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Relationship among Guanine Nucleotide Exchange, GTP Hydrolysis, and Transforming Potential of Mutated *ras* Proteins

LARRY A. FEIG[1] AND GEOFFREY M. COOPER[2]*

Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111,[1] and Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115[2]

Received 4 January 1988/Accepted 9 March 1988

The effect of a series of mutations on the transforming potential of normal human *ras*^{H} has been compared with their effects on GTPase and guanine nucleotide exchange rates of p21. The mutation Val-146 resulted in partial activation of transforming potential which could be attributed to a >1,000-fold-increased rate of nucleotide exchange in the absence of an effect on GTPase. In contrast, the more modest enhancement of exchange rate (~100-fold) which resulted from the mutation Met-14 did not affect biological activity. The partially activating mutation Thr-59 was found to result in both a 5-fold reduction in GTPase and a 10-fold increase in nucleotide exchange. However, the nontransforming mutant Ile-59 displayed a comparable decrease in GTPase without an effect on nucleotide exchange. The activating effect of the Thr-59 mutation may thus represent a combined effect of reduced GTPase and increased exchange. Similarly, the strongly activating mutation Leu-61 resulted in a fivefold increase in nucleotide exchange in addition to decreased GTPase, whereas weakly activating mutations at position 61 (Trp and Pro) resulted only in decreased GTPase without affecting nucleotide exchange rates. Finally, combining the two mutations Met-14 and Ile-59, which alone had no effect on biological activity, yielded a double mutant with a 20-fold increased transforming potential, demonstrating a synergistic effect of these two mutations. Overall, these results indicate that large increases in nucleotide exchange can activate *ras* transforming potential in the absence of decreased GTPase and that relatively modest increases in nucleotide exchange can act synergistically with decreased GTPase to contribute to *ras* activation.

The cellular *ras* genes encode plasma membrane-associated proteins (p21s) of 189 amino acids which bind GDP and GTP with high affinity and display a low level of GTPase activity (see reference 1 for a review). These properties of p21 are analogous to the G proteins, which transduce signals from a variety of cell surface receptors to enzymes which affect metabolism of second messengers (see reference 11 for a review). It is therefore an attractive hypothesis that p21s function analogously to the G proteins to affect second messenger pathway(s) which can result in abnormal cell proliferation.

One approach to understanding the function of p21 has been comparative analysis of the biological and biochemical activities of the products of mutated *ras* genes. Activation of the transforming potential of *ras* genes in human tumors has been found to result from single amino acid substitutions at positions 12, 13, and 61 (1). The viral *ras*^{H} and *ras*^{K} genes also contain activating mutations at position 59 (1). Activating mutations at positions 12 and 61 decrease the GTPase activity of p21 approximately 10-fold (6, 10, 14, 15, 18). Since GTP hydrolysis mediates physiological deactivation of the G proteins, these results are consistent with the G-protein analogy. However, reduced GTPase does not correlate quantitatively with the transforming potencies of *ras* genes mutated at either codon 12 or 61, suggesting that other factors also affect biological activity (6, 20). Furthermore, the role of GTPase in activation by mutation at codon 59 is unclear; although one study has reported that substitution of threonine at this position results in reduced GTPase (10), a subsequent study reported that the GTPase activity of Thr-59 p21 was indistinguishable from that of the normal protein (13).

* Corresponding author.

Mutations which alter the affinity of p21 for GTP have also been identified by in vitro mutagenesis (3, 4, 7, 8, 17, 21). These mutations decrease the equilibrium binding affinity of p21 for GTP primarily by increasing the dissociation rate of bound nucleotide, thereby resulting in an increased rate of nucleotide exchange (8). Since exchange of bound GDP for free GTP is a limiting step in physiological activation of the G proteins (11), mutations in p21 which increase guanine nucleotide exchange rates might be predicted to increase *ras* transforming potential. Consistent with this prediction, two mutations at codons 116 and 119 which decrease the GTP-binding affinity of p21 have been reported to increase transforming activity, although other mutants which decreased GTP binding did not alter biological activity (17, 21). However, the effect of these mutations on GTPase was not determined, nor was their presumed effect on nucleotide exchange measured. It has also been reported that Thr-59 p21 displays an increased rate of guanine nucleotide exchange, which has been suggested to account for the activating effect of this mutation in the absence of an effect on GTPase (12).

