MOLECULAR AND CELLULAR BIOLOGY, Oct. 1991, p. 4822–4829
0270-7306/91/104822-08$02.00/0

Vol. 11, No. 10

Dominant Inhibitory Mutations in the Mg^{2+}-Binding Site of Ras^H Prevent Its Activation by GTP

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Received 1 May 1991/Accepted 27 June 1991

We have previously demonstrated that substitution of Asn for Ser at position 17 of Ras^H yields a dominant inhibitory protein whose expression in cells interferes with endogenous Ras function (L. A. Feig, and G. M. Cooper, Mol. Cell. Biol. 8:3235–3243, 1988). Subsequent structural studies have shown that the hydroxyl group of Ser-17 contributes to the binding of Mg^{2+} associated with bound nucleotide. In this report, we show that more subtle amino acid substitutions at this site that would be expected to interfere with complexing Mg^{2+}, such as Cys or Ala, also generated dominant inhibitory mutants. In contrast, a Thr substitution that conserves a reactive hydroxyl group maintained normal Ras function. These results argue that the defect responsible for the inhibitory activity is improper coordination of Mg^{2+}. Preferential affinity for GDP, observed in the original Asn-17 mutant, was found exclusively in inhibitory mutants. However, this binding specificity did not completely block the mutant proteins from binding GTP in vivo since introduction of the autophosphorylation site, Thr-59, in 17N Ras resulted in the phosphorylation of the double mutant in cells. Furthermore, inhibitory mutants failed to activate a model downstream target, yeast adenylate cyclase, even when bound to GTP. Thus, the consequence of improper complexing of Mg^{2+} was to lock the protein in a constitutively inactive state. A model is presented to explain how these properties could cause the mutant protein to inhibit the activation of endogenous Ras by competing for a guanine nucleotide-releasing factor.

One of the most powerful experimental approaches to define the physiological function of a protein is to specifically inhibit its activity in vivo. In yeast cells, this can be accomplished by inactivating specific genes through homologous recombination. In mammalian cells, this approach is becoming more feasible, but it is still not applicable in many cases. Antisense RNA expression and antibody microinjection have also been successful for some genes products. An alternative approach that has evolved from structure-function studies of proteins is the creation of dominant inhibitory mutants that interfere with the function of their normal protein counterparts.

One such dominant inhibitory protein is a form of Ras^H protein, with a substitution of Asn for Ser at amino acid 17 (S17N) (8). Expression of this protein in cells generated phenotypes that were indistinguishable from those produced by microinjection of anti-Ras antibodies. For example, NIH 3T3 cell proliferation was inhibited, and this effect was overcome by oncogene products that are thought to act downstream of Ras in a growth regulatory pathway, such as the cytoplasmic Raf and Mos proteins. In contrast, cell surface tyrosine kinases, which are thought to act upstream of Ras, such as Src, Fms, and Fes failed to overcome this block in cell proliferation (8). More recently, neurite outgrowth induced by nerve growth factor (NGF) in PC12 cells was also shown to be inhibited by S17N Ras^H expression (30). It is not yet clear, however, whether this mutant Ras^H protein interferes specifically with endogenous Ras^H or with all family members, including Ras^N and Ras^K. Because of its unique phenotype, 17N Ras is potentially a very powerful reagent for deciphering the role of Ras in normal signal transduction. For example, the mutant has already been used to show that Ras proteins are involved in at least two parallel signal transduction pathways (3, 30). Therefore, it is important to understand the biochemical defect underlying the dominant inhibitory phenotype of this mutant.

Like other GTP-binding proteins, Ras proteins (p21) are activated by the replacement of GDP for GTP (1), a process that is catalyzed by a guanine nucleotide-releasing factor (GNRF) (32, 34). Ras is inactivated by an intrinsic GTPase activity that is catalyzed by a GTPase-activating protein (GAP) (31). The original characterization of 17N Ras^H showed a change in the protein's specificity for nucleotide binding from equal affinity for GTP and GDP to preference for GDP (8). This finding suggested that the sole reason the protein is inactive is that it does not bind GTP in vivo because of competition from GDP. Subsequent structural studies suggested that this defect could be due to improper Mg^{2+} binding, since Ser-17 was found to be one of three amino acids that are involved in binding this metal, which is associated with bound nucleotide (16, 18, 19). In this study, we investigated the consequence of additional mutations at position 17 and other amino acids involved in metal binding and found that improper complexing of Mg^{2+} is, in fact, most likely responsible for the dominant inhibitory phenotype of these mutants. Furthermore, the inhibitory mutants were found to be locked in an inactive conformation regardless of whether they were bound to GTP or GDP. Such a phenotype is consistent with the protein acting as a competitive inhibitor of normal Ras in cells for interactions with a nucleotide-releasing factor.

MATERIALS AND METHODS

Mutant isolation. Site-directed mutagenesis was used to introduce various amino acid substitutions into a composite ras^H gene described previously (9). The gene consisted of the 5′ end of viral ras^H, which was converted to the coding sequence of cellular ras^H by site-directed mutagenesis, and the 3′ end of a cellular *ras* cDNA clone. All mutations were verified by dideoxy sequencing.

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Purification of Ras proteins. The ~900-bp *Hin*dIII-*Bam*HI fragment of various mutant *ras*^{H} genes was excised from M13 double-stranded DNA and inserted in place of the comparable fragment removed from the bacterial expression vector pXCR (10). Normal and mutant Ras proteins were expressed in *Escherichia coli* and purified in soluble form to >80% purity as previously described (7). Routinely, 2 to 3 mg of protein was obtained per liter of bacteria, and approximately 10 to 20% of the protein was cleaved at the carboxy terminus. Proteins, which were >70% active as assessed by GTP binding, were stored at −70°C.

