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Biochemical Properties of Ha-ras Encoded p21 Mutants and Mechanism of the Autophosphorylation Reaction*

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Kinetic studies performed on $p21^H$ guanine nucleotide complexes with and without Mg^{2+} show that point mutations at positions 12, 59, and 61 each have a different effect on the rate of nucleotide dissociation. Double mutants with a combination of these amino acid substitutions reveal that the effects of each mutation on these kinetics are interactive (nonadditive) for positions 12 and 59 and approximately additive for the positions 12 and 61. The magnitude and direction of the effects seen are dependent on the nature of the nucleotide and whether or not the complexes contain Mg^{2+}. All the mutants have reduced GTPase activity.

It is also shown that the autophosphorylation reaction velocity is of first order with respect to the protein concentration and that this reaction is an intramolecular one, which takes place as a side reaction of the GTPase reaction. The autophosphorylation is not reversible under the experimental conditions. The covalently bound phosphate does not decrease the nucleotide-binding ability of the protein nor does it change the relative affinity of the protein for GTP *versus* GDP. The results are discussed in terms of the structural model and function of $p21^H$.

Mutations of the ras proto-oncogenes have been found in many human tumors and in the ras genes of the acutely transforming animal retroviruses Harvey murine sarcoma virus and Kirsten murine sarcoma virus (for reviews see Refs. 1–3). These mutations were found to be single or double point mutations and could be localized in all cases to two different regions of the amino acid sequence of the p21 proteins. Thus, either amino acids 12–13 or amino acids 59/61 were found to be mutated. The retroviral p21 proteins of the Kirsten and Harvey strain of MuSV contain a double mutation involving glycine 12 and alanine 59, where alanine 59 is changed to threonine in both cases (4–6). These double mutations have so far only been found in retroviral ras oncogenes. No mutation at position 59 alone has been found so far. The significance of this is not known. The ras genes have been frequently and reproducibly activated during chemically induced carcinogenesis in animal model systems. It has been established that mutations of ras genes are involved in the early steps of cancerogenesis (7–13). Evidence is accumulating that in certain human tumors the ras gene mutation precedes the development of neoplasia and is not a consequence of neoplastic growth (14, 15).

Little is known about the structure of p21 proteins, and numerous questions regarding the structural and functional consequences of mutations at positions 12/13 and 59/61 remain unanswered. Thus, it has been shown that these mutants have a reduced GTPase activity, are able to transform cultured fibroblasts, and yet have an apparently unaltered GDP/GTP binding (16–19). There is also conflicting evidence regarding the quantitative relationship between a reduced GTPase activity and the transforming properties of the mutants (20–24). The GTPase activity of the Gly^{12},Thr^{59} mutant has been reported to be reduced by Gibbs *et al.* (24) and normal by Lacal *et al.* (20), in spite of its ability to transform NIH3T3 cells (20, 24). Furthermore, it has been suggested that the nucleotide binding site of the transforming mutants is unaltered, which seems unlikely on the basis of their reduced GTPase activities (2, 16, 18, 20, 22, 26, 27). Finally, numerous investigators have reported that p21 mutants with threonine at position 59 become "autophosphorylated" at this residue (16–18, 24, 25), yet failed to show that this reaction is indeed an intramolecular reaction and what the kinetic mechanism of this reaction is.

Here, we have addressed ourselves to these questions. We show that a mutation at positions 12, 59, and 61, in each case, has a characteristic effect on the rate of GDP/GTP release. The effects of the substitution of Gly^{12} and Ala^{59} are opposed to each other and influence each other in the double mutants. We show that the GTPase activity of all Thr^{59} mutants is lower when compared to cellular p21 and that the phosphorylation of Thr^{59} is indeed an intramolecular autophosphorylation reaction.

MATERIALS AND METHODS

[8-^{3}H]GDP (485 GBq/mmol, Amersham Buchler) was diluted with unlabeled GDP (Pharma Waldhof) to the desired specific activity (200–300 cpm/pmol). [γ-^{32}P]GTP (400 GBq/mmol, Amersham Buchler) was diluted with unlabeled GTP (Pharma Waldhof) to the desired specific activity (1500–2000 cpm/pmol or 300–400 cpm/pmol). Nitrocellulose filters, type BA85, 0.45 μm were from Schleicher and Schuell. Isopropyl-β-D-thiogalactoside was from Serva Heidelberg. QAE-Sepharose Fast Flow and G-50 Sephadex Fine were from Pharmacia LKB Biotechnology Inc., while Ultrogel-AcA 54 was from LKB. desoxycytidin-5′-*O*-(thiotriphosphate) was synthesized according to Goody and Isakov (28) and was a kind gift of Dr. R. S. Goody (Max-Planck-Institut). All other chemicals were of the highest purity available.

Mutation of ras-expressing Plasmids—Restriction endonucleases, T4 DNA ligase, polynucleotide kinase, and dNTPs were from Boehringer Mannheim. The above reagents were used as described in the laboratory manual of Maniatis *et al.* (29). Transformation was done according to the method of Hanahan with frozen cells (30) or according to the $CaCl_2$ method (29).

The construction of the plasmids ptac-ras$_v$, ptac-cHras, and ptac-T24 has been described (31). The plasmids ptac-rasA, ptac-rasB, and ptac-rasD were constructed by *in vitro* recombination, as shown in Fig. 1. The rasA gene codes for p21(Gly^{12},Thr^{59}), ras8 for p21(Val^{12},Thr^{59}) and rasD for p21(Arg^{12},Ala^{59}).

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Site-directed mutagenesis was performed according to the method of Eckstein and co-workers (32) using exonuclease III from New England Biolabs and DNA polymerase I (Klenow fragment) from Du Pont-New England Nuclear. Oligonucleotides were synthesized on a DNA synthesizer (Cyclone, Biosearch) according to the phosphoamidite method (33). The DNA sequence of the mutants was verified with the dideoxy method (34).

Protein Purification—The cells were grown in a 10-liter air-lift fermenter. The cell culture medium has been described previously (31). Induction with isopropyl-β-D-thiogalactoside lasted 12–15 h and was initiated when the optical density (660 nm) of the cell culture was approximately 0.7. Cells were harvested, washed with 50 mM Tris·HCl, pH 7.5, shock-frozen, and stored at −70 °C.

