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A Dominant Activating Mutation in the Effector Region of RAS Abolishes IRA2 Sensitivity

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Previously described mutations in *RAS* genes that cause a dominant activated phenotype affect the intrinsic biochemical properties of RAS proteins, either decreasing the intrinsic GTPase or reducing the affinity for guanine nucleotides. In this report, we describe a novel activating mutation in the *RAS2* gene of *Saccharomyces cerevisiae* that does not alter intrinsic biochemical properties of the mutant RAS2 protein. Rather, this mutation, *RAS2-P41S* (proline 41 to serine), which lies in the effector region of RAS, is shown to abolish the ability of the IRA2 protein to stimulate the GTPase activity of the mutant RAS protein. This mutation also modestly reduced the ability of the mutant protein to stimulate the target adenylate cyclase in an in vitro assay, although in vivo the phenotypes it induced suggest that it retains potency in stimulation of adenylate cyclase. Our results demonstrate that although the effector region of RAS appears to be important for interaction with both target effector and negative regulators of RAS, it is possible to eliminate negative regulator responsiveness and retain potency in effector stimulation.

RAS has been dubbed a molecular switch because it alternates between a GTP-bound on state and a GDP-bound off state. In mammalian cells, RAS in the on state activates an unknown effector that leads to cell division. In *Saccharomyces cerevisiae* the effector is adenylate cyclase, which controls progression through the cell division cycle (3, 17, 40). Activated *RAS* mutations share the property that they result in a greater amount of cellular RAS-GTP (3, 17, 18, 40). Two different mechanisms are known for RAS activation due to mutations within the *RAS* gene: impairment of GTPase activity (3, 17) and reduction of nucleotide binding (15, 24, 39).

Proteins such as mammalian GTPase-activating protein (GAP) and yeast IRA1 and IRA2 interact with RAS and stimulate the intrinsic GTPase (41, 46). Recently the neurofibromatosis type 1 gene product was also shown to stimulate RAS GTPase (2, 27, 51). GAP has been shown to interact with Ha-RAS amino acids 30 to 40. This region, termed the effector domain or switch I, of the RAS oncoprotein is one of two regions that has a significantly different conformation in the GDP- and GTP-bound states (30, 37). Mutations within this region can abrogate effector interaction and prevent stimulation by GAP or IRA proteins. Heretofore, *RAS* effector region mutations that prevent GAP stimulation have encoded proteins unable to activate the effector in mammalian cells sufficiently to induce transformation (29).

Mutations in *IRA1* or *IRA2* also cause RAS-GTP accumulation (43). Increased RAS-GTP levels lead to greater adenylate cyclase activation, resulting in cyclic AMP (cAMP)-dependent protein kinase hyperactivity. Elevated cAMP-dependent protein phosphorylation reduces glycogen accumulation, increases sensitivity to heat shock, and promotes intolerance to nutrient starvation (17, 28, 40). Because IRA proteins are required to stimulate RAS GTPase in wild-type cells, *RAS* activation could also result from abrogation of IRA-RAS interaction or from a failure of RAS to respond to IRA stimulation. We isolated a large set of activated *RAS2* mutations to test this hypothesis (10). Characterization of one of these mutations, *RAS2-P41S*, is described in this work.

MATERIALS AND METHODS

Yeast strains and media. The strains JC482 (*MATα ura3-52 leu2 his4*) and JC302-26B (*MATα ura3-52 leu2 his4 ras2::LEU2*) have been described previously (8). Diploid derivatives of these strains were made by HO induction (8). JC746 is a diploid homozygous for *ura3-52 leu2 his3 trp1 can1*. Diploid JC877 is heterozygous for *cdc25::LEU2* and was derived from JC746 by transformation with a *Pvu*II-*Hin*dIII fragment from pL113 (33). TK161-R2V (*MATα ura3 leu2 trp1 his3 ade8 RAS2-G19V*) was used as a host for pKT16 to overexpress IRA2 (41). TK-B111, a gift from T. Kataoka via K. Matsumoto, was used for membrane preparation (16). Yeast media have been described previously (8, 18).

Dominant activated *RAS2* mutations. The plasmid p1045 has a 2.7-kb *Eco*RI-*Hin*dIII *RAS2* fragment cloned in YCp50. This yeast-*Escherichia coli* shuttle vector replicates as a low-copy-number episome in yeast cells because of centromere sequences and has a wild-type *URA3* gene as a selectable marker (34). Hydroxylamine mutagenesis of p1045 was performed at 37, 42, and 50°C (35), and the DNA was directly used to transform JC482 (22). Transformants were selected on uracil-deficient medium and replica plated to yeast extract-peptone (YEP)-glucose. The YEP-glucose plate was inverted over iodine crystals to stain cellular glycogen (11). DNA from yellow iodine-stained colonies was retrieved by transformation of *E. coli* and retested by a second JC482 transformation.

Analysis of guanine nucleotides bound to RAS in vivo. RAS

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proteins were expressed at high levels by using alcohol dehydrogenase promoter (ADHp)-driven expression vectors. Plasmids used were AAH5::*HaRAS* (13), p1982 (*ADHp*:: *RAS2* on YEp351 [21, 25]), and p2002 (identical to p1982 but with a *RAS2-P41S* mutation introduced by site-directed mutagenesis). JC746 transformed with these plasmids was grown in leucine-deficient minimal medium and subsequently labeled with 500 μCi of $^{32}P_i$ per ml as described previously (18). Cell extraction, RAS immunoprecipitation, and chromatography of eluted nucleotides on polyethyleneimine (PEI)-cellulose thin layers was done exactly as described previously (43) except that nucleotides were quantitated by using a Molecular Dynamics PhosphorImager.

