MOLECULAR AND CELLULAR BIOLOGY, Dec. 1993, p. 7311-7320
0270-7306/93/127311-10$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 13, No. 12

Effector Domain Mutations Dissociate p21^{ras} Effector Function and GTPase-Activating Protein Interaction

JAMES C. STONE,* MICHAEL COLLETON, AND DRELL BOTTORFF

Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Received 17 July 1993/Returned for modification 23 August 1993/Accepted 1 September 1993

The GTPase activity of p21^{ras} is stimulated by GTPase-activating proteins (GAPs) such as p120GAP and the product of the neurofibromatosis 1 gene, which may negatively regulate p21 function. GAPs are also proposed effectors of *ras*. We have sought activating substitutions in c-H-*ras* in the region encoding the effector domain, on the rationale that such mutations would dissociate effector function from negative regulation by GAP. One such activating mutation, Pro-34→Arg, encodes protein that is substantially bound to GTP in vivo. In vitro, this protein is not stimulated by GAPs, and its binding to p120GAP is grossly impaired. The results support the idea that the p21 structural requirements for effector function and GAP interaction are quite different and suggest that some molecule(s) other than p120GAP serves as the *ras* effector. In contrast to the results obtained with p120GAP, the Pro-34→Arg p21 species is effectively coupled to the *raf-1* product, as judged from electrophoretic mobility shifts of the Raf-1 phosphoprotein.

The products of *ras* genes (p21) function as GTP-dependent signal transducers in growth and development (7, 8). The equilibrium between the GTP-bound "on" and GDP-bound "off" forms of p21 protein is controlled by the rates of GTP hydrolysis and nucleotide exchange. These rates of hydrolysis and exchange are intrinsic properties of the p21 species but can be influenced by GTPase-activating proteins (GAPs) and guanyl nucleotide dissociation factors. Mammalian cells contain two GAPs that act on p21, p120GAP (51) and the product of *NF1*, the neurofibromatosis type 1 gene (2, 31, 57). Acceleration of hydrolysis presumably attenuates the p21-transduced signal.

Interest in *ras* proteins has been focused by the discovery that many human tumors are heterozygous for dominant *ras* oncogenes (6). Most frequently observed are single amino substitutions at codons 12, 13, and 61. These substitutions result in constitutively activated p21 species that have reduced GTPase as a result of both lower intrinsic and GAP-stimulated hydrolysis rates.

A central unresolved question concerns the nature of the *ras* target in vertebrate cells. In *Saccharomyces cerevisiae*, the *RAS* proteins regulate adenyl cyclase (9, 49), although this effector function is not conserved in other species. There is considerable evidence that a cascade of kinases is activated by p21 in vertebrates (48, 56). Very recently, evidence that *ras* can form a physical complex with the kinase encoded by Raf-1 as well as MAP kinase kinase has been obtained (32). Whether these molecules are effectors of *ras* has yet to be resolved.

Crystallographic studies of p21 have demonstrated that GTP binding induces a conformational shift in two regions, loops 2 and 4 (36, 39). In previous genetic studies, a conserved segment that overlaps loop 2 had been identified as an effector region (42, 54). Mutations in this region impair effector function in mammalian cells and in yeast cells without affecting any of the known biochemical properties of p21. Importantly, some of the conserved effector residues are also required for interaction with vertebrate GAPs, raising the possibility that these latter molecules might be downstream targets of *ras* as well as negative regulators (1, 10). Analysis of malignant schwannomas from NF1 patients has shown that loss of the *NF1*-encoded GAP is associated with an increase in the GTP content of cellular p21 (4, 11). This could be interpreted to mean that the *NF1* product is a negative regulator of p21, but since p21 signalling might promote differentiation rather than proliferation in neural crest derivatives, the results are consistent with the *NF1* product also being a target of *ras* action (4, 5, 11, 27).

The role of p120GAP is also unclear. This protein has a complex structure including two *src* homology 2 (SH2) domains and an SH3 domain in addition to the catalytic domain (52). The SH2 domains apparently facilitate binding to tyrosine-phosphorylated proteins such as the autophosphorylated platelet-derived growth factor receptor (23, 24), while the SH3 domain might be involved in coupling to yet other signalling molecules (37). In lysates from cells transformed by tyrosine kinases, p120GAP is found complexed with a 190-kDa protein that acts as a GAP for members of the rho class of GTPase and a 62-kDa protein that appears to be related to RNA binding proteins (14, 41, 55). Although p120GAP can function as a negative regulator of c-H-*ras* transformation in gene transfer experiments (59), other studies have supported a role for p120GAP in propagating *ras*-transduced signals (13, 40, 58).

We and others have reasoned that if GAPs were solely negative regulators and not the targets of *ras* action, then it should be possible to isolate mutations affecting the p21 effector domain that dissociate effector and GAP interaction. For example, a mutation temperature sensitive for transformation is not temperature sensitive for GTPase stimulation by p120GAP (12). A dissociating mutation of another type would activate the proto-oncogene by reducing negative regulation while leaving effector function intact. In this report, we describe the isolation of activating mutations affecting the effector domain of c-H-*ras*, and the interaction of one mutant p21 species with p120GAP and *NF1*-encoded GAP is described.

* Corresponding author.

MATERIALS AND METHODS

Cell lines and vectors. Rat2 cells (50) were used to study the transforming properties of *ras* genes, and ψ-2 cells (28) were used to create helper-free viral stocks. Moloney murine leukemia virus was used in virus rescue experiments. The pBabePuro vector (33), which encodes puromycin resistance, was used to transmit the mutant c-H-*ras* sequences. For the comparison of cell morphologies, p21 levels, and intracellular guanyl nucleotide content of p21, two rat cell clones, R2/RT8 (tk^+ vector) and R2/1631-1 (neo^+ vector), were used because these had been previously shown to express appreciable amounts of virus-transmitted $p21^{v\text{-}H\text{-}ras}$ and $p21^{c\text{-}H\text{-}ras}$, respectively. For the comparison of focus-forming unit/CFU ratios, however, the v-H-*ras* and c-H-*ras* were transferred into the pBabePuro vector by using polymerase chain reaction (PCR) methods, and the entire sequence of the transferred *ras* genes was shown to be correct.

Construction of a c-H-*ras* mutation library. In vitro mutagenesis of c-H-*ras* in the region encoding the p21 effector region was performed by a PCR protocol that employs chemically doped oligonucleotides. pBW1631 (44) was cut with *Cla*I, which recognizes a site outside the c-H-*ras* sequence, and 1-ng samples were used in two separate PCRs. Reaction A employed primers 1 (5′GGAGATCTCAA GAGCTCCTGGTTTGGCAGC3′) and 2 (5′CGAGTATGAT CCCACTATAGAGGACTCCTACCGG3′). Reaction B employed primers 3 (5′GTTTCCGGTAGGAGTCCTCTATAG TGGGATCATA3′) and 4 (5′GCACAAAGGACATGACTG ACC3′). Primer 1 corresponds to the sense strand of the region just upsteam of the c-H-*ras* coding sequence with a *Bgl*II site and a two-nucleotide cap engineered at the 5′ end to facilitate cleavage by *Bgl*II. Primer 2 represents the antisense sequence of c-H-*ras* corresponding to codons 32 to 40 as well as parts of codons 31 and 41. Similarly, primer 3 represents the sense strand of the region corresponding to the effector domain, while primer 4 is based on vector sequence from the opposite strand downstream of c-H-*ras* coding sequence.

Primers 2 and 3, representing the region where mutations were sought, were synthesized after cross-contaminating each of the four phosphoramadite solutions with 2% of each of the other three solutions (22). The conditions of synthesis were designed to give wild-type, single-mutant, double-mutant, and multiple-mutant frequencies, calculated from the appropriate term of a polynomial equation ($n = 32$), of about 14, 28, 28, and 30%, respectively. In this situation, we therefore obtain a good yield of single and double substitutions, which are probably the most useful for this sort of study.