Cell lysis and protein purification were performed as described previously (31, 35), except that ion exchange chromatography was performed with QAE-Sepharose fast-flow (Pharmacia) instead of DEAE-Sepharose CL-68 (Pharmacia). The salt gradient remained identical. We obtained a higher purification factor for p21 with QAE-Sepharose Fast-Flow than with DEAE-Sepharose CL-6B and a better separation of p21 from truncated EF-Tu[1] (35). Gel filtration chromatography was performed with Ultrogel-AcA 54 (Pharmacia LKB Biotechnology Inc.), as previously described, or with G-50-Sephadex Fine (Pharmacia). In the latter case, a higher salt concentration in the mobile phase (400 mM NaCl instead of 200 mM NaCl) was found to be useful.

The purified protein was more than 99% pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, whereas its specific activity, as determined by the [8-^{3}H]GDP binding assay, was between 90 and 95%. A specific activity of 100% corresponds to 47,619 pmol of [8-^{3}H]GDP bound per mg of protein. Protein concentrations were determined by the method of Bradford (36), using bovine serum albumin as a standard.

Autokinase Activity—5 μM p21, 50 μM [γ-^{32}P]GTP (1500–2000 cpm/pmol) in buffer B (64 mM Tris·HCl pH 7.6, 10 mM $MgCl_2$, 1 mM dithioerythritol, and 1 mM NaN_3) was incubated at 37 °C in a total volume of 100 μl. At the indicated times, 10-μl portions were removed and quenched with 100 μl of solution C (2 mM GTP, 2 mM GDP, 5 mM KP_i, 5 mM EDTA, pH 7.6, 64 mM Tris·HCl, pH 7.6, 1 mM dithioerythritol) for 10 min at 37 °C. Under these conditions all noncovalently bound ligands (*e.g.* [γ-^{32}P]GTP and $^{32}P_i$) were completely exchanged within 2 min ($\tau \leq 10$ s, data not shown). The incubation mixture was filtered through nitrocellulose filters, washed with 3 × 2 ml rinse buffer (64 mM Tris·HCl, pH 7.6, 5 mM $MgCl_2$, 100 mM KCl, 10 mM NH_4Cl, and 1 mM β-mercaptoethanol) and the filter-bound radioactivity determined by liquid scintillation counting. Essentially similar results were obtained with trichloroacetic acid precipitation, although the background was roughly 50 × higher.

Kinetics of Nucleotide Dissociation—2 μM p21, 10 μM [8-^{3}H]GDP (200–300 cpm/pmol), or 10 μM [γ-^{32}P]GTP (300–400 cpm/pmol), 0.9 mM EDTA, pH 7.6, in buffer A (buffer B, without Mg^{2+}) in a total volume of 1 ml were incubated for 30 min at 21 °C. If required, the Mg^{2+} concentration was brought to 10 mM and the temperature adjusted to 21 °C or 37 °C. A 200-fold excess of unlabeled GDP or GTP, pH 7.5, was added, and 50-μl portions were removed at defined times and diluted with 2 ml of rinse buffer. The mixtures were filtered immediately through nitrocellulose filters, washed, and the filter-bound radioactivity determined by liquid scintillation counting. Dissociation rate constants were determined using nonlinear least squares fits to single exponentials. The percentage error in double or triplicate determinations never exceeded 10% and was on average ±5%.

GTPase Assay—5 μM p21, 50 μM [γ-^{32}P]GTP (1500–2000 cpm/pmol) in buffer B in a total volume of 200 μl was incubated at 37 °C. The production of $^{32}P_i$ was measured as described previously (37).

RESULTS

We have described an inducible bacterial expression system for the Ha-ras encoded p21 proteins $p21_C$ (Gly^{12},Ala^{59}), $p21_V$(Arg^{12},Thr^{59}) and $p21_T$(Val^{12},Ala^{59}), using plasmids containing the tac promoter (31). With this system, large amounts of soluble and highly active p21 proteins are synthesized following induction with isopropyl-β-D-thiogalactoside. These proteins were subsequently used to measure the kinetics of association and dissociation of GDP to p21 at 4 °C. We found that the binding constant of cellular p21 for GDP is to the order of 1×10^{11} M^{-1} at 4 °C (38). We also found that the kinetics of dissociation at 4 °C differ somewhat between $p21_C$, $p21_T$, and $p21_V$.

Using the expression vectors described earlier (31), we created by exchange of restriction fragments the following single and double mutants, p21(Gly^{12},Thr^{59}), p21(Arg^{12},Ala^{59}), and p21(Val^{12},Ala^{59}), as outlined in Fig. 1. The single mutant (Gly^{12},His^{61}) was created by replacing glutamine 61 of cellular p21 with histidine by site-directed mutagenesis (see "Materials and Methods"). The double mutant p21(Val^{12},His^{61}) was made by exchanging the appropriate restriction fragments as described in Fig. 1.

These mutant proteins were expressed in *Escherichia coli*, with 15–20% of the total soluble protein being p21 (data not shown). They were purified to homogeneity using a slight modification of the previously described two-column procedure (see "Materials and Methods" and Ref. 31). The purity of the proteins was greater than 99%, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and all bound [8-^{3}H]GDP in the [8-^{3}H]GDP exchange assay between 90 and 95% of the theoretical maximum (47,619 pmol of GDP/mg of protein).

GDP Dissociation Rate Constants—One of the elementary steps in the enzymatic hydrolysis of GTP is the release of nucleotides from the enzyme nucleotide complex. We have examined the GDP dissociation reaction:

$$p21 \cdot GDP \xrightarrow{k_{-1}} p21 + GDP$$

and determined the effect of amino acid substitutions at positions 12, 59, and 61 on the velocity of this reaction, both in the presence and absence of Mg^{2+}.

