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# Proton NMR Studies of Transforming and Nontransforming H-*ras* p21 Mutants

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ABSTRACT: One- and two-dimensional nuclear magnetic resonance spectroscopy (1D and 2D NMR) and site-directed mutagenesis were used to study the influence of mutations on the conformation of the H-*ras* oncogene product p21. No severe structural differences between the different mutants, whether they were transforming or nontransforming, could be detected. Initially, selective incorporation of 3,5-deuterated tyrosyl residues into p21 and 2D NMR were used to identify the resonances representing the spin systems of the imidazole rings of the three histidyl residues in the protein, of six of the nine tyrosyl rings, and of four of the five phenylalanyl rings. The spin systems of the phenyl rings of $Phe^{28}$, $Phe^{78}$, and $Phe^{82}$ could be assigned by using mutant proteins, since no severe structure-induced spectral changes in the aromatic part of the spectra of the mutant proteins were detected. Sequence-specific assignments of the histidine imidazole resonances could be obtained by comparison of the distance information obtained by nuclear Overhauser enhancement spectroscopy (NOESY) experiments with the crystal structure. The change in the chemical shift values of the H1′ proton and the $\alpha$-phosphate of the bound GDP in the NMR spectra of the p21(F28L) mutant and the 28-fold increase in the GDP dissociation rate constants of this mutant suggest a strong interaction between $Phe^{28}$ and the p21-bound nucleotide. In solution, the p21-bound GDP·$Mg^{2+}$ has an anti conformation, and the phenyl ring of $Phe^{28}$ is close to the ribose of the bound GDP·$Mg^{2+}$.

The products of the *ras* gene family are highly related proteins of molecular weight 21 000 termed p21. They have chain lengths of 189 amino acids; their sequences are identical for the N-terminal 80 amino acids and over 85% identical up to amino acid 164/165. The C-terminal 25 amino acids are very divergent, except for the Cys-A-A-X-OH motif at the end of the chain (A being an aliphatic residue). Normal (cellular) *ras* genes acquire transforming properties by single point mutations within their coding sequences. *ras* genes carrying these mutations have been detected in a significant fraction of human cancers as well as in experimentally induced animal tumors [for a recent review, see, e.g., Barbacid (1987)]. p21 proteins are thus believed to play an essential role in cellular growth and/or development (Bishop, 1983). They bind guanine nucleotides with high affinity and specificity and exhibit low GTPase activity (Feuerstein et al., 1987; Gibbs et al., 1984; McGrath et al., 1984; Sweet et al., 1984; Manne et al., 1985). Due to their significant sequence homology and biochemical similarities to guanine nucleotide (G) binding proteins such as transducin and the bacterial elongation factor Tu (EF-Tu),[1]

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[1] Abbreviations: 1D, one dimensional; 2D, two dimensional; COSY, correlated spectroscopy; DQF, double quantum filtered; DSS, sodium 2,2-dimethyl-2-silapentanesulfonate; EDTA, ethylenediaminetetraacetic acid; EF-Tu, bacterial elongation factor EF-Tu; GMP, guanosine 5′-monophosphate; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; H-ras, Harvey *ras*; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; NMR, nuclear magnetic resonance; $p21_c$, cellular H-*ras* p21; $p21_v$, viral H-*ras* p21 (G12R, A59T); $pK_a$, apparent p*K* value; TPPI, time-proportional incrementation method.

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it is assumed that the p21 proteins are structurally and functionally related to this group of proteins (Gay & Walker, 1983; Wierenga & Hol, 1983; Lebermann & Egner, 1984; Halliday, 1983; Hurley et al., 1984; Bourne, 1985).

It is characteristic for G binding proteins that their regulatory function is modulated by GTP·$Mg^{2+}$ and GDP·$Mg^{2+}$ binding [for reviews, see, e.g., Kaziro (1978), Gilman (1984), and Allende (1988)]. The GTPase activity of p21 is lowered in oncogenic forms of the protein with amino acid replacements at position 12 (McGrath et al., 1984; Gibbs et al., 1984; Sweet et al., 1984; Manne et al., 1985) and other positions, e.g., 61 (Der et al., 1986). These substitutions probably cause structural differences close to the nucleotide binding site which are responsible for the altered GTPase activity and nucleotide binding characteristics (John et al., 1988; Pincus & Brandt-Rauf, 1985; Murakami, 1985; de Vos et al., 1988). Obviously, knowledge of the structural properties of the nucleotide binding site is essential for the understanding of these differences.

In order to get information on structural features of proteins in solution, the method of choice is currently proton nuclear magnetic resonance (NMR) spectroscopy (Wüthrich, 1986). Recently, we were able to characterize structural changes caused by mutations in the nucleotide binding loop in *Escherichia coli* adenylate kinase by NMR spectroscopy (Reinstein et al., 1988); hence, we employed this method to study mutants of p21. In particular, two-dimensional (2D) nuclear Overhauser enhancement spectroscopy (NOESY) can be used to determine spatial relations between protons even in some larger proteins due to the fact that the cross-peak intensities are proportional to the inverse sixth power of mutual proton distances (Wüthrich, 1986). Thus, we were able to show earlier that a phenylalanyl side chain is within 0.5 nm of the ribose C1′–H of the p21 bound GDP·$Mg^{2+}$ (Schlichting et al., 1988). To characterize structural features of the protein in solution as well as monitor structural changes induced by transforming mutations and by exchanging GDP·$Mg^{2+}$ and GTP·$Mg^{2+}$, it is preferable to assign at least some resonances in the proton NMR spectrum to specific residues in the protein sequence. Here we describe the use of site-directed mutagenesis and incorporation of isotopically labeled amino acids into p21 to tackle the assignment problem and at the same time study the influence of mutations leading to transforming and nontransforming variants of the protein.

