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Purification of a factor capable of stimulating the guanine nucleotide exchange reaction of ras proteins and its effect on ras-related small molecular mass G proteins

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ABSTRACT **We have previously identified a membrane factor capable of stimulating guanine nucleotide exchange activity for ras p21 proteins. The ras guanine nucleotide exchange factor (rGEF) was purified from bovine brain to near homogeneity by successive chromatographies on DE52 DEAE-cellulose, Sepharose 6B, hydroxylapatite, and FPLC phenyl-Superose resins. SDS/polyacrylamide gel electrophoresis of the purified rGEF showed a single major protein with a molecular mass of 35 kDa. rGEF increased the exchange rate of GDP in normal [Gly^{12}]p21 or oncogenic [Val^{12}]p21 up to 30- to 40-fold under physiological concentrations of Mg^{2+}. Since the factor was free from GDP/GTP binding activity and nonspecific GDP hydrolytic activity, we propose that rGEF may regulate GDP/GTP exchange reaction of ras proteins in response to external growth signals. Moreover, rGEF enhanced the dissociation of bound GDP from some of ras-like G proteins, R-ras, rap1-A, rab1-B, and rho proteins, raising the possibility that rGEF may affect the activities of these proteins.**

Mammalian ras p21 proteins are regarded as one of the regulatory components in the metabolic pathway related to cell proliferation and cell transformation. Normal p21 proteins, such as guanine nucleotide-binding regulatory (G) proteins, bind GDP and GTP and have an intrinsic GTPase activity (1). Although it is assumed that, in response to a cell proliferation signal, p21 exchanges bound GDP for GTP and that GTP·p21 activates its effector molecule, the precise mechanism of the ras regulation remains unclear. A cytoplasmic protein, GTPase activating protein (GAP), has been identified (2, 3) that accelerates the GTP hydrolysis in normal p21 but not in oncogenic p21. GAP could, as an upstream signal terminator, convert active GTP·p21 to inactive GDP·p21 through the hydrolysis of GTP bound to the p21 or act as a downstream effector, similar to the translation elongation factor EF-Tu (4). The later model is favored by the fact that GAP cannot induce the hydrolysis of GTP bound to mutant p21 proteins, which fail to interact with their effector. However, some observations raise the possibility that the guanine nucleotide exchange reaction is also an important event for p21 function. The GDP–GTP exchange reaction of p21 was inhibited by Y13-259 ras-neutralizing antibody, which also blocked the induction of cellular DNA synthesis (5, 6). In yeast *Saccharomyces cerevisiae* cells, the *CDC25* gene product was postulated to catalyze, either directly or indirectly, the GDP–GTP exchange reaction for the RAS2 protein, which is structurally related to mammalian ras proteins (7). Furthermore, the proliferation of NIH 3T3 cells was inhibited by the [Asp^{17}]p21 mutant with preferential affinity for GDP. This down-regulation could come from the competition between mutant and normal p21 proteins for coupling with the regulatory cellular components that catalyze the nucleotide exchange reaction (8).

We have identified (9) a membrane factor that markedly enhanced the exchange of GDP bound to Ha-ras p21 proteins for external GTP and designated the factor as a ras guanine nucleotide exchange factor (rGEF). rGEF activity was detected in cells of a variety of species, including NIH 3T3 and *Xenopus* oocytes, and the activity was completely blocked by Y13-259 ras-neutralizing antibody (9). From these data, it is conceivable that rGEF might be involved in the control of rate-limiting GDP–GTP exchange reaction of ras proteins. Evidence that growth factors, such as epidermal growth factor, may affect the guanine nucleotide binding activity and GTP-dependent phosphorylation of ras proteins supports this proposal (10). Another group (11) has also reported the presence of guanine nucleotide exchange factor for ras proteins in rat brain cytosol. To further characterize rGEF, we purified to near homogeneity rGEF from bovine brain tissues. The relationship of rGEF with small molecular mass G proteins is also discussed.

