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Purification of ras GTPase activating protein from bovine brain

(ras regulation/GTP-binding regulatory proteins/oncogenes/YPT1)

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ABSTRACT **In cytosolic extracts of bovine brain, we detected ras GTPase activating protein (GAP) activity that stimulated the GTP hydrolytic activity of normal c-Ha-ras p21 but not that of the oncogenic [Val^{12}]p21 variant. GAP was purified 19,500-fold by a five-column procedure involving DEAE-Sephacel, Sepharose 6B, orange dye and green dye matrices, and Mono Q resins. A single major protein band of 125 kDa was observed on NaDodSO$_4$/polyacrylamide gels that correlated with the elution of GAP activity on Mono Q. Purified GAP was devoid of inherent GTP hydrolytic activity, suggesting that it was a regulator of ras intrinsic GTPase activity. Under submaximal velocity conditions, the second-order rate constant of GTP hydrolysis at 24°C for p21–GTP + GAP (4.5×10^6 M^{-1}·sec^{-1}) was at least 1000-fold greater than that for [Val^{12}]p21–GTP + GAP ($<3 \times 10^3$ M^{-1}·sec^{-1}).**

The proposed actions of the ras proteins have been modeled after the known guanine nucleotide-binding regulatory proteins (G proteins). Like the G proteins, ras binds GTP and GDP specifically and has a GTP hydrolytic activity (1). Furthermore, only the GTP complexes of the ras proteins are biologically and biochemically active (2–7). Activating mutations in ras can impair GTPase activity or stimulate nucleotide exchange (8). These observations suggest that ras action is normally controlled by regulation of a GTP/GDP cycle.

We have previously identified the guanine nucleotides bound to ras-encoded proteins in growing *Saccharomyces cerevisiae* cells (5). Whereas the 38-kDa normal yeast RAS proteins were bound entirely to GDP, the 21-kDa normal mammalian c-Ha-ras protein (p21) expressed in yeast was bound to near equimolar GTP and GDP. This observation suggested that different mechanisms might be influencing the distribution of nucleotides complexed to these proteins in yeast cells. When normal c-Ha-ras was microinjected into ^{32}P-labeled *Xenopus* oocytes, only GDP was observed to be bound (J.B.G., M.D.S., and I.S.S., unpublished observations). The oncogenic [Val^{12},Thr^{59}]p21 that has impaired GTPase activity was complexed to >75% GTP in both yeast cells and frog oocytes. These results indicated that yeast and vertebrate cells could be distinguished by the ability to regulate the guanine nucleotides bound to p21.

Recently, Trahey and McCormick (6) observed that the GTP hydrolytic rate of N-ras was much greater upon microinjection into *Xenopus* oocytes than *in vitro*. Furthermore, this effect could be mimicked *in vitro* upon the addition of oocyte cytosol. Forms of N-ras protein with impaired GTPase were unaffected by the factor from the cytosolic fraction, suggesting that this activity might be important in the physiological regulation of normal ras action. Trahey and McCormick (6) have termed this factor GTPase activating protein (GAP). As a further step toward the characterization of GAP, we report here its purification from bovine brain.

METHODS

Assays. GTP hydrolytic activity was detected by preequilibrating ras proteins with [γ-^{32}P]GTP, incubating the reaction mixtures at 24°C and then quantitating the decrease in radiolabeled ras–GTP complex upon nitrocellulose filtration. This method readily distinguishes the GTP hydrolytic rates of p21 vs. [Val^{12}]p21 (8). *Escherichia coli*-expressed ras proteins were purified as described (9). Preequilibration was performed at 30°C for 15 min in buffer A {100 mM sodium phosphate, pH 6.8/0.5 mM EDTA/0.5 mg of bovine serum albumin per ml/0.5 mM dithiothreitol/0.005% sodium cholate/0.5 μM ras protein/0.5 μM [γ-^{32}P]GTP (1500–3000 Ci/mmol; 1 Ci = 37 GBq; ICN)}. Under these conditions, nucleotide binding was essentially complete within 5 min without any apparent GTP hydrolysis.

