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Induction of Neurite Formation in PC12 Cells by Microinjection of Proto-Oncogenic Ha-*ras* Protein Preincubated with Guanosine-5′-*O*-(3-Thiotriphosphate)

TAKAYA SATOH, SHUN NAKAMURA, AND YOSHITO KAZIRO*

Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108, Japan

Received 1 June 1987/Accepted 24 August 1987

Rat pheochromocytoma (PC12) cells differentiate to neuronal cells in response to nerve growth factor. It has been shown that microinjection of oncogenic but not proto-oncogenic p21 protein induces morphological differentiation in PC12 cells (D. Bar-Sagi and J. R. Feramisco, Cell 42:841–848, 1985). In this paper we describe a recombinant human proto-oncogenic Ha-*ras* protein which can effectively induce neurite extension of PC12 cells when microinjected as a complex with guanosine-5′-*O*-(3-thiotriphosphate). The protein was found to be less effective when complexed with GTP. On the other hand, an oncogenic *ras* protein coinjected with guanosine-5′-*O*-(2-thiodiphosphate) was entirely inactive. These results indicate that the binary p21-GTP complex, but not the p21-GDP complex, is effective in inducing differentiation in PC12 cells, irrespective of the oncogenic or the proto-oncogenic protein.

Several lines of evidence suggest that the protein products (p21) of the *ras* gene family have a regulatory role in signal transduction processes elicited by growth or differentiation factors (2, 7, 14, 15, 21, 23, 26). Transfection of the oncogenic *ras* gene or microinjection of its protein product induces transformation and proliferation of NIH 3T3 cells (8, 17, 24; see also reference 18 for a review). A line of rat pheochromocytoma cells (PC12 cells) which differentiate to neuronal cells in response to nerve growth factor (12) are differentiated morphologically by infection with Kirsten murine sarcoma virus (22), by transfection with the activated N-*ras* gene (13), or by microinjection of oncogenic p21 (1). On the other hand, the normal proto-oncogenic *ras* gene can neither transform NIH 3T3 cells (3, 4) nor induce differentiation of PC12 cells (13) unless it is expressed at elevated levels. It has also been shown that microinjection of the normal *ras* p21 into NIH 3T3 (8, 24) or PC12 (1) cells at an amount comparable to its activated form does not cause any biological effect.

From extensive genetic and biochemical studies, it has been revealed that the guanine nucleotide-binding domains are indispensable for transforming activity and that a single amino acid substitution around position 12 or 61 activates the transforming potential of *ras* p21 protein (see references 11 and 18 for reviews). Decreased in vitro GTPase activity has been found to be associated with such *ras* p21 mutants (10, 19, 20, 25).

Our previous studies on the role of GTP in protein biosynthesis have indicated that GTP-binding proteins involved in translational processes exist in two forms, i.e., a GTP-bound form and a GDP-bound form. The GTP-bound form is an active conformation which promotes the reaction, and the cleavage of GTP is required to shift the conformation to an inactive, GDP-bound form (see reference 16 for a review). An analogous scheme can be envisaged for the possible reaction mechanism of the *ras* p21 protein. The binary *ras* p21-GTP complex may be an active form, whereas the complex containing *ras* p21-GDP may be inactive in promoting signal transduction. However, no direct evidence for this hypothesis has yet been provided. In this paper, we microinjected *ras* p21 proteins together with guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) or guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) into PC12 cells to examine their effect on neuronal differentiation.

Both normal (Gly-12) and oncogenic (Val-12) p21s were expressed in *Escherichia coli*, and the proteins were purified as described elsewhere (T. Satoh, S. Nakamura, M. Nakafuku, and Y. Kaziro, Biochim. Biophys. Acta, in press). The purified protein contained 1 mol of tightly bound GDP per mol of protein. The dissociation constants (K_d) of p21(Gly-12) and p21(Val-12) for guanine nucleotides were determined (Table 1).

First, we injected p21 containing GDP (p21-GDP) into PC12 cells (Fig. 1; see also Table 2). About 5×10^{-9} μl of p21(Val-12)-GDP (3 mg/ml) was injected per cell, and after 24 h, 77 to 79% of the injected cells extended neurites (Fig. 1B). In a control experiment, a solution containing bovine serum albumin (3 mg/ml) was injected, but no morphological change was induced (Fig. 1C). p21(Gly-12)-GDP did not induce neurite formation even at a higher concentration (5.6 mg/ml) (Fig. 1A), in agreement with the results of Bar-Sagi and Feramisco (1).

