*Proc. Natl. Acad. Sci. USA*
Vol. 87, pp. 3562–3565, May 1990
Biochemistry

# Hydrolysis of GTP by p21$^{NRAS}$, the *NRAS* protooncogene product, is accompanied by a conformational change in the wild-type protein: Use of a single fluorescent probe at the catalytic site

SUSAN E. NEAL, JOHN F. ECCLESTON, AND MARTIN R. WEBB*

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

*Communicated by Mildred Cohn, February 20, 1990*

**ABSTRACT** **2′(3′)-*O*-(*N*-Methyl)anthraniloylguanosine 5′-triphosphate (mantGTP) is a fluorescent analogue of GTP that has similar properties to the physiological substrate in terms of its binding constant and the kinetics of its interactions with p21$^{NRAS}$, the *NRAS* protooncogene product. There is a 3-fold increase in fluorescence intensity when mantGTP binds to p21$^{NRAS}$. The rate constant for the cleavage of mantGTP complexed with the protein is similar to that of GTP, and cleavage is accompanied by a fluorescence intensity change in the wild-type protein complex. A two-phase fluorescence change also occurs when the nonhydrolyzable analogue 2′(3′)-*O*-(*N*-methyl)anthraniloylguanosine 5′-[β,γ-imido]triphosphate (mantp[NH]ppG) binds to wild-type p21$^{NRAS}$. The second phase occurs at the same rate as the second phase observed after mantGTP binding. Thus this second phase is probably a conformation change of the p21$^{NRAS}$·nucleoside triphosphate complex and that the change controls the rate of GTP hydrolysis on the protein. With a transforming mutant, [Asp$^{12}$]-p21$^{NRAS}$, there is no second phase of the fluorescence change after mantGTP or mantp[NH]ppG binding, even though mantGTP is hydrolyzed. This shows that an equivalent conformational change does not occur and thus the mutant may stay in a "GTP-like" conformation throughout the GTPase cycle. These results are discussed in terms of the proposed role of p21$^{NRAS}$ in signal transduction and the transforming properties of the mutant.**

p21$^{ras}$ is a $M_r$ 21,000 protein, whose function is not well understood but is likely to have a role in signal transduction associated with cell growth and a mechanism analogous to guanine nucleotide-binding regulatory proteins (for review, see refs. 1 and 2). Three RAS genes are present in the human genome, and this paper uses protein (p21$^{NRAS}$) expressed from *NRAS* in *Escherichia coli*.

Certain single-point mutants are found in many types of cancer cell and such mutants can cause cell transformation, although the mechanism is not understood (3). By analogy with guanine nucleotide-binding regulatory proteins and other guanine nucleotide binding proteins, such as elongation factor Tu, the activation of the protein is thought to be associated with GTP binding. The protein in this state can activate an effector molecule in the signal cascade and hydrolysis to GDP is associated with deactivation. Single-point mutations of p21$^{ras}$ may interfere with this mechanism. The GTPase-activating protein (GAP) that activates GTP hydrolysis by the wild-type protein but not the oncogenic mutants may well be part of the effector system (4–6).

We have defined (7) a minimal GTP hydrolysis mechanism for p21$^{NRAS}$ (Scheme I, where R represents p21$^{NRAS}$) and obtained the rate constants for nucleotide binding, hydrolysis, and release for the wild-type protein ([Gly$^{12}$]p21$^{NRAS}$ and

$$R + GTP \underset{1}{\rightleftharpoons} R\cdot GTP \underset{2}{\rightleftharpoons} R\cdot GDP\cdot P_i \underset{3\ P_i}{\rightleftharpoons} R\cdot GDP \underset{4}{\rightleftharpoons} R + GDP$$

Scheme I

one mutant [Asp$^{12}$]p21$^{NRAS}$) (7). Each step in Scheme I is numbered so that the forward and reverse rate constants for step $i$ are $k_{+i}$ and $k_{-i}$, respectively. These measurements used radiolabeled GTP and single turnover conditions with the HPLC-purified apoprotein. This minimal GTPase scheme includes only intermediates with different chemical states. Hydrolysis step 2 probably occurs by an in-line displacement of GDP by a water oxygen with no phosphoenzyme intermediate (8). Although the kinetic and mechanistic measurements gave no evidence for steps other than those in Scheme I, it is likely that conformation changes impart biologically active and inactive states on the protein–nucleotide complexes.

