A Cytosolic Protein Catalyzes the Release of GDP from p21^{ras}

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The rate of release of guanine nucleotides from the *ras* proteins (Ras) is extremely slow in the presence of Mg^{2+}. It seemed likely, therefore that a factor might exist to accelerate the release of guanosine diphosphate (GDP), and hence the exchange of GDP for guanosine triphosphate (GTP). Such a factor has now been discovered in rat brain cytosol. Brain cytosol was found to catalyze, by orders of magnitude, the release of guanine nucleotides from recombinant v-H-Ras protein bound with [α-^{32}P]GDP. This effect occurred even in the presence of a large excess of Mg^{2+}, but was destroyed by heat or by incubation of the cytosol for an hour at 37°C in the absence of phosphatase inhibitors. The effect was observed with either v-H-Ras or c-H-Ras, but not with p25^{rab3A}, a small G protein with about 30% similarity to Ras. The effect could not be mimicked by addition of recombinant Ras-GAP or purified GEF, a guanine nucleotide exchange factor involved in the regulation of eukaryotic protein synthesis. By gel filtration chromatography, the factor appears to possess a molecular size between 100,000 and 160,000 daltons. This protein (Ras-guanine nucleotide-releasing factor, or Ras-GRF) may be involved in the activation of p21^{ras}.

THE *ras* PROTO-ONCOGENE FAMILY codes for several small guanine nucleotide-binding proteins with low intrinsic GTPase activities (*1*). Mutations inhibiting this activity produce a transforming protein, an effect that suggests that *ras* proteins (Ras) act as key molecular switches to control cell proliferation (*1, 2*). GTP binding generates the "on state," which is then switched off by the hydrolysis of the bound GTP to GDP. A 125-kD cytosolic factor that accelerates this hydrolysis (Ras-GAP) has been described (*2, 3*). The effect of GAP, together with the slow intrinsic off rate for GDP from Ras in the presence of physiological concentrations of Mg^{2+} (*4*), suggest that cellular p21^{ras}, in the absence of any mechanism for catalyzing guanine nucleotide exchange, would be almost exclusively associated with GDP in vivo, and therefore in the off state.

In *Saccharomyces cerevisiae*, the *CDC25* gene product appears to function upstream of yeast *ras*, but it is not yet clear whether it acts as an exchange factor (*5*). The exchange of bound GDP for GTP by the trimeric G proteins such as G_i (inhibitory) and G_s (stimulatory) is enhanced through association with ligand-bound receptor (*6*). However, a more closely analogous model for the p21^{ras} family is perhaps provided by eukaryotic initiation factor 2 (eIF-2). eIF-2 is a GTP-binding protein possessing off rates for guanine nucleotides that are similar to those for p21^{ras} (*7*). In this case, a large, cytosolic, multisubunit complex catalyzes guanine nucleotide exchange. Understanding the oncogenic potential of *ras* requires a knowledge of the mechanisms that balance the active and inactive states of the protein. Toward this end we searched for a cellular factor that would accelerate release of GDP from p21^{ras}, thereby catalyzing generation of active p21^{ras}-GTP complex.

Cytosol was prepared (at 4°C) from rat brain homogenates by differential centrifugation, in the presence of multiple protease and phosphatase inhibitors (*8*), and used immediately. Recombinant c-H-Ras or v-H-Ras were produced in *Escherichia coli* under the control of the *tac* promoter and purified essentially as described by Tucker *et al.* (*9*). The resulting proteins were >95% pure (as determined by gel electrophoresis) and >37% active (as determined from [α-^{32}P]GDP binding of known quantities of the purified p21^{ras}). Clearing experiments with anti-p21^{ras} revealed no other guanine nucleotide-binding proteins in the purified preparations. To assay for guanine nucleotide-releasing activity (Fig. 1), a p21^{ras}-[α-^{32}P]GDP complex (*10*) stabilized by addition of 10 m*M* Mg^{2+} was incubated with homogenization buffer or cytosol, excess $MgCl_2$, and unlabeled GDP. Under these conditions, in the absence of cytosol, no decrease in bound [α-^{32}P]GDP was detectable over the period of the assay. This result was expected given that in the presence of millimolar Mg^{2+} concentrations the off rate for GDP from Ras at 25°C is >>>60 min (*4*). However, the addition of brain cytosol (15 mg of protein per milliliter) decreased the half-life of the p21^{ras}-[α-^{32}P]GDP to about 2 min (Fig. 1A). A similar release of GDP was obtained with the c-H-Ras protein (Fig. 1A). The dose-response curve (Fig. 1B) was sharply sigmoidal in shape, having a Hill coefficient of 3.2 (Fig. 1B, inset), which indicates that the interaction of p21^{ras} and the protein responsible for guanine nucleotide-releasing activity is highly cooperative.