MATERIALS AND METHODS

rGEF Assay. Normal [Gly^{12}]p21 and oncogenic T24 [Val^{12}]p21 Ha-ras proteins expressed in *Escherichia coli* cells were purified in the absence of a denaturant, as described (12). To assay rGEF activity, purified [Gly^{12}]p21 (200 ng) was incubated with 2 μM [^{3}H]GDP (11 Ci/mmol, 1 Ci = 37 GBq; Amersham) in 50 μl of reaction buffer (25 mM Tris Cl, pH 7.5/2 mM $MgCl_2$/100 mM KCl/0.5 mM dithiothreitol/bovine serum albumin (100 μg/ml) for 15 min at 37°C. Then 20 μl of the eluted fractions, at each step of purification, and 0.3 mM unlabeled GTP were added to the preincubated samples at 37°C for 10 min. The samples were then collected on the nitrocellulose filters (Schleicher & Schuell, 0.45 μm, pore size), and the radioactivity of [^{3}H]GDP bound to p21 was quantitated by scintillation counting, as described (9). rGEF activity was calculated from the decrease of radioactivity, as compared to that of reaction performed in the absence of rGEF. Activity was enhanced with increasing amounts of factor in a dose-dependent manner. There was no hydrolysis of [^{3}H]GDP bound to p21 by the factor (see below).

Purification Procedure. All steps were performed at 4°C. Cerebra isolated from four to six fresh bovine brains were washed with 2 liters of ice-cold isotonic phosphate-buffered saline and then homogenized in a Waring Blendor in 3 liters of buffer A [25 mM Tris-Cl, pH 7.5/1 mM $MgCl_2$/1 mM EGTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/leupeptin (10 μg/ml)/pepstatin (10 μg/ml). The ho-

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Abbreviations: rGEF, ras guanine nucleotide exchange factor; G protein, guanine nucleotide-binding regulatory protein; GAP, GTPase activating protein.

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mogenates were centrifuged at 1500 × *g* for 15 min, and the supernatants were further centrifuged in a Beckman type 19 rotor at 19,000 rpm for 45 min. The pellets were solubilized by stirring in 1 liter of buffer A containing 1% Triton X-100 for 30 min. The solubilized sample was centrifuged in a Beckman J14 rotor at 14,000 rpm for 20 min, and the resulting supernatant was used as the crude membrane extract. The cleared supernatant was incubated with 400 ml of DE52 DEAE-cellulose (Whatman) for 90 min, and the resin was twice washed with 1 liter of buffer B (buffer A plus 0.5% Triton X-100) by using a low-speed centrifugation at 1000 × *g* for 10 min. The resin was poured into a 5 × 30 cm column and washed with an additional 1 liter of buffer B. The proteins were eluted with a linear NaCl gradient (0–0.3 M) in buffer B, and 20-ml fractions were collected. The peak of rGEF activity eluted between 85 mM and 120 mM NaCl, as determined by conductivity. Ammonium sulfate [55% (wt/vol)] was added to the pooled peak fractions (250 ml) for 30 min. After centrifugation in a Beckman J14 rotor at 14,000 rpm for 15 min, the protein pellets were resuspended in 20 ml of buffer B, and the resuspended sample was dialyzed against buffer B for 1 hr. The dialysate was applied to a Sepharose 6B (Pharmacia LKB) column (5 × 100 cm) equilibrated with buffer C (buffer B plus 50 mM NaCl). Elution was continued with buffer C, and 15-ml fractions were collected. A single peak of rGEF activity was eluted at 100 kDa (≈1.3 liter of buffer C), as determined by molecular mass standards, catalase (220 kDa), aldolase (158 kDa), and bovine serum albumin (67 kDa). The peak fractions (120 ml) were pooled and concentrated to 30 ml in an ultrafiltration container (Amicon) with a YM30 membrane. The concentrated sample was mixed with 5 ml of hydroxylapatite resin (Bio-Rad) by a rotary shaker for 30 min. After centrifugation at 1000 × *g* for 10 min, the supernatant containing rGEF activity was concentrated to 5 ml. One milliliter of the sample was brought up to 5 ml by adding 0.75 M KH_2PO_4, pH 6.8/0.5 M NaCl and 2.2 ml of H_2O, and the mixture was applied to an FPLC phenyl-Superose column (Pharmacia LKB) equilibrated with 1 M NaCl/buffer A at 0.5 ml/min. After the 10-min stepwise wash with 1 M NaCl and 0.5 M NaCl, elution was continued at 0.5 ml/min with a decreasing NaCl gradient from 0.5 M to 0 M in buffer A and an increasing sodium cholate gradient from 0 to 1% in buffer A. A major peak of rGEF activity was eluted at 0.1 M NaCl and 0.8% sodium cholate. The peak fractions (5 ml) contained some Triton X-100, which was not completely separated by this procedure, but the detergent had no influence on the ras nucleotide exchange reaction. SDS/PAGE revealed that a single major protein of 35 kDa was eluted corresponding with rGEF activity. A minor peak of rGEF activity was also detected at 0.45 M NaCl and 0.1% sodium cholate, but the fractions contained contaminating proteins in addition to a small amount of a 35-kDa rGEF protein. Therefore, we used the major peak fractions as purified rGEF. The samples were concentrated in a Centricon microconcentrator (molecular mass cut off, 30 kDa, Amicon) to 0.5 ml and stored at −70°C.

