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## Two Types of RAS Mutants That Dominantly Interfere with Activators of RAS

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**In the fission yeast *Schizosaccharomyces pombe*, *ras1* regulates both sexual development (conjugation and sporulation) and cellular morphology. Two types of dominant interfering mutants were isolated in a genetic screen for *ras1* mutants that blocked sexual development. The first type of mutation, at Ser-22, analogous to the H-ras<sup>Asn-17</sup> mutant (L. A. Feig and G. M. Cooper, Mol. Cell. Biol. 8:3235–3243, 1988), blocked only conjugation, whereas a second type of mutation, at Asp-62, interfered with conjugation, sporulation, and cellular morphology. Analogous mutations at position 64 of *Saccharomyces cerevisiae* RAS2 or position 57 of human H-ras also resulted in dominant interfering mutants that interfered specifically and more profoundly than mutants of the first type with RAS-associated pathways in both *S. pombe* or *S. cerevisiae*. Genetic evidence indicating that both types of interfering mutants function upstream of RAS is provided. Biochemical evidence showing that the mutants are altered in their interaction with the CDC25 class of exchange factors is presented. We show that both H-ras<sup>Asn-17</sup> and H-ras<sup>Tyr-57</sup>, compared with wild-type H-ras, are defective in their guanine nucleotide-dependent release from human *cdc25* and that this defect is more severe for the H-ras<sup>Tyr-57</sup> mutant. Such a defect would allow the interfering mutants to remain bound to, thereby sequestering RAS exchange factors. The more severe interference phenotype of this novel interfering mutant suggests that it functions by titrating out other positive regulators of RAS besides those encoded by *ste6* and *CDC25*.**

Mutant versions of RAS proteins are found in a wide variety of human tumors (3). The normal vertebrate RAS regulates cellular growth, morphology, and differentiation (4). In the yeast *Saccharomyces cerevisiae*, RAS is required for vegetative growth (5, 46). In the yeast *Schizosaccharomyces pombe*, RAS is involved in pathways controlling sexual differentiation and cellular morphology (19, 40, 41). In each of these diverged species, it is evident that the nucleotide-bound state of RAS proteins regulates its activity. The conversion of the inactive RAS · GDP to the active RAS · GTP is regulated by guanine nucleotide exchange factors (GEFs) structurally and functionally related to the yeast *S. cerevisiae* *CDC25* gene product (13).

Previously, RAS mutants which dominantly interfere with wild-type RAS functions in the yeast *S. cerevisiae* and in animal cells, namely, H-ras<sup>Ala-15</sup> or its yeast homolog RAS2<sup>Ala-22</sup> (48) and H-ras<sup>Asn-17</sup> (15), have been identified. The H-ras<sup>Ala-15</sup> and RAS2<sup>Ala-22</sup> mutants likely act by titrating out upstream RAS GEFs. Evidence for this comes from the observation that the dominant interference of either mutant expressed in *S. cerevisiae* can be suppressed by overexpression of the RAS-GEF encoded by *CDC25* (45). Furthermore, in a two-hybrid assay, yeast *CDC25* was found to bind the RAS2<sup>Ala-22</sup> mutant more strongly than wild-type RAS2 (38). The mechanism by which dominant interfering mutants titrate out GEFs remains unclear, but there is evidence to suggest that the interfering mutants are locked into an inactive conformation that binds to and sequesters the intracellular pool of GEFs (14, 28, 38).

The goal of our current study was to probe the function of RAS in the fission yeast *S. pombe* by generating dominant interfering RAS mutants. The RAS pathways in *S. pombe* are not mediated by adenylyl cyclase as they are in *S. cerevisiae* (5, 56). The organism *S. pombe* has a single RAS homolog, *ras1* (19, 41), that is not involved in vegetative growth but rather is required for sexual differentiation and cellular morphology (40). A positive regulator of *ras1*, encoded by *ste6* (26), is structurally and functionally related to the *S. cerevisiae* RAS-GEF encoded by *CDC25*. In *S. pombe*, two signals, nutrient starvation and pheromones, lead to conjugation between the two mating types *h*<sup>+</sup> and *h*<sup>−</sup>. The conjugated cells then sporulate to form zygotic asci. *ras1*<sup>null</sup> cells are viable but defective in sporulation and conjugation; such cells also are rounded, whereas wild-type cells exhibit an elongated morphology (20, 40). In contrast to *ras1*<sup>null</sup> cells, *ste6*<sup>null</sup> cells are defective in conjugation but are normal with respect to both sporulation and cellular morphology. Disruption of the *ras1* gene, therefore, has a more pronounced effect than disruption of this upstream activator. The search for interfering mutants of *ras1* in *S. pombe* is attractive since *ras1* is not an essential gene and its protein product potentially regulates more than one pathway, as suggested by its roles in both sexual differentiation and cellular morphology. Here we describe the characterization of a novel dominant interfering mutant of *S. pombe* *ras1* containing substitutions at Asp-62. We have made analogous mutants of *S. cerevisiae* RAS2 and vertebrate H-ras and have characterized their interfering properties as well.

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### MATERIALS AND METHODS

**Plasmids, yeast strains, and reagents.** All strains and plasmids used in this study are described in Tables 1 and 2.

TABLE 1. *S. pombe* and *S. cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
<i>S. pombe</i>		
SP66	<i>h<sup>90</sup> leu1-32 ade6-216</i>	David Beach
SP66-R1C22	<i>h<sup>90</sup> leu1-32 ade6-216 ura4::adh-SPras1<sup>Cys-22</sup></i>	This study
SP66-R1Y62	<i>h<sup>90</sup> leu1-32 ade6-216 ura4::SPras1<sup>Tyr-62</sup></i>	This study
SP593	<i>h<sup>90</sup> leu1-32 ade6-216 ras1::ras1<sup>Val-17</sup></i>	David Beach
SP870	<i>h<sup>90</sup> leu1-32 ade6-210 ura4-D18</i>	David Beach
SPR2A	<i>h<sup>90</sup> leu1-32 ade6-216 ura4::RAS2<sup>Ala-22</sup></i>	59
SPRU	<i>h<sup>90</sup> leu1-32 ade6-210 ura4-D18 ras1::ura4</i>	59
<i>S. cerevisiae</i>		
IR-1	<i>MATa his3 leu2 ura3 trp1 ade8 can1 ira1::HIS3</i>	2
IR2.5	<i>MATa his3 leu2 ura3 trp1 ade8 can1 ira2::ADE8</i>	1a
IR2.53	<i>MATa his3 leu2 ura3 trp1 ade8 can1 ira1::HIS3 ira2::ADE8</i>	25
RB5	<i>MATα his3 leu2 ura3 trp1 ade8 can1 ira1::HIS3 ira2::ADE8 cdc25::URA3</i>	Roymarie Ballester
SP1	<i>MATa his3 leu2 ura3 trp1 ade8 can1</i>	56
STS1	<i>MATa his3 leu2 ura3 trp1 ade8 can1 ras1::URA3 ras2<sup>ts</sup></i>	48
TK161-R2V	<i>MATa his3 leu2 ura3 trp1 ad8 can1 RAS2<sup>Val-19</sup></i>	56

Plasmid pAAUN-ras1, expressing a cDNA for *S. pombe* *ras1*, was obtained from Hao-Peng Xu. Plasmid pART3-*ste6*, expressing the exchange factor for Ras1, and plasmid pUC-*ral2*, containing the genomic clone of *ral2*, a putative upstream regulator of Ras1 in *S. pombe* (21), were the kind gifts of M. Yamamoto. Plasmid pART1-*ral2*, expressing the cDNA of the *ral2* gene, was obtained by PCR using plasmid pUC-*ral2* as the template and two primers containing internal *Bam*HI sites (in boldface) to facilitate cloning into plasmid pART1: 5'-CTTATTAACGTCGGGATCCTAAACAGT-3' and 5'-GATAGAGCTACGGAGGATCCAAATGCA-3'.

*S. pombe* SP66-R1C22 and SP66-R1Y62, containing the integrated dominant interfering mutants *SPras1<sup>Cys-22</sup>* and

*SPras1<sup>Tyr-62</sup>*, respectively, were generated by transforming *S. pombe* SP66 with a *Not*I fragment derived from plasmids pVINCE1-*SPras1C22* and pVIN1-*SPras1Y62* (see below), respectively. *S. cerevisiae* RB5 was constructed by mating a *cdc25<sup>null</sup>* strain to an *ira1<sup>null</sup> ira2<sup>null</sup>* strain, sporulating, and selecting for complementation of auxotrophic markers.

