MOLECULAR AND CELLULAR BIOLOGY, Sept. 1986, p. 3291–3294
0270-7306/86/093291-04$02.00/0

Vol. 6, No. 9

ras^H Mutants Deficient in GTP Binding

CHANNING J. DER,† BIN-TAO PAN, AND GEOFFREY M. COOPER*

Laboratory of Molecular Carcinogenesis, Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

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-^{32}P]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 [^{32}P]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-

* Corresponding author.
† Present address: La Jolla Cancer Research Foundation, La Jolla, CA 92037.

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

Protein		Sequence[a]							Reference
Human *ras*^{H,K,N}	115	Gly	Asn	Lys	Cys	Asp	Leu	120	1, 2, 35
Yeast *RAS1*	122	Gly	Asn	Lys	Ser	Asp	Leu	127	27
Yeast *RAS2*	122	Gly	Asn	Lys	Ser	Asp	Leu	127	27
Drosophila Dras1	115	Gly	Asn	Lys	Cys	Asp	Leu	120	24
Drosophila Dras2	112	Gly	Asn	Lys	Cys	Asp	Leu	117	24
Dictyostelium Dd-*ras*	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 *Sac*I 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 μg 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

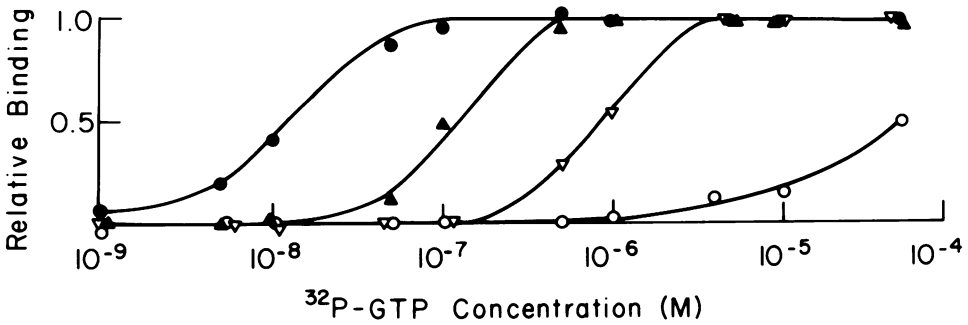

FIG. 1. Equilibrium binding of [^{32}P]GTP by purified p21 proteins. Between 50 and 500 ng of p21 was suspended in 50 μl of assay buffer (20 mM Tris hydrochloride [pH 7.4], 0.35 M guanidine hydrochloride, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 40 μg of bovine serum albumin per ml) and incubated with various amounts of [α-^{32}P]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.

LITERATURE CITED

1. **Capon, D. J., E. Y. Chen, A. D. Levinson, P. H. Seeburg, and D. V. Goeddel.** 1983. Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. Nature (London) **302**:33–37.
2. **Capon, D. J., P. H. Seeburg, J. P. McGrath, J. S. Hayflick, U. Edman, A. D. Levinson, and D. V. Goeddel.** 1983. Activation of Ki-*ras* 2 gene in human colon and lung carcinomas by two different point mutations. Nature (London) **304**:507–513.
3. **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.
4. **Chang, E. H., M. E. Furth, E. M. Scolnick, and D. R. Lowy.** 1982. Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. Nature (London) **297**:479–483.
5. **Cooper, G. M., and M. A. Lane.** 1984. Cellular transforming genes and oncogenesis. Biochim. Biophys. Acta **738**:9–20.
6. **Copeland, N. G., and G. M. Cooper.** 1979. Transfection by exogenous and endogenous murine retrovirus DNAs. Cell **16**:347–356.
7. **Der, C. J., and G. M. Cooper.** 1983. Altered gene products are associated with activation of cellular ras^K-genes in human lung and colon carcinomas. Cell **32**:201–208.
8. **Der, C., T. Finkel, and G. M. Cooper.** 1986. Biological and biochemical properties of human ras^H genes mutated at codon 61. Cell **44**:167–176.

8a. **Feig, L. A., B. T. Tan, T. M. Roberts, and G. M. Cooper.** 1986. Isolation of *ras* GTP-binding mutants using an *in situ* colony bind assay. Proc. Natl. Acad. Sci. USA **83**:4607–4611.

