The N-Terminal Half of Cdc25 Is Essential for Processing Glucose Signaling in *Saccharomyces cerevisiae*

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ABSTRACT: *Saccharomyces cerevisiae* Cdc25 is the prototype Ras GDP/GTP exchange protein. Its C-terminal catalytic domain was found to be highly conserved in the homologues $p140^{ras-GRF}$ and Sos. The regulatory domains in each Ras exchanger mediate the signals arriving from upstream elements such as tyrosine kinases for Sos, or Ca^{2+} and G proteins for $p140.^{Ras-GRF}$ In this study, we show that the N-terminal half (NTH) of *S. cerevisiae* Cdc25**,** as well as the C-terminal 37 amino acids, is essential for processing the elevation of cAMP in response to glucose. The mammalian $p140^{ras-GRF}$ catalytic domain (CGRF) restores glucose signaling in *S. cerevisiae* only if tethered between the N-terminal half (NTH) of *S. cerevisiae* Cdc25 and the C-terminal 37 amino acids. The glucose-induced transient elevation in cAMP is nullified or severely hampered by the deletion of domains within the NTH of Cdc25. These deletions, however, do not modify the intrinsic GDP/GTP exchange activity of mutant proteins as compared to native Cdc25. We also show that 7 Ser to Ala mutations at the cAMP-dependent protein kinase putative phosphorylation sites within the NTH of Cdc25 eliminate the descending portion of the glucose response curve, responsible for signal termination. These findings support a dual role of the NTH of Cdc25 in both enabling the glucose signal and being responsible for its attenuation.

Genetic and biochemical evidence have demonstrated that the *CDC25* gene product is a Ras guanyl nucleotide exchange factor (ras-GEF) that functions upstream to the Ras proteins in *Saccharomyces cerevisiae* (*1*−*4*). Furthermore, Cdc25 is essential for the glucose-induced rise in intracellular levels of cyclic AMP (cAMP), suggesting that the protein functions as a glucose-mediated Ras-GTP/GDP exchange protein (*5*).

The molecular mechanisms responsible for glucose-induced activation of the Cdc25 exchange activity are unknown. Similarly, the precise molecular mechanisms responsible for the activation of the Cdc25 mammalian homologue $p140^{ras-GRF}$ in response to Ca^{2+} and G proteins (*6*−*9*) remain elusive. In the case of *mammalian* Sos it was shown that its recruitment to the membrane and consequent juxtaposition to Ras are sufficient for its activation (*10*), although experiments on Sos suggest a more complex mechanism (*11*−*14*). The detailed study of the role of regulatory domains within Cdc25 in the regulation of Ras-dependent activation of adenylyl cyclase in *S. cerevisiae* may shed light on the mechanism of Ras exchangers in general. Thus, for example, it has already been shown that the attenuation of the exchange activity of yeast Cdc25 (*15*) as well as of Sos (*16*, *17*) is mediated by phosphorylation. Yeast Cdc25 and mammalian $p140^{ras-GRF}$, unlike Sos, do not lie downstream to and respond to signals from protein tyrosine kinases. Both yeast Cdc25 and mammalian $p140^{ras-GRF}$ are large proteins (140 to 180 kDa, respectively) in which a nonconserved noncatalytic N-terminal domain, which comprises about two-thirds of the molecule, is followed by a conserved catalytic region.

It is therefore likely that the N-terminal noncatalytic domains of ras-GRFs play an important regulatory role in both yeast and mammalian systems, in view of their long lengths, which have been conserved through evolution. These noncatalytic regions contain an SH3 domain in yeast Ras exchangers and a PH domain in mammalian ras-GRFs (*18*, *19*). The phosphorylation of Cdc25 at its N-terminal domain seems to attenuate the glucose response (*15*). In this study, we further analyze the role of the N-terminal half (NTH) of the Cdc25 molecule. We present a structure−function analysis of the Cdc25 NTH and show that it plays a crucial role in processing the glucose signal, which induces the transient rise in intracellular cAMP.

EXPERIMENTAL PROCEDURES

Strains, Media, and Genetic Techniques. S. cerevisiae strains used in this study are described in Table 1. Yeast cells were grown either in YEPD medium (1% yeast extract, 2% peptone, and 2% dextrose) or in SD medium (0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, and 2% dextrose) supplemented with the required amino acids. Starvation medium is YEPD medium containing 0.02% dextrose instead of 2%. For acetate growth curves, the cells were grown in YPA (1% yeast extract, 2% peptone, and 1% potassium acetate). Solid media contained 2% agar. The permissive incubation temperature for thermosensitive strains was 23 °C and the restrictive temperature was 37 °C. Genetic techniques were according to Sambrook et al. (*20*).

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Table 1: Strains Used in Study

strain	genotype	short name
TT1-YNP	*MATa his3 leu2 ura3 trp1 ade8 cdc25::URA3*, with plasmid pYNPala (H-ras 2μm *TRP1*)	p21
TT1-CDC25	*MATa his3 leu2 ura3 trp1 ade8 cdc25::URA3*, with plasmid pRS413-25 (*CDC25 CEN HIS3*)	CDC25/FL
TT1-CDC25−7m	*MATa his3 leu2 ura3 trp1 ade8 cdc25::URA3*, with plasmid pRS413−25−7m (*CDC25-7m CEN HIS3*)	cdc25-7m
TT1-CDC25-dH	*MATa his3 leu2 ura3 trp1 ade8 cdc25::URA3*, with plasmid pRS413-25-dH (*CDC25-dH CEN HIS3*)	dH
TT1-CDC25-dH.1	*MATa his3 leu2 ura3 trp1 ade8 cdc25::URA3*, with plasmid pRS413-25-dH.1 (*CDC25-dH.1 CEN HIS3*)	dH.1
TT1-CDC25-dH.2	*MATa his3 leu2 ura3 trp1 ade8 cdc25::URA3*, with plasmid pRS413-25-dH.2 (*CDC25-dH.2 CEN HIS3*)	dH.2
TT1-CDC25-dB	*MATa his3 leu2 ura3 trp1 ade8 cdc25::URA3*, with plasmid pRS413-25-dB (*CDC25-dB CEN HIS3*)	dB
TT1−25-Ct	*MATa his3 leu2 ura3 trp1 ade8 cdc25::URA3*, with plasmid pRS413-25-Ct (*CDC25-Ct CEN HIS3*)	Ct
TT1−25-Ct (2μm)	*MATa his3 leu2 ura3 trp1 ade8 cdc25::URA3*, with plasmid pRS413-25-Ct (*CDC25-Ct 2μm HIS3*)	Ct (2μ)
MS-LL1	*MATa cdc25-2 ura3-52 lys2 leu2-3,112 trp1 his3d-200 ade2*	cdc25^{ts}
TT1-Cat-RasGRF	*MATa his3 leu2 ura3 trp1 ade8 cdc25::URA3*, with plasmid pad4-GEF-Ct (rasGEF-Ct *2μm LEU2*)	CGRF (2μ)
TT1-N25/CGEF	*MATa his3 leu2 ura3 trp1 ade8 cdc25::URA3*, with plasmid pRS413-25/GEF (N25/CGRF *CEN HIS3*)	N25/CGRF
TT1-N25/CGRF/37	MATa his3 leu2 ura3 trp1 ade8 cdc25:URA3, with plasmid pRS413-25/GRF/37 (N25/CGRF/37 CEN HIS3)	N25/CGRF/37
TT1-Cdc25Δ37	MATa his3 leu2 ura3 trp1 ade8 cdc25:URA3, with plasmid pRS413-cdc25Δ37 (cdc25 Δ37 CEN HIS3)	cdc25Δ37

PCR reactions were performed with Taq polymerase (Advanced Biotech) using the recommended procedure. PCR of long fragments was performed with Taq Plus (Stratagene). Yeast transformation was performed according to Ito et al. (*21*). The strain TT1-YNP, disrupted in its endogenous *CDC25* gene (see Table 1), was used for transforming all the plasmids carrying deletion mutants and point mutants of the *CDC25* gene. Transformants were chosen that had lost the plasmid pTRP-H-ras, and gained a pHIS-cdc25 plasmid.