RAS GTPase and GDP dissociation assays. Wild-type RAS2, RAS2(G19V), RAS2(T42S), and RAS2(P41S) proteins lacking the carboxy-terminal 113 amino acids were purified from *E. coli* essentially as described previously (41). RAS2-[γ-^{32}P]GTP complex was prepared by incubating 20 pmol of purified RAS2 protein in 50 μl of a nucleotide exchange buffer containing 40 nM [γ-^{32}P]GTP (5,000 Ci/mmol; Amersham), 50 mM 2-(*N*-morpholino)ethanesulfonate (MES)-NaOH (pH 6.5), 1 mM EDTA, 0.5 mM $MgCl_2$, 2 mM dithiothreitol, and 300 μg of bovine serum albumin per ml at 30°C for 10 min. Nucleotide-bound RAS was separated from GTP by passing through two Sephadex G-25 columns in 50 mM MES-NaOH (pH 6.5)–2 mM dithiothreitol. GTPase activity was monitored by incubating the RAS-GTP complex at 30°C after adding $MgCl_2$ and bovine serum albumin to final concentrations of 2 mM and 1 mg/ml, respectively. For IRA2 activation of RAS GTPase, 10 pmol of RAS2-[γ-^{32}P]GTP, prepared by the above-described procedure but without Sephadex chromatography, was incubated in 180 μl of prewarmed GTPase assay buffer (50 mM MES-NaOH[pH 6.5], 2 mM $MgCl_2$, 2 mM dithiothreitol, 1 mg of bovine serum albumin per ml) at 30°C. The GTPase activation assay was initiated by adding IRA2 protein to this mixture. Aliquots were removed at each time point and added to excess ice-cold stop buffer (20 mM MES-NaOH[pH 6.5], 5 mM $MgCl_2$). These mixtures were applied to nitrocellulose filters (SM 11306; 0.45-μm pore size; Sartorius), rinsed three times with stop buffer, and scintillation counted. Alternatively, [α-^{32}P]GTP was used instead of [γ-^{32}P]GTP. After reaction mixtures were filtered, guanine nucleotides bound to RAS were eluted from the filters into 25 mM Tris-HCl (pH 7.4)–20 mM EDTA–2% sodium dodecyl sulfate–1 mM GDP–1 mM GTP at 65°C for 10 min and were analyzed by PEI-cellulose chromatography followed by autoradiography as described previously (41). GDP dissociation from RAS was measured by using ^{3}H[GDP] essentially as described previously (12).

Preparation of IRA2 protein. Two sources of IRA2 protein were prepared and used as described previously (41): glutathione-*S*-transferase-IRA2 (GST-IRA2) fusion protein which contains the catalytic domain of IRA2 (amino acids 1665 to 2025) fused to the carboxy-terminal end of GST and crude extracts of TK161-R2V cells transformed with pKT16 (IRA2 amino acids 528 to 2255).

Adenylate cyclase assays. Membranes from TK-B111 cells were prepared as previously described (45) with the exception that the cells were broken with glass beads. Assays were performed in a 100-μl reaction mixture containing 250 to 300 μg of membrane protein per ml, 20 mM MES (pH 6.2), 1 mM mercaptoethanol, 2.5 mM $MgCl_2$, 0.5 mM cAMP, 10 mM theophylline, 20 mM creatine phosphate, 20 U of creatine phosphokinase per ml, 0.5 mM [α-^{32}P]ATP (40 Ci/mol), and various amounts of RAS2 proteins complexed to GppNHp (guanylylimidodiphosphate). [^{32}P]cAMP produced was determined as described previously (36).

RESULTS

Dominant activated *RAS2* mutations. Previously isolated activated *RAS* mutations displayed several dominant traits: asporogeny, heat shock sensitivity, starvation intolerance, and reduced glycogen accumulation. These traits stem from increased activity of cAMP-dependent protein kinase, and an identical collection of traits result from mutations that increase cAMP-dependent protein kinase activity by other means (8, 45). Among these traits, the reduction in glycogen accumulation is the most sensitive to modest increases in protein kinase activity (9).

Plasmid p1045 has *RAS2* cloned in YCp50. This DNA was mutagenized, and derivatives that conferred a dominant reduction of glycogen were isolated (as described in Materials and Methods). DNA from the entire *RAS2* coding region in p1045 and mutant p1045 derivatives was sequenced. Wild-type *RAS2* in p1045 has the same sequence as previously reported (32) with the exception that codon 255 codes for Asp (GAT) instead of Val (GTT). This is a phenotypically silent mutation in a region of RAS that is not conserved in the RAS family (3, 4). Plasmid p1045-536 contains a transition mutation in codon 41 converting it from Pro (CCC) to Ser (TCC). This mutation is in the effector region (RAS2 amino acids 37 to 47). Many previous mutations described in this region of Ha-*ras* and *RAS2* have been unable to activate the yeast effector adenylate cyclase (26, 38). The reduction of glycogen for *RAS2-P41S* is consistent with the opposite effect, i.e., greater in vivo adenylate cyclase activation.

Comparison of *RAS2-P41S* with other activated *RAS2* mutations. We compared the traits of *RAS2-P41S* with those of two other activated *RAS2* mutations: *RAS2-A66T* (homologous to *A59T* in Ha-*ras*), which has a GTPase deficiency and reduced nucleotide binding (15, 19, 24), and *RAS2-D126N* (homologous to *D119N* in Ha-*ras*), which has reduced nucleotide binding (39). All three activated *RAS2* mutations were isolated on p1045 derivatives as described in Materials and Methods. We scored traits in strains with and without wild-type *RAS2* to reveal dominance. These mutant *RAS2* genes were transformed into haploid and diploid derivatives of JC482 and JC302-26B either on episomal p1045 derivatives or after transferring to YIp5 for chromosomal integration. We found no differences in phenotype between integrated and episomal locations. The glycogen accumulation, sporulation, and heat shock sensitivity traits were examined for at least three independent transformants.

