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Biochemistry

Ha-*ras* proteins exhibit GTPase activity: Point mutations that activate Ha-*ras* gene products result in decreased GTPase activity

(p21 proteins/oncogenic mutations/GTP binding and hydrolysis)

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ABSTRACT **Several *ras* genes have been expressed at high levels in *Escherichia coli* and the resultant *ras* proteins were shown to be functional with respect to their well-known specific, high-affinity, GDP/GTP binding. We were able to detect a weak GTPase activity associated with the purified proteins. The normal cellular *ras* protein (p21N) exhibits ≈10 times higher GTPase activity than the "activated" proteins. Even though the turnover rate of the reaction is very low (0.02 mol of GTP hydrolyzed per mol of p21N protein per minute), the reaction appears to be catalytic; one molecule of p21N hydrolyzes more than one molecule of GTP. The GTPase and the GDP binding activities both have been recovered from a M_r 23,000 protein eluted following NaDodSO$_4$/polyacrylamide gel electrophoresis, suggesting that these two activities are associated with the same protein. Mg^{2+} ions and dithiotheitol are required for GTPase activity and the optimal pH is between 7 and 8. Guanidine·HCl, which is required for solubilizing bacterially expressed *ras* protein, is strongly inhibitory to GTPase activity at concentrations higher than 0.5 M.**

Members of the *ras* gene family encode closely related proteins of ≈189 amino acid residues and M_r 21,000 termed p21 *ras* (1, 2). The *ras* genes were first identified as the oncogenic sequences of certain strains of acute transforming retroviruses (3). Their normal counterparts (cellular *ras* genes) are highly conserved in eukaryotic cells (4–10). Cellular *ras* genes acquire transforming properties by single point mutations within their coding sequences (11–20) and the "activated" *ras* genes are detected in a significant fraction of human cancers and in experimentally obtained animal tumors (21–23). The observed conservation of cellular *ras* genes and analysis of their expression (24, 25) have led to speculations that p21 proteins are essential components of normal cells involved in cell division and differentiation (26). It has also been observed that elevated expression of the normal cellular p21 gene can induce transformation (27). More recently, Stacey and Kung (28) observed that both mutated and normal p21 proteins can induce transformation of NIH 3T3 cells, when introduced by microinjection, but that higher concentrations of the normal protein were required. These observations strongly suggest that high levels of cellular p21 protein, as well as low amounts of activated p21 protein have the same effect on NIH 3T3 cells and this might be due to altered or lack of regulation of cellular p21 activity consequent to its "activation" by mutation. However, various studies aimed at understanding the biochemical basis for the transforming activity of *ras* gene products gave no major differences in their subcellular localization, post-translational modification, and *in vitro* guanine nucleotide binding properties of normal and activated *ras* proteins (29, 30).

We have shown recently that bacterially produced p21 protein binds guanine nucleotides with high affinity using a simple nitrocellulose filter assay (31). In the present study we show that GTPase activity is associated with p21 *ras* proteins and that cellular p21 *ras* protein (p21N) exhibits ≈10 times more GTPase activity than its activated counterparts.

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MATERIALS AND METHODS

Preparations. Table 1 describes the five different *ras* proteins we used in this study. All were produced in *Escherichia coli* and highly purified by the following procedure. Ten grams of *E. coli* cells was suspended in 25 ml of phosphate-buffered saline (P_i/NaCl) containing 5 mM EDTA, 25% sucrose, 1% Triton X-100, and 25 mg of lysozyme. The suspension was spun in a vortex and the homogeneous suspension was frozen and quickly thawed three times. DNase I (≈4 mg) was added to the lysate. After incubation at 0°C for 20 min, the lysate was centrifuged at 25,000 × *g* for 15 min. The pellet was washed four times with 1% Triton X-100/25% sucrose. The washed pellet was then dissolved in P_i/NaCl containing 3.5 M guanidine·HCl and extensively dialyzed against 3.5 M guanidine·HCl. Any material that precipitated after dialysis was removed by centrifugation at 15,000 × *g* for 10 min and the clear supernatant containing p21 protein was stored at −20°C. These preparations are stable for months without any significant loss of activity.

