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Regulatory Function of the *Saccharomyces cerevisiae* *RAS* C-Terminus

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Activating mutations (valine 19 or leucine 68) were introduced into the *Saccharomyces cerevisiae RAS1* and *RAS2* genes. In addition, a deletion was introduced into the wild-type gene and into an activated *RAS2* gene, removing the segment of the coding region for the unique C-terminal domain that lies between the N-terminal 174 residues and the penultimate 8-residue membrane attachment site. At low levels of expression, a dominant activated phenotype, characterized by low glycogen levels and poor sporulation efficiency, was observed for both full-length *RAS1* and *RAS2* variants having impaired GTP hydrolytic activity. Lethal *CDC25* mutations were bypassed by the expression of mutant *RAS1* or *RAS2* proteins with activating amino acid substitutions, by expression of *RAS2* proteins lacking the C-terminal domain, or by normal and oncogenic mammalian Harvey *ras* proteins. Biochemical measurements of adenylate cyclase in membrane preparations showed that the expression of *RAS2* proteins lacking the C-terminal domain can restore adenylate cyclase activity to *cdc25* membranes.

The yeast *Saccharomyces cerevisiae* contains two genes, *RAS1* and *RAS2*, that encode proteins related to the mammalian *ras* oncogene proteins (6, 22). The N-terminal 180 amino acids of the *RAS1* and *RAS2* proteins are 60% homologous with the mammalian Harvey *ras* (Ha-*ras*) protein (Ha). The *S. cerevisiae RAS1* and *RAS2* proteins differ from their mammalian homologs by having additional C-terminal domains 120 and 131 amino acids in length, respectively. At the extreme C terminus of these domains is a four-residue sequence that contains a cysteine residue believed to be involved in membrane attachment in a manner similar to that observed for mammalian *ras* proteins (10).

The yeast *RAS* proteins share the intrinsic biochemical properties of the mammalian *ras* proteins that are common to G-regulatory proteins (28, 30). Like the mammalian *ras* proteins, the yeast *RAS* proteins specifically bind GTP or GDP. Normal *RAS* proteins have a low-level GTP hydrolytic activity that is reduced 10- to 50-fold by the introduction of oncogenic mutations (12, 20, 27, 30). Similar amino acid substitutions in yeast *RAS* proteins also reduce or eliminate their GTP hydrolytic activity (30).

Whereas the function of the mammalian *ras* proteins is unknown, the yeast *RAS* proteins have been shown to control adenylate cyclase (2, 31). In *S. cerevisiae*, cyclic AMP (cAMP) is known to influence both entry into the G_1 phase of the cell cycle and sporulation (19, 31). Double-gene disruptions of both *RAS1* and *RAS2* are lethal unless the cell also contains secondary mutations which either increase the intracellular level of cAMP or bypass the requirement for cAMP in stimulating cell growth (4, 31). The *RAS2*[Val-19] allele (where valine 19 is analogous to the oncogenic valine 12 substitution of Ha-*ras*) gives a dominant "activated" phenotype characterized by low levels of storage carbohydrates in the cell as well as hyposporulation and poor viability (31). Low glycogen and trehalose levels are directly related to high intracellular cAMP levels that activate degradative enzymes by cAMP-dependent phosphorylation (2, 8, 9, 31, 33, 34, 36).

It has been observed that the *CDC25* gene affects intracellular cAMP levels and is required for entry into the cell cycle (3, 18, 23, 32). It has also been demonstrated that the *RAS2*[Ala-18, Val-19] gene will bypass the growth arrest of a *CDC25* temperature-sensitive mutation as well as a *CDC25* gene disruption (24). These results suggest that the *CDC25* gene product is involved in the RAS2-dependent regulation of adenylate cyclase.

In this report we have investigated the function of the C terminus of *S. cerevisiae RAS* proteins by examining the abilities of wild-type and mutant alleles of *RAS1*, *RAS2*, *RAS2*Δ (C-terminal domain deleted), and mammalian Ha-*ras* to affect glycogen and sporulation levels in *S. cerevisiae*, to bypass *CDC25* mutations, and to stimulate adenylate cyclase in membrane preparations.

MATERIALS AND METHODS

Yeast strains, bacterial strains, and media. Strains of *S. cerevisiae* used in this study are listed in Table 1. Bacterial cloning and plasmid maintenance were performed in *Escherichia coli* strains HB101 and DH5. *S. cerevisiae* strains were grown in minimal SD supplemented with required amino acids (25) or rich medium (1% yeast extract [Difco], 2% soy peptone, and 2% dextrose). Bacteria were grown in Luria broth (17) supplemented with 100 μg of ampicillin per ml.