It is the purpose of this paper to investigate whether kinetically distinguishable conformation changes occur within the GTPase mechanism, using a fluorescent analogue of GTP, 2′(3′)-*O*-(*N*-methyl)anthraniloylguanosine 5′-triphosphate (mantGTP) (9) (Fig. 1) and its nonhydrolyzable analogue, 2′(3′)-*O*-(*N*-methyl)anthraniloylguanosine 5′-[β,γ-imido]triphosphate (mantp[NH]ppG). Fluorescence methodologies coupled with the use of nonhydrolyzable nucleotides provide sensitive probes for detecting conformation changes in protein–nucleotide complexes. Modifications of the ribose moiety of guanine nucleotides have little effect on the binding affinity to p21$^{NRAS}$, unlike modifications of the purine base (10). A preliminary report of part of the work in this report has been presented (11).

## MATERIALS AND METHODS

p21$^{NRAS}$ (wild-type [Gly$^{12}$]p21$^{NRAS}$ or mutant [Asp$^{12}$]-p21$^{NRAS}$) was isolated as a complex with GDP from overproducing strains of *E. coli* (12) and further purified as an apoprotein by hydrophobic interaction HPLC on a Spherogel TSK-phenyl-5PW column (75 × 7.5 mm, Beckman) (7, 13). The protein was used immediately. p21$^{NRAS}$ activity was measured by its ability to bind [$^3$H]GDP by using a filter binding assay (12).

MantGTP, mantGDP, and mantp[NH]ppG were synthesized by reaction of the parent nucleotide with *N*-methylisatoic anhydride (Molecular Probes) (9) but purified on DEAE-cellulose using a gradient of triethylammonium bicarbonate. Nucleotides were analyzed by anion-exchange HPLC on a Partisil-10 SAX column (250 × 4.6 mm, Whatman) with 0.6 M $(NH_4)H_2PO_4$, adjusted to pH 4.0 with

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: mantGTP, mantGDP, etc., 2′(3′)-*O*-(*N*-methyl)anthraniloylguanosine 5′-triphosphate, 2′(3′)-*O*-(*N*-methyl)anthraniloylguanosine 5′-diphosphate, etc.; p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate.

*To whom reprint requests should be addressed.

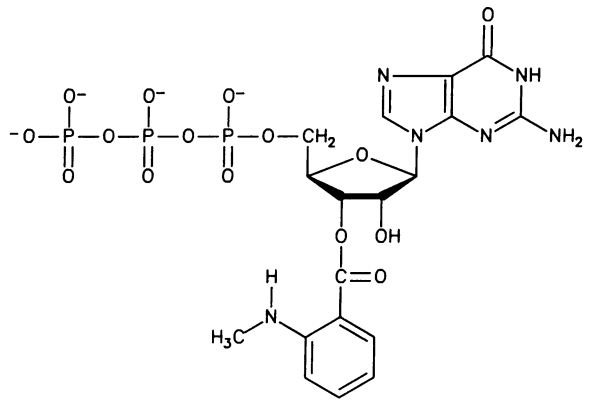

FIG. 1. Structure of mantGTP. $^1$H NMR experiments show that this type of molecule exists as 2'- and 3'-isomers in a ratio of 40:60, in slow exchange (<1 $s^{-1}$) at pH 7.0 and 22°C (J.F.E., unpublished data).