Transfections. The 0.7-kb *Bgl*II-*Bam*HI fragment of pXCR was subcloned into the *Bam*HI site of the mammalian expression vector pZIPneoSV(X) (4). Samples of various mutant *ras*^{H} genes in pZIPneoSV(X) (10 to 1,000 ng) along with 20 μg of carrier calf thymus DNA were used to transfect NIH 3T3 cells by the calcium phosphate precipitation method as previously described (10), and foci of transformed cells were quantitated 14 days later. In some cases, the cells were split 3 days after transfection and exposed to G418 (400 μg/ml). Drug-resistant colonies were pooled 20 days later and used in experiments described below. For cotransfection experiments, clones pM1 (a 6-kb genomic cellular *ras*^{H} clone with a 61L substitution cloned into pBR), psrc 11 (v-*src*) (26), and pF4 (v-*raf*) (21) were used.

Immunoprecipitations. NIH 3T3 cells transfected with various *ras*^{H} mutants were metabolically labeled with [^{35}S]methionine (Trans label; 200 μCi/ml; ICN) for 18 h in methionine-free medium containing 10% calf serum. Ras^H proteins were immunoprecipitated with anti-Ras monoclonal antibody YA6-172 as previously described (10). For autophosphorylation experiments, cells were labeled for 18 h with $^{32}PO_4$ and Ras proteins were immunoprecipitated with anti-Ras antibody YA6-259 as previously described (10).

Interactions with guanine nucleotides. Normal and mutant Ras^H proteins (50 ng) were incubated in binding buffer (50 mM Tris-HCl [pH 7.5], 1.0 mM $MgCl_2$, 1.0 mM dithiothreitol, 150 mM NaCl, 40 μg of bovine serum albumin per ml) for 1 h at 32°C with 10 μM [^{35}S]GDP (1,259 Ci/mmol) or [^{3}H]GDP (10 Ci/mmol) in the presence of various amounts of unlabeled GDP or GTP. Bound radioactive GDP was assayed by membrane filtration as previously described (9).

Stimulation of yeast adenylate cyclase. Crude membranes fractions were prepared from *Saccharomyces cerevisiae* TKB-111 (kindly provided by M. Wigler) as described previously (11) except that cells were lysed by two passages through a French press. Ras proteins were preloaded with either GTP or GDPβS by incubation of 60 μM protein solutions with 1.0 mM nucleotide in exchange buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA) for 20 min at 30°C. $MgCl_2$ was then added to a final concentration of 5 mM; when necessary, the proteins were concentrated in a Centricon 10 microconcentrator (Amicon Corp). Various amounts of protein were added to 10 μg of yeast membranes, and cyclic AMP (cAMP) production was measured for 20 min at 30°C by a modification of the method of Salomon (22). Each tube (final volume, 50 μl) contained 20 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.2), 5 mM $MgCl_2$, 20 mM creatine phosphate, 10 IU of creatine phosphokinase, 1 mM [^{3}H]cAMP (5×10^3 cpm; 36 Ci/mmol; Dupont NEN), and 0.50 mM [α-^{32}P]ATP (3×10^6 to 6×10^6 cpm; 800 Ci/mmol; ICN). To assay total cyclase activity in membranes, 5 mM $MnCl_2$ was substituted for $MgCl_2$.

To document that the proteins were loaded with the appropriate nucleotide, [α-^{32}P]GTP was included in some binding reactions, and after separation of bound from free nucleotide by filtering through nitrocellulose, bound nucleotide was released from the filter by exposure to 0.1 N HCl for 1 min. The pH was then adjusted to 7.5 by the addition of Tris base. Nonradioactive GTP (final concentration, 0.1 mM) was immediately added, and the radioactivity released from the filter was chromatographed on poly(ethylene)imine thin-layer chromatography plates with 0.75 M KH_2PO_4 (pH 3.5). GTP and GDP were visualized by autoradiography.

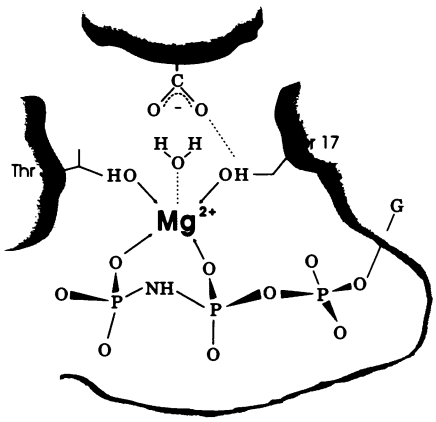

FIG. 1. Interactions of Mg^{2+} with amino acid side groups of Ras bound to the GTP analog GppNHp. The side chain hydroxyl of Ser-17 binds Mg^{2+} in both the GTP and GDP forms, whereas Thr-35 is complexed to the metal only when Ras is bound to GTP. Asp-57 complexes Mg^{2+} indirectly through a water molecule which contributes the third uncharged oxygen. This amino acid also forms a hydrogen bond with the side group of Ser-17. Mg^{2+} is also coordinated to one charged oxygen in each of the β- and γ-phosphates of GTP (not shown) (19).

RESULTS

Biological activity of *ras*^{H} mutants with various substitutions at position 17. It has been shown previously that an Asn substitution for Ser at position 17 of Ras^H p21 yields a dominant inhibitory protein whose expression in cells interferes with the function of endogenous Ras proteins. Subsequently, structural analysis based on X-ray crystallography has shown that the hydroxyl group of Ser-17 of Ras is involved in complexing Mg^{2+} associated with bound GTP and GDP (Fig. 1). Therefore, we tested whether the properties of Ras proteins with other amino acid substitutions at this site were consistent with the notion that the dominant inhibitory phenotype was a consequence of altered interactions with Mg^{2+}. These substitutions included Thr (S17T), which retains a reactive oxygen, Cys (S17C), which merely replaces the oxygen in the hydroxyl group with sulfur, Ala (S17A), which removes the hydroxyl group, and Gly (S17G), which removes the entire side group. *ras* genes with these mutations were subcloned into the mammalian expression vector pZIPneoSV(X) (4) and tested for either transforming activity or activity consistent with inhibition of endogenous Ras function in NIH 3T3 cells. DNA transfection experiments showed that S17T and S17G *ras*^{H} retained the ability to induce transformed foci in these cells although their activities, 5 and 6 foci per μg of DNA, respectively, were moderately lower than the activity of normal ras^H (25 foci per μg). In contrast, like the original S17N *ras*, S17C and S17A *ras* displayed undetectable transforming activity, rais-