Plasmid construction and transformation. The plasmids YCp50 and YCp50-*RAS2* were kindly provided by K. Tatchell, and AAH5 was a gift from B. Hall. *RAS1* gene variants were subcloned into YCp50 as *Hin*dIII fragments or into AAH5 by digesting the *RAS1* gene with restriction endonuclease *Bbv*I, ligating *Hin*dIII linkers (17), and cloning into the AAH5 *Hin*dIII expression site. *RAS2* gene variants were subcloned into YCp50 as *Eco*RI-*Hin*dIII fragments or into the *Hin*dIII site of AAH5 by *Hpa*I digestion of the *RAS2* gene followed by the addition of *Hin*dIII linkers. The construction of plasmids for the expression of Ha proteins is fully detailed elsewhere (7, 26). Competent HB101 and DH5 cells were purchased from Bethesda Research Laboratories and transformed according to protocol. *S. cerevisiae* was transformed by the lithium acetate method of Ito et al. (15).

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TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Source (reference)
112	α *leu2-3,112 ura3-1 can1-100 ade2-1 his3*	(7)
112.699	α *ras2-699*[a] *leu2-3,112 ura3-1 can1-100 ade2-1 his3*	(7)
HR125-5D	**a** *leu2-3,112 ura3-52 trp1-1 his3 his4*	G. Casperson
561-10D	**a** *cdc25-1 leu2-3,112 ura3-52 ade2 his4*	L. Robinson
610-113c	**a**/α *CDC25/cdc25-113:LEU2 leu2/leu2 ura3/ura3 his4/his4*	L. Robinson

[a] The *ras2-699* allele is a disruption of *RAS2* by the *HIS3* gene at the *Pst*I site (7).

Enzymes were purchased from New England Biolabs, and standard cloning methodology was followed (17).

Mutagenesis of *RAS1* and *RAS2*. Site-specific mutations were introduced into the *RAS1* and *RAS2* genes by oligonucleotide-directed mutagenesis as previously described (12, 26). The *RAS2*Δ mutant gene was constructed by oligonucleotide-directed loop-out and deletion of the gene segment encoding amino acids 174 to 300 (35). The *RAS2*[Ala-18, Val-19]Δ mutant gene was made by recombining *RAS2*[Ala-18, Val-19], from the 5′ *Eco*RI site to the internal *Pst*I site, with the *Pst*I-to-*Hin*dIII fragment of *RAS2*Δ.

Glycogen and sporulation assays. Relative glycogen levels were measured by iodine vapor staining (4). Appropriate yeast strains were grown on rich plates for 18 to 20 h at 30°C. After incubation, the plates were inverted over crystalline iodine for approximately 4 min and were immediately photographed. Glycogen levels were measured quantitatively by using the method of Gunja-Smith et al. (13) on cells grown exactly as described above. Sporulation efficiency was measured by mating transformed strain 112 clones with strain HR125-5D according to standard methodology (25). Three independent diploid isolates from each cross were taken and patched fresh from minimal plates to 1% potassium acetate sporulation plates (25) supplemented with histidine. After 3 days, at least 300 cells and tetrads were counted for each sporulated diploid, and the percentage of sporulated cells was calculated.

Adenylate cyclase assays. Membrane fractions were prepared from cultures grown to an A_{660} of 0.5 to 1.0 by glusulase digestion (5), Dounce homogenization of the spheroplasts in buffer containing 50 mM sodium MES [2(*N*-morpholino)ethanesulfonic acid] (pH 6.0)–0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]–0.1 mM $MgCl_2$–1 mM phenylmethylsulfonyl fluoride–1 mM dithiothreitol, and then centrifugation at 105,000 × *g* for 30 min at 4°C. The buffer used to prepare membranes from a strain expressing Ha[Asn-16]-*ras* also included 50 μM GDP to maintain biochemical activity of this Ha-ras mutant (26), and this solution was diluted 20-fold in the adenylate cyclase assay. Adenylate cyclase assays (100-μl reaction volume) measured the conversion of [α-^{32}P]ATP to [^{32}P]cAMP at 30°C as described (5), using 10 to 40 μg of membrane protein. Product was separated from reactants by sequential column chromatography on Dowex AG 50W-X4 (200 to 400 mesh; Bio-Rad) and neutral alumina (Sigma, type WN-3) (see reference 5). Guanine nucleotides were purchased from Boehringer Mannheim. In the indicated experiments, the guanine nucleotides were preincubated with the membranes at 500 μM in the absence of $MgCl_2$ for 10 min at 4°C and then for 10 min at 22°C in a 20-μl volume before dilution with the assay components. These conditions facilitated *RAS* protein-guanine nucleotide exchange.

Immunoblot analysis. Yeast membrane fractions were prepared as described above, and 30 μg of protein was electrophoresed at 25 mA on a 13.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate, using a Bio-Rad "baby" gel apparatus. The gel was washed for 15 min in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) before electrophoretic transfer at 4°C onto nitrocellulose (Schleicher and Schuell BA83, 0.2 μm) at 24 V for 15 h and then at 250 mA for 1 h. The filter was incubated with hybridization buffer containing 25 mM Tris chloride (pH 7.5)–150 mM NaCl–0.4% powdered milk–0.5% bovine serum albumin–1 mM $CaCl_2$–20 μM EDTA for 30 min at 4°C. The filter was then incubated at 4°C sequentially with *ras* antibody Y13-259 (11) (1:100 dilution for 14 h) and ^{125}I-labeled sheep anti-rat immunoglobulin G (0.4 μCi/ml for 5 h). Washes were performed with buffer containing 25 mM Tris chloride (pH 7.5)–150 mM NaCl–0.1% Tween-20. ^{125}I-labeled sheep anti-rat immunoglobulin G was from Amersham. Immunoblots were exposed on Kodak XAR-2 film at −70°C.