A standard GAP assay was performed for 5 min at 24°C. Reactions (final volume, 50 μl) contained 25 μl of 40 mM sodium Hepes (pH 7.5)/2.0 mM $MgCl_2$, 5 μl of bovine serum albumin (10 mg/ml), 10 μl of water, 5 μl of p21-[γ-^{32}P]GTP from the reaction above, and 5 μl of GAP preparation. The reaction was stopped on ice upon the addition of 1 ml of cold 25 mM Tris·HCl, pH 7.5/0.25 mM $MgCl_2$. Samples were collected on nitrocellulose filters (Schleicher & Schuell, BA 85; 25 mm) and quantitated by Cerenkov counting. Hydrolysis was calculated from the decrease of radioactivity compared to reactions performed in the absence of GAP. Hydrolysis of p21–GTP in the absence of GAP was negligible during the time frame of the assay. Activity was linear with time as long as <50% p21–GTP was hydrolyzed. In reactions performed with p21–[α-^{32}P]GTP or p21–[γ-^{32}P]GTP, the sole reaction products were GDP bound to p21 and free orthophosphate as determined by immunoprecipitation and thin-layer chromatography analyses (see below).

Protein concentrations were determined by the Coomassie dye method as supplied by Bio-Rad or by the amido black staining method of Schaffner and Weissman (10). Bovine serum albumin (Sigma A7030 or A7906) was used as the standard reference protein.

Purification Procedure. All steps were carried out at 4°C. Fresh bovine brains were obtained from a local slaughterhouse. The cerebra were isolated and washed in ice-cold 0.9% NaCl. The cerebra from four to six animals were homogenized in a Waring blender in 3 liters of buffer B (25 mM Tris·HCl, pH 7.5/1 mM $MgCl_2$/1 mM EGTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride). The homogenate was passed through five layers of cheesecloth and then centrifuged at 5000 × *g* for 20 min. The resulting supernatant was then centrifuged at 100,000 × *g* for 15 min to obtain a clarified supernatant fraction.

The high-speed supernatant fraction was incubated in a batch with 400 ml of DEAE-Sephacel (Pharmacia LKB) for at least 2 hr and then washed by using a Buchner funnel with

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Abbreviations: GAP, GTPase activating protein; G protein, GTP-binding regulatory protein.

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2 liters of buffer B. The resin was poured into a 5 × 30 cm column and washed with an additional 1 liter of buffer B. Elution was achieved with a 2-liter linear 0–0.3 M NaCl gradient. Flow rate was 60 ml/hr, and 20-ml fractions were collected. A single symmetrical peak of GAP activity eluted between 70 and 90 mM NaCl as determined by conductivity (model 32 conductance meter, Yellow Springs Instrument). The pooled peak fractions (200–300 ml) were concentrated to 30 ml by filtration with an Amicon PM-30 membrane in a stirred cell.

The concentrated pool was applied to two 5-cm columns linked in tandem (total resin length, 150 cm) containing Sepharose 6B (Pharmacia LKB). Elution was continued at 60 ml/hr with buffer B, and 17.5-ml fractions were collected. A single peak of GAP activity eluted at 1.80 liters of buffer B. For reference, the standard proteins alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa) eluted at 1.78 and 1.90 liters, respectively.

The Sepharose 6B pool was applied to a 2.5 × 30 cm column of orange dye matrix (Amicon). The column was then washed with buffer B until the absorbance at 280 nm reached baseline. Elution was achieved with a 400-ml linear 0–0.4 M NaCl gradient at 30 ml/hr and 3-ml fractions were collected. A single peak of GAP activity was detected between 40 and 70 mM NaCl.

