# Kinetic and Structural Analysis of the $Mg^{2+}$-binding Site of the Guanine Nucleotide-binding Protein p21$^{H\text{-}ras}$*

(Received for publication, March 4, 1992)

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**The coordination and binding of the $Mg^{2+}$ ion in the nucleotide-binding site of p21 have been investigated using site-directed mutagenesis, kinetic methods, and phosphorous NMR. $Mg^{2+}$ in the p21·nucleotide·$Mg^{2+}$ complex appears to be in fast equilibrium with the solvent. The dissociation constant between $Mg^{2+}$ and the p21·GDP complex was determined to be 2.8 μM. It decreases 30- or 16-fold on substituting Ser-17 or Asp-57 with alanine, respectively, whereas the T35A mutation has no effect. All three mutations influence the dissociation constants and the association and dissociation rate constants of the interaction between guanine nucleotides and p21, but to a different degree. We conclude that Thr-35 is only complexed to $Mg^{2+}$ in the GTP conformation and both Asp-57 and Ser-17 appear to be critical for both GDP and GTP binding. $^{31}P$ NMR spectra of the GDP and Gpp(NH)p (guanosine-5′-(β,γ-imido)triphosphate) complexes of mutated p21 show a remarkable perturbation of the guanine nucleotide-binding site compared to wild-type protein. The mutant proteins show reduced GTPase rates, which are not stimulated by the GTPase-activating protein GAP. p21(S17A) has been reported to function just as p21(S17N) as a dominant negative inhibitor of normal p21. We find that it inhibits oncogenic p21-induced survival of primary neurons.**

Proteins involved in phosphoryl transfer from or to nucleotides usually bind these nucleotides as their $Mg^{2+}$ complexes. The enzymatic process is absolutely dependent on the presence of divalent metal ions. In solution, the metal ion is complexed to nucleoside di- and triphosphates in a number of different modes that are in rapid equilibrium with each other. On the protein, the coordination between metal ion and nucleotide is presumably restricted to one mode, and this may or may not change during the enzymatic process. Many reports on the structure of metal-nucleotide complexes on the enzyme have used exchange inert Cr(III) or Co(III) complexes (Cleland, 1982), the EPR superhyperfine interaction between stereospecific $^{17}O$ nucleotide analogs and $Mn^{2+}$ as an analog of $Mg^{2+}$ (Reed and Markham, 1984; Kalbitzer, 1987), phosphorothioates (Jaffe and Cohn, 1979; Eckstein, 1985), or NMR measurements (Ray *et al.*, 1988; Nageswara Rao, 1990).

Guanine nucleotide-binding (GNB)[1] proteins such as the ribosomal elongation factors, the Gα subunits of heterotrimeric G-proteins involved in signal transduction and the small GNB proteins such as the *ras* oncogene-encoded protein p21[2] bind guanine nucleotides with high specificity and high affinity (for reviews, see Gilman (1987), Hall (1990b), Bourne *et al.* (1990, 1991), and Grand and Owen (1991)). The affinities of guanine nucleotides are usually increased and their dissociation rates decreased in the presence of divalent metal ions. In p21, the difference in affinity between p21 and GDP or GTP in the presence or absence of $Mg^{2+}$ is >500-fold (Tucker *et al.*, 1986; Hall and Self, 1986; Feuerstein *et al.*, 1987; John *et al.*, 1988), and the same applies to other small GNB proteins such as p24$^{ral}$ (Frech *et al.*, 1990b).

The three-dimensional structure of p21 complexed to the GTP analogue Gpp(NH)p has recently been solved to high resolution in our laboratory (Pai *et al.*, 1989; 1990). In the highly refined three-dimensional structure, the $Mg^{2+}$ ion is coordinated to 6 oxygen atoms in a perfect octahedral arrangement. This is shown schematically in Fig. 1. $Mg^{2+}$ is complexed to two phosphate oxygens of Gpp(NH)p as a β,γ-bidentate complex. Two additional bonds are formed to the side chain oxygens of Ser-17 and Thr-35, both of which are highly conserved in all guanine nucleotide-binding proteins. The two apical positions of the octahedron are occupied by oxygens from water molecules. One of these water molecules is coordinated to the α-phosphate oxygen and the other to Asp-57, another highly conserved residue of GNB proteins. In the structure of p21·GDP (Tong *et al.*, 1991) Ser-17, the β-phosphate and four water molecules have been identified as ligands of $Mg^{2+}$ (Fig. 1), but discrepancies persist as to the role of Asp-57 in the binding of the metal ion (Milburn *et al.*, 1990; Schlichting *et al.*, 1990b; Tong *et al.*, 1991). The existence of four water ligands has also been independently shown by EPR measurements using $[^{17}O]H_2O$ (Smithers *et al.*, 1990).

Since it is of interest to examine the interaction between $Mg^{2+}$ ions and p21-nucleotide complexes in solution as well as in crystals, we have used methods to do so employing radiolabeled and fluorescent GDP and GTP analogs. We have mutated the residues involved in binding $Mg^{2+}$ and investigated the effect on the interaction with nucleotides and $Mg^{2+}$, but also on the GTPase activity in the presence or absence of the GTPase-activating protein GAP (McCormick, 1989; Hall,

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[1] The abbreviations used are: GNB, guanine nucleotide-binding protein; NMR, nuclear magnetic resonance; Gpp(NH)p, guanosine-5′-(βγ-imido)triphosphate; mant-GDP/Gpp(NH)p, 2′,3′-*O*-(*N*-methylanthraniloyl)-GDP/Gpp(NH)p; mant-dGDP/dGpp(NH)p, 3′-mant-2′-deoxyGDP/Gpp(NH)p; NGF, nerve growth factor; GAP, GTPase-activating protein.