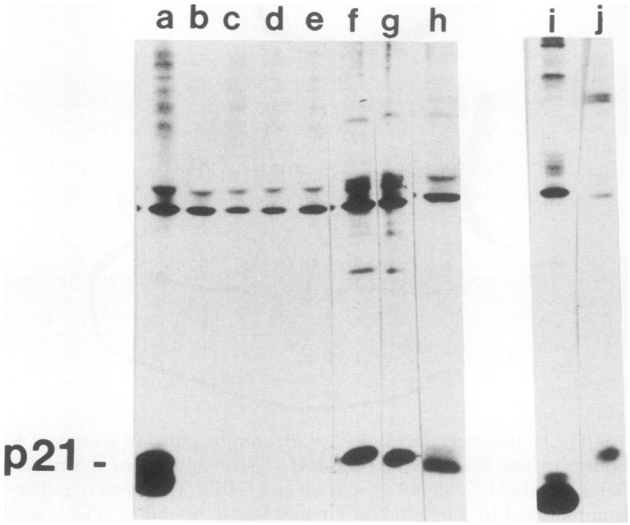

FIG. 2. Expression of mutant Ras proteins in NIH 3T3 cells. Pools of NIH 3T3 cells transfected with various mutant Ras genes were metabolically labeled with [^{35}S]methionine for 18 h, and Ras^{H} proteins were immunoprecipitated with anti-Ras antibody YA6-172. Samples were electrophoresed in sodium dodecyl sulfate-polyacrylamide gels and autoradiographed. Lanes: a, cells transfected with normal *ras*; b, untransfected cells; c to e, cells transfected with 17N *ras*, 17C *ras*, and 17A *ras*, respectively; f, cells transfected with 17G *ras*; g, cells transfected with 17T *ras*; h, cells transfected with 35N *ras*; i, cells transfected with 57N *ras*; j, cells cotransfected with 17A *ras* and v-*raf*.

ing the possibility that they were also dominant inhibitory genes.

To test this possibility, NIH 3T3 cells transfected with these genes were selected in G418-containing medium and the number of drug-resistant colonies that appeared was quantitated. As found originally for S17N *ras* (8), S17C and S17A *ras* genes generated ~10% of the colonies generated by normal *ras* (data not shown). Moreover, when those few colonies that did arise were metabolically labeled with [^{35}S]methionine and immunoprecipitated with anti-Ras antibodies, mutant Ras expression could not be visualized (Fig. 2, lanes c to e) above the background found in nontransfected NIH cells (lane b). This result contrasts the high level of Ras expression seen when a normal *ras* gene or functional S17T and S17G *ras* genes were transfected (lanes a, f, and g, respectively). These results suggested that the expression of 17A and 17C *ras* genes was incompatible with the outgrowth of drug-resistant colonies.

It has been shown previously that transformation by v-*src* but not v-*raf* is blocked in cells whose endogenous Ras proteins are inhibited. This has been documented by microinjection of anti-Ras antibodies into transformed cells (28) and by cotransfection of S17N ras along with either v-*src* or v-*raf* (8). In the latter experiment, S17N *ras* has been shown to inhibit the outgrowth of transformed foci induced by v-*src* but not v-*raf* (8). Table 1 shows that like the original inhibitory S17N *ras*, cotransfection of S17A or S17C *ras* also inhibited the outgrowth of foci induced by *src* but not v-*raf* genes in NIH 3T3 cells. Moreover, the ability of v-Raf to relieve growth inhibition by S17A Ras was confirmed by demonstrating that cells cotransfected with S17A *ras* and v-*raf* expressed significant amounts of S17A p21 (Fig. 2, lane j).

TABLE 1. Focus inhibition by mutant *ras* genes[a]

Cotransfected plasmid	No. of foci/dish for:	
	v-*src*	v-*raf*
S17C *ras*	1	53
S17A *ras*	2	47
Vector only	20	53

[a] NIH 3T3 cells were transfected with 100 ng of transforming plasmids, v-*src* and v-*raf* either alone or together with 1 μg of S17C *ras*^{H} or S17A *ras*^{H} in the expression vector pZIPneoSV(X), or vector alone. Foci were counted 14 days after transfection. Data represent averages of at least two experiments, each performed in duplicate.

Functional Ras proteins have been shown to be required for NGF stimulation of neurite outgrowth in the pheochromocytoma cell line PC12 (12). Consistent with the notion that S17N p21 inhibits endogenous Ras function is the observation that its expression in PC12 cells blocks NGF-induced neurite outgrowth (30). We have confirmed that S17C Ras behaves as a dominant inhibitory protein by showing that its expression in PC12 cells also inhibited NGF-induced neurite outgrowth (data not shown). The growth inhibition in NIH 3T3 cells and the inhibition of differentiated function in PC12 cells all support the notion that S17A and S17C inhibit endogenous Ras function in cells.

Taken together, the biological activities of mutant Ras proteins with Thr, Ala, and Cys substitutions document the importance of a reactive oxygen at position 17 of Ras. Since the function of this species is to complex Mg^{2+}, these results are consistent with a defect in this function being responsible for the dominant inhibitory phenotype of these mutant proteins.

At first, the results obtained with S17G Ras appear to contradict this interpretation, since this protein has no side group yet retains transforming activity. It is likely, however, that the removal of the entire side group by this amino acid substitution generates enough room for an H_2O molecule to fit into the nucleotide-binding pocket at this site and contribute an oxygen for Mg^{2+} complexing.

Substitutions at other sites involved in Mg^{2+} binding. Ser-17 is not the only amino acid in Ras involved in complexing Mg^{2+} associated with bound nucleotide. X-ray crystallography studies also implicate Asp-57 and Thr-35 (Fig. 1) (16, 18). We therefore were interested in determining whether dominant inhibitory proteins could be generated by Asn substitutions at these sites. These mutant *ras* genes were transfected into NIH 3T3 cells, and it was determined that D57N *ras* retained the ability to transform cells at levels comparable (5 foci per μg of DNA) to those of normal *ras* (25 foci per μg of DNA). The protein could also be expressed at levels comparable to those of normal Ras (Fig. 2, lane i). In contrast, T35N *ras* was completely devoid of measurable transforming activity; however, it did not inhibit endogenous Ras function in cells, since a normal number of G418-resistant colonies arose after transfection (data not shown) and these cells expressed large amounts of the mutant protein (Fig. 2, lane h). Therefore, substitutions at each of the three amino acids whose side groups appear to be involved in complexing Mg^{2+} gave three different phenotypes. Only changes at amino acid 17 generated dominant inhibitory proteins.