Assays. The GDP binding was measured as described (31). GTPase activity was determined by the release of $^{32}P_i$ from [γ-^{32}P]GTP as follows. Method A: The reaction mixtures (50 μl) contained 20 mM 3-(*N*-morpholino)propanesulfonic acid at pH 7.5, 0.2 mM $MgCl_2$, 5 mM dithiothreitol, 350 mM guanidine·HCl, 1 μg of bovine serum albumin, and 2 μM [γ-^{32}P]GTP (4–17 × 10^5 cpm per assay) (Amersham or New England Nuclear) and were incubated at 37°C for 60 min. The reaction was stopped by addition of 200 μl of ice-cold 0.7 M perchloric acid containing 25 mM KH_2PO_4 and 4% active charcoal. The mixture was then centrifuged for 10 min in an Eppendorf centrifuge. The supernatant, 50 μl, was carefully taken out and mixed with 10 ml of Hydrofluor (National Diagnostics, Somerville, NJ), and the radioactivity was determined in a Beckman LS 7800 liquid scintillation counter. Method B: Alternatively, the method of Wahler and Wollenberger (34), which is based on the extraction of $^{32}P_i$-labeled dodecamolybdate complex into isopropylacetate, was used. The reaction was stopped with 50 μl of 1 M $HClO_4$ containing 1 mM KH_2PO_4, and 150 μl of 20 mM sodium molybdate and 300 μl of isopropyl acetate were added. After vigorous mixing for 30 sec and centrifugation for 5 min in an Eppendorf tabletop centrifuge, 100 μl of organic phase was mixed with 10 ml of Hydrofluor and the radioactivity was counted in a Beckman LS7800 liquid scintillation counter. Both methods yielded consistent results and the identity of reaction products, GDP and P_i, was confirmed by thin-layer chromatography on PEI-cellulose plates. Protein was determined by the method of Bradford (35) using bovine serum albumin as the standard. Electrophoresis of proteins at pH 8.8 was carried out in 12.5% or 12–17% gradient NaDodSO$_4$/polyacrylamide gels as described by Laemmli (36).

Table 1. Ha-*ras* p21 proteins

Protein	Viral origin	NH_2 terminus†	Position 12	Position 59	Position 143	Source or ref.
p21T	BALB MSV	a	Lys	Ala	Lys	‡
p21N	BALB MSV	a	Gly	Ala	Lys	32
p21T*	BALB MSV	b	Lys	Ala	Lys	32
p21H	Harvey MSV	a	Arg	Thr	Glu	33
p21H*	Harvey MSV	b	Arg	Thr	Glu	§

MSV, murine sarcoma virus.

†a: Met-Thr-Glu-Tyr-Lys-Leu-Val-Val-Val-Gly-Ala-
b: Met-Asn-Glu-Phe-Gly-Ser-Ile-Asp-Lys-Leu-Val-Val-Val-Gly-Ala-

‡E. P. Reddy, personal communication.
§Unpublished data.

RESULTS

Purity of Bacterial *ras* Proteins. Fig. 1 shows the NaDodSO$_4$/polyacrylamide gel electrophoresis pattern of the five different *ras* protein preparations used in the present study. p21N, p21T, and p21H migrate in these 12–17% gradient gels as $M_r \approx 23{,}000$ proteins, whereas p21T* and p21H* show slightly slower mobility, probably due to the presence of four extra amino acid residues at their NH_2 termini. One distinct feature of all of these *ras* proteins expressed in *E. coli* RRI cells under the transcriptional control of the P_L promoter of bacteriophage λ is that they are aggregated into large inclusion bodies inside the *E. coli* cell as seen under the electron microscope (37). We have exploited this property for purifying these proteins from *E. coli* cells (see *Materials and Methods*). Because of their density, these inclusion bodies can be separated rapidly from bacterial membranes and the cytosolic fraction by centrifugation through a 25% sucrose cushion. With this single step, it is possible to obtain p21 protein preparations that are at least 80% pure as judged by densitometric scanning of the NaDodSO$_4$/polyacrylamide gels.