All three *RAS2* mutations are dominant for their reduction in glycogen accumulation scored by iodine staining (Fig. 1A). Iodine stains the glycogen within yeast colonies (11). Quantitation of glycogen in these strains confirmed a reduction for the three mutant *RAS2* genes tested (data not shown). Sporulation was also reduced in a dominant fashion, although *RAS2-D126N* was less impaired than the other mutations for this function.

These traits differentiate these three activated *RAS2* mutations. This is clearly illustrated by the 30-min heat shock experiment, results of which are shown in Fig. 1B. The *RAS2-D126N* mutation is most severe because it displays the greatest intolerance to heat shock irrespective of RAS2 expression. The *RAS2-P41S* mutation is as strong as *RAS2-D126N* in the presence of wild-type *RAS2* but is the weakest of these three activated mutations in the absence of wild-

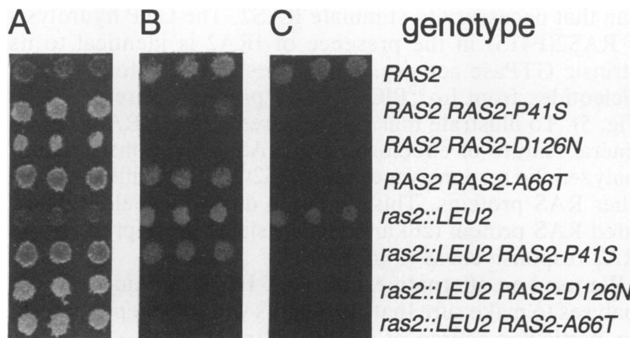

FIG. 1. Traits of activated RAS2 mutations in yeast cells. JC482 ($RAS2^+$) and JC302-26B (*ras2*::*LEU2*) were transformed with activated *RAS2* genes on YIp5 vectors, resulting in their integration at the *RAS2* locus. Three transformants were gridded on a master plate, grown overnight, and replica plated. (A) Colonies grown overnight on YEP-glucose were strained with iodine vapor to visualize glycogen storage. Dark colonies (*RAS2* and *ras2*::*LEU2*) were brown from stored glycogen; light colonies were yellow from glycogen reduction. (B and C) After replica plating, YEP-glucose plates were exposed for 30 and 60 min, respectively, to a temperature of 50°C and then grown overnight at 30°C to score heat shock sensitivity.

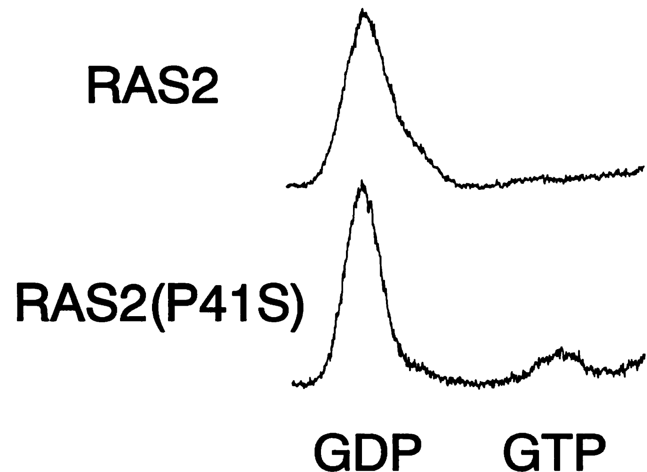

FIG. 2. In vivo RAS nucleotide analysis. JC746 yeast cells with *RAS* genes expressed from the *ADH1* promoter were grown in $^{32}P_i$ medium. RAS protein was immunoprecipitated from the extract, and eluted ^{32}P-labeled nucleotides were separated by PEI-cellulose thin-layer chromatography (as described in Materials and Methods). The origin is on the right. Radioactivity was quantitated with a Molecular Dynamics PhosphorImager. Shown are representative scans from cells expressing wild-type RAS2 or RAS2(P41S). The percentage of GTP, corrected for the number of phosphates, is 1.1 ± 0.7 (two experiments) and 15.2 ± 1.2 (three experiments) (average ± standard error of measurement) for RAS2 and RAS2(P41S), respectively. Only background levels of radioactivity were detected for immunoprecipitations without anti-RAS antibody (data not shown).

type *RAS2*. The presence or absence of wild-type *RAS2* appears to modulate the phenotype of *RAS2-P41S* and *RAS2-A66T* differently.

RAS2-P41S bypasses CDC25 function. The *CDC25* gene product facilitates RAS nucleotide exchange and is essential in otherwise wild-type cells (5, 7, 23, 33). Several mutations, including *ras2*::*LEU2* suppressors (8) and *ira1* (42), *ira2* (44), and activated *RAS2* mutations (5, 33), can bypass CDC25 function (allow viability of *cdc25*::*LEU2* cells). To test whether RAS2-P41S could bypass the requirement for CDC25 function, diploid JC877, heterozygous for *cdc25*::*LEU2*, was transformed with p1045 or p1045 derivatives bearing *RAS2-P41S*, *RAS2-A66T*, or *RAS2-D126N*. These diploids were sporulated, and approximately 30 tetrads were dissected. Only two viable spores were found for each tetrad when the p1045 transformant was analyzed. Furthermore, none of the viable haploid progeny were Leu^+ (*cdc25*::*LEU2*), indicating the lethality of the *cdc25*::*LEU2* mutation. In contrast, many viable Leu^+ Ura^+ haploid progeny were found when the other three transformants were analyzed. All Leu^+ haploids were also Ura^+, which means that *cdc25*::*LEU2* cells were only viable when the p1045 derivative plasmid was present (i.e., when activated *RAS2* genes were present). This result indicates that *RAS2-P41S* bypasses CDC25 function like other activated *RAS2* mutations.