Control experiments were designed to confirm that the observed accelerated GDP off rate was not a result of such artifacts as complexation of the Mg^{2+} or saturation of the nitrocellulose filters by the high cytosol-

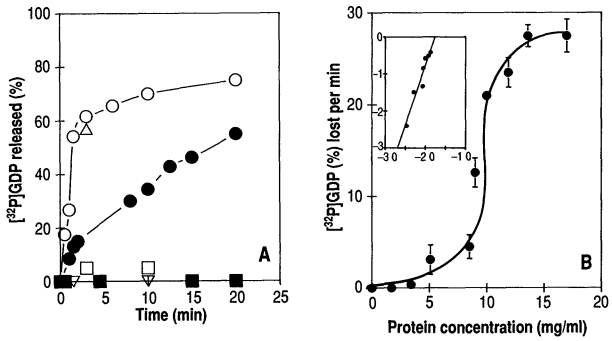

Fig. 1. Catalysis of release of [α-^{32}P]GDP from H-Ras proteins by rat brain cytosol. (**A**) Time course of release of [α-^{32}P]GDP at 4°C. Purified recombinant v-H-Ras (3 μg, circles), c-H-Ras (open triangle), or purified p25^{rab3A} proteins (*13*) (inverted open triangles) were loaded with [α-^{32}P]GDP (20 μCi/pmol) in the absence of Mg^{2+} as described previously (*10*), with the addition of oxidized insulin chain A (400 μg/ml) (Sigma) to prevent p21^{ras} sticking to the plastic microfuge tubes. The complexes were stabilized by addition of 10 m*M* $MgCl_2$. Cytosol was prepared from rat brains in the presence of protease and phosphatase inhibitors as described (*8*) and used immediately. Ras protein (1.7 μl, 0.1 μg) was added per 50 μl of cytosol (or homogenization buffer, closed squares) at 25°C, containing concentrations of either 8.5 mg/ml (closed circles), 17 mg/ml (open circles) protein, or purified GEF (open squares), 8.4 m*M* $MgCl_2$, and 2 m*M* GDP. (Unlabeled GDP is added to decrease the amount of [α-^{32}P]GDP that binds to cytosolic components during the assay.) At intervals, 10-μl samples were removed and filtered through double nitrocellulose (Millipore; 0.45 μm) filters, which were washed with 10 ml of ice-cold buffer containing 20 m*M* sodium phosphate, *p*H 6.5, 2.5 m*M* $MgCl_2$, 1 m*M* DTT, and 1 m*M* adenosine triphosphate (ATP). The filters were then placed in scintillation fluid and counted for ^{32}P. A value of 0% release represents 70,000 dpm bound. Results are representation of four independent determinations. (**B**) Dose-response for GDP-releasing activity of cytosol. The release assay was performed as in (A), at the indicated protein concentrations. Two-minute time points were taken and the amount of [α-^{32}P]GDP lost per minute is plotted as a function of protein. Error bars represent ±SD (n = 4). A value of 0% release represents 107,000 dpm bound.

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ic protein concentration. Increasing the total $MgCl_2$ concentration from 1 m*M* (*11*) to 10 m*M* did not block the GDP release. Moreover, a number of different controls provide convincing evidence that the effect was not a filter-binding artifact. First, we tested the effect of substituting either histone H1 (15 mg/ml) or lysozyme (15 mg/ml) for the cytosol. As shown in Table 1, neither of these proteins, at the same concentration as the cytosol, had any effect on GDP release from $p21^{v\text{-}ras}$. Recently, Ohga *et al.* (*12*) identified a protein factor (GDI) in rabbit intestinal tissue that, when complexed to a Ras-like protein, *rho*B, allows the *rho*B-GDP-GDI complex to pass through certain brands of nitrocellulose. However, they found that filters obtained from Schleicher & Schuell did bind the *rho*B-GDP-GDI. We observed the same level of guanine nucleotide release with nitrocellulose filters from either Millipore or Schleicher & Schuell (Table 1). Finally, the rate of guanine nucleotide exchange by $p25^{rab3A}$ (*13*), which is a small G protein with about 30% similarity to *ras*, was not altered in the presence of active cytosolic extract (Fig. 1A). This result confirms the validity of the release assay and in addition demonstrates the specificity of this factor for $p21^{ras}$. We have also shown that $p21^{ras}$ does not interact with GEF (Fig. 1A), the guanine nucleotide-exchange factor involved in the regulation of eukaryotic protein synthesis (7).