[2] For simplicity the product of the H-ras protoncogene is called p21, instead of p21$^{H\text{-}ras}$.

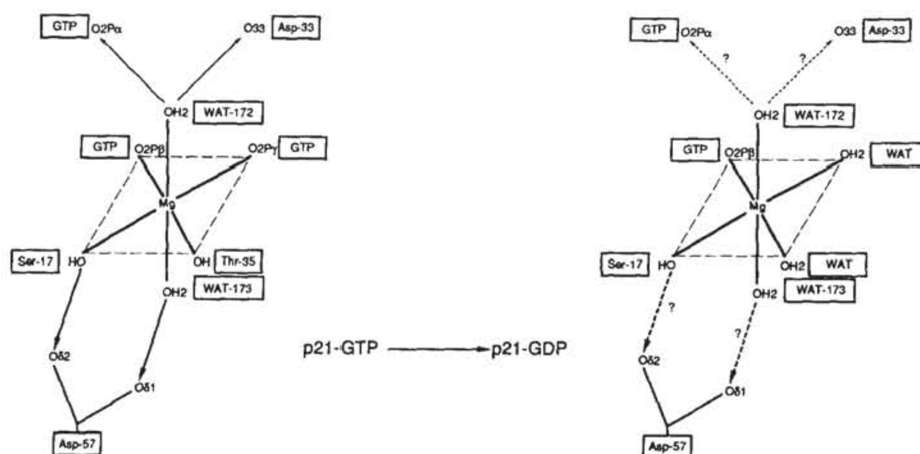


FIG. 1. **Scheme for the hexa-coordination of $Mg^{2+}$ in the complex with p21·Gpp(NH)p and GDP as determined by x-ray crystallography (Pai *et al.*, 1990; Tong *et al.*, 1991).**

1990a; Gibbs *et al.*, 1988) which stimulates the intrinsic GTPase activity of wild-type p21 $10^5$-fold (Gideon *et al.*, 1992).

## MATERIALS AND METHODS

*Cloning Techniques and Mutagenesis*—Restriction endonucleases, T4 DNA ligase, polynucleotide kinase, and dNTPs were from Boehringer (Mannheim, Germany). The above reagents were used as described by Sambrook *et al.* (1989). Site-directed mutagenesis was performed according to the method of Taylor *et al.* (1985), using exonuclease III from New England Biolabs and DNA polymerase (Klenow fragment) from Du Pont-New England Nuclear. Desoxycytidine-5'-*O*-(thiotriphosphate) was synthesized according to the method of Goody and Isakov (1986). Briefly, the *Eco*RI-*Pst*I fragment (680 base pairs) of the ptac-cHras cDNA (Tucker *et al.*, 1986) was cloned into M13 mp9 and single-strand DNA for mutagenesis was prepared. The oligonucleotides used for mutagenesis are as follows: 5'-GCC-GGC-GGT-GTG-GGC-AAG-*GCA-GCG-CTG-ACC-ATC-CAG-CTG-3' for S17A and 5'-C-ATC-CTG-GCT-ACC-G*CC-G-3' for D57A, with the asterisk to the left of the mutated residue. The DNA sequence of the mutated gene was verified by DNA sequencing. Following mutagenesis, the *Eco*RI-*Pst*I fragment was cloned back into the expression vector ptac-ras32 (John *et al.*, 1988). The *Escherichia coli* strain CK600K was used as a host, which is K12 wild-type CK600 containing the plasmid pDMI.1 (Certa *et al.*, 1986) carrying the *lacI*$^q$ gene and a kanamycin resistance gene (K). This plasmid is compatible with the expression plasmids described here.

*Protein Purification*—Protein purification was performed essentially as described previously (Tucker *et al.*, 1986; John *et al.*, 1988): The final purity of the proteins was >95%, as judged from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins contained 1 mol of guanine nucleotide bound/mol of protein, 85–95% of which could be exchanged against external GDP. Protein concentrations were determined with the Bradford assay using bovine serum albumin as standard (Bradford, 1976), while [8-$^3$H]GDP binding activity was determined by the filter binding assay (Tucker *et al.*, 1986). Standard buffer was always, unless stated otherwise, 64 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 1 mM sodium azide.

*GTPase Activity*—GTPase activity measurements were performed essentially as described previously (John *et al.*, 1989a). Briefly, p21·GDP (2 $\mu$M) was preincubated with [$\gamma$-$^{32}$P]GTP (40 $\mu$M) for 30 min at room temperature in a total volume of 1 ml in 1 mM EDTA, 64 mM Tris-HCl, pH 7.6, 2 mM dithiothreitol, 1 mM sodium azide. The concentration of $MgCl_2$ was brought to 10 mM and the temperature to 37 °C. 50-$\mu$l portions were removed at defined time intervals, and the production of liberated $^{32}P_i$ was measured as described. The experimental errors for GTPase and other rate measurements were between 10 and 20%.

*Kinetics of Nucleotide Dissociation and Association*—The rate of dissociation of [8-$^3$H]GDP from the p21·[8-$^3$H]GDP complex was measured with the filter binding assay as described previously (John *et al.*, 1988). Nitrocellulose filters from Schleicher & Schüll (BA 85, pore size = 0.45 $\mu$M) were used.

The measurement of association rates was done as described (John *et al.*, 1990) using a p21-guanosine complex, which was prepared as described (John *et al.*, 1990) and frozen in aliquots at −70 °C. Protein (0.5–1 $\mu$M) was reacted with excess *N*-methylanthraniloyl-GDP (mant-GDP) under various conditions in a stopped-flow apparatus (HighTech Scientific, Salisbury, United Kingdom). Excitation of fluorescence of mant-GDP was at 366 nm, and detection was through a filter with a cut-off at 445 nm. Data were collected using an analog-digital converter in an Apple II computer, or in a Nicolet 2090 digital storage oscilloscope. Values for $k_{off}$ (GDP) and $k_{on}$ (GTP) for wild-type p21 (John *et al.*, 1990) had to be corrected.