Peak fractions from the orange dye matrix column (30–40 ml) were applied to a 1.5 × 27 cm column of green dye matrix (Amicon). After application, the column was washed in buffer B containing 20% (vol/vol) glycerol and 0.3 M NaCl. Elution of GAP activity was achieved with a 400-ml linear 0.3–1.0 M NaCl gradient in buffer B plus 20% glycerol. Fractions (2 ml) were collected at 15 ml/hr. GAP activity was detected in a broad peak between 600 and 620 mM NaCl. The peak activity fractions (20–60 ml) were pooled and dialyzed overnight against 1 liter of buffer B plus 20% glycerol but lacking the phenylmethylsulfonyl fluoride.

The dialyzed material was applied to a Mono Q HR 5/5 column (Pharmacia LKB) at 0.7 ml/min. Reservoir A contained 25 mM Tris·HCl, pH 7.5/1 mM EGTA/1 mM $MgCl_2$/1 mM dithiothreitol/10% glycerol, and reservoir B contained the same buffer plus 1.0 M NaCl. Elution was achieved with an increasing NaCl gradient from 0 to 70 mM for 5 min, 70 to 150 mM for 30 min, and 150 to 1000 mM for 10 min. Flow rate was 0.7 ml/min and 0.5-min fractions were collected. A single peak of GAP activity corresponding to a single peak of A_{280} absorbing material eluted at ≈120 mM NaCl. The fractions were stored on ice at 4°C. Activity was more stable at 4°C upon the addition of bovine serum albumin (0.5 mg/ml). Dilutions of GAP prior to assay were done with buffer B plus bovine serum albumin (0.5 mg/ml).

Other Methods. $NaDodSO_4$/polyacrylamide gel electrophoresis with 7.5% acrylamide gels was done at 25–30 mA constant current using a Bio-Rad baby gel apparatus. Proteins were visualized with Coomassie blue R-250 or silver stain (Bio-Rad).

Immunoprecipitations of ras proteins bound to [α-^{32}P]GTP (3000 Ci/mmol; New England Nuclear) were done as described (5) with monoclonal antibody Y13-259 (11). Nucleotides were analyzed by chromatography on poly(ethyle·e)-imine-cellulose (Baker) developed with 1 M KH_2PO_4 and followed by autoradiography. For control hydrolysis reactions, potato acid phosphatase (Sigma P1146) and *Crotalus atrox* venom 5′ nucleotidase (Sigma N5880) were used with the unit activities stated by the supplier.

RESULTS

Detection and Isolation of GAP Activity. GAP activity was first described by Trahey and McCormick (6) using cytosolic extracts from *Xenopus* oocytes and cultured mammalian cells. We confirmed their observation in frog oocytes by using both their assay procedure as well as the one described in *Methods*. For the purpose of identifying a tissue source for the purification of GAP, the cytosolic fractions of various rat organs were assayed for GAP activity. Highest activity was measured in brain and testis. Intermediate activity was detected in kidney, spleen, lung, liver, and heart, and low activity was present in hind limb skeletal muscle. The detected activity promoted GTP hydrolysis of p21–GTP without having an effect on [Val^{12}]p21–GTP. GAP activity remained in the supernatant fraction after centrifugation at 100,000 × *g* for 45 min, and no activity was detected in the particulate fraction. The GAP activity detected in organ tissue and in frog oocytes had identical elution profiles when chromatographed on Mono Q or Superose 12 (Pharmacia LKB), and the effects of these activities were inhibited by ras monoclonal antibody Y13-259 (data not shown). By these criteria, it appeared that the GAP activities in frog oocyte and rat tissue were similar. Based on the survey of GAP activity in rat, bovine brain was chosen for the purification procedure because large amounts of starting material could be obtained.

