

ras^H Mutants Deficient in GTP Binding

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Received 17 March 1986/Accepted 22 May 1986

Single amino acid substitutions were introduced into a region of the *ras*^H protein (residues 116, 117, and 119) homologous to a variety of diverse GTP-binding proteins. Each of the mutant p21 proteins displayed a significant reduction (10- to 5,000-fold) in GTP binding affinity. Activated *ras*^H proteins deficient in GTP binding were unaltered in their ability to morphologically transform NIH 3T3 cells.

The human *ras* family consists of three known genes (*ras*^H, *ras*^K, and *ras*^N) which have been identified as active transforming genes in a variety of human neoplasms (5). The cellular *ras* genes encode closely related proteins designated p21s. These plasma-membrane-associated proteins bind guanine nucleotides with high affinity (9, 26, 32) and display a low GTPase activity (11, 19, 22, 34). Amino acid sequence comparisons have identified homologies between the *ras* proteins and other proteins which exhibit a high specific affinity for guanine nucleotides, including bacterial elongation and initiation factors, tubulin, and members of the G protein family (16, 18). Of these regions of shared sequence homology, the region represented by *ras* amino acids 110 through 120 is the most striking. We therefore used site-directed mutagenesis to evaluate the possible role of these *ras* sequences in guanine nucleotide binding.

A comparison of amino acid sequences representing a variety of guanine-nucleotide-binding proteins is shown in Table 1. A consensus sequence of Asn-Lys-X-Asp (human *ras* residues 116 through 119) was found in all GTP-binding proteins examined. To directly evaluate the involvement of these amino acids in the binding of GTP, oligonucleotide-directed mutagenesis (37, 38) was used to introduce single amino acid substitutions into a cDNA clone of a human *ras*^H gene whose transforming potential was activated by substitution of leucine for glutamine at position 61 (*ras*^H [61-Leu]; 8). Seventeen-base synthetic oligonucleotides, synthesized by the modified triester method, were used to introduce single nucleotide substitutions into a M13mp8 clone containing the *ras*^H cDNA sequence. The following three mutations were isolated: *ras*^H(61-Leu, 116-His), in which codon 116 was changed from AAC (Asn) to CAC (His); *ras*^H(61-Leu, 117-Glu), in which codon 117 was changed from AAG (Lys) to GAG (Glu); and *ras*^H(61-Leu, 119-His), in which codon 119 was changed from GAC (Asp) to CAC (His). The nucleotide sequences of the mutant clones were verified by dideoxy sequencing (31).

To evaluate the guanine-nucleotide-binding properties of the mutated *ras*^H proteins, each *ras*^H mutation was constructed into a bacterial expression vector pXVR (8a), which directs synthesis of authentic mammalian p21, and then introduced into *Escherichia coli* PR13-Q. Upon induction with isopropylthio- β -D-galactoside, approximately 30% of the total bacterial protein was represented by p21. Bacterium-expressed p21s were purified as previously described

(8a, 19), with the resulting p21 preparations suspended in 3.5 M guanidine hydrochloride (>80% homogeneous, as determined by Coomassie blue staining on sodium dodecyl sulfate-polyacrylamide gels). Purified preparations of *ras*^H(61-Leu) and the three mutant protein were incubated with [α -³²P]GTP (>600 Ci/mmol; New England Nuclear Corp.) at concentrations from 1×10^{-9} to 5×10^{-5} M at 30°C for 60 min. The amount of bound nucleotide was determined by a nitrocellulose filter binding assay and quantitated by liquid scintillation counting (Fig. 1) (8a, 20). In agreement with previous studies (8, 9, 20, 32), the concentration for half-maximal GTP binding of the *ras*^H(61-Leu) p21 was approximately 10^{-8} M. In contrast, all three *ras*^H mutant proteins possessed significantly reduced affinities for GTP. The GTP concentration for half-maximal binding was 10^{-7} M to *ras*^H(61-Leu, 116-His) p21 and 10^{-6} M to *ras*^H(61-Leu, 119-His) p21. Compared with equivalent amounts of the *ras*^H(61-Leu) p21, GTP binding to *ras*^H(61-Leu, 117-Glu) p21 was half-maximal at approximately 5×10^{-5} M. Excess ATP (10^{-3} M) did not compete for binding of [³²P]GTP to either *ras*^H(61-Leu) or any of the p21 mutants (data not shown). Thus, each amino acid substitution resulted in a 10- to 5,000-fold reduction in binding affinity for GTP without altering the specificity of p21 for guanine versus adenine nucleotides.