Guanine nucleotides bound to RAS2(P41S) in vivo. One common result of activated *RAS* mutations is that they result in greater quantities of GTP bound to RAS in vivo. We assayed nucleotides bound to RAS2(P41S) in vivo by growing cells in $^{32}P_i$-containing medium, isolating RAS protein by immunoprecipitation, and eluting and quantitating the bound nucleotides (as described in Materials and Methods). Immunoprecipitations done without anti-RAS monoclonal antibody Y13-259 isolated no ^{32}P-labeled nucleotides by this procedure. Controls for this experiment were wild-type RAS2 and c-Ha-RAS, which have been previously assayed (18, 43). We found very little GTP bound to wild-type RAS2 (Fig. 2), as has been reported. In contrast, RAS2(P41S) bound about 15 times more GTP than did wild-type RAS2. High levels of GTP bound to c-Ha-RAS were detected (data not shown). Therefore, RAS2(P41S) has increased GTP binding in vivo like other activated RAS proteins.

Intrinsic RAS GTPase and guanine nucleotide binding activities. We expressed and purified proteins encoding 210 amino-terminal amino acids of wild-type RAS2, RAS2 (P41S), and RAS2(G19V) for biochemical analysis. Biochemical properties of truncated RAS2 proteins are very similar to those of the full-length species in vitro (16). Initially we assayed the intrinsic GTPase activity of these proteins because this is the most common defect associated with RAS activation (3, 17) and proline 41 is proximal to other amino acids critical for GTP hydrolysis based on the Ha-RAS tertiary structure (30, 31, 37). As shown in Fig. 3, the intrinsic GTPase activity for wild-type RAS2 and RAS2 (P41S) proteins were almost identical, while RAS2(G19V) protein has a reduced activity. The rate of GTP hydrolysis by RAS2(P41S) was about 8 mmol of GTP hydrolyzed mol of $RAS2^{-1}$ min^{-1}, similar to the rate reported for wild-type RAS2 (12).

RAS proteins impaired in guanine nucleotide binding also exhibit activated phenotypes in vivo (39). Impairment of nucleotide binding for other RAS mutants resulted primarily from an increase in nucleotide dissociation rate. Therefore, we measured the rate of GDP dissociation of RAS2(P41S); again, it was similar to that of the wild-type protein. Apparent GDP dissociation constants, measured as described previously (12), for wild-type RAS2 and RAS2(P41S) were $(1.6 \pm 0.1) \times 10^{-2}$ min^{-1} and $(1.9 \pm 0.1) \times 10^{-2}$ min^{-1}, respectively. These data indicate that the guanine nucleotide binding is not significantly affected in the RAS2(P41S) protein.

RAS2(P41S) GTPase is insensitive to IRA2 stimulation. The above-described findings suggest that the *RAS2-P41S* mutation activates RAS2 by a novel mechanism because all

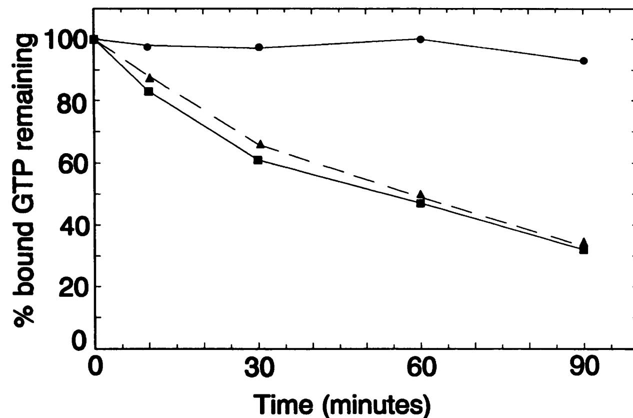

FIG. 3. Intrinsic GTPase activity is not affected in the RAS2(P41S) protein. Intrinsic GTPase activity of RAS2 (■), RAS2(G19V) (●), or RAS2(P41S) (▲) was assayed by the filter binding method as described in Materials and Methods. The percentage of initial radioactivity at each time point is shown. All values are the average of two determinations with a variation of less than 10%. Essentially identical results were found by using RAS-[α-^{32}P]GTP with subsequent analysis by PEI-cellulose chromatography as described in Materials and Methods (data not shown).

previously described activated RAS proteins had reductions in GTPase activity and/or guanine nucleotide binding. A potential mechanism could be insensitivity of the mutant protein to GTPase stimulation by the IRA1 and IRA2 proteins. These proteins, like their mammalian counterparts GAP and NF1, stimulate the intrinsic GTPase activity of RAS (41).

We compared the responsiveness of RAS2 and RAS2 (P41S) with the GTPase stimulatory activity of IRA2. Recently we have expressed the IRA2 domain (amino acids 1665 to 2025), which is homologous with the catalytic domain of mammalian GAP, as a GST fusion protein in *E. coli* (41). Affinity-purified GST-IRA2 fusion protein was added to RAS2 proteins bound to [γ-^{32}P]GTP, and the GTP at various times was quantitated by filter binding (Fig. 4). Wild-type RAS GTPase was greatly stimulated, whereas RAS2(P41S) was nonresponsive to IRA2 protein. The amount of IRA2 protein added was at least 10 times higher than that necessary to stimulate RAS2. The GTP hydrolysis of RAS2(P41S) in the presence of IRA2 is identical to its intrinsic GTPase activity. Similar results were found when nucleotides from [α-^{32}P]GTP RAS proteins were analyzed (Fig. 5). To illustrate that failure to respond to IRA2 is not a general feature of effector region RAS2 mutations, we also analyzed the responsiveness of RAS2(T42S) purified like the other RAS proteins. This mutation does not yield an activated RAS protein (26) and is only slightly compromised in IRA2 responsiveness (Fig. 5).

