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Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules

(p21 purification/oncogenic mutation/valine/threonine/autophosphorylating activity)

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ABSTRACT **The 21-kilodalton protein (p21) encoded by normal cellular Harvey-*ras* has been expressed in *Escherichia coli* as a fusion protein by using the pUC8 vector and has been purified to >95% homogeneity by ion-exchange chromatography and gel filtration. The purified protein molecules possess intrinsic GTPase activity on the basis of the following criteria: (*i*) elution of the GTPase activity with p21 GDP-binding activity in two different chromatography systems, (*ii*) parallel thermal inactivation of GTPase activity and p21 GTP-binding activity, and (*iii*) immunoprecipitation of the GTPase activity with monoclonal antibodies to p21. At 37°C, the rate of GTP hydrolysis by the purified normal p21 assayed in solution was 5.3–6.6 mmol/min per mol of p21. The rate of GTP hydrolysis by a form of p21 [Val^{12}] encoded by a human oncogene was significantly lower (1.4–1.9 mmol/min per mol of p21). The presence of a threonine phosphate acceptor site at residue 59 also decreased p21 GTPase activity. For regulatory proteins that use GTP as part of their biochemical mechanism, the hydrolysis of GTP to GDP reverses the biological activity of the respective proteins. The observation that oncogenic forms of p21 lose GTPase activity suggests that GTP hydrolysis may be a biochemical event that inactivates the growth-promoting effects of a p21·GTP complex.**

The *ras* oncogenes constitute a multigene family that transform cells through the action of a 21-kilodalton protein termed p21 (1, 2). p21 can be detected in normal mammalian cells (3), and *ras*-related genes and proteins are found in *Drosophila* and yeast (4, 5). These observations provide evidence that *ras*-encoded proteins may have essential and evolutionarily conserved normal cellular functions. Transformation of some mammalian cells can occur if p21 is expressed at an abnormally high level (6, 7) or if mutations of the *ras* gene alter the p21 primary structure (2, 8–10). A particularly potent mutation is one that changes a glycine at residue 12 of normal p21 to a valine in the oncogenic form of p21 (8).

p21 interacts with the plasma membrane and lipid molecules (11, 12), binds guanine nucleotides specifically (13, 14), and possesses an autophosphorylating activity if a threonine phosphate acceptor replaces the alanine at cellular p21 residue 59 (14–16). Previously, we demonstrated that p21 autophosphorylating activity distinguished the glycine form of p21 from the valine form *in vitro* and in intact NIH 3T3 mouse fibroblast cells (17). Since cellular p21 molecules with an alanine at residue 59 do not autophosphorylate (16), we speculated (17) that autophosphorylating activity may be a marker for some other p21 property such as GTPase activity. GTPase activity is a common property of other guanine nucleotide-binding proteins, including elongation factor (EF)-G (18, 19), EF-Tu (18, 20), tubulin (21), and the membrane-bound G components of the adenylate cyclase (22) and transducin (23) systems. In this report, we present evidence that purified [Gly^{12}, Ala^{59}]p21 molecules possess intrinsic GTPase activity that is decreased by a valine at residue 12 or a threonine at residue 59.

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MATERIALS AND METHODS

DNA Biochemistry. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. The large (Klenow) fragment of DNA polymerase I was from Bethesda Research Laboratories. DNA fragments were isolated from 5% polyacrylamide gels by electroelution (24). Ligation reactions were performed at 4°C with 0.05 pmol of DNA fragments in 0.025 ml of 20 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/10 mM 2-mercaptoethanol/1 mM rATP and 400 units of T4 DNA ligase. Oligonucleotides were synthesized on an Applied Biosystem 380A-02 DNA synthesizer.

The v-Ha-*ras* expression plasmid pRAS (25) and the recombinant plasmids pEC/v-Ha and pEJ/v-Ha (17) have been described. To express EC/v-Ha and EJ/v-Ha, the 661-base-pair (bp) *Hae* II/*Pst* I fragment of pRAS encoding v-Ha-*ras* p21 amino acids 11–189 was replaced by the analogous section of pEC/v-Ha or pEJ/v-Ha producing pRAS [Gly^{12}, Thr^{59}] and pRAS [Val^{12}, Thr^{59}], respectively. These substitutions were accomplished by three-way ligations of the 67-bp *Bam*HI/*Hae* II fragment of pRAS, the 661-bp *Hae* II/*Pst* I fragment of pEC/v-Ha or pEJ/v-Ha, and the large *Bam*HI/*Pst* I fragment of pUC8 (26).

