Amino Acid Residues in the CDC25 Guanine Nucleotide Exchange Factor Critical for Interaction with Ras

WEONMEE PARK,¹ RAYMOND D. MOSTELLER,² AND DANIEL BROEK^{2,3*}

Department of Biological Sciences, University of Southern California, Los Angeles, California 90038,¹ and Department of Biochemistry and Molecular Biology² and USC/Norris Comprehensive Cancer Center,³ University of Southern California School of Medicine, Los Angeles, California 90033

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Previously we found that negatively charged residues at positions 62, 63, and 69 of H-Ras are involved in binding to the CDC25 guanine nucleotide exchange factor (GEF). Using site-directed mutagenesis, we have changed conserved, positively charged residues of CDC25^{GEF} to glutamic acid. We find the nonfunctional CDC25^{R1374E} mutant and the nonfunctional H-Ras^{E63K} mutant cooperate in suppression of the loss of CDC25 function in *Saccharomyces cerevisiae*. Also, peptides corresponding to residues 1364 to 1383 of CDC25^{GEF} inhibit interaction between GEFs and H-Ras. We propose that residues 1374 of CDC25^{GEF} and 63 of H-Ras form an ion pair and that when this ion pair is reversed, functional interaction can still occur.

Ras proteins, when bound to GTP, activate a variety of cellular phenotypic changes, while the GDP-bound forms of Ras proteins are biologically inactive (1, 11). Ras-specific guanine nucleotide-exchange factors (GEFs), first identified in the yeast *Saccharomyces cerevisiae* and subsequently identified in other organisms, including humans, convert inactive Ras proteins to their active GTP-bound state (3, 13, 19, 20). A large number of distinct mammalian growth factor receptors are known to mediate a variety of cellular responses due in part to the activation of Ras proteins (4, 7, 11). However, for most of these receptors, it is not known which Ras-specific GEF (SOS1^{GEF}, SOS2^{GEF}, or CDC25^{GEF}) is used to mediate activation of the Ras pathway.

The yeast Ras-specific GEF, CDC25^{GEF}, is able to interact with the human H-Ras protein, at H-Ras amino acid residues 62 to 74, to promote nucleotide exchange (15). Mutations in H-Ras resulting in replacement of a negatively charged residue at position 62, 63, or 69 with a positively charged or polar residue results in proteins which are not activated by CDC25^{GEF} (15). The H-Ras^{E62K}, H-Ras^{E63K}, or H-Ras^{D69N} mutant can function in vivo, i.e., activate a downstream target, under conditions in which the GTPase activity of Ras is low, thus allowing Ras to become activated via a CDC25-independent mechanism (15). This finding indicates that this group of H-Ras mutants are defective in receiving an upstream signal but not defective in emitting an output signal.

The catalytic domain of the yeast CDC25^{GEF} has been mapped by molecular genetic analysis to lie between amino acid residues 1300 and 1541 (9). This 241-amino-acid domain shows a high degree of homology with all known Ras-specific GEFs. We reasoned that a highly conserved, positively charged residue in the catalytic domain of the yeast CDC25^{GEF} might interact directly with the negatively charged residues at position 62, 63, or 69 of H-Ras. Alignment of the catalytic domain of CDC25^{GEF} with the corresponding domains of 11 other Ras-specific GEFs revealed several positively charged residues which are highly conserved in evolution (3, 4, 9, 10, 13, 19, 20). We mutated a functional fragment of CDC25 to create five

mutants such that the conserved positively charged residue would be changed to a negatively charged amino acid. We found one of these mutants, CDC25^{R1374E}, to be an allele-specific suppressor of the H-Ras^{E63K} mutant. We show that while CDC25^{R1374E} and H-Ras^{E63K} cannot bind to wild-type H-Ras and CDC25^{GEF}, respectively, we demonstrate that the mutant CDC25^{R1374E} and H-Ras^{E63K} proteins can interact directly. Also, peptides with sequences surrounding residue 1374 of yeast CDC25 or the corresponding region from human CDC25^{GEF} can inhibit the interaction between CDC25 and H-Ras protein in vitro. The results presented here suggest that the activation of Ras proteins is in part due to positions 1374 of CDC25^{GEF} and 63 of H-Ras interacting as an ionic pair.