**Construction of mutant *S. pombe* *ras1* library.** Mutagenesis of *S. pombe* *ras1* was carried out by PCR as described by Zhou et al. (66). The following 5' and 3' oligonucleotide primers were used: 5'-CTTAGTGTGCTTTATAGGATCCTGAA-3' and 5'-GATCGGTGCATTACCAAAAGAGCTCA-3'. These oligonucleotides contain, respectively, *Bam*HI and *Sst*I sites (in boldface) for directional cloning into identical sites in the *S.*

TABLE 2. Vectors and plasmids used in this study

Plasmid	Comments	Reference or source
<i>S. pombe</i>		
pAIS1	<i>byr2</i> expression	59
pART1- <i>byr1</i>	<i>byr1</i> expression	59
pART1	<i>ARS, LEU2, adh</i> promoter	35
pART1-HRY57	<i>H-ras<sup>Tyr-57</sup></i> expression	This paper
pART1-HRY57S186	<i>H-ras<sup>Tyr-57, Ser-186</sup></i> expression	This paper
pART1- <i>ral2</i>	Upstream positive regulator of <i>ras</i>	This paper
pART1- <i>SPras1</i>	<i>S. pombe</i> <i>ras1</i> expression	Yan Wang
pART1- <i>SPras1C22</i>	<i>S. pombe</i> <i>ras1<sup>Cys-22</sup></i> expression	This paper
pART1- <i>SPras1N22</i>	<i>S. pombe</i> <i>ras1<sup>Asn-22</sup></i> expression	This paper
pART1- <i>SPras1G62</i>	<i>S. pombe</i> <i>ras1<sup>Gly-62</sup></i> expression	This paper
pART1- <i>SPras1Y62</i>	<i>S. pombe</i> <i>ras1<sup>Tyr-62</sup></i> expression	This paper
pART1- <i>ste6</i>	<i>S. pombe</i> <i>ras1</i> nucleotide exchange factor	26
pVIN1	Vector for integration of genomic DNA	This paper
pVIN1- <i>SPras1Y62</i>	<i>S. pombe</i> <i>ras1<sup>Tyr-62</sup></i> expression	This paper
pVINCE1	Vector for integration and expression of cDNA	This paper
pVINCE1-HR	<i>H-ras</i> expression	This paper
pVINCE1-HRA35	<i>H-ras<sup>Ala-35</sup></i> expression	This paper
pVINCE1-HRY57	<i>H-ras<sup>Tyr-57</sup></i> expression	This paper
pVINCE1-HRA35Y57	<i>H-ras<sup>Ala-35, Tyr-57</sup></i> expression	This paper
pVINCE1- <i>SPras1C22</i>	<i>S. pombe</i> <i>ras1<sup>Cys-62</sup></i> expression	This paper
pVINCE1- <i>SPras1Y62</i>	<i>S. pombe</i> <i>ras1<sup>Tyr-62</sup></i> expression	This paper
<i>S. cerevisiae</i>		
pAD4Δ	2μm, <i>LEU2</i>	2
pAD54	2μm, <i>LEU2</i> , Lerner epitope tagged	17
pAD4Δ-HRY57	<i>H-ras<sup>Tyr-57</sup></i> expression	This paper
pAD4Δ-R2A22	<i>RAS2<sup>Ala-22</sup></i> expression	This paper
pAD4Δ-R2Y64	<i>RAS2<sup>Tyr-64</sup></i> expression	This paper
pAD54-R2Y64	Epitope-tagged <i>RAS2<sup>Tyr-64</sup></i> expression	This paper

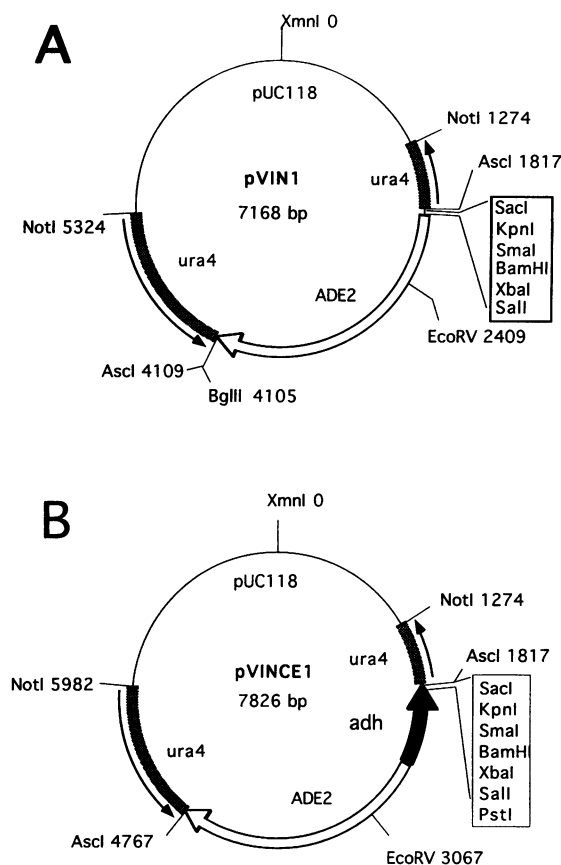


FIG. 1. Plasmids pVIN1 and pVINCE1 were constructed as described in the text. All sites except for *XbaI* are unique in the polylinker. These vectors contain the *S. cerevisiae ADE2* gene, a polylinker, or an *S. pombe adh* promoter flanked by *S. pombe ura4* segments. The entire insert can be removed by *NotI* digestion for transformation into *S. pombe*. Integration into the *ura4* locus can be verified by complementation of the adenine auxotrophy and loss of uracil prototrophy.

*pombe* expression vector pART1 (35). A library of over 10<sup>7</sup> clones was generated from a pool of eight separate 50- $\mu$ l PCRs.

**Selection of *S. pombe ras1* dominant interfering mutants.** DNA from the mutant *S. pombe ras1* library was isolated and transformed by the lithium acetate method (27) into wild-type *S. pombe* SP870. Cells were plated such that approximately 200 to 300 transformants grew per selective plate. Colonies defective for conjugation or sporulation were detected by the iodine vapor staining method (32). Plasmid DNA was isolated from these cells and retransformed into wild-type cells to confirm that the interference was due to the presence of the plasmid. The mutations in *S. pombe ras1* were identified by Sanger sequencing (49) of the full length of the gene.

**Construction of pVINCE1 and pVIN1.** pVINCE-1 (vector for integration and cDNA expression; (Fig. 1) was designed to facilitate integration of a cDNA expressed under *adh* promoter control into the *ura4* locus of *S. pombe* and was constructed as follows. Plasmid pUCN was constructed from plasmid pUC118 by removing the polylinker with *HindIII* and *EcoRI* digestion, blunt ending with Klenow polymerase, and ligating to *NotI* linkers. The *ura4*<sup>+</sup> gene was excised from vector pAIL (29) by *HindIII* digestion and treated with Klenow polymerase, and

*NotI* linkers were added for cloning into the vector pUCN, generating plasmid pUC-*ura4*. An *Ascl* restriction site was introduced into the internal *EcoRV* site of *ura4* by the use of linkers. *Ascl* linkers were then added onto a *HindIII-EcoRI* fragment obtained from pART1 (35) containing an *adh* promoter with a polylinker. The fragment was cloned into the *Ascl* site of pUC-*ura4*, thereby disrupting the *ura4* gene and generating plasmid *pura4-adh*. An *SphI* site, just upstream of the *adh* promoter, was converted into a *BclI* site by linkers. An *ADE2* gene was excised from plasmid pAZ11 (53) by restriction with *BglII* and cloned directly into the *BclI* site to generate pVINCE-1. The *ADE2* gene (53) of *S. cerevisiae* can complement the *S. pombe ade6* auxotrophy (33, 42). After cloning of the desired cDNA into the polylinker, the integrating fragment can be excised by *NotI* digestion for transformation. Successful disruption of the *ura4* locus can be identified by growth in adenine-free plates and failure to grow in uracil-free plates.

pVIN1 (vector for integration; Fig. 1) is similar to pVINCE1 except that it was designed for integration of cloned genomic fragments. This plasmid was constructed by a partial *BglII* digestion of pVINCE1 followed by *PstI* digestion to remove the *adh* promoter, then blunt ended by T4 polymerase, and self-religated.

The dominant interfering *SPras1*<sup>Cys-22</sup> and *SPras1*<sup>Tyr-62</sup> mutants were cloned into pVINCE-1, using restriction enzyme sites *BamHI* and *SstI* to create plasmids pVINCE1-*SPras1*C22 and pVINCE1-*SPras1*Y62, respectively.

