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Influence of Guanine Nucleotides on Complex Formation between Ras and CDC25 Proteins

CHAR-CHANG LAI,[1] MARK BOGUSKI,[2] DANIEL BROEK,[3]* AND SCOTT POWERS[1,4]†

Graduate Program in Biochemistry, Rutgers University, and Department of Biochemistry, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854[1]; National Center for Biotechnology Information National Library of Medicine, National Institutes of Health, Bethesda, Maryland 20894[2]; and Department of Biochemistry and Norris Cancer Center, University of Southern California School of Medicine, Los Angeles, California 90033,[3] and Onyx Pharmaceuticals, Richmond, California 94806[4]

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The *Saccharomyces cerevisiae CDC25* gene and closely homologous genes in other eukaryotes encode guanine nucleotide exchange factors for Ras proteins. We have determined the minimal region of the budding yeast *CDC25* gene capable of activity in vivo. The region required for full biological activity is approximately 450 residues and contains two segments homologous to other proteins: one found in both Ras-specific exchange factors and the more distant Bud5 and Ltel proteins, and a smaller segment of 48 amino acids found only in the Ras-specific exchange factors. When expressed in *Escherichia coli* as a fusion protein, this region of CDC25 was found to be a potent catalyst of GDP-GTP exchange on yeast Ras2 as well as human $p21^{H\text{-}ras}$ but inactive in promoting exchange on the Ras-related proteins Ypt1 and Rsr1. The CDC25 fusion protein catalyzed replacement of GDP-bound to Ras2 with GTP (activation) more efficiently than that of the reverse reaction of replacement of GTP for GDP (deactivation), consistent with prior genetic analysis of *CDC25* which indicated a positive role in the activation of Ras. To more directly study the physical interaction of CDC25 and Ras proteins, we developed a protein-protein binding assay. We determined that CDC25 binds tightly to Ras2 protein only in the absence of guanine nucleotides. This higher affinity of CDC25 for the nucleotide-free form than for either the GDP- or GTP-bound form suggests that CDC25 catalyzes exchange of guanine nucleotides bound to Ras proteins by stabilization of the transitory nucleotide-free state.

Ras proteins, like trimeric G proteins, are active when bound to GTP and inactive when bound to GDP (7). Activation of Ras proteins in vivo may be mediated by proteins referred to as guanine nucleotide exchange factors (also referred to as guanine nucleotide dissociation stimulators or releasing factors) (7). The first such exchange factor for Ras proteins to be identified was the *CDC25* gene product of *Saccharomyces cerevisiae* (10, 28). The essential function of *CDC25* is bypassed by mutations in *RAS2* that stabilize or promote the GTP-bound form of Ras2 (10, 12, 28), suggesting that the major cellular function of CDC25 is to activate normal Ras proteins. Biochemical analysis of CDC25 partially purified from yeast cells indicates that it activates Ras proteins by catalysis of GDP-GTP exchange (21).

CDC25 homologs that act upstream to activate Ras proteins have been identified by genetic means in two other eukaryotic organisms (5, 19, 30). In *Schizosaccharomyces pombe*, the *CDC25* homolog *ste6* operates upstream of Ras in the mating pheromone response pathway, and in *Drosophila melanogaster*, the *CDC25* homolog *SOS* operates upstream of Ras in the developmental signaling pathway initiated by the Sevenless protein tyrosine kinase (5, 19, 30). Homologs of *CDC25* have also been identified by molecular cloning methods in mice, humans, and rats (6, 23, 29, 34). Such conservation suggests that CDC25-type exchange factors play a central role in the control of eukaryotic Ras signaling pathways.

Other close relatives of Ras, within the larger family of Ras-like proteins, may also have corresponding GDP-GTP exchange factors that share more limited homology with *CDC25*. In budding yeast cells, two genes that share partial homology with *CDC25* have been identified, *BUD5* and *LTE1* (11, 26, 35). Neither of these genes appears to function in the *RAS* pathway, but genetic analysis suggests that the *BUD5* gene product acts as a GDP-GTP exchange factor for the yeast Rap homolog encoded by *RSR1/BUD1* (1, 11). A much closer homolog of *CDC25* exists in *S. cerevisiae*, the *SDC25* gene (8). A C-terminal fragment of *SDC25* expressed in *Escherichia coli* is a potent catalyst of GDP-GTP exchange for yeast Ras2 and mammalian $p21^{H\text{-}ras}$ proteins, yet genetic analysis of *SDC25* has not as yet revealed any cellular function for *SDC25* in the control of Ras activity (13, 14). Perhaps two distinct CDC25-like exchange factors in budding yeast cells allow Ras proteins to transduce signals from different growth stimuli.

We undertook this study to determine more precisely which region of the lengthy *CDC25* gene (1,589 codons) is required for essential cellular function and to use this information to test recombinant CDC25 protein fragments for GDP-GTP exchange activity. It has been suggested, on the basis of failure of prior attempts to obtain a biochemically active recombinant CDC25 protein, that CDC25 may require some other yeast proteins for GDP-GTP exchange activity (13). However, we show in this report that CDC25 protein produced in *E. coli* can catalyze GDP-GTP exchange on Ras proteins. Furthermore, we show that this activity is specific for Ras proteins and that CDC25 is more potent at activation of Ras proteins than at deactivation, which was predicted from prior genetic analysis (10, 28). Additionally, we have developed a simple protein-protein binding assay for Ras and CDC25 that will be useful in deciphering the regions of the two molecules involved in their physical interaction.

* Corresponding author.
† Present address: Onyx Pharmaceuticals, Richmond, CA 94806.