Table 1. Effect of treatments on release of [α-^{32}P]GDP from recombinant c-H-Ras as measured by a filter-binding assay (*10*). Each set of data is representative of least two similar experiments. The extent of [α-^{32}P]GDP release was variable from preparation to preparation of cytosol. The reason for this variability may be related to the thermal lability of the protein or to inactivation by dephosphorylation.

Conditions	Assay time (min)	[α-^{32}P]-GDP released (%)
Experiment 1		
Buffer (no addition)	10	0*
+Brain cytosol (15.3 mg/ml)	10	77
+Lysozyme (15 mg/ml)	10	0
+Histone H1 (15 mg/ml)	10	8
Experiment 2		
Buffer (no addition)	10	0†
+Brain cytosol	10	88
+Cytosol (100°C/30 min)	10	0
+Cytosol (65°C/30 min)	10	0
+Cytosol (37°C/60 min)	10	9
+Membranes (0.5% CHAPS)	10	0
+Recombinant Ras-GAP (2.5 μg)	10	0
Experiment 3		
Buffer (no addition)	3	0‡
+Cytosol (+phosphatase inhibitors)§	3	61
+Cytosol (no phosphatase inhibitors)§	3	25
Experiment 4		
No addition	3	0‖
Cytosol (Millipore)	3	60
Cytosol (Schleicher & Schuell)	3	63
Cytosol + CHAPS (0.5%)	3	60

*153,000 dpm bound. †180,000 dpm bound. ‡215,000 dpm bound. §Phosphatase inhibitors (phosphoserine, phosphotyrosine, phosphothreonine, and *p*-nitrophenylphosphate) were present (during preparation of the cytosol and during the assay) or were absent. ‖94,500 dpm bound.

To determine whether the GDP-releasing factor (Ras-GRF) was heat-stable, we incubated the freshly prepared cytosol for 30 min at 100°C or 65°C, before incubation with the [α-^{32}P]GDP-$p21^{c\text{-}ras}$. Both treatments destroyed all detectable releasing activity (Table 1). Moreover, incubating the cytosol preparation at 37°C for 1 hour also destroyed the releasing activity. Addition of Ras-GAP to bound $p21^{ras}$-[α-^{32}P]GDP had no effect on the GDP off rate (Table 1). These results suggest that the factor is a heat-labile protein distinct from Ras-GAP. To ascertain its subcellular distribution, we also performed release experiments on rat brain membrane proteins solubilized with 0.5% CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate} (*10*). No GDP-releasing activity was detectable, suggesting the protein is located exclusively in the cytosol. Addition of CHAPS (final concentration of 0.5%) to active cytosol did not inhibit the releasing factor activity (Table 1). This confirms that releasing activity in the solubilized membranes was not being masked by inhibitory effects of the detergent.

The assays described above only measure the accelerated release of GDP from $p21^{ras}$. To be of physiological significance, it was important to show that this cytosolic factor would also catalyze guanine nucleotide exchange by $p21^{ras}$. Therefore, we also assayed both accelerated release and exchange of guanine nucleotides by immunoprecipitation. Immunoprecipitation was used to assay for GDP release from a prebound $p21^{ras}$-[^{32}P]GDP complex (Fig. 2A) and accelerated exchange of both GDP and guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S) in the presence of cytosol (Fig. 2B). Sixty-two percent of the bound [^{32}P]GDP was released from Ras after incubation with cytosol for 10 min at room temperature. In the opposing assay for accelerated exchange, a 60-fold increase in the amount of [^{32}P]GDP associated with the immunocomplex was detected in the presence of cytosol. This effect was completely abolished by the addition of GTP-γ-S (2 m*M*). This result shows that cytosolic extracts accelerate GDP/GDP exchange and, more importantly, the competition by GTP-γ-S shows accelerated GDP/GTP exchange by Ras. Moreover, these data confirm that guanine nucleotide-releasing activity cannot be attributed to an artifact of the nitrocellulose filter-binding assays. Control experiments confirmed identical amounts (93% of total) of Ras were immunoprecipitated from reactions containing buffer and cytosol (*11*).