Then we studied the effect of microinjection of p21 (Gly-12) complexed with GTPγS. The binary p21-GTPγS complex was prepared by incubation of p21-GDP with an excess of GTPγS (see legend to Fig. 3). Under this condition, p21-GDP was stoichiometrically converted to the p21-GTPγS complex within 5 min as shown in the experiment of Fig. 2, in which the kinetics of formation of p21-[^{35}S]GTPγS was followed by the nitrocellulose membrane filter assay. The binding of GTPγS to p21 is noncovalent, since washing with 5% trichloroacetic acid released all the radioactivity from the filter. The p21(Gly-12)-GTPγS complex was injected into PC12 cells, and after 24 h we observed neurite extension in 41 to 58% of the injected cells (Fig. 3A; Table 2). As controls, we injected p21(Gly-12)-GDP, preincubated

* Corresponding author.

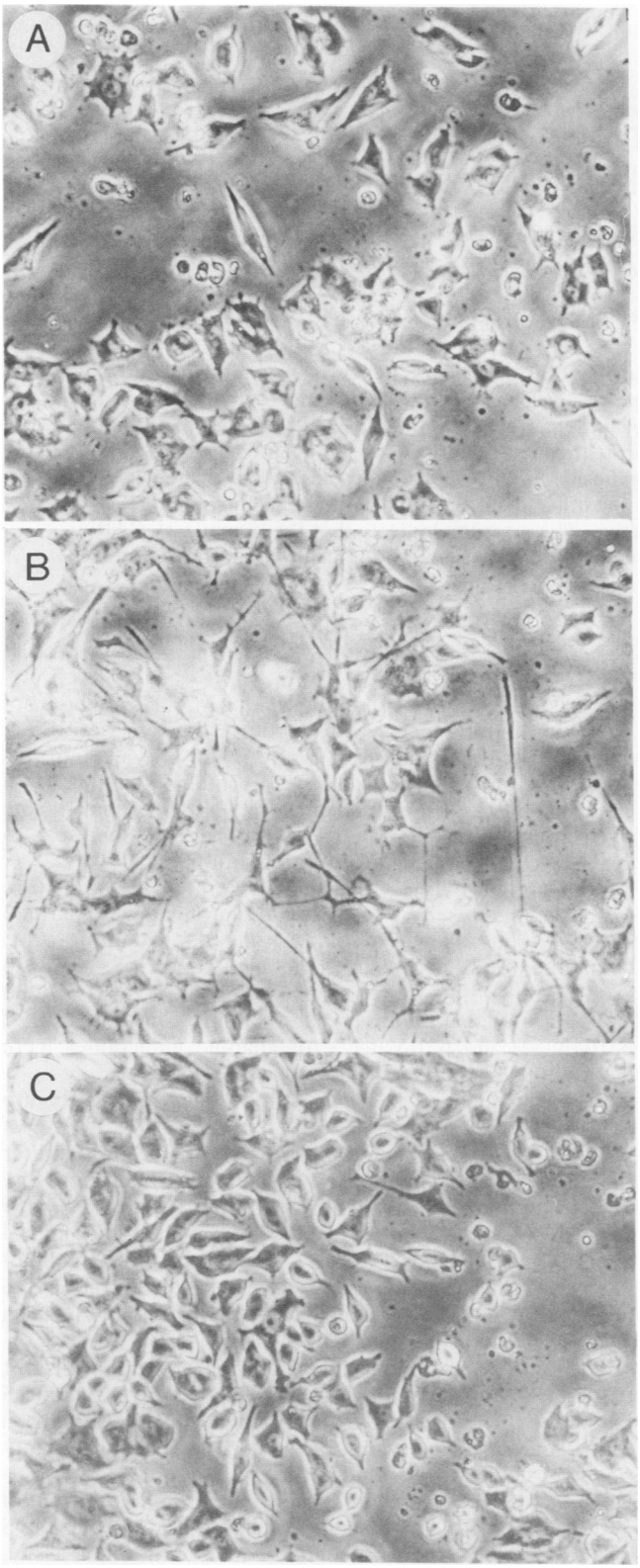

FIG. 1. Induction of the neuronal morphology of PC12 cells after microinjection of p21-GDP. p21(Gly-12)-GDP and p21(Val-12)-GDP were purified as described elsewhere (Satoh et al., in press). PC12 cells were grown in Dulbecco minimal essential medium supplemented with 10% fetal calf serum and 5% horse serum and incubated at 37°C in 5% CO_2. The cells were plated on 35-mm plastic petri dishes and were microinjected 24 h after plating. p21(Gly-12)-GDP (5.6 mg/ml) (A), p21(Val-12)-GDP (3 mg/ml) (B), and bovine serum albumin (3 mg/ml) (C) were injected into the cells. We estimate that about 5×10^{-9} μl was injected into each cell. The photographs were taken with a phase-contrast microscope 24 h after microinjection (magnification, ×1,500).

TABLE 1. Dissociation constants (K_d) of p21(Gly-12) and p21(Val-12) for GDP, GTP, and their derivatives[a]

Compound	K_d (M) of:	
	Gly-12	Val-12
GDP	1.2×10^{-8}	3.1×10^{-9}
GDPβS	2.8×10^{-8}	1.1×10^{-8}
GTP	2.1×10^{-9}	1.3×10^{-9}
GTPγS	1.2×10^{-9}	2.1×10^{-9}

[a] The K_d values for GDP were determined by Scatchard analysis; those for GTP, GDPβS, and GTPγS were determined by competition assay described elsewhere (Satoh et al., in press).