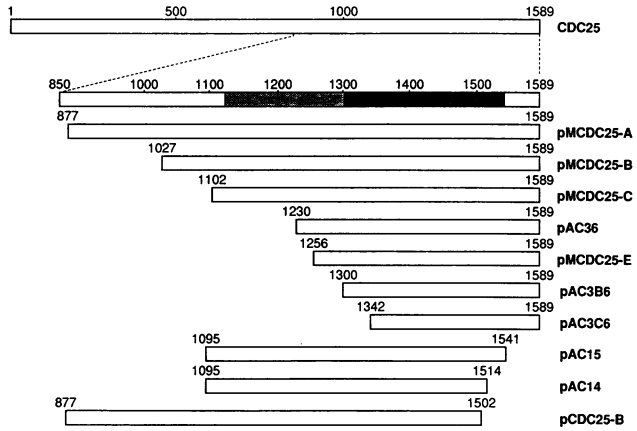

FIG. 1. Map of the different *CDC25* fragments used in complementation studies. Shown below the full-length *CDC25* coding region is an enlargement of a segment containing the previously determined minimal region (codons 877 to 1552) required for activity in vivo (10). The lightly shaded areas represent those portions of *CDC25* that are homologous to other genes. Dark shading corresponds to shared homology between Ras-specific guanine nucleotide exchange factors encoded by *CDC25*, *ste6*, *SOS*, and the rodent homologs of *CDC25*, the putative Rap-specific GRF encoded by the *BUD5* gene, and the *LTE1* gene (5, 11, 19, 23, 26, 29, 30). Light shading corresponds to shared homology between Ras-specific guanine nucleotide exchange factors that is not shared with *BUD5* or *LTE1*. Shown below are the regions of *CDC25* contained within the different plasmids used in this report to determine more precisely the minimal essential region of *CDC25*.

(This work was performed in partial fulfillment of the requirements for a doctoral thesis at Rutgers University by C.-C. Lai.)

MATERIALS AND METHODS

Yeast strains and complementation assays. LRA26, provided by K. Tatchell, contains the *cdc25-5* allele (*MATα leu2 ura3 his4 cdc25-5*) (25). T179-1AH (*MATα leu2 ura3 his3 trp1 ade8 cdc25::URA3* p*HIS3-TPK1*) was constructed by replacing the p*CDC25*(*TRP1*)-1 plasmid harbored within strain TT1A-1 with a high-copy-number plasmid, p*HIS3-TPK1*, containing the *HIS3* and *TPK1* genes (10). Transformation of yeast with DNA was performed by the lithium acetate method, with the modification that 25 mM dithiothreitol or 25 mM β-mercaptoethanol was included in the transformation buffers. Complementation assays were performed as described in the footnotes to Table 1.

Plasmid constructions. Plasmids pALCDC25 and p*HIS3-TPK1* were provided by M. Wigler's laboratory. Plasmids pMCDC25-A, pMCDC25-B, pMCDC25-C, pMCDC25-E, and pCDC25-B were constructed by appropriate modifications and insertion of restriction endonuclease fragments of *CDC25* into the polylinker region that follows the initiator codon and HA1 epitope encoded by the *ADH1*-based expression vector pAD5 (16). The restriction sites within the *CDC25* locus used were *Bgl*II and *Pvu*II (pMCDC25-A), *Bcl*I and *Pvu*II (pMCDC25-B), *Nco*I and *Pvu*II (pMCDC25-C), *Bam*HI and *Pvu*II (pMCDC25-E), and *Bgl*II and *Hin*dIII (pCDC25-B) (Fig. 1). Plasmids pAC36, pAC3B6, pAC3C6, pAC15, and pAC14 were constructed by polymerase chain reaction procedures, using fragments generated with appropriate primers derived from the *CDC25* sequence and insertion into pAD5. Plasmids pGTCD-1, pGTCD-6, pGTCD-7, and pGTCD-8 were constructed with polymerase chain reaction-derived *CDC25* fragments as indicated in Table 2 and insertion into pGEX-2T (31).

Purification of GST-CDC25 fusion proteins. Plasmids encoding glutathione *S*-transferase (GST) alone or GST-CDC25 fusion proteins were transformed into *E. coli* BL21. Induction of expression and purification of the GST fusion proteins were performed essentially as described previously (31) with the modification that cells were cultured at 25°C, which allowed for more efficient production of soluble forms of the fusion proteins. The final concentration and purity of the proteins were determined by the Bio-Rad assay and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The apparent molecular sizes of the soluble GST-CDC25 proteins ranged from 73 kDa (from pGTCD-1) to 58 kDa (from pGTCD-8). Specific yields from 100-ml cultures ranged from 0.2 to 1.4 mg. The soluble GST-CDC25 fusion proteins did not possess intrinsic GDP or GTP binding properties, as judged by nitrocellulose filter binding (9).

Guanine nucleotide dissociation assays. Recombinant Ras2 and p21^{ras} (H-*ras*$^{Val\text{-}12}$) proteins were purified as described previously (9). Purified Ypt1 protein was provided by S. Ferro-Novick. GST-Rsr1 protein was purified by standard procedures (31) from a strain provided to us by A. Bender. Nucleotide-free Ras2 protein was prepared by dialysis of samples for 6 days at 4°C against Mg^{2+}-free buffer containing 50% glycerol (50 mM Tris [pH 7.5], 50 mM KCl, 1 mM dithiothreitol). Control experiments with Ras2 proteins bound to [^{3}H]GDP indicated that this procedure removes up to 70% of bound nucleotide. Ras and Ras-like proteins were loaded with either [^{3}H]GDP (12.4 Ci/mmol; Amersham) or [^{3}H]GTP (9 Ci/mmol; Amersham) essentially as described previously (15). GDP-GTP exchange reactions were performed as described in the footnote to Table 2 and legend to Fig. 4. The amount of [^{3}H]GDP released from Ras2 protein was linearly proportional to the amount of added soluble GST-CDC25 protein (from pGTCD-1) over the range tested of 0.2 to 2 pmol, using 5-min reactions containing 25 pmol of Ras2-GDP.