The purification of GAP is documented in Table 1. The method gave a 19,500-fold purification of the activity in the cytosolic extract with a 4.2% yield. In six trials, 7–25 μg of purified GAP per brain could be isolated. The critical steps were sequential chromatography on the orange and green dye matrix columns. The interaction of GAP with these resins was apparently independent of the nucleotide-like properties of the dyes because elution was not effected by 1 mM concentrations of ATP, GTP, NAD, or EDTA. The final chromatography step on a Mono Q column resolved GAP from the remaining contaminants and concentrated the sample volume. As shown in Fig. 1, GAP activity eluted from Mono Q with a single A_{280} absorbing peak that was well resolved from the other peaks. The purified GAP preparation contained a single major polypeptide of 125 kDa as detected by $NaDodSO_4$/polyacrylamide gel electrophoresis on 7.5% polyacrylamide gels and Coomassie blue staining (Fig. 1 *Inset*) that coeluted with activity. Silver staining of these fractions indicated that purity was 90% (data not shown). No bands were observed in the 12- to 43-kDa range upon analysis with

Table 1. Purification of GAP from bovine brain

Step	Volume, ml	Protein, mg	Activity, pmol/min	Specific activity, pmol·min^{-1}·mg^{-1}	Purification, -fold	Yield, %
1. Cytosolic fraction	1400	13,100	1300	0.099	—	—
2. DEAE-Sephacel	140	393	1470	3.74	38	113
3. Sepharose 6B	110	75	480	6.4	65	37
4. Orange dye	38	5.3	600	113	1,140	46
5. Green dye	55	0.74	190	257	2,600	15
6. Mono Q	1.75	0.028	54	1930	19,500	4.2

Cerebra from four bovine brains were used as starting material. Activity was measured by stimulation of p21–GTP hydrolysis. Specific activity is defined as the amount of p21–GDP produced from p21–GTP per amount of GAP protein preparation (mg) added to the assay.

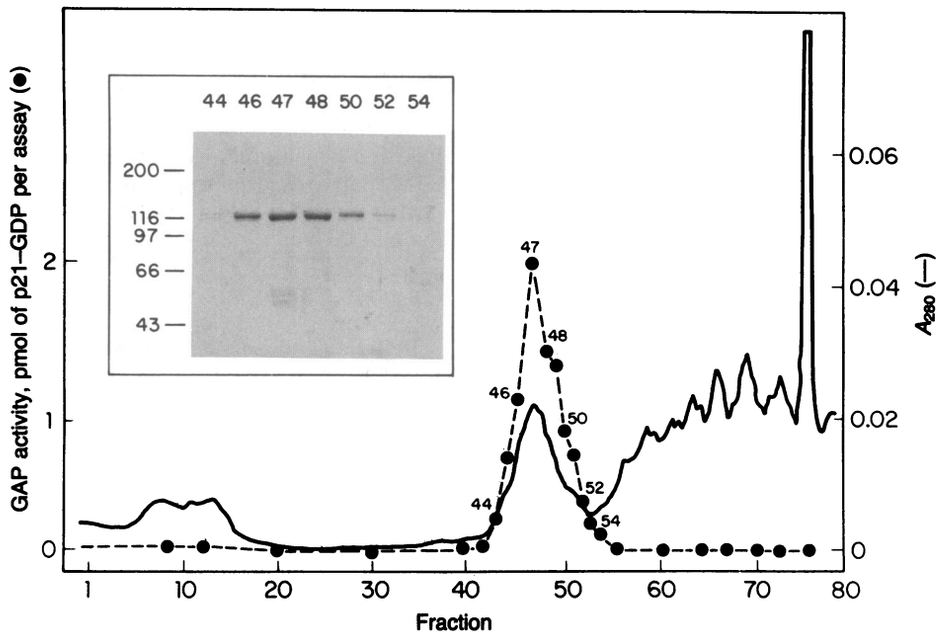

FIG. 1. Elution of GAP from Mono Q. Protein from the green dye column eluate was applied to a Mono Q HR 5/5 column and eluted. Fractions were 0.35 ml. GAP activity was assayed at 24°C. Aliquots of the peak GAP activity fractions were diluted 1:5 to 1:30 to maintain assay linearity. (*Inset*) The indicated fractions (15-μl aliquots) were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis on 7.5% acrylamide gels and stained with Coomassie blue R-250. Numbers on left are in kDa.