RESULTS

***RAS1* with oncogenic mutations has a dominant activated phenotype.** *RAS1* gene variants *RAS1*[Ala-18, Val-19] and *RAS1*[Leu-68] were constructed by oligonucleotide-directed mutagenesis and cloned into the centromere plasmid YCp50 along with 0.6 kilobase of 5′ *RAS1* noncoding sequence. The *RAS1*[Leu-68] substitution is analogous to the oncogenic leucine 61 substitution in Ha-*ras* and has been shown to impair GTP hydrolytic activity (30). DNA encoding the proteins RAS2 or RAS2[Ala-18, Val-19], together with more than 1 kilobase of 5′ *RAS2* noncoding sequence, was also cloned into the centromere plasmid YCp50. The resulting plasmids were transformed into the *RAS1 RAS2* strain 112. The introduction of oncogenic substitutions into the *RAS1* gene did not result in the overt manifestation of the activated phenotype associated with similar substitutions in *RAS2*, i.e., loss of viability, low glycogen storage, or poor sporulation. However, when glycogen levels were monitored at different growth stages, we observed that glycogen levels in cells expressing the *RAS1*[Leu-68] protein were dramatically reduced during the initial growth but returned to normal levels after 24 h of growth. The results in Fig. 1 show the effect of *RAS1*[Leu-68] on iodine staining measured after 15 h of growth. Direct measurement of intracellular glycogen levels confirmed the reduction of glycogen in the *RAS1*[Leu-

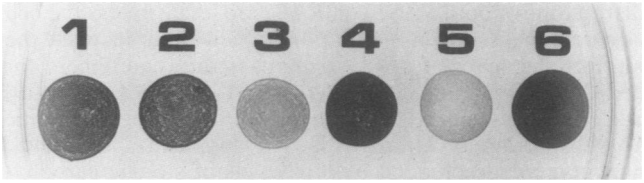

FIG. 1. Effects of *RAS1*[Leu-68] on glycogen storage. Strain 112 transformed with the described *RAS* plasmids was spotted onto rich agar. The cells were grown for 15 h at 30°C and then stained by iodine vapors. (1) YCp50; (2) YCp50-*RAS1*; (3) YCp50-*RAS1*[Leu-68]; (4) YCp50-*RAS2*; (5) YCp50-*RAS2*[Ala-18, Val-19]; (6) AAH5-*RAS1*. The patches of strain 112 transformed with YCp50-*RAS1*[Leu-68] and YCp50-*RAS2*[Ala-18, Val-19] stained a distinctive yellow color compared to the remaining patches, which all stained a rich brown color.

68] strain (Table 2). Recently, Breviario et al. have reported that reduction of *RAS1* expression occurs during logarithmic-phase growth and that the synthesis of RAS1 is limited by ethanol (1). Our observation of a transient activated phenotype for the *RAS1*[Leu-68] variant is consistent with this regulated expression.

For constitutive expression, the genes for RAS1, RAS1[Ala-18, Val-19], and RAS1[Leu-68] were cloned into the vector AAH5 and transformed into the *RAS1 RAS2* strain 112. Cells transformed with AAH5 expressing mutant *RAS1* genes failed to grow even though high expression of wild-type RAS1 had no effect on cell growth. To circumvent these toxic phenotypes, the ADH-*RAS1* and ADH-*RAS1* [Ala-18, Val-19] promoter-gene fusions from the AAH5 plasmids were subcloned as *Bam*HI fragments into the single-copy YCp50 vector, and these constructs were transformed into the *RAS1 RAS2* strain 112. At single copy, the ADH-expressed *RAS1*[Ala-18, Val-19] protein lowered glycogen levels and sporulation efficiency to the same degree seen for single-copy *RAS2*[Ala-18, Val-19] in the same *RAS1 RAS2* host strain (Table 2). The corresponding ADH-*RAS1* construct did not affect glycogen levels or sporulation efficiencies. Apparently, activated alleles of *RAS1*, like those of *RAS2*, have a dominant effect over both wild-type *RAS1* and *RAS2*. The YCpADH-expressed *RAS1* and *RAS1*[Ala-18, Val-19] protein levels were at least fivefold greater than those of endogenous *RAS1* protein (Fig. 2, lanes 1 to 3). Both RAS1 and RAS1[Ala-18, Val-19] are observed in yeast membranes as 34- and 39-kilodalton (kDa) species. Published results indicate that the 34-kDa species is a precursor of the 39-kDa form and that the 39-kDa form is phosphorylated (29).