These studies would suggest that mutational activation of p21 could result either from decreased GTPase or from increased rates of guanine nucleotide exchange, both of which would have the effect of increasing the level of the presumably active p21-GTP complex. However, GTPase activities alone do not account for the spectrum of transforming activities of mutant *ras* genes (6, 20), and studies of activating mutations which increase nucleotide exchange either have not determined the effect of these mutations on GTPase (17, 21) or have yielded controversial results (10, 13). To further investigate the role of both guanine nucleotide exchange and hydrolysis as determinants of biological activity, we have undertaken a comparative analysis of the

transforming potentials, GTPase activities, and guanine nucleotide exchange rates of p21s encoded by *ras* genes bearing several mutations in different molecular regions.

MATERIALS AND METHODS

Mutant isolation. GTP-binding mutants were generated by randomly mutagenizing a normal cellular ras^H bacterial expression vector with hydroxylamine and then screening for mutants with a bacterial colony GTP-binding assay as described previously (8, 9). The vector (pXCR) was generated by site-directed mutagenesis of a cDNA clone encoding a human ras^H gene activated by a Leu-61 substitution to restore the normal coding sequence (Gln-61) and insertion of this cDNA in place of viral ras^H in the pXVR vector (6, 7). Nitrocellulose filter replicas of plates containing transformed bacterial colonies expressing p21 were exposed to 10^{-8} M [α-^{32}P]GTP (New England Nuclear Corp.; 600 Ci/mmol), washed, and autoradiographed to detect bound guanine nucleotides (8, 9). The *ras* coding sequence of GTP-binding mutants was determined by the dideoxy method (16).

Recombination of fragments on either side of a unique *Pvu*II restriction site at codon 24 of ras^H was used to generate additional mutants. For example, Ile-59 c-ras^H was produced by replacing the first 24 codons of Ile-59 v-ras^H (9), which contains an activating substitution at position 12, with the corresponding fragment of normal human ras^H from pXCR. Similarly, the double mutants Met-14, Leu-61 ras^H and Met-14, Ile-59 ras^H were generated using the respective 3' and 5' *Pvu*II fragments of pXCR containing each of these single mutations.

Transfection assays. To assess biological activity, an 800-base-pair *Bgl*II-*Bam*HI fragment of pXCR containing the entire coding sequence of each of the mutant genes was subcloned into the mammalian expression vector pZIPneo SV(X)1 (2). Samples (1 to 1,000 ng) of each of these DNAs with 20 μg of NIH 3T3 carrier DNA were used to transfect NIH 3T3 cells, and the foci of transformed cells were quantitated 10 to 14 days later (5). Clonal populations of NIH cells expressing various mutant *ras* genes were isolated by selecting transfected cells for neomycin resistance by growth in 400 μg of G418 per ml.

Interactions with guanine nucleotides. Mutant proteins were expressed in bacteria and purified to greater than 80% homogeneity as previously described (8, 9). Binding affinities were evaluated by incubating 5 to 50 ng of protein with various concentrations of [α-^{32}P]GTP or [α-^{32}P]GDP (3,000 Ci/mmol) for 60 min at 32°C in 50 μl of binding buffer (20 mM Tris hydrochloride [pH 7.5], 0.3 M guanidine hydrochloride, 1 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, 40 μg of bovine serum albumin per ml). Nucleotides bound to p21 were collected by filtration through nitrocellulose and quantitated by scintillation counting (8). Nucleotide exchange rates were assessed by allowing *ras* proteins to bind [α-^{32}P]GDP at concentrations that were comparable to the K_d of the protein being studied. Samples were then diluted into 2 ml of binding buffer containing >1,000-fold excess unlabeled GTP. The rate at which cold GTP replaced [^{32}P]GDP bound to p21 was quantitated by using the filter-binding assay.

GTPase activity of p21s with normal affinities for guanine nucleotides was measured by incubating 5 μg of protein with 5×10^{-8} M [α-^{32}P]GTP (600 Ci/mmol) in 50 μl of reaction buffer (20 mM Tris hydrochloride [pH 7.4], 0.2 mM $MgCl_2$, 5 mM dithiothreitol, 20 μg of bovine serum albumin per ml) for various times. The conversion of GTP to GDP was determined by chromatographing 2 μl of each sample on polyethyleneimine plates (Brinkman Instruments, Inc., Westbury, N.Y.) as previously described (6). Labeled nucleotides were excised from the plates and quantitated by scintillation counting. For proteins with decreased affinities for GTP higher concentrations of [γ-^{32}P]GTP (36 Ci/mmol) were used, and the release of $^{32}P_i$ was assayed by the same chromatographic procedure.