PCRs A and B produced single products of 173 and 654 bp, respectively. After these two products were desalted by centrifugation through 1-ml spin columns filled with Sephadex G-50, the fragments were treated with Klenow polymerase to remove 3′ extensions that are created by the *Taq*I polymerase. PCR C was performed with about 0.5 μg of each of the two fragments to act as primers and templates for each other. The products of this reaction were resolved by electrophoresis on an agarose gel which did not contain ethidium bromide. A strip of this gel was stained with ethidium bromide to visualize molecular weight markers and one lane containing a fraction of the PCR products. A PCR product of 793 bp in length, apparently formed by the joining of the products of reactions A and B, was clearly visible. The unstained portion of the gel judged to contain the remainder of the 793-bp product was excised, frozen at −80°C, thawed, and then centrifuged at 14,000 × *g* for 5 min. The crushed gel fragment and associated liquid were then stored at 4°C overnight to facilitate elution of the 793-bp fragment. Two microliters of the eluted fragment served as template for PCR D that employed the primers 1 and 4 described above. This reaction served to generate about 0.1 μg of full-length c-H-*ras* fragment. After this product was desalted, the fragment was digested with *Bgl*II, which cuts within the first primer, and *Xho*I, which cuts between the 3′ end of c-H-*ras* and the fourth primer. The *Bgl*II-*Xho*I fragment was then subcloned into a *Bam*HI-*Sal*I double-digested retroviral vector, pBabePuro. The vector had been sequentially digested with *Bam*HI and *Sal*I and treated with calf intestine phosphatase, and the cut vector had then been purified by electrophoresis in an agarose gel to remove the small polylinker fragment. Conditions for ligation of the cut vector to the c-H-*ras* fragment that gave at least 50% of the transformants containing c-H-*ras* were established. A scaled-up ligation was desalted by ethanol precipitation and introduced by electroporation into competent *Escherichia coli* DH10B (Electromax; Bethesda Research Laboratories). A sample of the recovered cell mixture was plated to determine the number of ampicillin-resistant CFU, and the remainder was diluted into 1 liter of 2× YT medium (16 g of Bacto Tryptone per liter, 10 g of yeast extract per liter, 5 g of NACl per liter) containing 100 μg of ampicillin per ml. After growth for 24 h at 37°C, the plasmids were harvested from the liquid culture and purified by equilibrium banding in cesium chloride. A 150-μg sample of this plasmid preparation was subjected to electrophoresis in a 0.7% agarose gel (20 by 20 cm) at 40 V for 18 h. Under these conditions, the monomeric, supercoiled plasmids are resolved into two bands. The more slowly migrating, *ras*-containing band was recovered by electroelution. Several independent plasmid clones were subjected to sequence analysis to verify the presence of c-H-*ras* sequences, as opposed to v-H-*ras* sequences, that might have contaminated the PCRs, and to establish that effector domain mutations were recovered at approximately the expected frequency.

By using the calcium phosphate method (20), 50 μg of plasmid was transfected into 20 separate 75-cm^2 flasks of ψ-2 cells (28) that had been seeded with 2×10^6 cells the previous day. Virus produced at 48 h posttransfection was collected, and the ψ-2 cells were subcultured into medium containing puromycin. Drug-resistant ψ-2 colonies in each flask were pooled and plated in nonselective medium to generate additional virus stocks. Rat2 cells were infected with virus, and infected cells were subcultured into puromycin-containing medium 48 h later. Transformed proviral clones were then isolated. In some cases, infected Rat2 cells were left in nonselective medium and foci were isolated.

To determine whether the transformed proviral clones harbored the transforming versions of c-H-*ras*, retroviral sequences were rescued by infection with Moloney murine leukemia virus. Rat2 cells were infected with rescued virus, proviral clones were selected in puromycin-containing medium, and the morphologies of these secondary proviral clones were used to identify those virus stocks with significant transforming activity.

Genomic DNA extracted from these secondary proviral clones was used for blot hybridization analysis and for DNA sequence analysis. Duplicate samples of DNA were digested separately with *Bam*HI and *Eco*RI, which do not cut the proviral sequence, and these DNA samples were probed with a v-H-*ras* sequence in a Southern blot protocol to establish that a single provirus was present. Genomic DNA

also served as a template for PCRs that specifically recovered the proviral sequence. The primers in this reaction were the *Bgl*II-sensitive primer 1 described above and a *Xho*I-containing primer (5'GGCTCGAGCCACACCCTAACTGA CACAC3') based on the simian virus 40 origin that is downstream of the c-H-*ras* sequence in the pBabePuro constructs. PCR products were purified by electrophoresis, digested with *Bgl*II and *Xho*I, and subcloned into *Bam*HI and *Sal*I double-digested M13mp19 (35). The c-H-*ras* sequence in individual M13 clones was determined by the dideoxy method. When novel mutations were discovered, the sequence of the entire *ras* coding sequence was determined, and in most cases, two templates were studied. The Glu-37→Asp sequence was confirmed in three templates, and the Pro-34→Arg sequence was confirmed in four templates.

To construct the Pro-34→Arg mutation in v-H-*ras*, the cassette mutagenesis procedure previously described was used (44). Two plasmids were constructed, and the viruses derived from each were shown to have the same properties.

Biological assays. For focus assays, Rat2 cells were plated at 2.5×10^5 per 25-cm^2 culture flask the day before infection with pBabePuro-*ras* virus complexed with Moloney murine leukemia virus in the presence of 8 μg of Polybrene per ml. After 48 h of incubation, one half of the infected cells were subcultured in puromycin-containing medium, and one half were plated in nonselective medium. Drug-resistant colonies were enumerated after 8 days. Nonselective medium was changed on day 8, and foci were enumerated on day 12. Soft-agar growth assays were performed as described previously (43). For tumorigenicity studies, 2×10^5 cells were injected subcutaneously into *scid* mice.

Analysis of mutant p21 species. For selected clones, the p21 levels were compared by immunoprecipitation with monoclonal antibody Y13-259 (17) and then by electrophoresis on a 15% polyacrylamide gel as described previously (43). For analysis of guanyl nucleotides bound to p21 in vivo, cells were seeded at 10^6 or 10^5 per 25-cm^2 culture flask and then incubated for several hours to allow attachment. Cells were labelled overnight with 1 mCi of $^{32}P_i$ in 2.0 ml of phosphate-free Dulbecco's modified Eagle medium containing 10% fetal bovine serum that had been dialyzed against 0.9% NaCl. Lysates were prepared, p21 species were precipitated, and p21-associated guanyl nucleotide was analyzed on polyethyleneimine cellulose as previously described (18). The identity of radiolabelled GTP was confirmed by parallel analysis of pure GTP.

Biochemical properties of Pro-34→Arg. To examine the possible interaction of Pro-34→Arg with p120GAP directly, the viral *ras* sequence was transferred from the S11 cell line into a c-H-*ras* expression vector (26). The recombinant clone was sequenced to confirm the presence of the single Pro-34→Arg substitution. Recombinant protein was then produced after heat induction of the expression vector. Wild-type c-H-*ras* and v-H-*ras* were similarly produced. Lysates were subjected to gel exclusion column chromatography with Sephadex G-75 and then to ion-exchange chromatography with Q Sepharose to purify p21 species.