The dissociation rate contants were determined as described under "Materials and Methods." We show in Fig. 2 that the rate of GDP dissociation for p21(Val^{12},Ala^{59}) is roughly 3.5 times slower than for cellular p21 (Gly^{12},Ala^{59}) at 37 °C. On the other hand, we (38) and others (39, 40) found earlier that the viral (v-Ha-ras) p21(Arg^{12},Thr^{59}), which is a double mutant, releases its GDP faster than the cellular p21 (Gly^{12},Ala^{59}). Fig. 2 and Table I show that the rate is five times faster at 37 °C. Since viral p21(Arg^{12},Thr^{59}) is a double mutant, we wanted to determine the effect of each individual mutation on the GDP dissociation rate constant k_{-1}. We thus created the single mutants p21(Arg^{12},Ala^{59}) and p21 (Gly^{12}, Thr^{59}), as outlined above. It was also of interest to examine the biochemical properties of the double mutant p21(Val^{12}, Thr^{59}) and to see if mutations at positions 12 and 59 influence the GDP dissociation rate of the double mutant in a strictly additive manner or not.

As shown in Table I, the substitution of glycine 12 alone with arginine also decreases the GDP dissociation rate, the effect being somewhat smaller than with the valine 12 mutation. The substitution of alanine 59 with threonine leads to a 5.5-fold increase in the GDP dissociation rate at 37 °C. The substitution of glycine 12 with valine in cellular p21-(Gly^{12},Ala^{59}) and in p21(Gly^{12},Thr^{59}) reduces the GDP dissociation rate at 37 °C by a factor of 2.1 and 3.4, respectively, as shown in Table I.

A closer examination of the dissociation rates for the double mutants at positions 12 and 59 reveals that the effect of each individual mutation is not strictly additive. This is based on the following argument: let the GDP dissociation rate con-

[1] The abbreviations used are: EF-Tu, bacterial elongation factor Tu; $p21^H$, protein product of the Ha-ras oncogene; P_i, inorganic phosphate.

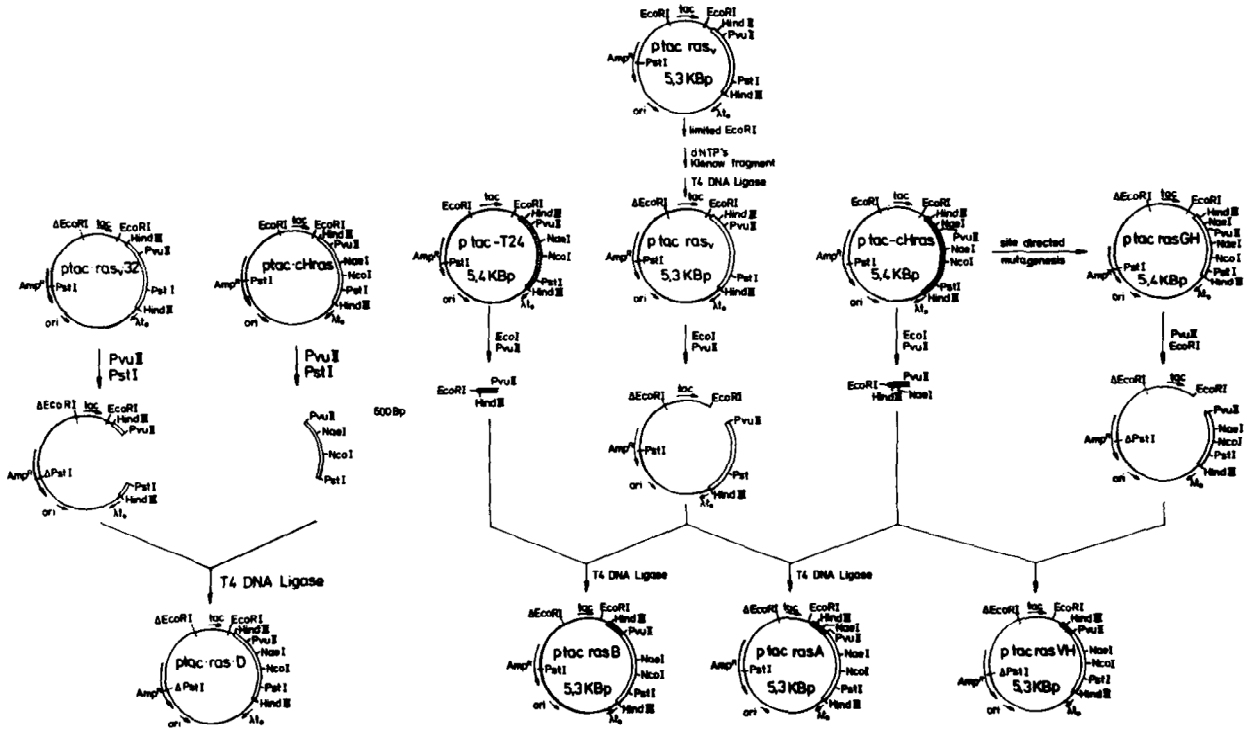


FIG. 1. **Construction of ras mutants and expression vectors.** The plasmids ptac-cHras, ptac-T24, and ptac-ras, were described previously (38). ptac-rasA expresses p21(Gly^{12},Thr^{59}); ptac-rasB expresses p21(Val^{12},Thr^{59}); ptac-rasD expresses p21(Arg^{12},Ala^{59}); ptac-rasGH expresses p21(Gly^{12},His^{61}), and ptac-rasVH expresses p21(Val^{12},His^{61}). Restriction digestion, ligation, and site-directed mutagenesis were performed as described under "Materials and Methods." The *PstI* site in the amp^R gene of ptac-ras 31 was removed by replacing the 250-base pair *BglI-PvuI* fragment of this gene with the corresponding fragment from the plasmid pUC18 (data not shown). *KBp*, kilobase pair(s).