Preferential affinity for GDP correlates with dominant inhibitory activity. A distinguishing characteristic of inhibitory S17N p21 was that it displayed preferential affinity for

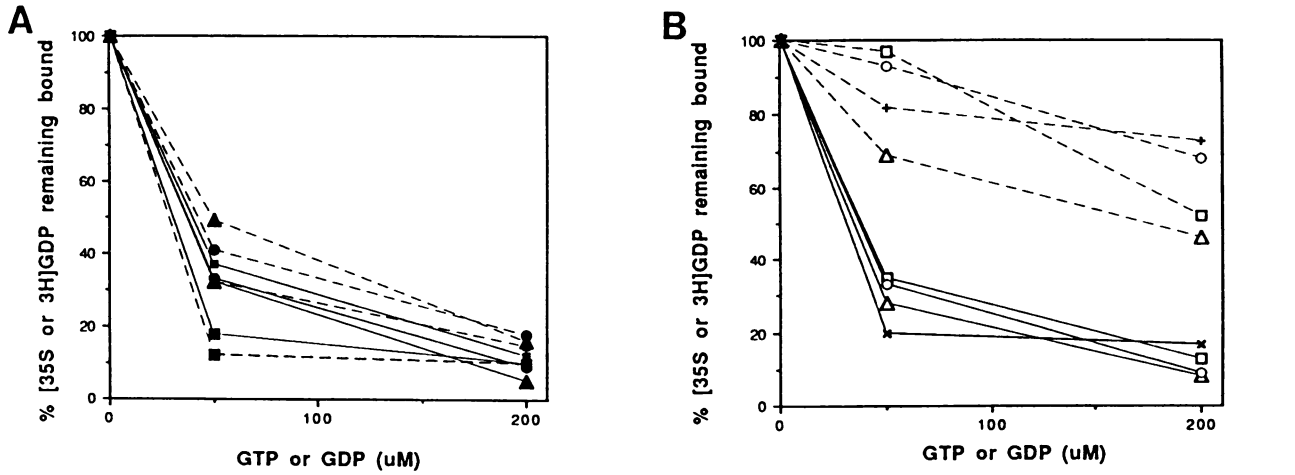

FIG. 3. Relative affinities of mutant Ras proteins for GTP and GDP. Samples (50 ng) of normal (■), S17G (●), T35N (⊞), or D57N (▲) Ras (A) and of S17N (△), S17A (□), S17C (○), or S17N/A59T (X) Ras (B) were incubated for 1 h at 32°C with 10 μM [^{35}S]GDP (1,259 Ci/mmol) or [^{3}H]GDP (10 Ci/mmol) in the presence of the indicated amount of unlabeled GDP (———) or GTP (– – – –). Bound radioactive GDP was assayed by membrane filtration. Data are expressed as the percentage of [^{35}S]GDP bound in the presence of unlabeled nucleotide compared with that in the absence of unlabeled nucleotide and represent the averages of duplicate experiments.

GDP (8). We tested whether this feature was retained in the dominant inhibitory S17A and S17C proteins by comparing the affinity of these mutants for GTP and GDP in competition assays (Fig. 3). [^{35}S]GDP or [^{3}H]GDP (10 μM) was incubated with normal and mutant proteins in the presence of various amounts of excess unlabeled GDP or GTP, and the ability of these nucleotides to compete with radioactive GDP for binding to Ras was compared. Normal Ras has equal affinity for GTP and GDP and, as expected, these nucleotides competed equally. The same was true for the functional S17G and D57N mutants as well as the nonfunctional T35N protein (Fig. 3A). However, GDP competed ~20 times better than GTP for binding to inhibitory S17A and S17C Ras, similar to the previously characterized S17N inhibitory Ras protein (Fig. 3B). The 20-fold preference for GDP is a minimum because contaminating GDP in GTP preparations would artificially reduce the actual difference in affinity. These results demonstrate a tight correlation between preferential affinity for GDP and dominant inhibitory activities in this set of Ras proteins. This unique binding specificity of the mutant could not be returned to normal by raising Mg^{2+} concentrations to 20 mM, nor could it be produced in wild-type Ras by chelating Mg^{2+} with EDTA (data not shown), suggesting the defect in the proteins is not a failure to bind Mg^{2+}.

Inhibitory Ras proteins retain the ability to bind GTP in vivo. It is clear that to function as dominant inhibitory proteins, these mutants must themselves be defective in signal transduction. The unique nucleotide-binding property of these inhibitory mutants suggests that competition from GDP prevents the proteins from ever binding GTP in vivo. This model implies that the GTP form of these proteins would function normally. An alternative explanation is that these mutations lock Ras in an inactive conformation regardless of whether it binds GDP or GTP. The preferential affinity for GDP would therefore not be the sole cause of the protein's inactivity. It correlates with inhibitory activity because it is a property of the inactive conformation of Ras. As an initial attempt to distinguish between these models, we determined whether inhibitory proteins ever bound GTP in cells. To do this, an A59T substitution was introduced into S17N Ras to generate a site that is autophosphorylated in vitro and in vivo (27). This double mutant displayed preferential affinity for GDP (Fig. 3B) and inhibitory activity (8) comparable to that of 17N p21.