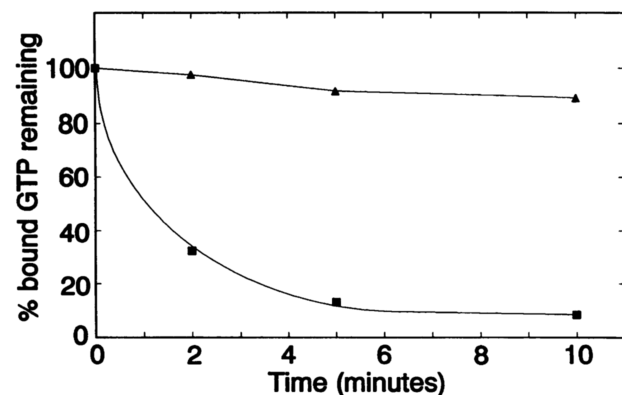

FIG. 4. IRA2 cannot stimulate the intrinsic GTPase activity of RAS2(P41S). Ten micrograms of GST-IRA2 fusion protein was added to a GTPase assay mixture containing RAS2 (■) or RAS2(P41S) (▲) complexed with [γ-^{32}P]GTP. GTP hydrolysis was followed by filter binding as described in the legend to Fig. 3.

We used an alternative source of IRA2 protein for these analyses to make sure that our results were not dependent on the particular source of IRA2. Extracts from yeast cells overexpressing IRA2 amino acids 528 to 2255 also exhibit RAS GTPase stimulation activity (41). These extracts were also unable to stimulate RAS2(P41S), yet they stimulated wild-type RAS2 GTPase (data not shown).

Adenylate cyclase activation. *S. cerevisiae* adenylate cyclase is stimulated by RAS-GTP in vivo. This stimulation can be demonstrated in vitro to be dependent upon the GTP-bound form of RAS (16). The nonhydrolyzable GTP analog, GppNHp, is used in these experiments to eliminate the complications of RAS intrinsic GTPase activity.

In vitro adenylate cyclase assays utilize membranes from yeast cells that overexpress adenylate cyclase (16). Using these membranes, we assayed adenylate cyclase stimulated by various amounts of wild-type RAS2, RAS2(P41S), and RAS2(T42S) proteins bound to GppNHp. The results indicate that RAS2(P41S) is capable of stimulating adenylate cyclase, although the level of maximal stimulation by RAS2(P41S) is lower than that by wild-type RAS2 (Fig. 6). In contrast, there was undetectable stimulation by RAS2(T42S) (Fig. 6) or by wild-type RAS2 or RAS2(P41S) bound to guanosine-5′-*O*-[2-thiodiphosphate](GDP-β-S) (data not shown). In conclusion, the effect of the *RAS2-P41S* mutation is to primarily abolish IRA2 responsiveness with a minor decrease in adenylate cyclase stimulation. This is in contrast to the *RAS2-T42S* mutation, which is unable to stimulate adenylate cyclase yet is responsive to IRA2 (Fig. 5). These data show that effector region amino acids are important (proline 41) or essential (threonine 42) for wild-type levels of adenylate cyclase stimulation.

DISCUSSION

The amino acid sequence of the RAS effector region is conserved in all members of the RAS family (4). This region of RAS is speculated to interact with the target of RAS (the effector) because alterations of these amino acids in viral Ha-RAS impair capacity to transform (38, 49, 50). Consistent with this idea, effector region mutations also prevent RAS activation of the *S. cerevisiae* RAS effector, adenylate cyclase (26, 38).

The mammalian GAP stimulates the low intrinsic GTPase activity of RAS (46, 47). Two classes of GAP nonresponsive RAS mutations have been found: effector region mutations and mutations that inhibit the intrinsic GTPase of RAS (like G12V or Q61L in Ha-RAS) (1, 6, 46, 47). The effector region of RAS therefore appears to be important for response to GTPase-activating proteins as well as effector stimulation.

Effector region mutations do not abrogate GAP responsiveness and effector stimulation equally. Although most effector region mutations reduce both GAP responsiveness and effector stimulation, there are a number of mutant proteins (altered at Ha-RAS amino acids 35, 36, 38, or 40) that respond to GAP yet fail to stimulate the effector (49).

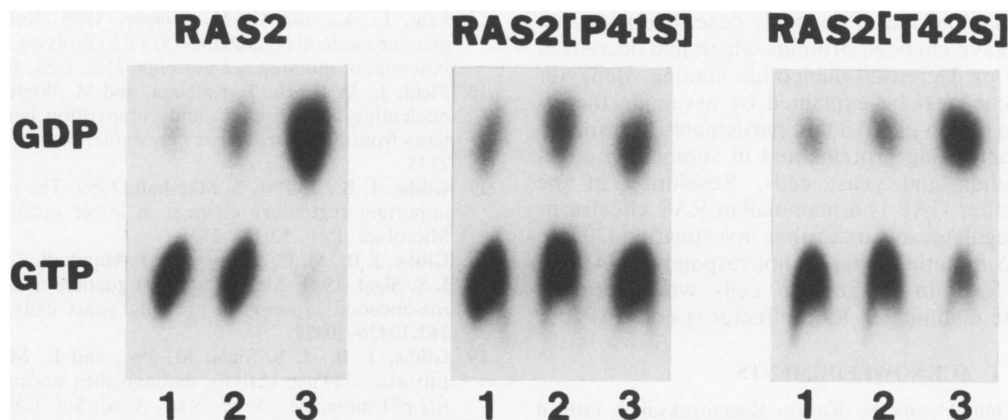

FIG. 5. Guanine nucleotide analysis of GST-IRA2 stimulation of RAS GTPase. RAS-bound guanine nucleotides were analyzed for RAS2, RAS2(P41S), and RAS2(T42S) proteins complexed with [α-^{32}P]GTP immediately (lane 1), after 10 min at 30°C with 10 μg of GST control protein (lane 2), or after 10 min at 30°C with 10 μg of GST-IRA2 (lane 3). After reaction mixtures were filtered, guanine nucleotides were analyzed by PEI-cellulose chromatography followed by autoradiography. Migration of GDP and GTP standards is shown on the left.