Biological activity of RAS2 proteins lacking the C-terminal domain. To investigate the role of the C terminus of RAS2, a mutant form of *RAS2* was constructed that did not encode the C-terminal intervening domain. Using oligonucleotide-directed mutagenesis, the region encoding residues 174 to 300 was deleted, forming the mutant gene *RAS2*Δ which encodes a polypeptide that still retained the C-terminal membrane attachment site. *RAS2*Δ was then recombined with *RAS2*[Ala-18, Val-19] to give *RAS2*[Ala-18, Val-19]Δ.

TABLE 2. Effects of activating *RAS1* mutations and *RAS2* C-terminal deletions on *S. cerevisiae* biology[a]

Plasmid	Iodine staining	Glycogen (μg/mg of protein)	Sporulation (fraction of control)
YCp50	+	63	1.0
YCp50-*RAS1*	+	76	ND
YCp50-*RAS1*[Ala-18, Val-19]	+	ND	ND
YCp50*RAS1*[Leu-68]	−	12	ND
YCp50-*RAS2*	+	61	1.6
YCp50-*RAS2*Δ	+	ND	ND
YCp50-*RAS2*[Ala-18, Val-19]	−	14	0.1
YCp50-*RAS2*[Ala-18, Val-19]Δ	+	30	0.8
AAH5–Ha-*ras*	+	ND	ND
AAH5-Ha [Val-12, Thr-59]-*ras*	+	ND	ND
YCpADH-*RAS1*	+	52	1.1
YCpADH-*RAS1*[Ala-18, Val-19]	−	6.7	0.1
YCpADH-*RAS2*	+	ND	1.0
YCpADH-*RAS2*Δ	−	ND	0.1
YCpADH-*RAS2*[Ala-18, Val-19]	−	ND	0.4
YCpADH-*RAS2*[Ala-18, Val-19]Δ	−	ND	0.2

[a] Plasmids expressing wild-type and mutant *RAS1*, *RAS2*, and Ha-*ras* were transformed into the *RAS1 RAS2* strain 112. The effects of the expressed *RAS* genes on glycogen storage were determined by iodine staining relative to isogenic *ras2*, *RAS2*, and *RAS2*[Ala-18, Val-19] controls. All plasmids were found, after replica plating to selective media, to be stably maintained under the growth conditions required for glycogen measurement. Quantitative glycogen measurements are from one experiment representative of three performed. ND, not determined.

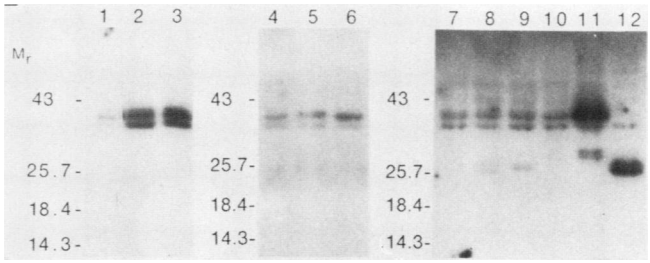

FIG. 2. *RAS* proteins in yeast membranes. The expression of *RAS* proteins, using the YCpADH and YCp50 vectors, was compared with that of endogenous RAS1 and RAS2 as detected by immunoblot. Strains 112 (lanes 1 through 6 and 10) and 112.699 (lanes 7 through 9, 11, and 12) were transformed with the indicated plasmids. Lanes: (1) YCp50, (2) YCpADH50-*RAS1*, (3) YCpRAS1-*RAS1*[Ala-18, Val-19], (4) YCp50, (5) YCp50-*RAS2*[Ala-18, Val-19], (6) YCp50-*RAS2*, (7) YCp50, (8) YCp50-*RAS2*Δ, (9) YCp50-*RAS2*[Ala-18, Val-19]Δ, (10) YCp50, (11) AAH5-*RAS2* (the 28-kDa band is a proteolytic degradation product), (12) AAH5-*RAS2*[Ala-18, Val-19]Δ. Whereas *RAS* genes in the vectors YCpADH and AAH5 expressed high protein levels (lanes 2, 3, 11, and 12), *RAS* genes in the vector YCp50 expressed protein at levels within a factor of 2 compared to endogenous *RAS* protein levels (lanes 5, 6, 8, and 9). YCp50-expressed RAS2[Ala-18, Val-19] (lane 5) was detected as a novel band migrating slightly slower than endogenous RAS2, and YCp50-expressed RAS2 (lane 6) was observed as a twofold more intense RAS2 band. The approximately 26-kDa bands corresponding to RAS2Δ (lane 8) and RAS2[Ala-18, Val-19]Δ (lane 9) were one-half the intensity of endogenous RAS protein.