*$Mg^{2+}$ Binding*—For the measurement of the binding affinities of $Mg^{2+}$ to the p21(S17A)·GDP complex, 10 $\mu$l of 80 $\mu$M p21(S17A)·GDP in standard buffer (10 mM $Mg^{2+}$) was first added to 1 ml of 1 $\mu$M mant-GDP, 100 $\mu$M EDTA in standard buffer without $Mg^{2+}$ at 25 °C. At equilibrium (15 min, room temperature) this solution contained equimolar amounts of p21(S17A) complexed to GDP and mant-GDP. It was titrated with increasing concentrations of $Mg^{2+}$. The fluorescence increase data were fitted to a hyperbolic binding curve with the program Enzfitter.

For the other mutants and for wild-type p21, the affinity of $Mg^{2+}$ to the p21·GDP complex was determined by measuring the GDP exchange rate ($k_{obs}$) at various $Mg^{2+}$ concentrations. To 1 ml of 10 $\mu$M [8-$^3$H]GDP in standard buffer containing variable amounts of $Mg^{2+}$, 10 $\mu$l of 0.2 mM p21·GDP in standard buffer (with 10 mM $MgCl_2$) was added. The GDP exchange rate was measured at 25 °C by the nitrocellulose filter binding assay. The exponential exchange reaction was analyzed with the program Enzfitter. The observed GDP dissociation rate constants were plotted against $Mg^{2+}$ concentration as described above.

The affinities of $Mg^{2+}$ for wild-type p21·GDP and p21·Gpp(NH)p were also determined by titrating 1 $\mu$M p21·mant-dGDP or p21·mant-dGpp(NH)p in standard buffer with 1 mM EDTA at pH 7.6 with $Mg^{2+}$ and following the increase in fluorescence, which was approximately 30% for mant-dGDP and 23% with mant-dGpp(NH)p. The data were fitted as described above using a value of 1.2 $\mu$M for the dissociation constant between $Mg^{2+}$ and EDTA at pH 7.6.

*Interaction with GAP*—Complexes between p21 and [$\gamma$-$^{32}$P]GTP were formed by incubating the protein with the radioactive nucleotide in the presence of 1 mM EDTA and 64 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol. Excess nucleotide was removed by gel filtration on a small Sephadex G-25 column (NAP-5, Pharmacia LKB) equilibrated with the GAP reaction buffer: 20 mM Hepes-NaOH, pH 7.5, 1 mM sodium azide, 1 mM dithiothreitol. The protein was eluted from this column with the same buffer. The GTPase reaction was started by addition of 2 mM $MgCl_2$ and the appropriate amount of recombinant human GAP (>2.5 nM). The GTPase reaction was measured as described (Gibbs *et al.*, 1988; Vogel *et al.*, 1988; Frech *et al.*, 1990a; Gideon *et al.*, 1992) by following the decrease of the concentration of [$\gamma$-$^{32}$P]GTP bound to p21 by filtration of the reaction mixture through nitrocellulose filters (pore size 0.45 $\mu$m). The initial rates were determined from the decrease of p21·GTP concentration with time.

*Biological Activity of p21*—A technique termed trituration or pressure loading was used to introduce p21 proteins into nerve cells from dorsal root ganglia of chicken embryos or into PC12 cells (Borasio *et al.*, 1989). 40 $\mu$l of a p21 protein solution in standard buffer with the concentration indicated was used. The protein is introduced into the cytoplasm by pushing the cells through a yellow pipette tip held at the bottom of the tube. This creates enough pressure to slightly rupture the cell membrane so that the protein can enter the cell. The

cells were screened for survival in the presence and absence of 20 ng/ml nerve growth factor (NGF) as described before (Borasio *et al.*, 1989). Oncogenic p21(G12V) (5 mg/ml) was used either alone or together with p21(S17A) as indicated.

The pheochromocytoma cell line PC12 (Greene and Tischler, 1982) was also used for assessing the biological activity as described previously (John *et al.*, 1989a). Approximately $5 \times 10^4$ cells were collected by centrifugation in an Eppendorf tube, and protein was introduced as described above. The cells were monitored for neurite outgrowth as described previously (Bar-Sagi and Feramisco, 1985; John *et al.*, 1989a). Studies on the inhibition of the NGF pathway were done in the presence of 20 ng/ml growth factor.

*NMR Spectroscopy*—For phosphorous NMR of the p21·GDP complex, the protein samples were used as isolated. The p21·Gpp(NH)p complexes were prepared by exchange of the protein bound GDP for Gpp(NH)p as described recently (John *et al.*, 1990). The samples were dialyzed against standard buffer (64 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10–20 mM dithiothreitol). Protein concentration was 0.5–1 mM.

NMR experiments were performed on a Bruker AM 500 spectrometer operating at a phosphorous resonance frequency of 202 MHz. 2.2-ml aliquots of sample in 10-mm sample tubes and a 10-mm probe were used. The phosphorous spectra are referenced to external 85% $H_3PO_4$. Sample temperature was kept at 303 K with a stream of dry air, which was temperature-regulated with a standard Bruker VT100 unit. Standard procedures and commercially available software on an Aspect 3000 computer were used throughout.