**Site-specific mutagenesis of *RAS* genes and cloning into expression vectors.** Site-directed mutagenesis by the double-primer method (12) was used to modify *RAS* genes (Trans-former mutagenesis kit; Clontech, Palo Alto, Calif.). Primer 5'-TGGACATTTTGTATACGGCAGGGCAGGAAG-3' was used to change residue 64 of *S. cerevisiae RAS2*, cloned into plasmid YEplac112 (23), from an aspartic acid to a tyrosine, to create plasmid YEplac112-R2Y64. Amino acid residue 57 of human H-ras in plasmid pAAU-HR (59) was changed from an aspartic acid to tyrosine, using the oligonucleotide 5'-CTGGC CGGCGGTATACAGGATGTCCAA-3' to create plasmid pAAU-HRY57. Position 35 of the effector loop (52, 64) in wild-type H-ras (H-ras<sup>WT</sup>) and H-ras<sup>Tyr-57</sup> was changed from a threonine to an alanine by mutagenesis of plasmids pVINCE1-HR and pVINCE1-HRY57 by using the oligonucleotide 5'-ACGAATACGACCCCGCTATAGAGGATTC-3', thereby creating plasmids pVINCE1-HRA35 and pVINCE1-HRA35Y57, respectively.

Plasmid pAL-*SPras1*Y62 contains the genomic *ras1* gene with a Tyr-62 mutation and was constructed by exchanging a *BsaBI-SstI* fragment of *ras1* from pART1-*SPras1*Y62 into plasmid pALR (59) containing a genomic clone of *ras1*. The genomic clone of *SPras1*<sup>Tyr-62</sup> was then transferred into vector pVIN1 to generate plasmid pVIN1-*SPras1*Y62.

*RAS2*<sup>Tyr-64</sup> was amplified by PCR from plasmid YEplac112-R2Y64 by using two oligonucleotides, 5'-GAAAGGAGATATACAGAGTCGACAATGCCT-3' and 5'-GTGAAAATGGATGTGATTGTGCGACTCTCTG-3', that contain internal *SalI* sites (in boldface) for insertion into yeast expression vectors pAD4 $\Delta$  or pAD54 (2, 17) to generate plasmids pAD4 $\Delta$ -R2Y64 and pAD54-R2Y64, respectively. Plasmid pAD4 $\Delta$ -R2A22 was generated similarly, using as the template the genomic clone of the *RAS2*<sup>Ala-22</sup> allele (48). The H-ras<sup>Tyr-57</sup> mutant gene was excised as a *BamHI-SstI* fragment from plasmid pAAU-HRY57 and cloned into vectors pART1 and pVINCE1, to generate pART1-HRY57 and pVINCE1-HRY57, respectively, or as a *SalI-SstI* fragment for cloning into pAD4 $\Delta$  to generate plasmid pAD4 $\Delta$ -HRY57.

Cys-186 of the CAAX processing motif (62, 63) of H-ras<sup>Tyr-57</sup> was changed to a serine by PCR-directed mutagenesis using oligonucle-

otides 5'-CCTCAGGATCCGAATGACGGAATATAAGCT-3' and 5'-CCATGGTCTGACTCAGGAGACACACTT-3'. The *Bam*HI and *Sal*I sites (in boldface) allowed for cloning into identical sites in vector pART1 to create pART1-HRY57S186. The complement of ACT (underlined) encodes the serine mutation.

**Conjugation and sporulation frequencies for *S. pombe*.** After cells were transformed, the colonies were grown and starved for 1 week. Haploid cells of opposite mating types will conjugate to form zygotic asci that can be easily distinguished by their boomerang morphology, the angle representing their point of conjugation (see Fig. 2 and Results). In the case of cells that are conjugation defective, diploids can be generated by cell fusion. The sporulation of these diploidized cells generates azygotic asci (see Fig. 2D). The frequencies of zygotic or azygotic ascus formation, determined by microscopic observation, were used to calculate, respectively, the conjugation and sporulation efficiencies.

**Heat shock assays for *S. cerevisiae*.** The heat shock assays (50, 56) were performed by replica plating patches of cells that had been starved for 2 to 3 days onto plates that were preheated for 1 h at 52 or 55°C, incubated for 2.5, 5, and 10 min at the indicated temperature, and then transferred to 30°C for 2 to 3 days.

**Two-hybrid system for studies of RAS interactions.** We used the modified two-hybrid system developed by S. M. Hollenberg, H. R. Sternglanz, and H. Weintraub as described by Vojtek et al. (58) to analyze interactions of RAS with its effectors. Reporter *S. cerevisiae* strain L40 and plasmids pLexA-Ras<sup>WT</sup>, pLexA-Ras<sup>V12</sup>, pLexA-Ras<sup>A15</sup>, pVP16, pVP16-CYR, and pVP16-RAF were the generous gifts of Anne Vojtek. The gene encoding H-ras<sup>Tyr-57</sup> was amplified by PCR using the two oligonucleotides 5'-CCTCAGGATCCGAATGACGGAATATAAGCT-3' and 5'-CCATGGTCTGACTCAGGAGACACACTT-3'. These oligonucleotides contain internal *Bam*HI and *Sal*I sites (in boldface), respectively, to permit cloning into vector pBTM116 (58), creating pLexA-HRY57.

**Purification of H-ras proteins and guanine nucleotide binding.** H-ras<sup>WT</sup> and dominant interfering H-ras<sup>Tyr-57</sup> were cloned into the pTrcHis Xpress System (Invitrogen, San Diego, Calif.) vectors for expression of His<sub>6</sub>-tagged proteins and transformed into *Escherichia coli* DH10B (Bethesda Research Laboratories, Gaithersburg, Md.). Protein was purified as described by the manufacturers except that 1 mM MgCl<sub>2</sub> was included in all buffer conditions. The supernatant was passed through Ni<sup>2+</sup>-charged Sepharose ProBond resin (Invitrogen) and eluted by using step gradients of lysis buffer containing 40, 60, 100, 200, 500, and 1,000 mM imidazole. The H-ras proteins eluted at 200 mM imidazole at greater than 90% purity, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The proteins were concentrated by Centricon (Amicon, Lexington, Mass.) centrifugation, adjusted to 40% glycerol, and stored at -20°C. Nucleotide-free H-ras proteins were prepared essentially as described by Crechet et al. (11) except that after EDTA treatment, buffer was exchanged by Centricon centrifugation.

Relative affinities for nucleotides were determined as described by Farnsworth and Feig (14) by incubating 5 pmol of purified nucleotide-free H-ras proteins in 100  $\mu$ l of binding buffer containing 0.5  $\mu$ M [<sup>3</sup>H]GDP and various concentrations of unlabeled GDP or GTP as described for Fig. 5. Bound counts were determined by a nitrocellulose filter assay (16).

**Interaction of H-ras proteins with the catalytic domain of the human cdc25<sup>GEF</sup>.** A plasmid designed to express glutathione S-transferase (GST) fused to a human RAS guanine nucleotide exchange factor, cdc25<sup>GEF</sup> (60), was made by cloning of codons 863 to 1275 of cdc25<sup>GEF</sup> into vector pGEX-2T (Pharmacia, Piscataway, N.J.). Induction and bind-

ing of the GST-cdc25<sup>GEF</sup> to glutathione-agarose were performed as described previously (31). Plasmids for expression of His<sub>6</sub>-tagged H-ras<sup>WT</sup> and H-ras<sup>Tyr-57</sup> proteins are described above. The H-ras<sup>Asn-17</sup> expression plasmid, pAT-rasH(17N), was kindly provided by C. Der. *E. coli* cells containing each of the H-ras expression plasmids were grown to an optical density at 600 nm of 0.4. Isopropylthiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the cultures were incubated overnight at 28°C. Triton X-100-solubilized extracts were prepared as described previously (31).

To examine the interaction of the H-ras proteins with the catalytic domain of cdc25<sup>GEF</sup>, suspensions of glutathione-agarose beads complexed to 30  $\mu$ g of GST-cdc25<sup>GEF</sup> were incubated at 4°C while rotating with 1 ml of a phosphate-buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub> [pH 7.4]) solution containing 0.5 mM EDTA (to ensure that H-ras proteins remain nucleotide free), 1% Triton X-100, and a final 0.5- $\mu$ g/ml concentration of H-ras<sup>WT</sup>, H-ras<sup>Asn-17</sup>, or H-ras<sup>Tyr-57</sup> protein. After 2-h incubations, each of the suspensions was divided equally into three tubes and then pelleted and washed three times with 1 ml of PBS containing 1% Triton X-100 without EDTA. Proteins bound to the glutathione-agarose were eluted from one set of triplicate pellets by addition of 25  $\mu$ l of 5 mM glutathione, after which the samples were incubated at 22°C for 1 h and overnight at 4°C (sample 1 in Fig. 6). To the other two samples were added 25  $\mu$ l of PBS containing 10 mM MgCl<sub>2</sub> and either 10 mM GTP (sample 2 in Fig. 6) or 10 mM GDP (sample 3 in Fig. 6), after which the samples were incubated at 22°C for 1 h and overnight at 4°C. After removal of the material eluted by conditions used for samples 2 and 3, the H-ras protein remaining bound to the glutathione-agarose-GST-cdc25<sup>GEF</sup> matrix was released by treatment with 25  $\mu$ l of 5 mM glutathione for 1 h at 22°C and overnight at 4°C (samples 4 and 5, respectively). Samples were analyzed by Western blot (immunoblot) analysis, using the Immunolite assay kit (Bio-Rad, Richmond, Calif.) and anti-RAS antibody A6-1 (Oncogene Sciences, Uniondale, N.Y.) as the primary antibody.