## Materials and Methods

*Protein Purification.* p21 was purified as the p21·GDP·$Mg^{2+}$ complex as described by Tucker et al. (1986). The protein was more than 95% pure as judged by SDS–PAGE, while its specific activity was 85–93% of the theoretical maximum (47691 pmol of GDP/mg of protein) as determined by the [8-$^3$H]GDP binding assay. [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). [$\gamma$-$^{32}$P]GTP (400 GBq/mmol, Amersham Buchler) was diluted with unlabeled GTP (Pharma Waldhof) to the desired specific activity (1500–2000 or 300–400 cpm/pmol). Nitrocellulose filters (type BA85, 0.45 $\mu$m) were obtained from Schleicher & Schuell. Isopropyl $\beta$-D-thiogalactoside was purchased from Biomol. $D_2O$ (99.96%) was obtained from Sigma. All chemicals were of the highest purity commerically available.

*Mutation of ras-Expressing Plasmids.* Restriction endonucleases, T4 DNA ligase, polynucleotide kinase, and dNTPs were from Boehringer, Mannheim. dCTP$\alpha$S was synthesized according to Goody and Isakov (1986). These reagents were used as described in the laboratory manual of Maniatis et al. (1982). Transformation was done according to the method of Hanahan (1983) or by the $CaCl_2$ method (Maniatias et al., 1982). Site-directed mutagenesis was performed according to Taylor et al. (1985) using exonuclease III from New England Biolabs and DNA polymerase I (Klenow fragment) from NEN. Oligonucleotides were synthesized on a DNA synthesizer (Cyclone, Biosearch) with the phosphoamidite method (Gait, 1984). The sequences we synthesized were

p21F28L: 5′ AG CTG ATC CAG AAC CAG* C*TA* GTG GAC GAA TAC 3′

p21F78Y: 5′ AAA CAC ACA CAG GT*A C*CCCTC CCC GGT G 3′

p21F82Y: 5′ TGT GTG TA*T GCC ATC 3′

p21Y71F: 5′ G CGG GAC CAG TT*C ATG CGC AC 3′

where an asterisk denotes the position of the mismatches. The DNA sequence of the mutants were verified with the dideoxy method (Sanger et al., 1977).

*Selective Deuteration of Tyrosines.* As in phenols and hydroxyaromatics, the hydrogens ortho to the hydroxyl group in L-tyrosine can be specifically exchanged with deuterium in acidic medium (Martin & Morlino, 1965). After a 0.3 M solution of L-tyrosine in 10% DCl in $D_2O$ was refluxed for 24 h, the pH of the cooled solution was adjusted to 5.6, and the precipitate was analyzed by $^1$H NMR. There was no significant racemization (the $C_\alpha$–H was not exchanged), and the hydrogens at positions 3 and 5 of the aromatic ring were exchanged to over 98%.

For the preparation of the selectively deuterated enzyme, the CGSC strain 6662 {λ–, *tyrA16*::*Tn10*, *IN*(*rrnD-rrnE*)1} was transformed with the p21 expression vector p*tac*-cH*ras* (Tucker et al., 1986) and the lac repressor containing PDMI,1 plasmid (Certa et al., 1986). This strain carries a mutation in the chorismate mutase T–prephenate dehydrogenase. Thus, this strain was expected to be tyrosine auxotrophic. In spite of that, the bacteria are also growing in a tyrosine-deficient medium, albeit 4–6 times more slowly than in a tyrosine-rich medium. Bacteria were grown in a 10-L air-lift fermenter in a medium containing 60 mg/L L-[2,5-$^2$H]tyrosine, 5 g/L protonated amino acids (except tyrosine) composed with the relative frequency of amino acids in *E. coli* proteins after Lehninger (1970), 5 g/L $K_2HPO_4/KH_2PO_4$, 0.5 g/L NaCl, 1 g/L $NH_4Cl$, 1.8% glycerol, 4 g/L glucose, 0.1 mM $CaCl_2$, 1 g/L $MgSO_4$, traces of $FeSO_4$, 50 mg/L kanamycin, and 100 mg/L ampicillin. Expression of p21 was induced with 100 $\mu$M IPTG when the optical density (660 nm) of the cell culture was about 0.7. After 12 h of induction, the cells were harvested and washed with 30 mM Tris, pH 7.6, 5 mM EDTA, 5 mM $MgCl_2$, 0.5 mM $NaN_3$, and 0.5 mM DTE.

*NMR Spectroscopy.* For preparation of the NMR samples, the protein solution was freeze-dried and redissolved in $D_2O$ twice in order to exchange labile protons. The samples contained approximately 45 mM potassium phosphate, 45 mM $MgCl_2$, 8 mM DTE, and 4 mM $NaN_3$. The pH was 6.5–6.6, except for the p21(Y71F) sample which was at pH 7.0. The protein concentration was 3.0–3.5 mM.

NMR experiments were performed on a commercial Bruker AM 500 spectrometer working at a proton resonance frequency of 500 MHz; 5-mm sample tubes were used for all experiments except the pH titrations, where 10-mm tubes and a 10-mm probe were used for convenience. All spectra are referenced to internal sodium 2,2-dimethyl-2-silapentanesulfonate (DSS). Sample temperature was kept at 303 K with a precooled