as described above but without GTPγS, or the solution containing GTPγS alone without p21(Gly-12). In both cases, no neurite extension was observed (data not shown). To confirm that the observed neurite formation was due to the injected p21(Gly-12)-GTPγS, we injected rat monoclonal anti-p21 antibody Y13-259 (9) (10 mg/ml) immediately before the injection of p21(Gly-12)-GTPγS. The preinjection of the antibody completely blocked neurite formation (Fig. 3B), indicating a critical role of p21(Gly-12)-GTPγS. To exclude the possibility that double injection by itself might disrupt differentiation, we carried out a control experiment in which we injected first bovine serum albumin and then p21(Gly-12)-GTPγS. The efficiency of neurite formation was about the same as with the single injection of p21(Gly-12)-GTPγS (data not shown).

Induction of morphological differentiation was also observed upon injection of p21(Gly-12)-GTP. The p21(Gly-12)-GTP was prepared in a manner similar to that for p21-GTPγS (see the legend to Fig. 3) and injected into PC12 cells. The cells were differentiated morphologically after 24 h (Fig. 3C), although the efficiency (20 to 37% of the injected cells being differentiated) was lower than when induced by p21(Gly-12)-GTPγS (Table 2). This may be due to the hydrolysis of GTP bound to p21 within the cells. The kinetics of morphological differentiation with p21(Gly-12)-GTPγS and p21(Gly-12)-GTP were similar to those with p21(Val-12)-GDP (data not shown). These results indicate that p21-GTP is an active form that can induce morphological differentiation of PC12 cells.

Although p21(Gly-12)-GTPγS and p21(Gly-12)-GTP were able to induce differentiation of PC12 cells, the efficiency was lower than that with p21(Val-12)-GDP (Table 2). We observed that the ability of p21(Val-12)-GDP to induce neurite extension was much decreased (to as low as 14 to 26%) by incubation for 10 min at 30°C (data not shown). This indicates that the low efficiency of differentiation of p21(Gly-12)-GTPγS or p21(Gly-12)-GTP may be due, at least in part, to inactivation, during incubation to exchange the bound GDP with GTP or GTPγS, of the ability to induce differentiation without causing a loss of the GDP-binding activity of p21 protein.