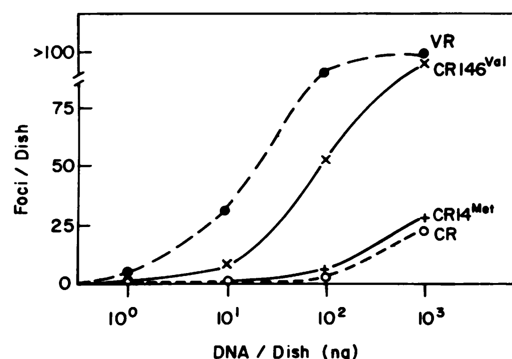

FIG. 1. Transforming activity of mutant *ras* proteins with decreased affinity for guanine nucleotides. NIH 3T3 cells were transfected with mutant *ras* genes in the mammalian expression vector pZipneoSV(X)1. Samples of 1 to 1,000 ng of each DNA were used, and transformed foci were quantitated 10 to 14 days later. Data represent the average of at least three independent assays. Symbols: (●) v-ras^H, (○) normal cell c-ras^H, (+) Met-14 c-ras^H, (×) Val-146 c-ras^H.

RESULTS

GTP-binding mutations at codons 14 and 146. Two GTP-binding mutants of normal human ras^H were isolated after random mutagenesis of a bacterial expression vector, pXCR, with hydroxylamine as previously described (8). Nucleotide sequence analysis identified a single mutation in one of the genes, the substitution of Val (GTC) for Ala (GCC) at position 146. The other gene contained three mutations: Met (ATG) in place of Val (GTG) at position 14, Gln (CAG) for Arg (CGG) at position 68, and Asn (AAC) for Asp (GAC) at position 108. Since previous studies had implicated amino acid 16 in GTP binding (17), and since amino acid 14 is also conserved among GTP-binding proteins (11), the effect of the mutation at position 14 was tested separately by constructing a recombinant which contained only this mutation. The equilibrium binding affinities of partially purified Met-14 and Val-146 p21s were 10^{-6} and 10^{-7} M, respectively, for both GTP and GDP. In comparison, the affinity of normal p21 for both nucleotides was 2×10^{-8} M.

The biological activities of ras^H genes bearing the Met-14 and Val-146 mutations were evaluated by inserting these mutant ras^H genes into the mammalian expression vector pZIPneoSV(X)1 and assaying transforming activity by transfection of NIH 3T3 cells (Fig. 1). The transforming activity of Met-14 ras^H was indistinguishable from that of the normal ras^H gene, corresponding to an efficiency of approximately 20 foci per μg of DNA. In contrast, the transforming activity of Val-146 ras^H was increased to approximately 500 foci per μg of DNA, although this transforming efficiency was still 5- to 10-fold lower than that of viral ras^H (Arg-12, Thr-59).

The rates of guanine nucleotide exchange exhibited by these mutant p21s were determined by assaying the ex-