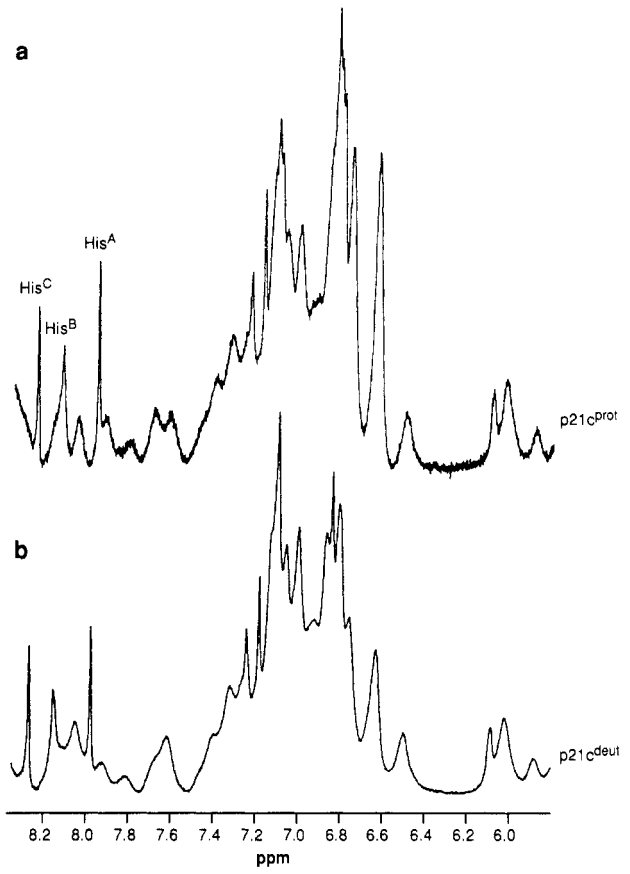


FIGURE 1: Proton NMR spectra of the aromatic side chains of $p21_c$ (top trace) and $p21_c$ partially composed of 3,5-deuterated tyrosine.

stream of dry air which was temperature-regulated with a standard Bruker VT1000 unit. The residual HDO resonance was suppressed by continuous (except acquisition) selective irradiation at the HDO frequency. Quadrature detection was used in all experiments. The two-dimensional experiments were performed in the phase-sensitive mode using the time-proportional incrementation method (TPPI; Marion & Wüthrich, 1983). For COSY and NOESY spectra, usually 128 transients of 4K data points were collected for each of 512 increments with a relaxation delay of 1.1 s between successive transients. The NOESY spectra were recorded with a mixing time of 0.15 s which was randomly varied by 15%. A sweep width of 4545.45 Hz was used in both dimensions. Prior to Fourier transformation, apodization was carried out in both dimensions using an unshifted sine-bell filter for the double-quantum-filtered COSY spectra and a $\pi/32$-shifted sine-bell filter for the NOESY spectra. After zero-filling in $t_1$, the digital resolution was 8.8 Hz/point. Standard procedures and commercially available software were used throughout. In addition, for data evaluation, a software package in part supplied by R. Kaptein, Utrecht, implemented in the C-programming language under the UNIX operating system on a Convex C 210 computer was used. 2D difference spectra were obtained by subtracting weighed frequency domain matrices.

Samples for $^{31}P$ NMR and pH titrations contained 1 mM protein in 50 mM $D_2O$/Tris or $D_2O$/Tris–maleate buffer, 10 mM $MgCl_2$, 2 mM DTE, and 1 mM $NaN_3$. pH values were adjusted with NaOD and DCl. Reported pH values represent direct meter readings. In order to control the integrity of the protein structure, binding of GDP·$Mg^{2+}$ was constantly verified by $^{31}P$ NMR. Therefore, $^{31}P$ NMR spectra were recorded every 1.5 pH units. All pH titrations were performed in 10-mm sample tubes with a 10-mm probe. $pK_a$ values were determined by fitting the Henderson–Hasselbalch equation to the experimental data.

*Other Methods.* GDP and GTP dissociation rates in the presence and absence of $Mg^{2+}$ at 21 and 37 °C as well as GTPase rate constants were determined as described previously (John et al., 1988).

## Results

*Identification of Spin Systems of $p21_c$.* Although the one-dimensional spectra of p21 and all the mutants studied here are rather ill-resolved as compared to those of other proteins in this molecular weight range, three singlet resonances—arbitrarily termed A, B, and C—in the imidazole C2–H resonance region can be discerned easily (Figure 1). The chemical shifts of all three resonances are pH dependent with $pK_a$ values of 6.3, 6.7, and 7.2, respectively, as expected for imidazole rings of histidyl residues (Table I). Thus, they represent the imidazole C2–H resonances of the three histidyl residues in $p21_c$. In addition to the $pK_a$ value of 7.2, we found a second $pK_a$ value of 5.6 for the imidazole ring of $His^C$ (Figure 2a and Table I).

In contrast to the poor quality of the 1D spectra, the phase-sensitive two-dimensional spectra are surprisingly well resolved. The complete imidazole spin systems of the three