MATERIALS AND METHODS

Site-specific mutagenesis of CDC25. The catalytic domain of the wild-type yeast CDC25 gene (codons 1100 to 1589) was PCR amplified by using the appropriate primers with *SalI* and *SacI* endonuclease restriction sites at the 5' and 3' ends of the coding sequence, respectively, and subcloned into the *SalI* and *SacI* sites of pAD5 (23) to create pCDC25-C. Site-directed mutagenesis by recombinant PCR was used to modify the CDC25 gene in pCDC25-C as described previously (14). Briefly, primers in both the 5'-3' and 3'-5' directions, encoding amino acid residues 1369 to 1379, 1406 to 1416, 1409 to 1419, 1439 to 1449, or 1484 to 1494 of the CDC25 gene, having a codon in place of the arginine or lysine, were used to modify the CDC25 gene at position 1374, 1411, 1414, 1444, or 1489, respectively, to Glu. For changing arginine to aspartic acid or leucine at position 1374, primers in both 5'-3' and 3'-5' directions encoding amino acid residues 1369 to 1379 having Asp or Leu in place of Arg were used. Overlapping fragments of the coding region were amplified and then combined by a second round of PCR amplification using primers encoding *SalI* and *SacI* endonuclease restriction sites at the 5' and 3' ends of the coding sequence, respectively, and subcloned into the *SalI* and *SacI* sites of pAD5 to create plasmids pcdc25^{R1374E}, pcdc25^{R1411E}, pcdc25^{K1414E}, pcdc25^{R1444E}, pcdc25^{R1489E}, pcdc25^{R1374E}, and pcdc25^{R1374L}. Each of these plasmids was subjected to sequence analysis to confirm that the desired single-codon change had occurred. Plasmids generated

* Corresponding author. Mailing address: NOR 524, 1441 Eastlake Ave., Los Angeles, CA 90033-0800. Phone: (213) 224-6532. Fax: (213) 224-6417.

from independent PCRs gave similar results when expressed in *S. cerevisiae*. The template DNA used was pCDC25-C.

Subcloning of H-ras cDNA into a yeast expression vector. The H-ras and mutant H-ras yeast expression plasmids (in the LEU2-based vector pAD4) have been previously described (15). Site-directed mutagenesis by the recombinant PCR method was used (14) to change codon 63 of H-ras to Arg, using oligonucleotides, in both 5'-3' and 3'-5' directions, encoding amino acid residues 59 to 67 of H-Ras containing an Arg codon at position 63 in place of Glu. Subsequently, a primer encoding the first five N-terminal amino acid residues of H-Ras (containing a *SalI* site at the 5' end) and a primer antisense to the last five C-terminal amino acid codons of H-Ras (containing a *SacI* site at the 5' end) were used for PCR amplifying the H-ras cDNA with Arg at position 63, which was then cloned into pAD4. Wild-type and mutant *BamHI-SacI* fragments of H-ras from the pAD4 plasmids were subcloned into a *TRP*-based yeast expression vector pTT29, kindly provided by Takashi Toda.

Construction of two-hybrid system vectors and assay for β -galactosidase. The H-ras plasmids for the two-hybrid system vector were previously described (15). DNA sequences of the yeast *CDC25* or various mutants of *CDC25* (codons 1100 to 1589) were PCR amplified by using appropriate primers with *SalI* and *SacI* endonuclease restriction sites at the 5' ends and cloned into the pGEM-T vector (Promega, Madison, Wis.). The *SalI* restriction fragments of *CDC25* of the resulting plasmids were then further subcloned into the *GAL4* activation domain vector, pGADGH, to create plasmids pGADGH-*CDC25*^{wt}, pGADGH-cdc25^{R1374E}, pGADGH-cdc25^{R1411E}, pGADGH-*CDC25*^{K1414E}, pGADGH-cdc25^{R1444E}, and pGADGH-cdc25^{R1489E}. Transformants of yeast strain PCY2 (5, 16), containing the two-hybrid system vectors, were tested for β -galactosidase activity by patching onto SC^{-Leu-Trp} (SC medium lacking leucine and tryptophan) plates containing 50 mM KPO₄ (pH 7.0), 2% sucrose, and 100 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) per ml (6). Plasmids pGADGH and pGBT9 were kindly provided by Linda van Aelst and Michael Wigler.