By oligonucleotide mutagenesis (27) the Thr-59 codon of Ha-*ras* was altered to that of Ala-59 as described below. The *Bam*HI/*Pst* I 728-bp fragment of pRas [Gly^{12}, Thr^{59}] was first inserted into M13mp9 (28). Single-stranded recombinant phage DNA (1 μg) was annealed with 0.1 μg of 5′-phosphorylated oligonucleotide d(C-T-T-A-G-A-C-A-C-A-G-C-A-G-G-T-C) in 0.030 ml of 20 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/50 mM NaCl at 55°C for 15 min. After 1 hr at room temperature, the solution was adjusted to 0.25 mM each dATP, dCTP, dGTP, and dTTP/10 mM 2-mercaptoethanol/0.5 mM rATP, and T4 DNA ligase (400 units) and Klenow fragment (4 units) were added. After a further 4 hr at room temperature, the reaction was quenched by heating to 70°C for 10 min. The solution was then treated for 2 hr at 37°C with 8 μg of proteinase K (Boehringer Mannheim) in 0.5% $NaDodSO_4$ and 0.5 M NaCl. Double-stranded DNA was recovered by ethanol precipitation after phenol extraction and incubated for 30 min with 5 units each of *Eco*RI and *Pst* I. The mutagenized Ha-*ras* fragment was inserted into pBR322 and used to transform *Escherichia coli* MM 294 (29). Selection of mutant [Ala^{59}] clones was by oligonucleotide hybridization (55°C) using the above primer (27). The mutant *Bam*HI/*Pst* I Ha-*ras* fragment was then recloned in pUC8 to give pRAS [Gly^{12}, Ala^{59}]. Finally, the corresponding pRAS

Abbreviations: bp, base pairs; v-Ha-*ras*, viral Harvey-*ras*; EF, elongation factor.

[Val^{12}, Ala^{59}] was constructed by a three-way ligation between the 101-bp *Bam*HI/*Pvu* II fragment of pRAS [Val^{12}, Thr^{59}], the 627-bp *Pvu* II/*Pst* I fragment of pRAS [Gly^{12}, Ala^{59}], and the large *Bam*HI/*Pst* I fragment of pUC8.

Protein Purification. *E. coli* (strain JM101) containing the expression plasmid were grown in 1-liter cultures for 20 hr at 37°C in the presence of 0.25 mM isopropyl β-D-thiogalactoside (Bethesda Research Laboratories) and ampicillin at 50 μg/ml. Bacteria were lysed with lysozyme (Sigma) at 100 μg/ml and 0.01% Nonidet P-40 (Sigma) as described (25) except that NaCl was omitted from the DNase digestion step. The prepared lysate was diluted 1:2 with cold buffer A (50 mM sodium Hepes, pH 7.5/1 mM sodium EDTA/1 mM dithiothreitol/0.01% *n*-octyl glucoside) and applied to a column (1.5 × 20 cm) of DEAE-Sephacel (Pharmacia) previously equilibrated with buffer A at 4°C. Protein was eluted with a 120-ml linear 0–0.6 M NaCl gradient in buffer A, and 2-ml fractions were collected. The peak p21 fractions were pooled (10 ml or less total volume) and applied to a column (2.6 × 34 cm) of Sephadex G-75 equilibrated with buffer A. The p21 that eluted from the Sephadex G-75 column was >95% homogeneous as analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis on 12% acrylamide gels (13), and 10–40 mg of purified protein was readily obtained from a 1-liter bacterial culture. During purification, p21 elution was followed by one of the assays described below, $NaDodSO_4$/polyacrylamide gel electrophoresis, or UV absorbance.