Plasmids. The centromeric plasmid pRS413 (22) harboring the *HIS3* gene was used for the expression of the full-length CDC25, the deletion mutants and the point mutants of CDC25, and the chimeras between CDC25 and rasGRF. pRS413-25 carries the full length *CDC25* gene (a *Sal*I−*Pvu*II fragment encoding amino acids 1−1588); pRS413-25-7m carries seven point mutations in the classical cAMP dependent kinase (cAPK) phosphorylation sites; pRS413-25-dH carries a deletion of the *Hpa*I−*Hpa*I fragment (encoding amino acids 113−348); pRS413-dH.1 carries a deletion of a fragment encoding amino acids 231−348; pRS413-dH.2 carries a deletion of a fragment encoding amino acids 113−230; pRS413-dB carries a deletion of the *Bgl*II−*Bgl*II fragment (encoding amino acids 352−875); pRS413-25-Ct carries the 3′ end of *CDC25* (a *Bgl*II−*Pvu*II fragment encoding amino acids 875−1588); pRS423-25-Ct is identical to pRS413-25-Ct plasmid except that it carries the 2 μm origin of replication (*22*).

pAD4-GRF-Ct carries the 3′ end of Ras-GRF (a *Xho*I−*Xho*I fragment encoding amino acids 844−1260). pAD4 is a shuttle vector harboring the yeast ADH promoter and terminator, the *LEU2* gene and the 2 μm origin of replication (*23*).

pRS413-N25/CGRF carries a chimeric gene comprising the 5′ end of *CDC25* (a *Sal*I−*Bam*HI fragment encoding amino acids 1−1253) and the 3′ end of Ras-GRF (a PCR fragment encoding amino acids 1005−1260).

The chimera N25/CGRF/37 consist of a *Sma*I−*Bam*HI fragment from the 5′ end of CDC25 followed by a PCR rasGRF fragment encoding amino acids 1005−1259 and the 3′ end encoding the last 37 amino acids of Cdc25. The chimera was cloned in pRS413.

The cdc25Δ37 plasmid consists of pRS413-25 in which the *Sph*I−*Not*I fragment (encoding the last 37 amino acids of Cdc25) was deleted.

pYNPala carries the mammalian H-ras gene. This plasmid harbors the yeast PGK promoter and the *TRP1* gene (*24*).

Site-Directed Mutagenesis, DNA Sequencing, and Sequence Analysis. Templates for site-directed mutagenesis were prepared by cloning genomic DNA fragments into Bluescript plasmids (Stratagene). Site-directed mutagenesis was performed with the Mutagenesis version 2.1 kit (Amersham) using the recommended procedure, and the mutagenesis plasmid were transformed into *Escherichia coli* TG1. The oligonucleotides used for creating the mutations were labeled with 32P and used to screen for point mutations, by colony hybridization.

DNA sequencing of the positive clones was performed by the chain-termination method of Sanger et al. (*25*) using the Sequence version 2.0 kit (USB). Single-stranded DNA was prepared by super-infection of *E. coli* TG1 cells with R408 helper phage (Stratagene). Sequencing reaction products were run on a Long ranger (AT Biochem) gel using the recommended procedure. Sequence analysis was done with the aid of the GCG sequence analysis software package, version 7.0 (26).

Preparation of Protein Extracts and Western Blotting. Cell lysates and membranes for Western blot analysis were prepared as described by Garreau et al. (*27*). Yeast cells were broken by vigorous vortexing with glass beads (0.4−0.6 mm diameter) in ice-cold buffer: 50 mM 4-morpholinethansul-

fonic acid (Mes), pH 6, 0.1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM 2-mercaptoethanol, and the following protease inhibitors, 313 μg/mL benzamidine, 1.36 μg/mL pepstatin-A, 5 μg/mL leupeptin, 2 μg/mL antipain, 2 μg/mL chymostatin, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, 2 mM phenylmethanesulfonyl fluoride, and 4 mM *o*-phenanthroline (all from Sigma). The mixture was then centrifuged at 800*g* for 5 min at 4 °C to remove unbroken cells and glass beads. The supernatant was referred to as the lysate. Crude membranes were prepared by centrifuging the lysate at 300000*g* for 10 min at 4 °C. Protein concentrations were determined according to Lowry et al. (*28*) or according to Peterson (*29*).

Lysates or membrane samples containing ~30 μg of protein were boiled in Laemmli sample buffer (*30*), and subjected to SDS−polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose using a semidry blotter (LKB) at 0.8 mA/cm^2 for 2 h. Blots were incubated for 1 h at room temperature with Tris-buffered saline containing 5% powdered skimmed milk (Cadbury) and 0.3% Tween 20 and allowed to react overnight at 4 °C with the primary antibody in the same solution. The immunopurified anti-Cdc25 antibody was used at a 1:100 dilution (*31*), and the anti-Ras-GRF antibody (Santa Cruz) was used at 1:500 dilution. Blots were washed seven times for 5−10 min, each time with Tris-buffered saline containing 0.2% Tween 20, and incubated with the second antibody for 2 h at room temperature in the same solution used for the first antibody. Horseradish-peroxidase-conjugated mouse anti-rabbit antibodies (Santa Cruz) were used at a 1:20000 dilution. The blots were visualized using the ECL detection kit (Amersham).

Gel Shift Assay. Yeast cells were grown in 10 mL of YEPD medium at 30 °C to a density of 1 to 2 × 10^7 cells/mL and then harvested and transferred to starvation medium (see above). After overnight incubation, glucose was added to a final concentration of 2% for 30 s and 1 mL aliquots were transferred to test tubes on ice containing 20 mM NaN_3 and 600 μg/mL cyclohexamide. The cells were broken by vigorous vortexing with glass beads as described above. The protein samples were separated on 7.5% acrylamide/0.4% bis-acrylamide gels in order to obtain clear separation between the phosphorylated and nonphosphorylated form of Cdc25.

cAMP Assay. Yeast cells were grown in 50 mL of YEPD medium at 30 °C to a density of 10^7 cells/mL and then harvested and transferred to starvation medium, as described by Kaibuchi et al. (*32*). After overnight incubation, the cells were washed once, resuspended in 50 mL of 50 mM Mes, pH 6.0, and incubated for 15 min at 30 °C while shaking, prior to glucose addition. Glucose was added to a final concentration of 2% and 1 mL aliquots were transferred at different time points to test tubes, each containing 0.3 mL of 20% perchloric acid. The tubes were incubated on ice for 2 h and centrifuged and 1 mL of the supernatant was transferred to a new tube and neutralized by adding 0.4 mL of 2 M $KHCO_3$. The neutralized samples were frozen at −20 °C for 2 h, thawed to ensure the release of CO_2, and centrifuged. One milliliter of the supernatant was subsequently transferred to a new tube and acetylation of the samples was performed with triethylamine/acetic anhydride (2:1). cAMP was determined by radioimunoassay using ^{125}I-labeled anti-(acetylated cAMP) antibodies (Sigma).

