MOLECULAR AND CELLULAR BIOLOGY, Oct. 1987, p. 3857–3861
0270-7306/87/103857-05$02.00/0

Vol. 7, No. 10

The Activation of Adenylate Cyclase by Guanyl Nucleotides in *Saccharomyces cerevisiae* Is Controlled by the *CDC25* Start Gene Product

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Received 2 March 1987/Accepted 8 June 1987

In the thermosensitive *cdc25* start mutant of *Saccharomyces cerevisiae*, the regulation of adenylate cyclase by guanyl nucleotides was rapidly nullified when the enzyme was prepared from nonsynchronized cells shifted to the restrictive temperature. In agreement with previous in vivo complementation studies, this biochemical defect was fully suppressed by the expression of either the whole cloned *CDC25* gene or its C-terminal portion. Moreover, membranes prepared from *cdc25*(Ts) cells grown at the permissive temperature evinced an altered regulation of adenylate cyclase by guanyl nucleotides. These results indicate that the CDC25 protein, together with RAS, is involved in the regulation of adenylate cyclase by guanyl nucleotides and raise the possibility that adenylate cyclase might form a ternary complex with RAS and CDC25.

Cyclic AMP is a key regulatory molecule in the life cycle of the yeast *Saccharomyces cerevisiae*, being involved in the control of cell proliferation and sporulation. Mutations in the adenylate cyclase *CDC35* (12, 13, 15) gene lead to growth arrest at the G1 phase of the cell cycle (15), and to induction of sporulation even though the medium is not appropriate for sporulation of the wild type (16). The phenotype of these mutants can be reverted either by addition of cyclic AMP to the medium (13) or by compensatory changes in the cyclic AMP pathway (11, 13). Also, two oncogene homologs of *S. cerevisiae RAS1* and *RAS2* (7, 8, 14), were found to code for GTP-GDP-binding proteins (9, 17) and to be involved in the activation of adenylate cyclase by guanyl nucleotides (1, 18, 19). Still another gene, *CDC25*, appears to be related to the cyclic AMP pathway. In fact, the phenotype of *cdc25*(Ts) mutants is very similar to that of *cdc35*(Ts) mutants (15, 16). Moreover, as recently described, the level of cyclic AMP decreases significantly in *cdc25*(Ts) cells when they are shifted to the restrictive temperature, and the addition of cyclic AMP to the medium suppresses the growth thermosensitivity of these cells (3). We have thus studied the adenylate cyclase activity in membranes prepared from *cdc25*(Ts) cells harvested before and after a shift to the restrictive temperature. Results presented here strongly suggest that the CDC25 protein, together with RAS, is involved in the regulation of adenylate cyclase by guanyl nucleotides.

Essentially two strains were used throughout this work: a *cdc25*(Ts) mutant and a transformant derivative carrying a centromeric plasmid (YCp50-2) containing the whole *CDC25* gene, thus enabling the cell to grow at a high temperature (5, 6). Strains were grown exponentially at 24°C in SD medium (yeast nitrogen base with ammonium sulfate and no amino acids [Difco Laboratories] plus 2% glucose) supplemented with the required nutrients (6). After a concentration of 1.4×10^7 to 1.7×10^7 cells per ml was reached, half of the culture (about 100 ml) was filtered through a nitrocellulose membrane (0.45-μm pore size; Millipore Corp.), whereas the other half was shifted to 34°C for 19 min (i.e., 15 min after temperature equilibration) before being filtered. Cell lysates and membranes were prepared (at temperatures not exceeding 22°C), and the adenylate cyclase activity of membranes was assayed, essentially as described previously (4). For both strains grown at the permissive temperature, there was a two- to threefold stimulation of the adenylate cyclase by Gpp(NH)p in the presence of Mg^{2+}, in agreement with the value reported by Casperson et al. (4) (Fig. 1). However, after the cultures were shifted to the restrictive temperature for 19 min, the stimulation of adenylate cyclase by Gpp(NH)p was nearly completely abolished in the *cdc25*(Ts) mutant, whereas it remained unaltered in the derivative carrying the centromeric suppressor plasmid. This difference between the two strains was even more dramatic after a 60-min shift at the restrictive temperature (Fig. 2, top and middle). We also did a temperature shift experiment with *cdc25*(Ts) derivatives carrying a multicopy plasmid containing either the active C-terminal portion of the *CDC25* gene (pFl1-2-2GX) or the whole *CDC25* gene (pFl1-2-2SX) (5). The results with these strains were basically similar to those found with the strain carrying the YCp50-2 centromeric plasmid (Fig. 2, middle and bottom).