## RESULTS

*Mechanism of Nucleotide Dissociation and $Mg^{2+}$ Affinity*—GNB proteins bind the nucleotides GDP or GTP as $Mg^{2+}$-nucleotide complexes. It has been shown by us and others that GDP or GTP is released from p21 very slowly in the presence of $Mg^{2+}$ and more rapidly in the presence of excess EDTA, which complexes the metal ion. Thus one can formally describe the dissociation of, for example, GDP from the protein as shown by the following scheme.

$$\begin{array}{ccc} p21\cdot GDP\cdot Mg^{2+} & \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} & p21\cdot GDP + Mg^{2+} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} p21 + GDP \\ k_{-3}\downarrow\uparrow k_3 & & \\ p21 + Mg^{2+}\cdot GDP & & \end{array}$$

SCHEME 1

$k_{-2}$ and $k_{-3}$ are the dissociation rate constants of GDP and of Mg·GDP, respectively. The rate constant at infinite $Mg^{2+}$ concentration is $k_{-3}$, and the rate constant in the absence of metal ion is $k_{-2}$. At intermediate $Mg^{2+}$ concentrations, the nucleotide dissociation rate is dependent on the $Mg^{2+}$ concentration. If the dependence of the observed dissociation rate constant $k_{obs}$ on the $Mg^{2+}$ concentration is measured (see Fig. 3), it is seen that $k_{obs}$ decreases approximately hyperbolically with increasing metal ion concentration, but that a plateau is reached at high $Mg^{2+}$. In order to interpret the influence of the metal ion concentration on kinetics of GDP exchange, it is necessary to establish whether metal ion dissociation is fast on the time scale of the observed exchange kinetics. Fig. 2 shows that p21.GDP in the presence of 1 mM $Mg^{2+}$ dissociates very slowly, with a half-life of 16.5 h at 21 °C. The exchange reaction is immediately (within 15 s) accelerated to the rate characteristic of metal ion free p21·GDP if the p21·GDP·$Mg^{2+}$ complex is treated with EDTA (half-life 2 min at 21 °C). We therefore conclude that bound $Mg^{2+}$ is in rapid equilibrium with the solvent. On addition of EDTA it is immediately complexed and must thus dissociate much faster than (and independently of) GDP.

Using this information, it can be shown that $k_{obs}$, the measured rate constant of GDP release, is dependent on the metal ion concentration and its affinity to the p21·GDP

FIG. 2. **$Mg^{2+}$-jump experiment.** 1.8 μM p21 complexed with [$^3$H] GDP was incubated with either 0.1 μM or 1 mM $Mg^{2+}$. The dissociation rates were measured as described after adding a large excess of unlabeled GDP. After 10 min 9 mM EDTA was added to the reaction mixture containing 1 mM metal ion and the resulting dissociation rate was measured immediately (first time point after 15 s).

complex $K_D$ as shown in Equation 1,

$$k_{obs} = \frac{k_{-3}}{1 + K_D/[Mg^{2+}]} + \frac{k_{-2}}{1 + [Mg^{2+}]/K_D} \quad \text{(Eq. 1)}$$

where $K_D = k_{-1}/k_1$. Thus, plotting $k_{obs}$ against the $Mg^{2+}$ concentration should (according to Equation 1) lead to a curve which varies between the extreme values of $k_{obs} = k_{-2}$ at low and $k_{obs} = k_{-3}$ at high $Mg^{2+}$ concentrations. If, as is qualitatively clear, $k_{-2} >> k_{-3}$, the form of the curve will be dominated by the second term in Equation 1, so that there should be an approximately hyperbolic decrease from $k_{-2}$ to $k_{-3}$. The curves shown in Fig. 3 are computer fits to Equation 1. The values obtained are $2.2 \times 10^{-1}$ min$^{-1}$ for $k_{-2}$, $9.7 \times 10^{-4}$ min$^{-1}$ for $k_{-3}$, and 2.8 μM for $K_D$.

*Properties of Ser-17, Thr-35, and Asp-57 Mutants*—The crystal structure of p21·Gpp(NH)p has revealed the $Mg^{2+}$ coordination shown in Fig. 1 (Pai *et al.*, 1990). It also appeared from time-resolved x-ray crystallographic studies that Asp-57 in the GDP conformation, which results from the GTPase reaction of p21·GTP in the crystal, is directly involved in the coordination of the metal ion (Schlichting *et al.*, 1990b), although this has not been found in the structure of the p21·GDP complex determined directly (Tong *et al.*, 1991; Milburn *et al.*, 1990). We have mutated Ser-17 and Asp-57 to alanine in the expression system ptac-cHras (Tucker *et al.*, 1986) in order to determine how the disturbance of the $Mg^{2+}$-binding site perturbs the $Mg^{2+}$ and nucleotide binding properties and the GTPase activity in the presence or absence of GAP. We also investigated the properties of the T35A mutant, which is derived from the p21-fusion protein described by Gibbs *et al.* (1984) with 20 additional N-terminal amino acids. A number of control experiments have been performed[3] to show that these additional amino acids do not influence the known biochemical properties of p21.

*Nucleotide Interactions of Mutant Proteins*—Table I shows

[3] J. John, H. Rensland, I. Schlichting, I. Vetter, G. D. Borasio, R. S. Goody, A. Wittinghofer, and J. Gibbs, unpublished data.