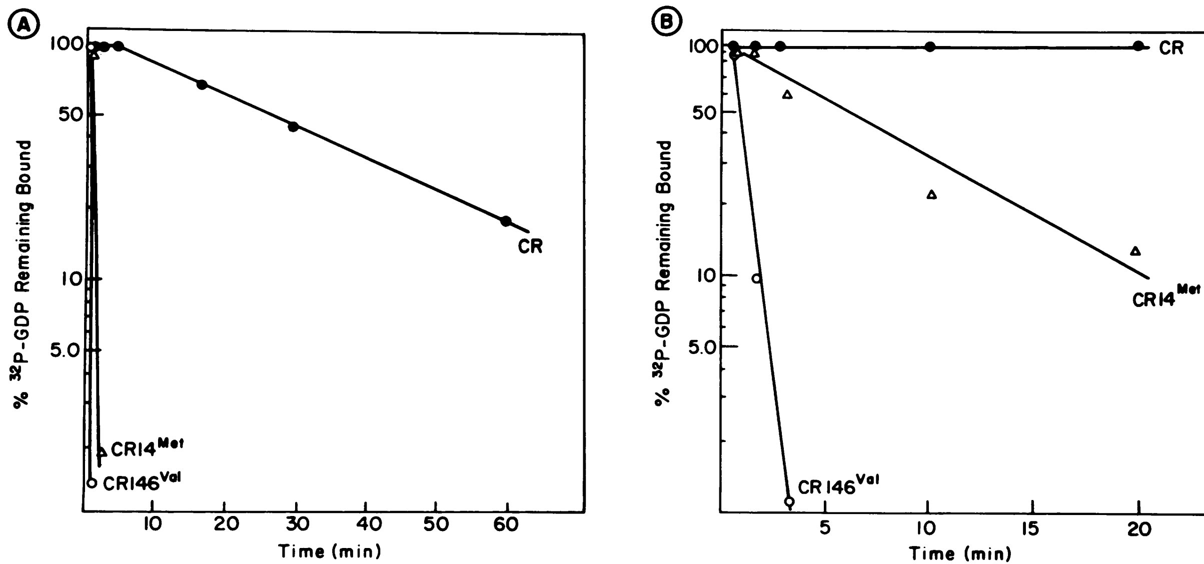

FIG. 2. Rate of exchange of GTP for GDP bound to mutant *ras* proteins. Samples of 50 ng of each mutant p21 were incubated at 32°C with 50 μl of binding buffer containing 5×10^{-7} M [α-^{32}P]GDP (3,000 Ci/mmol) until equilibrium was reached (1 h). Then the sample was diluted into 2 ml of binding buffer containing 500 μM unlabeled GTP and incubated at either 32°C (A) or 0°C (B). The percentage of [^{32}P]GDP remaining bound to p21 was determined as a function of time by quantitating p21-associated radioactivity trapped after filtration through nitrocellulose. Symbols: (●) normal cell ras^H p21, (△) Met-14 p21, (○) Val-146 p21.

change of bound [^{32}P]GDP for unlabeled GTP (Fig. 2). At 32°C the rate of exchange exhibited by both Met-14 and Val-146 p21s was at least 100-fold greater than that of normal p21 (~0.02/min) but was too high to quantitate (Fig. 2A). The exchange rates of these two mutant p21s were therefore further compared at 0°C (Fig. 2B). At this temperature, no exchange of GDP bound to normal p21 was detected. The rate of exchange for Met-14 p21 at 0°C was ~0.1/min, whereas the exchange rate exhibited by Val-146 p21 was still too high to measure (>1/min). Thus, the Val-146 mutation, which activated transforming potential, resulted in at least a 10-fold greater increase in nucleotide exchange than the nonactivating Met-14 mutation.

To determine whether the increased transforming potential of Val-146 ras^H was related to an alteration in GTPase as well as in nucleotide exchange, we compared the GTPase activities of normal, Val-146, and Leu-61 p21s. To normalize for the reduced binding affinity of Val-146 p21 (K_d, ~10^{-7} M), GTPase assays were performed with 5×10^{-7} M [γ-^{32}P]GTP (Fig. 3). (The GTPase of Met-14 p21 could not be assayed due to the still greater reduction in its GTP-binding affinity.) The GTPase activity of Val-146 p21 was indistinguishable from that of normal p21 (Fig. 3), indicating that the increased transforming potential of this mutant ras^H gene was associated with an effect on guanine nucleotide exchange independent of any effect on GTP hydrolysis.

The stability of both Met-14 and Val-146 p21s in NIH cells was indistinguishable from that of normal p21, indicating that the difference in biological activity of the two mutants was not a consequence of aberrant turnover of intracellular Met-14 p21 (data not shown). In addition, a recombinant Met-14, Leu-61 ras^H gene possessed the same high transforming activity (~10^4 foci per μg of DNA) as activated Leu-61 ras^H, indicating that the Met-14 mutation did not suppress the biological activity of an independently activated ras^H gene.

Effect of mutations at codon 59 on nucleotide exchange, GTPase, and transforming activity. Conflicting data have been reported concerning the biochemical properties of ras^H genes activated by the substitution of Thr for Ala at position 59 (10, 13). Because of this discrepancy, we compared the properties of normal ras^H (Ala-59) and ras^H Thr-59 with a