To test whether S17N/A59T p21 was phosphorylated in vivo, NIH 3T3 cells transformed by v-*raf* were transfected with this double mutant *ras* gene in pZIPneoSV(X) and G418-resistant colonies were selected. Since *raf*-transformed cells are resistant to Ras inhibition, they express inhibitory Ras mutants efficiently. Two independent colonies along with control cells with labeled with either [^{35}S]methionine or $^{32}PO_4$, and Ras^H proteins were immunoprecipitated. Both cell lines expressing S17N/A59T Ras (Fig. 4, lanes c and d) demonstrated that the mutant was indeed phosphorylated (Fig. 4, lanes g and h). These results were similar to those obtained for cells expressing v-Ras^H (G12R/A59T), which is known to be phosphorylated (Fig. 4, lanes a and e), and contrast with those for cells overexpressing normal Ras^H, which is known not to be phosphorylated (Fig. 4, lanes b and f). The fact that the inhibitory protein is phosphorylated in vivo argues that it has not totally lost the capacity to bind GTP in vivo.

Inhibitory proteins fail to stimulate downstream targets. The previous results suggested that the inhibitory mutants contained an additional defect, such as the inability to stimulate downstream targets even when bound to GTP. It is not possible to test this model directly because a target molecule in mammalian cells that responds to GTP-bound Ras has yet to be identified. However, mammalian Ras can substitute for yeast RAS in stimulating adenylyl cyclase (14), and that enzyme clearly distinguishes between the active GTP and inactive GDP forms of the mammalian protein (7). Mutations in the effector domain of Ras that inhibit transforming activity of oncogenic Ras also inhibit the ability of the protein to stimulate yeast adenylyl cyclase (24). While it is clear that Ras does not control adenylyl cyclase in mammalian cells, this yeast system appears to provide an accurate reflection of the coupling efficiency to a mammalian effector. We therefore tested whether these mutant proteins were defective in downstream coupling by assaying for yeast cyclase stimulation in vitro.

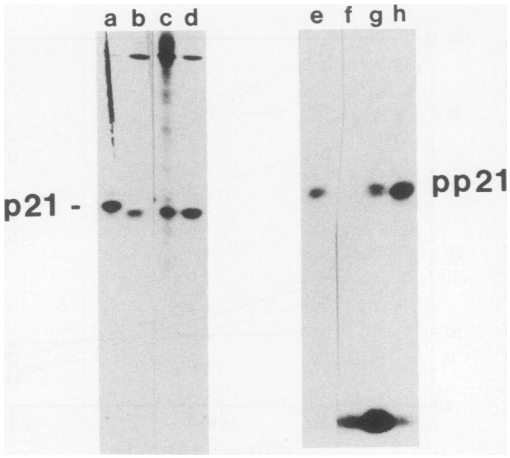

FIG. 4. In vivo phosphorylation of mutant Ras proteins. Cells were labeled for 18 h with either [^{35}S]methionine (lanes a to d) or $^{32}PO_4$ (lanes e to h). Ras proteins were immunoprecipitated with anti-Ras antibody YA6-172. The samples were electrophoresed in sodium dodecyl sulfate-polyacrylamide gels and autoradiographed. Lanes: a and e, cells expressing v-Ras^H; b and f, cells expressing normal Ras; c, d, g, and h, two independent cell lines expressing 17N/59T Ras.

Various Ras proteins were preloaded with either GTP or GDPβS at concentrations that ensured saturation binding even for the mutant proteins that had reduced affinity for GTP. We confirmed that S17N p21 did in fact bind GTP under these conditions despite its preferential affinity for GDP. [α-^{32}P]GTP was included in similar binding reactions, and filter-binding assays showed that radioactivity associated with mutant Ras was comparable to that observed for normal Ras (data not shown). That this radioactivity derived primarily from GTP was documented by eluting the nucleotide from the protein bound to the filter and characterizing it by thin-layer chromatography (data not shown).

The nucleotide-bound proteins were then incubated with membrane preparations from the yeast strain TKB-111, which expresses elevated levels of adenylyl cyclase in the absence of either of the two yeast Ras proteins (11). Yeast adenylyl cyclase was stimulated by only the active GTP-bound form of mammalian Ras, with half-maximal stimulation occurring at ~300 nM p21 (Fig. 5). In contrast, no significant activity was observed with S17N, S17A, or S17C p21 bound to GTP. A similar result was obtained for 17N p21 bound to GTPγS (data not shown). These findings demonstrate that these inhibitory proteins are locked in an inactive conformation even when bound to GTP. Inability to stimulate a downstream target in the GTP-bound state was not unique to Ras proteins mutated at amino acid 17. As stated previously, mutations in the GAP-binding/effector domain between amino acids 30 and 40 also display this phenotype. In fact, T35N p21 discussed earlier is mutated in this domain and also fails to activate yeast adenylate cyclase at the concentrations of protein tested in this study (Fig. 6). Therefore, the dominant inhibitory phenotype is not merely a consequence of the inability of the protein to transmit a downstream signal.

DISCUSSION

The results presented argue that the biochemical defect in 17N Ras responsible for its dominant inhibitory phenotype is improper complexing of Mg^{2+} associated with bound nucleotide. This explanation was first suggested by X-ray crystallography studies of p21 showing that the hydroxyl group of Ser-17 contributed to the binding of Mg^{2+} bound to both GDP and GTP (16, 18, 19). In this study, we showed that only those amino acid substitutions at position 17 that would be expected to disturb this interaction generated dominant inhibitory proteins. One inhibitory protein was made by changing Ser to Cys. This result is consistent with the fact that sulfur is known to bind Mg^{2+} much less efficiently than oxygen. Another inhibitory protein was generated by a Ser-to-Ala change that removed the reactive oxygen. As expected, a Ser-to-Thr substitution that retained the hydroxyl residue did not drastically alter Ras function. This