To test for the sensitivity of the Pro-34→Arg p21 to stimulation by recombinant GAPs, p21 was loaded with [α-^{32}P]GTP (800 Ci/mmol; New England Nuclear), and GTP-bound p21 was isolated after chromatography on G-50 Sephadex columns. Reaction mixtures containing approximately 10^6 cpm were incubated with the indicated amounts of recombinant p120GAP or NF1-GRD in a solution of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.3), 5 mM $MgCl_2$, 2 mM dithiothreitol, and 500 μg of bovine serum albumin (BSA) per ml for 30 min at room temperature in final volumes of 60 or 100 μl. Reactions were terminated by the addition of 1 μg of antibody Y13-259 and then chilling on ice. After 30 min on ice, 20 μl of protein A-linked Sepharose bound to rabbit anti-rat immunoglobulin G was added, and the incubation was continued with occasional shaking for a further 30 min on ice. Immunoprecipitates were washed, and bound guanyl nucleotides were analyzed by chromatography on polyethyleneimine plates as described for material labelled in vivo. For experiments with rat cell GAP, lysates were prepared from 10 confluent 10-cm plates of Rat2 cells. Cells were scraped in phosphate-buffered saline, centrifuged at low speed, and lysed in 1 packed-cell volume of 20 mM HEPES (pH 7.3)–5 mM $MgCl_2$–0.5% Nonidet P-40–2 mM phenylmethyl sulfonate. After removal of nuclei by centrifugation, lysates were stored at −80°C. Reaction mixtures were as described above but contained 1.2 mg of total cell protein per ml in place of recombinant GAP.

[α-^{32}P]GTP-bound p21 was also used to study the rate of nucleotide dissociation. Samples in GAP reaction buffer (minus the BSA) were diluted with an excess of unlabelled GTP, and after the mixtures were incubated at 37°C for various times, aliquots were collected on nitrocellulose filters and washed.

To determine whether Pro-34→Arg was capable of binding to p120GAP, a competition assay was used (5, 52). To prepare substrate, 70 pmol of wild-type p21$^{c\text{-}H\text{-}ras}$ was bound to 7 pmol of [γ-^{32}P]GTP (4,500 Ci/mmol; ICN Biochemicals), and unbound nucleotide was removed by chromatography on G-50 at 4°C. Each reaction mixture contained approximately 2×10^5 cpm of [γ-^{32}P]GTP-bound p21$^{c\text{-}H\text{-}ras}$ 1 nM recombinant p120GAP, and various amounts of either wild-type or Pro-34→Arg mutant p21$^{c\text{-}H\text{-}ras}$ bound to either GDP or GTPγS, a nonhydrolyzable GTP analog. After 10 min at room temperature, the reactions were halted and the extent of the GTPase reaction was determined by a phosphate release assay. The relative rates of the GTPase reactions were taken from the extent of hydrolysis (which was less than 30%), corrected by subtracting intrinsic hydrolysis, and expressed as a percentage of GAP-stimulated hydrolysis in the absence of competitor p21.

Analysis of Raf-1. To determine whether the Pro-34→Arg mutation was capable of inducing hyperphosphorylation of Raf-1, cell lysates were analyzed by a Western blot (immunoblot) protocol. Cells were plated at a density of 5×10^6/10-cm dish. The next day, cells were rinsed and lysed in 1.0 ml of sample buffer containing sodium dodecyl sulfate. One-tenth of the lysate was resolved in an 8% polyacrylamide gel (58.3:1, acrylamide-bis) and transferred to nitrocellulose in 20 mM sodium phosphate buffer (pH 6.8). The primary antibody was a 1:2,000 dilution of an anti-Raf-1 antiserum directed against the carboxy-terminal 12 residues of Raf-1. Antigen-bound primary antibody was detected by using the enhanced chemiluminescence system as recommended by the supplier (Amersham).

RESULTS

Isolation of effector domain mutations that activate c-H-*ras*. A plasmid library of 10^5 independent c-H-*ras* mutations consisting of substitutions in the region encoding the conserved portion of p21 effector domain was constructed in the pBabePuro retrovirus vector (33). After mobilization of the retroviral sequences by using the ψ-2 packaging system (28),

TABLE 1. Sequences of activated c-H-*ras* mutations

Cell line	Substitution(s)[a]
S3	Glu-37→Asp
S5	Glu-37→Asp
S6	Glu-37→Asp
S8	Lys-42→Arg, Gly-138→Asp, Phe-156→Val
S10	Thr-20→Ala, Lys-101→Glu, Val-103→Ala, Asn-116→Ser
S11	Pro-34→Arg
S14	Lys-147→Glu
S17	Glu-63→Lys, Arg-73→Pro
S19	Pro-34→Arg
PR20	Glu-37→Asp
7ff	Asp-33→Glu, Gln-61→Arg
8f	Leu-53→Pro, Glu-63→Lys
15ff	Ala-146→Val
17ff	Pro-34→His, Leu-113→Pro
18ff	Glu-37→Asp
S13ff	Asp-33→Tyr, Ala-59→Thr, Asn-86→Ser
PR3	Asp-33→Tyr

[a] When more than one substitution was observed, the one(s) most probably involved in activation is underlined.

Rat2 cells were infected, and then transformed proviral clones were selected. To establish that the transformed proviral clones did in fact harbor transforming versions of c-H-*ras*, retroviral sequences were rescued by infecting these clones with a nontransforming helper virus. Rescued viruses were then assayed for transforming activity by infecting Rat2 cells. Of 19 transformed cell lines analyzed, 1 produced a nontransforming, *puro*-transducing virus; 1 produced no detectable virus; and the remaining 17 produced *puro*-expressing virus that had substantial transforming activity. In most cases, many of the secondary clones were overtly transformed, as are clones that express wild-type v-H-*ras* in this vector. Clones that express c-H-*ras* from this vector usually are not morphologically transformed.

To investigate the structural basis of the c-H-*ras* mutations, proviral sequences were recovered from genomic DNA of secondary proviral clones by PCR, and the sequence of each mutant c-H-*ras* gene was determined. The results are presented in Table 1. In each cell line analyzed, genomic DNA blot hybridization analysis with two separate restriction enzyme digests of proviral clone DNA revealed a single proviral band (data not shown).

Consistent with our expectations, several transforming versions of c-H-*ras* had single substitutions within the effector region, and since no other mutations were observed in the coding sequence, these substitutions were deemed responsible for the activation of the proto-oncogene. It is noteworthy that Glu-37→Asp was recovered from 5 of 20 independent ψ-2 transfections. Substitution of Pro-34 by Arg is also capable of activating c-H-*ras* and was recovered twice. The other viral genomes either were relatively weakly transforming or harbored mutations outside the region of interest. Further studies reported here concentrated on the Pro-34→Arg and Glu-37→Asp mutations.

To verify unequivocally that the recovered Glu-37→Asp and Pro-34→Arg *ras* sequences were identical to those causing the transformation, proviral *ras* sequences were recovered from cell lines S6 and S11 by PCR and incorporated into the retroviral vector. The plasmids were subjected to sequence analysis and converted to virus. Both reconstructed mutant viruses were transforming.

The two selected mutations are capable of causing overt morphological transformation in Rat2 cells (Fig. 1). By comparison, a typical clone of Rat2 cells expressing c-H-*ras* from a retroviral vector forms a flat, contact-inhibited monolayer. Furthermore, clones expressing Pro-34→Arg exhibited a robust capacity for anchorage-independent growth. The degrees of transformation conferred by Glu-37→Asp and Pro-34→Arg were also assessed by using a focus assay. The ratios of focus-forming units to puromycin-resistant CFU units were as follows: v-H-*ras*, 1.43; c-H-*ras* 0.01; Glu-37→Asp, 0.49; and Pro-34→Arg, 0.83. These observations support the thesis that the mutations significantly activate the c-H-*ras* proto-oncogene.