Fractionation of yeast extracts and immunoblotting of epitope-tagged CDC25 proteins. Cultures (100 ml) of LRA26 strains transformed with plasmids expressing full-length or deletion mutant CDC25 proteins were grown in synthetic medium to late log phase. The cells were pelleted and washed with ice-cold buffer E (0.3 M sorbitol, 0.1 M NaCl, 5 mM $MgCl_2$, 10 mM Tris [pH 7.4]), resuspended in 500 μl of buffer E containing 1 mM phenylmethylsulfonyl fluoride and 1 mg of aprotinin per ml, and lysed with glass beads and vortexing. The lysate was cleared by centrifugation at 1,500 × *g* for 10 min and then centrifuged at 15,000 × *g* for 15 min to obtain the supernatant (fraction A). The pellet was resuspended in 300 μl of buffer E with the addition of 10% Triton X-100; after 1 h, the mixture was centrifuged as before, and the supernatant was collected (fraction B). The remaining pellet was resuspended in 300 μl of buffer E plus 0.5% deoxycholate and 0.1% SDS to obtain fraction C. Fifteen microliters of each fraction was mixed with 15 μl of 2× sample buffer, subjected to SDS-PAGE, and transferred to nitrocellulose paper. Immunoblotting with 12CA5 antibody was performed as described below.

Protein-protein binding assay. Samples of 75 to 300 ng of GST or GST-CDC25 fusion protein were diluted into 2.5 ml of buffer B (150 mM NaCl, 20 mM sodium phosphate [pH 7.4]) before addition of 30 μl of a 50% glutathione-agarose slurry (Sigma). The mixture was rotated at 4°C for 15 min

TABLE 1. Complementation of *cdc25* alleles by expression of *CDC25* gene fragments

LEU2$^+$ transforming plasmid	Amino acids of *CDC25* encoded	Suppression of *cdc25*[a]	Suppression of *cdc25-5*[b]
pAD5	None	0/381	−
pALCDC25	1–1589	290/386	+++
pMCDC25-A	877–1589	216/332	+++
pMCDC25-C	1102–1589	230/421	+++
pAC36	1230–1589	16/316	++
pMCDC25-E	1256–1589	20/320	++
pAC3B6	1300–1589	0/386	+
pAC3C6	1342–1589	0/328	−
pAC15	1095–1541	206/345	+++
pAC14	1095–1514	0/363	−
pCDC25-B	877–1502	0/318	−

[a] Strain T159-5AH, harboring a *cdc25::URA3* allele suppressed by a *HIS3*$^+$ plasmid that overexpresses the protein kinase A gene *TPK1* (10), was transformed with *LEU2*$^+$ plasmids expressing the indicated fragment of *CDC25*. After growth of the Leu$^+$ His$^+$ transformants without selection for 1 day, cells were cultured on YPD plates to obtain single colonies. The presence of the His$^+$ plasmid in the resultant colonies was determined by replica plating. The stability of the His$^+$ plasmid is expressed as the ratio of the number of His$^-$ colonies to the total number of colonies.

[b] Strain LRA26 was transformed with the same plasmids, and five independent Leu$^+$ transformants were purified, patched onto SC-Leu plates, and tested for growth on YPD at 36°C by replica plating.

and pelleted, and the beads were washed three times with 5 ml of buffer B. The GST- or GST-CDC25-coated beads were resuspended in 400 μl of buffer A containing 5 mM $MgCl_2$, 1 μg of nucleotide-free Ras2 protein, 1% Triton X-100, and 0.2% bovine serum albumin. The binding reaction was rotated at 4°C for 30 min, and the beads were then pelleted and washed three times with 5 ml of buffer B containing 1% Triton X-100. Fifteen microliters of sample buffer was added to the washed and pelleted beads; eluted proteins were then separated by SDS-PAGE and electrophoretically transferred to nitrocellulose paper. Ras2 protein was detected by blotting with anti-Ras antibody (DuPont) and reagents from the ECL kit (Amersham).

RESULTS

Minimal region of *CDC25* required for activity in vivo. Previously it has been reported that of the 1,589 amino acids encoded by *CDC25*, expression of a region spanning codons 877 to 1552 sufficed for essential function (10), yet the reported areas of significant homology between *CDC25* and genes encoding other putative Ras-specific GDP-GTP exchange factors such as *SOS* are considerably smaller, comprising codons 1121 to 1540 (Fig. 1). This finding suggested to us that the minimal region of *CDC25* required for activity in vivo might be smaller than the previously determined region of codons 877 to 1552. To test this possibility, we constructed plasmids that used the *ADH1* promoter to express various fragments of the *CDC25* gene and examined the ability of these plasmids to complement both a temperature-sensitive *cdc25* allele (*cdc25-5*) and a null allele (*cdc25::URA3*) (10, 25). We found that expression of regions corresponding to either codons 1102 to 1589 or codons 1095 to 1541 was sufficient for full complementation of both alleles (Table 1), demonstrating that the minimal region of *CDC25* required for full activity in vivo is no larger than codons 1102 to 1541. This region corresponds closely to the region of homology shared between *CDC25* and other Ras-specific GDP-GTP exchange factors (Fig. 1).

More extensive deletions of the C-terminal portion of this smaller region of *CDC25* resulted in complete loss of activity (Table 1). However, more extensive deletions of the N-terminal portion of this region resulted in only partial loss of activity (Table 1). Expression of a fragment encoding amino acids 1230 to 1589 still retained the ability to weakly complement both *cdc25-5* and *cdc25::URA3*, and expression of a segment corresponding to codons 1300 to 1589 was unable to complement *cdc25::URA3* but weakly complemented *cdc25-5* (Table 1). A more extensive deletion of the N-terminal portion, which removed codons 1300 to 1341, resulted in complete loss of activity (Table 1). We conclude that a region of no larger than 1300 to 1541 suffices for residual activity in vivo. This region corresponds closely to the only homologous region between *CDC25* and the putative Rap GDP-GTP exchange factor encoded by *BUD5* (Fig. 1).