Preparation of Membranes and Adenylyl Cyclase Assay. Yeast cells were grown in 1 L of YEPD to a density of 1 to 2 × 10^7 cells/mL, and the membranes were prepared as described by Casperson et al. (*33*) with a few changes. The cells were harvested by centrifugation and washed once with double-distilled water and once with 0.9 M Sorbitol. The cells were then resuspended in 20 mL of 0.9 M Sorbitol, and the cell wall was digested by the addition of 1 mL of glusulase (NEN) and incubation at 25 °C for 1−3 h. Spheroplasts were washed once with buffer B (0.8 M sorbitol, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) pH 7, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1 mM EDTA pH 8, 1 mM $MnCl_2$) and resuspended in 3 mL of buffer B and 7 mL of buffer C (50 mM Mes, pH 6, 0.1 mM $MgCl_2$, 0.1 mM EDTA (pH 8), and 1 mM PMSF (phenylmethanesulfonyl fluoride). Spheroplasts were broken with a tight-fit dounce homogenizer (5−20 strokes), and the crude lysate was stored at −70 °C until use. The membrane preparation was obtained by centrifugation of the crude extract at 200*g* for 10 min followed by centrifugation of the supernatant at 300000**g** for 10 min. The membrane pellet was suspended in buffer C to a final concentration of ~3 mg/mL.

Adenylyl cyclase activity in membrane fractions was assayed as described by Casparson et al. (*33*). The reaction was carried out at 30 °C for 60 min and started by the addition of ~100 μg of protein to the reaction mixture that contained 50 mM Mes, pH 6, 1 mM [^{3}H]cAMP (10 000 cpm), 1 mM [α-^{32}P]ATP (20−50 cpm/pmol), 20 units/mL creatine phosphokinase, 20 mM creatine phosphate, 2 mM 2-mercaptoethanol, and 0.1 mM EGTA, supplemented with either 2.5 mM Mn^{2+} or 10 mM Mg^{2+} with 0.1 mM guanosine 5′-[β,γ-imido]triphosphate (Gpp(NH)p) or 0.1 mM guanosine 5′-*O*-(2′-thiodiphosphate) (GDPβ-S) in a final volume of 150 μL. The reaction was terminated by the addition of 100 μL of stopping solution (2% SDS, 1 mM cAMP, and 12 mM ATP). Kinetic experiments were performed as described by Engelberg et al. (*34*). [^{32}P]cAMP levels were determined as described by Solomon et al. (*35*).

RESULTS

Deletion of the Cdc25 N-Terminal Domain Nullifies the Glucose Response in Vivo without Affecting Guanyl Nucleotide-Dependent Cyclase Activity in Vitro. We compared in detail the effects of various mutations in the NTH of Cdc25 on intracellular levels of cAMP in response to the reintroduction of glucose following overnight starvation ("glucose response") and on the efficacy of GDP/GTP exchange in vitro. To this end, a series of *cdc25*-carrying plasmids, in which all or part of the NTH of Cdc25 was deleted, were introduced into a *cdc25*-disrupted mutant, within a centromeric plasmid (Figures 1 and 2). All the constructs allow the *cdc25*-disrupted mutant to grow and also allow a *cdc25^{ts}* strain to grow at a nonpermissive temperature (data not shown), demonstrating that the ras pathway remains active. However, in cells in which the entire NTH domain of Cdc25 was deleted (Ct, Δ1−875), the level of cAMP was very low. These cells did not respond to glucose with a rise in cAMP, unlike cells that expressed the full-length Cdc25, which showed a sharp rise in cAMP levels in response to the reintroduction of glucose into the medium (FL; Figure 3a). Adenylyl cyclase activation appeared to be unaffected in the

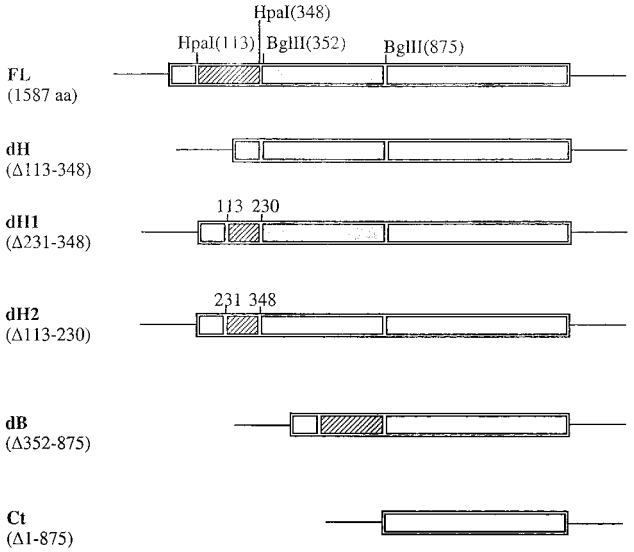

FIGURE 1: Scheme of the *cdc25* mutant protein products, numbers refer to amino acids in Cdc25, sites of DNA restriction enzymes used to make the deletions are shown. All the constructs were cloned on a centromeric plasmid unless mentioned.

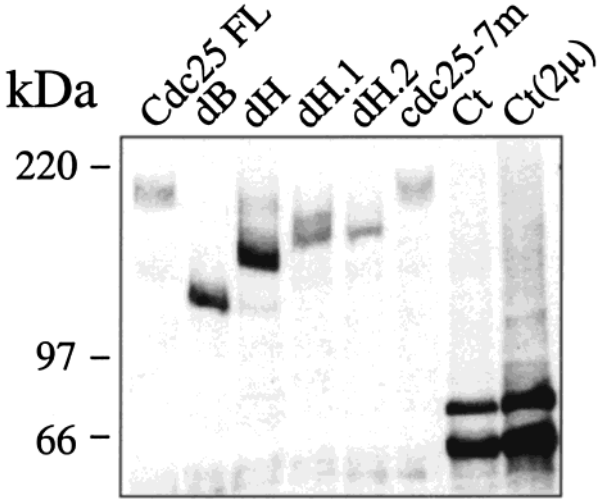

FIGURE 2: Immunoblot analysis of the Cdc25 deletion mutants depicted in Figure 1. Ct-cen, the Ct construct cloned on a centromeric plasmid; Ct-2μ, the Ct construct cloned in a multicopy plasmid. The numbers on the left refer to kilodaltons. The same amount of total protein was loaded in each lane.

mutant. In membranes prepared from the two strains, we found no significant differences in the Mg^{2+}-Gpp(NH)p-dependent cyclase activities, nor was there a significant difference in the ratios of cyclase activity in the presence of GppNHp to activity in the presence of GDPβS (Figure 4b and Table 2). Furthermore, the values of $k_{obs,}$ namely the rate of cyclase activation in the presence of Gpp(NH)p, did not differ significantly between the two strains (data not shown). These parameters measure quantitatively the rate of GDP/GppNHp exchange and were measured as previously described (*33*). The glucose response curves using intact cells suggest that the NTH is essential for processing the glucose response, but the kinetic analyses using isolated membranes suggest that the C-terminus on its own suffices for the intrinsic efficacy of the Cdc25/Ras/cyclase interaction in vitro.