The *RAS2* mutation reported in this work, *RAS2-P41S*, is an effector region mutation of the complementary type. It has undetectable responsiveness to the yeast GTPase-activating protein, IRA2, yet it is capable of activating yeast adenylate cyclase sufficiently to display an activated RAS phenotype.

Inability of RAS2(P41S) to respond to yeast IRA proteins has been demonstrated in this paper in vitro as well as in vivo. Our analysis showed that RAS2(P41S) had 15% GTP bound in vivo. Wild-type RAS2 had less than 1% bound. This is because the yeast IRA1 and IRA2 proteins, which stimulate RAS GTPase, are sufficiently active in vivo to keep RAS2 GTP levels low. Inactivation of IRA proteins (i.e., in a *ira1*::*LEU2 ira2*::*HIS3* strain) increases RAS2 GTP levels to about 15% (43). Even though we measured RAS2(P41S) responsiveness only to IRA2 in vitro, the in vivo results suggest that RAS2(P41S) is nonresponsive to both IRA proteins.

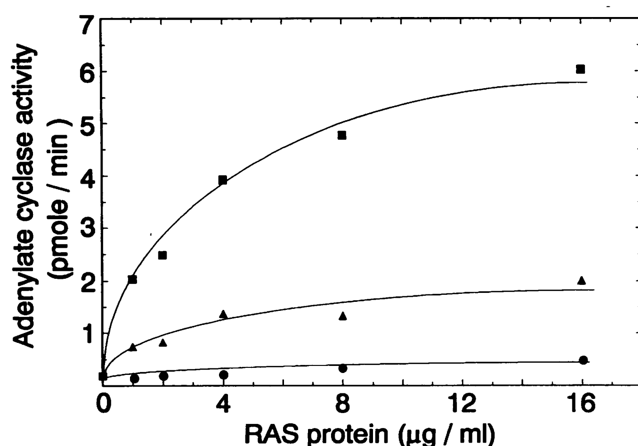

FIG. 6. Adenylate cyclase activation by RAS proteins. Adenylate cyclase assays were performed as described in Materials and Methods with various concentrations of wild-type RAS2 (■), RAS2(P41S) (▲), and RAS2(T42S) (●). These three proteins had approximately the same nucleotide binding equivalents per microgram of protein. In the presence of 2.5 mM $MnCl_2$, adenylate cyclase activity was 5.12 pmol/min, and without added RAS protein it was 0.18 pmol/min. All values are the average of two determinations with a variation of less than 10%.

Similar to other effector region mutations, *RAS2-P41S* has an impairment in effector stimulation. Maximal adenylate cyclase stimulation by RAS2(P41S) is reduced to 40% of the activity stimulated by wild-type RAS2 (Fig. 6). It is interesting that RAS2(P41S) behaves differently than other effector region mutants. Other mutant RAS proteins with reductions in adenylate cyclase stimulation apparently have diminished affinity for adenylate cyclase because at greater concentrations of RAS, adenylate cyclase activity plateaus to the same level (14). The results for RAS2(P41S) suggest that adenylate cyclase affinity is not reduced; rather, stimulatory competence is compromised.

The traits displayed by a mutant RAS protein that has impairments in both effector stimulation and GTPase-activating protein response depend upon the relative magnitudes of the two impairments. If the effector stimulation defect is greater, it cannot be offset by the increased amount of active, GTP-bound RAS that accumulates from the GTPase activation handicap. Such a mutant RAS will not exhibit activated traits. The double mutation *D33H P34S* in Ha-RAS reported recently is an example of such a *RAS* mutation (14). It has significantly reduced adenylate cyclase affinity and does not display activated RAS traits in mammalian cells even though it has increased levels of GTP bound (14). The RAS2(P41S) mutant protein described in this paper apparently retains a sufficient capacity to activate the effector such that its elevated amount of GTP bound in vivo translates into greater net effector stimulation than that elicited from wild-type RAS2. Therefore, RAS2(P41S) displays traits of an activated RAS. The traits documented here include reduction of glycogen accumulation, increased heat shock sensitivity, and ability to bypass CDC25 function. It is currently unclear what the significant differences are between Ha-*RAS D33H P34S* and *RAS2-P41S* that result in their different in vivo phenotypes. Ha-RAS amino acids 33 and 34 correspond to RAS2 amino acids 40 and 41; the effector regions of these two RAS proteins have identical amino acid sequences. It is possible that a single *P34S* mutation in Ha-RAS may transform mammalian cells if the *D33H* mutation was responsible for reducing effector stimulation in addition to that caused by *P34S*.

In summary, we believe that *RAS2-P41S* illustrates a novel mechanism of RAS activation; impairment of GTPase-activating response while others intrinsic properties of the

RAS protein remain normal. Previously described activated *RAS* mutations have encoded proteins which had decreased GTPase activity or decreased nucleotide binding. Behavior of *RAS2-P41S* can best be explained by assuming the effector region is a region of RAS that participates in responding to GTPase-activating proteins and in stimulating effectors in mammalian and yeast cells. Resolution of the question of whether GAP is a mammalian RAS effector or merely a RAS regulator awaits further investigation (20, 29, 48). Clearly *RAS* mutations that do not respond to GAP yet could activate RAS in mammalian cells would provide evidence that the mammalian RAS effector is not GAP.