**Binding of guanine nucleotides to preformed complexes of H-ras proteins and the catalytic domain of human cdc25<sup>GEF</sup>.** H-ras<sup>WT</sup>, H-ras<sup>Asn-17</sup>, and H-ras<sup>Tyr-57</sup> proteins were bound to glutathione-agarose-GST-cdc25<sup>GEF</sup> as described above. The final pellets were resuspended in PBS without EDTA. Aliquots of these suspensions, containing equivalent amounts of H-ras<sup>WT</sup>, H-ras<sup>Asn-17</sup>, and H-ras<sup>Tyr-57</sup>, as judged by SDS-PAGE and Coomassie blue staining, were incubated with 1  $\mu$ M [<sup>3</sup>H]GDP or [<sup>3</sup>H]GTP (10 Ci/mmol; New England Nuclear) in the presence of 5 mM MgCl<sub>2</sub> for 30 min at 22°C and then 5 min at 0°C. The entire reaction mixture was passed through a nitrocellulose filter as described previously (5) to determine the <sup>3</sup>H-labeled guanine nucleotides bound to H-ras proteins. The bound counts therefore reflect the guanine nucleotides bound to H-ras proteins both complexed to and dissociated from GST-cdc25<sup>GEF</sup>. At 1  $\mu$ M guanine nucleotide, there was no detectable dissociation of either H-ras<sup>Asn-17</sup> or H-ras<sup>Tyr-57</sup> protein from the complex with GST-cdc25<sup>GEF</sup>, whereas H-ras<sup>WT</sup> protein was dissociated from GST-cdc25<sup>GEF</sup> to near completion (data not shown). In control reactions containing glutathione-agarose-GST-cdc25<sup>GEF</sup>, less than 40 cpm of <sup>3</sup>H-labeled guanine nucleotides was found to bind.

## RESULTS

**Isolation of dominant interfering *S. pombe* ras1 mutants.** Upon starvation and stimulation by pheromones, *S. pombe* cells of opposite mating types will conjugate and then undergo

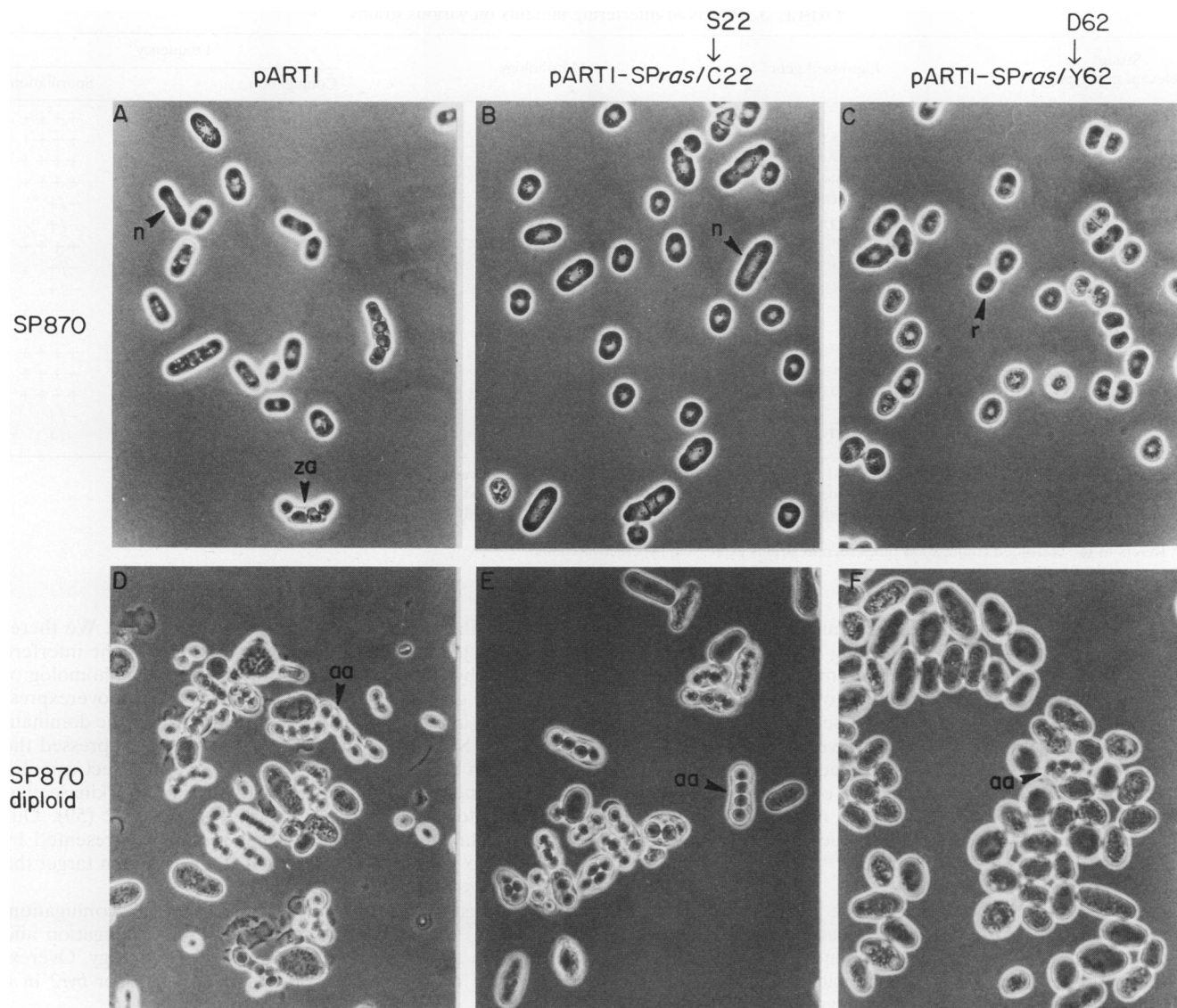


FIG. 2. Suppression of sexual and morphological development by interfering mutants of *S. pombe ras1*. The dominant interfering mutant *SPras1*<sup>Cys-22</sup> (B and E) or *SPras1*<sup>Tyr-57</sup> (C and F) was expressed in either haploid (A to C) or diploidized (D to F) wild-type *S. pombe* SP870. Wild-type cells, transformed with the control vector pART1, normally exhibit an elongated morphology (n; panel A). Wild-type haploid cells, under starvation conditions, will conjugate and then sporulate to form zygotic asci (za [A]), whereas diploid cells will sporulate to form azygotic asci (aa [D]). Wild-type cells expressing the *SPras1*<sup>Cys-22</sup> interfering mutant are defective in conjugation (B) but not sporulation (E). Wild-type cells expressing the *SPras1*<sup>Tyr-57</sup> interfering mutant are defective in conjugation (C) and sporulation (F). Furthermore, cells containing *SPras1*<sup>Tyr-57</sup> are morphologically defective. They are rounded (r) instead of elongated (n).

sporulation and ascus formation (Fig. 2A). Carbohydrate accumulation is a hallmark of the sporulation process and can be detected by staining with iodine vapor (32). Since *S. pombe ras1* regulates conjugation and sporulation, cells with a defect in the *ras1* pathway will not stain with iodine. Cells containing a hyperactivated *ras1* are also defective in conjugation and will not stain with iodine (40). The iodine staining method was therefore used to isolate mutants of *S. pombe ras1* that can block the conjugation-sporulation process. An expression library for mutants of *ras1* was generated by PCR as described in Materials and Methods. Of 26,000 colonies established by transformation of wild-type *S. pombe* with the library, 69 failed to stain positively for starch accumulation by iodine vapor. Of the 69, 45 were determined microscopically to have a pheno-

type indistinguishable from that induced by activated *ras1* mutants. *S. pombe* cells containing an activated *ras1* allele exhibit an exaggerated sexual response characterized by heightened agglutination, the formation of elongated conjugation tubes, and a failure to conjugate under starvation conditions (20, 40). These characteristics are referred to as a hypersexed phenotype. Plasmid DNA was successfully rescued from 21 of the remaining 24 isolates. Of these, 13 were wild type on retransformation and 8 dominantly interfered with conjugation or sporulation. These eight interfering mutants were equally distributed into two phenotypically distinct categories. The first type of interfering mutants conferred a *ste6*<sup>null</sup> phenotype onto wild-type *S. pombe* cells (Fig. 2B and E). *ste6* encodes the *S. pombe* homolog of *S. cerevisiae* RAS-GEF,

