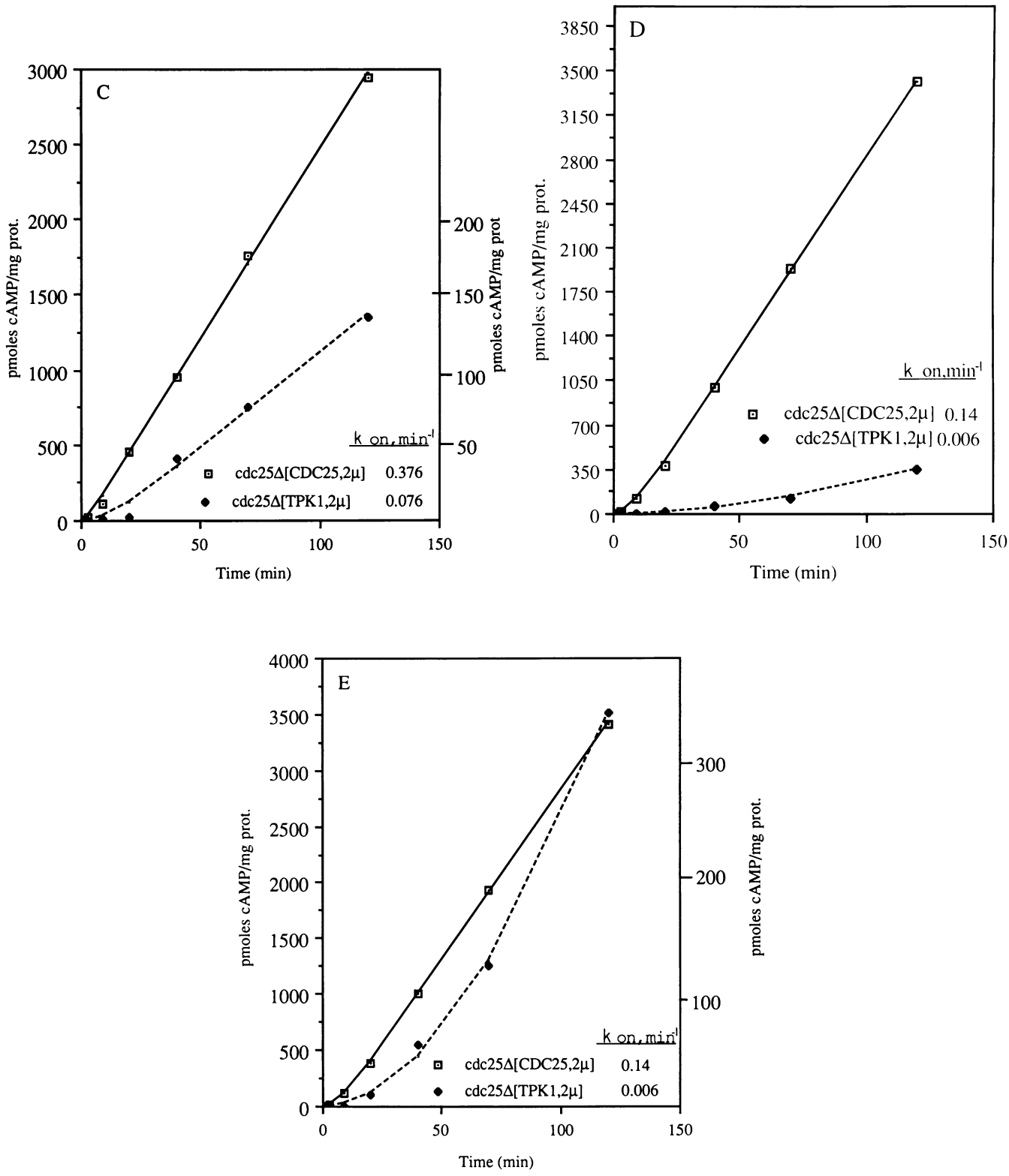



**Fig. 5.** Kinetics of cAMP formation in lysates of cdc25Δ[TPK1, 2 μ] and cdc25Δ[CDC25, 2 μ]. The kinetics of the adenylyl cyclase was measured as described in Materials and methods and in the legend to Figure 4. Experiments were performed either in the presence of 2.5 mM $Mn^{2+}$ $(acetate)_2$ (**A**), 10 mM $Mg^{2+}$ $(acetate)_2$ (**B** and **C**) or 10 mM $Mg^{2+}$ $(acetate)_2$ plus 100 μM GppNHp (**D** and **E**). The kinetics were measured in lysates prepared from strains TT1A-3 (◆ – – – –) and TT1A-4 (⊡———). Lines represents computer calculated curves according to kinetic parameters that best fit the experimental results, while symbols represent actual experimental results. The calculated parameters of several such experiments are shown in Table III. Each experiment was repeated 3–4 times and the data was pooled to compute the kinetic parameters depicted in Table II. A typical experiment is depicted in the figure. **C** and **E** show the same results as in **B** and **D** respectively, but the results of the TT1A-3 strain are depicted using different scale for the *Y* axis. This is done to emphasize the first order character of the reaction.

**Table II.