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REFERENCES

1. **Adari, H., D. R. Lowy, B. M. Willumsen, C. J. Der, and F. McCormick.** 1988. Guanosine triphosphate activating protein (GAP) interacts with the p21 effector binding domain. Science **240**:518–521.
2. **Ballester, R., D. Marchuk, M. Boguski, A. Saulino, R. Letcher, M. Wigler, and F. Collins.** 1990. The *NF1* locus encodes a protein functionally related to mammalian GAP and yeast *IRA* proteins. Cell **63**:851–859.
3. **Barbacid, M.** 1987. *ras* genes. Annu. Rev. Biochem. **56**:779–827.
4. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature (London) **349**:117–127.
5. **Broek, D., T. Toda, T. Michaeli, L. Levin, C. Birchmeier, M. Zoller, S. Powers, and M. Wigler.** 1987. The *S. cerevisiae* CDC25 gene product regulates the *RAS*/adenylate cyclase pathway. Cell **48**:789–799.
6. **Cales, C., J. F. Hancock, C. J. Marshall, and A. Hall.** 1988. The cytoplasmic protein GAP is implicated as the target for regulation by the *ras* gene product. Nature (London) **332**:548–551.
7. **Camonis, J. H., H. Kalekine, B. Gondre, H. Garreau, E. Boy-Marcotte, and M. Jacquet.** 1986. Characterization, cloning and sequence analysis of the *CDC25* gene which controls the cyclic AMP level of *Saccharomyces cerevisiae*. EMBO J. **5**:375–380.
8. **Cannon, J. F., J. B. Gibbs, and K. Tatchell.** 1986. Suppressors of the *ras2* mutation in *Saccharomyces cerevisiae*. Genetics **113**:247–264.
9. **Cannon, J. F., R. Gitan, and K. Tatchell.** 1990. Yeast cAMP-dependent protein kinase regulatory subunit mutations display a variety of phenotypes. J. Biol. Chem. **265**:11897–11904.
10. **Cannon, J. F., B. A. Wilson, and M. Khalil.** Unpublished data.
11. **Chester, V.** 1968. Heritable glycogen storage deficiency in yeast and its induction by ultraviolet light. J. Gen. Microbiol. **51**:49–56.
12. **Crechet, J.-B., P. Poullet, J. Camanois, M. Jacquet, and A. Parmeggiani.** 1990. Different kinetic properties of the two mutants, RAS2ile152 and RAS2Val19, that suppress the *CDC25* requirement in RAS/adenylate cyclase pathway in *Saccharomyces cerevisiae*. J. Biol. Chem. **265**:1563–1568.
13. **DeFeo-Jones, D., K. Tatchell, L. C. Robinson, I. S. Sigal, W. C. Vass, D. R. Lowy, and E. M. Scolnick.** 1985. Mammalian and yeast *ras* gene products: biological function in their heterologous systems. Science **228**:179–184.
14. **Farnsworth, C. L., M. S. Marshall, J. B. Gibbs, D. W. Stacey, and L. A. Feig.** 1991. Preferential inhibition of the oncogenic form of Ras^H by mutations in the GAP building/"effector" domain. Cell **64**:625–633.
15. **Feig, L. A., and G. M. Cooper.** 1988. Relationship among guanine nucleotide exchange, GTP hydrolysis, and transforming potential of mutated *ras* proteins. Mol. Cell. Biol. **8**:2472–2478.
16. **Field, J., D. Broek, T. Kataoka, and M. Wigler.** 1987. Guanine nucleotide activation of, and competition between, *RAS* proteins from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7**:2128–2133.
17. **Gibbs, J. B., and M. S. Marshall.** 1989. The *ras* oncogene—an important regulatory element in lower eucaryotic organisms. Microbiol. Rev. **53**:171–185.
18. **Gibbs, J. B., M. D. Schaber, M. S. Marshall, E. M. Scolnick, and I. S. Sigal.** 1987. Identification of guanine nucleotides bound to *ras*-encoded proteins in growing yeast cells. J. Biol. Chem. **262**:10426–10429.
19. **Gibbs, J. B., I. S. Sigal, M. Poe, and E. M. Scolnick.** 1984. Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. Proc. Natl. Acad. Sci. USA **81**:5704–5708.
20. **Hall, A.** 1990. *ras* and GAP—who's controlling whom? Cell **61**:921–923.
21. **Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagaloff.** 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast **2**:163–167.
22. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
23. **Jones, S., M.-L. Vignais, and J. R. Broach.** 1991. The *CDC25* protein of *Saccharomyces cerevisiae* promotes exchange of guanine nucleotides bound to RAS. Mol. Cell. Biol. **11**:2641–2646.
24. **Lacal, J. C., and S. A. Aaronson.** 1986. Activation of *ras* p21 transforming properties associated with an increase in the release rate of bound guanine nucleotide. Mol. Cell. Biol. **6**:4214–4220.
25. **Marshall, M. S., J. B. Gibbs, E. M. Scolnick, and I. S. Sigal.** 1987. Regulatory function of the *Saccharomyces cerevisiae RAS* c-terminus. Mol. Cell. Biol. **7**:2309–2315.
26. **Marshall, M. S., J. B. Gibbs, E. M. Scolnick, and I. S. Sigal.** 1988. An adenylate cyclase from *Saccharomyces cerevisiae* that is stimulated by RAS proteins with effector mutations. Mol. Cell. Biol. **8**:52–61.
27. **Martin, G. A., D. Viskochill, G. Bollag, P. C. McCabe, W. J. Crosier, H. Haubruck, L. Conroy, R. Clark, P. O'Connell, R. M. Crawthon, M. A. Innis, and F. McCormick.** 1991. The GAP-related domain of the neurofibromatosis type 1 gene product interacts with *ras* p21. Cell **63**:843–849.
28. **Matsumoto, K., I. Uno, and T. Ishikawa.** 1985. Genetic analysis of the role of cAMP in yeast. Yeast **1**:15–24.
29. **McCormick, F.** 1989. *ras* GTPase activating protein: signal transmitter and signal terminator. Cell **56**:5–8.
30. **Milburn, M. V., L. Tong, A. M. DeVos, A. Brunger, Z. Yamaizumi, S. Nishimura, and S.-H. Kim.** 1990. Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic *ras* proteins. Science **247**:939–945.
31. **Pai, E. F., U. Krengel, G. Petsko, R. S. Goody, W. Kabsch, and A. Wittinghofer.** 1990. Refined crystal structure of the triphosphate conformation of H-*ras* p21 at 1.35 A resolution: implications for the mechanism of GTP hydrolysis. EMBO J. **9**:2351–2359.
32. **Powers, S., T. Kataoka, O. Fasano, M. Goldfarb, J. Strathern, J. Broach, and M. Wigler.** 1984. Genes in *S. cerevisiae* encoding proteins with domains homologous to the mammalian *ras* proteins. Cell **36**:607–612.
33. **Robinson, L. C., J. B. Gibbs, M. S. Marshall, I. S. Sigal, and K. Tatchell.** 1987. *CDC25*: a component of the *RAS*-adenylate cyclase pathway in *Saccharomyces cerevisiae*. Science **235**:1218–1221.
34. **Rose, M. D.** 1987. Isolation of genes by complementation. Methods Enzymol. **152**:481–504.
35. **Rose, M. D., and G. R. Fink.** 1987. *KAR1*, a gene required for function of both intranuclear extranuclear microtubules in yeast. Cell **48**:1047–1060.
36. **Salomon, Y., C. Londos, and M. Rodbell.** 1974. A highly