TABLE 3. Effects of interfering mutants on various strains

Strain (relevant genotype)	Expressed gene <sup>a</sup>	Morphology	Frequency <sup>b</sup>	
			Conjugation	Sporulation <sup>c</sup>
SP1 (wild type)	—	Elongated	++++	++++
	<i>SPras1</i> <sup>Cys-22</sup>	Elongated	—	++++
	<i>SPras1</i> <sup>Asn-22</sup>	Elongated	—	++++
	<i>RAS2</i> <sup>Ala-22</sup>	Elongated	—	++++
	<i>SPras1</i> <sup>Gly-62</sup>	Rounded	—	-/+
	<i>SPras1</i> <sup>Tyr-62</sup>	Rounded	—	-/+
	<i>H-ras</i> <sup>Ala-35d</sup>	Elongated	++++	++++
	<i>H-ras</i> <sup>Tyr-57</sup>	Rounded	—	-/+
	<i>H-ras</i> <sup>Ala-35, Tyr-57d</sup>	Rounded	—	-/+
	<i>H-ras</i> <sup>Tyr-57, Ser-186</sup>	Elongated	++++	++++
SP593 ( <i>ras1</i> <sup>Val-17</sup> )	—	Hypersexed <sup>e</sup>	—	++++
	<i>RAS2</i> <sup>Ala-22</sup>	Hypersexed	—	++++
	<i>SPras1</i> <sup>Cys-22</sup>	Hypersexed	—	++++
	<i>SPras1</i> <sup>Tyr-62</sup>	Hypersexed	—	++++
SPRU ( <i>ras1</i> <sup>null</sup> )	—	Rounded	—	-/+
	<i>SPras1</i> <sup>Tyr-62</sup>	Rounded	—	-/+

<sup>a</sup> Genes were expressed from the vector pART1 unless otherwise specified. — indicates control vector.

<sup>b</sup> Relative to the wild-type frequency. Each + designates an increment of 25%; -/+ indicates less than 1%.

<sup>c</sup> For conjugation-defective cells, polyethylene glycol fusion was used to generate diploids for analysis of sporulation efficiencies.

<sup>d</sup> Expressed from the vector pVINCE1.

<sup>e</sup> Refers to the elongated conjugation tubes seen in strains expressing hyperactivated *ras1*.

CDC25, and its disruption manifests as a conjugation defect (26). The second type of interfering mutants conferred a *ras1*<sup>null</sup> phenotype when transformed into wild-type cells (Fig. 2C and F). Similar to the first type of interfering mutants, wild-type cells transformed with the second type of mutants were also conjugation defective. However, unlike the first type of mutants, they caused additional defects: cells were rounded rather than elongated, and sporulation of diploid cells was also suppressed. Sequence analysis of the mutant *ras1* genes revealed that only four of the eight clones had single missense mutations. Each of the *ste6*<sup>null</sup> or *ras1*<sup>null</sup> category of interfering mutants had two members.

There was a correlation between the positions of the mutations and the interfering phenotypes that they induced (Fig. 2). The two mutants that conferred a phenotype similar to a *ste6* disruption were altered at amino acid 22, where a serine encoded by AGT was changed into a cysteine (TGT, transversion, *SPras1*<sup>Cys-22</sup>) or into an asparagine (AAT, transition, *SPras1*<sup>Asn-22</sup>). These are analogous to the Asn-17 mutation of vertebrate H-ras (15). Those interfering mutants that conferred a phenotype similar to a *ras1* disruption were altered at amino acid position 62, where an aspartic acid encoded by GAC was changed to a glycine (GGC, transition, *SPras1*<sup>Gly-62</sup>) or to a tyrosine (TAC, transversion, *SPras1*<sup>Tyr-62</sup>). Others have previously introduced mutations into the analogous Asp-57 of H-ras, but interfering mutants were not created thereby (14, 28).

**Genetic analysis of the *S. pombe* interfering mutants.** We observed that neither the *SPras1*<sup>Cys-22</sup> nor the *SPras1*<sup>Tyr-62</sup> mutant blocked the hypersexed phenotype associated with an activated *ras1*<sup>Val-17</sup> (Table 3), thereby indicating that they both interfere upstream of *ras1*. Position 22 of *S. pombe* *ras1* corresponds to position 17 of vertebrate H-ras. Mutations at position 17 of vertebrate H-ras have been shown to dominantly interfere with RAS function in vertebrates (14, 15). A similar mutation at nearby position 15 of H-ras, or the analogous position 22 of *S. cerevisiae* RAS2, also creates a dominant interfering mutant that likely functions by sequestration of the RAS-GEF in *S. cerevisiae* encoded by *CDC25* (38, 48). In *S. pombe*, the interfering property of *SPras1*<sup>Cys-22</sup> is indistinguish-

able from that observed for *RAS2*<sup>Ala-22</sup> (Table 4). We therefore predicted that the *SPras1*<sup>Cys-22</sup> mutant might interfere with Ras1 by similarly sequestering the *S. pombe* homolog of the *CDC25* gene product encoded by *ste6*. Indeed, overexpression of *ste6* in a wild-type strain containing either the dominant interfering *SPras1*<sup>Cys-22</sup> or *RAS2*<sup>Ala-22</sup> mutant suppressed the conjugation defect (Table 4). The conjugation defect can also be overcome by overexpression of *ras1* or the kinase that functions downstream of *ras1*, *byr1* (39), or *byr2* (59). Our genetic data therefore suggest that mutants represented by positions 15 and 17 of H-ras have as their common target the CDC25 class of regulatory exchange factors.

In contrast to *SPras1*<sup>Cys-22</sup>, which blocked only conjugation, the *SPras1*<sup>Tyr-62</sup> interfering mutant blocked conjugation and sporulation and interfered with cellular morphology. Overexpression of the protein kinase encoded by *byr1* or *byr2* in a

TABLE 4. Suppression of interference by various genes<sup>a</sup>

Strain	Expressed gene	Morphology	Frequency	
			Conjugation	Sporulation
SPR2A	—	Elongated	—	++++
	<i>byr1</i>	Elongated	+++	++++
	<i>byr2</i>	Elongated	+++	++++
	<i>ras1</i>	Elongated	+++	++++
	<i>ste6</i>	Elongated	+++	++++
SP66-R1C22	—	Elongated	—	++++
	<i>byr1</i>	Elongated	+++	++++
	<i>byr2</i>	Elongated	+++	++++
	<i>ras1</i>	Elongated	+++	++++
	<i>ste6</i>	Elongated	+++	++++
	<i>ral2</i>	Elongated	—	++++
SP66-R1Y62	—	Rounded	—	-/+
	<i>byr1</i>	Rounded	—	+++
	<i>byr2</i>	Rounded	—	+++
	<i>ras1</i>	Elongated	+++	+++
	<i>ste6</i>	Rounded	—	-/+
	<i>ral2</i>	Rounded	—	-/+

<sup>a</sup> For details, see the footnotes to Table 3.



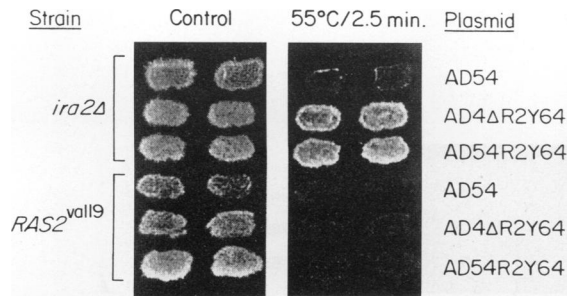


FIG. 3. Suppression of heat shock phenotype in *S. cerevisiae* containing hyperactivated RAS2. RAS is hyperactivated in these strains either because of disruption of the GTPase-activating protein encoded by *IRA2* (strain IR2.5) or because of the presence of the activated allele *RAS2*<sup>Val-19</sup> (strain TK161-R2V). Plasmids pAD4Δ and pAD54 are high-copy-number plasmids for expressing genes from an *adh* promoter (see Table 2).

SPras1<sup>Tyr-62</sup>-containing strain was only able to suppress the sporulation defect, whereas overexpression of wild-type *ras1* was able to overcome all SPras1<sup>Tyr-62</sup>-induced defects (Table 4). This suppression profile is indistinguishable from that observed for a strain with *ras1* deleted (39, 59). The ability of the SPras1<sup>Tyr-62</sup> mutant to block Ras1 function more profoundly than the SPras1<sup>Cys-22</sup> mutant suggests that Ras1 is regulated by other activators besides that encoded by *ste6*. *ral2* has been proposed to encode an alternate positive regulator of *ras1*, since it functions upstream of *ras1* and its disruption is phenotypically indistinguishable from a *ras1* disruption (22). We observed that *ral2* overexpression did not rescue interference by SPras1<sup>Cys-22</sup> or SPras1<sup>Tyr-62</sup> (Table 4).