Effector domain mutations in tumor samples have not been reported, yet the degree of activation exhibited by Pro-34→Arg is very substantial. To test for tumorigenic potential, a pool of proviral clones derived by infecting Rat2 cells with Pro-34→Arg helper-free virus was injected into *scid* mice. Tumors of 2 to 3 cm (maximum dimension) developed within 21 days in all mice ($n = 8$). No tumors developed in animals injected with parental Rat2 cells ($n = 7$) or the c-H-*ras*-expressing clone ($n = 10$) within 42 days, at which time the experiment was terminated.

Properties of mutant c-H-*ras* proteins expressed in fibroblasts. Analysis of p21 species was undertaken to investigate the mechanism of activation of our effector domain mutations at the protein level. Proviral clones expressing effector domain mutations were shown to express typical p21 species by immunoprecipitation of radiolabelled protein with monoclonal antibody Y13-259 (Fig. 2). For comparison, uninfected Rat2, a clone expressing v-H-*ras* and a clone expressing c-H-*ras* from retroviral vectors were studied. The proviral clones express similar amounts of virally encoded protein. Thus, enhanced expression of the effector domain mutations, as might be expected if the protein stability were increased, cannot account for their transforming properties.

The relative amounts of GDP and GTP bound to p21 were also determined in each of the proviral cell lines by immunoprecipitation of p21 from $^{32}P_i$-labelled cells (Fig. 3). Substantial amounts of radiolabelled GTP as well as GDP were coprecipitated from cells expressing v-H-*ras*, while the c-H-*ras* protein was found primarily complexed with GDP. Strikingly, the Pro-34→Arg protein behaves more like the v-H-*ras*-encoded protein in this assay. Glu-37→Asp p21 has a much lower amount of GTP, but apparently it has more than the wild-type c-H-*ras* protein. In an independent experiment using cells plated at a lower density, results qualitatively similar to those shown in Fig. 4 were obtained, although lower total amounts of radioactivity were recovered. No significant GTP or GDP was recovered when antibody was omitted (data not shown).

In vitro properties of Pro-34→Arg p21 expressed in *E. coli*. One possible explanation for the relatively high GTP content of the Pro-34→Arg p21 species is that this protein has a low in vivo GTPase activity as a consequence of its insensitivity to p120GAP. Accordingly, this sequence was expressed in an *E. coli* expression vector, and recombinant p21 complexed to [α-^{32}P]GTP was used as a substrate in a GAP assay. As can be seen in Fig. 4, wild-type c-H-*ras* protein has a substantial intrinsic GTPase, and this activity is enhanced by p120GAP. $p21^{v\text{-}H\text{-}ras}$ has a much lower intrinsic GTPase, and this activity is not accelerated by p120GAP. The Pro-34→Arg mutation has the relatively high intrinsic GTPase characteristic of the c-H-*ras* protein, but it is completely insensitive to p120GAP.

Since the Pro-34→Arg mutation is transforming in Rat2 cells, we were interested to determine which GAPs were

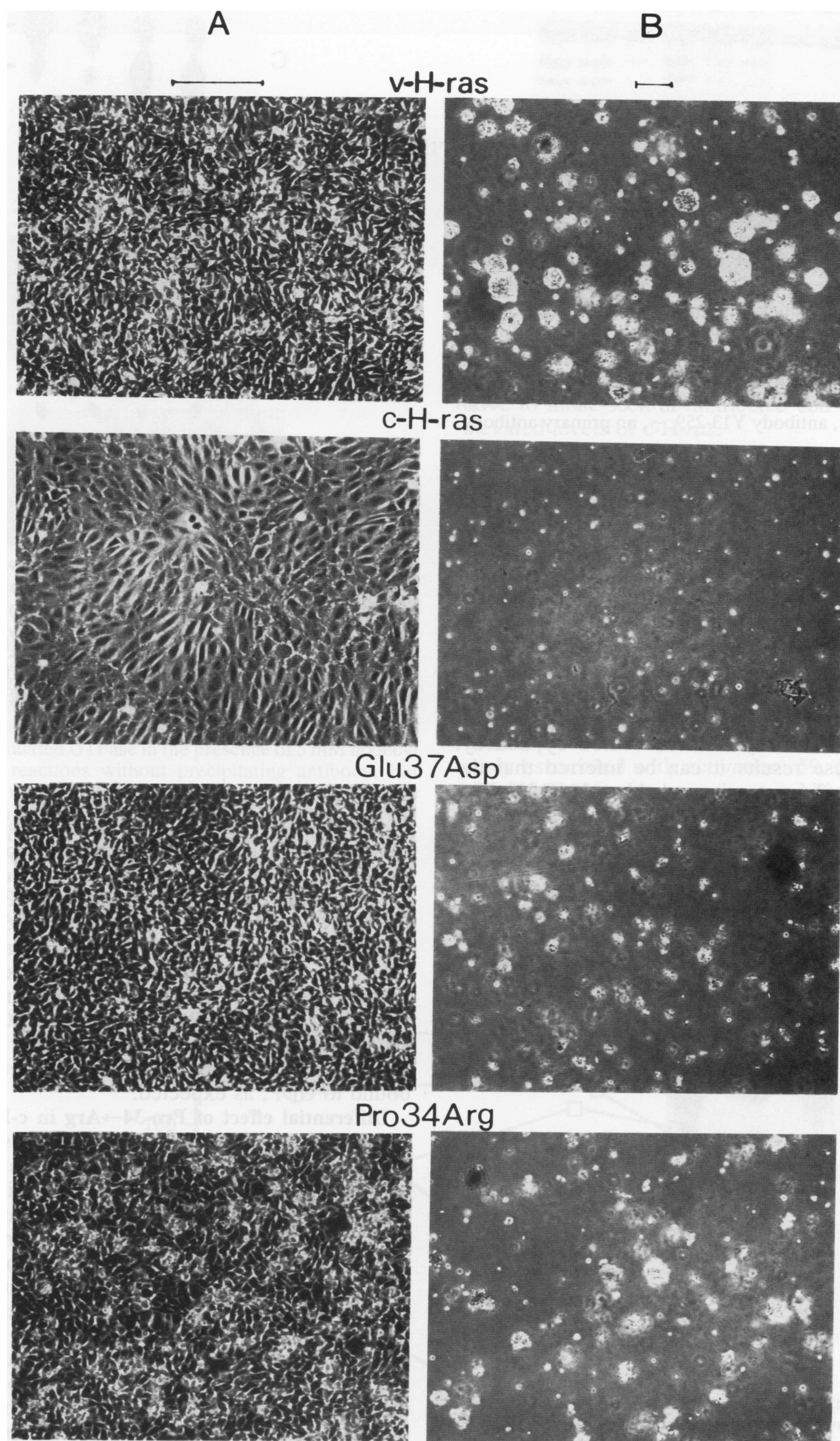

FIG. 1. Transformed phenotypes of Rat2 clones expressing activating effector domain mutations. Morphologies on plastic substrate (left) and the soft agar colonies (right) are shown for clones R2/RT8, R2/1631-1, S6, and S11, each expressing the indicated proteins. Bars, 1 mm. Photographs on the right were taken 10 days after seeding in soft agar.