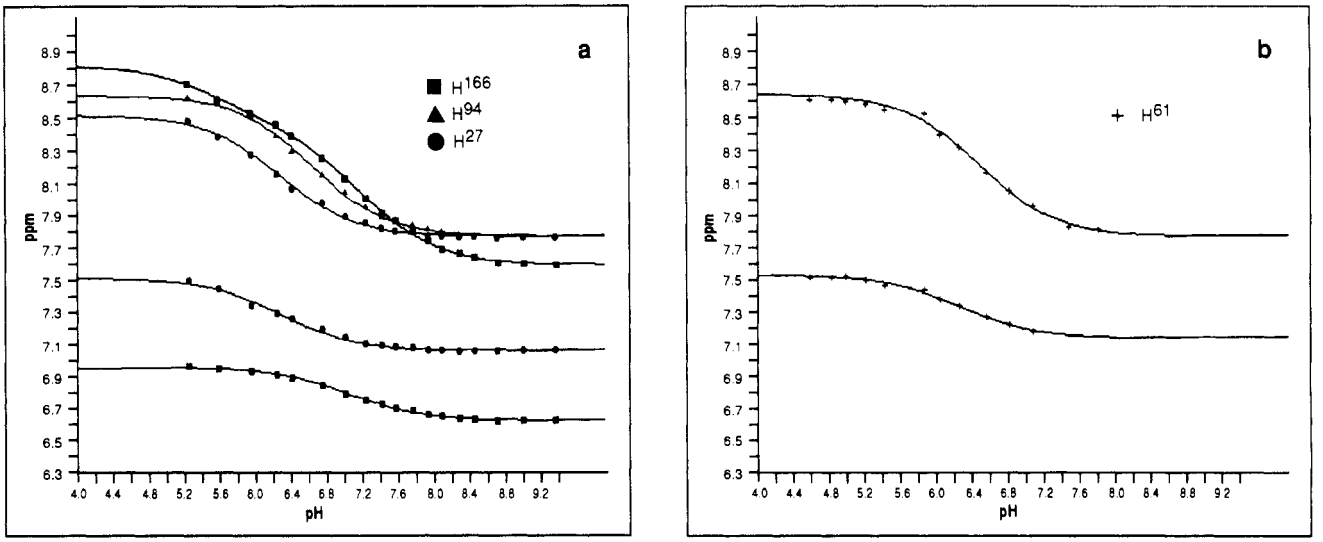


FIGURE 2: Dependence of the chemical shift of the imidazole C2–H and C4–H resonances of (a) $p21_c$ and (b) p21(Q61H) on the pH.

Table I: Titration Parameters for Histidyl Residues[a]

| | $pK_a$ | $\delta_{AH}$ | $\delta_{A^-}$ | $pK_a'$ | $\delta_{AH}'$ | $\delta_{A^-}'$ |
|---|---|---|---|---|---|---|
| | | $p21_c$ | | | | |
| $His^{A(94)}C2-H$ | 6.69 | 8.64 | 7.7 | | | |
| $His^{B(27)}C2-H$ | 6.33 | 8.52 | 7.7 | | | |
| $His^{B(27)}C4-H$ | | 7.51 | 7.0 | | | |
| $His^{C(166)}C2-H$ | 7.24 | 8.82 | 7.92 | 5.62 | 7.93 | 7.58 |
| $His^{C(166)}C4-H$ | | 6.95 | 6.59 | | | |
| | | p21(Q61H) | | | | |
| $His^{61}C2-H$ | 6.40 | 8.63 | 7.74 | | | |
| $His^{61}C4-H$ | | 7.52 | 7.11 | | | |

[a] The sequence specific assignments as derived in the main text are given in parentheses.

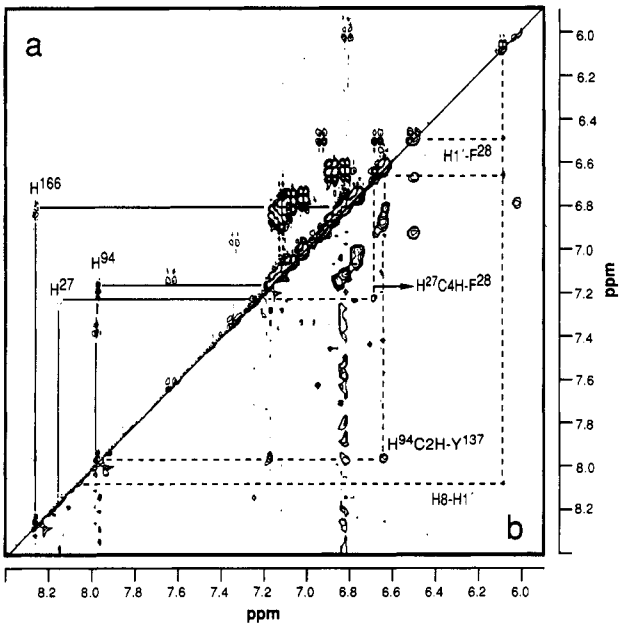


FIGURE 3: DQF-COSY (a) and NOESY (b) spectra of $p21_c$. Some NOESY connectivities within the aromatic region are indicated by dashed lines.

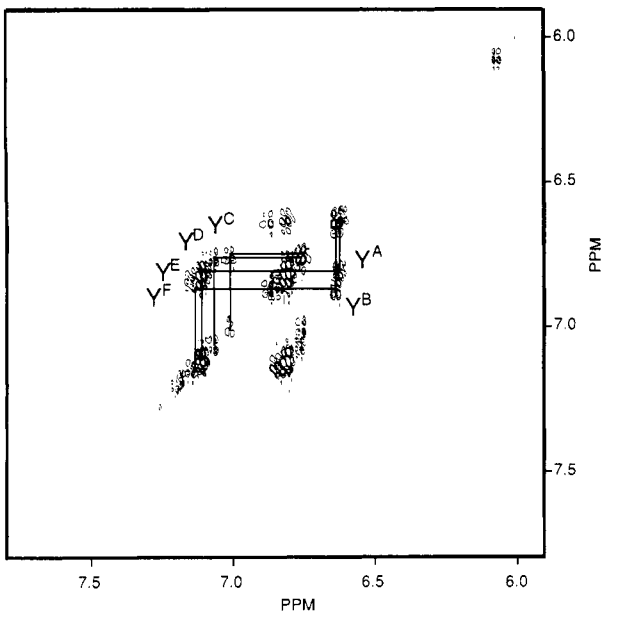


FIGURE 4: Difference spectrum between DQF-COSY spectra of $p21_c$ and $p21_c$ partially composed of 3,5-deuterated tyrosine. All remaining cross-peaks originate from tyrosines.