**Genetic analysis of *S. cerevisiae* *RAS2*<sup>Tyr-64</sup>.** In *S. cerevisiae*, products of *RAS* genes are involved in cell growth and regulation of cellular responses to nutrient signals. They are regulated in a positive fashion by the gene product of *CDC25* (6, 8), encoding its GEF, and negatively by products of *IRA1* and *IRA2*, encoding homologs of mammalian GTPase-activating proteins (55). *S. cerevisiae* cells containing a hyperactivated RAS exhibit a characteristic heat shock sensitivity (47).

A mutant analogous to SPras1<sup>Tyr-62</sup>, *RAS2*<sup>Tyr-64</sup>, was made from *RAS2* of *S. cerevisiae* to determine if it too has interfering properties. We found that *RAS2*<sup>Tyr-64</sup> inhibits growth of *S. cerevisiae* cells and that this inhibition can be relieved by overexpression of elements in the RAS-adenylyl cyclase pathway. *RAS2*<sup>Tyr-64</sup> did not inhibit the growth of a strain containing an activated *RAS2*<sup>Val-19</sup> allele, therefore showing that *RAS2*<sup>Tyr-64</sup>, like SPras1<sup>Tyr-62</sup> in *S. pombe*, functions upstream of RAS (data not shown). As further indication that the interference is upstream of RAS, *RAS2*<sup>Tyr-64</sup> cannot attenuate the heat shock sensitivity of a *RAS2*<sup>Val-19</sup>-containing strain (Fig. 3; Table 5).

We next tested if *RAS2*<sup>Tyr-64</sup> can attenuate the heat shock sensitivity resulting from RAS that is hyperactivated by disruption of the negative regulators encoded by *IRA1* and *IRA2*. *RAS2*<sup>Tyr-64</sup> was able to attenuate the heat shock sensitivity of strains deleted of either or both *IRA1* and *IRA2* (Fig. 3; Table 5). An *ira1*<sup>null</sup> *ira2*<sup>null</sup> strain is still heat shock sensitive when the positive regulator *CDC25* is also deleted (1). *RAS2*<sup>Tyr-64</sup> was capable of reversing the heat shock phenotype even in a *cdc25*<sup>null</sup> *ira1*<sup>null</sup> *ira2*<sup>null</sup> strain (Table 5). In contrast, we found that the interfering mutant *RAS2*<sup>Ala-22</sup> can attenuate the heat shock phenotype of an *ira1*<sup>null</sup> *ira2*<sup>null</sup> strain but not a *cdc25*<sup>null</sup> *ira1*<sup>null</sup> *ira2*<sup>null</sup> strain. We take this to mean that a positive

TABLE 5. Relative suppression of heat shock phenotype

Relevant genotype <sup>b</sup>	Suppression of heat shock sensitivity <sup>a</sup>		
	<i>RAS2</i> <sup>Ala-22</sup>	<i>RAS2</i> <sup>Tyr-64</sup>	H- <i>ras</i> <sup>Tyr-57</sup>
<i>ira1</i> <sup>null</sup>	+	+	+
<i>ira2</i> <sup>null</sup>	+	+	+
<i>ira1</i> <sup>null</sup> <i>ira2</i> <sup>null</sup>	+	+	—
<i>cdc25</i> <sup>null</sup> <i>ira1</i> <sup>null</sup> <i>ira2</i> <sup>null</sup>	—	+	—
<i>RAS2</i> <sup>Val-19</sup>	—	—	—

<sup>a</sup> +, attenuation of the heat shock sensitivity relative to the same strain containing a control plasmid; —, absence of effect on heat shock sensitivity. The vector used in the control and for expression of the interfering RAS mutants, resulted in no suppression of heat shock sensitivity.

<sup>b</sup> Heat shock assays were carried out at 55°C (*ira1*<sup>null</sup> and *ira2*<sup>null</sup>) or at 52°C (*ira1*<sup>null</sup> *ira2*<sup>null</sup>, *cdc25*<sup>null</sup> *ira1*<sup>null</sup> *ira2*<sup>null</sup>, and *RAS2*<sup>Val-19</sup>, because of the increased heat shock sensitivity of the strains).

regulator of *RAS2* other than *CDC25* exists in *S. cerevisiae* and is also the target of *RAS2*<sup>Tyr-64</sup>.

**Interfering properties of mammalian H-*ras*<sup>Tyr-57</sup>.** A mutation analogous to SPras1<sup>Tyr-62</sup> was made in H-*ras* at position 57, H-*ras*<sup>Tyr-57</sup>, and expressed in wild-type *S. pombe* or *S. cerevisiae* cells. The interference resulting from SPras1<sup>Tyr-62</sup> was mimicked by H-*ras*<sup>Tyr-57</sup> when expressed in *S. pombe* (Table 3). However, in *S. cerevisiae*, it interfered more weakly, failing to suppress the heat shock phenotype of strains deleted of both *IRA1* and *IRA2* (Table 5).

We also studied two double mutants of H-*ras* in *S. pombe*. To test if the interfering mutant was functioning via titration of upstream regulators rather than downstream effectors, we constructed a double mutant that contains both a Tyr-57 mutation and an effector loop mutation, Ala-35, that is believed to block downstream signaling from *ras* (52). This double mutant, H-*ras*<sup>Ala-35, Tyr-57</sup>, still induced a *ras1*<sup>null</sup> phenotype, indicating that the dominant interference is not due to blocking downstream elements of the *ras1* pathway (Table 3). The H-*ras*<sup>Ala-35</sup> mutant did not exhibit any interfering functions, which is consistent with observations by others that Thr-35 substitutions of H-*ras* block its own function without interfering with endogenous H-*ras* functions (14, 28). We also tested if membrane localization is required for the dominant interfering properties of H-*ras*<sup>Tyr-57</sup>. It was previously shown that blocking the posttranslational addition of lipid to Cys-186 of the CAAX motif (63) for the interfering mutant H-*ras*<sup>Asn-17</sup> abolished its interfering properties (15). Mutation of Cys-186 to an arginine in H-*ras*<sup>Tyr-57</sup> also blocked its interfering activities when tested in *S. pombe*. H-*ras*<sup>Tyr-57, Arg-186</sup> failed to inhibit sexual development or effect the cellular morphology of wild-type *S. pombe* cells (Table 3).

**Two-hybrid analyses of interactions between H-*ras* proteins and its effectors.** The two-hybrid system (18) has been used to study proteins that interact with H-*ras* (57, 58). The *S. cerevisiae* reporter strain L40 permits the detection of protein-protein interaction by transcriptional activation of both a *HIS3* and a *lacZ* gene. As reported previously (58), both H-*ras*<sup>WT</sup> and activated H-*ras*<sup>Val-12</sup> interacted with the catalytic domain of *S. cerevisiae* exchange factor *CDC25*, indicated by complementation of host histidine auxotrophy and by a β-galactosidase color assay (Fig. 4). They also interacted with two downstream effectors of RAS, budding yeast adenylyl cyclase and mammalian RAF (Fig. 4). In contrast, both the H-*ras*<sup>Ala-15</sup> and H-*ras*<sup>Tyr-57</sup> interfering mutants failed to interact with the downstream effectors. Relative to H-*ras*<sup>WT</sup>, the H-*ras*<sup>Ala-15</sup> and H-*ras*<sup>Tyr-57</sup> mutants interacted more strongly with *CDC25*, as indicated by the increased intensity from the β-galactosidase



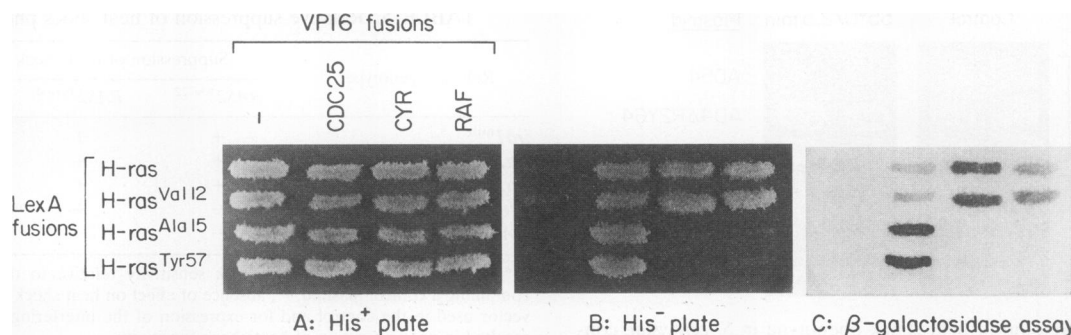


FIG. 4. Two-hybrid analysis of H-ras and mutant interactions with effectors. The reporter strain used in this system permits for detection of protein-protein interaction through transcriptional activation of both the *HIS3* and the *lacZ* gene. Cells were transformed with the plasmids noted in panel A. (A) Growth of transformants on histidine-containing plates. (B) Growth of transformants on histidine-free plates. (C) Growth of cells on histidine-containing plates followed by assay for  $\beta$ -galactosidase activity.

assay (Fig. 4). This finding confirms our genetic data suggesting that the interfering mutants function through sequestration of upstream activators of RAS.