We also studied the effect of GDPβS (5, 6) on differentiation of the PC12 cells by injecting p21(Val−12)-GDP (3 mg/ml) along with GDPβS (156 mM) (Fig 4). Coinjection of

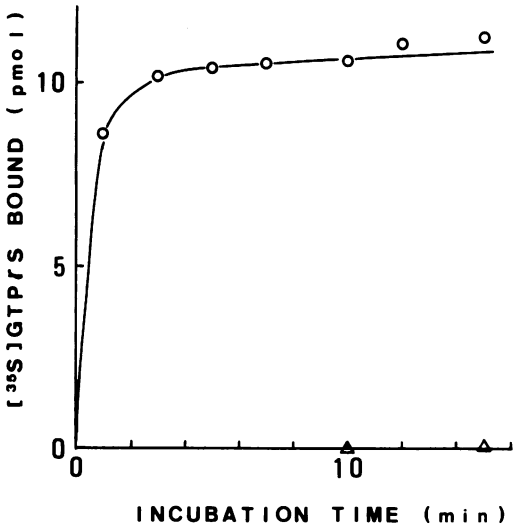

FIG. 2. Kinetics of formation of the p21(Gly-12)-[^{35}S]GTPγS complex. p21(Gly-12)-GDP was incubated in 50 mM Tris hydrochloride (pH 7.5)–2.8 mM $MgCl_2$–7.4 mM EDTA–148 mM NH_4Cl–10 mM 2-mercaptoethanol–0.5 mg of bovine serum albumin per ml–20 μM [^{35}S]GTPγS (250 μCi/μmol) at 30°C. At the specified times, samples containing 11 pmol of p21 were filtered through a nitrocellulose membrane filter. The filter was washed once with (△) or without (○) cold 5% trichloroacetic acid and then with 10 mM Tris hydrochloride (pH 7.5) containing 10 mM $MgCl_2$. The radioactivity of [^{35}S]GTPγS retained on the filter was determined.

TABLE 2. Effects of microinjection of p21 complexes on neurite extension in PC12 cells

Substance injected (concn)	Expt	No. of cells injected	No. of cells extending neurites	% Positive
Bovine serum albumin (3.0 mg/ml)	1	123	3	2.4
	2	185	6	3.2
p21(Gly-12)-GDP (5.6 mg/ml)	1	137	4	2.9
	2	169	7	4.1
p21(Val-12)-GDP (3.0 mg/ml)	1	156	123	79
	2	127	98	77
p21(Gly-12)-GTPγS (4.2 mg/ml)	1	131	62	47
	2	239	97	41
	3	310	179	58
p21(Gly-12)-GTP (4.2 mg/ml)	1	180	67	37
	2	169	55	33
	3	186	38	20
GDPβS (156 mM) + p21(Val-12)-GDP (3.0 mg/ml)	1	324	0	0
	2	227	5	2.2
	3	279	4	1.4