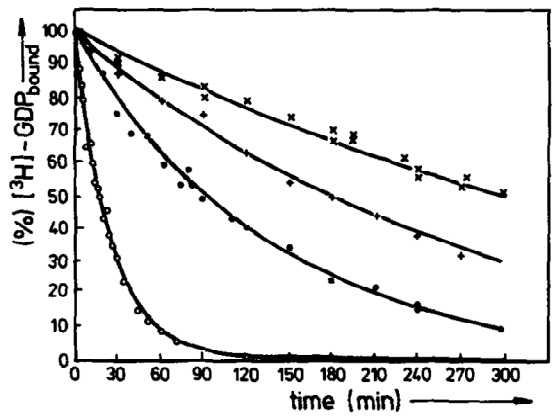


FIG. 2. **Kinetics of the dissociation of GDP from p21^H mutants.** GDP dissociation rates were measured at 37 °C in the presence of 10 mM Mg^{2+}. ●, cellular p21(Gly^{12},Ala^{59}); ○, viral p21(Arg^{12},Thr^{59}); +, p21(Val^{12},Ala^{59}); ×, p21(Arg^{12},Ala^{59}). 2 μM p21 and 10 μM [8-^{3}H] GDP in buffer A (see "Materials and Methods") were incubated with excess EDTA for 30 min at 21 °C. If required, the Mg^{2+} concentration was raised to 10 mM and the temperature adjusted to 21 or 37 °C. A 200-fold excess of GDP was added, and the displacement of [8-^{3}H] GDP from its complex with p21 was measured, as described under "Materials and Methods."

stants of the two mutants (1 and 2) with single amino acid substitutions be designated $k_{-1}{}^1$ and $k_{-1}{}^2$, the rate constant for wild type be $k_{-1}{}^0$, and the observed rate constant for the double mutant be $k_{-1}{}^{12}$.

In the case where the two amino acid substitutions change the free energy of activation (ΔG^A) in an additive manner, *i.e.* independently of each other, the observed rate constant $k_{-1}{}^{12}$ is given by:

$$k_{-1}{}^{12} = (k_{-1}{}^1 \times k_{-1}{}^2)/k_{-1}{}^0$$

as shown under "Appendix."

The rate constants for the double mutation calculated with the above equation are shown in Table I. One can see that these values tend to differ significantly from the observed dissociation rate constants. Although the introduction of the second mutation changes the dissociation rate in a predictable manner, the effects seen are in general not strictly additive. This suggests that the corresponding sequences, which are believed to be part of the active site (1–3), interact with each other in the three-dimensional structure of these double mutants.[2]

Point mutations at position 61, two amino acids away from position 59, have been found in several human tumors (2, 3). Der *et al.* (41) have shown that most mutants with amino acid substitutions at position 61 have transforming activity. It is quite likely that amino acids 59 and 61 are close to or part of the nucleotide binding site (this paper and also Footnote 2) and part of the same structural element.

We mutated glutamine 61 to histidine, a conservative mutation that has been found in a lung carcinoma and a rhabdomyosarcoma (42, 43). The GDP off rates for the mutant p21 (His^{61}) are very similar to those of normal p21^H. Thus, the substitution of glutamine 61 with histidine does not significantly affect the rate of GDP release, in contrast to the substitution of alanine 59 with threonine. The double mutant

[2] S. H. Kim, personal communication.

TABLE I

GDP dissociation rate constants for various single and double mutant p21^H proteins

The dissociation rates were determined as described in the legend to Fig. 1 and under "Materials and Methods."

Protein	GDP dissociation rate constants			Ratio of k_{-1} ($-Mg^{2+}$) to k_{-1} ($+Mg^{2+}$) at 21 °C
	37 °C, 10 mM Mg^{2+}	21 °C, 10 mM Mg^{2+}	21 °C, ≈0.1 μM Mg^{2+}	
	$k_{-1} \times 10^2$ (min^{-1})			
p21(Gly^{12},Ala^{59}) = cellular p21	0.79	0.07	35	500
p21(Val^{12},Ala^{59})	0.23	0.03	9	300
p21(Arg^{12},Ala^{59})	0.37	0.02	20	1000
p21(Gly^{12},Thr^{59})	4.4	0.58	145	250
p21(Arg^{12},Thr^{59}) = viral p21	4 (2.0 ± 0.13)[a]	0.82 (0.17 ± 0.029)[a]	100 (83 ± 11.7)[a]	121
p21(Val^{12},Thr^{59})	2.1 (1.3 ± 0.35)[a]	0.25 (0.25 ± 0.043)[a]	75 (37 ± 6.4)[a]	300
p21(Gly^{12},His^{61})	0.72	0.07	36	500
p21(Val^{12},His^{61})	0.20 (0.2 ± 0.02)[a]	0.02 (0.03 ± 0.005)[a]	12 (9.3 ± 0.93)[a]	600

[a] The numbers in parentheses are the rate constants for the double mutants that would be observed if the individual amino acid substitutions influence the rate constant in an additive manner (see "Appendix"). The errors for these constants were calculated with standard statistical methods using errors of 10% for the experimentally determined rate constants (see "Materials and Methods").

p21(Val^{12},His^{61}) has a decreased GDP dissociation rate, which is very similar to that of the single mutant p21(Val^{12},Gln^{61}). Thus, these double mutations influence each other in an additive manner, as far as the GDP off rate is concerned. We will show later on that this is not the case with the GTP off rates, which suggests an interesting change of conformation between the p21·GDP and p21·GTP forms of this particular mutant.

We have shown earlier that in the active site of EF-Tu and p21, the Mg^{2+} ion is coordinated as a monodentate complex to a nonbridging oxygen atom of the β-phosphate group of GDP (38, 50). In p21, this Mg^{2+} ion is exposed to the solvent, which enables EDTA to rapidly chelate and remove the metal ion from the nucleotide binding site. As we and others have shown, the release of GDP and GTP is accelerated in the presence of EDTA (31, 44–46). The GDP dissociation rates, measured in the presence and absence of Mg^{2+}, are listed in Table I. These rates, which are much higher in the absence of the coordinated metal ion, are in fact too fast to be measured conveniently at 37 °C, and were hence determined at 21 °C. The ratios of the dissociation rate constants measured in the absence and presence of magnesium vary between 120 and 1000 and are lower for proteins with threonine at position 59. Here also, p21(His^{61}) resembles cellular p21, whereas the p21(Val^{12},His^{61}) double mutant is exactly like the p21(Val^{12}) single mutant.