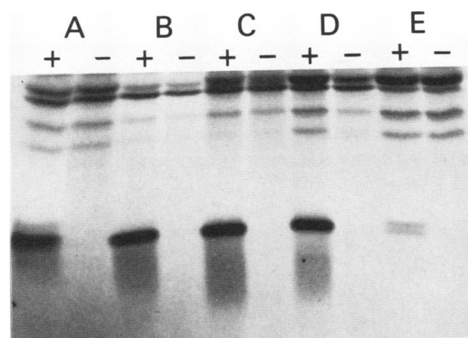

FIG. 2. Expression of p21 in rat cell clones. The proviral clones shown in Fig. 1 were labelled with [^{35}S]methionine, 4 × 10^7 cpm of radiolabelled lysate was immune precipitated, and precipitated proteins were resolved on SDS-polyacrylamide gels. Exposure was for 14 days with an intensifying screen. Lanes: A, R2/RT8 (v-H-*ras*); B, R2/1631-1 (c-H-*ras*); C, S6 (Glu-37→Asp); D, S11 (Pro-34→Arg); E, Rat2 (uninfected). +, antibody Y13-259; −, no primary antibody.

active in these cells. The detergent dodecyl maltoside has been shown to inhibit specifically the GAP encoded by *NF1*. Therefore, we assayed for GAP activity in lysates prepared from Rat2 cells in the presence and absence of this detergent. Control experiments demonstrated the efficacy of the detergent in distinguishing between the two GAPs: 5 mM dodecyl maltoside inhibited the activity of NF1-GRD, the recombinant GAP-related domain of *NF1* product, but did not affect p120GAP (Fig. 5). We were able to detect a modest amount of GAP activity towards the c-H-*ras* protein in rat cell lysates, and this activity was unaffected by dodecyl maltoside. From these results it can be inferred that the major activity in Rat2 lysates is probably p120GAP. The Pro-34→Arg protein was insensitive to NF1-GRD and rat lysate GAP as well as recombinant p120GAP (Fig. 5).

Using a filter binding assay, we found that both wild-type and Pro-34→Arg p21-[α-^{32}P]GTP complexes dissociate with rate constants of 0.006 min^{-1}. Thus, enhanced nucleotide exchange does not account for the oncogenic activation observed.

Effect of Pro-34→Arg substitution on p120GAP affinity. A

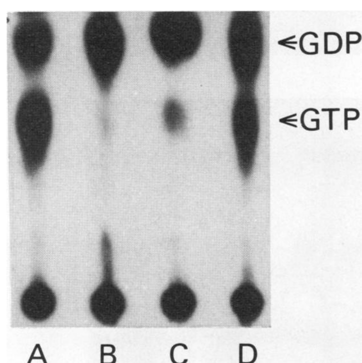

FIG. 3. In vivo guanyl nucleotide content of mutant p21 species. The proviral clones shown in Fig. 1 were labelled with ^{32}P$_i$, and p21 species were immunoprecipitated with Y13-259. Dissociated nucleotides were chromatographed on polyethyleneimine plates. Lanes: A, v-H-*ras*-expressing cells; B, c-H-*ras*-expressing cells; C, Glu-37→Asp-expressing cells; D, Pro-34→Arg-expressing cells. Positions of standards are shown. Exposure was for 30 h with an intensifying screen.

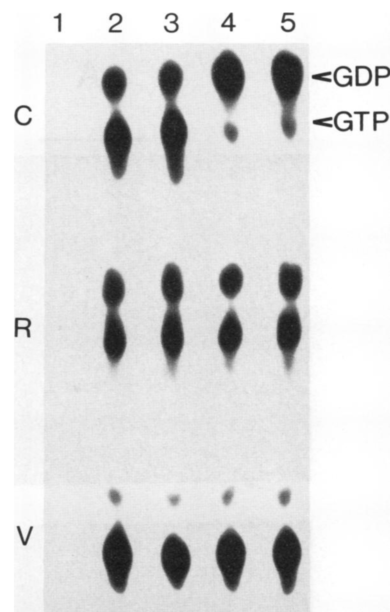

FIG. 4. Intrinsic and p120GAP-stimulated GTPase of recombinant p21 species. Recombinant p21 species were complexed with [α-^{32}P]GTP, and unbound nucleotide was removed by gel filtration chromatography. GTPase reactions were performed at 25°C without (lanes 1, 2, and 3) or with (lanes 4 and 5) 2 nM p120GAP and were terminated by the addition of antibody Y13-259 and then by incubation on ice. In lanes 1, antibody was omitted. C, c-H-*ras* protein; R, Pro-34→Arg protein; V, v-H-*ras* protein. Exposure was for 5 h with an intensifying screen.

competition assay (52) was employed to examine the effect of the Pro-34→Arg substitution on the physical interaction between p21 and p120GAP. The wild-type p21$^{c\text{-}H\text{-}ras}$ bound to GTPγS, a nonhydrolyzable GTP analog, was effective at inhibiting the GAP-stimulated GTPase of wild-type p21$^{c\text{-}H\text{-}ras}$ complexed to [γ-^{32}P]GTP (Fig. 6). Estimates of the K_d for the complex between p120GAP and wild-type p21-GTPγS ranged from 1.0 to 3.5 μM in three different experiments. The Pro-34→Arg substitution dramatically decreased the ability of p21-GTPγS to inhibit p120GAP activity, consistent with a large decrease in the affinity between p120GAP and the mutant p21 species. Neither protein was active when bound to GDP, as expected.

Differential effect of Pro-34→Arg in c-H-*ras* and v-H-*ras*. Previous work showed that a Glu-33→His Pro-34→Ser double mutation dramatically reduced the effector function of activated *ras*, while the same mutation in c-H-*ras* blocked GTPase stimulation by p120GAP without affecting the low transforming activity of the proto-oncogene in NIH 3T3 cells (15). This differential behavior of effector mutations in the two forms of *ras* prompted us to investigate the properties of Pro-34→Arg in v-H-*ras*. Two independent v-H-*ras* Pro-34→Arg viruses were found to be null in transformation assays. Analysis of pooled proviral clones failed to reveal any p21 expression above the level of the endogenous cellular p21, however. Apparently the Pro-34→Arg substitution confers protein instability when expressed in the context of v-H-*ras* sequences. By contrast, we previously found that Glu-37→Asp was highly transforming in v-H-*ras* (43).

Pro-34→Arg does not interfere with coupling of p21 and Raf-1. In cells that have been transformed by *ras*, the Raf-1 protein kinase becomes hyperphosphorylated, and it as-

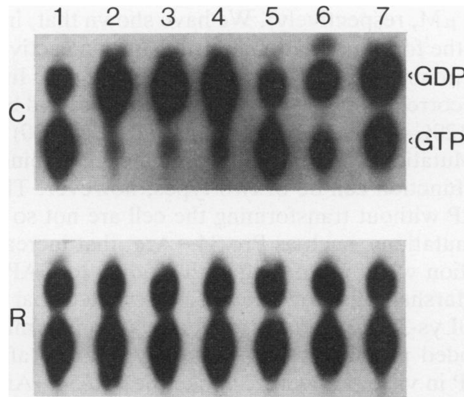

FIG. 5. Influence of p120GAP, NF1-GRD, and rat cell lysate GAP on the GTPase of wild-type and Pro-34→Arg c-H-*ras* protein. Reaction conditions were similar to those described in the legend to Fig. 4. Lanes 1, intrinsic GTPase; lanes 2, p120GAP-stimulated GTPase; lanes 3, p120GAP-stimulated GTPase in the presence of 5 mM dodecyl maltoside; lanes 4, NF1-GRD-stimulated GTPase; lanes 5, NF1-GRD-stimulated GTPase in the presence of 5 mM dodecyl maltoside; lanes 6, rat cell lysate-stimulated GTPase; lanes 7, rat cell lysate-stimulated GTPase in the presence of 5 mM dodecyl maltoside. Control reactions without precipitating antibody, but otherwise identical to those used to generate lanes 1, yielded no radioactivity (data not shown). p120GAP was used at 10 nM, and NF1-GRD was used at 20 nM. Exposure was for 5 h with an intensifying screen. C, p21$^{c\text{-}H\text{-}ras}$; R, Pro-34→Arg p21.

sumes a reduced electrophoretic mobility (34). This same response is observed with a wide variety of extracellular stimuli that are thought to signal through the *ras* pathway. To determine whether the Pro-34→Arg substitution influenced signalling between p21 and Raf-1, the electrophoretic mobility of the kinase was examined. As can be seen in Fig. 7, the mobilities of Raf-1 species in both v-H-*ras*- and Pro-34→Arg-transformed cells are significantly retarded relative to those seen in uninfected cell or in cells expressing elevated levels of c-H-*ras*.