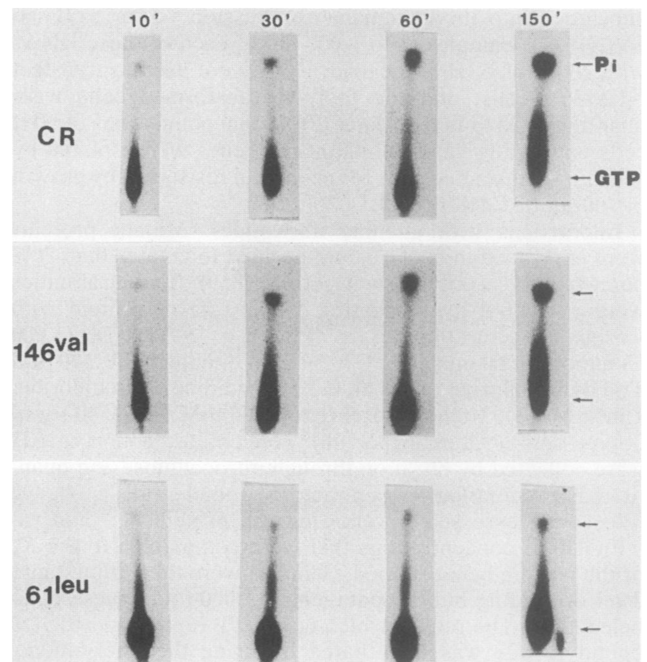

FIG. 3. GTPase activity of Val-146 p21. Normal cell ras^H (CR), Val-146, or Leu-61 p21 (5 μg) was incubated at 37°C with 5×10^{-7} M [γ-^{32}P]GTP (36 Ci/mmol) in a 50-μl reaction. Under these conditions, the proteins bound comparable amounts of GTP. After the indicated time interval (10, 30, 60, or 150 min), 2 μl of each sample was chromatographed on a polyethyleneimine plate to separate GTP from P_i.

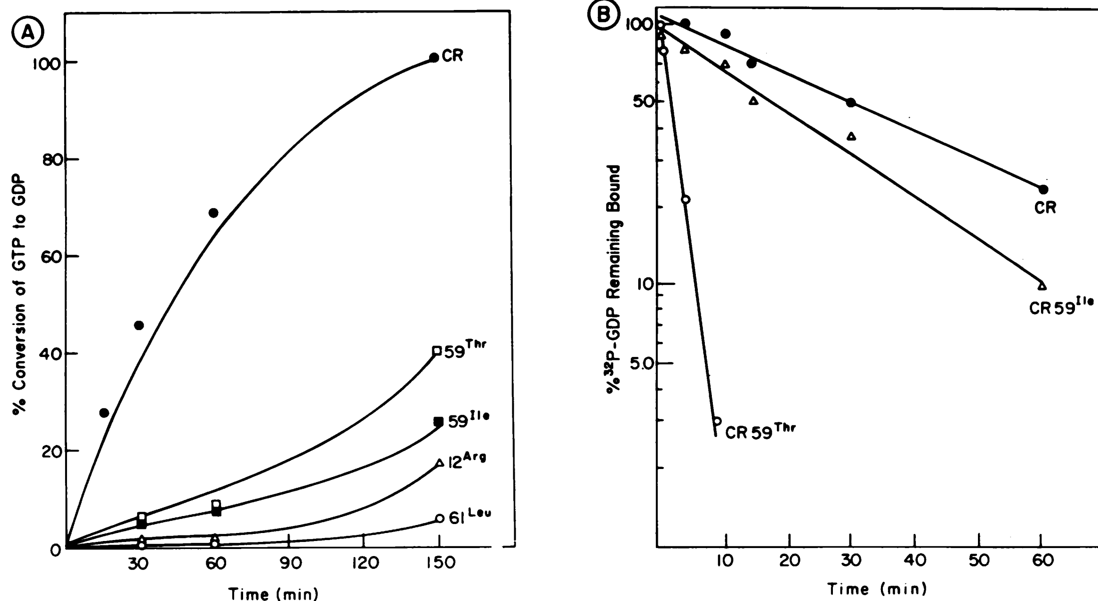

FIG. 4. GTPase activity and nucleotide exchange rates of *ras* proteins mutated at position 59. (A) p21 (5 μg) was incubated with 50 μl of 10^{-8} M [α-^{32}P]GTP (600 Ci/mmol) at 37°C. At the indicated times, 2 μl was chromatographed on a polyethyleneimine plate, which was subsequently autoradiographed. Labeled spots representing GTP and GDP were then excised, and radioactivity was quantitated by scintillation counting. Symbols: (●) normal cell c-*ras*^{H}, (□) Thr-59 c-*ras*^{H}, (■) Ile-59 c-*ras*^{H}, (△) Arg-12 c-*ras*^{H}, (○) Leu-61 c-*ras*^{H}. (B) p21 (50 ng) was incubated with 50 μl of 10^{-8} M [α-^{32}P]GDP until equilibrium binding was reached (1 h). The samples were then diluted into 2 ml of binding buffer containing 500 μM unlabeled GTP, and the percentage of [^{32}P]GDP remaining bound to p21 was determined as described in the legend to Fig. 2. Symbols: (●) normal cell c-*ras*^{H}, (△) Ile-59 c-*ras*^{H}, (○) Thr-59 c-*ras*^{H}.

ras^{H} Ile-59 mutant which we had previously isolated as a viral *ras*^{H} which was defective in autophosphorylation (9).