GTP Dissociation Rate Constants—Guanine nucleotide binding proteins are a class of enzymes that perform diverse functions by the same general principle, namely a conformational change between an active GTP state and an inactive GDP state which is triggered by the intrinsic GTPase activity of these proteins (46–49). We have described for EF-Tu the change in sterochemistry of the nucleotide binding site between the GDP-bound and the GTP-bound state (50). Here we examined the influence of mutations on the GTP-bound state in p21 by measuring the GTP dissociation rates of the mutant proteins (Table II). The reaction rates determined in the absence of Mg^{2+} show the same tendency as the corresponding GDP dissociation rates: *i.e.* a mutation in amino acid 12 reduces the GTP dissociation rate, whereas the Ala^{59} → Thr mutation drastically increases the GTP dissociation rates. The magnitude of the effects are, however, different from those shown in Table I for the GDP dissociation rates. The double mutations (positions 12, 59 and 12, 61) have an intermediate effect on the GTP dissociation rate and are again not strictly additive. It is interesting to note that the His^{61} mutation shows a reduction in the dissociation rate of GTP, but not of GDP. This suggests that the structural change is only evident in the GTP conformation. Furthermore, the GTP dissociation rate of the double mutant p21(Val^{12},His^{61}) is influenced by the individual mutations in a nonadditive manner.

To determine the dissociation rates of GTP in the presence of Mg^{2+}, we corrected for the loss of [γ-^{32}P]phospate from the protein due to GTP hydrolysis and the rapid rate of release of $^{32}P_i$.[3] The GTPase rate constants have been determined at 37 °C (see below and Table III), and these values were used to calculate k_{-1} for GTP. The single point mutants p21(Val^{12}) and p21(Arg^{12}) show the expected reduction in the GTP dissociation rate by a factor of 4.9 and 20, respectively. It was surprising to find that the introduction of threonine at position 59, p21(Gly^{12},Thr^{59}), led to a small increase in the GTP dissociation rate, in contrast to the large increase for the GDP dissociation rates shown in Table I. The substitution of a second amino acid, as in p21(Arg^{12},Thr^{59}) and p21(Val^{12},Thr^{59}), does not lead to a significant decrease in the GTP dissociation rate (37 °C, 10 mM Mg^{2+}). Instead, the rate constants are again much higher than what one would expect for a purely additive effect of the individual mutations. The His^{61} mutation shows a 6.2-fold reduction of the GTP dissociation rate which indicates that the structural difference between cellular p21 and p21(His^{61}) is greatest when the protein is in the p21·GTP·Mg^{2+} complex.

A common property of all the transforming mutants, except p21(Val^{12},Gln^{61}) is that the ratio of the GTP dissociation rate constants determined in the absence and presence of Mg^{2+}, determined for technical reasons at two different temperatures, is higher by a factor of 4–10 when compared to cellular p21. Although we cannot interpret this result in structural terms, this finding seems to indicate that when p21 is in the ternary p21·GTP·Mg^{2+} state, there is a characteristic difference between normal and transforming proteins.

GTPase Rate Constants—There have been conflicting reports regarding the GTPase activity of the threonine 59 mutants. It has been reported that the substitution of alanine 59 with threonine does not affect the rate of GTP hydrolysis (20), inspite of this mutant being able to transform NIH3T3 cells. On the other hand, Gibbs *et al.* (18) have shown that a p21 fusion protein with threonine 59 has a reduced GTPase activity. Therefore we tested the *in vitro* GTPase activity of

[3] J. John, M. Frech, and A. Wittinghofer, manuscript in preparation.

TABLE II

GTP dissociation rate constants

The rate constants were determined as described in the legend to Fig. 2 and under "Materials and Methods," except that [γ-^{32}P]GTP (≈200 cpm/pmol) was used instead of [8-^{3}H]GDP. In the presence of Mg^{2+}, the observed rate constant (k_0) is related to the GTP dissociation rate constant (k_{-1}) and the rate constant of GTP hydrolysis (k_{+2}) by the equation $k_0 = k_{-1} + k_{+2}$.

Protein	GTP dissociation rate constants		Ratio of k_{-1} ($-Mg^{2+}$, 21 °C) to k_{-1} ($+Mg^{2+}$, 37 °C)
	21 °C, ≈0.1 μM Mg^{2+}	37 °C, 10 mM Mg^{2+}	
	$k_{-1} \times 10^2$ (min^{-1})		
p21(Gly^{12},Ala^{59}) = cellular p21	17	≈2.3	7
p21(Val^{12},Ala^{59})	13	0.47	28
p21(Arg^{12},Ala^{59})	3.7	0.11	33
p21(Gly^{12},Thr^{59})	219	2.8	78
p21(Arg^{12},Thr^{59}) = viral p21	93 (48 ± 8.2)[a]	3.1 (0.13 ± 0.02)[a]	30
p21(Val^{12},Thr^{59})	69 (167 ± 29)[a]	2.3 (0.57 ± 0.1)[a]	30
p21(Gly^{12},His^{61})	13.6	0.37	36
p21(Val^{12},His^{61})	4.4 (10.4 ± 1.8)[a]	0.7 (0.075 ± 0.013)[a]	6

[a] As in Table I.

TABLE III

GTPase and autophosphorylation rate constants of various p21 proteins

The GTPase and autophosphorylation rate constants were determined at 37 °C. 5 μM p21 and 50 μM [γ-^{32}P]GTP (≈2000 cpm/pmol) in buffer B in a total volume of 200 μl were incubated at 37 °C. The production of $^{32}P_i$ and phosphorylated p21 was monitored as described under "Materials and Methods."