Table II: Chemical Shift Values of Resonances of Aromatic Side Chains in $p21_c$[a]

| resonance | chemical shift (ppm) | resonance | chemical shift (ppm) |
|---|---|---|---|
| $Tyr^A$ | 6.64/6.85 | $Phe^{C(28)}$ | 6.5/6.7/6.93 |
| $Tyr^B$ | 6.66/6.89 | $Phe^D$ | 7.34/6.97/? |
| $Tyr^C$ | 6.78/7.02 | $His^{A(94)}$ | 7.96/7.17 |
| $Tyr^D$ | 6.79/7.07 | $His^{B(27)}$ | 8.13/7.23 |
| $Tyr^E$ | 6.83/7.13 | $His^{C(166)}$ | 8.24/6.83 |
| $Tyr^F$ | 6.87/7.16 | C1′-H | 6.08 |
| $Phe^{A(78)}$ | 7.63/7.12/? | C8-H | 8.08 |
| $Phe^{B(82)}$ | 6.06/6.8/? | | |

[a] The sequence-specific assignments as derived in the main text are given in parentheses.

histidyl residues could be identified immediately from the double-quantum-filtered (DQF) COSY spectrum of $p21_c$ (Figure 3a). On the other hand, unequivocal identification of the tyrosyl and phenylalanyl ring spin systems based solely on COSY and 2D proton double-quantum (DQ) spectroscopy proved to be impossible. Thus, we tried to obtain this information by studying $p21_c$ partially composed of 3,5-deuterated tyrosine. As is evident even from the 1D spectrum of the aromatic residues of this protein, the intensity of several of the resonances in this spectral region decreased significantly (Figure 1). As expected, the difference spectrum between the DQF-COSY spectra of the completely protonated and the partially deuterated $p21_c$ shows only a few of the aromatic spin system detected in the DQF-COSY spectrum of completely protonated p21 (Figure 4), thus allowing identification of four out of the five phenylalanyl ring spin systems and six out of the nine tyrosyl ring spin systems in the protein (see Table II).

*Mutagenesis of p21.* At the outset of this work, p21 was proposed to have a three-dimensional structure similar to EF-Tu from *E. coli* (McCormick et al., 1985; Jurnak, 1985). On the basis of sequence homologies, we assumed that the phenylalanyl residue in close proximity to the C1′-H of the ribose of p21-bound GDP·$Mg^{2+}$ (Schlichting et al., 1988) could be $Phe^{78}$ or $Phe^{82}$, corresponding to $Ala^{101}$ or $Val^{105}$, respectively, in EF-Tu. The latter residues are close enough to the GDP molecule in the structure of EF-Tu as proposed by Jurnak (1985) to account for the observed nuclear Overhauser effects between the homologous residues in p21 and the ribose C1′-H of the p21-bound GDP·$Mg^{2+}$. Thus, we used site-directed mutagenesis to substitute phenylalanyl residues 78 and 82 with tyrosyl residues. Since the three-dimensional crystal structure of the p21·GDP as determined by DeVos et al. (1988) suggested that $Phe^{28}$ is close to the ribose ring of the p21-bound GDP·$Mg^{2+}$, we also mutated this residue. In addition, $Tyr^{71}$ was substituted with a phenylalanyl residue since $Tyr^{71}$ is highly conserved in all *ras*-related proteins and is therefore believed to be important in maintaining structural and functional properties (Touchot et al., 1987).

*Purification of Mutant Proteins.* Mutation of the corresponding p21-cDNA was performed by using the thionucleotide procedure of Taylor et al. (1985). Mutagenized fragments from the M13 vector were subcloned into the expression vector p-*tac*-c*Hras*3 described earlier (Tucker et al., 1985), where the expression is under the control of the *tac* promoter. All expression vectors could be induced to produce high amounts of protein that are in the soluble fraction of crude extracts. The proteins could be purified following our standard two-column procedure. They all contained bound guanine nucleotides, usually 70–90% GDP (including dGDP) and 10–30% GTP (including dGTP). The ability to exchange bound nucleotide against external radiolabeled GDP was between 80% and 93% of the theoretical maximum. This suggests that all the mutants described here have a well-defined structure and retain biochemical activity.

Table III: Kinetic Rate Constants

| | dissociation rate constants × $10^2$/min for | | | | |
|---|---|---|---|---|---|
| | GDP, 21 °C, in | | GTP | | GTPase rate × $10^2$/min at 37 °C for 10 mM $Mg^{2+}$ |
| | 0.1 μM $Mg^{2+}$ | 10 mM $Mg^{2+}$ | 21 °C in 0.1 μM $Mg^{2+}$ | 37 °C in 10 mM $Mg^{2+}$ | |
| $p21_c$[b] | 35 | 0.07 | 17 | 2.3 | 2.8 |
| F28L | nd[a] | 2.0 | 2[c] | 2[c] | 1.3 |
| Y71F | 50 | 0.09 | 20 | 2.3 | 1.9 |
| F78Y | 50 | 0.08 | 13 | 1.7 | 1.3 |
| F82Y | 50 | 0.08 | 18 | 2.6 | 1.6 |

[a] nd, not determined. [b] John et al. (1988). [c] 10 mM $Mg^{2+}$, 21 °C.

*p21(F78Y) Mutant.* The GDP and GTP dissociation rate constants in the presence as well as in the absence of $Mg^{2+}$ at 21 and 37 °C are very similar to the ones of the corresponding wild-type $p21_c$ complexes. The GTPase activity measured at 37 °C for this mutant was also similar to the corresponding wild-type activity (Table III). Comparison of the region of the aromatic amino acid resonances in the NOESY spectrum of the p21(F78Y)·GDP·$Mg^{2+}$ complex (Figure 6) with the corresponding region of the NOESY spectrum of $p21_c$ (Figure 3b) shows immediately the newly emerging cross-peak from $Tyr^{78}$ at 6.64/6.97 ppm. In addition, the cross-peak at 7.63/7.12 ppm—identified as a phenylalanine residue by the deuteration experiment and termed $Phe^A$—disappeared in the DQF-COSY as well as in the NOESY spectra. As the mutant and wild-type spectra are virtually identical otherwise, this yields the assignment of $Phe^A$ to $Phe^{78}$.

*p21(F82Y) Mutant.* The dissociation rate constants of the GDP·$Mg^{2+}$ and GTP·$Mg^{2+}$ complexes as well as the GTPase activity of the p21(F28Y) mutant are almost identical with the values for the corresponding $p21_c$ complexes under similar conditions (Table III). In the NOESY spectrum of the p21(F82Y)·GDP·$Mg^{2+}$ complex, the unusually high-field-shifted resonances from the aromatic ring protons of $Phe^B$ at 6.06 and 6.8 ppm can no longer be detected (Figure 6). A pair of resonances from *J*-coupled protons unobservable in the spectrum of the wild-type protein appears at 6.23 and 6.85 ppm. As the spectra of p21(F82Y)·GDP·$Mg^{2+}$ and $p21_c$·GDP·$Mg^{2+}$ are virtually identical otherwise, this yields the assignment of $Phe^B$ to $Phe^{82}$.