To determine the effects of these alterations in GTP binding on transforming activity, each *ras*^H(61-Leu) mutation was introduced into the *Bam*HI site of the retrovirus shuttle vector pZIP-NeoSV(x)1 (3). The introduction of the *ras*^H cDNA sequences into this retroviral plasmid resulted in the efficient expression of *ras*^H by using the 5' long terminal repeat as the promoter and the 3' long terminal repeat as the signal for polyadenylation (8a). Each pZIP-*ras*^H(61-Leu) mutant induced transformation of NIH 3T3 cells (6, 14) with efficiencies that were equivalent to that of the wild-type *ras*^H(61-Leu) retroviral construct (>50 foci per ng of DNA). Thus, the reduced binding affinities for GTP did not alter the focus-forming activity of the *ras*^H(61-Leu)-encoded mutant proteins.

Because long-terminal-repeat promoter constructs of the normal *ras*^H(61-Gln) gene were shown to transform NIH 3T3 cell via overexpression (4), the comparison of focus-forming activities of the different GTP-binding deficient mutants that used these retroviral *ras*^H constructs may possibly have masked partial reductions of *ras*^H transforming potential. To rule out the possibility that elevated expression was necessary for the transforming activity of the *ras*^H genes, the focus-forming efficiencies of these mutants were also compared by using M13-*ras*^H constructs which contain a 2.9-

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TABLE 1. Amino acid sequence homology among GTP-binding proteins

Protein	Sequence ^a	Reference
Human <i>ras</i> ^{H,K,N}	115 Gly Asn Lys Cys Asp Leu 120	1, 2, 35
Yeast <i>RAS1</i>	122 Gly Asn Lys Ser Asp Leu 127	27
Yeast <i>RAS2</i>	122 Gly Asn Lys Ser Asp Leu 127	27
<i>Drosophila Dras1</i>	115 Gly Asn Lys Cys Asp Leu 120	24
<i>Drosophila Dras2</i>	112 Gly Asn Lys Cys Asp Leu 117	24
<i>Dictyostelium Dd-ras</i>	115 Gly Asn Lys Ala Asp Leu 120	29
Bovine α -transducin	264 Leu Asn Lys Ile Asp Lys 269	23, 36
Bacterial EF-Tu	134 Leu Asn Lys Cys Asp Met 139	15
Bacterial EF-G	140 Val Asn Lys Met Asp Arg 145	25
Bacterial IF2	497 Val Asn Lys Ile Asp Lys 502	30

^a Numbers refer to amino acid sequences. Amino acid identities are boxed.

kilobase *SacI* fragment of genomic human *ras*^H and lack the flanking sequences required for efficient expression in transfected cells (28). Two of the mutations (116-His and 119-His) were introduced into a genomic M13-*ras*^H(61-Leu) clone (8) by site-directed mutagenesis, and the resulting double-stranded M13-*ras*^H mutant DNA was used for transfection analysis. In agreement with results obtained with the pZIP-*ras*^H constructs, both M13-*ras*^H(61-Leu) mutants (116-His and 119-His) displayed focus-forming activities that were indistinguishable from that of M13-*ras*^H(61-Leu) (50 foci per μ g of DNA). Immunoprecipitation analysis (7, 10) indicated that NIH cells transformed by M13-*ras*^H(61-Leu) expressed approximately fivefold lower levels of p21 than did NIH cells transformed by pZIP-*ras*^H(61-Leu) (data not shown). This level of p21 was equivalent to the level of p21 expressed by untransformed NIH cells, and comparable levels of p21 were detected in NIH cells transformed by the two GTP-binding mutants. Thus, induction of morphological transformation by the GTP mutants did not require elevated expression of p21.

The results presented here indicate that the domain of p21, including amino acids 116, 117, and 119, is involved in the ability of *ras* proteins to bind guanine nucleotides. X-ray crystallographic analysis of the GDP binding site of the bacterial elongation factor, EF-Tu, identified a region (residues 135 through 138) that directly interacts with the bound nucleotide, and it was proposed that the homologous region

in the *ras*^H protein is represented by *ras*^H residues 116 through 119 (17, 21). In particular, it was suggested that the side chains of Asn-116 and Asp-119 interact directly with the guanine ring of the bound nucleotide (17, 21). The results presented here are consistent with these predictions and additionally suggest that Lys-117 is important for guanine nucleotide binding.