Other Methods. SDS/PAGE was performed using 10% acrylamide, according to Laemmli's method (13). Proteins were stained with Coomassie brilliant blue R-250 or with a silver staining kit (Accurate Chemicals, Westbury, NY). Protein concentration was determined by using a Bio-Rad protein assay kit with bovine serum albumin (Sigma) as a standard. Recombinant rap1-A, R-*ras*, and the proteins were provided by A. Hall (Institute of Cancer Research, Great Britain). rab1-B protein expressed with baculovirus vector was provided by C. Der (La Jolla Cancer Research Foundation).

RESULTS

Purification of rGEF. rGEF activity was (9) identified in the membrane fraction but not in the cytosolic fraction, suggesting that the factor is a membrane-associated protein. The activity was determined by an immunoprecipitable [^{3}H]GDP binding assay, in which the anti-ras monoclonal antibody was used for immunoprecipitation after incubation of [^{3}H]GDP·p21 complex with the extract and excess unlabeled GTP. Brain contained the highest level of activity of [^{3}H]GDP released per mg of protein among bovine tissues examined, including lung, kidney, and liver (9). Therefore, we used a crude bovine brain membrane extract as starting material for the purification. The purification of rGEF is summarized in Table 1. Brain membrane fraction was solubilized by 1% Triton X-100, and the extract was fractionated by four steps of column chromatography. The crude membrane extract itself resulted in a high background of [^{3}H]GDP binding to the nitrocellulose filter, which made it difficult to determine the effect of crude extract on the ras nucleotide exchange activity by this assay method. Therefore, the overall yield of the activity and the purification factor were shown only with column chromatography steps. A 170-fold purification of the activity from DE52 to phenyl-Superose chromatography was achieved by this method. Approximately 10 μg of purified rGEF per brain could be obtained.

Although rGEF activity was eluted at ≈100 kDa from the gel filtration Sepharose 6B column, purified rGEF contained a single major polypeptide of 35 kDa (>90% pure), as detected by SDS/PAGE (Fig. 1). This difference in molecular mass estimation may be due to (*i*) multimer formation of rGEF or (*ii*) association of a cellular component(s), either protein or lipid, with rGEF. After loading the gel-filtration columns equilibrated with buffer C (Fig. 2), a Sepharose 6B sample and a purified rGEF were eluted at ≈100 kDa and at 40 kDa, respectively. This makes the former case unlikely. Furthermore, rGEF and *E. coli* ras proteins eluted separately when the mixtures of those proteins were passed through a gel-filtration column under the conditions as described above, suggesting that at least rGEF did not bind to p21 proteins tightly (unpublished data). The factor did not bind cation-exchange resin in the pH > 5 region; and green A-Sepharose, blue-Sepharose, wheat germ agglutinin-Sepharose, and hydroxylapatite resin did not have strong affinity for the factor in the presence of 0.2–0.5% Triton X-100. The homogeneous preparation of rGEF was stable at

Table 1. Purification of rGEF from bovine brain

Purification step	Volume, ml	Protein, mg	Total activity, pmol/sec	Specific activity, pmol per sec per mg	Purification, fold	Yield, %
Crude membrane extract	1000	4350	—	—	—	—
DE52	250	438	7700	18	1	100
Sepharose 6B	120	54	5300	98	5.6	69
Hydroxylapatite	30	3.8	1100	294	17	14
Phenyl-Superose	5	0.06	200	2986	170	2.6

Cerebra from five brains were used as starting materials. Samples from each step of purification were assayed for the ability to accelerate the replacement of [^{3}H]GDP bound to [Gly^{12}]p21 for unlabeled GTP. Specific activity was expressed as the amount of [^{3}H]GDP dissociated from the p21 (200 ng) per sec per mg of rGEF protein preparation.