These observations, therefore, suggest that the CDC25 protein, and more specifically its C-terminal portion, is somehow required for stabilizing the G protein-adenylate cyclase complex. Moreover, a 19-min shift to high temperature was found to be sufficient for nullifying most of the guanyl nucleotide regulation of the adenylate cyclase in the mutant. Since this time period represents less than 1/12 of the generation time of this strain, the stabilizing effect of the CDC25 protein is likely to occur not only at G1, but at all phases of the cell cycle.

To clarify further the role of the CDC25 protein, we studied the effect of various guanyl nucleotides and of

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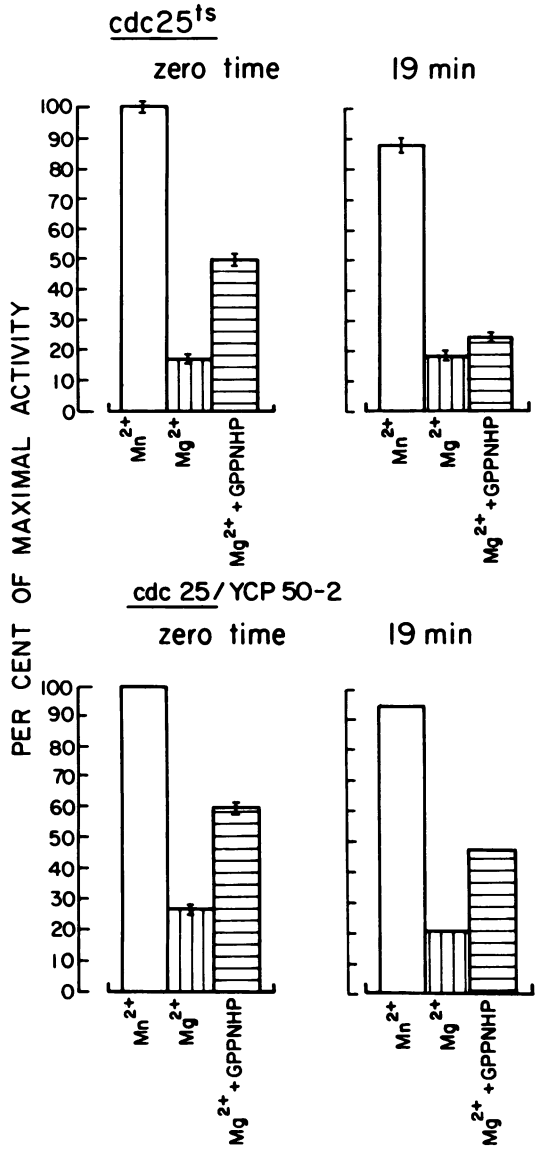

FIG. 1. Loss of guanyl nucleotide-sensitive adenylate cyclase activity of *cdc25*(Ts) cells after a temperature shift. Adenylate cyclase of membranes was assayed as described previously (4), except that reactions were done at 24°C and after the stop solution was added, samples were boiled for 3 min. Concentrations of divalent cations and effectors were as follows: 1 mM Mn^{2+}, 10 mM Mg^{2+}, and 0.1 mM Gpp(NH)p. The RAS-independent measure of adenylate cyclase activity obtained in the presence of Mn^{2+} from cells grown at the permissive temperature was taken as 100% and was 71 pmol/min per mg of protein for the *cdc25*(Ts) strain and 83 pmol/min per mg of protein for the *cdc25*(YCp50-2) strain. The bars reflect the standard deviation of three separate determinations.

temperature on the adenylate cyclase activity of membranes prepared from the *cdc25*(Ts) mutant grown at the permissive temperature. The results of a typical experiment are presented in Fig. 3. The activation of adenylate cyclase by the nonhydrolyzable GTP analog, Gpp(NH)p, whether assayed at 24 or 34°C, was almost as high in the *cdc25*(Ts) mutant as in the two control [wild-type and *cdc25*(Ts)(YCp50-2)] membranes. However, GTP, guanosine 5′-γ-thiotriphosphate, and mostly guanosine 5′-β-thiodiphosphate, at both temperatures, resulted in a significantly lower reproducible adenylate cyclase activity in the *cdc25*(Ts) mutant than in the control membranes.