Ala-59, Thr-59, and Ile-59 p21s all bound GTP and GDP with affinities of ~10^{-8} M (data not shown). The GTPase activities of these three p21s are shown in Fig. 4A. Under our assay conditions, both the Thr-59 and Ile-59 mutations reduced the GTPase activity of p21 as compared with that of the normal protein. However, the reduction in GTPase exhibited by Thr-59 and Ile-59 p21s (about 5-fold) was not as severe as that exhibited by p21s bearing the activating mutations Arg-12 or Leu-61 (10- to 20-fold). The nucleotide exchange rates of Ala-59, Thr-59, and Ile-59 p21s are compared in Fig. 4B. The rate of exchange exhibited by Thr-59 p21 was approximately 10-fold greater than that of Ala-59 p21. In contrast, Ile-59 p21 exhibited nearly the same rate of nucleotide exchange as the normal protein.

The transforming activities of Thr-59 and Ile-59 *ras*^{H} genes in the ZIPneo vector were assayed by transfection of NIH 3T3 cells (Fig. 5). The mutation Thr-59 partially activated transforming potential to an efficiency of approximately 5 × 10^{2} foci per μg of DNA. In contrast, the transforming activity of Ile-59 *ras*^{H} was lower than that of the normal gene (5 to 10 foci per μg of DNA). These results indicate that activation of transforming potential by the Thr-59 mutation is associated with both a reduction in GTPase and an increase in guanine nucleotide exchange. However, the lack of activation by the Ile-59 mutation indicates that a fivefold reduction in GTPase is not sufficient to increase transforming potential.

Effect of mutations at codon 61 on guanine nucleotide exchange. Previous analysis of *ras*^{H} genes bearing 17 different mutations at codon 61 indicated that all of these mutant p21s displayed reduced GTPase activity, although the different mutants varied over 1000-fold in transforming potency (6). Since differences in guanine nucleotide exchange correlated with the biological activities of Thr-59 and Ile-59 *ras*^{H} genes, we investigated the rates of guanine nucleotide exchange exhibited by three codon 61 mutant p21s which represented different classes of transforming potencies.

The transforming activities of these codon 61 mutants were previously compared by transfection of genomic clones in which *ras* gene expression was mediated by cellular promoter sequences (6). Under these conditions, *ras*^{H} Leu-61 was highly transforming (10^{5} foci per μg of DNA), *ras*^{H} Trp-61 was weakly transforming (10^{3} foci per μg of DNA), and *ras*^{H} Pro-61 was indistinguishable in transforming activity from normal *ras*^{H} (<10 foci per μg of DNA) (6). The rates of guanine nucleotide exchange displayed by Trp-61 and

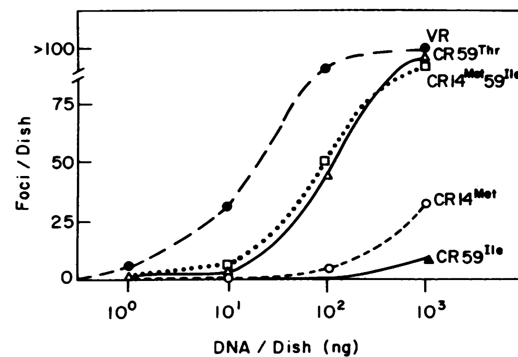

FIG. 5. Transforming activity of *ras* genes with mutations at codon 59. NIH cells were transfected with various *ras* mutants subcloned into the mammalian expression vector pZIPneoSV(X)1. Data are averages of at least two assays. Symbols: (●) v-*ras*^{H}, (△) Thr-59 c-*ras*^{H}, (□) Met-14, Ile-59 c-*ras*^{H}, (○) Met-14 c-*ras*^{H}, (▲) Ile-59 c-*ras*^{H}.

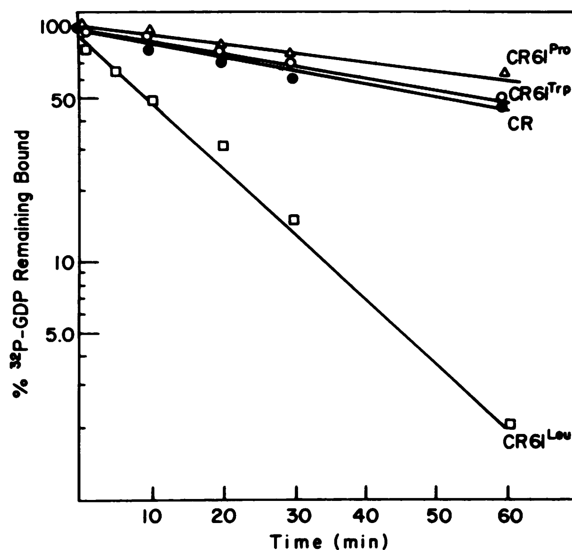

FIG. 6. Nucleotide exchange rates of *ras* proteins mutated at position 61. The rate at which [^{32}P]GDP bound to mutant p21s was replaced by unlabeled excess GTP was assayed as described in the legend to Fig. 4. Symbols: (●) c-ras^H, (△) Pro-61 c-ras^H, (○) Trp-61 c-ras^H, (□) Leu-61 c-ras^H.