** Kinetic parameters of adenylate cyclase activation in strains harboring different copy number of the CDC25 gene[a]

| Strain | Short name | Maximal specific activity (pmol cAMP/mg/min) | | | Rate constant of cyclase activation $k_{on}$ ($min^{-1}$) | | |
|---|---|---|---|---|---|---|---|
| | | $Mn^{2+}$ | $Mg^{2+}$ | $Mg^{2+}$/GppNHp | $Mn^{2+}$ | $Mg^{2+}$ | $Mg^{2+}$/GppNHp |
| 352-1-5A2 | $cdc25^{ts}$ | 24.2 ± 4.2 | 26.5 ± 3.4 | 82 ± 6.8 | 0.13 ± 0.26 | 0.03 ± 0.005 | 0.019 ± 0.001 |
| 352-1-5A2-1 | $cdc25^{ts}$[CDC25, CEN] | 24.6 ± 4.5 | 38.9 ± 8.8 | 56 ± 3.4 | 0.157 ± 0.02 | 0.084 ± 0.0045 | 0.099 ± 0.002 |
| 352-1-5A2-2 | $cdc25^{ts}$[CDC25, 2 μ] | 22.3 ± 5.4 | 25.7 ± 4.8 | 37.2 ± 1.5 | 0.34 ± 0.02 | 0.136 ± 0.015 | 0.133 ± 0.011 |
| TT1A-3 | cdc25Δ[TPK1, 2 μ] | 8.4 ± 1.5 | 2.0 ± 0.6 | 17.2 ± 1.7 | 0.11 ± 0.03 | 0.046 ± 0.02 | 0.0075 ± 0.001 |
| TT1A-4 | cdc25Δ[CDC25, 2 μ] | 13.4 ± 2.8 | 21.4 ± 2.7 | 27.5 ± 1.9 | 0.4 ± 0.1 | 0.25 ± 0.01 | 0.11 ± 0.02 |

[a]The kinetic parameters shown are the mean ± standard error of at least three experiments. Representative experiments are depicted in Figure 4 and Figure 5.

**Table III.** $Mn^{2+}$ dependent adenylate cyclase activity in cell fractions of ras1ras2bcy1 strain and ras1ras2bcy1 transformed with various plasmids[a]