Assays. All assays were done in a 0.050-ml reaction volume containing buffer B (50 mM sodium Hepes, pH 8.0/0.5 mM $MgSO_4$/100 mM NaCl/1 mM dithiothreitol/0.25 mM adenyl-5′-yl imidodiphosphate). Blank values were determined by assaying each experimental condition in the absence of protein. GTPase assays were performed at 37°C in buffer B with 4 μM [γ-^{32}P]GTP (ICN, 10–80 cpm/fmol). Reactions were analyzed after charcoal treatment as described (22). Radioactive material in the supernatant after charcoal treatment was soluble in trichloroacetic acid, and the assay reaction products were identified as orthophosphate and GDP by chromatography on poly(ethylenimine)-cellulose (14, 22). Contaminating [^{32}P]phosphate and nonenzymatic [γ-^{32}P]GTP hydrolysis were <5% of total isotope.

Autophosphorylating activity was assayed in buffer B plus 4 μM [γ-^{32}P]GTP at 37°C. Reactions were stopped with 1.5 ml of ice-cold 5% trichloroacetic acid after addition of 10 μg of bovine serum albumin as carrier protein. Precipitated protein was collected on Millipore HA filters (25 mm). The incorporated [^{32}P]phosphate was stable to boiling in 1% $NaDodSO_4$/0.01% 2-mercaptoethanol, and all incorporated radioactivity corresponded to p21 as analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. Assay blank values were <0.5% of total isotope.

Nucleotide binding activity was measured in buffer B plus 4 μM [^{3}H]GDP or [^{3}H]GTP (both from Amersham, 10–11 Ci/mmol, 2000 cpm/pmol; 1 Ci = 37 GBq) at 37°C or 50°C. Reactions were stopped at 4°C with 1.5 ml of ice-cold 50 mM sodium Hepes, pH 8.0/0.25 mM $MgSO_4$, and bound nucleotide was collected on Schleicher & Schuell BA85 filters (25 mm). Radioactivity retained on the filters was bound to p21 as analyzed by gel filtration of the reaction mixtures on Sephadex G-25. Nonspecific binding (defined in the presence of 0.1–1.0 mM GTP) was equivalent to the assay blank (<1000 cpm). Near-maximal nucleotide binding to p21 was observed after 90 min at 37°C or 30 min at 50°C, and this measurement was used to determine the amount of p21 in each assay. Radioactivity in all of the above assays was quantitated by liquid scintillation spectrometry in Filtron X (National Diagnostics, Somerville, NJ).

Protein concentration was determined by the method of Lowry *et al.* (30) or by using the p21 molar absorptivity coefficient (ε) of 17,500 cm^{-1} M^{-1} at 280 nm. (Calculation of ε utilized protein concentrations determined by amino acid analysis.)

Immunoprecipitation. Immunoprecipitation of purified p21 molecules with Harvey-*ras* specific monoclonal antibody 172 (3) and staphylococcal protein A-Sepharose (Pharmacia) coated with rabbit anti-rat IgG (Cappell Laboratories, Cochranville, PA) was done as previously described (17) with the following modifications. Antibody (25 μl) was incubated with 600 μl of 7% (dry wt/vol) protein A-Sepharose in buffer C (1% Triton X-100/0.5% sodium deoxycholate/0.1% $NaDodSO_4$/100 mM NaCl/1 mM EDTA/10 mM sodium phosphate, pH 8.0) for 30 min at 4°C, 10 min at 50°C, and then 10 min at 4°C. The complex was washed five times with cold buffer C and two times with 50 mM sodium Hepes, pH 8.0/0.25 mM $MgSO_4$. This procedure ensured a low blank in the GTPase assay. Purified p21 (2–5 μg) was incubated with the immune complex at 4°C for 45 min in 100 μl of buffer B minus dithiothreitol. The mixture was then centrifuged for 15 min at 2000 × *g*, and the supernatant was collected. The pellet was washed two times with 50 mM sodium Hepes, pH 8.0/0.25 $MgSO_4$ and resuspended in 100 μl of buffer B minus dithiothreitol. Assays were initiated at 37°C after addition of radioisotope to the supernatant and immunoprecipitate fractions.