Pro-61 p21s were not significantly different from normal, whereas Leu-61 p21 exhibited approximately a fivefold increase in exchange rate (Fig. 6). This difference in exchange rate correlated qualitatively with the high transforming activity of Leu-61 ras^H, but not with the difference in transforming activities of the Trp-61 and Pro-61 genes.

To further compare the biological activities of Trp-61, Pro-61, and normal ras^H genes, we inserted cDNAs of the Trp-61 and Pro-61 mutants into the ZIPneo vector. In this vector, *ras* transcription is mediated by the retroviral long terminal repeat promoter, resulting in levels of p21 expression higher than those obtained in the previous studies. Under these conditions, the transforming activity of Trp-61 ras^H was increased to the same level as that of Leu-61 ras^H (~5×10^3 foci per μg of DNA). The transforming activity of Pro-61 ras^H was also increased to approximately 2×10^2 foci per μg of DNA, which was about 10-fold higher than that of normal ras^H. The higher level of expression achieved in the ZIP vector thus appears to increase the activities of less potent transforming mutants, consistent with the previous observations that higher levels of such mutant p21s were needed to induce morphological transformation (6). Under these conditions, the Pro-61 mutant displayed partial activation of transforming potential, perhaps related to its reduced GTPase. However, the difference in biological activities of Pro-61 and Trp-61 ras^H genes was evident whether these genes were expressed from cellular or retroviral promoters and was not correlated with differences in either GTPase or nucleotide exchange rates of these mutant proteins.

Transforming activity of Met-14, Ile-59 double mutant. The results presented above indicated that p21s of some activated *ras* genes (Thr-59 and Leu-61) displayed both reduced GTPase activities and increased nucleotide exchange rates, suggesting synergistic effects of decreased GTPase and increased nucleotide exchange on transforming activity. It was therefore of interest to construct a recombinant which contained both Met-14 and Ile-59 mutations, neither of which alone increased transforming activity, although Met-14 increased nucleotide exchange 100-fold and Ile-59 decreased GTPase 5-fold. The Met-14, Ile-59 double mutant ras^H gene in ZIPneo induced transformation with an efficiency of approximately 5×10^2 foci per μg of DNA, similar to the transforming activity of Thr-59 ras^H (Fig. 5).

The double mutant Met-14, Ile-59 p21 was expressed in *Escherichia coli* for characterization of its nucleotide exchange rate and GTPase activity. However, the nucleotide-binding affinity of this double mutant fell below detectable levels (K_d, >10^{-5} M), precluding direct demonstration that its enhanced transforming activity was associated with both increased nucleotide exchange rate and decreased GTPase activity.

DISCUSSION

To evaluate the roles of guanine nucleotide exchange and GTPase as determinants of *ras* transforming activity, we have analyzed p21s encoded by several mutant *ras* genes with varied transforming potentials.

The activation of transforming potential by the Val-146 mutation provides a direct demonstration that increased guanine nucleotide exchange is sufficient to increase biological activity. This mutation decreased the equilibrium GTP-binding affinity of p21 only 10-fold, allowing us to quantitate both the rate of nucleotide exchange and the GTPase activity of the mutant protein. It was thus possible to show that the Val-146 substitution did not affect GTPase, indicating that the enhanced transforming activity of Val-146 p21 was associated only with increased nucleotide exchange. In contrast, the Met-14 mutation also increased guanine nucleotide exchange but did not affect transforming potential. This difference in biological activities may be related to the difference in nucleotide exchange rates exhibited by these two mutant p21s: the Met-14 mutation increased the rate of exchange by about 100-fold, whereas the Val-146 mutation had at least a 10-fold greater effect. It is of interest that these effects on exchange rate did not directly parallel the effect of these mutations on equilibrium binding affinities, which were 10^{-7} M for Val-146 p21 and 10^{-6} M for Met-14 p21. The fact that the Val-146 mutation has a greater effect on exchange rate (~1,000-fold) than on equilibrium binding affinity (10-fold) indicates that this mutation increases both the off rate and the on rate of GTP binding. Similar increases in both off rate and on rate are induced by Thr-59 and Leu-61 mutations, both of which increase exchange rates without altering equilibrium binding affinity.