DISCUSSION

We have used a novel mutagenesis scheme to isolate activating mutations in the region of c-H-*ras* that encodes the p21 effector domain on the rationale that such mutations might dissociate effector function from negative regulation by p120GAP. Before discussing the properties of the effector domain substitutions, the activating mutations that are outside the region of interest warrant comment. These mutations, presumably the product of *Taq* polymerase or reverse transcriptase errors, were found in or near codons previously associated with activation: 59, 61, 63, 113, 116, 146, and 147 (3, 16). In some cases, multiple substitutions were observed, and it is not possible to state with certainty which ones contribute to the activation. In the case of line S8 we suspect that Phe-156→Val is at least partially responsible for the activation. A similar change, Val-158→Leu, is the basis of *Roughened*, a dominant gain-of-function mutation in *Drosophila melanogaster* (21). *Roughened* appears to be an alteration in the *Drosophila* homolog of *Krev*-1, a mamma-

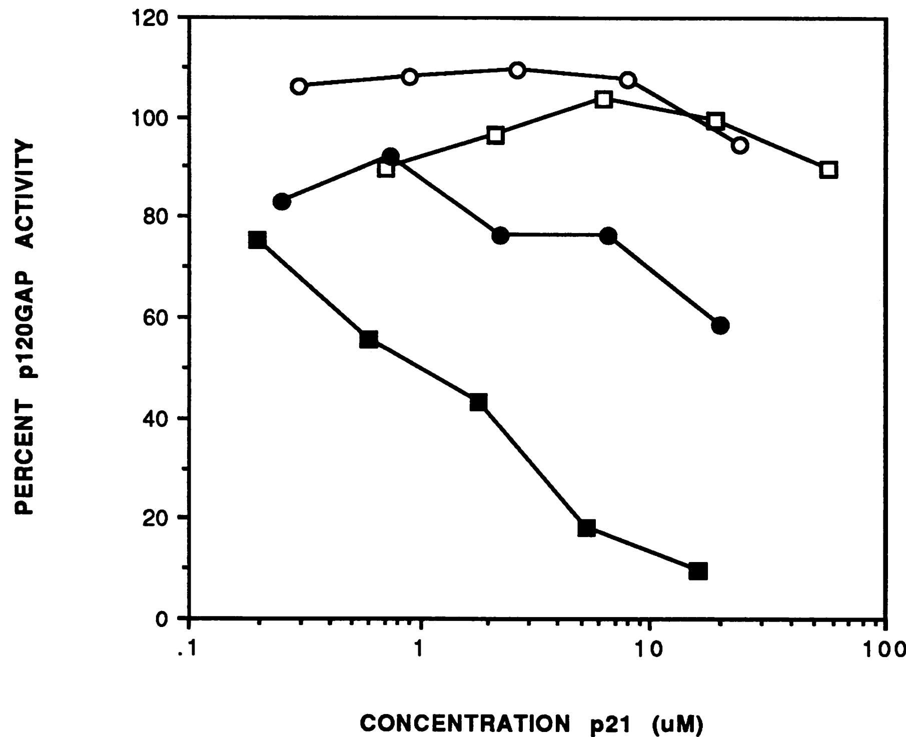

FIG. 6. Competitive interaction between p120GAP and p21 species. p120GAP stimulation of p21 GTPase was measured by using 1 nM recombinant human p120GAP, wild-type p21$^{c\text{-}H\text{-}ras}$ complexed with [γ-^{32}P]GTP, and various amounts of competitor protein complexed with either GDP or GTPγS; open squares, wild-type p21-GDP; closed squares, wild-type p21-GTPγS; open circles, Pro-34→Arg–GDP; closed circles, Pro-34→Arg–GTPγS.

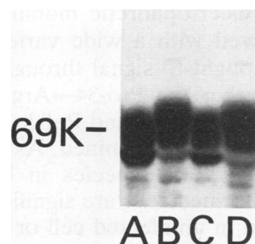

FIG. 7. Raf-1 mobility shifts induced by v-H-*ras* and Pro-34→Arg. Cell lysates were resolved on polyacrylamide gels, and Raf-1 was detected in a Western blot protocol. The position of the 69-kDa molecular mass marker is shown on the left. Lanes: A, uninfected Rat2 cells; B, R2/RT8 (v-H-*ras*); C, R2/1631-1 (c-H-*ras*); D, S11 (Pro-34→Arg).

lian *ras*-related gene that can cause reversion of *ras* transformation in cultured cells (25).

Since its discovery (51), p120GAP has been an attractive candidate p21 effector molecule. The fact that p120GAP did not act on oncogenic p21 was reconciled with the effector hypothesis by the demonstration that oncogenic p21 could bind to GAP in a competition assay (52). Likewise, the observation that GAP is primarily cytosolic while p21 needs to be located on the inner surface of the plasma membrane to function was countered by the demonstration that a significant fraction of cellular GAP associates with autophosphorylated membrane protein tyrosine kinases (23, 24). Initial evidence suggesting that p120GAP might be the target of p21 came from comparing the structural requirements for transformation with those for GAP interaction in vitro. In the N-*ras* proto-oncogene, for example, there is a good correlation between the ability of effector domain substitutions to influence transformation of NIH 3T3 cells and the ability of the encoded p21 species to be stimulated by cellular GAP (10). However, as pointed out by Farnsworth et al. (15), any decrease in GAP coupling to a mutant proto-oncogene form of p21 could be offset by a concomitant reduction in negative regulation. Consequently, one does not necessarily expect a good correlation between transforming activity and stimulation by p120GAP, even if this latter protein is the p21 effector. An alternative approach is to compare the effector function of mutant v-H-*ras* with the sensitivity of the cognate $p21^{c\text{-}H\text{-}ras}$ species to GAP stimulation (1). This strategy is compromised by the possibility that the structural properties of a substitution might be quite different in the two settings, as emphasized by our finding that the Pro-34→Arg substitution is nontransforming in v-H-*ras*. In any case, the influence of effector domain mutations on GAP stimulation of GTPase only addresses the possibility of regulation of p21 by GAP and not the hypothetical regulation of GAP by p21.

Attempts have also been made to correlate mutant *ras*-transforming activity with the ability of mutant p21 species to compete for substrate in p120GAP assays, on the rationale that this reflects binding to p120GAP. For example, the double substitutions Asp-33→His Pro-34→Ser (15) result in reduced affinity for p120GAP in both activated and proto-oncogene forms of p21, while only in the former context does the double substitution reduce transformation. As mentioned above, this differential effect has been interpreted to arise from the compensatory decrease in negative regulation by p120GAP in the case of the proto-oncogene. Gideon et al. (19) have reported that the Asp-38→Glu and Asp-38→Ala mutations encode p21 species with similar K_ds (7.5 and 10.0 μM, respectively). We have shown that, in v-H-*ras* at least, the former mutation has transforming activity under certain circumstances while the latter is null (43). In general, no good correlation between effector function and binding of p21 to p120GAP (29, 38, 53) or to *NF1* product (30) has been made. Mutations in *ras* that dissociate GAP binding and effector function can be of two types, however. Those that bind GAP without transforming the cell are not so informative as mutations, such as Pro-34→Arg, that increase effector function while interfering with binding to GAPs. Previously, Marshall and coworkers (29) reported that the Val-12Glu-30Lys-31 *ras* gene is moderately transforming while the encoded protein exhibits a greatly reduced affinity for p120GAP in vitro. However, unlike the Pro-34→Arg substitution reported here, the proto-oncogene form, Glu-30Lys-31, was nontransforming, and no evidence pertaining to the possible interaction of this protein with GAPs in vivo was presented.