One possible explanation for the failure of smaller *CDC25* fragments to complement *cdc25* mutations is that the corresponding CDC25 mutant proteins were not expressed at reasonable levels, or that they did not localize properly to their normal site of action. We analyzed the expression levels and subcellular fractionation profiles of full-length and different deletion mutants of CDC25 that affected residues N terminal to position 1342. By immunoblotting, full-length CDC25 was detected almost exclusively in the particulate fraction, suggesting membrane localization (Fig. 2A). We observed that CDC25 was resistant to solubilization with the nonionic detergent Triton X-100 but that it could be solubilized with ionic detergents (Fig. 2A). These findings are in agreement with a previous report that analyzed endogenous CDC25 protein in wild-type cells (18). Of potential biological significance, but irrelevant to the issue at hand, we found that the level of expression of full-length CDC25 was considerably lower than that of any of the deletion mutants that lacked residues 1 to 877 (Fig. 2B). We did not observe any significant differences in the expression levels of biologically active CDC25 deletion mutants and biologically inactive CDC25 deletion mutants (Fig. 2C). We also observed that both biologically active and inactive CDC25 deletion mutant proteins were predominantly localized to the particulate fraction (Fig. 2C). We conclude that those deletions affecting residues 877 to 1342 that impair the biological activity of CDC25 do not do so by affecting the levels of expression or cellular localization of CDC25. We have not done comparable analysis on deletions affecting C-terminal residues 1510 to 1589.

Minimal region of *CDC25* required for activity in vitro. Having more precisely determined the minimal region of *CDC25* required for full and partial activity in vivo, we wished to evaluate the ability of corresponding *CDC25* fragments expressed in *E. coli* to stimulate GDP-GTP exchange on Ras2 protein. For this purpose, we constructed bacterial expression plasmids that direct the synthesis of *CDC25* fragments fused to the GST gene (31). As shown in Table 2, we detected Ras2-GDP-releasing activity with a GST fusion protein that contained amino acids 1084 to 1589 of *CDC25*, which demonstrates that CDC25 protein by itself can function as a GDP-GTP exchange factor. Somewhat surprisingly, we could not detect any activity with smaller fusion proteins that corresponded to fragments that retained partial biological activity in yeast cells (Table 2). This observation does not appear to be due to limitations of our exchange assay, as when we increased the amount of the smaller GST-protein containing residues 1300 to 1589 fivefold and extended the exchange reaction to 20 min, we still did not observe any activity (22). Thus, only expression of

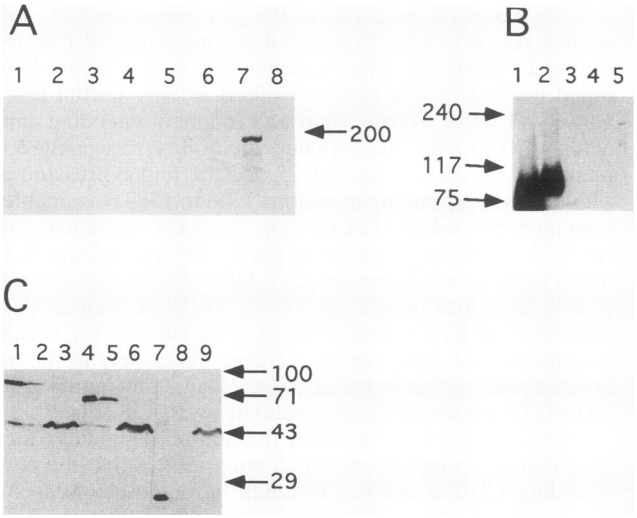

FIG. 2. Expression levels and localization of full-length and deleted CDC25 proteins. (A) Localization of CDC25 in yeast cells expressing an influenza virus HA1 epitope-tagged full-length CDC25 protein. Extracts were fractionated into soluble and particulate fractions as described in Materials and Methods and then subjected to SDS-PAGE and immunoblotting. Lanes: 1, soluble fraction of strain expressing epitope-tagged CDC25; 2, soluble fraction of a control strain not expressing epitope-tagged CDC25; 3, blank; 4 and 5, Triton X-100-extractable portions of the particulate fractions of the epitope-tagged CDC25 strain (lane 4) and the control strain (lane 5); 6, blank; 7 and 8, ionic detergent extracts of the particulate fractions of the epitope-tagged CDC25 strain (lane 7) and the control strain (lane 8). Shown to the left is the migration of the 200-kDa standard. (B) Relative expression levels of full-length and N-terminal deletion mutants of CDC25. Whole cell extracts of 25-ml cultures were made by including 0.5% deoxycholate and 0.1% SDS in the lysis buffer (see Materials and Methods), separated by SDS-PAGE, and analyzed by immunoblotting. Lanes: 1, yeast cells carrying pMCDC25-B (CDC25 amino acids 1027 to 1589); 2, yeast cells carrying pMCDC25-A (CDC25 amino acids 877 to 1589); 3 and 4, yeast cells carrying pAD5 (negative control); 5, yeast cells carrying pALCDC25 (full-length CDC25). Shown to the right is the migration of molecular weight standards (in kilodaltons). (C) Localization of deletion mutant CDC25 proteins in yeast cells. Analysis was carried out as described for panel A. Lanes: 1 to 3, yeast cells carrying pMCDC25-A (CDC25 amino acids 877 to 1589); 4 to 6, yeast cells carrying pMCDC25-B (CDC25 amino acids 1027 to 1589); 7 to 9, yeast cells carrying pAC3C6 (CDC25 amino acids 1342 to 1589; 1, 4, and 7, the Triton X-100-insoluble portion of particulate fractions; 2, 5, and 8, the Triton X-100-soluble portion of particulate fractions; 3, 6, and 9, the soluble fraction. Shown to the right is the migration of molecular weight standards (in kilodaltons). An endogenous yeast protein that is largely soluble, cross-reacts with the 12CA5 antibody, and migrates slightly more slowly than the 43-kDa marker protein was observed for all strains.

the region of *CDC25* required for full activity in yeast cells yielded a catalytically active molecule in *E. coli*, whereas expression of regions corresponding to those that suffice for weak activity in yeast cells resulted in what appears to be completely inactive exchange factors. These shorter CDC25 fusion proteins may not fold properly in *E. coli*. An alternative explanation is that their partial activity in yeast cells is due to some other property of CDC25, independent of intrinsic exchange activity.