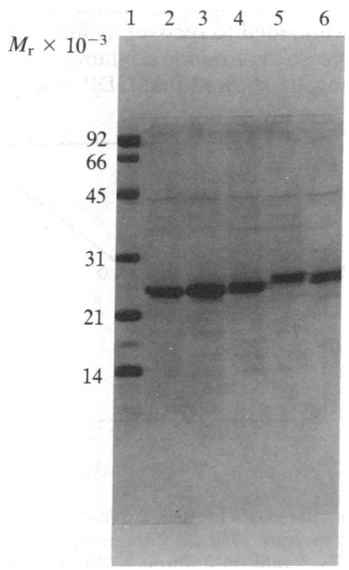

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of purified recombinant *ras* proteins. The indicated amounts of p21 *ras* proteins were subjected to electrophoresis under denaturing conditions through a 12–17% gradient NaDodSO$_4$/polyacrylamide gel (Separation Sciences, Attleboro, MA) followed by staining with Coomassie brilliant blue R-250 and were destained in 20% methanol containing 7% acetic acid. Lane 1, protein molecular weight markers: phosphorylase B (M_r 92,500), bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,400); lane 2, p21N (9.6 μg); lane 3, p21T (11 μg); lane 4, p21H (10 μg); lane 5, p21H* (7.8 μg); lane 6, p21T* (8 μg).

GTPase Acitivity of *ras* Proteins. We have compared the GTPase activity of the five different *ras* proteins, purified to the same level. Even though the GTPase reaction is slow, it is highly reproducible. The p21N *ras* protein that is closely related to the normal human Harvey-*ras* (Ha-*ras*) protein consistently gave ≈10 times more GTPase activity than the other four, which are activated *ras* proteins (Fig. 2). Fig. 2 also shows that the GTPase activity is proportional to the amount of *ras* protein present in the assay. Based on a M_r of 23,000, the turnover number for p21N is ≈1.2 mol of GTP hydrolyzed per mol of p21N protein per 60 min. The products of the reaction were characterized to be P_i and GDP (not shown).

GTPase Activity Requirements. Since the GTPase activity associated with all of the *ras* proteins is very low, we tested whether it could be improved by further manipulation of the assay conditions. We found that 100 mM NaCl, which we had routinely used, was inhibitory to the GTPase activity (50% inhibition). The optimal pH is between 7 and 8 (Fig. 3*A*), and Mg^{2+} ions and dithiothreitol are required for opti-

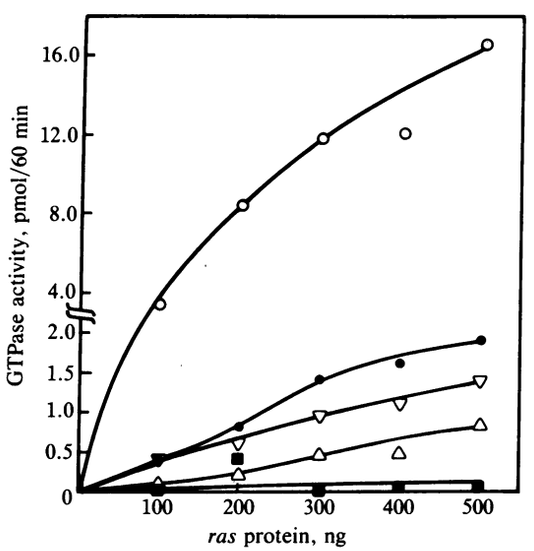

FIG. 2. GTPase activity of *ras* proteins. The GTPase activity was determined by method B at the indicated amounts of *ras* protein. Non-enzymatic hydrolysis during the 60-min incubation under identical assay conditions (2700 cpm or 0.56 pmol) was substracted from all of the values shown. ○, p21N; ●, p21T; ▽, p21H; △, p21H*; ■, p21T*. Simultaneously, 0.5 μg of each *ras* protein was assayed for [^{3}H]GDP binding as described (31). The amounts of [^{3}H]GDP bound are 5.7 pmol (p21N), 23 pmol (p21T), 11.1 pmol (p21H), and 6 pmol (p21H*). p21T* did not show significant [^{3}H]GDP binding.