The results presented in Fig. 3 thus indicate that the CDC25 protein somehow controls the regulation of adenylate cyclase by guanyl nucleotides. This is in agreement with a recent independent report (2). In addition, we showed that in *cdc25* cells shifted to high temperature, the activation of adenylate cyclase by guanyl nucleotides was rapidly lost (Fig. 1 and 2). This loss of activation might have resulted from the disruption of an unstable RAS-adenylate cyclase complex at high temperature in vivo that could not reform in vitro. This thermosensitive biochemical phenotype in vivo (Fig. 1 and 2) did not have an equivalent in vitro (Fig. 3). Basically, guanyl nucleotide regulation can be detected in membranes prepared from *cdc25*(Ts) cells, grown at the permissive temperature but assayed at the nonpermissive temperature. These results suggest that the CDC25 protein is already functionally defective at 24°C, leading to a weaker interaction between the CDC25 protein and the RAS-adenylate cyclase complex. At 34°C, this defect would lead to loss of the guanyl nucleotide regulation of the adenylate cyclase in vivo but not in vitro.

An intriguing possibility is that CDC25 might function as a detector protein, in a manner similar to that of the hormone receptors of higher eucaryotes. We previously raised this possibility in view of the regional homology we found between CDC25 and the putative ligand-binding site of cytochromes P450. We hypothesized that CDC25 might detect some intracellular metabolite and convey this signal to the RAS-adenylate cyclase complex (5). This speculation is supported by the observation that the main alteration seen in the adenylate cyclase from the *cdc25*(Ts) mutant, compared with that of the control strains, was its poor stimulation by guanosine 5′-β-thiodiphosphate and GTP, whereas stimulation by Gpp(NH)p remained relatively high (Fig. 3). This suggests that the normal CDC25 protein could act by facilitating the GDP-GTP exchange on RAS, in a manner similar to what occurs during the interaction between hormone receptors and G proteins in higher eucaryotes.

It is unlikely that the CDC25 protein acts by an indirect mechanism, such as by altering the membrane state or modulating the level of some metabolite. In fact, such indirect effects could not easily account for the differences in adenylate cyclase response toward various guanyl nucleotides observed in the *cdc25*(Ts) mutant, relative to the control strains. Two more likely alternative possibilities can be envisaged: the CDC25 protein either is an integral part of or is a modifying enzyme of the RAS-adenylate cyclase complex. We favor the first hypothesis since, as previously proposed (10), the regulation of adenylate cyclase by RAS protein(s) probably involves some additional component(s). Moreover, sequence data of the *CDC25* gene do not reveal any homology with known modifying enzymes (3, 5).

We plan to test these hypotheses with antibodies raised against the CDC25 protein. Also, since mammalian RAS can reconstitute yeast adenylate cyclase (1), we are considering