A significantly conserved region found only in Ras GDP-GTP exchange factors. Our results suggested to us that somewhere within CDC25 residues 1102 to 1300 there was a critical determinant required for full activity in vivo and perhaps strictly required for activity in vitro. Published alignments of *CDC25* with homologs such as *SOS* and *BUD5* indicated that this region was homologous to the corresponding region of *SOS* but not homologous to any regions of *BUD5* or *LTE1* (5, 11, 19, 23, 26, 29, 30). We wished to examine this matter more thoroughly and used the MACAW multiple alignment program to analyze this region in six Ras-specific CDC25 homologs as well as Bud5 and Lte1 (3). We found that the six Ras-specific homologs, but not Bud5 or Lte1, contained a significantly conserved region of 26 residues that corresponds to CDC25 residues 1143 to 1168 (Fig. 3). Immediately adjacent and N terminal to this conserved region, we observed by eye what appears to be a conserved extension of this region containing several amino acid identities (Fig. 3). It should be noted, however, that we failed to find highly significant conservation in this extension when all six Ras-specific homologs were grouped together and analyzed with MACAW. Downstream of this conserved region is a larger region of highly conserved residues found in Bud5 and Lte1 as well as the six Ras-specific CDC25 homologs (Fig. 3). This larger conserved region, in which four temperature-sensitive mutations of *CDC25* map (25), is likely to represent the core catalytic region of CDC25- and Bud5-type exchange factors (4). We observed that the number of residues between the N-terminal conserved region of Ras-specific exchange factors and the more widely conserved C-terminal region is variable, with the largest stretch found in the rodent CDC25 homolog Ras-GRF (Fig. 3). It should be noted that all of the active recombinant Ras exchange factors reported to date include the N-terminal conserved region that we have described here (13, 29).

TABLE 2. Guanine nucleotide-releasing activities of different GST-CDC25 fusion proteins[a]

Plasmid	Amino acids of *CDC25* encoded	Relative amt of [^{3}H]-GDP remaining bound to Ras2 protein (%)
pGEX-2T	None	100
pGTCD-1	1084–1589	35
pGTCD-6	1189–1589	101
pGTCD-7	1230–1589	103
pGTCD-8	1300–1589	100

[a] Ras2 protein (250 pmol) was equilibrated for 20 min at room temperature with an equimolar amount of [^{3}H]GDP in 150 ml of fast-exchange buffer (buffer A). $MgCl_2$ was added to a final concentration of 5 mM, and after 15 min at room temperature, the releasing assay was initiated by aliquoting 15 ml of the Ras2-GDP mixture into tubes that contained 2.5 nmol of cold GTP plus 1 ml of the indicated GST fusion protein. After 5 min at room temperature, duplicate samples were assayed for [^{3}H]GDP remaining bound to Ras2 protein by the nitrocellulose filter binding assay (9). The amounts of the various GST fusion proteins in each assay tube were 1 to 2 pmol, relative to 25 pmol of Ras2 protein. Values are expressed as the percentage of [^{3}H]GDP released relative to the value for the control (GST alone).

Characterization of guanine nucleotide-releasing activity of CDC25. Genetic analysis of *CDC25* has indicated that it functions as a positive regulator of normal Ras proteins (10, 28), and therefore one might predict that the exchange activity of its gene product would be more potent at activation of Ras protein (promoting release of bound GDP and replacement with GTP) than at deactivation (promoting release of bound GTP and replacement with GDP). We tested this prediction with the catalytically active GST-CDC25 protein. The results displayed in Fig. 4A and B show that under otherwise identical conditions, the recombinant CDC25 protein was considerably more potent at catalyzing