Preparation of Ras and CDC25 proteins. Glutathione S-transferase (GST)-*CDC25*^{GEF} (an approximately 78-kDa fusion protein), composed of GST and the catalytic domain of the *S. cerevisiae* *CDC25*^{GEF}, was prepared as previously described (9, 15). Plasmid pGST-*CDC25*^{GEF} was previously described (9, 15). H-ras sequences (codons 1 to 169) were cloned into pRSET, an expression system (Invitrogen, San Diego, Calif.) vector, for expression of His₆-tagged protein and transformed into *Escherichia coli* BL21 (DE3). Cells were grown to an optical density at 600 nm of 0.5 to 1.0 and then induced by addition of isopropylthiogalactopyranoside (IPTG; final concentration, 1 mM) and incubation at 28°C overnight. The cells were pelleted and resuspended in 4 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]) per g of cell pellet. The cells were lysed by sonication. The supernatant was cleared by centrifugation at 39,000 $\times$ g for 20 min and then purified by using nickel-agarose beads as suggested by the manufacturer (Novagen Inc., Madison, Wis.).

Ras-*CDC25*^{GEF} binding assay. Equal amounts (50 pmol each) of (i) GST-*CDC25*^{GEF} protein (9) bound to glutathione-agarose beads and (ii) purified nucleotide-free His₆-tagged H-Ras protein were incubated in 100 μ l of phosphate-buffered saline containing 0, 50, 125, 250, or 500 μ M peptide from yeast *CDC25* (1364 to 1383) or from human *cdc25* (1098 to 1117) or, as a control, scrambled peptides (see Fig. 2). Protein-protein binding assays were carried out as previously described (9, 10, 15). Western blot (immunoblot) analysis of H-Ras was carried

out with a mouse monoclonal antibody directed against H-Ras and by use of the Immune-Lite blot detection kit as described by the manufacturer (Bio-Rad, Hercules, Calif.).

Yeast strains. The temperature-sensitive yeast strains STS8 (*MATa his3 leu2 trp1 ura3 ade8 can1 ras1::URA3 ras^{ts}*) and LV25-5 (*MATa his3 leu2 trp1 ura3 ade8 can1 cdc25-5^{ts}*) were kindly provided by Roy-Marie Ballester and Michael Wigler. The STS8 or LV25-5 strain containing human GTPase-activating protein (H-GAP) cDNA was previously described (15).

Other materials and methods. Oligonucleotides were purchased from Operon Technologies (Alameda, Calif.). Yeast transformations were performed as previously described (2, 17, 18). *E. coli* transformations were performed as previously described (12). Yeast strains were grown in YPD medium or SC medium (18). *E. coli* strains were grown in LB medium containing 100 μ g of ampicillin per ml. Peptides used for these studies were provided by Andrew Welcher of Amgen Corporation.

RESULTS

Testing the functionality of *CDC25*^{GEF} with missense mutations at highly conserved positions in the catalytic domain.

Plasmids encoding residues 1100 through 1589 of the *S. cerevisiae* *CDC25* gene product are potent suppressors of yeast strains with a temperature-sensitive allele of *cdc25* (9). The shorter catalytic domain of *CDC25*^{GEF} (residues 1300 to 1589) is a weak suppression of the loss of *CDC25* function. We constructed five plasmids which can express residues 1100 through 1589 of *CDC25*^{GEF} with the point mutations Arg-1374 to Glu (R1374E), Arg-1411 to Glu (R1411E), Lys-1414 to Glu (K1414E), Arg-1444 to Glu (R1444E), or Arg-1489 to Glu (R1489E). These positions in the catalytic domain are conserved in most of the family members of Ras-specific GEFs. To determine whether these point mutations affected *CDC25*^{GEF} function, we tested each of the mutant plasmids for the ability to suppress a temperature-sensitive *cdc25-5* mutant strain (LV25-5). As shown in Fig. 1, substitutions at positions 1374, 1444, and 1489 resulted in nonfunctional *CDC25*^{GEFs}, as judged by their inability to suppress the *cdc25-5* mutant strain (Fig. 1). By contrast, expression of mutants with substitution at position 1411 or 1414 was able to suppress the *cdc25-5* mutant strain, although the 1411 mutant exhibited only weak suppression. The inability of these mutants to suppress the loss of *CDC25* function was not due to protein instability, since Western blot analysis using anti-yeast *CDC25*^{GEF} polyclonal antibodies detected similar levels of a 65-kDa protein in extracts of cells overexpressing each of these mutant truncated *CDC25* proteins (data not shown). Position 1414 of the yeast *CDC25*^{GEF} is not conserved in the SOS-like Ras-specific GEFs; thus, it is not surprising that a substitution at this position does not affect *CDC25* function.