More direct evidence that p120GAP can influence *ras* signalling comes from experiments that endeavor to modify p21 and p120GAP activity in model systems such as regulation of potassium channels in guinea pig atrial membranes (58), maturation of *Xenopus* oocytes (13), and transcription in cultured mammalian cells (40). Whether these effects relate to the normal function of p21 or p120GAP or their role in mammalian cell transformation is unclear. In NIH 3T3 cells, cotransfection of p120GAP plasmids reduced transformation by c-H-*ras* plasmids, suggesting a role for GAP in negative regulation of normal *ras* (59).

In this study we isolated mutations in c-H-*ras* in the region encoding the p21 effector domain that resulted in oncogenic activation. In the case of the Pro-34→Arg mutation, we have demonstrated that a substantial fraction of the encoded protein is GTP bound in vivo. Recombinant Pro-34→Arg p21 is resistant to p120GAP as well as to NF1-GRD in vitro. The mutant p21 species is also resistant to the major GAP activity in rat cell lysates which seems to be of the p120GAP type. In *S. cerevisiae*, a similar mutation was isolated after random mutagenesis (47). In fact, the Pro-41→Ser mutation affects the equivalent of the c-H-*ras* codon mutated in Pro-34→Arg. It was proposed that the mutant *RAS* protein was resistant to negative regulation by *IRA1* and *IRA2* products, which are GAPs related to p120GAP and *NF1*-encoded protein (45, 46).

We have shown that the Pro-34→Arg mutation dramatically decreases the ability of the protein to interfere with p120GAP stimulation of wild-type p21 GTPase. The most probable explanation for this result is that Pro-34→Arg p21 has a greatly reduced affinity for p120GAP. It is possible that cellular p120GAP is modified or complexed with other proteins in vivo and behaves differently or that the low affinity observed in vitro reflects an adequate affinity for activation of a hypothetical p120GAP effector. A simpler hypothesis is that some other molecule(s) serves as an effector in *ras* transformation. Certainly the p21 structural requirements for transforming function and GTPase stimulation by GAPs are quite different.

In contrast to the results with GAPs, the Pro-34→Arg p21 species appears to be effectively coupled to Raf-1, inasmuch as the shift to reduced mobility reflects activation of the kinase cascade by *ras*. It will be of interest to see whether the Pro-34→Arg substitution affects the ability of p21 to physically interact with Raf-1.

ACKNOWLEDGMENTS

We thank Alex Papageorge, Gideon Bollag, and Mark Marshall for helpful discussions. p120GAP and NF1-GRD were generous gifts from Onyx Pharmaceuticals. Anti-Raf-1 antibody was the generous gift of D Morrison.

This work was supported by operating grants from the National Cancer Institute of Canada and from the Alberta Heritage Foundation for Biomedical Research.

REFERENCES

1. **Adari, H., D. R. Lowy, B. M. Willumsen, C. J. Der, and F. McCormick.** 1988. Guanosine triphosphatase activating protein (GAP) interacts with the p21 *ras* effector binding domain. Science **240:**518–521.
2. **Ballester, R., D. Marchuk, M. Boguski, A. Saulino, R. Letcher, M. Wigler, and F. Collins.** 1990. The *NF1* locus encodes a protein functionally related to mammalian GAP and yeast *IRA* proteins. Cell **63:**851–859.
3. **Barbacid, M.** 1987. *ras* genes. Annu. Rev. Biochem. **56:**779–827.
4. **Basu, T. N., D. H. Gutmann, J. A. Fletcher, T. W. Glover, F. S. Collins, and J. Downward.** 1992. Aberrant regulation of *ras* proteins in malignant tumour cells from type 1 neurofibromatosis patients. Nature (London) **356:**713–715.
5. **Bollag, G., and F. McCormick.** 1991. Differential regulation of rasGAP and neurofibromatosis gene product activities. Nature (London) **351:**576–579.
6. **Bos, J. L.** 1989. *ras* oncogenes in human cancer: a review. Cancer Res. **49:**4682–4689.
7. **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.
8. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature (London) **349:**117–127.
9. **Broek, D., N. Samly, O. Fasano, A. Fujiyama, F. Tamanoi, J. Northup, and M. Wigler.** 1985. Differential activation of yeast adenylate cyclase by wild-type and mutant *RAS* proteins. Cell **41:**763–769.
10. **Calés, C., J. F. Hancock, C. J. Marshall, and A. Hall.** 1988. The cytoplasmic protein GAP is implicated as the target for regulation by the *ras* gene product. Nature (London) **332:**548–550.
11. **DeClue, J. E., A. G. Papageorge, J. A. Fletcher, S. R. Diehl, N. Ratner, W. C. Vass, and D. R. Lowy.** 1992. Abnormal regulation of mammalian p21^{ras} contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. Cell **69:**265–273.
12. **DeClue, J. E., J. C. Stone, R. A. Blanchard, A. G. Papageorge, P. Martin, K. Zhang, and D. R. Lowy.** 1991. A *ras* effector domain mutant which is temperature sensitive for cellular transformation: interactions with GTPase-activating protein and NF-1. Mol. Cell. Biol. **11:**3132–3138.
13. **Dominguez, I., M. S. Marshall, J. B. Gibbs, A. García de Herreros, M. E. Cornet, G. Graziani, M. T. Diaz-Meco, T. Johansen, F. McCormick, and J. Moscat.** 1991. Role of GTPase activating protein in mitogenic signalling through phosphatidylcholine-hydrolysing phospholipase C. EMBO J. **10:**3215–3220.
14. **Ellis, C., M. Moran, F. McCormick, and T. Pawson.** 1990. Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature (London) **343:** 377–380.
15. **Farnsworth, C. L., M. S. Marshall, J. B. Gibbs, D. W. Stacey, and L. A. Feig.** 1991. Preferential inhibition of the oncogenic form of Ras^{H} by mutations in the GAP binding/"effector" domain. Cell **64:**625–653.
16. **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.
17. **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.
18. **Gibbs, J. B., M. S. Marshall, E. M. Scolnick, R. A. F. Dixon, and U. S. Vogel.** 1990. Modulation of guanine nucleotides bound to ras in NIH3T3 cells by oncogenes, growth factors, and the GTPase activating protein (GAP). J. Biol. Chem. **265:**20437–20442.
19. **Gideon, P., J. John, M. Frech, A. Lautwein, L. Clark, J. E. Scheffler, and A. Wittinghoffer.** 1992. Mutational and kinetic analyses of the GTPase-activating protein (GAP)-p21 interaction: the C-terminal domain of GAP is not sufficient for full activity. Mol. Cell. Biol. **12:**2050–2056.
20. **Graham, F. L., S. Bacchetti, S. McKinnon, R. Stanners, C. Cordell, and H. M. Goodman.** 1980. Transformation of mammalian cell with DNA using the calcium technique, p. 3–25. *In* R. Baserga, C. Croce, and G. Rovera, (ed.), Introduction of macromolecules into viable mammalian cells. Alan R. Liss, Inc., New York.
21. **Hariharan, I. K., R. W. Carthew, and G. M. Rubin.** 1991. The Drosophila *roughened* mutation: activation of a rap homolog disrupts eye development and interferes with cell determination. Cell **67:**717–722.
22. **Hutchison, C. A., III, S. K. Nordeen, K. Vogt, and M. H. Edgell.** 1986. A complete library of point substitution mutations in the glucocorticoid response element of mouse mammary tumor virus. Proc. Natl. Acad. Sci. USA **83:**710–714.
23. **Kaplan, D. R., D. K. Morrison, G. Wong, F. McCormick, and L. T. Williams.** 1990. PDGF B-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex. Cell **61:**125–133.
24. **Kazlauskas, A., C. Ellis, T. Pawson, and J. A. Cooper.** 1990. Binding of GAP to activated PDGF receptors. Science **247:** 1578–1581.
25. **Kitayama, H., Y. Sugimoto, T. Matsuzaki, Y. Ikawa, and M. Noda.** 1989. A *ras*-related gene with transformation suppressor activity. Cell **56:**77–84.
26. **Lacal, J. C., E. Santos, V. Notario, M. Barbacid, S. Yamazaki, H.-F. Kung, C. Seamans, S. McAndrew, and R. Crowl.** 1984. Expression of normal and transforming H-*ras* genes in *Escherichia coli* and purification of their encoded p21 proteins. Proc. Natl. Acad. Sci. USA **81:**5305–5309.
27. **Li, Y., G. Bollag, R. Clark, J. Stevens, L. Conroy, D. Fults, K. Ward, E. Friedman, W. Samowitz, M. Robertson, P. Bradley, F. McCormick, R. White, and R. Cawthorn.** 1992. Somatic mutations in the neurofibromatosis 1 gene in human tumors. Cell **69:**275–281.
28. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33:**153–159.
29. **Marshall, M. S., L. J. Davis, R. D. Keys, S. D. Mosser, W. S. Hill, E. M. Scolnick, and J. B. Gibbs.** 1991. Identification of residues required for Ras p21 target activation. Mol. Cell. Biol. **11:**3997–4004.
30. **Marshall, M. S., and L. A. Hettich.** 1993. Characterization of ras effector mutant interactions with the NF1-GAP related domain. Oncogene **8:**425–431.
31. **Martin, G. A., D. Viskochil, G. Bollag, P. C. McCabe, W. J. Crosier, H. Haubruck, L. Conroy, R. Clark, P. O'Connell, R. M. Cawthon, M. A. Innis, and F. McCormick.** 1990. The GAP-related domain of the neurofibromatosis type 1 gene product interacts with *ras* p21. Cell **63:**843–849.
32. **Moodie, S. A., B. M. Willumsen, M. J. Weber, and A. Wolfman.** 1993. Complexes of ras-GTP with Raf-1 and mitogen-activated protein kinase kinase. Science **260:**1658–1660.
33. **Morgenstern, J. P., and H. Land.** 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. **18:**3587–3596.
34. **Morrison, D. K., D. R. Kaplan, U. Rapp, and T. M. Roberts.** 1988. Signal transduction from the membrane to cytoplasm: growth factors and membrane bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. Proc. Natl. Acad. Sci. USA **85:**8855–8859.
35. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. Gene **26:**101–106.