The DNAs encoding these mutant *RAS2* genes were subcloned into YCp50 as detailed for full-length *RAS2*. In addition, the genes encoding Ha and Ha[Val-12, Thr-59] were cloned into the multicopy expression plasmid AAH5, which contains the ADH promoter and terminator. These plasmids, along with YCp50-*RAS2* and YCp50-*RAS2*[Ala-18, Val-19], were transformed into the *S. cerevisiae RAS1 RAS2* strain 112, and the ability of each *RAS* protein to promote an activated phenotype was determined. Intracellular glycogen levels were determined by both iodine staining of colonies and direct biochemical measurement. Sporulation efficiency in diploids containing plasmids was measured as detailed in Materials and Methods. As shown in Table 2 for the YCp50-expressed proteins, low levels of glycogen and poor sporulation were observed only with expression of full-length *RAS2*[Ala-18, Val-19]. A 50% decrease in measured glycogen levels and sporulation efficiency was observed with the expression of *RAS2*[Ala-18, Val-19]Δ, although no decrease in glycogen was observed by iodine staining alone. The levels of expressed proteins RAS2[Ala-18, Val-19] and RAS2[Ala-18, Val-19]Δ, as detected by immunoblot analysis, were within twofold that of the endogenous RAS (Fig. 2, lanes 4 through 9). No effect on glycogen levels was observed for Ha[Val-12, Thr-59] and Ha, proteins lacking the *RAS* C-terminal region. Ha proteins were expressed at levels greater than threefold that of endogenous RAS (not shown). Similar results were obtained in the *ras2*$^-$ strain 112.699. All the *RAS* proteins described were functional in that they were able to complement a *ras2* disruption mutant as measured by growth on ethanol at 37°C (data not shown).

To determine the effects of the C-terminal deleted *RAS2*

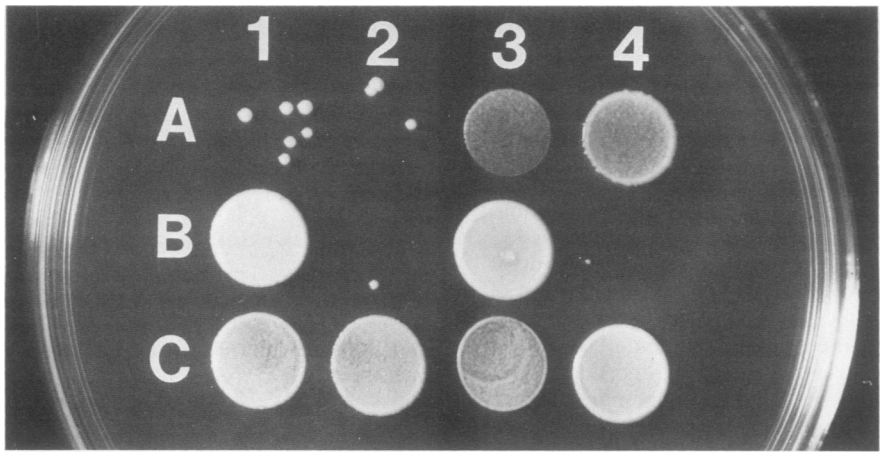

FIG. 3. *RAS* bypass of *cdc25-1* temperature-sensitive growth arrest. The haploid *cdc25-1* strain 561-10D, transformed with *RAS1*, *RAS2*, and Ha-*ras* variants, was spotted onto rich agar after overnight growth in selective medium at 30°C. Incubation was for 3 days at 37°C. Plasmid order from left to right is as follows: Row A: (1) YCp50, (2) YCp50-*RAS2*, (3) YCp50-*RAS2*Δ, (4) YCp50-*RAS2*[Ala-18, Val-19]. Row B: (1) YCp50-*RAS2*[Ala-18, Val-19]Δ, (2) YCp50-*RAS1*, (3) YCp50-*RAS1*[Leu-68], (4) AAH5. Row C: (1) AAH5–Ha-*ras*, (2) AAH5-Ha[Val-12, Thr-59]-*ras*, (3) AAH5-Ha[Asn-16]-*ras*, (4) AAH5-Ha[Ala-119]-*ras*.

proteins at high levels of expression, the genes encoding RAS2, RAS2Δ, RAS2[Ala-18, Val-19], and RAS2[Ala-18, Val-19]Δ were subcloned into the YCp50-ADH vector described previously. These constructs were transformed into strain 112, and their effect on glycogen storage and sporulation efficiency was determined. Only overproduced RAS2 did not affect glycogen levels or sporulation efficiency (Table 2). In addition, all of the overexpressed *RAS* proteins, with the exception of normal RAS2, decreased the cell growth rate and markedly reduced the ability of the cell to survive storage. Each of these constructs expressed protein at levels at least fivefold greater than endogenous RAS2 (data not shown).

Lethal and temperature-sensitive *CDC25* phenotypes can be complemented by *RAS* alleles encoding proteins that lack a C-terminal domain. The ability of the *RAS2* and *RAS1* variants described above to bypass *CDC25* mutations was investigated. The *RAS1 RAS2 cdc25-1* strain 561-10D was transformed with YCp50 subclones of *RAS1*, *RAS1*[Leu-68], *RAS2*, *RAS2*Δ, *RAS2*[Ala-18, Val-19], and *RAS2*[Ala-18, Val-19]Δ. In addition, AAH5 plasmids expressing Ha, Ha[Val-12, Thr-59], and two nucleotide-binding mutant proteins, Ha[Asn-16] and Ha[Ala-119] (26), were transformed into strain 561-10D. Purified clones from each transformation were grown overnight in liquid minimal medium, spotted onto rich plates, and incubated at a restrictive temperature of 37°C for 3 days (Fig. 3). Whereas expression of the wild-type *RAS1* and *RAS2* alleles did not permit growth at the restrictive temperature, suppression of *cdc25-1* growth arrest at 37°C was observed in strains expressing *RAS1* or *RAS2* alleles having activating mutations or lacking the unique *RAS* C-terminal region. Among the *RAS2* variants, *RAS2*Δ and *RAS2*[Ala-18, Val-19] were similar in suppressing the *cdc25* growth arrest and were surpassed by *RAS2*[Ala-18, Val-19]Δ. Expression of normal and oncogenic mammalian *ras* proteins, including the Ha[Asn-16] and Ha[Ala-119] nucleotide-binding mutants, also suppressed the *cdc25* mutation. Of the Ha variants, the oncogenic form Ha[Val-12, Thr-59] was reproducibly more effective than the wild-type Ha protein in bypassing the *cdc25* mutation.