| | Specific activity (pmol cAMP/min/mg protein) | Total activity (pmol cAMP/min) | % of total |
|---|---|---|---|
| *ras1ras2bcy1* | | | |
| membrane fraction[b] | 8.2 ± 0.8 | 67 ± 15.5 | 9.3 |
| soluble fraction | 16.5 ± 3.5 | 655 ± 83 | 90.7 |
| *ras1ras2bcy1[CDC25, 2 μ]* | | | |
| membrane fraction | 115 ± 3 | 585 ± 90 | 77.7 |
| soluble fraction | 16.2 ± 6 | 168 ± 25.5 | 22.3 |
| *ras1ras2bcy1[CDC25, CEN]* | | | |
| membrane fraction | 6.3 ± 1.8 | 112 ± 10 | 9.0 |
| soluble fraction | 18.8 ± 7.2 | 1140 ± 113 | 91.0 |
| *ras1ras2bcy1[CEN]* | | | |
| membrane fraction | 17.5 ± 1.2 | 139 ± 12 | 26.8 |
| soluble fraction | 6.5 ± 0.7 | 379 ± 26 | 73.2 |
| *ras1ras2bcy1[TPK1, 2 μ]* | | | |
| membrane fraction | 6.0 ± 2 | 51 ± 12 | 30.4 |
| soluble fraction | 4.5 ± 1.2 | 117 ± 23 | 69.6 |
| *wild-type* | | | |
| membrane fraction | 86 ± 7.4 | 1433 ± 184 | 75.9 |
| soluble fraction | 10.5 ± 2.2 | 455 ± 53 | 24.1 |

[a]The results shown are the mean ± standard error of at least three experiments, each performed in duplicate.
[b]Membrane fraction refers to the pellet obtained from centrifugation at 100 000 *g* for 30 min at 4°C of the whole lysate as described in Materials and methods. Soluble fraction is the 100 000 *g* supernatant.

CDC25 protein) provides direct evidence for the role of the CDC25 protein in this regulation. The mixing experiments cannot rule out the possible involvement of other molecules that in combination with RAS and CDC25 regulate the guanyl nucleotide sensitive adenylyl cyclase activity. However, the possibility of physical interaction between RAS and CDC25 is supported by independent findings (Powers *et al.*, 1989). The GTP regulated adenylyl cyclase activity in our reconstituted system is much higher than the arithmetic average calculated from the activities of the two strains alone (Figures 1 and 2) and reached a significant fraction of wild-type levels of activity. This may result from the presence of a defective, temperature sensitive, CDC25 protein which competes with the wild-type protein, thus slowing the 'on' GDP to GTP exchange. In the presence of GppNHp, however, reconstitution is more effective since the RAS protein, once activated, becomes permanently active. Thus even if the rate of exchange is slow, its mere occurrence, due to the presence of a native CDC25 protein, allows considerable activation within the duration of the experiment. Indeed kinetic data show (see below) that it is the rate of activation which seems to be influenced by CDC25. The efficiency of reconstitution is 21–46% of wild-type activity, where the fold activation by guanyl nucleotides is 2.5- to 7-fold. It is worth noting that when pure or crude components of the β-adrenoceptor dependent adenylyl cyclase are co-reconstituted stimulation of cyclase by the hormone and GTP or GppNHp is 2- to 6-fold where the maximal activity obtained is 28–35% of the native system (May *et al.*, 1985; Cerione *et al.*, 1985; Feder *et al.*, 1986). The reconstitution experiments described in this study can serve as the basis for a biochemical assay for the CDC25 protein. Our aim is to use this system in order to develop a CDC25/RAS/cyclase reconstitution *in vitro*, using more pure CDC25 preparations and recombinant CDC25 molecules. Also, this reconstitution system may provide the basis for characterizing biochemically CDC25 homologues from higher eucaryotes.

### *CDC25 as the catalyst for guanyl nucleotide exchange*

The activation of the stimulatory GTP binding proteins, Gs proteins, in avian and mammalian adenylyl cyclase is catalyzed by hormone-bound receptor. Upon interacting with the G protein the hormone-bound receptor catalyzes the exchange of GDP to GTP (Gilman, 1987; Levitzki, 1986,