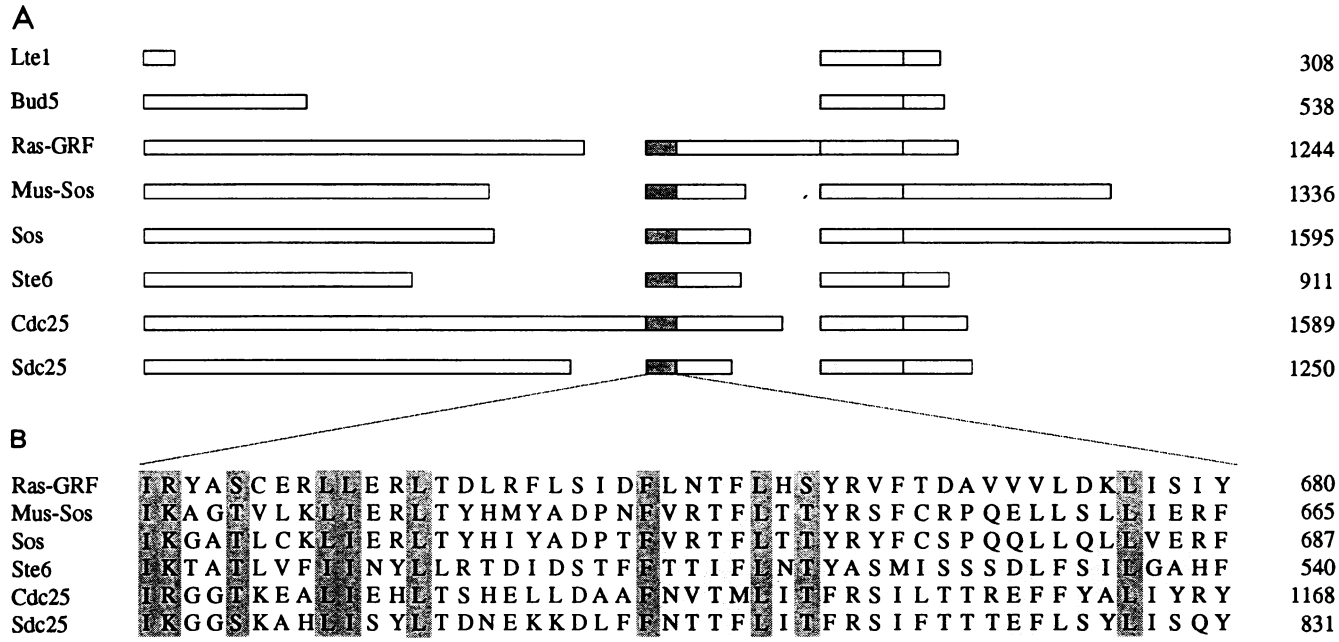

FIG. 3. Conserved domain specific for CDC25 homologs that act on Ras proteins. Sequences were analyzed with the multiple alignment tool kit and MACAW program (3). (A) Schematics of eight putative exchange factors with gaps to align the homology regions; the top two proteins (Lte1 and Bud5) presumably do not operate on Ras proteins themselves, whereas the bottom six have been shown genetically or biochemically to operate on Ras proteins. The gray rectangles shared by all eight proteins represents a highly conserved region that has recently been analyzed in detail (4). The smaller gray rectangles in the middle represent a region conserved only in the Ras-specific exchange factors and not found in Bud5 or Lte1. (B) Amino acid sequences within the latter region. Amino acid identities are displayed by dark shading. When all six proteins when analyzed together by MACAW, the C-terminal stretch of 26 amino acids (light shading) was the only homology within this region found to be statistically significant. To eliminate the overestimation of statistical significance due to the substantial pairwise similarities of CDC25/Sdc25 and murine Sos/Sos, the latter sequence from each pair was excluded from the calculations. The probability that the aligned 26 amino acids are related by chance was calculated to be 2.1×10^{-4} (3).

replacement of bound GDP with GTP (activation) than at the reverse reaction of replacement of bound GTP with GDP (deactivation). Similar results have been reported for both the *SDC25* C-terminal fragment and the rodent CDC25 homolog Ras-GRF (13, 29). Our results suggest that CDC25 protein may possess a higher affinity for the GDP-bound form of Ras2 than for the GTP-bound form (24; see Discussion).

Genetic analysis of *CDC25* has also indicated that its function in yeast cells is restricted to the activation of yeast Ras1 and Ras2 proteins (10, 28), but that under certain conditions its gene product can interact with mammalian $p21^{H\text{-}ras}$ (27). We therefore thought it possible that the GST-CDC25 protein would act as a GDP-GTP exchange catalyst for $p21^{H\text{-}ras}$ but not for yeast members of the Ras-like family distinct from Ras1 and Ras2. To test these possibilities, we analyzed the ability of the active GST-CDC25 protein to promote GDP-GTP exchange for $p21^{H\text{-}ras}$, the closely related yeast Ras-like protein Rsr1, and the more distantly related yeast Ras-like protein Ypt1 (1, 33). The results presented in Fig. 2 show that CDC25 is as potent at catalyzing GDP-GTP exchange for human $p21^{H\text{-}ras}$ as it is for yeast Ras2. On the other hand, CDC25 failed to significantly promote GDP-GTP exchange for the Ras-like proteins Rsr1 and Ypt1 (Fig. 4). We conclude that the ability of CDC25 to act as a GDP-GTP exchange factor is specific for Ras proteins.

Direct binding assay for CDC25 and Ras proteins. We wished to determine whether the affinity of CDC25 for Ras proteins was sufficiently strong so that a simple protein-protein binding assay could be developed. Since previous genetic results suggested that the interaction of CDC25 and Ras2 might be more avid when Ras2 protein was nucleotide free (26, 27), we first tested this possibility with nucleotide-free Ras2 protein (20). We mixed CDC25 and nucleotide-free Ras2 proteins together and then used the GST tag of the CDC25 fusion protein to specifically absorb CDC25 with glutathione-agarose beads. Following extensive washing, we collected the beads and analyzed the bound proteins by SDS-PAGE and immunoblotting with anti-Ras antibodies. By this method, we found that Ras2 binds to the GST-CDC25 protein but not to control GST protein (Fig. 5A). On the basis of titration of CDC25 and comparison with a given amount of Ras2 protein, we conclude that the binding of CDC25 to Ras2 under these conditions is close to stoichiometric (Fig. 5A). The catalytically inactive CDC25 fusion proteins, corresponding to fragments of *CDC25* that had partial biological activity in yeast cells, failed to bind to Ras2 under these conditions (Fig. 5C).

To test whether the guanine nucleotide bound state of Ras protein influenced this tight interaction with CDC25, we performed an experiment that examined the effect of inclusion of GDP or GTP in the binding buffer, as well as the effect of allowing binding to occur with nucleotide-free Ras2 protein but adding GDP or GTP at the washing step of the procedure. In all cases in which guanine nucleotides were added, either preceding or following the binding reaction, we observed that the tight interaction between Ras2 and CDC25 protein was abolished (Fig. 5B). We have also prepared nucleotide-free Ras proteins by denaturing conditions and