Since the exact nature of the *cdc25-1* mutation is not known, we tested the ability of activated and deleted forms of RAS2 to bypass a *CDC25* gene disruption, *cdc25-113:LEU2* (24). A diploid strain heterozygous for the *CDC25* disruption, 610-113 (*RAS1/RAS1*, *RAS2/RAS2*, *CDC25/cdc25-113:LEU2*) was transformed with YCp50 subclones of *RAS2*, *RAS2*Δ, *RAS2*[Ala-18, Val-19], and *RAS2*[Ala-18, Val-19]Δ. Each transformed diploid was sporulated on acetate medium selective for YCp50 (lacking uracil), and at least 10 tetrads from each cross were dissected. Viable progeny were scored for the presence of the plasmid (Ura^+), the presence of the *CDC25* disruption (Leu^+), and the coincidence of these two phenotypes. The results were similar to those observed using the *cdc25-1* mutant (Table 3). Viable Ura^+ Leu^+ progeny resulted only when *RAS2*Δ, *RAS2*[Ala-18, Val-19], or *RAS2*[Ala-18, Val-19]Δ was present. No viable Ura^+ Leu^+ spores were observed from sporulation of the diploid 610-113 with YCp50 alone or full-length *RAS2*, even though most tetrads showed segregation of plasmid. Both *RAS2*[Ala-18, Val-19] and *RAS2*[Ala-18, Val-19]Δ allowed *cdc25*$^-$ spores to germinate and grow normally. Consistent with the results seen for *cdc25-1*, *cdc25-113* progeny with *RAS2*Δ grew more slowly than wild-type sister spores.

In vitro adenylate cyclase activities. Adenylate cyclase activities were measured in membrane preparations isolated from the strains described above. *ras* proteins exhibit high affinities for GDP and GTP and, in the case of bacterially

TABLE 3. Bypass of *cdc25* lethality by deleted and activated alleles of *RAS2*[a]

Plasmid	No. of asci dissected	No. Ura^+[b] spores	No. Leu^+[c] spores	No. Ura^+ Leu^+ spores	No. of viable spores
YCp50	10	9	0	0	19
RAS2	19	13	0	0	34
*RAS2*Δ	10	16	7	7	26
RAS2[Ala-18, Val-191]	10	15	9	9	28
RAS2[Ala-18, Val-19]Δ	10	15	7	7	25

[a] Diploid strain 610-113C (*CDC25/cdc25-113:LEU2*), maintaining the indicated plasmids, was sporulated, and tetrads were dissected. Haploid progeny were scored for the presence of plasmids (Ura^+) and *cdc25-113* (Leu^+) and the cosegregation of both markers. Only four-spored asci were dissected.

[b] YCp50 carries the *URA3* marker.

[c] The *cdc25-113* disruption is marked with *LEU2*.

TABLE 4. *RAS*-mediated guanine nucleotide stimulation of yeast adenylate cyclase[a]

Expt	Plasmid	Adenylate cyclase activity (pmol of cAMP per min per mg)					Gpp(NH)p or GTPγS stimulation (fold)
		Mg^{2+}	Mg^{2+}, GDPβS	Mg^{2+}, GTPγS	Mg^{2+}, Gpp(NH)p	Mn^{2+}	
1	YCp50	1.5			7.7	60	5.1
	YCp50-*RAS2*	9.6			20	64	2.1
	YCp50-*RAS2*[Ala-18, Val-19]	17			32	82	1.9
	YCp50-*RAS2*Δ	42			47	64	1.1
	YCp50-*RAS2*[Ala-18, Val-19]Δ	62			55	98	0.9
	AAH5–Ha-*ras*	39			34	79	0.9
2	YCP50-*RAS2*	7.9	2.8	21			2.6
	YCp50-*RAS2*Δ	13	4.3	18			1.4
	AAH5-Ha[Asn-16]-*ras*	1.8	0.3	13			6.9
	AAH5	3.2	1.0	4.8			1.5