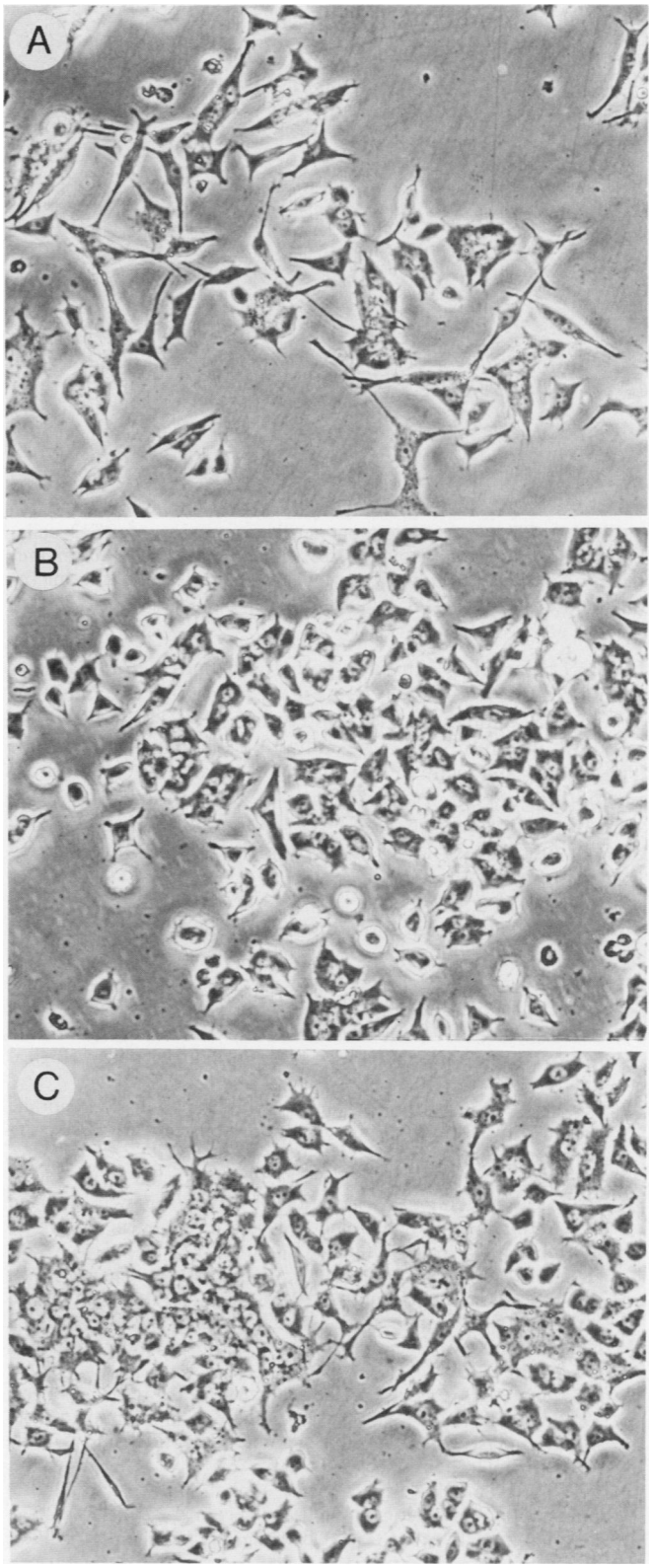

FIG. 3. Induction of neuronal morphology of PC12 cells by microinjection of p21(Gly-12)-GTPγS and p21(Gly-12)-GTP. Samples of 56 μg of p21(Gly-12)-GDP and 52.6 nmol (approximately 20-fold molar excess of p21) of GTPγS or GTP were incubated in a solution (13.5 μl) containing 50 mM Tris hydrochloride (pH 7.5), 2.8 mM $MgCl_2$, 7.4 mM EDTA, and 148 mM NH_4Cl at 30°C for 10 min. The resultant p21(Gly-12)-GTPγS (4.15 mg/ml) (A and B) or p21 (Gly-12)-GTP (4.15 mg/ml) (C) compound was microinjected. For panel B, monoclonal antibody Y13-259 (10 mg/ml), dissolved in 50 mM Tris hydrochloride (pH 7.5) and 10 mg of bovine serum albumin per ml, was injected into the same cells before the injection of p21(Gly-12)-GTPγS. Photographs were taken as described for Fig. 1.

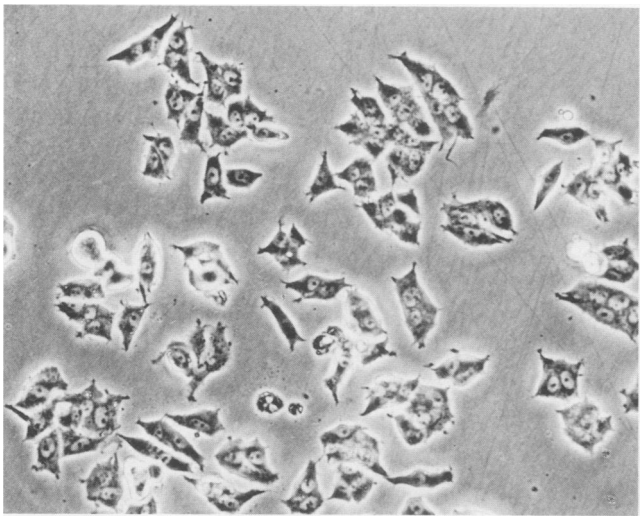

FIG. 4. Effect of GDPβS on neurite extension of PC12 cells. GDPβS (156 mM) was coinjected with p21(Val-12)-GDP (3 mg/ml) into PC12 cells. The photograph was taken 24 h after the microinjection as described for Fig. 1.

GDPβS completely inhibited neurite formation in PC12 cells induced by p21(Val-12)-GDP (Fig. 4; Table 2). The injection of GDPβS decreases the population of p21(Val-12)-GTP, resulting in inhibition of the induction of neurite formation. This result suggests that p21-GDP is probably inactive in promoting the signal transduction of differentiation.

In this report we present direct evidence that p21-GTP is active whereas p21-GDP inactive in the induction of neurite formation in PC12 cells.