The nonfunctional *cdc25*^{R1374E} mutant can complement the nonfunctional H-ras^{E63K} mutant in an allele-specific manner. The negatively charged amino acids of H-Ras, Glu-62 and Glu-63, have been shown to interact directly with Ras-specific nucleotide exchange factors (15). H-Ras proteins mutant at position 62, 63, 67, or 69 were judged to be *CDC25*^{GEF} unresponsive by molecular genetic analysis (15). Evidence in support of this conclusion was obtained by using a yeast two-hybrid system and in vitro biochemical protein-protein interaction analysis. Under conditions in which wild-type H-Ras could bind *CDC25*^{GEF}, the H-Ras^{E63K} mutant failed to bind *CDC25*^{GEF} (15).

We wished to test whether the loss of *CDC25*^{GEF} interaction with H-Ras^{E62K}, H-Ras^{E63K}, H-Ras^{M67I}, or H-Ras^{D69N} could

FIG. 1. Suppression of the *cdc25^{ts}* defect by mutant and wild-type *CDC25*. Growth of the temperature-sensitive *cdc25-5* strain (LV25-5) was examined after transformation with the plasmid containing the catalytic domain of wild-type *CDC25* or mutant *cdc25^{R1374E}*, *cdc25^{R1411E}*, *CDC25^{K1414E}*, *cdc25^{R1444E}*, or *cdc25^{R1489E}*. Two independent transformants of each transformation were patched onto selective medium (SC^{-Leu}) and grown for 3 days at 28°C. These plates were then replica plated onto two SC^{-Leu} plates; one was incubated at 28°C, and the other was incubated at 36°C. Substitutions are indicated at the left. wt, truncated wild-type *CDC25*.

be restored by the *CDC25^{GEF}* mutant *CDC25^{R1374E}*, *CDC25^{R1444E}*, or *CDC25^{R1489E}*. For this purpose, we cotransformed a yeast strain (LV25-5-H-GAP) defective in *CDC25* function (and expressing H-GAP necessary for negative control of H-Ras function) in *S. cerevisiae* with pairwise combinations of wild-type- and mutant H-ras-expressing plasmids and wild-type- and mutant *CDC25*-expressing plasmids (see Materials and Methods).

As indicated in Table 1, the coexpression of *cdc25^{R1374E}* and

H-ras^{E63K} can suppress the loss of *CDC25*. This suppression is allele specific, as shown by the inability of the coexpression of any other pairwise combination of nonfunctional H-ras and *cdc25* mutants (initially tested here) to suppress the *cdc25^{ts}* defect (Table 1). These results suggest that residue 63 of H-Ras may form an ion pair with residue 1374 of the yeast *CDC25* gene product, and if this putative ion pair is reversed, functional interaction can still occur. Assuming this proposal to be correct, we predicted that the charge of the side groups of the reversed ion pair would be critical for functional interaction. Furthermore, the length of the side groups involved in this reversed ion pair might also affect the relative abilities of these proteins to interact. A reversed ion pair with side groups of the same length as those observed in the wild-type ion pair might result in a better functional interaction than ion pairs with altered lengths of side groups.