RESULTS

Preparation of p21 Variants. Four variants of Harvey-*ras* p21 have been expressed in *E. coli* as fusion proteins with a 20 amino acid NH_2-terminal extension and purified as described in *Materials and Methods*. We identify the p21 forms as EC [Gly^{12}, Ala^{59}], EJ [Val^{12}, Ala^{59}], EC/v-Ha [Gly^{12}, Thr^{59}], and EJ/v-Ha [Val^{12}, Thr^{59}]. The amino acid position numbers refer to authentic p21 and not the actual fusion products. Although we refer to the proteins as p21, the actual sizes of the fusion products (calculated as 23,260 daltons) were observed as 25,000 daltons by $NaDodSO_4$/polyacrylamide gel electrophoresis and 25,000–28,000 daltons by gel filtration on Sephadex G-75 (not shown). Peptide sequencing confirmed the presence of glycine or valine at position 12 as well as the complete fusion leader sequence. The purified proteins exhibited the same subtle mobility differences on $NaDodSO_4$/polyacrylamide gels (not shown) as we observed previously for these p21 variants expressed in NIH 3T3 mouse fibroblast cells (17).

Identification of GTPase Activity as an Intrinsic Property of p21. During purification of EC p21 and EC/v-Ha p21 on Sephadex G-75, it was observed that GTPase activity was eluted with EC p21 GDP-binding activity and with EC/v-Ha p21 autophosphorylating activity. To verify the cochromatography of GTPase activity with p21, purified EC p21 was subjected to ion-exchange chromatography (Fig. 1*A*) and gel filtration chromatography (Fig. 1*B*). The chromatograms show that GTPase activity and p21 GDP-binding activity were eluted in parallel from both DEAE-Sephacel and Sephadex G-150. Also, the distribution of activity was proportional to the amount of p21 in each fraction as detected by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1 *Insets*). The results in Fig. 1*B* show that p21 GDP-binding activity could be assayed at 50°C. This high temperature facilitated the exchange of radioligand with the bound GDP that copurifies with p21 (unpublished observation). The GTPase activity that chromatographed with p21 GDP-binding activity could also be assayed at 50°C, and its elution paralleled the GTPase activity assayed at 37°C (not shown).

Next, the thermal stability of GTPase activity in the EC p21 and EC/v-Ha p21 preparations was compared directly with two previously described intrinsic p21 biochemical properties: nucleotide binding and autophosphorylating activities. As shown in Fig. 2, GTPase, autophosphorylating, and GTP-binding activities were stable at 50°C for at least 5