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LITERATURE CITED

1. **Bar-Sagi, D., and J. R. Feramisco.** 1985. Microinjection of the *ras* oncogene protein into PC12 cells induces morphological differentiation. Cell **42**:841–848.
2. **Bar-Sagi, D., and J. R. Feramisco.** 1986. Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by *ras* proteins. Science **233**:1061–1068.
3. **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.
4. **DeFeo, D., M. A. Gonda, H. A. Young, E. H. Chang, D. R. Lowy, E. M. Scolnick, and R. W. Ellis.** 1981. Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. Proc. Natl. Acad. Sci. USA **78**:3328–3332.
5. **Eckstein, F., D. Cassel, H. Levkovitz, M. Lowe, and Z. Selinger.** 1979. Guanosine 5′-*O*-(2-thiodiphosphate). An inhibitor of adenylate cyclase stimulation by guanine nucleotides and fluoride ions. J. Biol. Chem. **254**:9829–9834.
6. **Fein, A.** 1986. Blockade of visual excitation and adaptation in *Limulus* photoreceptor by GDP-β-S. Science **232**:1543–1545.
7. **Feramisco, J. R., R. Clark, G. Wong, N. Arnheim, R. Milley, and F. McCormick.** 1985. Transient reversion of *ras* oncogene-induced cell transformation by antibodies specific for amino acid 12 of *ras* protein. Nature (London) **314**:639–642.
8. **Feramisco, J. R., M. Gross, T. Kamata, M. Rosenberg, and R. W. Sweet.** 1984. Microinjection of the oncogene form of the human H-*ras* (T-24) protein results in rapid proliferation of quiescent cells. Cell **38**:109–117.
9. **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.
10. **Gibbs, J. B., I. S. Sigal, M. Poe, and E. M. Scolnick.** 1984. Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. Proc. Natl. Acad. Sci. USA **81**:5704–5708.
11. **Gibbs, J. B., I. S. Sigal, and E. M. Scolnick.** 1985. Biochemical properties of normal and oncogenic *ras* p21. Trends Biochem. Sci. **10**:350–353.
12. **Greene, L. A., and A. S. Tischler.** 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA **73**:2424–2428.
13. **Guerrero, I., H. Wong, A. Pellicer, and D. E. Burstein.** 1986. Activated N-*ras* gene induces neuronal differentiation of PC12 rat pheochromocytoma cells. J. Cell. Physiol. **129**:71–76.
14. **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.
15. **Kamata, T., and J. R. Feramisco.** 1984. Epidermal growth factor stimulates guanine nucleotide binding activity and phosphorylation of *ras* oncogene proteins. Nature (London) **310**:147–150.
16. **Kaziro, Y.** 1978. The role of guanosine 5′-triphosphate in polypeptide chain elongation. Biochim. Biophys. Acta **505**:95–127.
17. **Kung, H., M. R. Smith, E. Bekesi, V. Manne, and D. W. Stacey.** 1986. Reversal of transformed phenotype by monoclonal antibodies against Ha-*ras* p21 proteins. Exp. Cell Res. **162**:363–371.
18. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Cellular oncogenes and multistep carcinogenesis. Science **222**:771–778.
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. **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.
21. **Mulcahy, L. S., M. R. Smith, and D. W. Stacey.** 1985. Requirement for *ras* proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature (London) **313**:241–243.
22. **Noda, M., M. Ko, A. Ogura, D. Liu, T. Amano, T. Takano, and Y. Ikawa.** 1985. Sarcoma viruses carrying *ras* oncogenes induce differentiation-associated properties in a neuronal cell line. Nature (London) **318**:73–75.
23. **Smith, M. R., S. J. DeGudicibus, and D. W. Stacey.** 1986. Requirement for c-*ras* proteins during viral oncogene transformation. Nature (London) **320**:540–543.
24. **Stacey, D. W., and H. Kung.** 1984. Transformation of NIH 3T3 cells by microinjection of Ha-*ras* p21 protein. Nature (London) **310**:508–511.
25. **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.
26. **Wakelam, M. J. O., S. A. Davies, M. D. Houslay, I. McKay, C. J. Marshall, and A. Hall.** 1986. Normal $p21^{N\text{-}ras}$ couples bombesin and other growth factor receptors to inositol phosphate production. Nature (London) **323**:173–176.