1988). This exchange reaction can be monitored if non-hydrolysable analogs of GTP are used in the adenylyl cyclase assay. The rate of cyclase activation reflects the rate of guanyl nucleotide exchange (Hanski *et al.*, 1979). Strikingly, the kinetics of activation of the *S.cerevisiae* adenylyl cyclase by $Mg^{2+}$ or GTP analogs was found to be first order (Figures 4 and 5) as are the kinetics of activation of the mammalian enzyme. The first order rate constant $k_{obs}$ of adenylyl cyclase activation can be correlated to the copy number and state of the *CDC25* gene in the cell. It is high in a strain which contains a multicopy plasmid carrying *CDC25* and is very low in a strain that contains a mutated or disrupted *CDC25* gene (Figures 4 and 5; Table II). This observation strongly supports the view that the CDC25 protein facilitates the rate of the guanyl nucleotide exchange reaction. The kinetic parameters of the reaction in the presence of $Mn^{2+}$ are also affected, though less dramatically. This effect may result from the stabilizing effect of the CDC25 protein on the putative multiprotein adenylyl cyclase complex (see below). When $Mg^{2+}$/GppNHp was used in the reaction mixture, the maximal specific activity of strain 352-1-5A-2, containing the *CDC25* gene on a multicopy plasmid, was lower than that in the other two isogenic strains (352-1-5A and 352-1-5A-1), although its $k_{on}$ was the highest among the three (Table II). One possible explanation is that the presence of many molecules of CDC25 protein already facilitate significantly the back reaction, destabilizing the RAS−GppNHp complex, thus reducing the maximal specific activity observed. On the other hand the maximal specific activity in our $cdc25^{ts}$ strain is very high in the presence of $Mg^{2+}$/GppNHp although its $k_{on}$ is very low (Table II). We have no explanation for this observation. In the *CDC25* disrupted strain (TT1A-3) the calculated maximal specific activity in the presence of $Mg^{2+}$/GppNHp is higher than that with $Mn^{2+}$ despite the large differences in the $k_{on}$ (Table II). This result shows that in these cells too, the CDC25 protein affects the rate of the activation and not the intrinsic properties of the enzyme (Table III). It should be noted that in the CDC25 disrupted strain, TT1A-3, the maximal specific activities obtained are much lower than those obtained in the isogenic strain TT1A-4, which harbors a multicopy plasmid with *CDC25* (Figure 5, Table II). This phenomenon may result from feedback inhibition by the *TPK1* gene product (Nikawa *et al.*, 1987) or a direct consequence of the absence of the CDC25 protein which results in the destabilization of the adenylyl cyclase complex.

The role of the CDC25 product as the GDP/GTP exchange protein is analogous to the role of the mammalian and avian hormone-bound receptors in transmembrane signalling systems where heterotrimeric G proteins function as transducers. The nucleotide sequence of the *CDC25* gene (Broek *et al.*, 1987) does not suggest a putative transmembrane sequence, nor is the structure reminiscent of any known receptor. The CDC25 protein may, however, serve as an intracellular detector, which responds to an as yet unknown molecule produced in response to glucose, or may be activated by the glucose transport system. Recently it was shown that glucose signalling requires an intact CDC25 gene and that the C-terminal region of the CDC25 protein is involved in the down regulation of the signal. This finding strengthens the hypothesis that CDC25 functions as a detector or receptor protein (Munder and Kuntzel, 1989). Attempts to determine whether the signalling molecule for the yeast adenylyl cyclase is glucose or an early glucose metabolite have so far been unsuccessful (Purwin *et al.*, 1986).