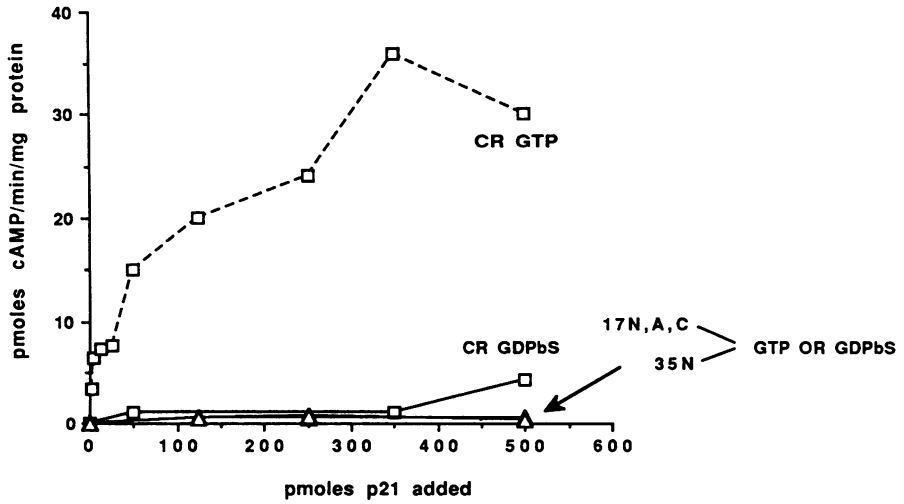

FIG. 5. Stimulation of yeast adenylate cyclase by mammalian Ras. Various amounts of either GTP- or GDPβS-bound Ras proteins were incubated for 20 min at 30°C with 10 μg of membranes from *S. cerevisiae* TKB-111. cAMP produced was assayed as described in Materials and Methods. Maximal stimulation of adenylate cyclase induced by $MnCl_2$ averaged 150 pmol/min/mg. Data represent the averages of at least two experiments, each performed in duplicate. Symbols: □----□, GTP-bound cellular Ras (CR); □——□, GDPβS-bound cellular Ras; △, 17N, 17C, 17A, or 35N Ras bound to either GTP or GDPβS.

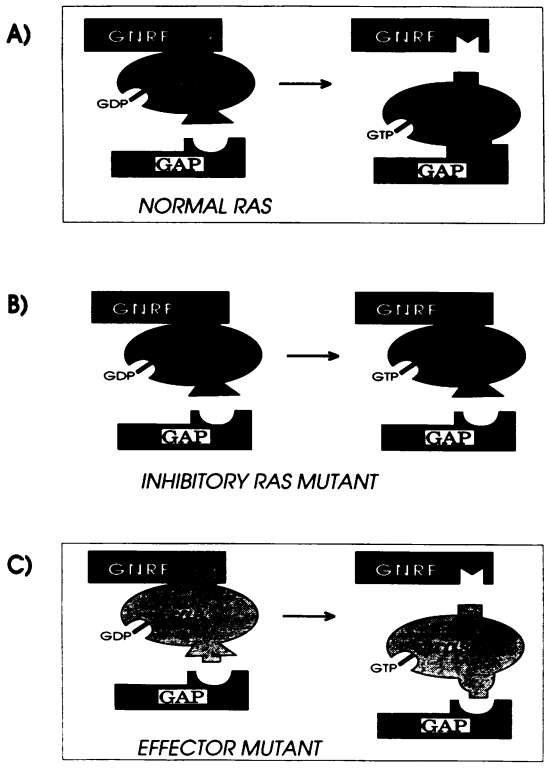

FIG. 6. Model to explain how 17N Ras might interfere with upstream activation of normal Ras by a guanine nucleotide-releasing factor. Normal Ras (A) replaces GTP for bound GDP in response to GNRF. This is associated with a change in conformation of the protein that leads to enhanced interaction with a downstream target of Ras, which may be GAP. It also leads to decreased interaction with GNRF. The data presented here argue that the inhibitory mutants are locked in an inactive conformation even when bound to GTP. This results in an inability to interact productively with a downstream target. Importantly, the protein retains high affinity for GNRF throughout the GTP-GDP cycle. In this way, it suppresses activation of endogenous Ras by competing effectively for GNRF. Effector mutants are not efficient dominant inhibitors because they likely maintain normal interactions with GNRF despite decreased ability to stimulate downstream targets.

result is also consistent with the fact that some GTP-binding proteins have Thr instead of Ser at this position. The ability of Ras with glycine at this site to function seems at first to contradict this interpretation. However, it is possible that the removal of the entire side group generated space for an additional H_2O molecule in the binding pocket which contributed an oxygen for Mg^{2+} complexing.

It is unlikely that the inhibitory mutants have lost the ability to bind Mg^{2+} at this site, since the defect in nucleotide binding by the inhibitory mutants could not be reversed by raising Mg^{2+} concentrations to as much 3 orders of magnitude higher than is necessary for GTPase activity. Moreover, preferential affinity for GDP was not observed for normal Ras when Mg^{2+} was chelated with EDTA. Structural studies of Ras have shown that other amino acids, such as Thr-35 and Asp-57, also interact with the metal, which could account for residual binding activity (16, 18). Asp-57 has also been shown to form a hydrogen bond with the side group of Ser-17. We thus cannot rule out the possibility that interruption of this interaction by these mutations contributes to the inhibitory phenotype. However, since Asp-57 contributes to Mg^{2+} binding, this model would also involve altered Mg^{2+} binding.

A unique feature of the original 17N Ras mutant was its switch in binding specificity from equal affinity for GTP and GDP to preferential affinity for GDP (8). This phenotype is apparently important for dominant inhibitory activity, because analysis of additional mutations at position 17 described here has shown a tight correlation between inhibitory phenotype and preferential affinity for GDP. This property suggests that the mutants do not function properly solely because they cannot bind GTP in vivo due to competition from GDP. However, the experiments described show that the mutant protein did not completely lose the ability to bind GTP in vivo despite its preference for GDP, since the double mutant, 17N/59T Ras, became phosphorylated in vivo. That phosphorylation in vivo was due to autophosphorylation rather than phosphorylation by other proteins in the cell is supported by previous observations showing that Ras proteins that have lost the ability to bind GTP do not become phosphorylated in vivo (5). Thus, the ability to be phosphorylated was most likely dependent on the mutant protein retaining the ability to bind GTP in cells. Second, even when 17N Ras was complexed to GTP or GTPγS, it failed to activate a model downstream target, yeast adenylyl cyclase. Thus, besides displaying altered nucleotide binding, the mutant protein was not activated by GTPs. These findings suggest an alternative model in which improper complexing of Mg^{2+} locks the protein in an inactive state. The preferential affinity for GDP represents a characteristic of this conformational state of Ras.