Protein	GTPase	Autophosphorylation
	$k \times 10^3$ (min^{-1})	$k \times 10^4$ (min^{-1})
p21(Gly^{12},Ala^{59})	10.2	
p21(Val^{12},Ala^{59})	2.0	
p21(Arg^{12},Thr^{59})	1.4	2.3
p21(Gly^{12},Thr^{59})	2.4	3.8
p21(Val^{12},Thr^{59})	1.8	5.8
p21(Arg^{12},Ala^{59})	1.4	
p21(Gly^{12},His^{61})	1.9	
p21(Val^{12},His^{61})	1.7	

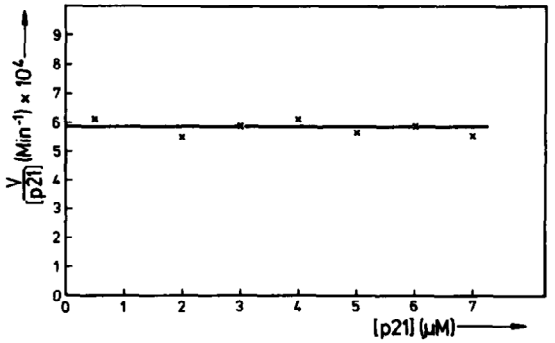


FIG. 3. **The reaction order of the autophosphorylation reaction.** The effective velocity $v/[E_0]$ is plotted against the protein concentration. 50 μM [γ-^{32}P]GTP and 0.5–7 μM p21(Val^{12},Thr^{59}) in buffer B (see "Materials and Methods") was incubated at 37 °C in a final volume of 100 μl. The reaction velocity (v) was measured as described in the legend to Fig. 4 and under "Materials and Methods."

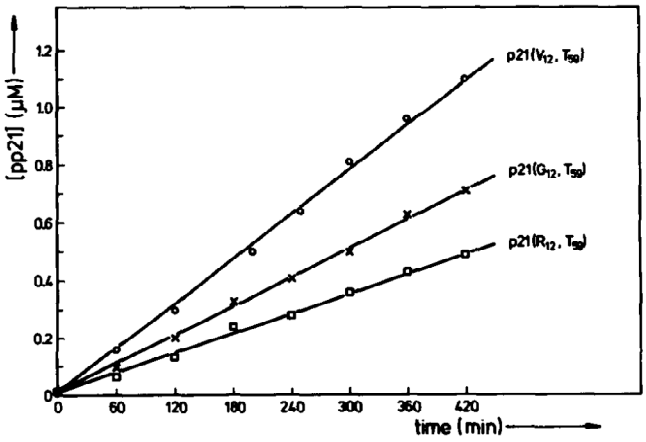


FIG. 4. **Autophosphorylation of p21^{H} mutants at threonine 59.** 5 μM p21 and 50 μM [γ-^{32}P]GTP in buffer B (see "Materials and Methods") was incubated at 37 °C in a final volume of 100 μl. 10 μl portions were removed at indicated times and covalently bound [^{32}P] phosphate was determined as described under "Materials and Methods." Abbreviations: G_{12} = glycine 12; V_{12} = valine 12; R_{12} = arginine 12; T_{59} = threonine 59; and *pp21* is phosphorylated p21.

p21(Gly^{12},Thr^{59}) and the other mutants. The results of these experiments are shown in Table III. They demonstrate that all single and double mutants with threonine at position 59 have a reduced GTPase activity. The GTPase activity of these mutants and the single mutants in amino acid 12 are reduced by a factor of 5–10. Besides this, the substitution of glutamine 61 with histidine also leads to a 5-fold decrease in the rate of GTP hydrolysis (Table III). Thus, amino acid substitutions at positions 12, 59, and 61 lead to a large decrease in the GTPase activity. The absolute values of the GTPase reaction rates of single and double mutants do not reveal any tendency as in the case of the GDP/GTP dissociation rate constants. However, since a single turnover of the GTPase reaction at 37 °C for the mutants takes about 500 min, the GTPase rates cannot be measured with an error of less than ±25%.

Rates and Mechanism of Autophosphorylation—It has previously been observed that p21 mutants with threonine at position 59 become covalently modified at this residue, due to the formation of a phosphothreonine ester on incubation with GTP (16–18, 24, 25). This reaction has been thought to be an autophosphorylation reaction. We have indeed found that the velocity of this reaction is of first order with respect to the protein concentration in the presence of excess GTP (Fig. 3). In other words, the effective reaction rate ($v/[E]_0$) is independent of the protein concentration. This demonstrates that the covalent attachment of the γ-phosphate group of bound GTP to threonine 59 is an intramolecular reaction, which implies that threonine 59 is situated close enough to participate in nucleophilic attack on the γ-phosphate group of GTP. Similar evidence has been presented for the autophosphory-

lation of protein kinase C by ATP (51).

The autophosphorylating activity of other threonine 59 mutants, *e.g.* p21(Val^{12},Thr^{59}), p21(Gly^{12},Thr^{59}), has been determined in crude extracts of NIH3T3 cells that were transformed with the corresponding ras oncogenes and with purified proteins (18, 24). However, the rates differ from each other by almost an order of magnitude (18, 24). We have determined the rate constants of the reaction using our purified p21 proteins (Fig. 4). The results are also shown in Table III. The reaction rate constants lie between 2.3×10^{-4} min^{-1} and 5.8×10^{-4} min^{-1}. p21(Val^{12},Thr^{59}) having the highest rate constant and p21(Arg^{12},Thr^{59}) the lowest. Thus, the reaction rate is modulated by substitutions at position 12, where the replacement of glycine 12 with valine increases the reaction rate, whereas a replacement with arginine leads to a decrease in the reaction rate. This has been observed in crude extracts (24), but other effects have been reported with purified proteins (18). In our hands, however, the GTPase reaction is always faster than the autophosphorylation reaction. This is also true for p21(Val^{12},Thr^{59}). The ratio of the GTPase reaction rate to the autophosphorylation reaction rate is approximately 6:1 for the p21(Gly^{12},Thr^{59}) and p21(Arg^{12},Thr^{59}) mutants and 3:1 for the p21(Val^{12},Thr^{59}) mutant.

The dephosphorylation rate, which has not been measured before, was estimated for the phosphoproteins pp21(Gly^{12},Thr^{59}), pp21(Val^{12},Thr^{59}), and pp21(Arg^{12},Thr^{59}). The rate constants of this reaction are at least 4 times slower than the forward rate of autophosphorylation and are in fact at the lower limit of detection ($\leq 6 \times 10^{-5}$ min^{-1}, data not shown). Thus, the dephosphorylation reaction can be neglected during the initial stages (8 h) of the autophosphorylation. Besides this, we observed that the phosphorylated threonine 59 of pp21 is a very poor substrate for alkaline phosphatase. Thus, no significant increase in the hydrolysis rate of the phosphorylated threonine in the above three phosphoproteins was observed when excess alkaline phosphatase was present in the reaction mixture (data not shown). This suggests that the phosphate group is not totally exposed to the solvent.