This interpretation was supported by the finding that overexpression of the C-terminal half of Cdc25 from a

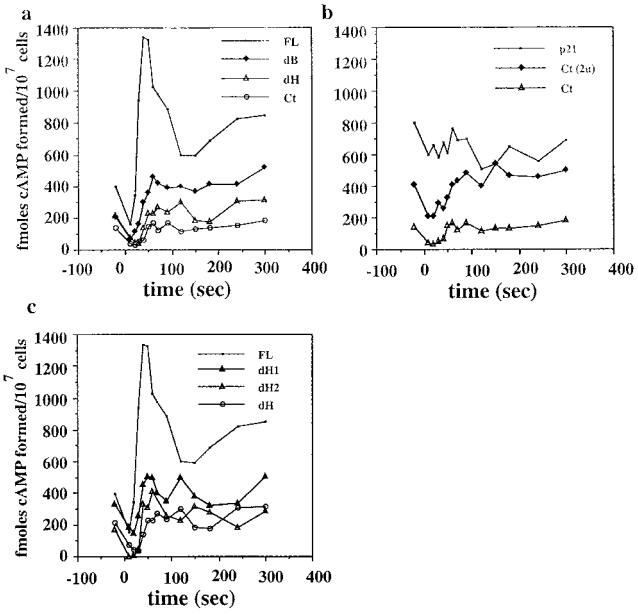

FIGURE 3: Glucose response of the mutant strains. a. FL, dB, dH, Ct deletion mutants depicted in Figure 1. (b) Mammalian p21 H-Ras, comparison between Ct (depicted in Figure 1) and Ct (2μ). (c) The dH deletion mutants.

multicopy plasmid [Ct (2μ)] led to elevated basal cAMP levels but not to a normal glucose-induced cAMP increase (Figure 3b, Table 3). In vitro, Ct (2μ) conferred high basal activity in the presence of GDPβS (Table 2). Figure 4b depicts an isogenic strain into which $p21^{H-ras}$ (p21) was introduced. $p21^{H-ras}$ is not recognized by Ira1 or Ira2, the GTPase activating proteins of S.cerevisiae and is constitutively active in yeast (*36, 37*). In this case high basal levels of cAMP are obtained with no glucose response (Figure 3b).

To identify the subdomains within the NTH that are involved in generating the glucose response, we examined the effects of four smaller in-frame deletions within the region (Figures 1 and 2). None of these deletions affected catalytic exchange in vitro (Figure 4a, Table 2, and see below), as expected from the behavior of the Ct construct. Yet, in vivo, the patterns of the glucose response curves differed among the various mutants (Figure 3, panels a and c). Deletion of the amino acid residues 352−875 (dB) led to low basal levels of cAMP, but fairly normal induction of cAMP in response to glucose (Figure 3a and Table 3). The difference in fold activation between the full-length Cdc25 (FL) and dB could also imply a minor involvement of this region in glucose induction, but to our understanding, this difference is in the range of error of the assay. On the other hand, deletion of amino acid residues 113−348 (dH) led to low basal cAMP levels and no induction of cAMP in response to glucose. The region defined by dH was further delineated, by looking at strains dH.1 (Δ231−348) and dH.2 (Δ113−230). dH.1 cells showed high basal levels of cAMP but poor induction of cAMP in response to glucose (Figure 3c and Table 3). dH.2 cells exhibited diminished basal and steady-state levels of cAMP, but some increase in response to glucose.

The differences in the basal and inducible cAMP level seem not to be related to the expression level of the constructs. The mutants dB, dH, and dH.2 show low basal cAMP levels, whereas the expression level varies between

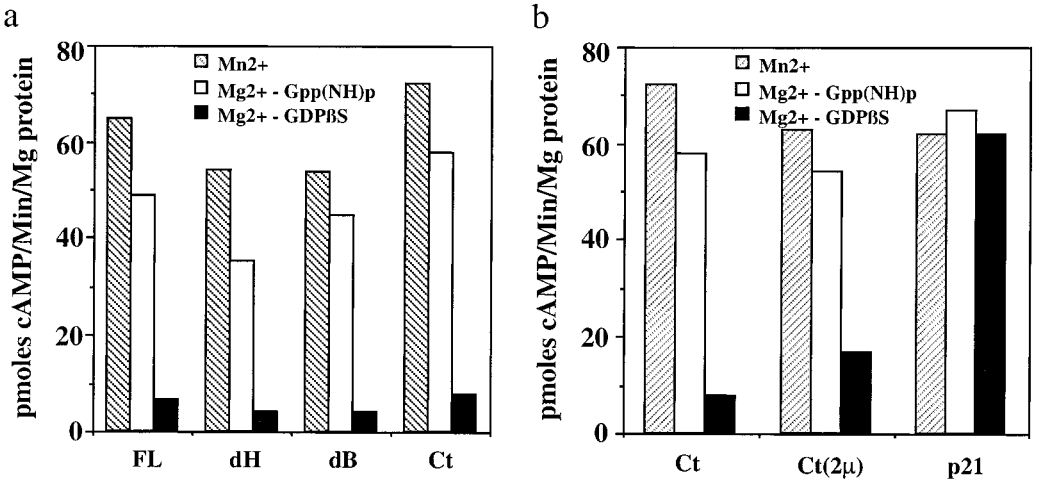

FIGURE 4: Adenylyl cyclase activity of the mutant strains. The data depicted is the average of two independent sets of experiments on parallel batches of cells. Results between the two sets were within 4−9% where each cAMP determination was conducted in triplicate. Repetitive experiments yield absolute values within 10% or less of the values depicted here. Other experimental details are given in the Experimental Procedures.

Table 2: Adenylyl Cyclase Activity of CDC25 Mutants[a]

	FL	dH	dB	Ct	Ct (2μ)	p21	dH.1	dH.2
Mn^{2+}	61.6 ± 7	49.4 ± 5	53.7 ± 3	72.2 ± 6.0	62.7 ± 7.1	61.8 ± 3.5	58 ± 5.6	68.6 ± 6.6
Mg^{2+} Gpp NHp	40.3 ± 7	31 ± 4	44.7 ± 5.1	57.9 ± 5.1	54.1 ± 3.1	67 ± 4.5	41.4 ± 3.4	47.9 ± 5.1
Mg^{2+} GDPβS	6.1 ± 1	3.9 ± 0.5	4.2 ± 0.5	7.9 ± 0.8	16.9 ± 1.1	62.2 ± 6.6	4.1 ± 0.3	7.6 ± 0.6
Gpp(NH)p GDPβS	7 ± 0.9	7.9 ± 1	10.6 ± 1.1	7.3 ± 1.1	3.2 ± 1.3	1.1 ± 0.1	10.1 ± 1.2	6.3 ± 0.5
k_{obs} (min^{-1})	0.13 ± 0.02	0.15 ± 0.03	ND	ND	0.29 ± 0.04	ND	0.07 ± 0.02	0.12 ± 0.03
V_{max} (pmol/min/mg)	40.3 ± 4.1	26.4 ± 2.1	ND	ND	67 ± 7.3	ND	43.1 ± 5.1	35.2 ± 2.8

[a] Data are from three or four independent experiments with standard error of the mean.

Table 3: Parameters of Glucose Signaling of Cdc25 Mutants[a]

	levels of cAMP							
	FL	dH	dB	Ct	Ct(2μ)	p21	dH.1	dH.2
starved	350 ± 65	246 ± 81	183 ± 90	143 ± 22	469 ± 68	796	332	171
glucose (3′)	637 ± 125	256 ± 60	337 ± 123	183 ± 43	498 ± 54	652	381	286
fold activation	3.8 ± 0.7	1.4 ± 0.6	2.87	1.62 ± 0.6	1.06 ± 0.1	0.96	1.53	2.4

[a] Numbers are in femtomoles of *cAMP* per 10^7 cells, data from Figure 3. Data were collected from four independent experiments with standard error of the mean. For experiments in which SEM is not given only two experiments were performed but there was less than 8% difference between the data from the two experiments. Starved: the level of cAMP (fmol/10^7 cells) before addition of glucose (−20 s). Glucose (3′): the level of cAMP 3 min after the addition of glucose. Fold activation: the ratio between the highest cAMP level (45−65 s after glucose addition) and the starvation cAMP level.

them. On the other hand dH.1 and dH.2 have similar expression levels but their effects are different (Figure 2). Our results suggest that the domain from amino acid 231 to 348 regulates the glucose response and the domains from amino acid 113 to 230 and from 352 to 875 regulate basal levels of cAMP (Figure 5). Interestingly, the 113−230 domain (dH, Figure 1) is the region phosphorylated in response to glucose (*15* and see below).