[a] The *RAS1 ras2* strain 112.699 was transformed with the indicated plasmids. Membrane fractions were prepared using buffer containing 50 mM sodium MES (pH 6.0)–1 mM EDTA–1 mM EGTA–1 mM phenylmethylsulfonyl fluoride–1 mM dithiothreitol experiment 1) or the buffer described in Materials and Methods (experiment 2). Guanine nucleotides were present at 100 μM; $MgCl_2$, at 10 mM; and $MnCl_2$, at 2.5 mM. In experiment 2, the nucleotides were preincubated with the membranes as described in Materials and Methods. An activity unit of 1.0 reflects measurements approximately 50% greater than the assay blanks (92 to 245 cpm). The results are typical of two or three experiments.

expressed *ras* proteins, copurify with bound nucleotide (21). In an attempt to measure the activity of the nucleotide complex of the *ras* proteins present in vivo, we measured the adenylate cyclase activities in the absence of exogenous GDP and GTP. The nucleotide dependence of the variants was then examined by the addition of the hydrolysis-resistant GTP analog guanosine β,γ-imidotriphosphate [Gpp(NH)p]. In agreement with previous reports (4, 31), the disruption of the *RAS2* gene in the *RAS1 ras2* strain 112.699 resulted in low adenylate cyclase activity (see Table 4, experiment 1). Expression of RAS2 or RAS2[Ala-18, Val-19] in strain 112.699 increased membrane adenylate cyclase activity, with the activated form being the more potent. Both of these activities were further stimulated twofold upon the addition of Gpp(NH)p (Table 4, experiment 1). Expression of either RAS2Δ or RAS2[Ala-18, Val-19]Δ resulted in increased adenylate cyclase activities that were insensitive to exogenous Gpp(NH)p. High adenylate cyclase activities that were insensitive to added Gpp(NH)p were also observed upon expression of Ha protein (Table 4, experiment 1). Activity with Mn^{2+} reflected similar levels of catalytic adenylate cyclase. Gpp(NH)p insensitivity was not a result of rate-limiting amounts of adenylate cyclase, since a five-fold overproduction of adenylate cyclase in strains expressing Ha and Ha[Val-12, Thr-59] did not alter the experimental results (data not shown).

The apparent Gpp(NH)p insensitivity of the deleted forms of *ras* protein could be caused by the proteins being isolated as their GTP complex or by their being fully active irrespective of being present as the GTP or GDP complex. To test between these two possibilities, membranes were preincubated with guanosine-5′-*O*-(2-thiodiphosphate) (GDPβS) or guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) under conditions that facilitate nucleotide exchange. GDPβS decreased the Mg^{2+} activity of both *RAS2* and *RAS2*Δ strains by 65%, whereas GTPγS stimulated activity only in the *RAS2* strain (Table 4, experiment 2). Near-complete inhibition of adenylate cyclase activity by GDPβS was observed by using membranes containing Ha[Asn-16], which exchanges nucleotide at least 50 times faster than normal Ha (26). In this experiment the stimulation of adenylate cyclase activity by GTPγS relative to the GDPβS level in the Ha[Asn-16] strain was 40-fold. In five other experiments, the observed stimulations were 11-, 12-, 19-, 24-, and 26-fold. The lower activity values in experiment 2 relative to those in experiment 1 (Table 4) are due to the preincubation step.

The effects of full-length and deleted *RAS* proteins and the *CDC25* gene product on adenylate cyclase activity were investigated in the *RAS1 RAS2* strains 112 (*CDC25*) and 561-10D (*cdc25-1*). The cells were grown at the permissive temperature (25°C) to an A_{660} of 0.5 and then incubated for 2 h at the restrictive temperature (35°C). In the wild-type strain 112, Mg^{2+} adenylate cyclase activity was observed for all forms of *RAS* tested, whereas appreciable activity was only measured in the *cdc25-1* strains expressing *RAS2*Δ, *RAS2*[Ala-18, Val-19], or *RAS2*[Ala-18, Val-19]Δ (Table 5). The order of increasing Mg^{2+} activity levels is consistent with the biological potency of these proteins for bypassing the *cdc25* defect. The addition of 100 μM Gpp(NH)p in the assays for membranes derived from the *RAS1 RAS2 cdc25* strain transformed with YCp50 or YCp50-*RAS2* increased adenylate cyclase activities to within 30 to 50% of wild-type values. The reduced adenylate cyclase activity assayed with Mg^{2+} for strain 112 transformed with YCp50-*RAS2*[Ala-18, Val-19] probably reflects the toxic effect of this mutant in this strain. We do not know whether the two- to threefold variation in adenylate cyclase activities assayed with Mn^{2+} reflects *RAS* protein influence on this activity or changes in the amount of adenylate cyclase protein.

DISCUSSION

It has been demonstrated previously that both *S. cerevisiae RAS* genes can singly promote cell viability as well as activate adenylate cyclase (2, 16, 31). Both genetic and biochemical evidence suggests that the *RAS2* protein is the primary controlling protein of adenylate cyclase and that RAS1 plays a secondary role in controlling cell growth through the cAMP pathway (2, 31). Our results indicate that once RAS1 expression becomes constitutive, variants with reduced GTP hydrolytic activity can affect cell biology exactly as do activated RAS2 variants. Further support for a common biochemical pathway for RAS1 and RAS2 is shown by the ability of activated alleles of both *RAS* genes to bypass the need for *CDC25*, a gene shown to regulate the cAMP pathway (3, 18, 24, 32).