Since we expect threonine 59 to be situated at or close to the nucleotide binding site, and fairly close to the γ-phosphate group of bound GTP, we wished to know whether the phosphate ester of threonine hinders the binding of GTP and/or GDP. To do this, we determined the relative affinity of p21 for GTP *versus* GDP during the autophosphorylation reaction. Fig. 5 shows the binding of an equimolar mixture of [8-^{3}H]GDP and [γ-^{32}P]GTP to p21(Arg^{12},Thr^{59}) and p21(Val^{12},Thr^{59}) during the time course of the reaction of these proteins with GTP. After 8 h at 37 °C, when 28% of p21(Val^{12},Thr^{59}) is phosphorylated at Thr^{59}, the ratio of bound GTP *versus* total nucleotide binding remains unchanged. Control experiments verified that total nucleotide binding does not change either during the time course of the experiment (data not shown). We conclude that the phosphorylation of threonine 59 has no measurable effect on the relative affinity of GTP, at least for these mutants. This suggests that the electrostatic repulsion between the γ-phosphate group of bound GTP and the phosphate of phosphothreonine is negligible, in relation to the binding energy of GTP. This could be due to a given flexibility of the threonine 59 residue and/or the peptide backbone.

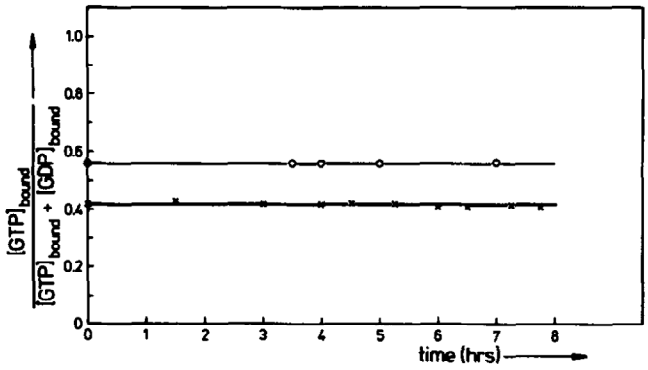


FIG. 5. **The influence of the autophosphorylation on the relative affinity of GTP.** ○, p21(Arg^{12},Thr^{59}); ×, p21(Val^{12},Thr^{59}). 5 μM p21, 50 μM GTP in buffer B (see "Materials and Methods") was incubated in a total volume of 1 ml at 37 °C. At the indicated times, 100-μl portions were removed and desalted on a 2-ml Sephadex-G25 column (NAP-5, Pharmacia) equilibrated in buffer A (see "Materials and Methods"). The desalted protein was collected (350 μl, [p21] ≈ 1.5 μM), the EDTA concentration adjusted to 0.9 mM, and saturated with 10 μM [8-^{3}H]GDP (specific activity ≈ 200 cpm/pmol) and 10 μM [γ-^{32}P]GTP (specific activity ≈ 200 cpm/pmol). After 20 min at 21 °C, nucleotide binding was determined by the filter binding test (see "Materials and Methods"). A control experiment showed no decrease of nucleotide binding during the incubation with [^{3}H]GTP over the same time range (data not shown).

DISCUSSION

We have shown here that three different single point mutations in $p21^H$ lead to different effects on the GDP/GTP binding site, as revealed by the analysis of guanine nucleotide dissociation rates, measured in the presence and absence of Mg^{2+} ions. At the same time, the GTPase reaction rate is of a similar magnitude for all the mutants, including p21(Gly^{12},Thr^{59}). In the structure proposed for p21, based on the structure of the GDP binding domain of EF-Tu (52, 53), glycine 12 and residues 59 and 61 are situated in two different loops that connect secondary structural elements. According to NMR measurements that have been performed on the cellular and transforming p21 mutants, the overall structure of all forms of $p21^H$ seems to be similar.[4] Thus, the structural changes caused by mutations at positions 59, 61, and 12 are most likely localized to the immediate surroundings of these amino acids. Our results presented here suggest that if amino acids 59/61 and 12 are localized in loops, as in the EF-Tu structure, the local chain tracing of these loops can be changed independently of the overall three-dimensional structure. The fact that in the double mutants the structural changes due to each single mutation are not additive seems to indicate that these structural elements are situated close to each other in the three-dimensional structure of p21. The results also show that these loops are probably in close proximity to the guanine nucleotide binding site, since the binding of nucleotides and/or the GTPase activity is affected by the replacement of these amino acids.

The explanation for the high incidence of glycine residues in the loop containing glycine 12 has been that this loop forms a tight turn, similar to the one observed in other mononucleotide binding proteins such as adenylate kinase (54–56). In adenylate kinase and EF-Tu, the phosphate groups of ATP and GDP are situated close to the glycine-rich loop. Several glycine residues in the glycine-rich loop of adenylate kinase have backbone (φ, ψ) dihedral angles that are unallowed for other amino acids (57). Replacement of such a glycine by most other amino acids would thus lead to an "opening up" of the loop. Our results on single amino acid substitutions of glycine 12 in p21 show that the mode of nucleotide binding is indeed changed and support the notion that the nucleotide is bound close to the glycine-rich loop in p21 as well. Studies

[4] I. Schlichting, J. John, A. Wittinghofer, and P. Rösch, manuscript in preparation.

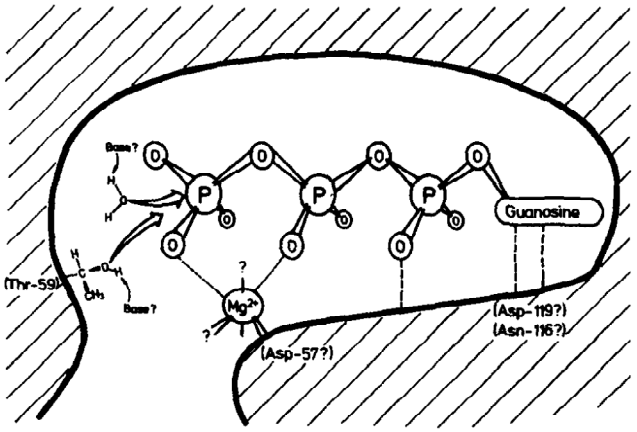


FIG. 6. **A model for the active site of p21.** The *continuous black line* represents the active site of the protein. From previous studies, we know that the Mg^{2+} ion is only coordinated with the β-phosphate group and that the pro-R oxygen atom of the α-phosphate group interacts with the protein (31, 38). This study shows that the γ-phosphate group of GTP is fairly close to the hydroxyl group of threonine 59.

leading to similar conclusions have been performed on the glycine-rich loop of adenylate kinase from *E. coli.* (58).