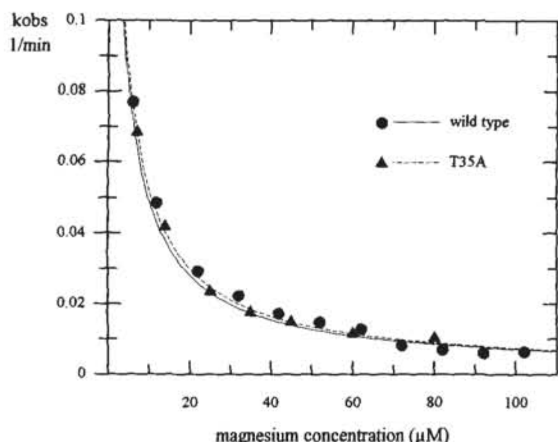


FIG. 3. **Hyberbolic decrease of the GDP dissociation rate constant $k_{obs}$ with increasing $Mg^{2+}$ concentration.** The dissociation of GDP from its complex with p21 was measured with 2 μM p21·GDP and 10 μM [³H]GDP at 25 °C and increasing concentrations of $Mg^{2+}$. The reaction was started by the addition of p21·GDP to 1 ml of [³H]GDP. The exchange rates were measured and analyzed as described under "Materials and Methods." Values of constants fitted according to Equation 1 are: for wild-type p21, $k_{-2} = 0.22\ min^{-1}$, $k_{-3} = 9.7 \times 10^{-4}\ min^{-1}$ and $K_D = 2.8\ \mu M$; for the T35A mutant, $k_{-2} = 0.23\ min^{-1}$, $k_{-3} = 7.5 \times 10^{-4}\ min^{-1}$, $K_D = 3.0\ \mu M$.

TABLE I

*Dissociation and association rate constants (at 2–4 °C), the equilibrium dissociation constant $K_D$, and in vitro GTPase (at 37 °C) rate constants of wild-type and mutant p21 proteins*

pH = 7.6, [$MgCl_2$] = 10 mM. NM, not measurable.

| | $p21_C$ | p21(D57A) | p21(T35A) | p21(S17A) |
|---|---|---|---|---|
| GDP | | | | |
| $k_{off}$ ($\times 10^6$, $s^{-1}$) | 6.3 | 43 | 6.2 | 360 |
| $k_{on}$ ($\times 10^{-5}$, $M^{-1}s^{-1}$) | 3.1 | 15 | 4.1 | 4.1 |
| $K_D$ (pM) | 20.3 | 29 | 15 | 8878 |
| GTP | | | | |
| $k_{off}$ ($\times 10^5$, $s^{-1}$) | 0.96[a] | 270 | 4.3 | 4600 |
| $k_{on}$ ($\times 10^{-5}$, $M^{-1}s^{-1}$) | 9 (5 °C) | 24 | 6.7 | 1.9 |
| $K_D$ (pM) | 10.7[b] | 1125 | 64 | 242,000 |
| GTPase ($\times 10^3$, $min^{-1}$) | 28 | 12 | 6 | NM |

[a] Calculated from the dissociation constant and the association rate constant.

[b] Calculated from the relative affinity of GDP/GTP to p21 (Scherer *et al.*, 1989).

the various dissociation and GTPase rate constants of wild-type and mutant proteins in the presence of excess (10 mM) $Mg^{2+}$. The S17A but not the D57A and T35A mutations have a drastic effect on the kinetics of the interaction between p21 and GDP. The affinity of p21(S17A) for GDP is reduced more than 400-fold, and this is due solely to the increased dissociation rate constant. The D57A mutation has some effect on both the dissociation and association rate constants, but they offset each other so that the dissociation constant is basically unchanged. The interaction between p21 and GTP is influenced by all three mutations but the magnitude of the effect is more pronounced in the order T35A, D57A, S17A. The mutations of Asp-57 and Ser-17 obviously have a much greater effect on the GTP interaction than on the GDP interaction and in both cases the effect is mainly caused by the increase in the dissociation rate of GTP. The ratios of affinities for GDP and GTP is 4-fold for T35A, 39-fold for p21(D57A), and 27-fold for p21(S17A) as compared to 0.54 for wild-type p21. This means that all mutant proteins bind GDP more strongly than GTP. The GTPase activity is slightly reduced for p21(D57A) and p21(T35A) and too low to be measured for p21(S17A).

We have shown above that the dissociation of GDP (and most probably GTP) can occur by two mechanisms. In one of these the Mg·GDP complex dissociates in a single step, in the other $Mg^{2+}$ release precedes GDP release. We wished to determine whether the mutations described above have any effect on the mechanism of guanine nucleotide dissociation. The results shown in Fig. 3 demonstrate that for p21(T35A), the dissociation rate constant is also dependent on the $Mg^{2+}$ concentration, and the results obtained are not significantly different from those for wild-type protein. For p21(S17A) however, $k_{obs}$ is increased to 1 $min^{-1}$ at low $Mg^{2+}$ concentration and does not change significantly on increasing the metal ion concentration to 10 mM (Table II). This suggests either that $Mg^{2+}$ binds so weakly to the p21(S17A)·GDP complex that the $Mg^{2+}$-binding site cannot be saturated under these conditions or that the rate of GDP is independent of the presence of $Mg^{2+}$ at the active site. These possibilities are distinguished by the experiments described below.

*$Mg^{2+}$ Ion Binding of Mutant Proteins*—The affinities between mutant p21·GDP and $Mg^{2+}$ were measured using either the dependence of $k_{obs}$ on $Mg^{2+}$ ion concentration as described above or by using the fluorescent analogue mant-dGDP. We have shown earlier that the binding to p21 of guanine nucleotides with a fluorescent *N*-methylanthraniloyl group on the 3′- or 2′-hydroxyl group produces large fluorescent changes, the sizes of which are dependent on whether or not metal ions are present (John *et al.*, 1989b, 1990). The magnitude of the fluorescent differences between the Mg-free and the Mg-bound complex are different for different nucleotides and also different for various p21 mutants. For wild-type p21 the difference in the signal is approximately 10% for mant-GDP and 30% for mant-dGDP. As shown in Fig. 4, the binding of $Mg^{2+}$ to the p21(S17A) mant-GDP complex can be easily monitored since this leads to a 80% fluorescent increase. The titration curve can be fitted for a single binding site with a