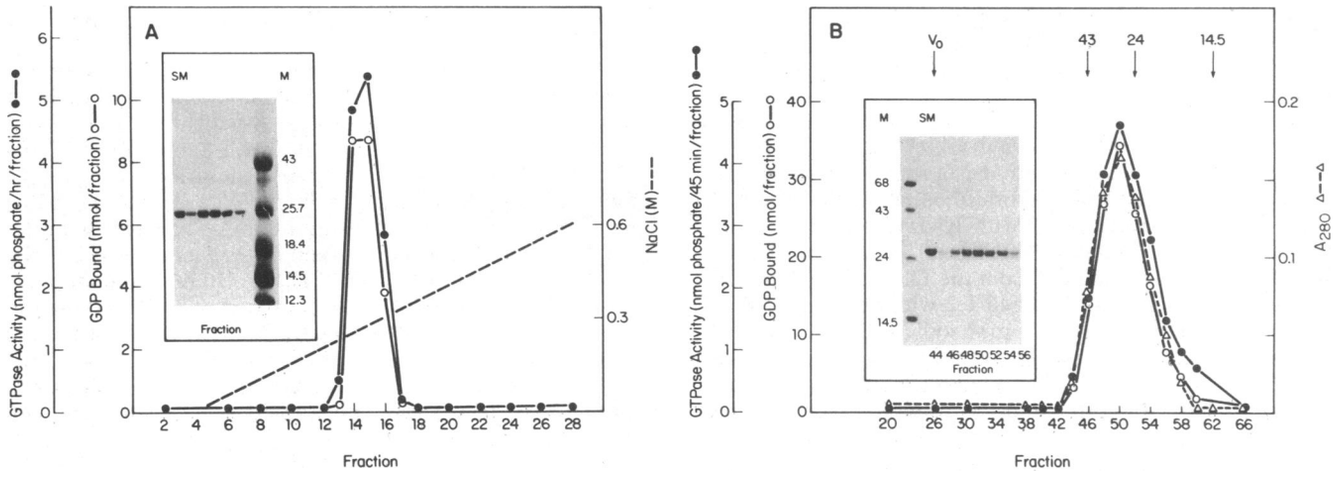

FIG. 1. Cochromatography of EC p21 GDP-binding and GTPase activities. (*A*) EC p21 was purified as described in *Materials and Methods* and applied to a column (1 × 6 cm) of DEAE-Sephacel. Protein was eluted with a 14-ml linear 0–0.6 M NaCl gradient in buffer A, and 0.5-ml fractions were collected. Aliquots of fractions were diluted 1:4 with buffer A before assay of GTPase activity (●) and [^{3}H]GDP binding activity (○) at 37°C for 60 min. The broken line indicates the theoretical concentration of NaCl. (*Inset*) The applied starting material (SM) and eluted fractions 13–17 were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis and protein was visualized by Coomassie blue stain. The positions of molecular size standards (kilodaltons) are denoted in lane M. Lane SM contains 9 μg of protein. (*B*) EC p21 was purified as described in *Materials and Methods* and applied to a column (2.6 × 34 cm) of Sephadex G-150. Protein was eluted with buffer A, and 2-ml fractions were collected. GTPase activity (●) was assayed at 37°C for 45 min; [^{3}H]GDP-binding activity (○) was assayed at 50°C for 30 min; and absorbance at 280 nm (△) was measured. The arrows indicate the elution of blue dextran (V_0), ovalbumin (43 kilodaltons), trypsinogen (24 kilodaltons), and lysozyme (14.5 kilodaltons). (*Inset*) The applied starting material (SM) and the indicated Sephadex G-150 fractions were analyzed by gel electrophoresis as in *A*. The recoveries of applied GDP-binding and GTPase activities in *A* and *B* were >88%. Assay blank values were <2% (binding) and <20% (GTPase) of the peak fraction activities.

min, and these activities decreased as a function of increasing temperature in an identical manner. Half-maximal inactivation of all three biochemical parameters occurred at approximately 60°C.

To further examine the intrinsic nature of the GTPase activity, we tested whether the GTPase activity was immunoprecipitable with p21 monoclonal antibody 172. This particular monoclonal antibody was chosen because it is highly specific for Harvey-*ras* p21; it does not recognize the related Kirsten-*ras* p21 (3) or the yeast-*ras*-encoded protein (5). Antibody 172 removed 94% of the GTP-binding activity and 81% of the GTPase activity from the EC p21 sample, as shown in Table 1, experiment 1. The activity removed from the supernatant was present in the immunoprecipitate, with overall recoveries of 65% and 70% for GTP-binding activity and GTPase activity, respectively. Immunoprecipitation of GTP-binding activity and GTPase activity was dependent on the presence of antibody (Table 1, experiment 1), and the amount of both activities in the precipitate increased in parallel as a function of limiting amounts of antibody used (not shown). The GTPase specific activities in the minus-antibody supernatant and the plus-antibody immunoprecipitate were 0.375 and 0.313 pmol of phosphate per 90 min per pmol of p21, respectively. In other experiments with EC p21, up to 95% of the GTPase activity was removed from the sample supernatant with antibody 172, and identical results were obtained when p21 monoclonal antibody 259 was used instead of antibody 172 (not shown). In EC/v-Ha p21 preparations, antibody 172 effectively immunoprecipitated both GTPase and autophosphorylating activities (Table 1, experiment 2). The ratio of GTPase activity to autophosphorylating activity in the minus-antibody supernatant (1.99) was similar to that ratio in the plus-antibody precipitate (2.19). It is unlikely that immunoprecipitated GTPase activity is due to another pro-