*p21(Y71F) Mutant.* The GDP and GTP dissociation rates of the p21(Y71F) mutant are slightly higher than those of $p21_c$, whereas the GTPase activity is very similar (Table III). In the NOESY spectrum of the p21(Y71F) mutant, two additional cross-peaks as compared to the NOESY spectrum of $p21_c$ could be detected at 7.18/7.33 ppm and 7.44/6.77 ppm (Figure 6). Since no missing cross-peaks could be identified, assignment of $Tyr^{71}$ was not possible with this experiment.

*p21(F28L) Mutant.* Due to a drastic increase in all dissociation rate constants, the rates in the absence of $Mg^{2+}$ at 21 °C as well as in the presence of $Mg^{2+}$ at 37 °C become too fast to be measured by the filter binding test for the p21(F28L) mutant. The GTPase activity of the mutant is similar to the wild-type activity (Table III). In the $^{31}P$ NMR spectra of $p21_c$ and p21(F28L), a clear difference in the chemical shift of the resonance of the α-phosphate of bound GDP·$Mg^{2+}$ can be seen, whereas the chemical shift values of the β-phosphate of the bound nucleotides are the same (Figure 7). It can be clearly seen in the NOESY spectrum as well as in the 1D spectrum of the p21(F28L) mutant that the resonances of $Phe^C$ are missing (Figure 5), whereas all other resonances are virtually identical. The ribose C1′–H and the guanine base C8–H resonances of the bound nucleotide have different chemical shifts in $p21_c$ and the p21(F28L) mutant, in the latter one 5.9 ppm (C1′–H) and 7.85 ppm (C8–H)

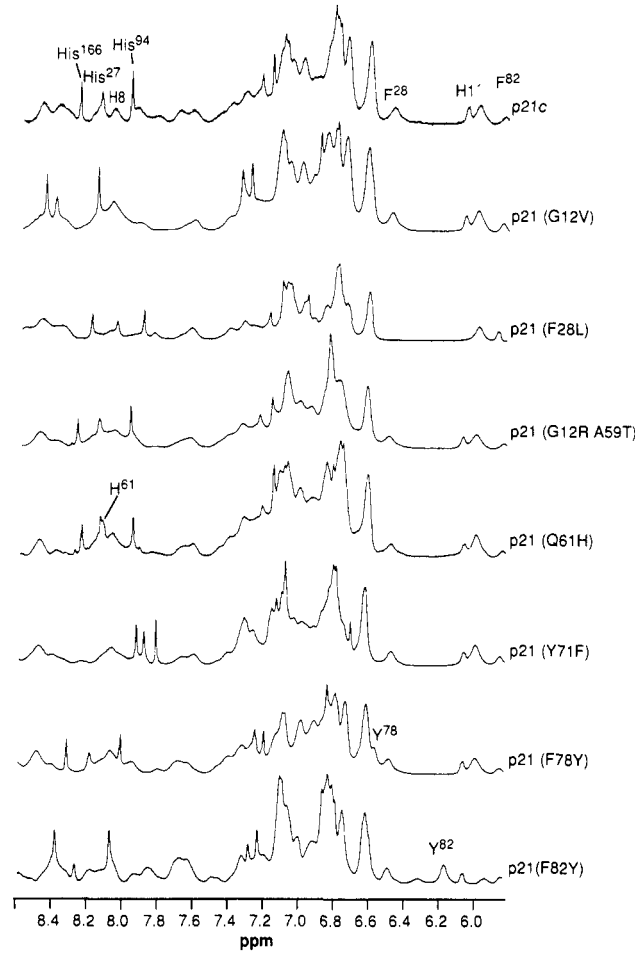


FIGURE 5: Proton NMR spectra of the aromatic side chains of $p21_c$ and mutant proteins. Differences in the chemical shifts of the imidazole proton resonances are due to minor differences in the pH values of the samples.

(Table II). No cross-peaks could be detected in the NOESY spectrum between the ribose C1′–H of the bound GDP·$Mg^{2+}$ and protein protons.

*Intramolecular NOESY Cross-Peaks.* From the intramolecular NOESY cross-peaks between different aromatic amino acid side chains, a few more assignments could be derived: We detected NOESY cross-peaks between the C4–H resonance of $His^B$ and the resonances of the phenyl ring of $Phe^{28}$ at 6.7 ppm (Figure 3b). The crystal structure of the G binding domain of p21 [amino acids 1–171, p21(1–171) (de Vos et al., 1988; Tong et al., 1989b); amino acids 1–166, p21(1–166) (Pai et al., 1989)] suggests that of the three imidazole rings of histidyl residues in the p21·nucleotide·$Mg^{2+}$ complex only the imidazole ring of $His^{27}$ would be close enough to $Phe^{28}$ to generate a detectable NOE. Thus, we assign $His^B$ to $His^{27}$. We also observed a NOE cross-peak between the C2–H imidazole resonance of $His^A$ and the resonances of the aromatic ring of a tyrosyl residue at 6.64 ppm. From the X-ray structure (Pai et al., 1989), there is only one His–Tyr pair with a mutual distance small enough to give rise to a NOESY cross-peak, namely, $His^{94}$ and $Tyr^{137}$. Thus, we assign $His^A$ to $His^{94}$. Consequently, $His^C$ is assigned to $His^{166}$.