The mechanism by which 17N Ras interferes with normal Ras in cells is not completely understood. However, evidence suggests that it inhibits upstream activation of Ras by competing for a nucleotide exchange factor. First, 17N Ras does not compete with normal Ras for a downstream target because the inhibitory phenotype of 17N Ras was not reduced by a second-site mutation that blocked downstream Ras function (29). Second, the mutant inhibited normal Ras much more efficiently than oncogenic Ras (29). This finding is relevant because in yeast cells, normal Ras is much more dependent on a putative nucleotide exchange factor, CDC25, than is oncogenic Ras (2). The weak inhibition of oncogenic Ras displayed by 17N Ras (3, 8, 29) most likely reflects the fact that even a GTPase-deficient mutant is dependent on exchange factor function for full activity. This view is consistent with the observation that a GTPase-deficient yeast RAS protein does not fully complement the loss of CDC25 activity (2). In contrast, inhibition of downstream function of Ras in mammalian cells suppresses oncogenic Ras more effectively than normal Ras (7, 29). Third, a neighboring mutation in yeast RAS has been shown to block endogenous RAS, and this was overcome by overexpression of CDC25 (20). Finally, 17N Ras has a higher affinity for a partially purified nucleotide exchange factor than does normal Ras (33).

A model to explain how the biochemical defects described for S17N Ras might lead to efficient competition for nucleotide exchange factor is schematized in Fig. 6. The GDP-bound form of normal Ras is thought to be converted to the GTP form by interaction with GNRF. X-ray crystallography has detected a conformational change associated with Ras activation that leads to enhanced interactions with GAP (Fig. 6A). It is likely, but not yet documented, that activation also decreases the affinity of Ras for GNRF, leading to the dissociation of the two proteins. This is consistent with the observation that a yeast nucleotide-releasing factor, SCD25,

has ~3-fold-higher affinity for GDP-Ras than GTP-Ras (6). For the inhibitory mutant (Fig. 6B), however, we propose that incorrect coordination of Mg^{2+} prevents the protein from switching to the active conformation upon binding GTP. This blocks stimulation of downstream targets, as we have demonstrated in yeast cyclase assays. Another predicted effect, particularly important for the dominant inhibitory phenotype, is that the mutant does not decrease its affinity for nucleotide exchange factor upon binding GTP. This could account for the fact that 17N Ras has ~3-fold-higher affinity than normal Ras for the putative Ras nucleotide exchange factor (33). Moreover, it has been shown by microinjection experiments that the inhibitory phenotype of 17N Ras can be overcome by a ~3-fold excess of normal Ras (7). Thus, by interacting efficiently but nonproductively with GNRF, 17N Ras apparently competes for this regulatory protein and suppresses the formation of the active GTP form of endogenous Ras.

Effector domain mutants may not interfere efficiently with normal Ras function because they likely retain the ability to switch to an active conformation upon binding GTP, at least in the regions that interact with GNRF (Fig. 6C). Thus, even though they cannot stimulate a downstream target efficiently because of a local perturbation in the effector domain, they do not compete as efficiently as 17N Ras for interactions with GNRF. Moreover, most effector domain mutants retain some ability to interact with GAP (7, 24). Therefore, normal Ras function may never by inhibited in vivo because when these mutants are expressed at high enough levels to compete with endogenous Ras for GNRF, they begin to stimulate downstream targets.

The analysis of these mutants illustrate the importance of proper Mg^{2+} binding for normal Ras function. Thr-35 is part of the effector domain that switches conformation upon GTP binding (16, 25). One aspect of this activation event is the binding of Thr-35 to Mg^{2+} (Fig. 1). An important role of Ser-17 may therefore be to ensure correct Mg^{2+} complexing in order to anchor Thr-35 when GTP is bound. This could explain why defective Mg^{2+} complexing by inhibitory mutants results in the mutant protein's inability to activate downstream effector molecules.

The critical role of Ser-17 in Ras function is likely conserved in other low-molecular-weight GTP-binding proteins, since the phosphate-binding loop in which this amino acid resides is highly conserved (23). In fact, Ser or Thr is found at this site in all GTP-binding proteins. This argues that mutations comparable to those described here for Ras would generate dominant inhibitory versions of other small GTP-binding proteins. Such mutants would be valuable tools for deciphering the function of this large family of proteins.

As described in the accompanying paper, we have characterized such a mutation in G_s α that couples hormones to adenylyl cyclase and ion channels (13). It belongs to a different class of GTP-binding proteins that are ~45 kDa and exist as part of a heterotrimer, containing inhibitory β and γ subunits. A Ser-to-Asn change at position 54 of that protein also changed the nucleotide-binding specificity of that protein to preferential affinity for GDP, suggesting it also altered Mg^{2+} binding. Moreover, like S17N Ras, the mutant G_s α protein had abnormal interactions with its nucleotide-releasing factor. However, unlike S17N Ras, S54N G_s α retained the ability to stimulate downstream targets when bound to GTP. This implies that the role of Mg^{2+} in the conversion of these proteins to the active conformation upon GTP binding is different. This may reflect the fact that the domain of Ras that interacts with downstream targets is directly involved in Mg^{2+} binding whereas the comparable domain of G_s α appears to be at the carboxyl terminus of the protein (15, 17).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA 47391 and American Cancer Society grant JFRA-222 to L.A.F.