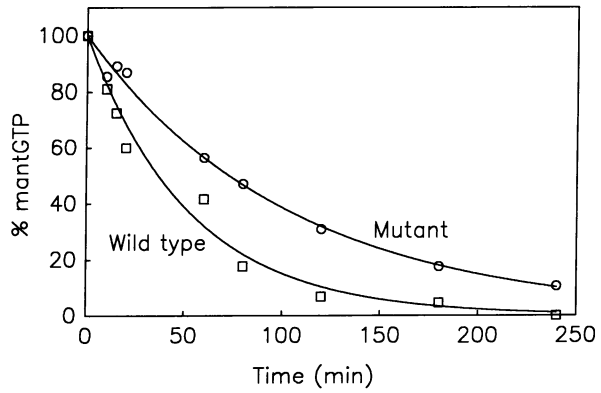


FIG. 2. Time course for cleavage of mantGTP complexed with wild-type (□) and mutant (○) proteins. mantGTP·protein complex (5 μM) was incubated at 37°C. Aliquots were taken at each time point and the percent mantGTP hydrolysis was measured by HPLC. Solid lines are the best-fit single-exponential curves.

HCl/15% (vol/vol) methanol at 2 ml/min. This showed that the nucleotide analogues contained <1% of the parent nucleotide. To determine purity further and to obtain information about the location of the ester linkage (14), $^1$H NMR spectra were obtained on a Bruker AM500 spectrometer.

The binding affinity of mantGDP relative to GDP was determined by measuring the competition between mantGDP and [$^3$H]GDP for binding to p21$^{NRAS}$. Solutions (final volume, 125 μl) containing 5 μM GDP·p21$^{NRAS}$ complex, 10 μM [$^3$H]GDP, 50 mM Hepes, 0.5 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 0.25 mM adenosine 5'-[β,γ-imido]triphosphate (added to prevent nonspecific binding by [$^3$H]GDP), pH 7.6, were incubated with a range of concentrations of the analogue (1 μM–1 mM) at 37°C for 90 min. Each measurement was done in triplicate. The amount of [$^3$H]GDP remaining bound to p21$^{NRAS}$ was then determined by binding to a cellulose nitrate filter. The data were analyzed as described (15).

To prepare nucleotide·p21$^{NRAS}$ complexes, a 2-fold excess of nucleotide was added to the apoprotein at 0°C. The protein was desalted and the excess nucleotide was removed using a Bio-Gel P4 column in a buffer containing 50 mM Tris·HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, and 10 mM dithiothreitol at 4°C. This procedure left the complex intact as shown by the coincidence of nucleotides and protein in the elution profile. All measurements were made in this buffer, unless otherwise indicated.

Fluorescence measurements were made using an SLM model 8000 photon-counting spectrofluorimeter. Kinetic measurements were at 37°C under the identical conditions described (7). Kinetic data were fitted to single exponential curves by using a nonlinear least squares procedure (16).

## RESULTS

To assess how closely mantGTP and mantGDP mimic GTP and GDP in their interaction with p21$^{NRAS}$, the binding affinity of mantGDP for p21$^{NRAS}$ and the rate constants for mantGTP and mantGDP dissociation and for the mantGTP cleavage step were measured under the identical conditions used (7) for GTP and GDP. MantGDP binds to wild-type p21$^{NRAS}$ 1.8 times weaker than GDP (association constant, 1 × $10^{11}$ $M^{-1}$; ref. 7), as determined by a competitive binding experiment with [$^3$H]GDP.

The rate constant for the cleavage of mantGTP·21$^{NRAS}$ was determined by following the time course of mantGDP formation after the addition of a substoichiometric amount of mantGTP to p21$^{NRAS}$ (Fig. 2). The rate constant for the dissociation of mantGTP from p21$^{NRAS}$ was determined by a similar experiment in which a large excess of GDP was added to the mantGTP·p21$^{NRAS}$ complex and the proportions of nucleotide were determined by HPLC. The rationale for these experiments has been described (7). The rate constant for the dissociation of mantGDP from p21$^{NRAS}$ was measured using the large enhancement of fluorescence that occurs on the binding of mantGDP to p21$^{NRAS}$ (see below). After the addition of a large excess of GDP to a solution of mantGDP·p21$^{NRAS}$ complexes, the fluorescence intensity was followed with time.

The results of these experiments are summarized in Table 1 and show that all of the rate constants determined for the mant-nucleotides are within a factor of 2 of those for the physiological nucleotides with both wild-type and mutant proteins.