**Guanine nucleotide binding characteristics of H-ras<sup>Tyr57</sup>.** Previously characterized dominant interfering mutants of RAS have been shown to have altered affinities for guanine nucleotides, exhibiting a preference for binding GDP over GTP (14, 28, 45). We therefore determined if this was a property shared by the H-ras<sup>Tyr57</sup> mutant. H-ras<sup>WT</sup> and H-ras<sup>Tyr57</sup> were purified as fusion proteins, and their nucleotide binding properties were determined by using a relative affinity assay as described by Farnsworth and Feig (14) (Fig. 5). Whereas H-ras<sup>WT</sup> bound GDP and GTP with similar affinities, the H-ras<sup>Tyr57</sup> mutant exhibited less than a 10-fold preference for GDP in the presence of GTP. Therefore, H-ras<sup>Tyr57</sup>, similar to other interfering mutants, binds preferably to GDP over GTP.

**Interaction of H-ras proteins with the catalytic domain of the human cdc25<sup>GEF</sup>.** We next determined if there were

biochemical differences between the interactions of wild-type or interfering mutant H-ras proteins and a human homolog of *S. cerevisiae* CDC25, cdc25<sup>GEF</sup>. A GST fusion with the catalytic domain of cdc25<sup>GEF</sup> was constructed as described in Materials and Methods. Wild-type or mutant H-ras protein, in the nucleotide-free state, readily associates with GST-cdc25<sup>GEF</sup> bound to glutathione agarose (Fig. 6, lane 1), confirming previous observations by Lai et al. (31). The H-ras<sup>WT</sup> protein could be readily released from the glutathione agarose-GST-cdc25<sup>GEF</sup> complex by addition of 10 mM GTP or GDP (Fig. 6). The H-ras<sup>Asn-17</sup> mutant could be only partially released from the agarose matrix by addition of 10 mM GTP or GDP. However, in marked contrast to H-ras<sup>WT</sup> and H-ras<sup>Asn-17</sup> proteins, 10 mM GTP or GDP allowed only a trace release of H-ras<sup>Tyr57</sup> protein (Fig. 6, lanes 2 and 3, respectively). Following elution of the H-ras proteins by GTP or GDP from the complexes, we treated each of the resulting complexes with 5 mM glutathione to determine the amounts of the various H-ras proteins remaining bound to GST-cdc25<sup>GEF</sup>. Western blot

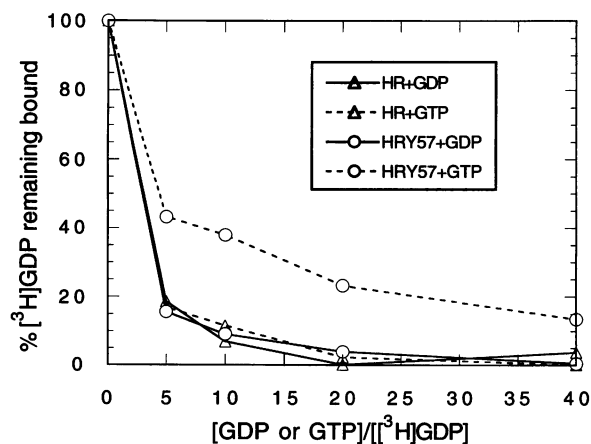


FIG. 5. Relative affinities of H-ras ( $\Delta$ ) and H-ras<sup>Tyr57</sup> ( $\circ$ ) for GDP (—) and GTP (---) were determined as described in Materials and Methods. A constant 0.5 mM [ $^3$ H]GDP was used in the presence of various concentrations of unlabeled GDP or GTP. The abscissa represents the concentration of unlabeled nucleotide used divided by the fixed concentration of labeled [ $^3$ H]GDP. Percentages of bound counts were determined from the ratio of bound counts attained in the presence of unlabeled nucleotide over that obtained without unlabeled nucleotides added. Results are averages of duplicate datum points which differed by no more than 20%.

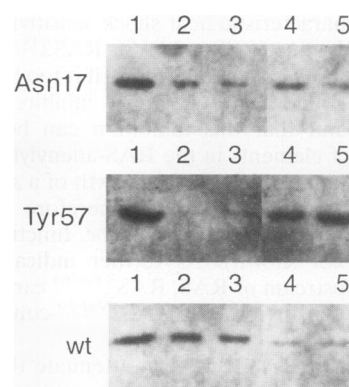


FIG. 6. Guanine nucleotide-dependent release of H-ras proteins from a complex with GST-cdc25<sup>GEF</sup>. H-ras proteins were bound to glutathione-agarose-GST-cdc25<sup>GEF</sup> and divided into three equivalent portions. For each portion, H-ras proteins were released by 5 mM glutathione (lane 1), 10 mM GTP and 10 mM MgCl<sub>2</sub> (lane 2), or 10 mM GDP and 10 mM MgCl<sub>2</sub> (lane 3). After elution with GTP or GDP, the remaining H-ras proteins bound to the glutathione-agarose-GST-cdc25<sup>GEF</sup> were eluted with 5 mM glutathione (lanes 4 and 5, respectively). The relative amounts of H-ras proteins eluted by these various conditions were determined by Western analysis as described in Materials and Methods.

TABLE 6. Binding of guanine nucleotides to RAS · GST-cdc25<sup>GEF</sup> complexes

Protein	cpm of nucleotide bound	
	GDP	GTP
H-ras <sup>WT</sup>	670	678
H-ras <sup>Asn-17</sup>	628	526
H-ras <sup>Tyr-57</sup>	633	709

<sup>a</sup> <sup>3</sup>H-labeled guanine nucleotide binding to matrices containing GST-cdc25<sup>GEF</sup> bound to the indicated H-ras protein was monitored in a nitrocellulose filter binding assay as described in Materials and Methods. Samples containing equivalent amounts of H-ras proteins were incubated with 1 μM [<sup>3</sup>H]GDP or [<sup>3</sup>H]GTP as indicated at 22°C for 30 min. Values are averages of duplicate datum points which did not differ by more than 15%. Less than 40 cpm of [<sup>3</sup>H]GDP or [<sup>3</sup>H]GTP was found to bind in negative control reactions containing glutathione-agarose bound only to GST-cdc25<sup>GEF</sup>. Similar results were obtained in two independent experiments.

analysis of these glutathione-released samples indicated that only trace amounts of H-ras<sup>WT</sup> remained bound to the matrix, whereas significant amounts of H-ras<sup>Tyr-57</sup> and H-ras<sup>Asn-17</sup> proteins remained bound after GDP or GTP treatment. The defect in the ability of guanine nucleotides to release H-ras<sup>Asn-17</sup> or H-ras<sup>Tyr-57</sup> from cdc25<sup>GEF</sup> might explain how interfering mutants sequester endogenous pools of GEF to block normal RAS functions. The more severe defect apparent in H-ras<sup>Tyr-57</sup> might explain why it is a stronger interfering mutant than H-ras<sup>Asn-17</sup>.

**Binding of guanine nucleotides to preformed complexes of H-ras proteins and the catalytic domain of human cdc25<sup>GEF</sup>.** GDP and GTP are unable to effectively disrupt a complex of nucleotide-free H-ras<sup>Tyr-57</sup> and GST-cdc25<sup>GEF</sup> under conditions in which H-ras<sup>WT</sup> is readily dissociated from GST-cdc25<sup>GEF</sup>. This observation indicates that either the H-ras<sup>Tyr-57</sup> mutant, when complexed with GST-cdc25<sup>GEF</sup>, has a very low affinity for guanine nucleotides and hence remains in a nucleotide-free state tightly complexed with GST-cdc25<sup>GEF</sup> or that the binding of guanine nucleotides to the H-ras<sup>Tyr-57</sup> · GST-cdc25<sup>GEF</sup> complex does not induce the conformational changes in H-ras<sup>Tyr-57</sup> necessary for its release from GST-cdc25<sup>GEF</sup>. We therefore examined the ability of complexes of GST-cdc25<sup>GEF</sup> and either H-ras<sup>WT</sup>, H-ras<sup>Asn-17</sup>, or H-ras<sup>Tyr-57</sup> protein to bind <sup>3</sup>H-labeled guanine nucleotides at a concentration of 1 μM. Previous work has shown that a GDP or GTP concentration of 1 μM is above that required for saturation of binding of the H-ras · GST-CDC25<sup>GEF</sup> complex (37). At this concentration of guanine nucleotides, H-ras<sup>WT</sup> protein is released to near completion (data not shown; see reference 37), whereas mutant H-ras proteins remain bound to GST-cdc25<sup>GEF</sup>. Therefore, we determined the total guanine nucleotide bound to H-ras proteins both complexed to and dissociated from GST-cdc25<sup>GEF</sup>. There was no significant change in the ability of H-ras<sup>WT</sup>, H-ras<sup>Asn-17</sup>, or H-ras<sup>Tyr-57</sup> protein, as a preformed complex with GST-cdc25<sup>GEF</sup>, to bind guanine nucleotides (Table 6). Thus, in this assay, H-ras<sup>Asn-17</sup> or H-ras<sup>Tyr-57</sup> protein complexed to GST-cdc25<sup>GEF</sup> does not have any apparent defect in the ability to bind guanine nucleotides. Rather, the binding of guanine nucleotides to these complexes does not allow the H-ras<sup>Asn-17</sup> or H-ras<sup>Tyr-57</sup> protein to dissociate from cdc25<sup>GEF</sup>.