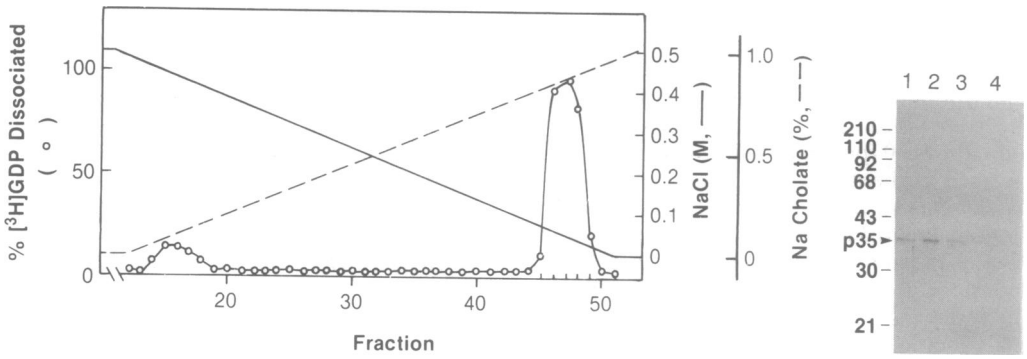

FIG. 1. Elution of rGEF from a phenyl-Superose column. Proteins in the unbound fraction from a hydroxylapatite column were applied to an FPLC phenyl-Superose column, 0.7-ml fractions were collected, and 20 μl of each fraction was assayed for rGEF activity. Activity is given as a percentage of bound [^{3}H]GDP dissociated. Approximately 8000 cpm of [^{3}H]GDP was bound to [Gly^{12}]p21 incubated without extracts. The samples of the indicated fractions (20 μl) were analyzed by SDS/PAGE using 10% polyacrylamide gels and stained with Coomassie brilliant blue. Numbers on left show the molecular mass in kDa. Lanes: 1, fraction 46; 2, fraction 47; 3, fraction 48; 4, fraction 49.

4°C for at least 3 weeks, and the addition of glycerol to 10% (vol/vol) increased the stability. The purified rGEF lost its activity by freezing–thawing but retained its activity during incubation at 55°C for 30 min, whereas rGEF partially purified by Sepharose 6B was not stable.

Effect of rGEF on ras GDP–GTP Exchange Reaction. We characterized the effect of purified rGEF on the p21 guanine nucleotide exchange reaction. For this purpose, the change in the nucleotide dissociation rate caused by rGEF was first examined. As described by others (14–16), a high concentration of $MgCl_2$ stabilized the GDP·p21 complex, and the half-life of the GDP·p21 was ≈30 min for normal [Gly^{12}]p21 and 75 min for oncogenic [Val^{12}]p21 in the presence of 2 mM $MgCl_2$ and excess unlabeled GTP. The addition of rGEF to the [^{3}H]GDP·[Gly^{12}]p21 or [^{3}H]GDP·[Val^{12}]p21 dramatically increased the dissociation rate of [^{3}H]GDP bound to the p21 proteins, and the reaction was subject to the first-order kinetics (Fig. 3). rGEF increased the dissociation rate constant (k_{-1}) as much as 30- to 40-fold, and the k_{-1} was dependent on the amount of added rGEF, as summarized in Fig. 3. The increased rate of [^{3}H]GDP release was close to that of stimulatory or inhibitory G protein activated by hormone–receptor complexes (17). By incubating 24 pmol of GDP·[Gly^{12}]p21 with 1 pmol of rGEF, 12 pmol of GDP·[Gly^{12}]p21 was converted to a GTP-bound form within 1 min. Approximately 12 mol of GTP·[Gly^{12}]p21 was generated per 1 mol of rGEF. This was comparable to the reaction rate of GAP-induced GTP hydrolysis in [Gly^{12}]p21 (3). Next, the dissociation constants (K_d) of p21 proteins were determined for GDP. Recombinant ras proteins produced in *E. coli* cells are not completely free of bound GDP, and equimolar p21·GDP complexes are detectable. Therefore, the K_d for GDP was determined by Scatchard analysis under the high concentration of GDP, and the K_d for GTP was measured by the competition assay, as described (14). The K_d (GDP) for [Gly^{12}]p21 and [Val^{12}]p21 were increased 8- and 5-fold by the addition of rGEF (10 nM), respectively (Table 2). Although the K_d (GTP) was also increased by rGEF, both [Gly^{12}]p21 and [Val^{12}]p21 had greater affinity for GTP than GDP in the presence of rGEF (Table 2). These results indicated that rGEF increased the dissociation constant for GDP and that this could be explained by the increase in dissociation rate constant k_{-1}