On binding to wild-type p21$^{NRAS}$, mantGTP shows a 3.2-fold enhancement of fluorescence intensity and mantGDP shows a 2.8-fold enhancement (Fig. 3). When either mantGTP or mantGDP bind to the mutant protein [Asp$^{12}$]p21$^{NRAS}$, a 3.2-fold fluorescence enhancement is observed (data not shown). We have monitored this change in fluorescence intensity for the wild-type protein by incubating mantGTP·p21$^{NRAS}$ complexes over the time course of the hydrolysis to mantGDP·p21$^{NRAS}$ complexes (Scheme I, steps 2 and 3) (Fig. 4*A*). The fluorescence intensity decreases by 10% for the wild-type protein with a rate constant of 3.1 × $10^{-4}$ $s^{-1}$, which is not significantly different from the rate constant in Table 1 controlling cleavage of mantGTP·p21$^{NRAS}$ or GTP·p21$^{NRAS}$. By contrast, there is no detectable change in the fluorescence intensity of the mutant mantGTP·[Asp$^{12}$]-p21$^{NRAS}$ complex over the time course of mantGTP cleavage (compare Figs. 2 and 4*A*).

Two controls were carried out to show that the complexes are resistant to nucleotide dissociation and protein denaturation throughout the time course. The fluorescence of wild-

Table 1. Rate constants for the p21$^{NRAS}$ GTPase scheme, obtained using either [$^3$H]guanine nucleotide or mant-nucleotide at 37°C for wild-type [Gly$^{12}$]p21$^{NRAS}$ or a mutant, [Asp$^{12}$]p21$^{NRAS}$

| Reactants | Rate constant (×$10^4$), $s^{-1}$ | | | |
|---|---|---|---|---|
| | $k_{-1}$ | $k_{+2}$ | $k_{-2}$ | $k_{+4}$ |
| Guanine nucleotide + [Gly$^{12}$]p21$^{NRAS}$ | 1.0 | 3.4 | <0.2 | 4.2 |
| Guanine nucleotide + [Asp$^{12}$]p21$^{NRAS}$ | 5.0 | 1.5 | <0.1 | 2.0 |
| Mant-nucleotide + [Gly$^{12}$]p21$^{NRAS}$ | 1.7 | 3.0 | <0.2 | 2.8 |
| Mant-nucleotide + [Asp$^{12}$]p21$^{NRAS}$ | 3.0 | 2.3 | <0.1 | 1.6 |

Rate constants refer to steps in the GTPase mechanism (Scheme I). Data for guanine nucleotide binding are from ref. 7. Data for mant-nucleotide binding are from this work.

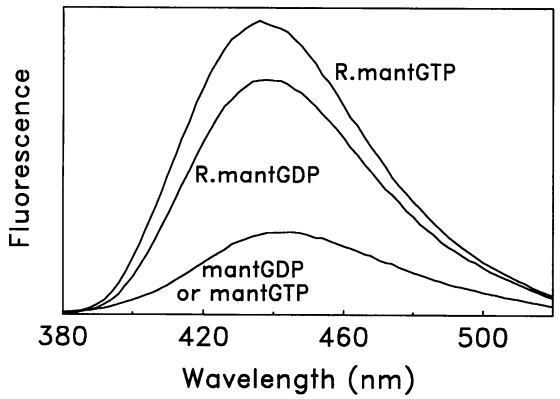


FIG. 3. Fluorescence enhancement on binding mantGDP and mantGTP to wild-type $p21^{NRAS}$ (R). To obtain an accurate assessment of this enhancement, the reaction was reversed: mantGTP was displaced from its complex with $p21^{NRAS}$ by excess GDP. Wild-type mantGTP·$p21^{NRAS}$ complex (5 $\mu$M) was incubated at 37°C in a cuvette in the fluorimeter. GDP (1 mM) was added and the emission was immediately scanned between 360 nm and 560 nm, with excitation at 350 nm (R.mantGTP spectrum). This solution was incubated at 37°C until all the mantGTP had dissociated and a final scan was taken (mantGTP spectrum). This process was repeated with mantGDP·$p21^{NRAS}$ complexes and the curves for this nucleotide were normalized so that the free nucleotides had the same intensity. This procedure was used to overcome any differences in concentration of the two initial complexes.

type mantGDP·$p21^{NRAS}$ was followed over the same time course, and there was no change in intensity (Fig. 4*C*). In the second control to test for protein denaturation, and hence formation of free nucleotide, a sample of the protein complex present at the end of the time course was passed through a Bio-Gel P4 gel-filtration column. For both wild type and mutant, there was no detectable free nucleotide: >99% of the fluorescence was eluted with the protein.