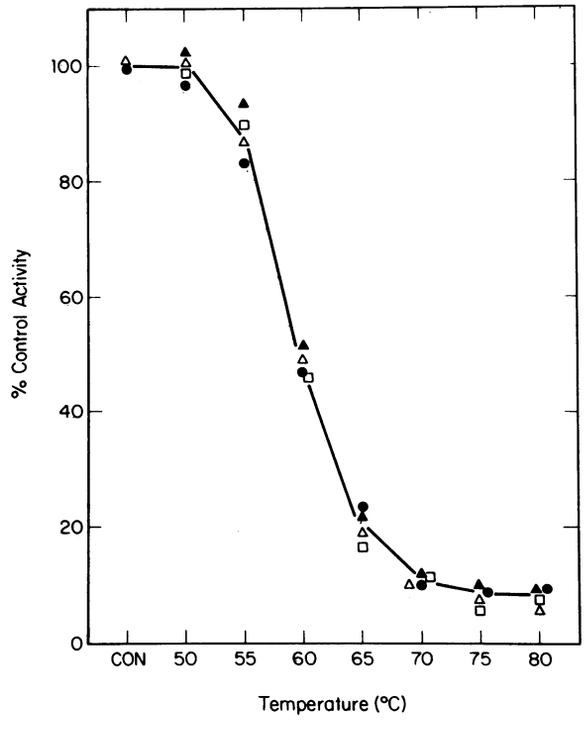

FIG. 2. Heat stability of p21 biochemical activities. Aliquots of purified EC and EC/v-Ha p21 in buffer A were heated at the indicated temperature for 5 min, quickly chilled in an ice bath, and then centrifuged (7000 × *g*) for 60 sec. The supernatant was then analyzed in buffer B plus 0.01% Nonidet P-40 at 50°C for 30 min. p21 is stable in buffer B at 50°C for at least 60 min (not shown). EC p21 [^{3}H]GTP-binding (●) and GTPase (▲) activities and EC/v-Ha p21 autophosphorylating (□) and GTPase (△) activities are expressed as percent of the control activity (CON) in samples with no heat treatment. The maximal amounts of p21 determined by [^{3}H]GTP binding were 98.8 pmol (EC) and 115 pmol (EC/v-Ha) per assay. Maximal GTPase activity values were 56.6 pmol of phosphate (EC) and 9.22 pmol of phosphate (EC/v-Ha), and maximal EC/v-Ha autophosphorylation was 6.44 pmol of phosphate. The subtracted blank values were 0.25 pmol (nucleotide binding), 3.66 pmol (GTPase), and 0.32 pmol (autophosphorylation).

Table 1. Immunoprecipitation of p21 biochemical activities

Exp.	p21 source	Assay	Anti-body	Activity, pmol sup	ppt	sup + ppt
1	EC	GTP binding	−	16.0	0	16.0
			+	0.58	9.78	10.4
	EC	GTPase	−	6.01	0	6.01
			+	1.17	3.06	4.23
2	EC/v-Ha	Autophos-phorylation	−	0.78	0	0.78
			+	0.11	0.58	0.69
	EC/v-Ha	GTPase	−	1.55	0	1.55
			+	0.17	1.27	1.44

Purified EC and EC/v-Ha p21 were immunoprecipitated (+) with Harvey-*ras* p21 monoclonal antibody 172 previously immobillzed on protein A-Sepharose. The minus-antibody condition (−) was protein A-Sepharose without antibody 172. [^{3}H]GTP binding, GTPase, and autophosphorylating activities were measured at 37°C for 90 min in the absence of dithiothreitol, and data are expressed as pmol of nucleotide bound or pmol of phosphate formed during the entire incubation period. The supernatant and precipitate fractions were obtained after the p21 sample was incubated with the antibody–protein A-Sepharose complex and centrifuged. In each experiment, the supernatant (sup) and precipitate (ppt) fractions were then divided into equal portions so that each assay was done with identical samples. An experimental blank value was determined for every measurement by assaying that condition in the absence of p21. The ranges of blank values were 0.82–1.2 pmol (binding), 7.0–8.4 pmol (GTPase), and 0.84–1.4 pmol (autophosphorylation). Immunoprecipitation experiments typically had higher blank values and lower activity values than normal solution-phase experiments. Similar activity distributions were observed in assays done at 50°C, in which blank values were 10–35% of the maximal activity values (not shown).

tein bound to p21 because p21 elutes from molecular sieving columns as a monomer species with the same size as that determined on $NaDodSO_4$/polyacrylamide gels (Fig. 1*B* and data not shown).