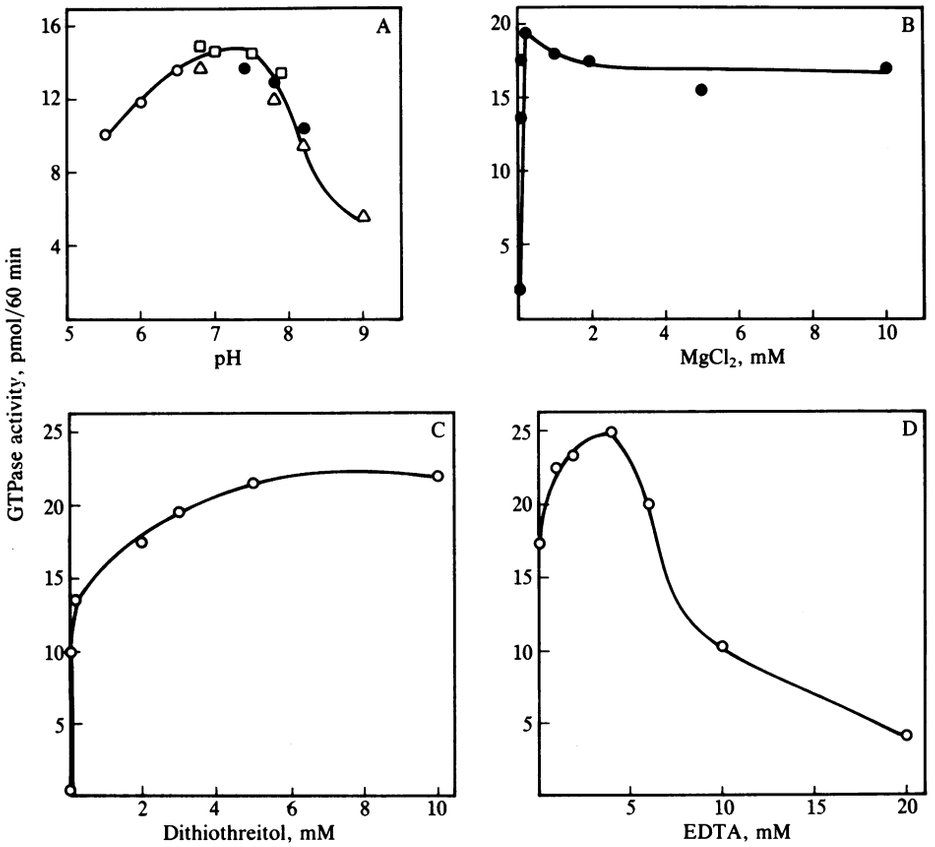

FIG. 3. Effects of pH, $MgCl_2$, dithiothreitol, and EDTA on GTPase activity of normal Ha-*ras* protein (p21N). (*A*) GTPase activity (method A) was determined by using 0.4 μg of p21N in the presence of 20 mM buffer of the indicated pH. ○, 2-(*N*-morpholino)ethanesulfonic acid; □, 3-(*N*-morpholino)propanesulfonic acid; ●, Hepes; △, Tris·HCl. (*B*) GTPase activity (method B) was determined by using 0.5 μg of p21N protein at the indicated concentrations of $MgCl_2$. (*C*) GTPase activity (method A) was determined by using 0.5 μg of p21N protein under standard assay conditions, except that the concentration of dithiothreitol was varied as indicated. (*D*) GTPase activity (method A) was determined by using 0.5 μg of p21N protein and 5 mM $MgCl_2$. EDTA was supplemented to the reaction mixtures as indicated.

mal activity (Fig. 3 *B* and *C*). In the absence of added $MgCl_2$, there is little basal activity (Fig. 3*B*) and this is abolished with 1 mM EDTA. High concentrations of $MgCl_2$ (2–10 mM) are slightly inhibitory, and this is confirmed by titration with EDTA of a reaction mixture containing 5 mM $MgCl_2$ (Fig. 3*D*). The GTPase activity was stimulated by EDTA concentrations between 1 and 4 mM and then sharply declined. This may be due to inhibition of GTPase activity by heavy metal ion(s).