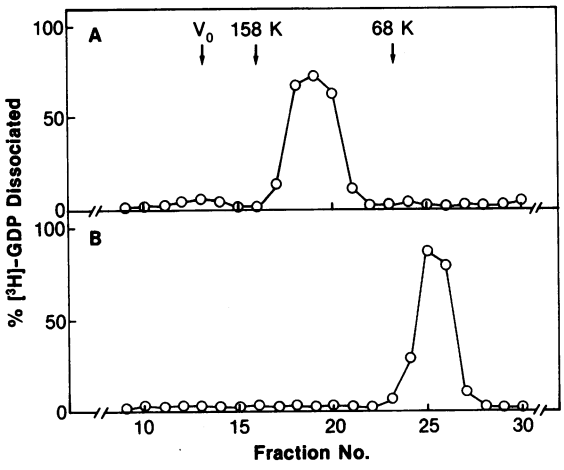

FIG. 2. Superose 12 molecular sizing of rGEF. Pooled Sepharose 6B rGEF peak fractions (200 μl, 100 μg) (*A*) or purified rGEF (200 μl, 2.5 μg) (*B*) was reloaded onto an FPLC Superose 12 column equilibrated with buffer C at the flow rate of 0.4 ml/min, 0.7-ml fractions were collected, and 20 μl of each fraction was assayed for the rGEF activity, as described in Fig. 1. Activity is given as a percentage of bound [^{3}H]GDP, and ≈9000 cpm of [^{3}H]GDP was bound to the p21 incubated with buffer alone. K, kDa; V_o, void volume.

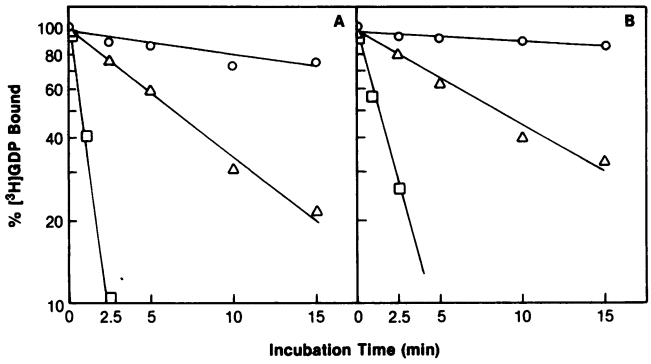

FIG. 3. Effect of rGEF on the dissociation rate of p21 for GDP. [^{3}H]GDP·[Gly^{12}]p21 (*A*) and [^{3}H]GDP·[Val^{12}]p21 (*B*) (200 ng) were prepared by the incubation at 37°C for 30 min and 60 min, respectively, and the binary complexes were incubated with 0 ng (○), 6 ng (1.8 nM) (△), or 36 ng (10 nM) (□) of purified rGEF in the presence of unlabeled GTP (300 μM) and 2 mM $MgCl_2$ at 37°C as indicated. The residual amount of [^{3}H]GDP bound to p21 was determined by nitrocellulose filter binding assays, as described in Fig. 1. The results were plotted as a percentage relative to the value for the samples harvested at 0 min. The values at 0 min for [Gly^{12}]p21 were 9500, 9400, and 9000 cpm for 0, 6, and 36 ng of the factor, and those for [Val^{12}]p21 were 8100, 8300, and 8700 cpm, respectively. The half-life of the [^{3}H]GDP·p21 complex ($t_{1/2}$) was determined from the figure, and the dissociation rate constant (k_{-1}) was calculated with the following equation: $k_{-1} = \log 2/t_{1/2}$. The unit of k_{-1} (GDP) value shown is s^{-1}. The data represent the mean value of duplicate experiments and similar results were obtained in the separate set of experiments. (*A*) k_{-1}: ○, 3.9×10^{-4} s^{-1}; △, 1.9×10^{-3} s^{-1}; □, 1×10^{-2} s^{-1}. (*B*) k_{-1}: ○, 1.5×10^{-4} s^{-1}; △, 1.2×10^{-3} s^{-1}; □, 0.65×10^{-2} s^{-1}.