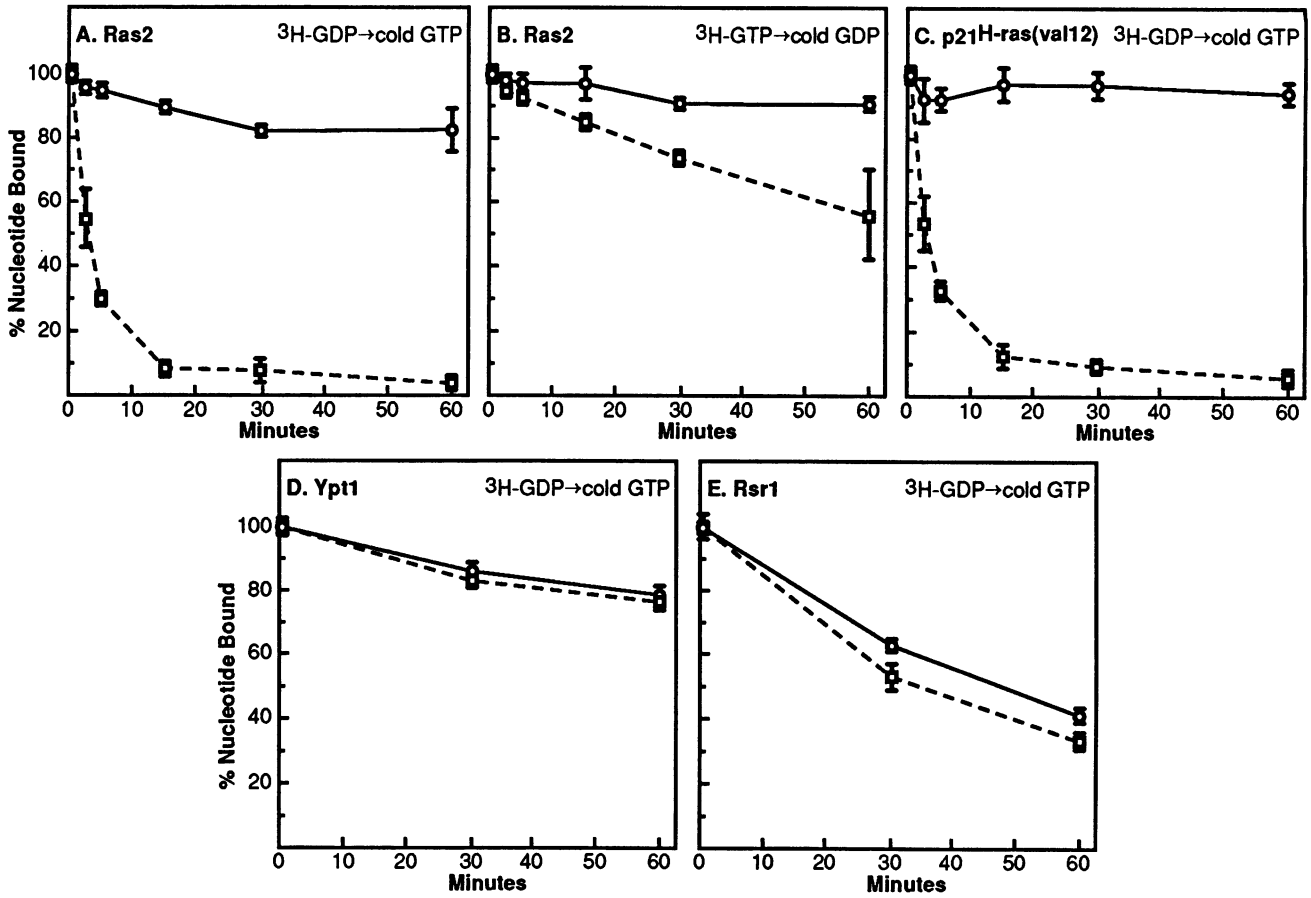

FIG. 4. Guanine nucleotide-releasing activity of GST-CDC25 fusion protein. Ras and Ras-like proteins (120 to 500 pmol) were loaded with either [^{3}H]GDP or [^{3}H]GTP as described in the footnote to Table 2. The releasing reactions contained 1 μl of the indicated cold guanine nucleotide plus either 1 μl of GST protein (circles) or 1 μl of GST-CDC25 protein from pGTCD-1 (boxes). At the indicated time point, the amount of radioactive guanine nucleotide bound to Ras or Ras-like protein was determined in duplicate. Values are expressed as the percentage of the average zero time point. (A) Each sample contained 25 pmol of Ras2, 1 pmol of GST or GST-CDC25, and 2.5 nmol of GTP. (B) Each sample contained 25 pmol of Ras2, 1 pmol of GST or GST-CDC25, and 2.5 nmol of GDP. (C) Each sample contained 20 pmol of p21^{ras} (H-*ras*$^{Val\text{-}12}$), 1 pmol of GST or GST-CDC25, and 2.5 nmol of GTP. (D) Each sample contained 10 pmol of Ypt1, 2 pmol of GST or GST-CDC25, and 2.5 nmol of GTP. (E) Each sample contained 27 pmol of GST-Rsr1, 2 pmol of GST or GST-CDC25, and 2.5 nmol of GTP.

have obtained similar results (22). Since the GST-CDC25 protein itself does not bind guanine nucleotides (see Materials and Methods), this effect is likely due to alteration of Ras2 protein conformation following its binding to guanine nucleotides. We conclude that CDC25 possesses higher affinity for the nucleotide-free form of Ras2 protein than for either the GDP- or GTP-bound form.

DISCUSSION

Activation of Ras in vivo is likely to be mediated in many cases by specific guanine nucleotide exchange factors. Genes encoding Ras-specific GDP-GTP exchange factors all contain a region that is structurally homologous to a portion of the *CDC25* gene of *S. cerevisiae* (5, 19, 23, 29, 30, 34). We have found that expression of this homologous region of approximately 450 codons is sufficient for complete complementing activity in yeast cells and yields a catalytically active protein when expressed in *E. coli* (see also references 23 and 29). Expression of a smaller C-terminal segment within this region, spanning approximately 250 amino acids, is sufficient for weak complementing activity in yeast cells but fails to yield a catalytically active exchange factor when expressed in *E. coli* (see also reference 23). This smaller segment corresponds to the area of homology shared between *CDC25* and putative GDP-GTP exchange factors encoded by *BUD5* and *LTE1* and is very likely the core catalytic region of these exchange factors (4, 11, 26, 35). However, this smaller segment does not contain a significantly conserved region of 26 residues that is found in all Ras-specific homologs of CDC25 (Fig. 3). We speculate that this latter region is involved in the specific recognition of Ras proteins by CDC25.