TABLE II

*Influence of the total $Mg^{2+}$ concentration on the GDP dissociation rate constants $k_{obs}$ ($min^{-1}$) for p21(S17A) and $p21_C$ at 25 °C*

| | [$Mg^{2+}$] (μM) | | |
|---|---|---|---|
| | <0.1 | 200 | 10,000 |
| p21(S17A) | 1 | 0.98 | 0.84 |
| $p21_C$ | 0.22 | | $9.7 \times 10^{-4}$ |

TABLE III

*Dissociation constants $K_D$, in μM, between $Mg^{2+}$ and protein-GDP complexes of wild-type and mutant p21, at 25 °C, measured using the $Mg^{2+}$ dependence of the observed dissociation rates (Equation 1)*

| Protein | $p21_C$ | p21(D57A) | p21(S17A) | p21(T35A) | p21(A59T) |
|---|---|---|---|---|---|
| | 2.8 | 45 | 83[a] | 3.0 | 5 |

[a] Measured by fluorescence titration as shown in Fig. 4.

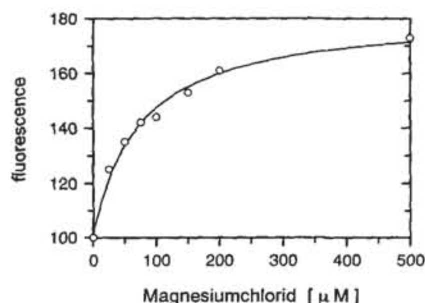


FIG. 4. **Fluorescence titration of the complex between p21(S17A) and the fluorescent GDP analogue mant-GDP (John *et al.*, 1990) with increasing concentration of $Mg^{2+}$.**

dissociation constant of 83 μM. Thus, although the affinity for $Mg^{2+}$ is much lower than for wild-type protein, it is easy to saturate the binding at available concentrations of $Mg^{2+}$. This suggests that the reason for the lack of significant influence of $Mg^{2+}$ on the rate of GDP release is that $k_{-3}$ in Scheme 1 is approximately similar to $k_{-2}$.

The affinities of $Mg^{2+}$ to the p21·GDP complexes are shown in Table III. The $Mg^{2+}$ dissociation constants are increased 30-fold for p21(S17A) and 16-fold for p21(D57A) in comparison with wild-type p21. Since the substitution Ala-59 → Thr makes Thr-59 a target for autophosphorylation and since Thr-59 could potentially be close enough to complex $Mg^{2+}$ (Pai *et al.*, 1989), we also measured the dissociation constant between $Mg^{2+}$ and the mutant p21(A59T)-GDP. It is 5 μM, almost unchanged from that of wild-type protein.

We have also measured the $Mg^{2+}$ affinity to wild-type p21·mant-dGDP and p21·mant-dGpp(NH)p complexes using the fluorescence increase and EDTA to buffer the $Mg^{2+}$ concentration. We find dissociation constants of 270 and 22 nM for the diphosphate and triphosphate complexes of $p21_C$, respectively. The constant for p21·GDP is different from the one shown in Table III, which was measured under different conditions, and could also partly be due to an incorrect estimation of the binding constant between $Mg^{2+}$ and EDTA. The data also show that under these circumstances the affinity of $Mg^{2+}$ to the triphosphate complex is about 1 order of magnitude higher than to the diphosphate complex.

*Interaction with GTPase-activating Protein (GAP)*—We have investigated the interaction of the mutant proteins with the GTPase-activating protein GAP. The p21·GTP complexes with the mutations S17A, T35A, and D57A can no longer be activated by GAP. The slow, intrinsic GTPase activities of p21(D57A) and p21(T35A) were reduced to 43 and 23% of the wild-type value and was too slow to be measured with p21(S17A) (Table IV). This supports the notion that the proper coordination of $Mg^{2+}$ is important for the GTPase reaction and that the reaction is absolutely dependent on the presence of a bivalent metal ion, also in the presence of GAP.

Table IV shows that the mutations also influence the affinity of the p21 proteins for GAP. These were measured by inhibiting the GAP stimulated GTPase activity of wild-type p21 with p21 mutants complexed to $GppCH_2p$ (S17A) or Gpp(NH)p (T35A, D57A). Whereas the D57A mutation has no effect on the affinity, S17A and T35A show drastically decreased affinities for GAP. This fits well with the observation that the structure of the effector domain (residues 32–40) in the three-dimensional structure of p21 is regulated by the coordination of Thr-35 to $Mg^{2+}$ (Pai *et al.*, 1990; Wittinghofer and Pai, 1991).

*$^{31}P$ NMR of p21 Nucleotide Complexes*—$^{31}P$ NMR spectra of protein bound nucleotide complexes have shown that the α-, β-, and γ-phosphates of GDP and GTP (or a GTP analogue such as Gpp(NH)p) show characteristic chemical shift differences when bound to the protein (Rösch *et al.*, 1986; Schlichting *et al.*, 1990a). Since $Mg^{2+}$ is bound to the β-phosphate in p21·GDP and to the βγ-phosphate and, via water, also to the α-phosphate in the GTP analogue complex, it was of interest to study the effect of the mutations of the $Mg^{2+}$-binding site on the $^{31}P$ NMR spectrum. For p21·GDP complexes the D57A mutation has an appreciable effect only on the α-phosphate and almost none on the β-phosphate, whereas T35A shows only a slight shift of the α-phosphate resonance. Only S17A has a drastic effect on both the α- and β-resonance. The mutant p21·Gpp(NH)p complexes all have $^{31}P$ NMR spectra different from that of wild-type protein. S17A, D57A, and T35A all show similar chemical shift changes whose sizes for the three phosphates are in general α- > β- > γ-phosphate. For p21(T35A) we find two peaks for each phosphate resonance. Table V lists the chemical shift values of all the phosphate resonances. Although the chemical shifts of the $^{31}P$ nucleus are difficult to interpret, the NMR measurements show that the correct binding of the $Mg^{2+}$ nucleotide in the active site of the protein is important for the proper function of the protein.