### *The CDC25 − RAS − cyclase complex*

The nature of the interaction between the known components of the system (CDC25, RAS and CDC35) is currently unclear. The relocalization of adenylyl cyclase activity to the membrane fraction by the CDC25 product (Table III), in the complete absence of RAS, with a concomitant increase in the intracellular cAMP level, suggests a CDC25 to CDC35 cross-talk in the absence of RAS. At present it is not clear whether there is a direct interaction between the cyclase and the CDC25 protein. The absence of RAS proteins seems to affect not only the GTP regulation of cyclase, but also its stability in the membrane. These three protein components of the system may generate a complex in which the CDC25 protein interacts directly with the cyclase in the cell membrane, although mechanistically it activates the cyclase through RAS. The small but significant influence of the mutated *CDC25* gene product (Figure 4A) or its absence (Figure 5A), on the $Mn^{2+}$ dependent adenylyl cyclase activity supports this hypothesis. The presence of relatively high $Mn^{2+}$ dependent adenylyl cyclase activity in the soluble fraction of our $cdc25^{ts}$ strain after temperature shift to the restrictive temperature (data not shown) also supports this notion. Studies on the hydrodynamic properties of the yeast adenylyl cyclase and its activation by trypsin may also support the idea of a multicomponent enzymatic complex (Heideman *et al.*, 1987). The low total activity observed in strain ras1ras2bcy1[TPK1, 2 $\mu$] (Table III) can be accounted for by the strong feedback inhibition observed in strains harboring active protein kinases (Nikawa *et al.*, 1987). We have currently no explanation, however, for the relatively low total activity observed in ras1ras2bcy1[CEN] and relatively high total activity observed in ras1ras2bcy1[CDC25, CEN] (Table III).

An alternative explanation for the results shown in Table III could be differential activation of a soluble and a membrane bound adenylyl cyclase enzyme rather than relocalization. Since there has been no reported evidence of a soluble adenylyl cyclase enzyme in *S.cerevisiae* and in purification experiment only one adenylyl cyclase enzyme was purified (Field *et al.*, 1988) we favor the relocalization hypothesis. Yet, the purified adenylyl cyclase enzyme appeared to be a multisubunit complex of 200 kd and 70 kd proteins, none of which is either RAS or CDC25 (Field *et al.*, 1988). It is possible that the conditions used for purification favor partial complex dissociation. Clearly, more studies are required in order to reveal the existence of such a putative complex.

Our failure to reconstitute the GTP-induced activity using the cyr1 mutant with either $cdc25^{ts}$, or ras1ras2bcy1 strains may be due to the fact that the lesion in the large adenylyl cyclase protein (Kataoka *et al.*, 1985b) caused by the *cyr1* mutation, loosens the enzymatic complex, and as a result the other protein components of the system are inactivated.

## Materials and methods

### *Strains and media*

Yeast strains used are listed in Table I. Wild-type relevant genes are denoted by capital letters and recessive mutations by lower case letters. Medium commonly used was SD (0.17% yeast nitrogen base without amino acids,

0.5% amonium sulfate and 2% glucose) plus the appropriate metabolites necessary for each strain. Strains TT1A-3 and TT1A-4 were grown on YEPD medium (1% yeast extract, 2% peptone and 2% glucose). Cultures were grown under vigorous shaking at 30°C. Temperature sensitive mutants were grown at 23°C as the permissive temperature. Shifts to the restrictive temperature were performed by growing cells at 23°C to $1 \times 10^7$ cells/ml and then transferring the culture to 36°C for 2 h.

### *Plasmids*

The plasmid YCp50-2 is a centromeric (single copy) plasmid derived from plasmid YCp50 (Rose *et al.*, 1987) and contains an insert of the *S.cerevisiae* genome with the gene *CDC25*. The plasmid pFl1-6 is a multicopy plasmid, derived from the 2 $\mu$ based plasmid YEp24 (Botstein *et al.*, 1979) also containing an insert with *CDC25*. The plasmid pFL1-8 is derived from the plasmid YEp24 and contains an insert of the *S.cerevisiae* genome with the gene *TPK1*. Plasmids YCp50-2, pFl1-6 and pFl1-8 were described previously (Daniel and Simchen, 1986).

Plasmids pTPK1 and pCDC25 which are present in strains TT1A-3 and TT1A-4 respectively (see Table I) are described by Broek *et al.* (1987). Both plasmids are derived from the plasmid YEp13 which is a 2 $\mu$ based plasmid (Sherman *et al.*, 1982). pTPK1 contains the gene *TPK1* and pCDC25 contains the gene *CDC25*.