REFERENCES

1. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1990. The GTPase superfamily: a conserved switch for diverse cell functions. Nature (London) **348**:125–132.
2. **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 Ral/adenylate cyclase pathway. Cell **48**:789–799.
3. **Cai, H., J. Szeberenyi, and G. M. Cooper.** 1990. Effect of a dominant inhibitory Ha-Ras mutation on mitogenic signal transduction in NIH 3T3 cells. Mol. Cell. Biol. **10**:5314–5323.
4. **Cepko, C. L., B. Roberts, and R. C. Mulligan.** 1984. Construction and applications of a highly transmissible murine retrovirus shuttle vector. Cell **37**:1053–1062.
5. **Clanton, D. J., S. Hattori, and T. Y. Shih.** 1986. Mutations of the ras gene product p21 abolish guanine nucleotide binding. Proc. Natl. Acad. Sci. USA **83**:5076–5080.
6. **Crechet, J.-B., P. Poullet, M. Y. Mistou, A. Parmeggiani, J. Camonis, E. Boy-Marcotte, F. Damak, and M. Jacquet.** 1990. Enhancement of the GDP-GTP exchange of Ras proteins by the carboxyl-terminal domain of SCD25. Science **248**:866–868.
7. **Farnsworth, C. L., M. S. Marshal, J. B. Gibbs, D. W. Stacey, and L. A. Feig.** 1991. Preferential inhibition of the oncogenic form of RasH by mutations in the GAP binding/"effector" domain. Cell **64**:625–633.
8. **Feig, L. A., and G. M. Cooper.** 1988. Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. Mol. Cell. Biol. **8**:3235–3243.
9. **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.
10. **Feig, L. A., B. T. Pan, T. M. Roberts, and G. M. Cooper.** 1986. Isolation of ras GTP-binding mutants using an in situ colony-binding assay. Proc. Natl. Acad. Sci. USA **83**:4607–4611.
11. **Field, F., D. Broek, T. Kataoka, and M. Wigler.** 1987. Guanine nucleotide activation of, and competition between, RAS proteins from *Saccaromyces cerevisiae*. Mol. Cell. Biol. **7**:2128–2133.
12. **Hagag, N., S. Halegoua, and M. Viola.** 1986. Inhibition of growth factor-induced differentiation of PC12 cells by microinjection of antibody to ras p21. Nature (London) **319**:680–682.
13. **Hildebrandt, J. D., R. Day, C. L. Farnsworth, and L. A. Feig.** 1991. A mutation in the putative Mg^{2+}-binding site of G_s α prevents its activation by receptors. Mol. Cell. Biol. **11**:4830–4838.
14. **Kataoka, T., S. Powers, S. Cameron, O. Fasano, M. Goldfarb, J. Broach, and M. Wigler.** 1985. Functional homology of mammalian and yeast RAS genes. Cell **40**:19–26.
15. **Masters, S. B., K. A. Sullivan, R. T. Miller, B. Beiderman, N. G. Lopez, J. Ramachandran, and H. R. Bourne.** 1988. Carboxyl terminal domains of Gsα specifies coupling of receptors to stimulation of adenylyl cyclase. Science **241**:939–945.
16. **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.
17. **Osawa, S. N., N. Dhanasekaran, C. W. Woon, and G. L. Johnson.** 1990. $G\alpha_i$-$G\alpha_s$ chimera define the function of α chain domains in control of G protein activation and βγ subunit interactions. Cell **63**:697–706.
18. **Pai, E. F., U. Krengel, G. A. Petsko, R. S. Goody, W. Kabsch, and A. Wittinghofer.** 1990. Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 Å resolution: implications for the mechanism of GTP hydrolysis. EMBO J.

9:2351–2359.
19. **Pai, F. E., W. Kabsch, U. Krengel, K. C. Holmes, J. John, and A. Wittinghofer.** 1989. Structure of the guanine-nucleotide-binding domain of the Ha-ras oncogene product p21 in the triphosphate conformation. Nature (London) **341**:209–214.
20. **Powers, S., K. O'Neill, and M. Wigler.** 1989. Dominant yeast and mammalian Ras mutants that interfere with the CDC25-dependent activation of wild-type RAS in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9**:390–395.
21. **Rapp, U. R., M. D. Goldborough, G. E. Mark, T. I. Bonna, J. Groffen, F. H. Reynolds, and J. R. Stephenson.** 1983. Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. Proc. Natl. Acad. Sci. USA **80**:4218–4222.
22. **Salomon, Y.** 1979. Adenylyl cyclase assay. Adv. Cyclic Nucleotide Res. **10**:35–54.
23. **Saraste, M., P. R. Sibbald, and A. Wittinghofer.** 1990. The P-loop—a common motif in ATP- and GTP-binding proteins. Trends Biochem. Sci. **15**:430–434.
24. **Schaber, M. D., V. M. Garsky, D. B. Boylon, W. S. Hill, E. M. Scolnick, M. S. Marshall, I. S. Sigal, and J. B. Gibbs.** 1989. Ras interactions with the GTPase-activating protein (GAP). Proteins Struct. Funct. Genet. **6**:306–315.
25. **Schlichting, 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.** 1980. Time-resolved X-ray crystallographic study of the conformational change in Ha-Ras p21 protein on GTP hydrolysis. Nature (London) **345**:309–316.
26. **Shalloway, D., A. D. Zelenetz, and G. M. Cooper.** 1980. Molecular cloning and characterization of the chicken gene homologous to the transforming gene of Rous sarcoma virus. Cell **24**:531–541.
27. **Shih, T. Y., A. G. Papageorge, P. E. Stokes, M. O. Weeks, and E. M. Scolnick.** 1980. Guanine nucleotide-binding and autophosphorylating activities associated with the p21src protein of Harvey murine sarcoma virus. Nature (London) **287**:686–691.
28. **Smith, M. R., S. J. DeGudicibus, and D. W. Stacey.** 1986. Requirement for c-ras proteins during viral oncogene transformation. Nature **320**:540–543.
29. **Stacey, D. W., L. A. Feig, and J. B. Gibbs.** 1991. Dominant inhibitory Ras mutants selectively inhibit the activity of either cellular or oncogenic Ras. Mol. Cell. Biol. **11**:4053–4064.
30. **Szeberenyi, J., H. Cai, and G. M. Cooper.** 1990. Effect of a dominant inhibitory Ha-*ras* mutation on neuronal differentiation of PC12 cells. Mol. Cell. Biol. **10**:5324–5332.
31. **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.
32. **West, M., H. F. Kung, and T. Kamata.** 1990. A novel membrane factor stimulates guanine nucleotide exchange reaction of ras proteins. FEB Lett. **259**:245–248.
33. **Wolfman, A., and L. A. Feig.** Unpublished data.
34. **Wolfman, A., and I. G. Macara.** 1990. A cytosolic protein catalyzes the release of GDP from p21. Science **248**:247–249.