Time Course and Catalytic Nature of the Reaction. Since the GTPase activity exhibited even by the normal *ras* protein is very weak when compared to well-known GTPases, it is interesting to know whether the reaction is catalytic or simply stoichiometric in nature. Long incubations showed the reaction to be fairly linear over periods of several hours (Fig. 4). There is an initial lag period of about 10 min. As suggested earlier, this may reflect the fact that the *ras* protein from guanidine·HCl solution is renaturing (31). After 3 hr, ≈35% of the substrate GTP (35 pmol) is hydrolyzed by 14 pmol of p21N protein. Thus, we conclude that the reaction is catalytic.

GTPase Activity Is an Inherent Activity of p21N Protein. The fact that activated p21 *ras* protein preparations having identical contaminants as p21N *ras* protein exhibit 1/10th as much GTPase activity suggests that GTPase activity is probably associated with the p21N protein. In addition, other recombinant proteins of similar size (e.g., immune interferon) made in *E. coli* under the control of the same expression plasmid and solubilized from the particulate fractions of bacterial cell extracts in an identical manner to that used for p21 had minor contaminants similar to those in the p21 preparations shown in Fig. 1 yet exhibited no trace of GTPase activity.

To eliminate the possibility that the GTPase detected in the *ras* preparations is not due to a trace contaminant, Na$DodSO_4$/polyacrylamide gel-purified proteins were analyzed. p21N protein was subjected to electrophoresis in a 12.5% Na$DodSO_4$/polyacrylamide gel after heat denaturation of the sample in Na$DodSO_4$ sample buffer. The gel was intentionally overloaded to recover as much protein and activity as feasible so that minor contaminants would be detectable. The results showed that GDP binding activity and

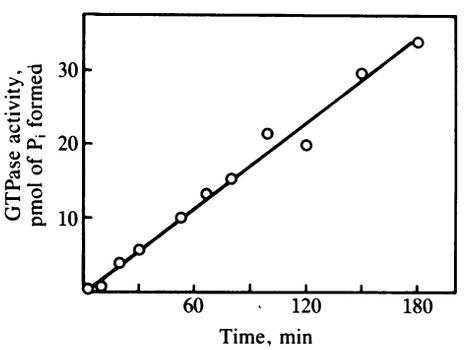

FIG. 4. Time course of GTPase activity exhibited by p21N protein. A reaction mixture (750 μl) containing 20 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM dithiothreitol, 350 mM guanidine·HCl, 15 μg of bovine serum albumin (Bethesda Research Laboratories), 7.5 μg of p21N *ras* protein, and 2 μM [γ-^{32}P]GTP (9452 cpm/pmol) was incubated at 37°C. At the indicated intervals of time, 50-μl aliquots were withdrawn into 200 μl of ice-cold 0.7 M perchloric acid containing 25 mM KH_2PO_4 and 4% active charcoal and kept on ice until samples from all time points were collected for subsequent processing. A control reaction mixture with identical composition but without p21N protein was incubated simultaneously at 37°C and 50-μl aliquots were removed at the same time intervals as for the reaction mixture containing p21N proteins and processed as above. The values shown are for 50-μl aliquots and have been obtained after substracting the nonenzymatic hydrolysis at the corresponding intervals of time.

GTPase activity were present in the gel slice that contained p21N protein (Fig. 5). However, ≈25% of the GDP binding/GTPase activity was also detected in a fraction from the top of the gel. On re-electrophoresis of this fraction, a protein band was detected at p21N position (Fig. 5*C*). Thus, we conclude that the activity probably reflects aggregation or precipitation of part of the p21N sample at the top of the gel. This is not unexpected since p21 is fairly insoluble in the absence of guanidine·HCl. These results strongly suggest that both GDP binding and GTPase activities are intrinsic to the p21N protein.