### *Cell transformation*

Yeast cells were transformed with plasmid DNA using the lithium acetate method (Itoh *et al.*, 1983).

### *Preparation of lysates and membranes*

Yeast cultures were grown in a volume of 1 l to a concentration of $1-2 \times 10^7$ cells/ml and then crude extracts were prepared as described by Casperson *et al.* (1983). Cells were collected by centrifugation and washed with double distilled water (DDW) and then with 0.9 M sorbitol. Cells were incubated in 20 ml 0.9 M sorbitol plus 0.5 ml glusulase (NEN) at 25°C for 1 h to digest cell wall. Spheroplasts were washed with 0.8 M sorbitol, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM $MnCl_2$ and 50 mM HEPES pH 7.0 (buffer b) and resuspended in 5.0 ml buffer b plus 15 ml of 50 mM MES pH 6.0, 0.1 mM EDTA, 0.1 mM $MgCl_2$ and 1 mM phenylmethylsulfonylfluoride (PMSF) (buffer c). Spheroplasts were broken either with a dounce homogenizer (4 strokes), or in a cavitation bomb for 30 s under 500 p.s.i. nitrogen (we found no essential difference in the activity obtained by the two methods) and stored at −70°C until use. Lysate used was a supernatant of 200 *g* centrifugation for 10 min at 4°C of the crude preparation. Membranes used were obtained by centrifuging the lysate at 100 000 *g* for 30 min at 4°C and were suspended in buffer c to a final concentration of 3 mg/ml.

### *Adenylyl cyclase assay*

Adenylyl cyclase activity was assayed as described by Casperson *et al.* (1983) and [$^{32}$P]cAMP produced was determined as described by Solomon *et al.* (1974). The reaction mixture always contained ~50 $\mu$g protein in 50 mM MES pH 6.0, 0.1 mM EDTA, 20 mM creatine phosphate, 20 units/ml of creatine phosphokinase, 2.0 mM 2-mercaptoethanol, 1.0 mM [$\alpha$-$^{32}$P]ATP (10–20 c.p.m./pmol) (Amersham) and 1.0 mM [$^3$H]cAMP (12 000 c.p.m.) (Amersham) in a final volume of 150 $\mu$l. Divalent cations and nucleotides were added as described in the figure legends. The reaction was stopped by addition of 10 times stopping solution (2% SDS, 1 mM cAMP and 12 mM ATP), boiling for 3 min and addition of 1 ml of ice cold DDW. For kinetic experiments the reaction was peformed in a volume of 1.2 ml and at desired time points 150 $\mu$l samples were aliquoted into tubes containing 100 $\mu$l of 10 times stopping solution and treated as described above. For mixing membranes or lysates the procedure of Toda *et al.* (1985) was used with modifications. Lysates or membranes of the strains chosen for mixing (2 mg/ml) were dissolved in 0.06% lubrol PX with or without 400 $\mu$M GppNHp, and equal aliquots of each preparation were mixed together. Mixed and non-mixed preparations were incubated at 4°C for 15 min and at 25°C for 15 min before being added to the adenylyl cyclase reaction mixture and assayed.

### *cAMP assay*

Yeast cultures were grown in SD media to a concentration of $1 \times 10^7$ cells/ml and were extracted in 0.5 M $HClO_4$. The extracts were prepared for cAMP assay with the Amersham cAMP determination kit.

### *Analysis of kinetic data (equation 1)*

The curve and function that best fit the experimental data and the kinetic parameters of this curve were calculated using the ENZFITTER computer program for IBM-PC computer (ELSVIER-BIOSOFT).

## Acknowledgements

We wish to thank Dr K.Matsumoto and Dr M.Wigler for providing yeast strains. We also thank Mr H.Abeliovich for performing cAMP assays. This work was partially supported by a grant from the U.S.-Israel Binational Research Foundation, Jerusalem, Israel.

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*Received on September 25, 1989; revised on November 15, 1989*