Membranes prepared from cells harboring these *cdc25* mutations and membranes from cells harboring full-length Cdc25 exhibited identical Gpp(NH)p-dependent adenylyl cyclase activity, similar ratios between the Gpp(NH)p- and GDPβS-dependent activities and similar rates of cyclase activation (k_{obs}) by Mg^{2+}-Gpp(NH)p in vitro (Figure 4, Table 2). Hence, the N-terminal deletions do not affect the ability of the mutant Cdc25 proteins to catalyze guanyl nucleotide exchange on Ras proteins, but rather affect transmission of the glucose signal to Ras proteins in the intact cell.

NTH and the C-Terminal 37 Amino Acids of Yeast Cdc25 Mediate the Transmission of External Signals to the Catalytic Region of the Mammalian CGRF. To further evaluate the role of the N-terminal region of *S. cerevisiae* Cdc25, we constructed a chimeric gene comprising the NTH of CDC25 (N25) and the C-terminal catalytic domain of the mammalian Cdc25 homologue ($Cdc25^{Mm}$/$p140^{ras-GRF}$) (*38*) depicted as CGRF (Figures 6 and 7). Overexpression from a multicopy plasmid or expression from a centromeric plasmid of CGRF alone permitted a $cdc25^{ts}$ mutant to grow at the nonpermissive temperature (not shown) as shown earlier by Martegani et al. (1992) and as also shown for the C-terminal region of *Drosophila* Sos (*39*). Expression of CGRF did not, however, confer the ability to elevate intracellular cAMP levels in response to glucose, and the basal cAMP levels in these cells were very low (Figure 8a). Expression of the chimeric gene from a centromeric plasmid in which CGRF was fused to the NTH of *S. cerevisiae* Cdc25 (N25) enabled cells to respond to glucose with a rise in cAMP levels (Figures 6 and 7), but the descending portion of the response curve was missing (Figure 8a). Comparison of the amino acid sequences of *S. cerevisiae* Cdc25 and $p140^{ras-GRF}$ showed that the most

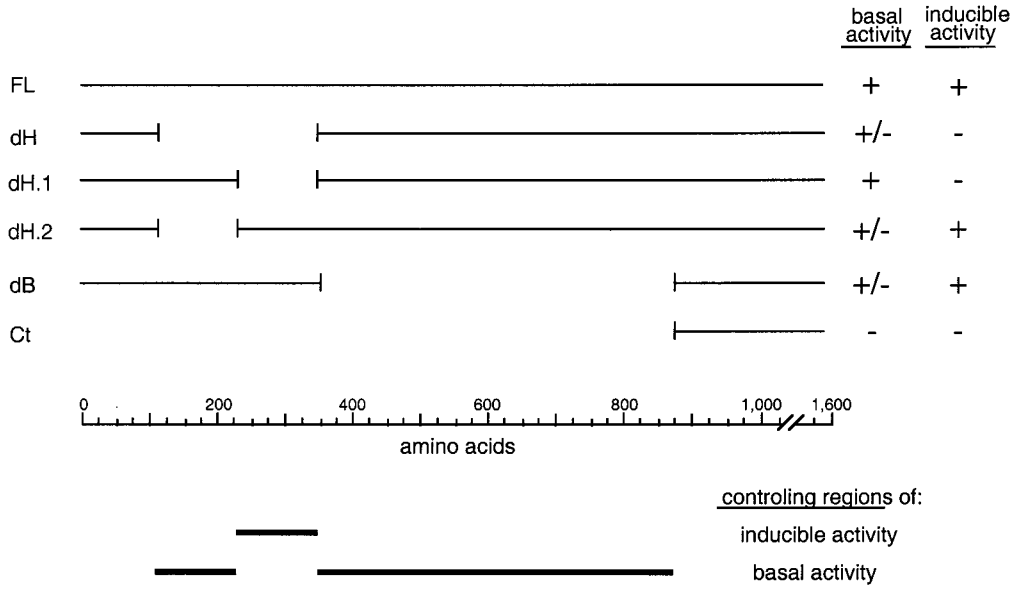

FIGURE 5: Domains in Cdc25 which regulate inducible and basal adenylyl cyclase activity. Data from Figure 1 and Table 2 suggest that one may differentiate between the role of the domains in sustaining the basal level of cAMP and the glucose induced rise in cAMP.

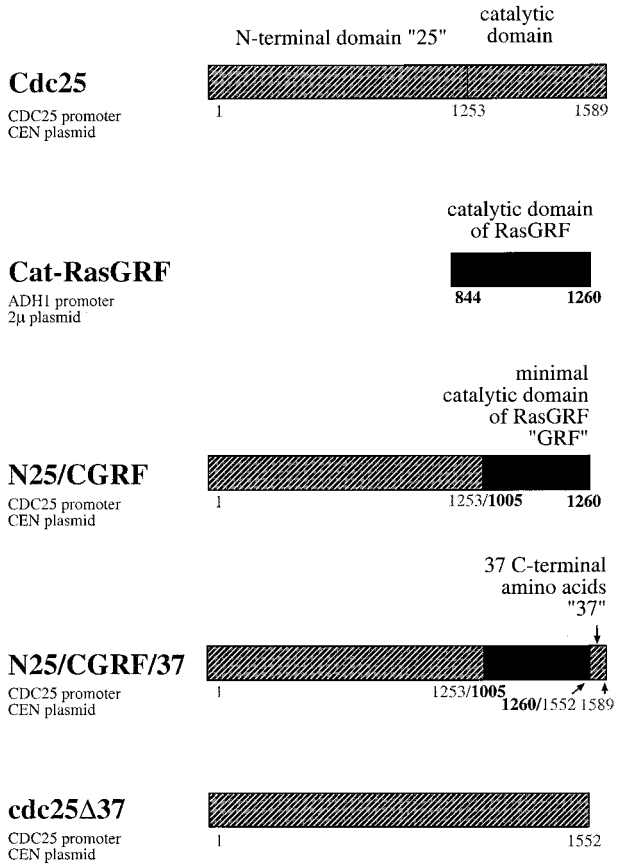

FIGURE 6: Chimeras between CDC25 and p140-rasGRF. Numbers refer to the respective protein amino acids.

terminal 37 amino acid residues of Cdc25 are very divergent. Furthermore, truncation of these 37 amino acids from Cdc25 was reported to lead to an altered glucose response (*5, 40*). We therefore constructed another chimeric gene, to express a fusion protein including the NTH of Cdc25 (N25), the catalytic domain of rasGRF (CGRF) and the terminal 37 amino acid residues of Cdc25, in that order (N25/CGRF/37). This chimera expressed from a centromeric plasmid (Figures 6 and 7) led to a glucose response approaching that of cells harboring the wild-type plasmid (Figure 8b); intracellular cAMP levels rose sharply upon reintroduction of glucose into the medium and thereafter decreased to the steady-state induced level.