## DISCUSSION

Dominant interfering mutants of *ras1* that block *ras1* functions when expressed in wild-type *S. pombe* cells have been isolated. These mutants fall into two categories. Type I confers

a *ste6*<sup>null</sup> phenotype. Type II confers a *ras1*<sup>null</sup> phenotype. Type I mutants, analogous to the previously described H-ras<sup>Ala-15</sup> (48) and H-ras<sup>Ala-17</sup> mutants (15), likely function by titrating out some positive regulators of RAS, e.g., the nucleotide exchange factors encoded by the *S. pombe ste6* gene and the *S. cerevisiae CDC25* gene. Evidence for this comes from our observation that the interfering phenotypes induced by the SPras1<sup>Cys-22</sup> mutant or the RAS2<sup>Ala-22</sup> mutant can be completely reversed by overexpression of *ste6*. In a two-hybrid protein-protein interaction experiment, the type I human H-ras<sup>Ala-15</sup> mutant binds the yeast *S. cerevisiae* exchange factor, CDC25, more strongly than H-ras<sup>WT</sup>. We also demonstrate that the H-ras<sup>Asn-17</sup> protein binds more stably to the catalytic domain of human *cdc25* gene product, cdc25<sup>GEF</sup>, at concentrations of guanine nucleotides that will effectively dissociate H-ras<sup>WT</sup> from a similar complex.

The type II mutants, like the type I mutants, also function upstream of RAS and not by titrating out downstream effectors of RAS. First, SPras1<sup>Tyr-62</sup> did not diminish the hypersexed phenotype of *S. pombe* cells containing activated Ras1<sup>Val-17</sup>, and RAS2<sup>Tyr-64</sup> did not block the heat shock phenotype in *S. cerevisiae* cells containing activated RAS2<sup>Val-19</sup>. These observations also indicate that it is unlikely that these mutants interfere with RAS processing, as the mechanism for interference. Second, we also constructed a double mutant, H-ras<sup>Ala-35, Tyr-57</sup>, containing an Ala-35 mutation that reduces biological activity of H-ras by disrupting interaction with the immediate downstream target (52). The double mutant maintained its ability to block *ras1* function. Third, in a two-hybrid study, it was found that the H-ras<sup>Tyr-57</sup> mutant was not able to interact with two downstream effectors, adenylyl cyclase and RAF, but bound CDC25 more strongly. Both the double-mutant experiment and the results from the two-hybrid system support the conclusion that the H-ras<sup>Tyr-57</sup> mutant is interfering by titrating out upstream activators of RAS.

The type II mutants represent a novel class of interfering mutants with properties distinguishing them from the type I mutants. They exhibit an interference more pronounced than that observed for the type I mutants. Type II mutants can inhibit all *ras1*-associated phenotypes in *S. pombe*, whereas type I mutants, similar to a *ste6* disruption, can block conjugation but cannot interfere with sporulation or cell morphology. The type I interfering mutants can be overcome by overexpression of *ste6*, suggesting that *ste6* is its target. Interference by type II mutants cannot be overcome by overexpression of *ste6*. In *S. cerevisiae*, a type II mutant can attenuate the heat shock sensitivity of an *ira1*<sup>null</sup> *ira2*<sup>null</sup> or a *cdc25*<sup>null</sup> *ira1*<sup>null</sup> *ira2*<sup>null</sup> strain. In contrast, a type I mutant attenuated the heat shock sensitivity of an *ira1*<sup>null</sup> *ira2*<sup>null</sup> strain but not a *cdc25*<sup>null</sup> *ira1*<sup>null</sup> *ira2*<sup>null</sup> strain. The type II mutant is also biochemically distinguishable from the type I mutant in a cdc25<sup>GEF</sup> binding and release assay. We showed that a complex of H-ras<sup>WT</sup>, in its nucleotide-free state, and cdc25<sup>GEF</sup> can be readily disrupted in the presence of guanine nucleotides. Under identical conditions, a complex containing H-ras<sup>Tyr-57</sup> is more severely impaired in the ability to dissociate from cdc25<sup>GEF</sup> than is a similar complex containing H-ras<sup>Asn-17</sup>. The more severe defect in dissociation for type II mutants may explain why it interferes more strongly than type I mutants. The more severe defect, however, does not explain the genetic data indicating that the type II mutants interfere more profoundly with RAS functions than do the type I mutants and that they do so even in the absence of the known GEFs. This suggests that type I and type II interfering mutants have different specificities, with the type II mutants targeting other activators of RAS besides

those encoded by *ste6* in *S. pombe* and its homolog *CDC25* in *S. cerevisiae*.

The type II mutants have amino acid substitutions at a site previously undescribed for a RAS dominant interfering mutant, namely, that corresponding to position 57 of human H-ras. Asp-57 of H-ras localizes to a DX<sub>2</sub>G motif that is strictly conserved not only for the RAS superfamily but also for the GTPase superfamily (4). This indicates a structural and functional conservation of this region in this class of proteins. Ser-17, Thr-35, and Asp-57 of H-ras represent three amino acids critical for Mg<sup>2+</sup> interaction (36, 43, 44). The importance of Mg<sup>2+</sup> coordination in H-ras regulation is emphasized by the fact that appropriate alterations of either Ser-17 or Asp-57 can generate dominant interfering mutants. Previous reports have suggested that the deliberate manipulation of position 57 might generate interfering mutants of H-ras. Neither an Ala-57 (28) nor an Asn-57 (14) mutation proved interfering, thus indicating that the generation of interfering mutants at this position is dependent on the amino acid substitution.

The mechanism of interference is not fully understood. It has been shown that the nucleotide-free or GDP-bound form of RAS complexes more tightly to GEFs than the GTP bound form of RAS (31). Defects in the ability of RAS to bind guanine nucleotides might therefore explain how interfering mutants function. We have shown here that whereas wild-type H-ras binds GDP and GTP with similar affinities, a type II interfering mutant binds guanine nucleotides, but with a preference for GDP over GTP. This is a property shared by type I interfering mutants (14, 15, 28). Such a bias, however, is insufficient for predicting whether a RAS mutant will be interfering, since an Ala-57 mutation (28) was not interfering although it exhibited a 39-fold bias for GDP over GTP. A defect in nucleotide binding to RAS proteins also cannot account for dominant interference properties, since activated mutants of H-ras that exhibit profound defects in nucleotide binding have been described (16, 30). From the work presented here, showing that the mutants are impaired in the ability to be dissociated from cdc25<sup>GEF</sup> by guanine nucleotides, and the work of others (14, 28), we suggest that a common defect of dominant interfering mutants in RAS proteins may be the inability to adopt a guanine nucleotide-bound conformation necessary for release from cdc25<sup>GEF</sup>-related molecules.

The discovery of type II interfering mutants opens new opportunities for further investigations. First, the high-affinity interaction between the type II mutants and GEFs might serve as a biochemical tool for purification and characterization of these alternate activators. These other activators of RAS might also be found by screening for suppressors of the type II interfering mutants in either *S. cerevisiae* or *S. pombe*. Second, since all G proteins contain the DX<sub>2</sub>G motif (4) in which the type II mutation is localized, it would be useful to determine if manipulation of this conserved aspartic acid in other G proteins will constitute a general strategy for generating interfering mutants. Third, type I interfering mutants have been used to dissect RAS function in mammalian systems (7, 54). Since our genetic analyses in two yeast systems indicate that the type I mutants do not completely abolish RAS functions, a conservative interpretation of the effects of type I mutants in mammalian systems is warranted until similar mammalian studies are carried out with type II interfering mutants. The ability of the two forms of RAS mutants to interfere in a discriminatory manner with RAS pathways suggests the interesting possibility that the RAS effector is coupled with the upstream activator with which RAS associates. The presently available repertoire of RAS exchange factors isolated from mammalian cells (9, 10,

24, 34, 51, 61, 65) and the availability of these two forms of interfering RAS mutants should allow us to test this possibility.

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