TABLE 5. Influence of the *CDC25* gene product on adenylate cyclase activity[a]

Strain	Plasmid	Adenylate cyclase activity (pmol of cAMP per min per mg)		
		Mg^{2+}	Mg^{2+}, Gpp(NH)p	Mn^{2+}
112 (*CDC25*)	YCp50	9.0	18	35
	YCp50-*RAS2*	9.9	17	52
	YCp50-*RAS2*Δ	12	21	37
	YCp50-*RAS2*[Ala-18, Val-19]	6.4	8.9	47
	YCp50-*RAS2*[Ala-18, Val-19]Δ	18	30	88
561-10D (*cdc25*)	YCp50	1.9	11	34
	YCp50-*RAS2*	0.9	5.7	35
	YCp50-*RAS2*Δ	6.8	24	82
	YCp50-*RAS2*[Ala-18, Val-19]	12	23	83
	YCp50-*RAS2*[Ala-18, Val-19]Δ	31	30	96

[a] The *RAS1 RAS2* strains 112 (*CDC25*) and 561-10D (*cdc25-1*) were transformed with the indicated plasmids. Membranes were prepared in buffer as described in the text and then assayed for adenylate cyclase activity at 25°C for 40 min with 10 mM $MgCl_2$ or 2.5 mM $MnCl_2$. Gpp(NH)p (100 μM final concentration) was preincubated with the membranes before the assay as described in Materials and Methods. An activity unit of 1.0 reflects a measurement of approximately 50 cpm over an assay blank of 117 cpm. The results are typical of five experiments using three membrane preparations.

The role of the unique C terminus of the *RAS* proteins was explored, using the RAS2Δ variant in which this domain was deleted. Whereas at low levels of expression of either RAS2Δ or RAS2[Ala-18 Val-19]Δ, an activated phenotype was not observed, an activated phenotype was detected at higher levels of expression of these proteins. In contrast, overexpression of RAS2 protein did not noticeably alter the phenotype of the cells. Expression of *ras* proteins lacking the *RAS* C-terminal domain resulted in high levels of adenylate cyclase activity in membrane preparations. As seen most clearly for *ras2*$^-$ strains (Table 4), these activities did not require the addition of exogenous GTP analogs and were reduced upon the addition of GDP analogs. Experiment 2 of Table 4 clearly shows that GTP and not GDP is required for the *ras*-dependent stimulation of adenylate cyclase in membranes. As previously observed (2, 31), in the in vitro assay, the addition of exogenous GTP was necessary for the full stimulation of the adenylate cyclase by RAS2. These results might be expected if in vivo more of the GTP complex occurs for the *ras* proteins that lack the *RAS*-specific C-terminal domain than occurs for the full-length *RAS* proteins.

The ability of the *ras* proteins to bypass *CDC25* mutations correlates well with the relative adenylate cyclase activities observed in the membrane preparations of the respective strains when assayed in the absence of added GTP analogs. Under these conditions, the full-length, normal *RAS* proteins were inactive whereas the variants that have mutations which reduce GTP hydrolytic activity or those that lack the *RAS* unique C-terminal domain were active. The observation that addition of GTP analogs restored the ability of the full-length, normal *RAS* proteins to stimulate adenylate cyclase suggests that the primary defect resulting from the absence of the *CDC25* gene product may be a low level of the GTP complex of the *RAS* proteins in vivo. The ability of the C-terminal truncated forms to bypass *cdc25* is consistent with the hypothesis that these proteins are bound to more GTP in vivo than the full-length forms. It should be noted that the variant that was most effective in bypassing the *cdc25-1* mutation contained a deletion of the C-terminal domain and had reduced GTPase activity. Several mechanisms for the function of the *CDC25* gene product are consistent with these data. It has been proposed that the *CDC25* protein is an exchange factor similar to Ts for EF-Tu (14, 24). Proteins which form more stable in vivo complexes with GTP could function in the absence of such an exchange factor. Alternatively, the *CDC25* protein might act together with *RAS* proteins in stimulating the adenylate cyclase. The presence of a greater amount of the active GTP complex of the *RAS* variants might alleviate the need for additional stimulating factors such as the *CDC25* protein.

In conclusion, our studies show that both *RAS1* and *RAS2* proteins of *S. cerevisiae* are functionally similar and that the unique C-terminal domain of *RAS2* protein acts as a modulator of *RAS* activity. A negative regulatory role for the C-terminal domain of the *RAS* protein is explicitly revealed by the observation that *ras* proteins lacking this domain, but not the full-length wild-type *RAS* proteins, can bypass *CDC25* mutations. On the basis of the in vitro biochemistry we have suggested that the C-terminal domain and the *CDC25* gene product operate to control the formation of the GTP complex of the *RAS* proteins. It can be envisioned that distinct proteins in mammalian cells might perform the functions of the yeast *RAS* C terminus and the *CDC25* gene product in regulating *ras* protein interaction with its mammalian target.

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