13.5% polyacrylamide gels. The apparent size for GAP upon gel filtration on Sepharose 6B was 130 kDa (see *Methods*). Based on the apparent size of GAP (125 kDa), we calculate 8 nmol of GAP per mg of GAP protein. Subsequent expressions of mol of GAP are corrected for the purity of the preparation and assume 100% active protein.

GAP activity was stable for at least 1 month at 4°C in the presence of 10% glycerol at protein concentrations >20 μg/ml. Freeze–thawing of the purified material destroyed activity. GAP retained activity after incubation at 42°C for 30 min; however, incubation at 56°C for 30 min abolished detectable activity. Activity was reversibly inhibited by NaCl (IC_{50}, 40 mM). This effect of NaCl necessitated assaying the green dye matrix column eluate (600–620 mM NaCl) at higher temperature (30°C) and longer time (10–20 min) to detect GAP activity.

Effect of GAP on ras Proteins. We tested whether purified GAP might be acting on p21–GTP as a nonspecific phosphatase. As shown in Fig. 2, the conversion of p21–GTP to p21–GDP was markedly stimulated upon addition of GAP but was only slightly affected by potato acid phosphatase or snake venom 5′ nucleotidase (lanes 1–4). In the presence of GAP (lane 2), the p21–GTP was near-quantitatively converted to p21–GDP. No GMP was detected and only small amounts of [^{32}P]orthophosphate were present. In the absence of p21, free GTP was not a substrate for GAP, whereas acid phosphatase and 5′ nucleotidase readily hydrolyzed the nucleotide (lanes 5–8). These results suggested that GAP was stimulating the intrinsic GTPase activity of p21 without exerting a nonspecific hydrolyzing activity.

GAP assay activity increased as a function of temperature over the range 10–37°C; however, no activity was detected at 0°C. The velocity of product formation (p21–GDP) was proportional to both p21–GTP and GAP concentrations (data not shown). In an experimental mixture containing 56 fmol of GAP and 1900 fmol of p21–GTP, 530 fmol of p21–GDP was formed under linear conditions within 1 min at 24°C. Nearly 10 mol of p21–GDP was produced per 1 mol of GAP. In this experiment, the apparent first-order rate constant for the conversion of p21–GTP to p21–GDP was 0.28 min^{-1}. The same rate (0.30 ± 0.05 min^{-1}) was observed over a p21–[γ-^{32}P]GTP range of 50 to 1900 fmol when 56 fmol (1.1 nM) of GAP was present. The apparent first-order rate constant was dependent on the amount of GAP in the assay (0.18 min^{-1} and 0.5 min^{-1} in the presence of 0.56 and 1.7 nM GAP, respectively). Under these submaximal velocity conditions, the second-order rate constant at 24°C for p21–GTP plus GAP was calculated to be 4.5×10^6 $M^{-1} \cdot sec^{-1}$.

Purified GAP was assayed with various ras proteins, and the results are summarized in Table 2. In addition to stimulating the GTPase activity of p21, GAP increased the rate of the yeast RAS1 185 amino acid N-terminal domain [RAS1