To test this prediction, we created additional point mutations at codon 63 of H-ras and at codon 1374 of *CDC25* (see Materials and Methods). Substitution of Glu at codon 63 of H-ras with Arg resulted in *CDC25^{GEF}*-unresponsive protein, as judged by its inability to suppress *ras^{ts}* mutant strain expressing H-GAP (data not shown), and it can suppress the *ras^{ts}* strain in the absence of H-GAP. Also, substitution of Arg at codon 1374 of yeast *CDC25^{GEF}* with either Asp or Leu resulted in nonfunctional protein, as judged by its inability to suppress the *cdc25-5* mutant strain (Fig. 1). We then examined the ability of wild-type or nonfunctional mutants in H-ras (wild-type or E63K or E63R substitution) and *CDC25* (wild-type or R1374E, R1374D, or R1374L substitution) to cooperate in the suppression of the loss of *CDC25* function (Table 1 and Fig. 1). The *cdc25^{R1374L}* mutant was unable to cooperate with H-ras, H-ras^{E63K}, or H-ras^{E63R} in suppression of the loss of *CDC25* function (Table 1). The combination of H-ras^{E63R} and *cdc25^{R1374E}*, with side chain lengths the same as those of the wild-type ion pair, was the strongest suppressor of the loss of *CDC25* function (Table 1). By contrast, the combination of H-ras^{E63K} and *cdc25^{R1374E}* or H-ras^{E63R} and *cdc25^{R1374D}*, whose reversed ion pair side chain lengths are slightly shorter than those of the wild-type ion pair, did suppress the loss of *CDC25* function, although suppression was weaker than that observed with H-ras^{E63R} and *cdc25^{R1374E}*.

TABLE 1. Ability of cotransformation of the *CDC25*- and H-ras-containing plasmids to suppress the defect of a *cdc25-5^{ts}* strain (LV25-5-H-GAP)^a

CDC25 mutation	Suppression ^b					
	No H-Ras mutation	E62K	E63K	E63R	M67I	D69N
None	+++++ ^c	+++++ ^c	+++++ ^c	+++++ ^c	+++++ ^c	+++++ ^c
R1374E	—	—	++ ^d	+++ ^e	—	—
R1444E	—	—	—	ND	—	—
R1489E	—	—	—	ND	—	—
R1374D	—	—	+ ^f	++ ^g	—	—
R1374L	—	—	—	—	—	—

^a The *cdc25-5^{ts}* strain was cotransformed with the indicated pairwise combinations of the indicated H-ras-containing plasmids in combination with the *CDC25* plasmid with the indicated mutations. Each resulting transformation was plated out on two selective plates (SC^{-Leu-Trp}). One plate was incubated at 28°C for 3 days, while the other was incubated at 34°C for 5 days.

^b The diameters of 30 random colonies from the 34°C plates were measured from photographs (fivefold enlargements) of the plates, and the mean was determined. +++++, suppression at wild-type levels of growth; +++, ++, and +, successively weaker suppression than that of wild-type *CDC25*; —, no detectable growth even after prolonged incubation at the nonpermissive temperature; ND, not determined. The groups of means designated by superscripts c, d, e, and f are statistically significantly different from each other at the 0.05 level of significance, i.e., $P < 0.01$.

^c These means (1.95 to 2.06 mm) are not statistically different from each other at the 0.05 level of significance, i.e., $P > 0.05$ (95% confidence interval, 1.87 to 2.10).

^d 95% confidence interval (0.68 to 0.80 mm), mean = 0.74.

^e 95% confidence interval (0.98 to 1.08 mm), mean = 1.03.

^f 95% confidence interval (0.26 to 0.33 mm), mean = 0.29.

^g 95% confidence interval (0.57 to 0.66 mm), mean = 0.62.

TABLE 2. Interaction of mutant and wild-type H-Ras proteins with mutant and wild-type CDC25 proteins^a

CDC25 mutation	Intensity of blue colonies ^b					
	No H-Ras mutation	E62K	E63K	E63R	M67I	D69N
None	+++++	—	—	—	—	—
R1374E	—	—	+	++	—	—
R1411E	++	—	—	ND	—	—
K1414E	+++	—	—	ND	—	—
R1444E	—	—	—	ND	—	—
R1489E	+++++	—	—	ND	—	—

^a The PCY2 strain was transformed with a pGADGH-based plasmid containing the indicated CDC25 fragment, and the resulting transformants were transformed with pGBT9-based plasmids containing the indicated H-ras fragments.

^b β -Galactosidase activity was scored by streaking independent transformants resulting from each transformation onto selective plates containing X-Gal. The relative degree of intensity of the blue colonies is indicated as follows: +++++, intense blue colonies; +, very pale blue colonies; —, no detectable blueness of colonies even after prolonged incubation as described in the text; ND, not determined. In three independent experiments, similar results were obtained.