9. **Finkel, T., C. J. Der, and G. M. Cooper.** 1984. Activation of *ras* genes in human tumors does not affect subcellular localization, post-translational modification or guanine nucleotide binding properties of p21. Cell **37**:151–158.
10. **Furth, M. E., L. J. Davis, B. Fleurdelys, and E. M. Scolnick.** 1982. Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular *ras* gene family. J. Virol. **43**:294–304.
11. **Gibbs, J. B., R. W. Ellis, and E. M. Scolnick.** 1984. Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. Proc. Natl. Acad. Sci. USA **81**:5704–5708.
12. **Gilman, A. G.** 1984. G proteins and dual control of adenylate cyclase. Cell **36**:577–579.
13. **Goodrich, G. A., and H. R. Burrell.** 1982. Micromeasurement of nucleotide 5′-triphosphates using coupled bioluminescence. Anal. Bioch. **127**:395–401.
14. **Graham, F. L., and A. J. Van der Eb.** 1973. Transformation of rat cells by DNA of human adenovirus 5. Virology **54**:536–539.
15. **Halliday, K.** 1984. Regional homology in GTP-binding proto-oncogene products and elongation factors. J. Cyclic Nucleotide Res. **9**:431–448.
16. **Hurley, J. B., M. I. Simon, D. B. Teplow, J. D. Robishaw, and A. G. Gilman.** 1984. Homologies between signal transducing G proteins and *ras* gene products. Science **226**:860–862.
17. **Jurnak, F.** 1985. Structure of the GDP domain of EF-Tu and location of the amino acids homologous to *ras* oncogene proteins. Science **230**:32–36.
18. **Leberman, R., and U. Egner.** 1984. Homologies in the primary structure of GTP-binding proteins: the nucleotide-binding site of EF-Tu and p21. EMBO J. **3**:339–341.
19. **Manne, V., E. Bekesi, and H. Kung.** 1985. Ha-*ras* proteins exhibit GTPase activity: point mutations that activate Ha-*ras* gene products result in decreased GTPase activity. Proc. Natl. Acad. Sci. USA **82**:376–380.
20. **Manne, V., S. Yamazaki, and H. Kung.** 1984. Guanosine nucleotide binding by highly purified Ha-*ras*-encoded p21 protein produced in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81**:6953–6957.
21. **McCormick, F., B. F. Clark, T. F. M. la Cour, M. Kjeldgoard, L. Norskov-Lauritsen, and J. Nyborg.** 1985. A model for the tertiary structure of p21, the product of the *ras* oncogene. Science **230**:78–82.
22. **McGrath, J. P., D. J. Capon, D. V. Goeddel, and A. D. Levinson.** 1984. Comparative biochemical properties of normal and activated human *ras* p21 protein. Nature (London) **310**:644–649.
23. **Medynski, D. C., K. Sullivan, D. Smith, C. Van Dop, F. Chang, B. Fung, P. H. Seeburg, and H. R. Bourne.** 1985. Amino acid sequence of the alpha subunit of transducin deduced from the cDNA sequence. Proc. Natl. Acad. Sci. USA **82**:4311–4315.
24. **Neuman-Silberberg, F. S., E. Schejter, F. M. Hoffmann, and B. Z. Shilo.** 1984. The Drosophila *ras* oncogenes: structure and nucleotide sequence. Cell **37**:1027–1033.
25. **Ovchinnikov, Y. A., Y. B. Alakhov, Y. P. Bundulis, M. A. Bundule, N. V. Dougas, V. P. Kozlov, L. P. Motuz, and L. M. Vinokurov.** 1982. The primary structure of elongation factor G from *Escherichia coli*. FEBS Lett. **139**:130–135.
26. **Papageorge, A., D. Lowy, and E. M. Scolnick.** 1982. Comparative biochemical properties of p21 *ras* molecules coded for by viral and cellular *ras* genes. J. Virol. **44**:509–519.
27. **Powers, S., T. Kataoka, O. Fasano, M. Goldfarb, J. Strathern, J. Breach, and M. Wigler.** 1984. Genes in *S. cerevisiae* encoding proteins with domains homologous to the mammalian *ras* proteins. Cell **36**:607–612.
28. **Puga, A., J. Gomez-Marquez, P. R. Brayton, E. M. Cantin,**

L. K. Long, M. Barbacid, and A. L. Notkins. 1985. The immediate-early enhancer element of herpes simplex virus type 1 can replace a regulatory region of the c-Ha-*ras*1 oncogene required for transformation. J. Virol. **54**:879–881.
29. **Reymond, C. D., R. H. Gomer, M. C. Mehdy, and R. A. Firtel.** 1984. Developmental regulation of a Dictyostelium gene encoding a protein homologous to mammalian *ras* protein. Cell **39**:141–149.
30. **Sacerdot, C., P. Dessen, J. W. B. Hershey, J. A. Plumbridge, and M. Grunberg-Manago.** 1984. Sequence of the initiation factor IF2 gene: unusual protein features and homologies with elongation factors. Proc. Natl. Acad. Sci. USA **81**:7787–7891.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
32. **Scolnick, E. M., A. G. Papageorge, and T. Y. Shih.** 1979. Guanine nucleotide-binding activity as an assay for *src* protein of rat-derived murine sarocma viruses. Proc. Natl. Acad. Sci. USA **76**:5355–5359.
33. **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.
34. **Sweet, R. W., S. Yokoyama, T. Kamata, J. R. Feramisco, M. Rosenberg, and M. Gross.** 1984. The product of *ras* is a GTPase and the T24 oncogenic mutant is deficient in this activity. Nature (London) **311**:273–275.
35. **Taparowsky, E., K. Shimizu, M. Goldfarb, and M. Wigler.** 1983. Structure and activation of the human N-*ras* gene. Cell **34**:581–586.
36. **Yatsunami, K., and G. Khorana.** 1985. GTPase of bovine rod outer segments: the amino acid sequence of the alpha subunit as derived from the cDNA sequence. Proc. Natl. Acad. Sci. USA **82**:4316–4320.
37. **Zoller, M. J., and M. Smith.** 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. **100**:468–500.
38. **Zoller, M. J., and M. Smith.** 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA **3**:479–488.