Intramolecular cross-peaks between the C1′–H of the ribose and the ribose C3′–H as well as the guanine base C8–H resonance of the bound GDP·$Mg^{2+}$ could be discerned easily in the $p21_c$ spectrum. Similarly, in the NOESY spectrum of the p21(F78Y) mutant, intramolecular NOESY cross-peaks between the resonance of the ribose C2′–H at 4.72 ppm, the ribose C3′–H at 4.51 ppm, and the base C8–H at 8.09 ppm

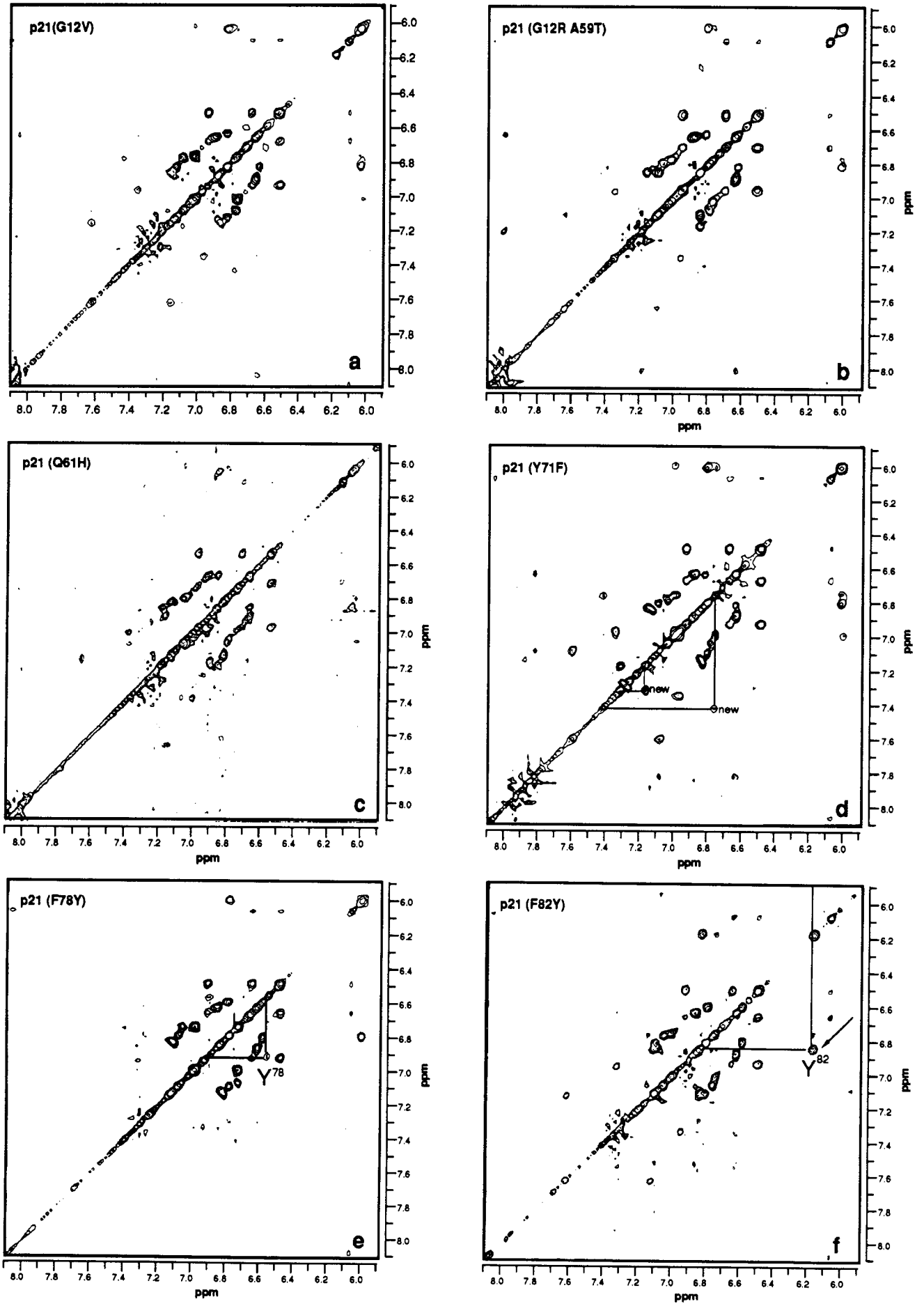


FIGURE 6: NOESY spectra of p21 mutants as indicated.

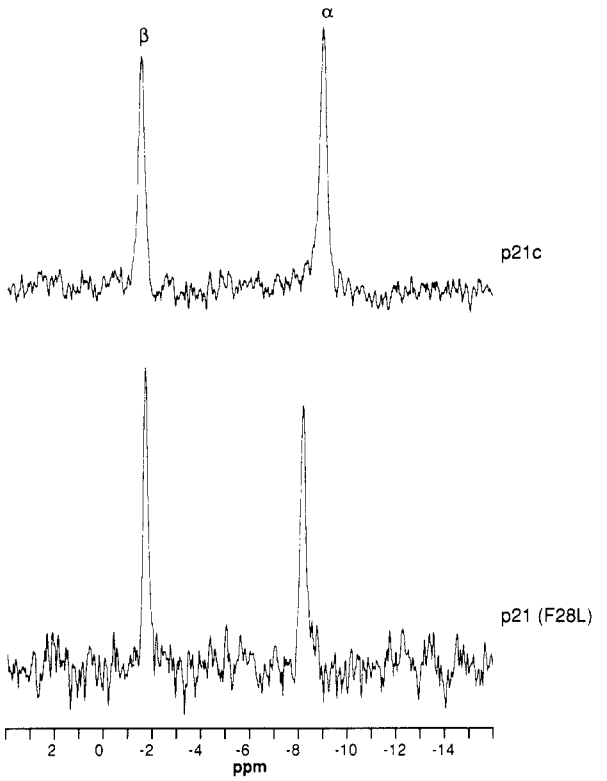


FIGURE 7: $^{31}P$ NMR spectra of $p21_c$ and p21(F28L).

of very similar magnitude could be detected. Due to the $r^{-6}$ dependence of the cross-peak intensities, this corresponds to nearly identical distances between these pairs of protons, indicating an anti conformation of the glycosidic bond of the bound nucleotide.