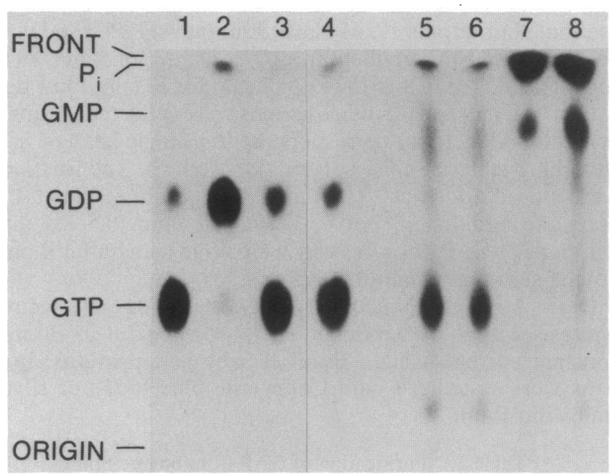

FIG. 2. Stimulation of p21–GTP hydrolysis by purified GAP. Reaction mixtures contained buffer B (lanes 1 and 5), 1 pmol of GAP (lanes 2 and 6), 0.02 unit of potato acid phosphatase (lanes 3 and 7), or 1.0 unit of snake venom 5′ nucleotidase (lanes 4 and 8). The components were incubated at 24°C for 10 min with 0.7 pmol of p21-[α-^{32}P]GTP (lanes 1–4) or 3.3 pmol of [α-^{32}P]GTP (lanes 5–8) under the conditions of the GAP assay and then placed on ice. Reaction mixtures containing p21–GTP were immunoprecipitated with monoclonal antibody Y13-259 and treated as described (5). Sample aliquots (2000 cpm) were spotted onto poly(ethylene)imine-cellulose, developed with 1 M KH_2PO_4, and visualized by autoradiography. The migration of nucleotide standards is indicated.

Table 2. Effect of purified GAP on mutant ras proteins and YPT1

Protein	k, min^{-1}		-fold stimulation
	− GAP	+ GAP	
p21	0.0020	0.19	95
RAS1 (term. 185)	0.0052	0.34	65
[Val^{12}]p21	<0.0002	<0.0002	—
[Leu^{61}]p21	<0.0002	<0.0002	—
[Pro^{11}]p21	0.0045	0.25	55
[Pro^{12}]p21	0.0055	0.0067	1.2
[Asn^{11},Ser^{12}]p21	0.0013	0.0013	—
YPT1	0.0013	0.0013	—

Purified proteins bound to [γ-^{32}P]GTP (0.4–0.8 pmol) were incubated in the absence and presence of 40 fmol of GAP at 24°C. Buffer solutions were prewarmed to 24°C, protein–GTP was added, and reactions were initiated upon addition of GAP. Reactions were linear over the times tested (30, 60, and 90 min or 1, 2, and 3 min for assays minus or plus GAP, respectively). Results are the first-order rates for conversion of protein–GTP to protein–GDP at the stated concentration of GAP. At 0.8 nM GAP (40 fmol), the reaction is not at maximal velocity.

(term. 185); ref. 12]. No stimulation was observed with the activated proteins [Val^{12}]p21 and [Leu^{61}]p21 that have impaired intrinsic GTP hydrolytic activity (1, 12). The second-order rate constant for [Val^{12}]p21–GTP + GAP was $<3 \times 10^3$ $M^{-1}\cdot sec^{-1}$, which is at least 1000 times lower than that for p21–GTP + GAP. No hydrolysis of [Val^{12}]p21–GTP was observed by increasing the assay temperature to 37°C or incubating with GAP for 90 min. Both [Pro^{11}]p21 and [Pro^{12}]-p21 have GTP hydrolytic rates in the absence of GAP that are 2-fold greater than that of normal p21. However, [Pro^{11}]p21 was sensitive to GAP, whereas [Pro^{12}]p21 was not. GAP also had no effect on the yeast GTP-binding protein YPT1 (13) or [Asn^{11},Ser^{12}]p21, which has the substitutions found at YPT1 residues 16 and 17.

DISCUSSION

The ras GTPase activating protein activity in bovine brain analogous to that described in *Xenopus* oocytes (6) was purified to near homogeneity by a five-column procedure. The 19,500-fold purification of GAP indicated that it constituted <0.01% of total soluble protein in bovine brain cerebra. The increase in total GAP activity units was reproducibly observed during the early column purification steps (Table 1) and possibly reflects GAP alterations and modifications. The major protein in our final preparation was 125 kDa as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis and was assigned as GAP based on its coelution with GAP activity on Mono Q. The similarity in size between the 125-kDa band and the molecular size of GAP activity as chromatographed on Sepharose 6B (130 kDa) suggested that GAP was a monomeric polypeptide.