To characterize the fluorescence change further, we used the analogue mantp[NH]ppG, which is not hydrolyzed by $p21^{NRAS}$ under the conditions of this experiment (see below). Mantp[NH]ppG binds to $p21^{NRAS}$ with affinity 19 times weaker than GDP (data not shown). Thus the association constant for mantp[NH]ppG is $5 \times 10^9$ $M^{-1}$. The rate of mantp[NH]ppG release from its complex with $p21^{NRAS}$, measured as described above for mantGDP, is $9 \times 10^{-4}$ $s^{-1}$ at 37°C.

There is a small decrease in fluorescence intensity on incubating the wild-type mantp[NH]ppG·$p21^{NRAS}$ complex with a rate constant, $2.2 \times 10^{-4}$ $s^{-1}$, similar to that with mantGTP although the amplitude is smaller (Fig. 4*B*). During the time course of this experiment, HPLC analysis shows that no hydrolysis of the analogue occurs and gel filtration shows no dissociation of the complex.

## DISCUSSION

MantGTP and mantGDP closely mimic GTP and GDP in their interactions with $p21^{NRAS}$: both binding affinity and the measured rate constants in the GTPase mechanism are within a factor of 2 for the two types of nucleotide. This small effect of the modification of the ribose moiety of the nucleotides is consistent with the crystal structure of $p21^{HRAS}$, which shows the 2′,3′-hydroxyl groups of the nucleotide project away from the protein toward the solvent (17–19).

The mant-nucleotides are environmentally sensitive (9) and on binding to $p21^{NRAS}$ there is a large enhancement of their fluorescence. With the wild-type protein, this is 3.2-fold for mantGTP and 2.8-fold for mantGDP. This suggests that the mant-fluorophore is in a slightly different environment in

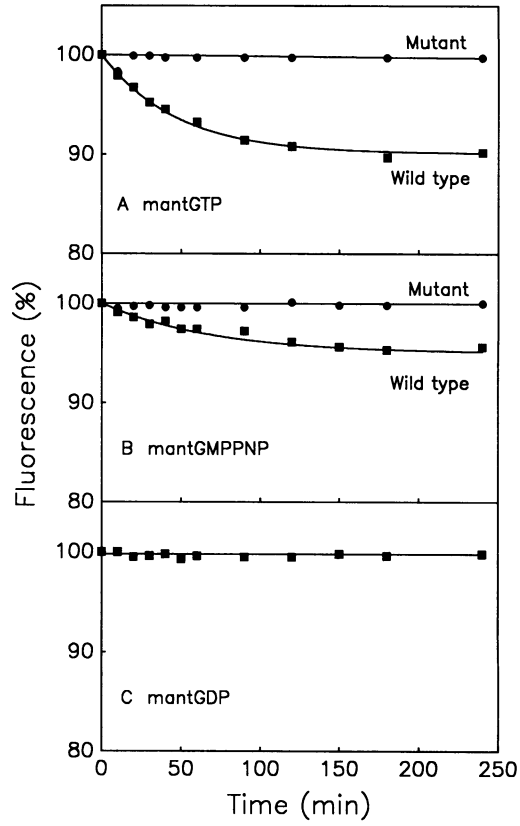


FIG. 4. Change in fluorescence intensity on incubating complexes of $p21^{NRAS}$ with mantGTP, mantGDP, or mantp[NH]ppG. Emission intensities were measured at 442 nm, and excitation was at 350 nm. Solutions of complexes (5 $\mu$M) prepared at 4°C were placed in a stirred cell and allowed to reach 37°C (typically within 2 min) before the zero-time measurement was made. The excitation light shutter was closed between measurements to minimize photobleaching. Each set of data was normalized to this zero time measurement. (*A*) mantGTP-protein complexes for wild-type and mutant proteins. Aliquots from these reaction mixtures were analyzed by HPLC to obtain Fig. 2. (*B*) mantp[NH]ppG-protein complexes (mantGMPPNP) for wild-type and mutant proteins. (*C*) mantGDP-protein complexes for wild-type protein.