Purified p21 GTPase and autophosphorylating activities had identical guanine nucleotide specificities, and [γ-^{32}P]ATP was not a substrate for these reactions (data not shown). Both activities required magnesium ion, but the activities were independent of magnesium concentration (0.5–50 mM).

We also tested whether our EC p21 preparations were contaminated by the GTPase activity of EF-Tu (43 kilodaltons) or EF-G (80 kilodaltons) because these proteins constitute 1–6% of total *E. coli* protein (18). The low level GTPase activities of EF-Tu and EF-G are stimulated by ribosomes (18–20). However, the GTPase activity in our EC p21 preparations was not affected by *E. coli* NH_4Cl-washed ribosomes under assay conditions that showed an effective stimulation of EF-G GTPase activity (data not shown).

p21 GTPase Activity Is Decreased by Mutations. Since amino acid mutations alter p21 biology and autophosphorylating activity, it was tested whether mutations also modulated p21 GTPase activity. The rate of GTP hydrolysis by EC p21 [Gly^{12}, Ala^{59}] was 4.3- to 5.0-fold greater than the rate of GTP hydrolysis by EJ p21 [Val^{12}, Ala^{59}] (Fig. 3). Results similar to those in Fig. 3 were also observed when the GTPase activities were expressed relative to the amount of [^{3}H]GTP bound at each time point (data not shown). Previous data (17) showed that the Val-12 mutation increased p21 autophosphorylating activity when the Thr-59 phosphate acceptor was present. Therefore, the GTPase and autophosphorylating activities of the purified EC, EJ, EC/v-Ha, and EJ/v-Ha p21 variants were directly compared (Table 2). It is clear that the conversion of GTP to GDP by purified EC p21 assayed in solution is greater than that by the other three p21 variants even when autophosphorylating and GTPase activities are added together (Table 2, column C). EC/v-Ha p21 GTPase activity was 6.5- to 8.5-fold greater than that for

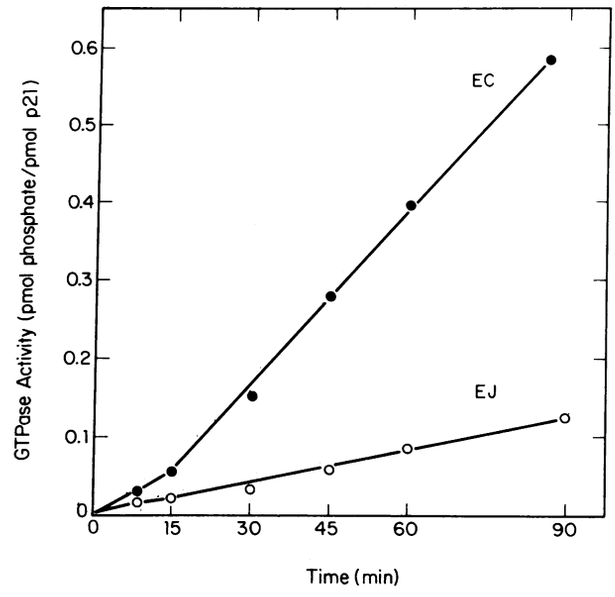

FIG. 3. Time course for GTPase activities of EC p21 and EJ p21. Assay mixtures (325 μl) containing 2 μM [γ-^{32}P]GTP and EC p21 (●) or EJ p21 (○) at 19 μg/ml were prepared on ice, and reactions were initiated at 37°C. At the times indicated, a 50-μl aliquot was removed and treated with charcoal at 4°C. The GTPase assay blank values were <7% (EC) and <38% (EJ) of the activities measured at the 90-min time point. The amounts of p21 per 50-μl aliquot (determined by [^{3}H]GTP binding at 37°C for 90 min) were 18.4 pmol (EC) and 10.5 pmol (EJ). The assay lag period is abolished if the assay mixtures are prewarmed to 37°C and then the reactions are initiated by addition of p21 (not shown).