*Biological Activity of Mutants*—The mutants described here, p21(S17A), p21(T35A), and p21(D57A), were all investigated for their biological activity. They were pressure loaded into PC12 cells, which can be induced to differentiate by oncogenic p21. None of the mutants induces neurite outgrowth. Thus it can be concluded that these proteins are probably not oncogenic, because it has been shown that oncogenic transformation of fibroblasts and neurite induction in PC12 cells are always correlated for the mutants investigated up to now (Sassone-Corsi *et al.*, 1989). In line with our observation on p21(D57A) Farnsworth and Feig (1991) have found that p21(D57N) is weakly transforming after transfection into NIH 3T3 cells, somewhat more weakly than normal p21, which again is much weaker than oncogenic p21. They also reported that p21(T35A) has no biological effect on NIH 3T3 cells.

It has been shown that the mutation S17N inhibits proliferation of NIH 3T3 cells by interfering with the function of normal ras proteins (Feig and Cooper, 1988; Stacey *et al.*, 1991; Szeberényi *et al.*, 1990) and that S17A and S17C also appear to be inhibitory (Farnsworth and Feig, 1991). We investigated the biological activity of p21(S17A) by loading the protein into PC12 cells and chick embryonic neurons. Neurite outgrowth in PC12 cells induced by NGF (data not shown) and the survival of NGF-responsive dorsal root ganglia (Fig. 5) was only weakly inhibited by loading the cells, via trituration (Borasio *et al.*, 1989), with high concentrations of p21(S17A). We have shown before that oncogenic p21(G12V) can mimick the action of NGF by promoting survival of various types of primary neurons. When equal

TABLE IV
*Interaction of wild-type and mutant proteins with GAP*

| | $p21_c$ | p21(D57A) | p21(S17A) | p21(T35A) |
|---|---|---|---|---|
| GTPase ($min^{-1}$) | 0.028 | 0.012 | ND | 0.0063 |
| GAP stimulation | + | – | – | – |
| GAP affinity (μM) | 8.4[a] | 7 | ~100 | ~100 |

[a] The affinity between wild-type p21 and GAP is measured as the $K_m$ of the GAP-stimulated GTPase with a constant amount of GAP (the enzyme) and increasing concentration of p21 (the substrate). It correlates closely with the true affinity (Gideon *et al.*, 1992). The affinities of the mutants S17A and T35A have been determined as inhibition constants (see text) with only one concentration of the proteins and are thus only approximate values. A full titration was done for p21(D57A).

TABLE V
*Chemical shift values of phosphate resonances in p21-nucleotide complexes*

| Protein | Chemical shift | | | | |
|---|---|---|---|---|---|
| | GDP-Mg | | GppNHp-Mg | | |
| | α | β | α | β | γ |
| Free nucleotide | −9.25 | −5.22 | −9.53 | −5.03 | −0.52 |
| $p21_C$ | −9.98 | −1.51 | −11.06 | −2.61 | +0.36 |
| p21(S17A) | −12.6 | −0.16 | −10.66 | −1.93 | +0.26 |
| p21(T35A) | −10.14 | −1.49 | −10.61 | −1.93 | +0.26 |
| | | | −11.70 | −2.40 | ? |
| p21(D57A) | −10.99 | −1.60 | −11.21 | −2.09 | +0.63 |

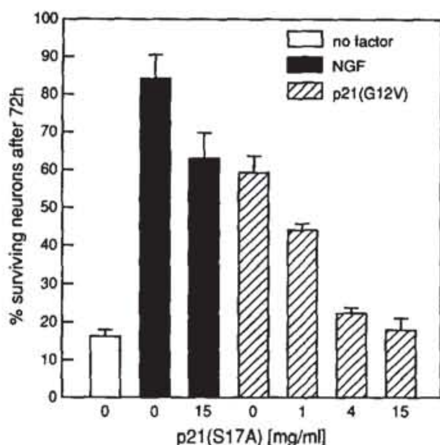


FIG. 5. **Biological activity of p21(S17A).** Dorsal root ganglions from chick embryos were cultured at embryonic day 9 and p21(S17A) was triturated into the neurons as described (Borasio *et al.*, 1989). The effects of the protein on neuronal survival induced by NGF (20 ng/ml, *solid columns*) or cotriturated oncogenic p21(G12V) (5 mg/ml, *hatched columns*) were monitored by counting live neurons 1 and 72 h after plating to determine survival rates.

amounts of p21(G12V) and p21(S17A) were triturated into dorsal root ganglia of chicken embryos, the action of the oncogenic p21 was completely inhibited by p21(S17A) (Fig. 5).

## DISCUSSION

The conformational change from the GTP-bound to the GDP-bound form is the critical reaction for the function of all guanine nucleotide-binding proteins. This reaction changes the $Mg^{2+}$-binding site as shown in Fig. 1, and the sequence elements which are involved in metal ion binding are completely conserved in all GNB proteins. The role of Asp-57 in the p21·Gpp(NH)p structure as shown in Fig. 1 has been clarified (Pai *et al.*, 1990) but has not been directly addressed in the p21·GDP structure (Tong *et al.*, 1991). In the structure of the p21·GDP complex resulting from GTP hydrolysis in the crystal, Asp-57 has been found to be directly coordinated to the metal ion (Schlichting *et al.*, 1990b). The function of this residue needs to be clarified by further structural investigations. In the structure of the G domain of EF-Tu (Jurnak, 1985; LaCour *et al.*, 1985), Asp-80 is 4.7 Å away from the metal ion.[4] Although water cannot be identified in the electron density map, it is plausible that a water ligand of the metal ion is bound by Asp-80.