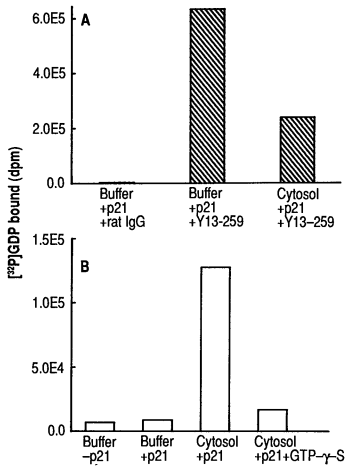

Fig. 2. Accelerated release and exchange of GDP and GTP-γ-S in the presence of cytosol. (**A**) Ras (3 μg) was loaded with [^{32}P]GDP (50 μCi/pmol) as described (*10*). $p21^{ras}$-[^{32}P]GDP (200 ng; 3.3 μl) was added to buffer or cytosol (50 μl; final 2 m*M* GDP and 10 m*M* Mg^{2+}). After 10 min at room temperature anti-Ras antibody Y13-259 (1 μg; Oncogene Science) was added and the samples were mixed for 30 min at 4°C. Protein A–Sepharose (100 μl), adsorbed with rabbit antibody to rat immunoglobulin G, was added to each sample and incubated for an additional 30 min at 4°C. Samples were layered onto a 700-μl sucrose cushion and centrifuged in a microfuge for 3 min. The cytosol was aspirated off to the interface, the walls of the microfuge tubes were washed with 2*M* urea, and the solutions were aspirated off down to the protein A–Sepharose pellet. The pellet was washed once with 20 m*M* MOPS, *p*H 7.2, 200 m*M* sucrose, 1 m*M* $MgCl_2$, and 1 m*M* DTT. Scintillation fluid (1 ml) was added and the amount of [α-^{32}P]GDP associated with the immuno-complex was determined. Data represent means of duplicate experiments; range of duplicates was within 10%. (**B**) Ras was bound with unlabeled GDP (final 200 μ*M*). Ras (0.2 μg) was then added to 50 μl of cytosolic filtrate, cytosol, or cytosol + 2 m*M* GTP-γ-S, each containing 20 μCi of [α-^{32}P]GDP. (Cytosolic filtrate was prepared by filtration through a Centricon 10, to remove the releasing factor but to retain endogenous GDP and GTP, which dilute the specific activity of added [^{32}P]GDP.) Exchange was allowed to proceed for 10 min at 25°C. The $p21^{ras}$-[^{32}P]GDP complex was immunoprecipitated as described above. Data represent the means of four independent determinations.

To estimate the approximate size of the Ras-GRF, we loaded cytosol (5 mg in 0.1 ml) onto a Superose 12 gel filtration column and fractions were assayed for GDP-releas-

ing activity. The GDP-releasing activity eluted as a single peak at an apparent molecular mass between 100,000 and 160,000 daltons (Fig. 3). However, because of the highly cooperative nature of the interaction between $p21^{ras}$ and Ras-GRF, it remains unclear whether this value represents the molecular size of the fully active factor.

We conclude that Ras-GRF is a novel protein, distinct from Ras-GAP, that enhances the off rate of guanine nucleotides from $p21^{ras}$. Although we do not yet understand the mechanisms that might regulate GDP-releasing activity, we observed that cytosol prepared in the absence of phosphatase inhibitors exhibited significantly less activity than that prepared in the presence of phospho-amino acids and *p*-nitrophenyl phosphate (Table 1). Moreover, the releasing activity did not remove all of the [α-^{32}P]GDP from Ras (Fig. 1), and the rate and extent of release were rather variable from preparation to preparation. These data suggest that the Ras-GRF is rapidly inactivated, perhaps by dephosphorylation, which might account for the difficulties others have encountered in trying to detect a GTP/GDP exchange activity for Ras.