EJ/v-Ha p21, and EJ/v-Ha p21 autophosphorylating activity was 2-fold greater than that for EC/v-Ha p21. However, the sums of GTPase and autophosphorylating activities for these two variants were not substantially different from each other or from that of EJ p21. At present, we do not know whether the differences observed for the EC/v-Ha and EJ/v-Ha p21 variants reflect a possible influence of p21 phosphorylation state on GTPase activity or simply a partitioning phenomenon between water and threonine-59 as phosphate acceptors.

Table 2. GTPase and autophosphorylating activities of p21 mutants

p21 preparation	Activity, fmol of phosphate per 90 min per pmol of p21 A. GTPase	B. Autophos-phorylation	C. (A + B)
1. EC	596 ± 170 (22)	0	596 ± 170
2. EC	481 ± 42 (7)	0	481 ± 42
3. EC	562 ± 190 (5)	0	562 ± 190
4. EJ	129 ± 50 (13)	0	129 ± 50
5. EJ	169 ± 14 (4)	0	169 ± 14
6. EC/v-Ha	153 ± 110 (4)	41 ± 3 (3)	194 ± 110
7. EC/v-Ha	118 ± 20 (5)	38 ± 2 (2)	156 ± 20
8. EJ/v-Ha	18 ± 10 (4)	81 ± 3 (3)	99 ± 10

Different preparations of the indicated p21 form were assayed in multiple experiments for GTPase and autophosphorylating activities at 37°C for 30–90 min. The amount of p21 per assay (7.24–112 pmol) was determined by [^{3}H]GTP binding at 37°C for 90 min or [^{3}H]GDP binding at 50°C for 30 min. Data are the mean ± SD for the number of determinations indicated in parentheses. GTPase assay blank values were 3–23% (EC), 10–42% (EJ), 9–28% (EC/v-Ha), and 32–70% (EJ/v-Ha) of the measured activities. Autophosphorylation assay blank values were <7%.

The rate at which p21 converts GTP to GDP is very slow when compared to typical enzymatic reactions. The calculated turnover numbers at 37°C for EC p21 and EJ p21 are 6.0 ± 2.1 and 1.6 ± 0.6 mmol of GTP hydrolyzed per min per mol of protein, respectively. Even at 50°C, the turnover number for EC p21 is only 19–26 mmol/min per mol of p21 (data not shown and from Fig. 2 legend).

DISCUSSION

The results in this report demonstrate that cellular p21 with alanine at position 59 is capable of hydrolyzing GTP. Since the oncogenic mutations Val-12 and Thr-59 alter p21 GTPase activity, it seems likely that this activity is important for p21 cellular function. Guanine nucleotide-binding proteins such as EF-G, EF-Tu, tubulin, and the G components of adenylate cyclase and transducin hydrolyze GTP slowly, with turnover numbers of 12–250 mmol/min per mol of protein at 37°C (19–23). The GTPase activities of these proteins are stimulated by other cellular components and physiological effectors, and one can speculate that the low GTPase activity of p21 may also be stimulated by a cellular component or effector. It is also possible that various effectors can differentially stimulate the GTPase activity of normal and oncogenic forms of p21.

The biochemical model that has emerged for guanine nucleotide-binding regulatory proteins is that the biologically active protein·GTP species are deactivated by GTP hydrolysis (reviewed in ref. 31). If p21 functions in a manner analogous to other G proteins and if we assume that oncogenic forms of p21 have decreased GTPase activity *in vivo*, then a model for p21-mediated cell transformation would involve an increase of the p21·GTP population either by mutations that reduce p21 GTPase activity or by overexpression of normal p21. Moreover, if GTP hydrolysis is rate limiting for the inactivation of p21·GTP, then this model predicts that mutated p21 molecules with reduced GTPase activity would be inherently more potent for transformation than more normal molecules of p21.

There is no direct proof yet that p21 regulates a biological process by a GTP hydrolysis mechanism, and it cannot be excluded that p21 functions in another manner (for example, phosphorylation of proteins or small molecules). Nevertheless, the observations on the GTPase activity of EC and EJ p21 support the hypothesis that p21-mediated cell transformation involves a biologically active p21·GTP complex. The experimental analogy to other GTP-utilizing proteins (19–23) makes us sanguine that an analogous biochemical cycle will soon be discovered for p21, involving cellular proteins and effectors with which p21 interacts to control the transformation of cells.

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