Effect of Guanidine·HCl. Because of their insolubility, the *ras* proteins are not active with respect to GDP/GTP binding or GTPase activity unless some guanidine·HCl or urea is present in the assay mixture. Fig. 6 shows results of our analysis of the effect of various concentrations of guanidine·HCl on the reaction. Amounts <200 mM appear to be insufficient to keep the protein in solution. Under these conditions GTPase activity is lost. Higher concentrations (>500 mM) inhibit the activity. The concentration of guanidine·HCl required for optimal GTPase activity depends to some extent on protein concentration in the assay mixture. We found that with 0.5 μg of protein in the assay mixture, 350 mM guanidine·HCl gives optimal and consistent results. The GTPase activity is linear up to 0.5 μg of protein in the assay.

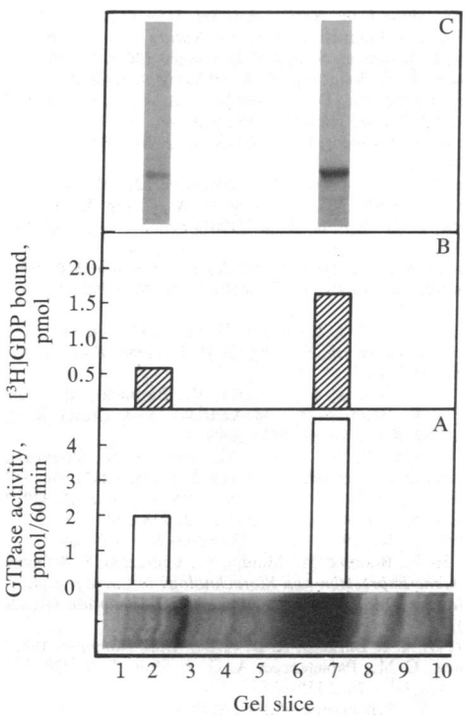

FIG. 5. Reconstitution of the GTPase and GDP binding activities of p21N protein following $NaDodSO_4$/polyacrylamide gel electrophoresis and re-electrophoresis of the recovered proteins with GDP binding/GTPase activities. Purified p21N *ras* protein, 500 μg, was heat denatured in the presence of $NaDodSO_4$ and 2-mercaptoethanol and was electrophoresed through a 3-mm-thick 12.5% $NaDodSO_4$/polyacrylamide gel in five lanes (each lane, 100 μg of protein). One lane was cut out for staining by Coomassie brilliant blue, and the rest of the gel was sliced into 1-cm pieces, proteins were eluted from the gel slices, $NaDodSO_4$ was removed by acetone precipitation, and the protein was renatured from guanidine·HCl solution essentially as described by Hager and Burgess (38). Small aliquots of sample from each fraction were assayed for GTPase activity by method B (*A*) and by [^{3}H]GDP binding (*B*). About 40% of the initially applied GTPase activity/GDP binding activity was recovered after reconstitution. One of the lanes stained for protein was shown at the bottom of *A*. The proteins recovered from slices 2 and 7 were re-electrophoresed in 12–17% $NaDodSO_4$ gels and are shown at the top in *C*.

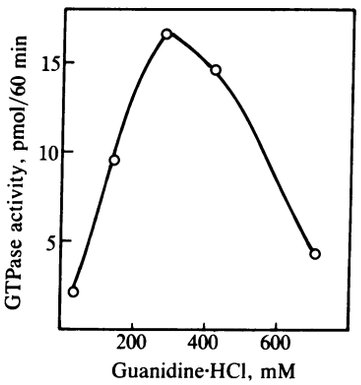

FIG. 6. Effect of guanidine·HCl on the GTPase activity of p21N *ras* protein. The GTPase activity of p21N protein (0.4 μg) was determined by method B at the indicated concentration of guanidine·HCl in the assay.

DISCUSSION

One of the well-characterized biochemical properties of *ras* proteins is their ability to bind guanine nucleotides with high specificity and affinity (29, 31, 39–41). It has been repeatedly suggested that the guanine nucleotide binding property of the *ras* proteins might be involved in the mechanism by which *ras* proteins bring about transformation (29, 39–41). However, a systematic comparison of normal cellular *ras* protein and activated counterparts failed to reveal any major differences in their guanine nucleotide binding characteristics (29, 30). Since the guanine nucleotide binding activity appears to be common to all p21 molecules, it seemed important to determine if any other biochemical parameters may be altered as a result of *ras* protein activation. The present study suggests one possibility; we have consistently observed that the normal cellular *ras* protein has at least 10 times higher GTPase activity than its activated counterparts.