36. **Pai, E. F., 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.
37. **Pawson, T., and G. D. Gish.** 1992. SH2 and SH3 domains: from structure to function. Cell **71:**359–362.
38. **Schaber, M. D., V. M. Garsky, D. Boylan, W. S. Hill, E. M. Scholnick, M. S. Marshal, I. S. Sigal, and J. B. Gibbs.** 1989. Proteins **6:**306–315.
39. **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.** 1990. Time-resolved X-ray crystallographic study of the conformational change in Ha-Ras p21 protein on GTP hydrolysis. Nature (London) **345:**309–315.
40. **Schweighoffer, F., I. Barlat, M.-C. Chevallier-Multon, and B. Tocque.** 1992. Implication of GAP in ras-dependent transactivation of a polyoma enhancer sequence. Science **256:**825–827.
41. **Settleman, J., V. Narasimhan, L. C. Foster, and R. A. Weinberg.** 1992. Molecular cloning of cDNAs encoding the GAP-associated protein p190: implications for a signalling pathway from ras to the nucleus. Cell **69:**539–549.
42. **Sigal, I. S., J. B. Gibbs, J. S. D'Alonzo, and E. M. Scolnick.** 1986. Identification of effector residues and a neutralizing epitope of Ha-*ras*-encoded p21. Proc. Natl. Acad. Sci. USA **83:**4725–4729.
43. **Stone, J. C., and R. A. Blanchard.** 1991. Genetic definition of *ras* effector elements. Mol. Cell. Biol. **11:**6158–6165.
44. **Stone, J. C., W. C. Vass, B. M. Willumsen, and D. R. Lowy.** 1988. p21-*ras* effector domain mutants constructed by "cassette" mutagenesis. Mol. Cell. Biol. **8:**3565–9.
45. **Tanaka, K., B. K. Lin, D. R. Wood, and F. Tamanoi.** 1991. IRA2, an upstream negative regulator of RAS in yeast, is a RAS GTPase-activating protein. Proc. Natl. Acad. Sci. USA **88:**468–472.
46. **Tanaka, K., K. Matsumoto, and A. Toh-E.** 1989. *IRA1*, an inhibitory regulator of the *RAS*-cyclic AMP pathway in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9:**757–768.
47. **Tanaka, K., D. R. Wood, B. K. Lin, M. Khalil, F. Tamanoi, and J. F. Cannon.** 1992. A dominant activating mutation in the effector region of RAS abolishes IRA2 sensitivity. Mol. Cell. Biol. **12:**631–637.
48. **Thomas, S. M., M. DeMarco, G. D'Arcangelo, S. Halegoua, and J. S. Brugge.** 1992. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. Cell **68:**1031–1040.
49. **Toda, T., I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broek, S. Cameron, J. Broach, K. Matsumoto, and M. Wigler.** 1985. In yeast, RAS proteins are controlling elements of the adenylate cyclase. Cell **40:**27–36.
50. **Topp, W. C.** 1981. Normal rat cell lines deficient in nuclear thymidine kinase. Virology **113:**408–411.
51. **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.
52. **Vogel, U. S., R. A. F. Dixon, M. D. Schaber, R. E. Diehl, M. S. Marshall, E. M. Scolnick, I. S. Sigal, and J. B. Gibbs.** 1988. Cloning of bovine gap and its interaction with oncogenic *ras* p21. Nature (London) **335:**90–93.
53. **Willumsen, B. M., H. Adair, K. Zhang, A. Papageorge, J. C. Stone, F. McCormick, and D. R. Lowy.** 1988. A mutation analysis of *ras* function. NATO ASI Ser. Ser. A **165:**1–13.
54. **Willumsen, B. M., A. G. Papageorge, H.-F. Kung, E. Bekesi, T. Robins, M. Johnsen, W. C. Vass, and D. R. Lowy.** 1986. Mutational analysis of a *ras* catalytic domain. Mol. Cell. Biol. **6:**2646–2654.
55. **Wong, G., O. Müller, R. Clark, L. Conroy, M. F. Moran, P. Polakis, and F. McCormick.** 1992. Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. Cell **69:**551–558.
56. **Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis.** 1992. *ras* mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, raf-1, and RSK. Cell **68:**1041–1050.
57. **Xu, G., B. Lin, K. Tanaka, D. Dunn, D. Wood, R. Gesteland, R. White, R. Weiss, and F. Tamanoi.** 1990. The catalytic domain of the neurofibromatosis type 1 gene product stimulates *ras* GTPase and complements *ira* mutants of *S. cerevisiae*. Cell **63:**835–841.
58. **Yatani, A., K. Okabe, P. Polakis, R. Halenbeck, F. McCormick, and A. M. Brown.** 1990. *ras* p21 and GAP inhibit coupling of muscarinic receptors to atrial K^+ channels. Cell **61:**769–776.
59. **Zhang, K., J. E. DeClue, W. C. Vass, A. G. Papageorge, F. McCormick, and D. R. Lowy.** 1990. Suppression of c-*ras* transformation by GTPase-activating protein. Nature (London) **346:**754–756.