sensitive adenylate cyclase assay. Anal. Biochem. **58**:541–548.

37. **Schlicting, I., S. C. Almo, G. Rapp, K. Wilson, K. Petratos, A. Lentfer, A. Wittinghofer, W. Kabsch, E. F. Pai, G. A. Petsko, and R. S. Goody.** 1990. Time-resolved X-ray crystallographic study of the conformational change in Ha-Ras p21 protein on GTP hydrolysis. Nature (London) **345**:309–315.
38. **Sigal, I. S., J. B. Gibbs, J. S. D'Alonzo, and E. M. Scolnick.** 1986. Identification of effector residues and a neutralizing epitope of Ha-*ras*-encoded p21. Proc. Natl. Acad. Sci. USA **83**:4725–4729.
39. **Sigal, I. S., J. B. Gibbs, J. S. D'Alonzo, G. L. Temeles, B. S. Wolanski, S. H. Socher, and E. M. Scolnick.** 1986. Mutant *ras*-encoded proteins with altered nucleotide binding exert dominant biological effects. Proc. Natl. Acad. Sci. USA **83**:952–956.
40. **Tamanoi, F.** 1988. Yeast *RAS* genes. Biochem. Biophys. Acta **948**:1–15.
41. **Tanaka, K., B. K. Lin, D. R. Wood, and F. Tamanoi.** 1991. IRA2, an upstream negative regulator of RAS in yeast, is a RAS GTPase activating protein (GAP). Proc. Natl. Acad. Sci. USA **88**:468–472.
42. **Tanaka, K., K. Matsumoto, and Toh-E.** 1989. *IRA1*, an inhibitory regulator of the *RAS*-cyclic AMP pathway in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9**:757–768.
43. **Tanaka, K., M. Nakafuku, T. Satoh, M. S. Marshall, J. B. Gibbs, K. Matsumoto, Y. Kaziro, and A. Toh-E.** 1990. *Saccharomyces cerevisiae* genes, *IRA1* and *IRA2*, encode proteins which may be functionally equivalent to mammalian *ras* GTPase activating protein (GAP). Cell **60**:803–807.
44. **Tanaka, K., M. Nakafuku, F. Tamanoi, Y. Kaziro, K. Matsumoto, and A. Toh-E.** 1990. *IRA2*, a second gene of *Saccharomyces cerevisiae* that encodes a protein with a domain homologous to mammalian *ras* GTPase-activating protein. Mol. Cell. Biol. **10**:4303–4313.
45. **Toda, T., I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broek, S. Cameron, J. Broach, K. Matsumoto, and M. Wigler.** 1985. In yeast, *RAS* proteins are controlling elements of adenylate cyclase. Cell **40**:27–36.
46. **Trahey, M., and F. McCormick.** 1987. A cytoplasmic protein stimulates normal N-*ras* p21 GTPase, but does not affect oncogenic mutants. Science **238**:542–545.
47. **Vogel, U., R. A. Dixon, M. R. Schaber, E. Diehl, M. S. Marshall, E. M. Scolnick, I. S. Sigal, and J. B. Gibbs.** 1988. Cloning of bovine GAP and its interactions with oncogenic Ras p21. Nature (London) **335**:90–93.
48. **Wigler, M. H.** 1990. GAPs in understanding Ras. Nature (London) **346**:696–697.
49. **Willumsen, B. M., H. Adari, K. Zhang, A. G. Papageorge, J. C. Stone, F. McCormick, and D. R. Lowy.** 1989. A mutational analysis of RAS function, p. 165–177. In L. Bosch, B. Kraal, and A. Parmeggiani (ed.), The guanine-nucleotide binding proteins: common structural and functional properties. Plenum Press, New York.
50. **Willumsen, B. M., A. G. Papageorge, H. Kung, E. Bekesi, T. Robins, M. Johnsen, W. C. Vass, and D. R. Lowy.** 1986. Mutational analysis of a RAS catalytic domain. Mol. Cell. Biol. **6**:2646–2654.
51. **Xu, G., B. Lin, K. Tanaka, D. Dunn, D. Wood, R. Gestland, R. White, R. Weiss, and F. Tamanoi.** 1990. The catalytic domain of the neurofibromatosis type 1 gene product stimulates *ras* GTPase and complements *ira* mutants of *S. cerevisiae*. Cell **63**:835–841.