The major complication in the present study is the very slow turnover rate of the reaction under the conditions employed. It is possible that guanidine·HCl, which is required to keep the protein soluble, may slow down the reaction due to its denaturing properties. We have tried 1 M urea in the assay mixtures without observing any significant difference. Earlier results have suggested that these denaturing agents do partially inhibit the reaction kinetics. In our hands, bacterial *ras* proteins cannot be solubilized with a variety of detergents (either alone or in combination). This contrasts with results reported by two other groups (42, 43), who were able to solubilize bacterial p21 proteins with detergents. The fact that the latter *ras* proteins are fusion proteins containing 14 and 20 extra amino acids, respectively, at their NH_2 termini may be significant. It is also possible that the slow GTPase activity might reflect the requirement for another component in the reaction or a different molecular environment. p21 is localized in the inner cytoplasmic membrane and a hydrophobic environment could be essential for optimal activity.

In the current context it may be worth considering the properties of the diverse group of proteins that exhibit a high specific affinity for guanosine nucleotides. It includes polypeptide chain initiation factors, polypeptide chain elongation factors, tubulin, transducin, and guanine nucleotide binding regulatory proteins (N proteins) of the eukaryotic adenyl cyclase system. Many of these proteins exhibit GTPase activity in conjunction with another protein(s). These GDP/GTP binding proteins (including ras protein) contain a conserved region of primary structure (44). The sequences for bacterial

elongation factor Tu (EF-Tu) and human Ha-*ras* protein are particularly striking in their degree of homology (50%). EF-Tu exhibits GTPase activity in the presence of aminoacyl tRNA and ribosomes (for a review, see ref. 45). It has long been suspected that the catalytic center for GTP hydrolysis is on EF-Tu itself. However, direct evidence was obtained only when it was found that kirromycin, an antibiotic which binds EF-Tu, induces GTPase in the absence of aminoacyl tRNA and ribosomes (46). It was later observed that a kirromycin-resistant EF-Tu exhibits weak GTPase activity (47). Elongation factor G and prokaryotic initiation factor 2 also exhibit GTPase activity in the presence of ribosomes only. Presumably, ribosomal proteins trigger the enzymatic activity in them. The inhibitory guanine nucleotide binding regulatory component of adenyl cyclase (Ni) has been shown recently to have a weak GTPase activity (48). The GTPase activities of transducin and tubulin are markedly increased by rhodopsin (49) and polymerizing conditions, respectively. Some of the components in the retinal light-activated cGMP-phosphodiesterase system and the hormone-sensitive adenyl cyclase system are interchangeable, suggesting that they are functionally analogous. For example, rhodopsin from retinal system strongly stimulates GTPase activity of Ni (50). All of these examples of guanine nucleotide binding proteins, which, by themselves, show very weak or no GTPase activity in the absence of an interacting component(s), suggest that the same may be true for p21 *ras* proteins. It will be important to determine if any components from the rhodopsin cycle or the adenyl cyclase system will stimulate GTPase activity of p21 *ras* proteins.

Our results considered against this background allow speculations concerning the manner by which *ras* proteins may cause transformation. It seems possible that the *ras* protein is a regulatory molecule controlling cell division or differentiation; its mechanism of action could possibly be similar to transducin or N protein. The active signal in such a case might be a p21·GTP complex that is dissipated as soon as the GTP molecule is hydrolyzed. If for some reason the activated protein were unable to hydrolyze GTP efficiently, the result could be persistent activation of the system, which might then produce uncontrolled cell division and transformation. The fact that normal *ras* protein has higher GTPase activity than its transforming counterparts lends some credence to this hypothesis. Higher amounts of normal *ras* protein cause transformation probably due to the persistent activation of the system. A component(s) that could interact with p21 *ras* proteins and specifically stimulate GTPase activity associated with *ras* proteins has yet to be identified. After submitting this paper, McGrath *et al.* (51) reported similar results.

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