Mutations of alanine 59 and glutamine 61 have been shown to lead to activated p21 proteins with transforming properties (1–3). Although mutations at both positions result in a reduced GTPase activity, the influence on the dissociation of guanine nucleotides is different. The substitution of alanine 59 with threonine leads to a 5-fold increase in the GDP dissociation rate, whereas the substitution of glutamine 61 with histidine has virtually no effect. On the other hand, the dissociation rate for GTP in the presence of Mg^{2+} is unaltered for p21(Gly^{12},Thr^{59}) and strongly reduced for p21(Gly^{12},His^{61}). The similar reduction of the rate of the GTPase is thus the result of different structural changes of the native p21 structure.

We have shown here that the autophosphorylation reaction at threonine 59 is an intramolecular reaction, which demonstrates that threonine 59 is close to the γ-phosphate group of GTP. The autophosphorylation reaction is always slower, although to a varying degree, than the GTPase reaction of the same mutant and has the same specificity for GTP as the GTPase reaction (59). This suggests that the autophosphorylation reaction is a side reaction of the GTPase reaction.

Based on this, we have developed a model of the active site, as shown in Fig. 6. According to this, GTP would either be hydrolyzed by nucleophilic attack of water, with inversion of configuration, as has been shown for EF-Tu (60) and p21,[5] or by the hydroxyl group of threonine 59. The rate constants for this reaction are given in Table III and show that the nature of the amino acid at position 12 influences the rate of autophosphorylation, as has been observed before (18, 24). There is, however, no correlation such that a slow GTPase reaction corresponds to a slow autophosphorylation reaction and vice versa.

Finally, we would like to address ourselves to the correlation between the transforming ability of various p21 proteins and their biochemical properties. The original hypothesis was that p21 acts as a signal-transducing molecule, where the mutated p21 proteins have a longer lasting signal due to their reduced GTPase activity. The latter presumably serves to switch off the growth-signalling pathway (1–3, 16–20, 47). This idea was questioned, because some transforming proteins were reported to have an apparently normal GTPase activity or bind GDP/GTP with much lower affinity (20, 61–63). We have shown here that all mutants, including p21(Gly^{12},Thr^{59}), have a reduced GTPase activity. Other transforming mutants exhibit a strongly decreased binding constant for GTP, due to an increased dissociation rate (63). It has been proposed that the fast exchange of guanine nucleotides is the physiologically relevant parameter and that cellular and transforming p21 proteins differ in this respect (63). Our results with the dissociation rates of various mutant p21 proteins show that their dissociation rates may be increased, decreased, or remain unchanged, whereas their GTPase activities are reduced. We, therefore, still favor the idea that it is the reduced GTPase activity that is responsible for the transforming activity of these mutants. It is, however, likely that the GTPase rates or nucleotide exchange kinetics measured *in vitro* are different from those prevailing in the natural system, where presumably the GDP/GTP exchange reaction is mediated by an activated receptor, whereas another protein influences the rate of GTP hydrolysis.

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APPENDIX

Derivation of the Equation $k_{-1}^{12} = (k_{-1}^{1} \times k_{-1}^{2})/k_{-1}^{0}$

From equilibrium thermodynamics and transition state theory we know that the rate constant (k_{-1}) of a first order reaction is related to the corresponding free energy of activation (ΔG^{A}) by the following equation:

$$k_{-1} = (kT/h)\exp(-\Delta G^{A}/RT) \quad (1)$$

where T is the absolute temperature, R the universal gas constant, k is the Boltzmann constant and h is Planck's constant (64).

Let k_{-1}^{0} be the rate constant for wild type, k_{-1}^{1} the rate constant for the single mutant 1, k_{-1}^{2} the rate constant for the single mutant 2, and k_{-1}^{12} the rate constant for the corresponding double mutant 12.

The corresponding free energy of activation are designated ΔG^{A}_{0}, ΔG^{A}_{1}, ΔG^{A}_{2}, and ΔG^{A}_{12}, respectively.

Thus, the change in the free energy of activation, with respect to wild type, for the two single mutants are, $\Delta G^{A}_{1} - \Delta G^{A}_{0}$ for mutant 1 and, $\Delta G^{A}_{2} - \Delta G^{A}_{0}$ for mutant 2.

If the individual amino acid substitutions in the double mutant influence the free energy of activation in an additive manner, *i.e.* independently of each other, then the free energy of activation for the double mutant (ΔG^{A}_{12}) is given by,

$$\Delta G^{A}_{12} = \Delta G^{A}_{0} + (\Delta G^{A}_{1} - \Delta G^{A}_{0}) + (\Delta G^{A}_{2} - \Delta G^{A}_{0}) \quad (2)$$

or,

$$\Delta G^{A}_{12} = \Delta G^{A}_{1} + \Delta G^{A}_{2} - \Delta G^{A}_{0} \quad (3)$$

based on this assumption, k_{-1}^{12} is given by,

$$k_{-1}^{12} = (kT/h)\exp\{-(\Delta G^{A}_{1} + \Delta G^{A}_{2} - \Delta G^{A}_{0})/RT\} \quad (4)$$

which can be rewritten as,

$$k_{-1}^{12} = \frac{(kT/h)\exp(-\Delta G^{A}_{1}/RT)\exp(-\Delta G^{A}_{2}/RT)}{\exp(-\Delta G^{A}_{0}/RT)} \quad (5)$$

which simplifies to,

$$k_{-1}^{12} = (k_{-1}^{1} \times k_{-1}^{2})/k_{-1}^{0}. \quad (6)$$

[5] M. R. Webb, J. Feuerstein, and R. S. Goody, unpublished data.

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