Two mutations (Ala-119 and Ile-116) which decreased the equilibrium GTP-binding affinity of p21 have previously been reported to activate transforming potential, although the effects of these mutations on GTPase were not determined (17, 21). In the same studies, two other GTP-binding mutations (Asn-16 and Gln-116) failed to increase transforming potential. This difference did not correlate with systematic differences in binding affinities, which were ~2×10^{-7} and >10^{-5} M for the two activating mutations (Ala-119 and Ile-116) compared with ~2×10^{-6} and ~10^{-7} M for the nonactivating mutations (Asn-16 and Gln-116) (17, 21). It would, however, be of interest to determine whether these differences in biological activity correlate with rates of nucleotide exchange, as appears to be the case for the Met-14 and Val-146 mutations.

Conflicting data have been published on the biochemical properties of p21 encoded by *ras* genes activated by the mutation Thr-59. Gibbs et al. (10) attributed the activating effect of this mutation to reduced GTPase activity. In contrast, Lacal et al. (13) reported that the Thr-59 mutation

did not affect GTPase and instead attributed activation by this mutation to an enhanced rate of nucleotide exchange (12). In our hands, Thr-59 p21 exhibited both a 5-fold reduction in GTPase activity and a 10-fold increase in nucleotide exchange. Interestingly, the mutation Ile-59 resulted in a comparable decrease in GTPase without an effect on nucleotide exchange and did not activate transforming potential. These results might suggest that the partial activating effect of the Thr-59 substitution is due to increased nucleotide exchange rather than reduced GTPase. However, the 10-fold-increased rate of nucleotide exchange exhibited by Thr-59 p21 is less than that of Met-14 p21, which displays normal biological activity. The activating effect of the Thr-59 substitution therefore suggests a synergistic result of combining relatively weak effects on both nucleotide exchange and GTPase.

As previously reported (12), we found that the strongly activating mutation Leu-61 resulted in a fivefold increase in the rate of nucleotide exchange as well as in decreased GTPase. In contrast, p21s encoded by two weakly activating mutations at codon 61 (Trp and Pro) (6) exhibited normal rates of nucleotide exchange. We had previously found that 17 different mutations at codon 61 resulted in *ras* genes with transforming potentials which varied over 1,000-fold (6). Since all of these mutant p21s displayed reduced GTPase activities, these results indicated that reduction in GTPase was not sufficient to account for transforming potential (6). The increased nucleotide exchange rate of the strongly transforming Leu-61 p21 thus further suggests the possibility of a synergistic effect of decreased GTPase and increased nucleotide exchange on transforming activity.

To directly test the combined effect of mutations which decreased GTPase and increased nucleotide exchange, we constructed a recombinant Met-14, Ile-59 double mutant. Each of these mutations alone either increased nucleotide exchange (Met-14) or decreased GTPase (Ile-59) without increasing transformation activity. In contrast, the double mutant *ras* gene displayed a significant activation of transforming potential, indicating a synergistic effect of the two single mutations. This likely represents a synergistic combination of effects on nucleotide exchange and GTPase. However, since the double mutant p21 displayed reduced GTP-binding affinity (and presumably increased nucleotide exchange), it is also possible that its transforming activity, like that of Val-146 *ras*^{H}, is the consequence of an extremely rapid exchange rate.

Overall, our results are consistent with an analogy between p21 and the G proteins in that mutations which increase biological activity are associated with biochemical alterations which would favor the formation (increased nucleotide exchange) or persistence (decreased GTPase) of the p21-GTP complex. The biological effects of increased nucleotide exchange appear less dramatic than decreased GTPase, in that large increases in exchange rate (>1,000-fold for Val-146) resulted in only partial activation of transforming potential even when the mutant *ras* gene was expressed at high levels from a retroviral long terminal repeat. However, relatively modest increases in nucleotide exchange (5- to 10-fold) may act synergistically with decreased GTPase in the case of Thr-59 and Leu-61 mutations. There remain several instances where the biological activities of mutant p21s are not directly correlated with their intrinsic biochemical activities. These include Pro-61 and Trp-61 mutants, which exhibit similar alterations in both GTPase and nucleotide exchange but differ at least 10-fold in transforming efficiency, as well as the Thr-59 mutant which displays only a partial activation of transforming potential in spite of the effect of this mutation on both nucleotide exchange and GTPase. These discrepancies may reflect nonphysiological in vitro assay conditions. Alternatively, some amino acid substitutions may directly affect the interaction of p21 with regulatory or effector proteins in vivo (19).

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