the two complexes although small changes in total fluorescent intensities are difficult to measure between different solutions. However, we have been able to measure the decrease in fluorescent intensity with time when mantGTP·$p21^{NRAS}$ hydrolyzes to mantGDP·$p21^{NRAS}$, and in this case solution and instrumental parameters are constant for all measurements. We have shown that this hydrolysis is accompanied by an exponential decrease in fluorescence with a first-order rate constant, the same within experimental error as that measured for the cleavage step. There are three possible interpretations of this result.

(*i*) A conformation change of the protein–nucleotide complex occurs during the cleavage step that changes the local environment of the fluorophore.

(*ii*) A conformation change occurs during the release of $P_i$ from the mantGDP·$P_i$·$p21^{NRAS}$ complex.

(*iii*) The rate of the cleavage step is limited by a conformation change of the mantGTP·$p21^{NRAS}$ complex, which precedes cleavage.

The decrease in fluorescence intensity of the complex of $p21^{NRAS}$ with mantp[NH]ppG, which is not hydrolyzed and so does not undergo step 2 (Scheme I), suggests that the third situation occurs. In this case the measured value of $k_{+2}$ reported here for mantGTP and that for GTP reported previously (7) is actually the rate of the isomerization step

and the subsequent cleavage step is controlled by this rate. The smaller fluorescence decrease with mantp[NH]ppG relative to mantGTP could be due to slightly different local environments of the fluorophore in the two complexes. Alternatively, there could be approximately equal fluorescence changes with mantGTP on both the proposed conformation change and on either the cleavage or phosphate release steps.

For the mutant protein, no change in fluorescence intensity occurs during the conversion of mantGTP·[$Asp^{12}$]$p21^{NRAS}$ to mantGDP·[$Asp^{12}$]$p21^{NRAS}$ or on incubation of the mantp[NH]ppG·[$Asp^{12}$]$p21^{NRAS}$ complex. Either the proposed isomerization step does not occur with the mutant protein or it does occur but the environment of the fluorophore does not change in the same way as in the wild-type protein. With the wild-type and mutant proteins, the GDP complexes undergo oxygen exchange between [$^{18}O_4$]$P_i$ and water faster than would be expected for the forward and reverse rate constants of the cleavage step (ref. 7; J. Hunter and M.R.W., unpublished data). This result lends support to the argument that a conformation change occurs prior to the cleavage step in wild-type and mutant protein.

It is possible that the proposed conformation change is important in determining the biologically active state of the protein. In the wild-type protein, this process results in the formation of an "inactive" state of the protein but with the mutant protein, although hydrolysis occurs, the protein stays in an "active" or GTP-like conformation.

There is evidence (19–23) of conformation differences, either between GTP- and GDP-bound forms or between wild-type and mutant proteins. Conformational differences between $p21^{NRAS}$·GTP and $p21^{NRAS}$·GDP have been shown using circular dichroism measurements (20). The measurement suggests a change in the percent $\alpha$-helix, although differences between the wild-type protein and mutants are small. Structural differences between $p21^{HRAS}$ (as the GDP complex) and a mutant ([$Val^{12}$]$p21^{NRAS}$) have been observed in the crystal structure (21). In particular a change in protein conformation about the GDP $\beta$-phosphate is seen. NMR data also suggests small structural differences between wild-type $p21^{NRAS}$ and the mutant [$Asp^{12}$]$p21^{NRAS}$ in the GDP states (22, 23). The crystal structure has now been obtained with p[NH]ppG bound (19), and this will be important in comparing different structural states due to different bound nucleotides.