Table 2. Dissociation constants of p21

	rGEF, nM	K_d (GDP), M	K_d (GTP), M
[Gly^{12}]p21	0	4.2×10^{-8}	1.8×10^{-8}
	10	3.0×10^{-7}	3.9×10^{-8}
[Val^{12}]p21	0	3.1×10^{-8}	2.1×10^{-8}
	10	1.4×10^{-7}	3.6×10^{-8}

K_d values of p21 proteins for GDP were determined by Scatchard analysis (14). For K_d (GDP), 20 pmol of purified [Gly^{12}]p21 or [Val^{12}]p21 was incubated in GDP exchange buffer containing various amounts of [^{3}H]GDP and reacted with 36 ng of rGEF equivalent to 10 nM in the presence of unlabeled GTP (1 mM), as described in Fig. 1. For K_d (GTP), the competition assay was performed under the same conditions, except that 20 pmol of [Gly^{12}]p21 or [Val^{12}]p21 was incubated with 50 μl of reaction buffer containing various amounts of [^{3}H]GDP, 20 pmol of unlabeled GTP, and 36 ng of rGEF.

(GDP). The rate-limiting step of the nucleotide exchange reaction was in the dissociation of the bound GDP from p21 proteins. The rGEF did not bind to GDP or GTP [<50 cpm of [^{3}H]GDP or 20 cpm of [^{3}H]GTP (10 Ci/mmol) was bound to 18 ng of rGEF], and the significant dissociation of [^{3}H]GDP was only detected in the presence of exogenous GDP or GTP (Table 3).

Furthermore, the incubation of p21·GDP complexes with [^{3}H]GTP in the presence of rGEF resulted in an increase in the amount of [^{3}H]GTP bound to p21 proteins (data not shown).

To test whether purified rGEF causes nonspecific degradation of [^{3}H]GDP bound to p21 proteins, reaction products of the GDP–GTP exchange reaction were examined. Most of the radioactivity released from [^{3}H]GDP·p21 by the addition of rGEF was quantitatively recovered as [^{3}H]GDP, and neither GMP nor GTP was detected (Table 4). These results indicated that the rGEF-induced nucleotide exchange activity was not due to hydrolysis of [^{3}H]GDP. As demonstrated for the partially purified rGEF (9), the activity of homogeneously purified rGEF was also inhibited by Y13-259 ras monoclonal antibody (data not shown).

Effect of rGEF on Small Molecular Mass G Proteins. To examine whether rGEF affects the nucleotide exchange activity of small molecular mass G proteins, recombinant R-*ras*, rap1-A, rho, and rab1-B proteins were incubated with [^{3}H]GDP and reacted with rGEF in the presence of excess unlabeled GTP. As shown in Fig. 4, rGEF enhanced the release of [^{3}H]GDP bound to these proteins in a manner similar to that of ras proteins.

DISCUSSION

We have purified a rGEF from bovine brain to near homogeneity by using a four-step column chromatography. The molecular mass of a single major protein in the final preparation was 35 kDa, as determined by SDS/PAGE. Although we cannot rule out the possibility that rGEF is associated with detergent-forming mycells, rGEF may be associated with other cellular components because its activity eluted at 100 kDa on the Sepharose 6B gel-filtration column. Computer analysis shows that there is no significant identity in amino acid sequence between microsequenced peptides of p35 and any known proteins (a detailed analysis will be published elsewhere). Most of rGEF activity was recovered in the membrane fraction. This seems consistent with the hypothesis that membrane localization is required for ras action (1). Additionally, a factor that accelerates the dissociation of GDP from ras proteins (ras–guanine nucleotide-releasing factor or ras-GRF) has been reported (11). Whereas rGEF and ras–GRF have a similar molecular size (100–200 kDa) on the gel-filtration column, most of ras–GRF activity, in contrast to rGEF, appears to be localized in the cytosol rather than the membrane. If the two factors represent an identical protein, then the discrepancy in determining cellular localization may come from different conditions in the two ex-