In addition to the *ras*^H amino acid sequence 110 through 120, sequence alignment comparisons identified three other principal regions of significant homology between *ras* and other GTP-binding proteins, including transducin and the bacterial elongation (EF-Tu and EF-G) and initiation (IF2) factors (15, 23, 36). These three regions of homology are represented by the cellular *ras* amino acid residues (i) 5 to 15, (ii) 55 to 65, and (iii) 72 to 85, and they appear in the same order in α -transducin and the bacterial elongation factors EF-Tu and EF-G. It was demonstrated that substitutions that activate *ras* transforming potential at positions 12 and 61 do not affect GTP binding affinity or specificity (8, 9) but do result in reduced GTP hydrolysis activities (8, 19, 22). In a recent study (8a) that involved random mutagenesis, amino acid substitutions at positions 83 (Ala to Thr), 119 (Asp to Asn), and 144 (Thr to Ile) of viral *ras*^H p21 were found to result in 25- to 100-fold reductions in guanine-nucleotide-binding affinities. This identification of a binding mutation at position 83 suggests that region iii is also involved in GTP binding. Although Thr-144 does not fall into any of the four

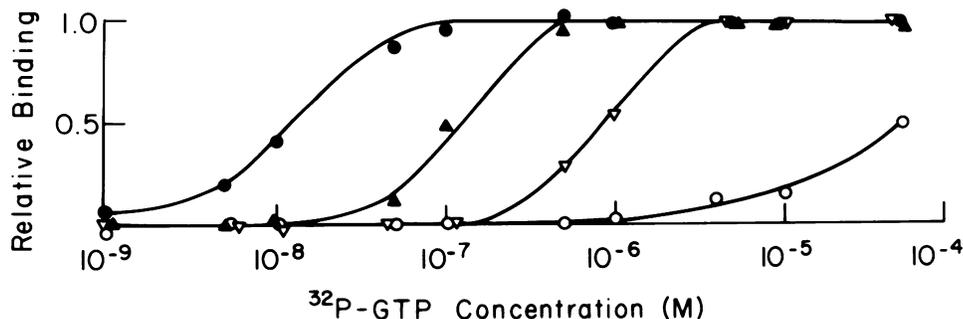


FIG. 1. Equilibrium binding of [³²P]GTP by purified p21 proteins. Between 50 and 500 ng of p21 was suspended in 50 μ l of assay buffer (20 mM Tris hydrochloride [pH 7.4], 0.35 M guanidine hydrochloride, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 40 μ g of bovine serum albumin per ml) and incubated with various amounts of [³²P]GTP. Shown in this figure is a representative result of multiple determinations, with the amount of bound nucleotide normalized to the amount of binding at maximal concentration of GTP. Symbols: ●, *ras*^H(61-Leu); ▲, *ras*^H(61-Leu, 116-His); ○, *ras*^H(61-Leu, 117-Glu); ▽, *ras*^H(61-Leu, 119-His).

regions of homology, X-ray crystallographic analysis does suggest that this region in EF-Tu is also part of the GTP-binding site (17). Sigal et al. (33) also recently reported that mutations at positions 16 and 119 reduce GTP binding. Thus, consistent with proposed models for the GTP-binding domain of *ras* proteins, these observations suggest that multiple regions of p21 are associated with the ability to bind guanine nucleotides.

The transforming activity of *ras*^H(61-Leu) was not compromised by as much as a 5,000-fold decreased GTP binding affinity. Since intracellular guanine nucleotide concentrations are in the millimolar range (13), one explanation of this result is that these mutated proteins may still be capable of binding GTP *in vivo*. Consistent with this possibility is the recent characterization of a viral *ras*^H GTP-binding mutant (119-Asn) (8a). Similar to the mutants described in this report, this viral *ras*^H mutant is reduced 100-fold in GTP-binding affinity yet displays a focus-forming activity unchanged from the wild-type *v-ras*^H gene. Immunoprecipitation analysis of p21 in NIH cells transformed by this viral mutant demonstrated that the *v-ras*^H(119-Asp)-encoded p21 was phosphorylated to the same degree as its nonmutated counterpart, suggesting significant *in vivo* binding of the phosphate donor, GTP (8a). Alternatively, *ras* transforming activity may not require the binding of GTP.

The structural and biochemical similarities between *ras* and the G proteins suggest that these proteins also share functional similarities as regulatory molecules (12). It is proposed that the binding and hydrolysis of GTP is involved in the regulation of the active and inactive states of these proteins. Although the observed reductions in guanine nucleotide binding affinities in the mutants studied here did not compromise *ras* transforming activity, high-affinity binding of guanine nucleotides may, as discussed elsewhere (8a), be required for normal function of the *ras* protein. The further biological characterization of these mutants will therefore be useful for defining the role of GTP binding in *ras* activity.

ACKNOWLEDGMENTS

We are grateful to M. Corbley and T. Roberts for the *ras*^H cDNA clone and to L. Feig for helpful discussions.

This research was supported by Public Health Service grants from the National Cancer Institute, by a fellowship to C.D. from the National Cancer Institute, and by a faculty research award to G.M.C. from the American Cancer Society.

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