C-Terminal 37 Amino Acids Are Not Essential for Native Cdc25. Deletion of the 37 terminal amino acids from native Cdc25 does not affect the glucose response of cells harboring this construct (Figures 7 and 8c). This is in contrast to a previous report (*5*). In this study, however, the gene was disrupted, but no molecular or biochemical characterization of the construct was reported. It is possible that the disruption actually resulted in a replacement of the 37 amino acids by a protein segment from the disrupting gene. Deletion of Cdc25 37 C-terminal amino acids did not affect the basal cAMP level. The small differences in this level in Figure 8c was not reproducible and is within the experimental error.

Cdc25 Is Mainly Phosphorylated in Its N-Terminal Domain. We have previously demonstrated that addition of glucose to glucose-starved cells induces rapid phosphorylation of Cdc25, probably by cAMP dependent kinase (cAPK; *15*). Deletion of amino acids 113−348 (mutant dH), which abolished the glucose response (Figure 3), also completely abolished the glucose-induced phosphorylation and mobility shift (*15*, Figure 9A). The mutated Cdc25 protein produced by strains harboring plasmid dH.1 (Δ231−348) apparently retains the sites which when phosphorylated in response to glucose, still induce mobility shift upon addition of glucose (Figure 9A). The mutated Cdc25 produced by plasmid dH.2 (Δ113−230), however, was only slightly upshifted following addition of glucose (Figure 9A). A computerized search of the Cdc25 sequence revealed seven "classical" cAPK phosphorylation sites, all serines residing in the N-terminal region. Four of these putative cAPK phosphorylation sites reside in the 113−230 region (Figure 10A, dH.1 in Figure 1). Mutating each of the four serines separately, or all four to alanines, did not abolish completely the glucose-induced phosphorylation of Cdc25 but eliminated most of the upshift in the electrophoretic mobility of the Cdc25 protein, after

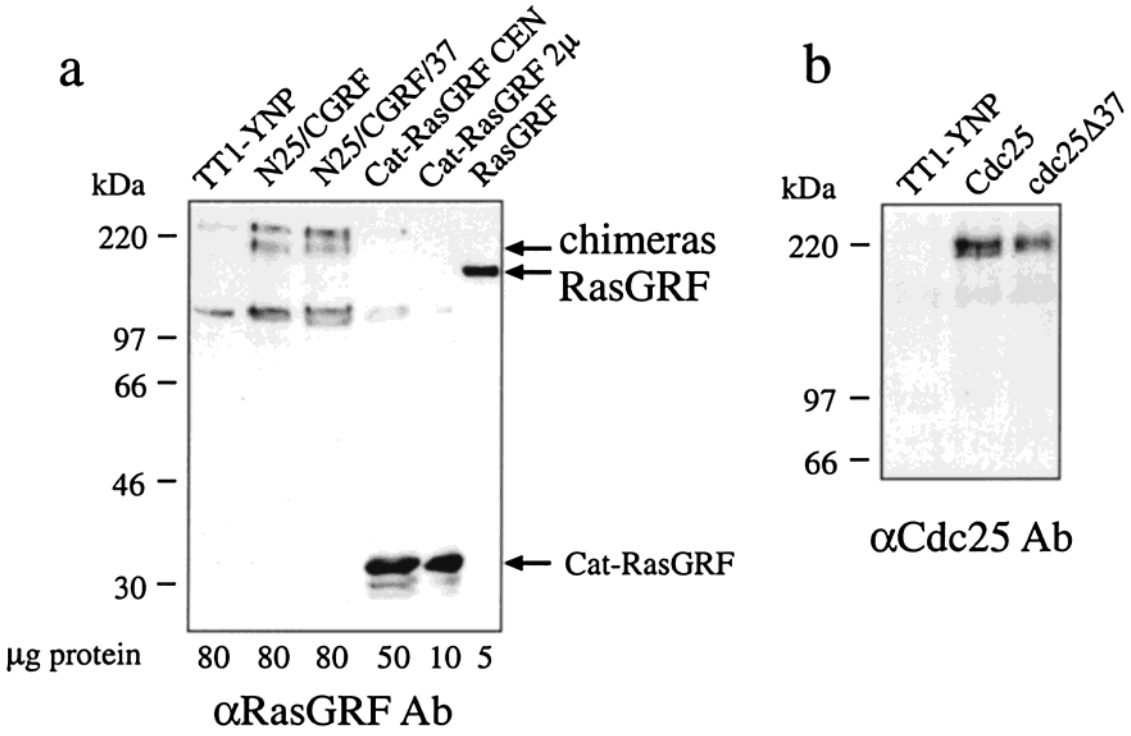

FIGURE 7: Immunoblot analysis of the chimeras in Figure 6.

glucose challenge (Gross, Ph.D.thesis, 1996, data not shown). On the other hand, mutating all seven serines to alanine (cdc25-7m) led to the abolishment of the descending portion of the typical glucose-induced cAMP signal (Figure 10B) concomitantly to the complete obliteration of the glucose-induced electrophoretic mobility upshift of the mutated Cdc25-7m (Figure 9B).

Cells with the cdc25-7m plasmid grew much more slowly on acetate medium than isogenic cells expressing wild-type Cdc25 (Figure 11). This phenotype is similar to that of the mutants with an overactive Ras-adenylyl cyclase pathway as mutants of bcy1 (the catalytic subunit of cAPK) or mutants expressing $Ras2^{Val19}$ (*41*).

DISCUSSION

Regulatory Role of the N-Terminal Domain. Although several Ras exchangers from yeast, flies, and mammals have been cloned and studied, their exact mode of regulation has not been completely elucidated. In this study, we show that the noncatalytic N-terminal half (NTH) of Cdc25 plays an important role in enabling yeast cells to respond to glucose and in sustaining basal levels of cAMP in unstimulated cells. Deletions within this region dramatically reduce the capacity of the cyclase system to respond to glucose (Figure 3) and/or sustain basal cAMP levels. These deletions do not affect the guanyl nucleotide-dependent cyclase activity in membranes prepared from these cells (Figure 4, Table 2). Furthermore, the ratio of GppNHp to GDPβS-dependent activity and the rate of cyclase activation by Mg^{2+}-GppNHp (*34*) are all very similar (Figure 4, Table 2). These findings rule out the trivial explanation that the deletions abolish the exchange activity of Cdc25 and therefore obliterate its ability to transmit the glucose signal to the Ras/cyclase complex. Our preferred explanation is that the NTH of Cdc25 participates in receiving the glucose signal, and transmits this signal to a macromolecular complex that includes Ras and the Cyr1/Cdc35 cyclase. Thus, deletions within this region as in dH, dH.1, dH.2, and dB (Figures 1 and 3) interfere with the ability of the Cdc25/Ras/cyclase complex to respond to glucose and to generate cAMP under normal physiological conditions. Deletions at the 5′-end of CDC25 have previously been shown to lead to defects in carbohydrate metabolism and in sporulation (*5, 40*), consistent with our proposal that such deletions lead to impaired ability to sense the presence/absence of glucose. The N-terminal region of Cdc25 has also been shown to interact directly with the cyclase Cyr1/Cdc35 (*44*). The finding that cells expressing the chimeric protein N25/CGRF/37 respond almost normally to glucose (Figure 8b) supports our hypothesis. The C-terminal 37 amino acids of *S. cerevisiae* Cdc25 also appear to be required for normal glucose signaling (Figure 8b) when the catalytic domain (CGRF) originates from the mammalian gene. These amino acid residues may confer on Cdc25 a conformation essential for the fidelity of the glucose response.

The N-terminal region of Cdc25 was shown to possess a cyclin destruction box (*42*). It is interesting to note that deletion of amino acids 113−348 (dH) or 352−875 (dB) as well as the whole NTH (Ct) induce overexpression of the protein whereas smaller deletions or mutations such as in dH.1, dH.2, or cdc25-7m do not change the expression level. This finding suggests that the domains regulating the stability of Cdc25 are dispersed through the entire NTH.

