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A novel membrane factor stimulates guanine nucleotide exchange reaction of *ras* proteins

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A factor with a molecular weight of 100 kDa, which markedly enhanced the guanine nucleotide exchange reaction of *ras* p21 proteins, was partially purified from bovine brain tissues. The factor was predominantly associated with the plasma membrane. When the partially purified factor and excess cold GTP were added to [^{3}H]GDP·Gly12 p21 or Val12 p21 in the presence of 2 mM $MgCl_2$, the nucleotide exchange rate was stimulated up to 25-fold. The stimulation of the p21-nucleotide exchange reaction by the factor was completely blocked by the Y13-259 *ras*-neutralizing antibody. Taken together, these results suggest that the factor may control the rate limiting GDP/GTP exchange step in recycling of p21 in *ras*-mediated signal transduction.

Ras protein; GDP/GTP exchange reaction; GTP-binding protein; Signal transduction

1. INTRODUCTION

Mammalian *ras* p21 proteins bind guanine nucleotides and have intrinsic GTPase activity which converts bound GTP to GDP [1]. In this respect, p21 may behave like regulatory G-proteins [2]. *Ras* proteins have been implicated in the malignant transformation of cells in the control of cell proliferation [1]. It is assumed that a growth signal stimulates the conversion of p21 · GDP to p21 · GTP at the plasma membrane, and that active p21 · GTP interacts with an effector molecule to transmit an internal signal. Recently, a cytoplasmic protein GAP, has been identified to activate the GTPase activity of normal p21 but not of oncogenic p21, implying that GAP plays a role as a signal terminator for p21 proteins [3-5]. While GAP converts active p21 · GTP to inactive p21 · GDP, it is not clear how inactive GDP-bound p21 is reactivated. We report here that mammalian cells contain a novel factor which stimulated the exchange of GDP bound to p21 for exogenous GTP. The possible role of the factor in recycling of p21-nucleotide exchange is discussed.

2. MATERIALS AND METHODS

Bovine brain tissues (10 g) were homogenized in 20 ml of buffer A (25 mM Tris-Cl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonylfluoride). The homogenates were centrifuged at 1500 × *g* for 10 min at 4°C, and the supernatants were further centrifuged at 100 000 × *g* for 20 min at 4°C. The supernatants (cytoplasmic fraction) were saved, and the pellets were resuspended in 2 ml of buffer A containing 1% Triton X-100. The resuspended samples were centrifuged at 12 000 × *g* for 10 min at 4°C, and the supernatants were used as crude membrane extract. Normal Gly12 and oncogenic T24 Val12 p21 proteins were expressed in *Escherichia coli* cells and purified, as described previously [6]. Purified p21 proteins (200 ng) were incubated with 2 μM [^{3}H]GDP (10-15 Ci/mmol, Amersham) in 50 μl of reaction buffer (25 mM Tris-Cl, pH 7.5, 1 mM $MgCl_2$, 100 mM KCl, 0.5 mM DTT, 100 μg/ml BSA). Then, indicated amounts of membrane or cytoplasmic extracts were added at 37°C for 10 min in the presence of 0.2 mM GTP. After incubation, the incubated mixtures were further reacted with 2 μl of anti-*ras* monoclonal antibody Y13-259 for 60 min at 4°C. The immune complexes were collected with anti-rat IgG-protein A-Sepharose for 30 min at 4°C and washed 4 times with 1 ml of washing buffer (25 mM Tris-Cl, pH 7.5, mM $MgCl_2$). Nucleotides were eluted from p21 proteins with 100 μl of 1% SDS, 20 mM EDTA at 60°C for 10 min, and the radioactivity was determined by scintillation counting.

For the protein purification, bovine membrane extracts (15 mg of protein) were applied to a Mono Q HR 5/5 ion-exchange column (Pharmacia/LKB), equilibrated with buffer B (buffer A plus 0.5% Triton X-100). After washing, bound proteins were eluted with a linear NaCl gradient (0-0.3 M NaCl), and 0.5 ml of fractions were collected. Normal Gly12 p21 (200 ng) was preincubated with 2 μM [^{3}H]GDP, as described above, and 20 μl of each fraction and 0.2 mM GTP were added to 50 μl of the reaction mixture containing the p21 · [^{3}H]GDP binary complex at 37°C for 10 min. After incubation, the reaction was stopped by adding 1 ml of ice-cold washing buffer, and the amount of radioactivity bound to the p21 was determined by Schleicher-Schuell nitrocellulose filtrations and scintillation counting. Peak fractions from a Mono Q column were concentrated by Centricon tubes (Amicon) to 0.4 ml. The concentrate was applied to a Superose 12 (Pharmacia/LKB) gel filtration column equilibrated with buffer B containing 100 mM NaCl. Fractions (0.4 ml) were collected, and 20 μl of each fraction was assayed for the activity to promote the nucleotide exchange reaction, as described above.