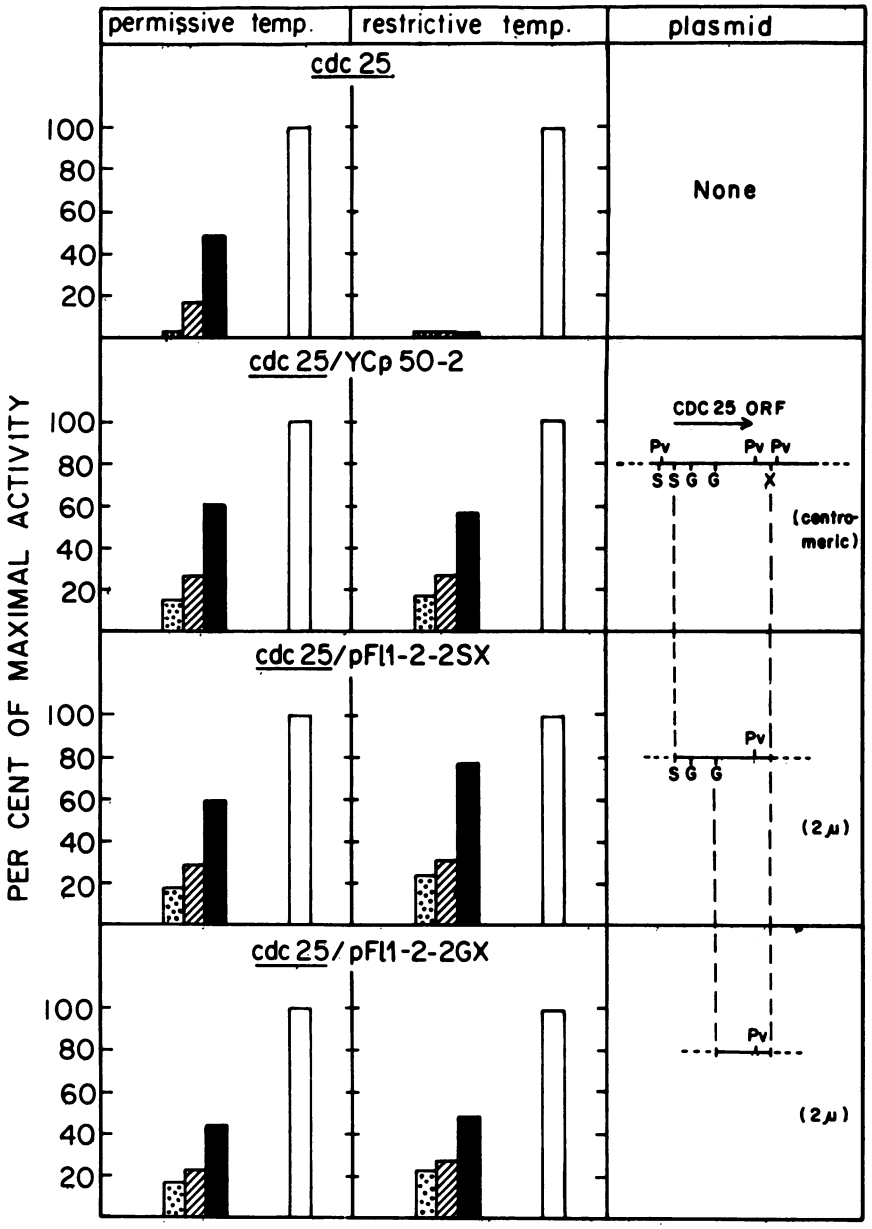

FIG. 2. Guanyl nucleotide-sensitive adenylate cyclase of *cdc25*(Ts) cells carrying different suppressor plasmids after a temperature shift. The relevant characteristics of the plasmids carried by the *cdc25*(Ts) strain are presented at the right side of figure. CDC25 ORF means CDC25 open reading frame. Restriction sites are as follows: Pv, *Pvu*II; S, *Sal*I; G, *Bgl*II; X, *Xho*I (6). Growth and temperature shift of strains and cell lysate and membranes preparation were done as described in the legend to Fig. 1, except the duration of the temperature shift to 34°C was 60 min. Concentrations of Mn^{2+}, Mg^{2+}, and Gpp(NH)p were the same as those described in the legend to Fig. 1. The concentration of guanosine 5'-β-thio diphosphate was 0.1 mM. Mn^{2+}-supported activity was taken as 100% in each case and was as follows for this particular experiment (in picomoles per minute per milligram of protein): for *cdc25*(Ts), 54 (24°C) and 10 (34°C); for *cdc25*(Ts)(YCp50-2), 40 (24°C) and 70 (34°C); for *cdc25*(Ts)(pFl1-2-2SX), 57 (24°C) and 34 (34°C); and for *cdc25*(Ts)(pFl1-2-2GX), 34 (24°C) and 41 (34°C). Results obtained with a *CDC25* wild-type strain, isogenic to the *cdc25*(Ts) strain except for the *CDC25* allele replacement, were basically similar to the *cdc25*(Ts) strains carrying the various plasmids. Symbols: ▭, Mg^{2+} plus guanosine 5'-β-thiodiphosphate; ▨, Mg^{2+}; ■, Mg^{2+} plus Gpp(NH)p; □, Mn^{2+}.