The noncatalytic domains of Sos also appear to play an important regulatory role in vivo. A detailed structure−function analysis of DSos in *Drosophila* embryos and in COS cells showed that the N-terminal noncatalytic region of Sos regulates the activity of its catalytic region, although it is still unclear whether the N-terminus plays a role in transducing signals (*11, 39*). Domains within this region (e.g.,

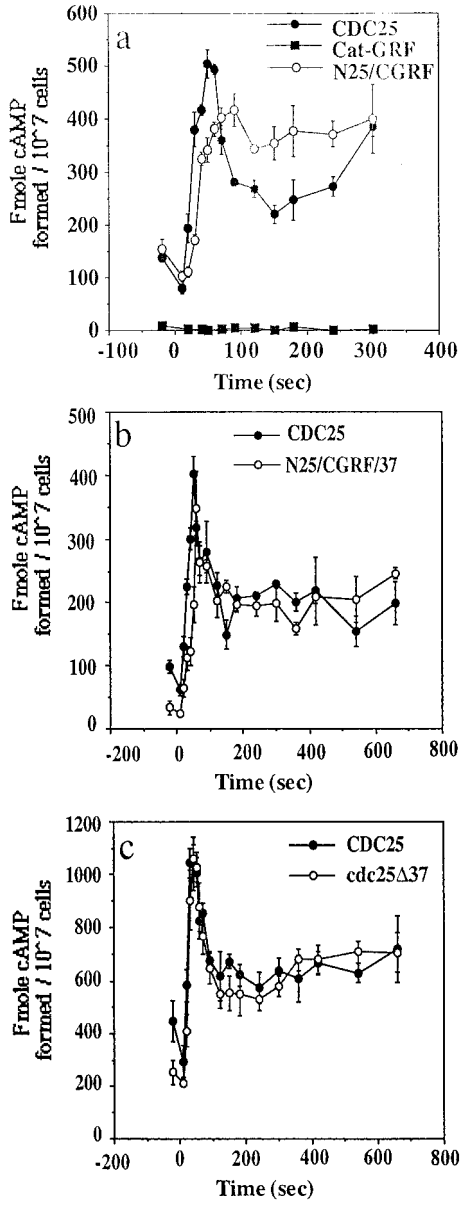

FIGURE 8: Glucose response of the chimeras in Figure 6. Experimental conditions are identical to those described in Figure 1 and in the Experimental Procedures.

the PH domain) may play a role in localizing the protein in the plasma 11). Another noncatalytic region, the C-terminus of mSos1, inhibits the activity of the protein in vivo and its deletion enhances transformation (*10, 12*).

Our findings demonstrates that the C-terminal domain of Cdc25 plays no role in transmission of the glucose signal, but is exclusively involved in catalyzing GDP/GTP exchange. Furthermore, the N-terminal domain appears to be exclusively involved in signal processing and not in catalytic activity. Thus, on one hand, yeast cells expressing the C-terminal domain of Cdc25 do not respond normally to glucose in vivo (Figure 3b). On the other hand, the intrinsic catalytic activity of the C-terminal domain of Cdc25 does not appear to be impaired by the truncations in the N-terminal domain, and membranes from such cells are fully competent to carry out GDP/GTP exchange in vitro (Figure 4). Similarly, Wang et al. (*12*) found no difference between the in vitro exchange activity of the full-length Sos compared to a C-terminus deleted Sos that retains the catalytic domain.

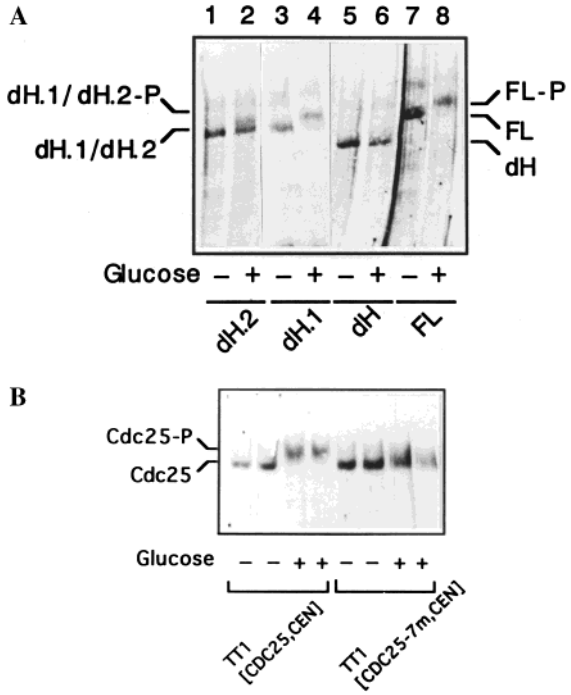

FIGURE 9: The electrophoretic mobility of truncated and mutated Cdc25 proteins. CDC25/dH, CDC25/dH1 and CDC25/dH2 were expressed from a centromeric plasmid in a *cdc25^{ts}* strain (Table 1). Cells were grown at 23 and 37° and then exposed to glucose, at the same temperature. Cdc25 proteins were analyzed on SDS−PAGE. The CDC25 gene was mutated in the 7 putative serine cAPK phosphorylation sites as described in Figure 10 and the Experimental Procedures. (A) Truncated Cdc25 proteins. (B) The Ser Ala mutated Cdc25 (CDC25-7m).

Thus, the effect of the regulatory domains is detectable in vivo but not in vitro. It is likely that relatively weak protein−protein interactions, which are functional in the context of the undisrupted intact cell, become disrupted upon cell breakage.

Overexpression of the catalytic region of p140$^{Ras-GRF}$ (CGRF) "bypasses" the need for Cdc25 and permits growth of yeast cells carrying the *cdc25^{ts}* mutation, confirming the original findings which led to the identification and cloning of p140$^{Ras-GRF}$ (*7*). Similarly, overexpression of the catalytic region of *Drosophila* Sos (DmSos) permits growth of *cdc25^{ts}* yeast mutants (*39*). The role of the N-terminal noncatalytic region of Cdc25 may have been overlooked because overexpression of the catalytic region alone is sufficient to functionally rescue the *cdc25^{ts}* phenotype. Another contributing factor was a report which claimed that yeast cells overexpressing the C-terminus of Cdc25 respond normally to glucose (*43*), a finding which we have been unable to confirm with our C-terminus construct (Figure 3, panels a and b). It is possible that there is a difference in the protein product between Van Aelst et al. work and ours because of the lack of promoter in our C-terminus construct. In Van Aelst et al. work, no characterization of the protein product was reported so a direct comparison cannot be made. The small difference in protein size might be the cause for the difference in the glucose-response result. We find that overexpression of the Ct-Cdc25 causes a dramatic rise in the basal levels of cAMP in the glucose-starved state, with the complete absence of the expected glucose-induced rise in cAMP (Figure 3, Table 3). Our data are in agreement with those of Munder et al. who showed that deletions within the