We find that both the D57A and S17A mutation change the interaction between p21 and GTP drastically, although S17A has a larger effect. Since only S17A has a comparable, albeit weaker, effect on GDP binding, one would assume that Asp-57 is not coordinated to $Mg^{2+}$ in a similar way in the p21·GDP conformation. T35A has no effect on the interaction with GDP, which supports the result from crystallography that it is not involved in $Mg^{2+}$ binding (Schlichting *et al.*, 1990b; Tong *et al.*, 1991). Its only moderate effect on GTP binding seems to suggest that Thr-35 can be easily replaced by a water ligand without great disturbance of the nucleotide-binding site, contrary to what is found for Ser-17. It is remarkable that even drastic differences in overall affinity are caused by minor changes in the association rate constants and large changes in the dissociation rate constants. The most drastic effect is produced by the S17A mutation, which changes the affinity to GDP and GTP by factors of 450- and 23,000-fold, respectively.

The importance of the $Mg^{2+}$-binding site for the GAP-stimulated and for the unstimulated GTPase is documented by the results presented here. All three mutations investigated have a reduced unstimulated GTPase, where the effect of D57A is relatively small as compared to the S17A and T35A effects. GAP cannot stimulate the GTPase of these proteins, which confirms the results of Calés *et al.* (1988) on $p21^{N\text{-}ras}$(T35A). We also show here that p21(D57A) binds to GAP with wild-type affinity, whereas p21(S17A) and p21(T35A) have a much lower affinity. Since on the basis of the three-dimensional structure it is unlikely that the Ser-17 and Thr-35 side chains interact directly with GAP, we assume, that there is a structural rearrangement of p21, which is recognized by GAP.

It has been shown before that p21(S17N) behaves as a suppressor of normal p21 (Feig and Cooper, 1988; Szeberényi *et al.*, 1990) and that p21(S17A) and p21(S17C) behave similarly (Farnsworth and Feig, 1991). Several explanations have been given for the mechanism of inhibition (Farnsworth and Feig, 1991); in one the mutant protein is able to bind GTP in the cell, but the structure of the protein-GTP complex is such that the upstream target molecule is blocked for its interaction with normal ras protein. The nucleotide exchange factor has been assumed to be the probable target for the inhibition by the S17N (or A) mutation, since Stacey *et al.* (1991) have shown that additional mutations in the effector loop of S17N do not influence its inhibitory activity and that oncogenic ras is less inhibited than normal ras, since normal p21 is more dependent on nucleotide exchange than oncogenic p21 for reloading. Other explanations assume, given the preferential affinity of the Ser-17 mutation for GDP, that p21(S17N) or p21(S17A) are complexed *in vivo* to GDP only, or that they are unable to bind $Mg^{2+}$.

The absolute values for the affinities of the S17N or S17A protein to GDP/GTP have so far not been determined. From competition experiments it appeared that the mutated proteins have a 20-fold higher affinity for GDP than for GTP (Farnsworth and Feig, 1991). We also find that the S17A mutation has a 27-fold higher affinity for GDP than for GTP, but that the dissociation constants of 243 nM for GTP and 8.9 nM for GDP are sufficiently low to saturate the protein with guanine nucleotide in the cellular environment. It also indicates that with an estimated GTP/GDP ration of 30 (Trahey and McCormick, 1987) and because GAP does not accelerate GTP hydrolysis, approximately 50% of p21(S17A) would be complexed to GDP. Since D57A has an even greater preferential activity for GDP but is not a dominant negative suppressor of normal ras function (data not shown), it appears that it is not the preferential affinity but rather a distinct change in structure of p21(S17N) or p21(S17A) which is responsible for its biological activity.

Our results also indicate that $Mg^{2+}$ is still bound by the mutant protein albeit with a weaker affinity. However, its binding does not change the dissociation rate constant of GDP, as is found for normal p21. The NMR studies also show that there is a rearrangement of the structure around the nucleotide-binding site which could contribute to its suppressing activity. In support of this it has been shown that p21(S17N) has a higher affinity for a partially purified exchange factor (Farnsworth and Feig, 1991). One possible mechanism of an exchange factor which accelerates the guanine nucleotide release from p21 could be to remove or complex the metal ion in the nucleotide-binding site. p21(S17A) has fast dissociation rates for guanine nucleotides, which are

[4] J. Nyborg, unpublished data.

independent of $Mg^{2+}$ and are not accelerated by the SDC25 exchange factor (Mistou *et al.*, 1992). It is thus possible that the presumed tight binding of this mutant to the exchange factor mimics some complex along the reaction pathway between p21-nucleotide and exchange factor.

We have shown here that p21(S17A) has suppressing activity in primary chicken neurons, where it can counteract the effect of oncogenic p21(G12V), which supports survival of the primary neurons in the absence of nerve growth factor (Borasio *et al.*, 1989). This is a further indication that $p21^{ras}$ in primary neurons is employed in the signal transduction pathway involving NGF. The fact that oncogenic ras can be inhibited by the dominant inhibitory mutant is somewhat at variance with earlier findings that p21(S17N) inhibits normal ras action much more efficiently than that of oncogenic ras (Stacey *et al.*, 1991) and maybe a special feature of neurons.

Neither the survival of these neurons nor the differentiation of PC12 cells by NGF is inhibited by loading the cells with p21(S17A). This is in line with other observations that the effects mediated by NGF and the NGF receptor can be transmitted via different signal transduction pathways, only one of which uses $p21^{ras}$ (Thompson *et al.*, 1990; Qin *et al.*, 1991).

*Acknowledgment*—We thank Prof. K. C. Holmes for continuous support.

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