GAP appeared to act in a catalytic manner to stimulate ras GTP hydrolytic activity. The observation that purified GAP was devoid of inherent GTP hydrolytic activity is consistent with a model in which GAP regulates ras GTPase. The apparent first-order rate for the conversion of p21–GTP to p21–GDP was independent of p21–GTP and dependent on GAP over the concentrations tested. In *Xenopus* oocytes, the rate for the conversion of N-ras–GTP to N-ras–GDP was determined to be 0.25–0.35 min^{-1} at room temperature (6). This rate corresponds to the values determined at 24°C for p21 *in vitro* when 56 fmol (1.1 nM) of purified GAP was present.

As shown in Table 2, the ability of GAP to stimulate ras GTP hydrolytic activity was dependent on the amino acid substitutions at ras residues 11, 12, and 61. Known activating mutations such as Val-12 and Leu-61 rendered ras insensitive to GAP. Under the conditions stated in Table 2, the GTP hydrolytic rate of p21 in the presence of GAP was at least 950-fold greater than those of [Val^{12}]p21 and [Leu^{61}]p21. The yeast RAS1 (term. 185) protein, which has the sequence Gly-Gly-Gly-Gly at residues analogous to p21 10–13 (Gly-Ala-Gly-Gly), [Pro^{11}]p21, and [Pro^{12}]p21 had GTP hydrolytic rates in the absence of GAP that were 2-fold greater than that of p21. The -fold stimulation in response to GAP was greatest for p21 followed by RAS1 (term. 185) and [Pro^{11}]p21, which were much greater than [Pro^{12}]p21. At present, we do not know whether the amino acids at residues 11, 12, and 61 influence the physical interaction of ras with GAP or whether this position influences an active conformation necessary for GAP-stimulated GTP hydrolytic activity. [Pro^{12}]p21, like p21, is unable to transform mammalian cells when present at low expression levels (14). Since [Pro^{12}]p21 did not respond to GAP *in vitro*, this result implies that ras intrinsic GTPase activity, independent of GAP, may also be a factor that determines biological potency. The inability of [Pro^{12}]p21 to respond to GAP would result in a [Pro^{12}]p21–GTP complex *in vivo* that would be more stable than a p21–GTP complex but less stable than a [Val^{12}]p21–GTP complex. Accordingly, [Pro^{12}]p21 might be expected to be biologically more potent than normal c-Ha-ras [Gly^{12}]p21.

GTP/GDP exchange of elongation factor Tu and the stimulatory G-protein component of adenylate cyclase is influenced by elongation factor Ts and agonist-occupied receptors, respectively (15, 16). The GTPase activity of elongation factor Tu is stimulated by ribosomes. An analogous factor has not yet been reported for the stimulatory G-protein component of adenylate cyclase. At present we do not know whether GAP is an upstream regulator or a downstream target of ras. If GAP functions *in vivo* to regulate ras action, then GAP may mediate some of the effects of growth factors or other proliferation signals. The inability of GAP to influence the GTP hydrolytic activity of YPT1 protein, another 20-kDa G protein in yeast cells and mammalian tissues (13, 17, 18), suggests that ras and YPT1 are subject to distinct regulatory mechanisms. Future experiments should be aimed at determining whether GAP is specific for ras or whether GAP can influence other G proteins having glycine at the position analogous to ras residue 12.

Transfection assays with the DNA of human tumors have identified only dominant oncogenes. If GAP serves as an upstream regulator to turn off ras function, then one would predict that GAP could be a recessive oncogene upon inactivation of its activity. This possibility can now be examined biochemically in various tumors and cell lines.

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