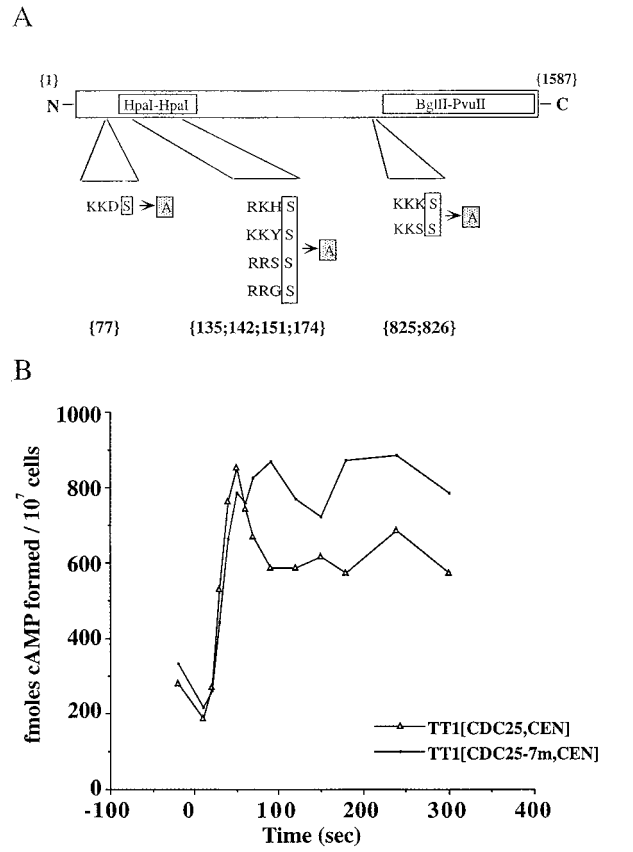

FIGURE 10: Glucose response of phosphorylation defective Cdc25. (A) The seven putative Ser cAPK phosphorylated sites mutated to Ala. Point mutations were introduced using Oligonucleotide-Directed in vitro Mutagenesis Kit version 2.1 (Amersham). The *Sal*1−*Eco*RV and *Eco*RV−*Bam*HI fragments of CDC25 were separately cloned into plasmid pBluescript II KS (+) (Stratagene). Point mutations were verified by DNA sequencing. Plasmid DNA was prepared from colonies carrying the point mutations and the *Sal*1−*Eco*RV and *Eco*RV−*Bam*HI fragments were used to replace the wild-type fragments of *CDC25*. Yeast cells were transformed with plasmids carrying the replaced fragments. Other details are described in the Experimental Procedures. (B) Glucose-induced cAMP signal of cells containing the mutated Cdc25. Glucose-induced cAMP levels of TT1 [CDC25, CEN] or TT1 [CDC25-7m, CEN] after overnight glucose-starvation. Cyclic AMP was determined by radioimmunoassay using ^{125}I-labeled anti-(acetylated-cAMP) antibodies (Sigma). The graphs shown represent an average of three independent experiments.

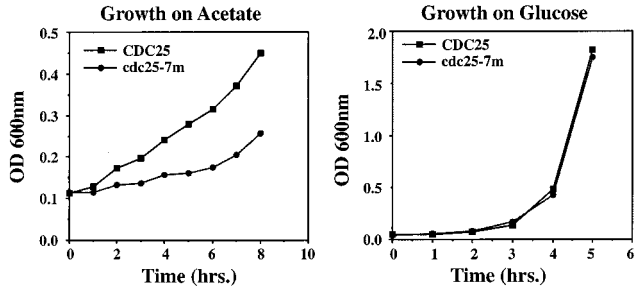

FIGURE 11: Growth curve on acetate medium of cells containing the full-length Cdc25 or the mutated Cdc25-7m. Cells were grown in acetate medium (1% yeast extract, 2% peptone, and 1% potassium acetate). Optical density (OD) was measured at 600 nm. Other experimental details are given in the Experimental Procedures.

N-terminus of Cdc25 completely abolished the glucose response (*5*).

One issue of interest is whether Cdc25 is itself the signal receiver or is actually part of a multimolecular complex, which processes the glucose signal. We have previously argued that Cdc25 is essential for allowing the Ras-activated cyclase (Cdc35/Cyr1) to respond to the glucose signal but is not itself the signal receiver. Indeed, an activated mutant of cyclase when expressed in yeast allows glucose response to occur, albeit in a somewhat aberrant form, in the absence of Cdc25 altogether (*45*). The present study suggests that the N-terminal domain of Cdc25 participates in processing the glucose signal. One possibility is that in addition to interactions with Cdc35/Cyr (*44*) this domain may interact with other proteins such as the glucose transporter, glucokinase, and/or hexokinase (*45*).

Possible Role of the 37 Terminal Amino Acids. The C-terminal 37 amino acids are essential for reinstating the declining portion of the glucose signal when elicited from a protein in which the catalytic domain is of mammalian origin (Figure 8b). The terminal 37 amino acids are highly enriched with positively charged amino acids. These amino acids may interact with the N-terminal phosphorylated amino acids after glucose exposure, bringing about a folding of Cdc25, resulting in the attenuation in its catalytic activity. This folding or conformational change could cause the observed translocation of Cdc25 from the membrane to the cytosol upon glucose signaling (*15*).The segment between amino acids 1488 and 1511 that possesses eight positively charged amino acids and only two negatively charged amino acids, may also participate in this interaction with the phosphorylated NTH. This segment is missing from the chimera N25/CGRF as compared to native Cdc25, which may account for this difference in behavior of the two constructs. At this stage, one cannot attribute a major role to the 37 terminal amino acids of Cdc25 since their deletion does not affect the glucose signal as compared to native Cdc25. It is however possible that this short domain contributes to the stability of the Cdc25/cyclase complex.

Possible Role of Phosphorylation of the NTH in Signal Termination. In addition to the role of the N-terminal regulatory domains of Cdc25 in glucose sensing, they appear to be involved in signal termination. Sequence analysis of this region revealed seven "classical" cAMP-dependent kinase (cAPK) phosphorylation sites (Figure 10A). We have already previously shown that Cdc25 undergoes phosphorylation, which results in the attenuation of the glucose signal and an upward mobility shift upon electrophoresis (*15*). Similarly, phosphorylation has been shown to play a role in the negative regulation of Sos. Sos phosphorylation by MAP kinase causes its dissociation from Grb2 (*16*, *17*, *46*−*49*). This phosphorylation probably weakens Sos localization to the membrane, thus attenuating its in vivo exchange activity without affecting its intrinsic catalytic activity. We propose that the glucose response is attenuated by phosphorylation of Cdc25. In our studies, deletion of amino acid residues 113−348 (plasmid dH) eliminated the glucose-induced phosphorylation of Cdc25 (Figure 9A and ref *15*). Deletion of the domain 113−230 (dH.2, Figure 1), which possesses four of the putative cAPK phosphorylation sites (Figure 10A), abolish the upward mobility shift (Figure 9A). Site-directed mutagenesis of all seven putative cAPK sites (Figure 10A) completely abolished the glucose-induced mobility shift (Figure 9B). This mutant did not alter the ascending portion of the glucose response curve, but abolished the decline in cAMP production that normally follows the transient rise in

cAMP (Figure 10B). These results support the suggestion that phosphorylation of Cdc25 attenuates its activity in the intact cell, leading to attenuation of the glucose-induced cAMP formation (*15*). Mutation of the phosphorylation sites did not affect the intrinsic catalytic activity of Cdc25. Membranes from cells harboring the *cdc25* plasmid in which all seven putative cAPK sites had been mutated exhibited normal Gpp(NH)p-dependent cyclase activity, a normal ratio of Gpp(NH)p-dependent to GDPβS-dependent cyclase activities and normal k_{obs} values (not shown). The phosphorylation sites in the N-terminal domain are close to the SH3 domain of the protein. It is possible that phosphorylation of these sites weakens the interaction of this domain with the adenylyl cyclase (Cdc35/Cyr1) (*44*), which in turn attenuates the response of the macromolecular complex to glucose.

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