The combination of H-ras^{E63K} and *cdc25*^{R1374D}, whose reversed ion pair side group lengths differ the most from those of the wild-type ion pair, was the weakest pair of suppressors (Table 1). These results indicate that a negatively charged residue at position 1374 of the nonfunctional CDC25 mutants is required to functionally interact with the nonfunctional H-Ras mutants with a positively charged residue at position 63 and that the length of the side groups in the putative reversed ion pairs affects the relative abilities of nonfunctional H-ras and *cdc25* mutants to cooperate in the suppression of the loss of CDC25 function.

Analysis of the interaction of mutant and wild-type H-ras and CDC25 molecules in the yeast two-hybrid system. Consistent with the findings of Munder and Furst (16), we have previously used a yeast two-hybrid system (15) to show an interaction between wild-type H-Ras and the yeast CDC25^{GEF} catalytic domain. By inserting codons 1 to 166 of H-ras into pGBT9 and codons 1100 to 1589 of the yeast CDC25 into pGADGH and cotransforming these vectors into strain PCY2, direct interaction can be assayed by observing the activation of the GAL4-dependent β -galactosidase reporter gene (see Materials and Methods).

We cotransformed strain PCY2 with pGBT9-based plasmids harboring H-ras (or with H-ras mutants defective in CDC25 binding) and pGADGH-based plasmids harboring wild-type or mutant CDC25. The pairwise cotransformations of the wild-type and mutant H-ras and CDC25 indicated in Table 2 were carried out. To ensure maintenance of both plasmids, independent transformants of each of the cotransformations were streaked out onto selective plates containing X-Gal (a colorimetric substrate for β -galactosidase). After 3 to 5 days of incubation at 28°C, β -galactosidase activity was observed by the blue color of colonies harboring wild-type CDC25, *cdc25*^{R1411E}, *CDC25*^{K1414E}, or *cdc25*^{R1489E} plasmids also containing wild-type H-ras (Table 2). After 5 days of incubation at 28°C, no blue colonies were detected in any of the other pairwise combinations of H-ras and CDC25 cotransformants, indicating no interaction or very weak interaction between the mutants. The plates were then incubated at 4°C for 10 days. After this prolonged incubation, strains harboring the H-ras^{E63K} or H-ras^{E63R} and the *cdc25*^{R1374E}-expressing plasmids showed blue colonies, indicating a weak interaction between nonfunctional proteins (Table 2). This finding sug-

FIG. 2. Synthetic peptides with sequences derived from Ras-specific GEFs inhibit binding of H-Ras to CDC25^{GEF}. GST-CDC25, bound to glutathione-agarose beads and was incubated with purified nucleotide-free H-Ras in the presence of the indicated amounts of synthetic peptide from either yeast CDC25^{GEF} (TIVKQADVKT SKLTQYFVT, residues 1364 to 1383) (A) or human CDC25^{GEF} (h-cdc25^{GEF}; EIIRNEDINARVSAIEKWVA, residues 1098 to 1117) (B). For controls, synthetic peptides were used with amino acid composition identical to residues 1364 to 1383 of the yeast CDC25^{GEF} or 1098 to 1117 of the human CDC25^{GEF}, but whose sequence was scrambled (*S. cerevisiae*, KRQDFYIKTSLTKQAV; human, DRAVESKENIRVIAIWENA). After incubation for 1 h at 4°C, the glutathione-agarose beads were washed three times with phosphate-buffered saline. The final pellets were eluted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer at 95°C; they were then subjected to Western blot analysis to detect H-Ras protein (see Materials and Methods).

gests the H-Ras^{E63K} and H-Ras^{E63R} mutant proteins are able to bind to the nonfunctional CDC25^{R1374E} protein but are otherwise nonfunctional in that they cannot interact with the wild-type proteins or other mutant versions of these proteins examined. Western blotting using anti-CDC25 rabbit polyclonal antisera demonstrated that all of the CDC25-GADGH fusion proteins were expressed at similar levels (data not shown). These results are consistent with the molecular genetic data presented above, suggesting that residue 1374 of the yeast CDC25 gene product may interact with residue 63 of the H-Ras protein.