Consistent with prior genetic analysis of *CDC25* which suggested that its cellular role was restricted to Ras function, CDC25 protein appears to interact specifically with the Ras subgroup of the superfamily of Ras-like proteins. We and others have determined that the ability of CDC25-type exchange factors to catalyze GDP-GTP exchange does not extend to other Ras-like proteins, including close relatives of Ras within the Ral and Rap subgroups (29). It is also clear from this and previous reports that CDC25-type exchange factors are more potent at replacement of GDP-bound to Ras with GTP (activation) than they are at replacement of GTP

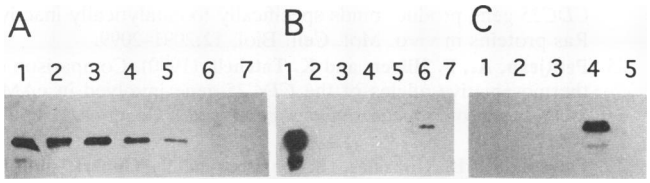

FIG. 5. Protein-protein binding assay for Ras2 and CDC25 proteins. (A) Immunoblot that detected the presence of Ras2 protein. Lanes: 1, 200 ng of Ras2 protein as a comparative standard; 2 to 7, samples in which 1 μg of nucleotide-free Ras2 protein was incubated with glutathione-agarose beads that had been precoated with 600 ng of GST-CDC25 (lane 2), 300 ng of GST-CDC25 (lane 3), 150 ng of GST-CDC25 (lane 4), 75 ng of GST-CDC25 (lane 5), 75 ng of GST alone (lane 6), or no protein (lane 7). After binding, the beads were washed extensively and bound proteins were eluted, separated by SDS-PAGE, and transferred to nitrocellulose paper. (B) Effect of guanine nucleotides on CDC25-Ras2 binding. Lane 1 contains 1 μg of Ras2 protein as a comparative standard. One microgram of nucleotide-free Ras2 protein was incubated with glutathione-agarose beads that had been precoated with the either 75 ng of GST protein (lane 7) or 75 ng of GST-CDC25 protein (lanes 2 to 6). For lanes 2 and 3, the binding buffer contained no guanine nucleotides, but either 1 mM GTP (lane 2) or 1 mM GDP (lane 3) was included in the washing buffer. For lanes 4 and 5, the binding buffer contained either 1 mM GTP (lane 4) or 1 mM GDP (lane 5). Lane 6 represents a control with no guanine nucleotides in the binding reaction or in the washing buffer. (C) Inability of shorter GST-CDC25 fusion proteins to bind to nucleotide-free Ras2. One microgram of nucleotide-free Ras2 protein was incubated with glutathione-agarose beads that had been precoated with 200 ng of the GST-CDC25 protein containing CDC25 amino acids 1300 to 1589 (lane 1), 200 ng of the GST-CDC25 protein containing CDC25 amino acids 1230 to 1589 (lane 2), 200 ng of the GST-CDC25 protein containing CDC25 amino acids 1189 to 1589 (lane 3), 200 ng of the GST-CDC25 protein containing CDC25 amino acids 1084 to 1589 (lane 4), or 200 ng of GST protein as a negative control (lane 5). After binding, the beads were treated and analyzed as described for panel A.

with GDP (deactivation) (13, 29). This finding confirms prior genetic analysis of *CDC25* that indicated a positive role in the activation of Ras proteins (10, 28).

Results obtained with a genetic two-hybrid system (24), similar to the method developed by Fields and Song (17), indicated that CDC25 protein possesses higher affinity for the GDP-bound form of Ras2 protein than for the GTP-bound form. Such differential affinity may underlie the inefficiency of CDC25 protein in catalyzing release of GTP-bound to Ras2 protein, relative to its ability to catalyze release of bound GDP. Additionally, with the in vivo two-hybrid system, the $Ras2^{Ala-22}$ mutant protein was found to be more effective at interaction with CDC25 than was wild-type Ras2 (24). We have previously reported that $Ras2^{Ala-22}$ protein is seriously impaired in its ability to bind guanine nucleotides (26), and it therefore is likely to spend more time in vivo in a nucleotide-free state.

This latter result is consistent with the findings that we have made by using an in vitro binding assay. We have found that nucleotide-free Ras proteins bind tightly to CDC25 protein in a coprecipitation assay. The addition of either GDP or GTP reverses this tight association, demonstrating that CDC25 possesses higher affinity for the nucleotide-free form of Ras than for either the GDP- or GTP-bound form. Although it is unlikely that normal Ras proteins spend an appreciable amount of time in the nucleotide-free state in vivo, our results are consistent with a model for CDC25 action that postulates catalysis of GDP-GTP exchange by stabilization of (higher affinity for) the transitional nucleotide-free state of Ras. Such a mechanism has been proposed to explain catalysis of GDP-GTP exchange-mediated by rhodopsin on the trimeric G-protein transducin (32).

It has been shown that another Ras-like protein, Ran1, is tightly bound to its exchange factor, Rcc1, in vivo (2). Presumably this tight interaction observed in vivo is between the GDP-bound form of Ran1 with Rcc1 (2). No such tight binding between the GDP-bound form of Ras2 with CDC25 was observed in this study, indicating that there may be important differences in the mechanisms by which different GDP-GTP exchange factors catalyze exchange on their respective Ras-like proteins.

The direct binding assay that we have developed will be useful in determining the regions of Ras and CDC25 that are involved in their physical association. Additionally, it may now be possible to isolate novel CDC25-type factors for Ras proteins, on the basis of the tight affinity of nucleotide-free Ras proteins for such GDP-GTP exchange factors.

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