Although it was surprising to detect Ras-GRF in the cytosol rather than associated with the plasma membrane, the result supports earlier suspicions that the Ras superfamily of proteins is structurally and functionally more closely related to factors involved in the control of vectorial processes such as EF-Tu and eIF-2, rather than to the trimeric G proteins (*10*). The guanine nucleotide-exchange factor for eIF-2 is a large cytosolic protein, the interaction of which with eIF-2 is governed by phosphorylation (*7*).

We therefore propose that Ras-GRF allows the rapid equilibration of $p21^{ras}$ with the free guanine nucleotide pools in the cell, to control the fraction of *ras* protein in the on state.

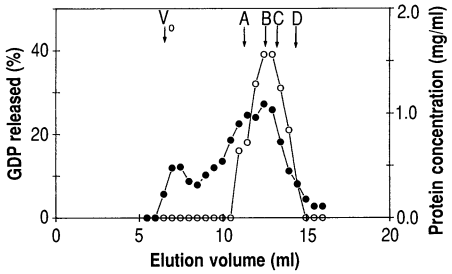

Fig. 3. Gel filtration chromatography of *ras*-GRF. Rat brain cytosol was concentrated in a Centricon 10 (approximately fivefold) and loaded onto a Superose 12 sizing column (5 mg in 0.1 ml). The column was eluted with 20 m*M* tris-HCl, *p*H 7.4, 125 m*M* NaCl, 10% glycerol, 1 m*M* $MgCl_2$, 1 m*M* DTT, plus phosphatase inhibitors (*8*). Fractions (0.5 ml) were assayed for GDP-releasing activity (open circles) as described in Fig. 1. Protein (closed circles) was measured by a modification of the method described by Lowry (*14*). The column was calibrated with (A) amylase (200 kD), (B) alcohol dehydrogenase (150 kD), (C) bovine serum albumin (66 kD), and (D) carbonic anhydrase (29 kD). The results are representative of two independent experiments. V_0, void volume.

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8. Rat brains were homogenized at 4°C in a Waring blender, then with a Dounce homogenizer, in 20 m*M* 2-(*N*-morpholino)propanesulfonic acid (MOPS), *p*H 7.2, 1 m*M* $MgCl_2$, 0.1 m*M* EDTA, 1 m*M* dithiothreitol (DTT), 200 m*M* sucrose, plus aprotinin and leupeptin (25 μg/ml each), phenylmethylsulfonyl fluoride (PMSF) (40 μg/ml), and pepstatin A (1.7 μg/ml) as protease inhibitors, plus 1.5 m*M* *p*-nitrophenyl phosphate, 0.5 m*M* phosphoserine, 0.5 m*M* phosphotyrosine, and 1 m*M* phosphothreonine as phosphatase inhibitors. The homogenates were centrifuged at 100,000*g* for 30 min. The supernatants were used immediately.
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Characterization of an Extremely Large, Ligand-Induced Conformational Change in Plasminogen

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Native human plasminogen has a radius of gyration of 39 angstroms. Upon occupation of a weak lysine binding site, the radius of gyration increases to 56 angstroms, an extremely large ligand-induced conformational change. There are no intermediate conformational states between the closed and open form. The conformational change is not accompanied by a change in secondary structure, hence the closed conformation is formed by interaction between domains that is abolished upon conversion to the open form. This reversible change in conformation, in which the shape of the protein changes from that best described by a prolate ellipsoid to a flexible structure best described by a Debye random coil, is physiologically relevant because a weak lysine binding site regulates the activation of plasminogen.

NATIVE HUMAN PLASMINOGEN IS A single-chain polypeptide of 790 amino acids (*1*) located within six domains (*2*). At the NH_2-terminus are five kringles, triple-loop structures of 80 to 100 amino acids constrained by three disulfide bridges, followed by the protease domain which is homologous to chymotrypsin. The kringles have a high degree of sequence similarity (35%) and are autonomous structural and folding domains (*3*) that have evolved by exon shuffling (*4*). Plasmin is formed upon cleavage by a plasminogen activator of the arginyl-valyl bond 560 amino acids from the NH_2-terminus of plasminogen (*5*). There are two classes of lysine binding sites on plasminogen. For the ligand 6-aminohexanoic acid (6-AHA) there is