Disruption of binding of CDC25 to wild-type H-Ras by peptides. We have previously used an in vitro assay to detect the interaction of CDC25^{GEF} and Ras proteins (10, 15). We made use of this in vitro protein-protein interaction assay to determine whether peptides with sequences identical to those surrounding residue 1374 of CDC25^{GEF} (residues 1364 to 1383) or the corresponding region of the human CDC25^{GEF} (residues 1098 to 1117) (9, 20) could disrupt the interactions between a GST-CDC25^{GEF} fusion protein and H-Ras protein (10, 15). We observed that a peptide identical to yeast CDC25^{GEF} residues 1364 to 1383, in increasing concentrations, effectively competed for GST-CDC25^{GEF}/H-Ras binding (Fig. 2). Also, a peptide identical to human CDC25^{GEF} residues 1098 to 1117 was able to compete for GST-CDC25^{GEF}/H-Ras binding (Fig. 2). Control peptides, with amino acid compositions identical to those of the CDC25^{GEF} peptides (but with scrambled sequences), at concentrations similar to those used for the CDC25^{GEF} peptides, had no detectable effect on GST-CDC25^{GEF}/H-Ras binding (Fig. 2).

GST protein alone under these conditions did not bind to H-Ras (data not shown). These results support our suggestion that the highly conserved, positively charged arginine residue at position 1374 of the yeast *CDC25* gene product, and likely surrounding amino acid residues, is involved in direct interactions with Ras proteins.

DISCUSSION

Evidence presented here and our previous results have shown that the H-Ras^{E63K} mutant is defective in interaction with wild-type *CDC25* (15), although this mutant H-Ras protein can adopt an active GTP-bound conformation. Evidence for an active conformation of the H-Ras^{E63K} mutant comes from our previous observation that a double-mutant H-Ras^{G12V,E63K} can suppress the loss of Ras function in yeast cells (15). Here we demonstrate that a *CDC25*^{R1374E} mutant is nonfunctional in yeast cells and cannot bind to wild-type H-Ras, as indicated by yeast two-hybrid analysis. Also, we demonstrate that the H-Ras^{E63K} and *cdc25*^{R1374E} mutants which cannot interact with wild-type *CDC25* and H-Ras, respectively, are able to functionally interact with each other. This conclusion is supported by the allele-specific suppression of the loss of *CDC25* function by these otherwise nonfunctional H-Ras^{E63K} and *cdc25*^{R1374E} mutants. Further, the yeast two-hybrid analysis presented here also supports this conclusion. The interaction between H-Ras^{E63K} and *CDC25*^{R1374E} is weak, as judged by the yeast two-hybrid analysis. Further, in *in vitro* experiments, we failed to detect protein-protein interactions by H-Ras^{E63K} and *CDC25*^{R1374E} proteins isolated from *E. coli* expression systems (data not shown).

The glutamic acid at position 63 of H-Ras has previously been shown to interact directly with *CDC25*^{GEF} (15). The H-Ras mutant with a change of charge substitution (namely, lysine) at position 63 disrupts interaction with *CDC25*^{GEF}. It is interesting to speculate that the wild-type glutamic acid at position 63 of H-Ras forms an ion pair with the positively charged arginine at position 1374 of *CDC25*^{GEF}. Thus, the allele-specific suppression of defects in the yeast RAS pathway and restoration of binding between the H-Ras^{E63K} and *CDC25*^{R1374E} may be due to recreating an ion pair, albeit distinct from that of the wild-type protein-protein interaction.

We note that each of the reversed ion pairs examined here is a weaker suppressor than the wild-type pair. Also, the restoration of binding by these H-Ras and *CDC25* mutants is weak, as indicated by the yeast two-hybrid system results. This is not surprising, given that a charged residue is affected by the environment in which it resides. Previous reports have yielded conflicting conclusions regarding the use of ion pair reversals in exploring protein-protein interactions (8, 21, 22). These studies presented here demonstrate the power of ion pair reversal mutagenesis in the study of some protein-protein interactions and indicate that the length of side groups may require alteration to achieve success with this approach.

We have begun to raise antisera against Ras-GEF peptides (SOS1^{GEF} residues 855 to 874, SOS2^{GEF} residues 790 to 809, and *CDC25*^{GEF} residues 1098 to 1117) which correspond to sequences surrounding residue 1374 of the yeast *CDC25*^{GEF}. We will prepare antibodies which are specific for each GEF. Microinjection of these antibodies into cells should neutralize a single Ras-specific GEF and thus prove useful in determining which mammalian Ras-specific GEFs are regulated by various growth factor receptors.

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