The only ligand–protein NOESY cross-peaks which could be identified in the aromatic region were the resonances of the ribose C1′–H of the bound GDP·$Mg^{2+}$ and the ring protons of $Phe^{28}$ (Figure 3b; Schlichting et al., 1988).

*NMR Spectra of Oncogenic p21 Mutants.* It has been shown that mutations in certain positions of the p21 sequence activate the potential of the protein to transform fibroblasts. The G12V mutation of p21 does not perturb the overall structure of the protein as determined by X-ray crystallography (Tong et al., 1989.). The structural change is in fact localized to the immediate surroundings of the β-phosphate group (Tong et al., 1989a). EPR investigations by Feuerstein et al. (1987) have shown that the metal ion coordination in the nucleotide binding site of p21 is indistinguishable between the cellular p21 and p21(G12V). To verify the generalization that only minor changes in the overall structure of the protein are required to induce transforming properties, we compared the NMR spectra as indicators of the solution structure of the transforming p21 mutants p21(G12V), p21(G12R,A59T) (corresponding to viral p21), and p21(Q61H) (Figures 5 and 6). In the 1D NMR spectra of the p21(Q61H) mutant, the imidazole C2–H resonance of the new histidine could be identified clearly by the appearance of a new singlet resonance in the imidazole C2–H region (Figure 5) and by a pH titration indicating an apparent p$K$ of 6.4 for this resonance, i.e., a p$K$ in the region expected for imidazole rings (Figure 2b; Table I). The cross-peak patterns in the region of the resonance of the aromatic ring protons of both the COSY and the NOESY spectra of the GDP·$Mg^{2+}$ complexes of $p21_c$, p21(G12R,A59T), p21(G12V), and p21(Q61H) are nearly identical (Figure 6a–f). In particular, the same NOESY cross-peaks between $Phe^{28}$ and the ribose C1′–H of the bound GDP·$Mg^{2+}$ can be observed. We have to conclude that neither the mutations in positions 12 and 59 nor the mutation in position 61 changes the overall structure of p21 in solution.

## Discussion

The presence of high-field-shifted methyl resonances in the 1D NMR spectra of p21 indicates that the preparation procedure conserves a well-defined tertiary structure of the p21·GDP·$Mg^{2+}$ complex (Schlichting et al., 1988). This correlates well with the finding that the proteins purified under native conditions have high GDP/GTP binding constants as well as GTPase activity (Tucker et al., 1986; Feuerstein et al., 1987; John et al., 1988).

NOESY cross-peaks can be expected to be observable under the usual conditions of protein NMR only if the mutual proton distance is less than about 0.5 nm. This is in agreement with our observation of a cross-peak between the ribose C1′–H and the guanine base C8–H resonance of the p21-bound GDP and their known mutual distance in the anti conformation about the glycosidic bond of 0.35 nm in the crystal structure of GMP (Emerson & Sundaralingam, 1980). From this, the distance between the ribose C1′–H and the protons of the aromatic ring of $Phe^{28}$ is also expected to be around 0.35 nm.

Crystals of the GDP complex of p21(1–171) have been prepared in 100 mM $CaCl_2$ without $Mg^{2+}$ (de Vos et al., 1988; Tong et al., 1989b). Thus, it is not clear from these studies whether or not GDP is bound with a complexed metal ion and what the nature of the metal ion is. Our NMR results nevertheless show that in solution the p21-bound GDP·$Mg^{2+}$ complex has an anti conformation and the guanosine moiety is close to the aromatic ring of $Phe^{28}$ as it is in the crystal structure of the p21·nucleotide·$Mg^{2+}$ complex (de Vos et al., 1988; Tong et al., 1989b; Pai et al., 1989). This may be an energetic minimum configuration for the two rings, as a perpendicular orientation of aromatic amino acid side chains is one of the common types of interaction between aromatic groups in protein crystals (Burley & Petsko, 1985). The stabilizing effect of the interaction between $Phe^{28}$ and the guanine base is evidenced by the high increase of the GDP and GTP dissociation rate constants of the p21(F28L) mutant.

Several experiments suggest that the region around amino acid position 80 is crucial for the functional and structural integrity of p21 (Fasano et al., 1988; Schmitt et al., 1988; Hattori et al., 1986; Feig et al., 1986). Indeed, de Vos et al. (1988), Tong et al. (1989b), and Pai et al. (1989) found that the nucleotide-spanning motif in the crystal structure of p21 is from residue 78 to residue 142. Our results, in addition, indicate that at least the conservative amino acid replacements phenylalanine ↔ tyrosine do not severely disturb the structure of the protein. Also, the kinetic rate constants (nucleotide dissociation rates and GTPase activity) together with the NMR data indicate that p21(Y71F), p21(F78Y), p21(F82Y) have structural and biochemical properties similar to those of the wild-type protein.

It should be pointed out that a straightforward general application of NMR in this context is the easy and rapid verification of the structural integrity of extrinsic proteins expressed in *E. coli*. NMR can also establish very quickly whether the structures of a protein and a mutant form are similar. From our NMR spectra, we conclude that the mutations in the nucleotide binding loops (positions 12, 59, and 61) of p21 that activate the transforming and tumorogenic potential of p21 only lead to local alterations in the p21 structure. Recently, the difference in the crystal structure between normal p21 and p21(G12V) was reported to be locally restricted to the surroundings of the β-phosphate group of the

bound nucleotide (Tong et al., 1989a). Our proton NMR measurements show that also in solution there is only a local perturbation in the structure of the G12V mutant protein. Our results further show that the other activating mutations in positions 59 and 61 only lead to local structural changes not perturbing the overall structure of the protein.

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