Table 3. Requirement of external GDP/GTP on the rGEF-induced dissociation of [^{3}H]GDP from p21 proteins

Addition(s)	[^{3}H]GDP bound, % control	
	[Gly^{12}]p21	[Val^{12}]p21
None	100	100
rGEF	96	98
rGEF + GDP	20	25
rGEF + GTP	11	13
GDP	89	94
GTP	85	90

[^{3}H]GDP·[Gly^{12}]p21 or [^{3}H]GDP·[Val^{12}]p21 binary complexes were incubated with rGEF (18 ng) in the presence or absence of unlabeled GDP or GTP (300 μM), as described in Fig. 1. At 100% control, [Gly^{12}]p21 bound 8500 cpm and [Val^{12}]p21 bound 9600 cpm.

Table 4. Analysis of nucleotide exchange reaction products

Addition	Recovered radioactivity, cpm					
	GMP		GDP		GTP	
	Sup	Pellet	Sup	Pellet	Sup	Pellet
None	0	0	710	4400	0	0
rGEF	0	0	4200	650	0	0

[Gly^{12}]p21 was incubated with [^{3}H]GDP at 37°C for 30 min, and [^{3}H]GDP·p21 complex was separated from free [^{3}H]GDP by an FPLC Superose 12 column under the same elution condition, as described (9). The binary complex (≈6000 cpm) was incubated, with or without the factor (10 ng), in 50 μl of reaction mixture containing unlabeled GTP, as described in Fig. 1. The p21 was immunoprecipitated by the Y13-259 antibody for 1 hr at 4°C, and the immunocomplexes retained on protein A-Sepharose beads were separated from the supernatant by a brief centrifugation. The samples extracted from the pelleted Sepharose beads, as described (9), and the samples of the supernatants were analyzed by chromatography on polyethyleneimmine-cellulose using guanine nucleotides as authentic markers (3). Sup, supernatant.

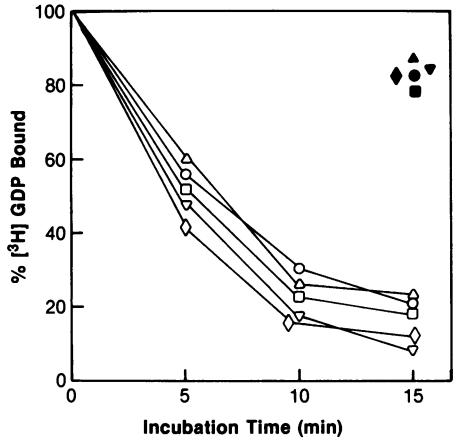

FIG. 4. Effect of rGEF on ras and ras-related small molecular mass G proteins. Approximately 200 ng of normal [Gly^{12}]p21 (□, ■), rap1-A (▽, ▼), R-ras (◇, ◆), rho (○, ●), and rab1-B (△, ▲) was preincubated with [^{3}H]GDP. After addition of purified rGEF (6 ng) and unlabeled GTP (300 μM), mixtures were incubated at 37°C and the residual amount of [^{3}H]GDP bound to proteins was determined. The assay was performed in the absence (solid symbols) or presence (open symbols) of purified rGEF. Activity is a percentage relative to the value for the samples harvested at 0 min. The values at 0 min for [Gly^{12}]p21, rap1-A, R-ras, rho, and rab1-B were 8200, 9900, 11,000, 7800, and 8300 cpm, respectively. The data represent the mean value of duplicate experiments.

perimental systems. The issue as to whether these factors are identical requires further characterization.