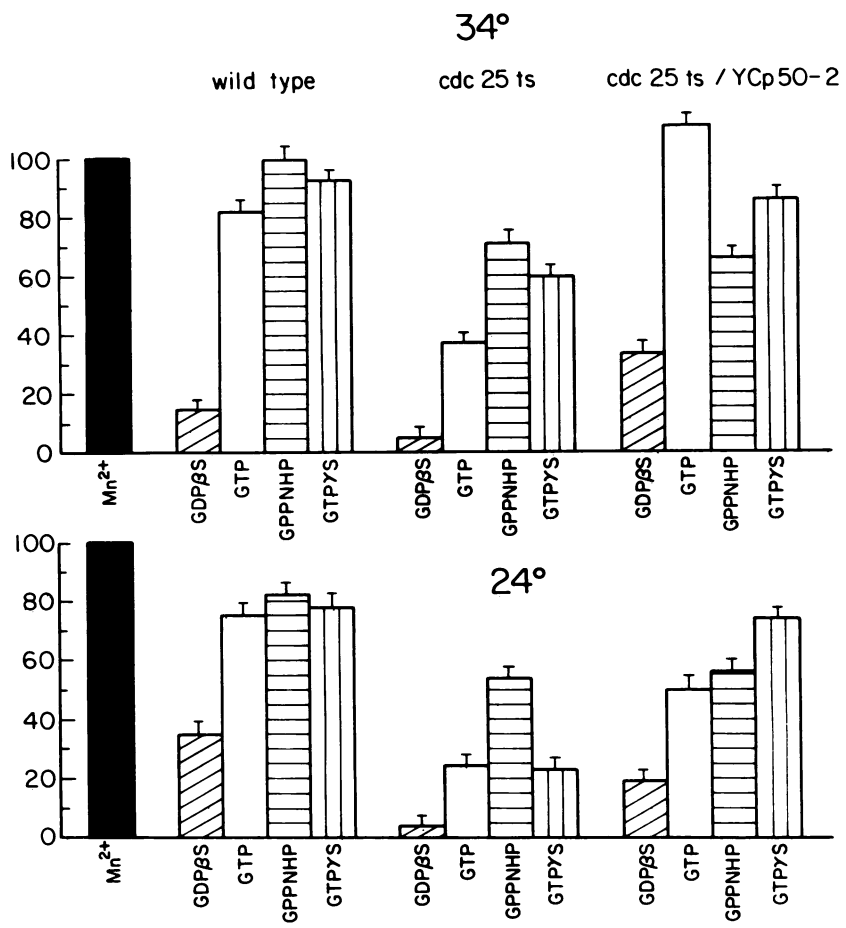

FIG. 3. Effect of various nucleotides and of temperature on the adenylate cyclase from the wild-type, *cdc25*(Ts), and *cdc25*(Ts)(YCp50-2) strains. Adenylate cyclase activity was measured at 34 (top) and 24°C (bottom) in membranes prepared from wild-type (see the legend to Fig. 2), *cdc25*(Ts), and *cdc25*(Ts)(YCp50-2) strains grown at the permissive temperature (24°C). Growth conditions at 24°C, membrane preparations, and adenylate cyclase assays were as described in the legend to Fig. 1. Concentrations of cations and effectors were as follows: 1 mM Mn^{2+}; 10 mM Mg^{2+}; 0.1 mM guanosine 5'-β-thiodiphosphate; 0.1 mM GTP; 0.1 mM Gpp(NH)p; and 0.1 mM guanosine 5'-γ-thiotriphosphate. Scale and bars described in the legend to Fig. 1. Mn^{2+}-supported activity at each assay temperature was taken as 100% and was (in picomoles per minute per milligram of protein) 142 (34°C) and 69 (24°C) for the wild type, 76 (34°C) and 29 (24°C) for *cdc25*(Ts) cells, and 120 (34°C) and 107 (24°C) for *cdc25*(YCp50-2) cells. Results presented are from one typical experiment among three independent experiments giving essentially identical results.

the possibility that a CDC25 homolog exists that is involved in the RAS system of mammalian cells. We are therefore searching for *CDC25*-related genes in higher eucaryotes.

J.M.B. was supported as a Fogarty Senior International Scholar, National Institutes of Health, during the course of this work.
J.D. is on leave from the Institut Pasteur (Paris).

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