The proposed conformation change requires a minimum of one other conformation change in the GTPase cycle to re-form the original conformation as in R·GTP (Scheme I). This could occur elsewhere in the GTPase mechanism or in nucleotide-free $p21^{NRAS}$, which could exist in two interconvertible states—one capable of binding GDP and the other capable of binding GTP. Further studies are required to determine where this second conformation change occurs. It will be of interest to determine which steps the GTPase-activating protein (GAP) modifies and whether its interactions with individual complexes are consistent with its postulated role as part of the effector system. The proposed conformation change is consistent with a simple model of GAP interaction with $p21^{NRAS}$. GAP can interact with wild-type or mutant proteins, but only with the former does the conformation change allow GAP to accelerate the hydrolysis and so deactivate the $p21^{NRAS}$.

We thank Dr. David Trentham (National Institute for Medical Research, London) for helpful discussions and Dr. A. Hall (Institute for Cancer Research, London) for a gift of the ras expression system. This work was supported by the Medical Research Council, U.K.

1. Stryer, L. & Bourne, H. R. (1986) *Annu. Rev. Cell Biol.* **2**, 391–419.
2. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649.
3. Marshall, C. J. (1986) *J. Cell. Sci.*, Suppl. 4, 417–430.
4. Trahey, M. & McCormick, F. (1987) *Science* **238**, 542–545.
5. Cales, C., Hancock, J. F., Marshall, C. J. & Hall, A. (1988) *Nature (London)* **332**, 548–550.
6. Adari, H., Lowy, D. R., Willumsen, B. M., Der, C. J. & McCormick, F. (1988) *Science* **240**, 518–521.
7. Neal, S. E., Eccleston, J. F., Hall, A. & Webb, M. R. (1988) *J. Biol. Chem.* **263**, 19718–19722.
8. Feuerstein, J., Goody, R. S. & Webb, M. R. (1989) *J. Biol. Chem.* **264**, 6188–6190.
9. Hiratsuka, T. (1983) *Biochim. Biophys. Acta* **742**, 496–508.
10. Eccleston, J. F., Kanagasabai, T. F., Molloy, D. P., Neal, S. E. & Webb, M. R. (1989) in *The Guanine Nucleotide Binding Proteins: Common Structural and Functional Properties*, NATO ASI Series A, eds. Bosch, L., Kraal, B. & Parmeggiani, A. (Plenum, New York), Vol. 165, pp. 87–97.
11. Neal, S. E., Hunter, J., Eccleston, J. F. & Webb, M. R. (1989) *Biophys. J.* **55**, 123a (abstr.).
12. Hall, A. & Self, A. (1986) *J. Biol. Chem.* **261**, 10963–10965.
13. Feuerstein, J., Kalbitzer, H. R., Goody, R. S. & Wittinghofer, A. (1987) *Eur. J. Biochem.* **162**, 49–55.
14. Cremo, C. R. & Yount, R. G. (1987) *Biochemistry* **26**, 7524–7534.
15. Eccleston, J. F. (1981) *Biochemistry* **20**, 6265–6272.
16. Edsall, J. T. & Gutfreund, H. (1983) *Biothermodynamics* (Wiley, New York).
17. de Vos, A. M., Tong, L., Milburn, M. V., Matias, P. M., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E. & Kim, S.-H. (1988) *Science* **239**, 888–893.
18. Tong, L., de Vos, A. M., Milburn, M. V., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E. & Kim, S.-H. (1989) *Nature (London)* **337**, 90–93.
19. Pai, E. F., Kabsch, W., Krengel, U., Holmes, K. C., John, J. & Wittinghofer, A. (1989) *Nature (London)* **341**, 209–214.
20. Pingoud, A., Wehrmann, M., Pieper, U., Gast, F., Urbanke, C., Alves, J., Feuerstein, J. & Wittinghofer, A. (1988) *Biochemistry* **27**, 4735–4740.
21. Tong, L., Milburn, M. V., de Vos, A. M. & Kim, S.-H. (1989) *Science* **245**, 244.
22. Campbell Burk, S., Papastavros, M. Z., McCormick, F. & Redfield, A. G. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 817–820.
23. Grand, R. J. A., Levine, B. A., Byrd, P. J. & Gallimore, P. H. (1989) *Oncogene* **4**, 355–361.