Although GDP·p21 was stable under the physiological Mg^{2+} concentration, the binary complex was unstable in the presence of rGEF. rGEF increased both the dissociation constant and the dissociation rate constant for GDP, and the affinity of [Gly^{12}]p21 for GTP as compared to GDP was also higher. In contrast to this observation, receptor-activated stimulatory G protein and light-activated transducin have equal binding affinities for GDP and GTP (18, 19). [Gly^{12}]p21 and [Val^{12}]p21 similarly responded to rGEF (Fig. 3), and the nucleotide exchange activity of viral [Thr^{59}]p21 was also enhanced by rGEF to the level seen with [Gly^{12}]p21 (data not shown). This implies that at least the oncogenic mutations at residues 12 and 59 do not influence the biochemical interaction of p21 proteins with rGEF. The effect of rGEF was similar to that of EDTA or sulfate ion, which increased the dissociation rate of p21·GDP (refs. 15 and 16; unpublished data). Although the mechanism of sulfate ion action is not known, it is postulated that EDTA depletes Mg^{2+} from the p21 and this may mimic the receptor-induced p21 activation *in vivo*. It would be of particular interest to examine whether the mode of rGEF action is the same as that of EDTA.

The GDP–GTP exchange reaction of normal [Gly^{12}]p21 may follow the equation.

$$\text{GDP·p21} + \text{GTP} \xrightarrow{k_{-1}} \text{GTP·p21} \xrightarrow{k_{cat}} \text{GDP·p21} + P_i$$

The association of GDP and p21 (the back reaction) could be negligible because of high ratio of GTP to GDP in intracellular nucleotide pools (19). At the first step, the p21 exchanges its bound GDP for external GTP. However, the dissociation rate of the p21 for GDP is slow under physiological concentrations of Mg^{2+} ($\approx$1 mM), which would not explain the rapid recycling of the p21 *in vivo* (14, 16). We propose that this rate-limiting step is catalyzed by rGEF. At the second step, the GTP bound to [Gly^{12}]p21 will be hydrolyzed, as GTP·[Gly^{12}]p21 activates its effector molecule. GAP would accelerate this reaction because the low intrinsic GTPase activity of [Gly^{12}]p21 ($\approx$1 mmol of GTP per min) (2) is not enough to generate the amount of GDP·p21 required at the first step. In contrast, mutated oncogenic [Val^{12}]p21 fails to hydrolyze its bound GTP even when it interacts with GAP and remains GTP-bound (2). The probability of replacement of bound GTP with external GDP would be very low because of the large excess of GTP over GDP in nucleotide pools (19). Thus, [Val^{12}]p21 persistently remains in an active state. Since the ratio of GTP and GDP on the [Gly^{12}]p21 at a steady state will be changed by the factors that affect either k_{-1} or k_{cat} for the p21, our finding raises the possibility that either rGEF or GAP controls the activation of the effector molecule through its interaction with GTP·p21. In analogy with ligand-occupied receptors for the stimulatory G protein subunit of adenylate cyclase and photoactivated rhodopsin for transducin (20), rGEF may catalyze the conversion of GDP·p21 to GTP·p21 in response to the external stimuli through the plasma membrane. It should be noted that the Val-12 mutation *per se* caused no significant qualitative change in the interaction of the p21 with rGEF (see Fig. 3) and that the introduction of rGEF alone cannot explain the cell activation by the mutated oncogenic ras protein unless the factor, such as GAP, is coupled with the process for p21 recycling.

The inability of rGEF to affect the nucleotide exchange activity of transducin and translation elongation factor EF-Tu suggests that at least ras proteins have the regulatory mechanism different from that of these G proteins (9). Some ras-related small molecular mass GTP-binding proteins, such as R-ras, appear to share the same GAP with ras proteins whereas rho uses a distinct GAP (21, 22). We tested whether rGEF is specific for ras proteins or influences the regulation of other types of small GTP-binding proteins. Our results indicate that the GDP–GTP exchange activity of R-ras, rho, rap1-A, and rab1-B proteins were stimulated by rGEF in a manner similar to ras proteins (Fig. 4). If these membrane proteins are closely related to ras proteins with amino acid sequence homology ($\approx$50% for R-ras and rap1-A and $\approx$30% for rho and rab1-B) (23), then it is possible that ras and the ras-related proteins may use a common regulatory mechanism despite their different biological effects. For example, the β subunit of stimulatory G protein in the adenylate cyclase system is known to affect the activities of its inhibitory G protein and transducin (20). Availability of purified rGEF will facilitate the investigation of a physiological role of rGEF in ras-mediated signal transduction.

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