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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 Lte1 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 Ras as well as human p21H-ras but inactive in promoting exchange on the Ras-related proteins Ypt1 and Rap1. 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 RSRI/BUD1 (1, 11). A much closer homolog of CDC25 exists in S. cerevisiae, the SCD25 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 p21H-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.

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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 pHIS3-TPK1) was constructed by replacing the pCDC25(TRP1)-1 plasmid harbored within strain T179-1 with a high-copy-number plasmid, pHIS3-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 pHIS3-TPK1 were provided by M. Wigler’s laboratory. Plasmids pMCDC25-A, pMCDC25-B, pMCDC25-C, pMCDC25-E, and pMCDC25-B were constructed by appropriate deletions 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 BglII and PvuII (pMCDC25-A), BglII and PvuII (pMCDC25-B), NcoI and PvuII (pMCDC25-C), BamHI and PvuII (pMCDC25-E), and BglII and HindIII (pMCDC25-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 p21ras (H-rasVal12) 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 MgCl2-free buffer containing 50% glycerol (50 mM Tris [pH 7.5], 50 mM KCl, 1 mM dithiothreitol). Control experiments with Ras2 proteins bound to [3H]GDP indicated that this procedure removes up to 70% of bound nucleotide. Ras and Ras-like proteins were loaded with either [3H]GDP (12.4 Ci/mmol; Amersham) or [3H]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 [3H]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.

Purification 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 MgCl2, 10 mM Tris [pH 7.4]), resuspended in 500 μl of buffer E containing 1 mM phenylmethylsulfonyl fluoride and 1 mM 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

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 1592) 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 Ras-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.

The work was performed in partial fulfillment of the requirements for a doctoral thesis at Rutgers University by C.-C. Lai.)
TABLE 1. Complementation of cdc25 alleles by expression of CDC25 gene fragments

<table>
<thead>
<tr>
<th>LEU2* transforming plasmid</th>
<th>Amino acids of CDC25 encoded</th>
<th>Suppression of cdc25*</th>
<th>Suppression of cdc25-5*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAD5</td>
<td>None</td>
<td>0/381</td>
<td>-</td>
</tr>
<tr>
<td>pACDC25</td>
<td>1-1589</td>
<td>290/386</td>
<td>++</td>
</tr>
<tr>
<td>pMCDC25-A</td>
<td>877-1589</td>
<td>216/332</td>
<td>+++</td>
</tr>
<tr>
<td>pMCDC25-C</td>
<td>1102-1589</td>
<td>230/421</td>
<td>+++</td>
</tr>
<tr>
<td>pAC36</td>
<td>1230-1589</td>
<td>16/316</td>
<td>+</td>
</tr>
<tr>
<td>pMCDC25-E</td>
<td>1256-1589</td>
<td>20/320</td>
<td>+</td>
</tr>
<tr>
<td>pAC3B6</td>
<td>1300-1589</td>
<td>0/386</td>
<td>+</td>
</tr>
<tr>
<td>pAC3C6</td>
<td>1342-1589</td>
<td>0/328</td>
<td>-</td>
</tr>
<tr>
<td>pAC15</td>
<td>1095-1514</td>
<td>206/345</td>
<td>++ +</td>
</tr>
<tr>
<td>pAC14</td>
<td>1095-1514</td>
<td>0/363</td>
<td>-</td>
</tr>
<tr>
<td>pCD25-B</td>
<td>877-1502</td>
<td>0/318</td>
<td>-</td>
</tr>
</tbody>
</table>

*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 LKA29 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 MgCl₂, 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 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, deletion of the C-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 potentially 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 these 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-relaxing activity with a GST fusion protein that contained amino acids 1084 to 1589 of CDC25, which demonstrated that the 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...
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 examined 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).

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

### Table 2. Guanine nucleotide-releasing activities of different GST-CDC25 fusion proteins

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Amino acids of CDC25 encoded</th>
<th>Relative amt of [3H]-GDP remaining bound to Ras2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEX-2T</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>pGTC-1</td>
<td>1084-1589</td>
<td>35</td>
</tr>
<tr>
<td>pGTC-6</td>
<td>1189-1589</td>
<td>101</td>
</tr>
<tr>
<td>pGTC-7</td>
<td>1230-1589</td>
<td>103</td>
</tr>
<tr>
<td>pGTC-8</td>
<td>1300-1589</td>
<td>100</td>
</tr>
</tbody>
</table>

* Ras2 protein (250 pmol) was equilibrated for 20 min at room temperature with an equimolar amount of [3H]-GDP in 150 ml of fast-exchange buffer (buffer A). MgCl2 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 [3H]-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 [3H]-GDP released relative to the value for the control (GST alone).
replacement of bound GDP with GTP (activation) than at the reverse reaction of replacement of bound GDP with GTP (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-ras} (27). We therefore thought it possible that the GST-CDC25 protein would act as a GDP-GTP exchange catalyst for p21^{H-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-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-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
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.
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 Ras2A<sup>99</sup>-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<sup>61A-22</sup> 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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1352 LAI ET AL.