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3. RESULTS

In order to test if the cells contained a factor(s) which stimulates the dissociation of bound GDP from *ras* p21 proteins, we prepared Triton X-100 solubilized membrane and cytoplasmic extracts from bovine brain tissues and examined the effect of extracts on the guanine nucleotide exchange reaction of p21. Both normal Gly12 and oncogenic T24 Val12 p21 were bacterially made, and the proteins were purified to homogeneity in the absence of protein denaturants [6]. To distinguish p21-specific guanine nucleotide binding from other GTP/GDP binding in the cell extracts, an immunoprecipitation technique was used. p21 was preincubated with 2 μM [^{3}H]GDP at 37°C for 15 min and ~70% of p21 molecules bound [^{3}H]GDP under the conditions used. The [^{3}H]GDP · p21 complex was further reacted with the extracts in the presence of excess unlabeled GTP (0.2 mM) at 37°C for 10 min. The p21 protein was immunoprecipitated by monoclonal anti-*ras* antibody (Y13-259), and the radioactivity of [^{3}H]GDP retained on the p21-antibody complex was determined. As shown in fig.1a, the addition of membrane extracts increased the dissociation of [^{3}H]GDP from normal Gly12 p21 in a dose-dependent manner. During the 10-min incubation, radiolabeled GDP bound to the p21 decreased by about 70%. A similar effect of the membrane extract was observed with oncogenic Val12 p21. In contrast, no significant replacement of [^{3}H]GDP bound to p21 proteins with cold GTP was detected when the cytoplasmic extract was added to the [^{3}H]GDP · p21 complex (fig.1B). These results indicate that most of the factor(s), which promotes the guanine nucleotide exchange reaction of p21, was localized in the brain cell membrane fraction and efficiently solubilized from the membrane with a detergent, such as Triton X-100. Moreover, Gly12 and Val12 p21 showed a similar exchange activity stimulated by the factor(s).

We next examined the distribution of the membrane-associated factor in various tissues. Membrane extracts were prepared from various bovine tissues including brain, lung, pancreas, spleen, liver, and kidney, and each extract was tested for the ability to promote the GDP/GTP exchange reaction of p21, as described above. Brain contained the highest specific activity among 8 tissues examined (unpublished data). This appears to correlate with the high level of *ras* p21 expression in brain [7]. The extract from mouse NIH 3T3 and *Xenopus* oocyte cells also contained the activity, suggesting that the factor is present in the cells of a variety of species.

To further characterize the membrane factor(s), the membrane extract made from bovine brain tissues was fractionated by FPLC Mono Q ion-exchange column chromatography with a linear NaCl gradient (0 to 0.3 M). The fractions were tested for the ability to

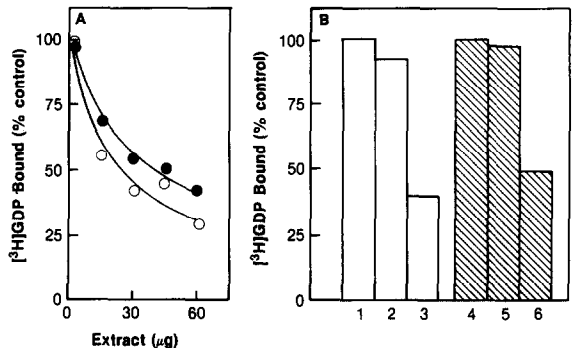

Fig.1. Effect of brain cell membrane or cytoplasmic extracts on the exchange of [^{3}H]GDP for GTP on p21 proteins. (A) Stimulation of [^{3}H]GDP release from p21 proteins by the addition of membrane extracts. Purified Gly12 (—○—) or Val12 p21 (—●—) was preincubated in the reaction buffer containing 2 μM [^{3}H]GDP, and the formed binary complex was incubated with the indicated amount of membrane extracts in the presence of cold GTP at 37°C for 10 min, as described in section 2. p21 proteins were immunoprecipitated with Y13-259 antibody. Radioactivity remaining bound is expressed as percent relative to the value obtained for the sample incubated without extracts (8300 cpm for Gly12 p21 and 9200 cpm for Val12 p21). (B) Comparison of membrane extract with cytoplasmic extract for the effect on p21-nucleotide exchange activity. 200 ng of Gly12 (open bars) and Val12 (hatched bars) p21 · [^{3}H]GDP were prepared, and then the nucleotide exchange reaction was started by the addition of cold GTP and 60 μg of membrane [3,6] or cytoplasmic [2,5] extracts, as described above. Radioactivity in immunoprecipitated p21 proteins is expressed as percent relative to the value for the control sample incubated without extracts [1,4] (8600 cpm for Gly12 p21 and 8900 cpm for Val12).

dissociate [^{3}H]GDP from normal Gly12 p21 in the presence of excess cold GTP by using a filter binding assay. The activity to stimulate [^{3}H]GDP dissociation eluted at approximately 0.1 M NaCl, and the pooled peak fractions were further chromatographed on a FPLC Superose 12 gel filtration column. The major activity eluted at 100 kDa, and two additional minor peaks (~250 kDa, ~160 kDa) were detected (fig.2). We focused on the characterization of the major peak (100 kDa factor). The activity of the partially purified 100 kDa factor was abolished by heating at 50°C for 5 min and was inactivated by chymotrypsin treatment (3 units, 6 h at 20°C) indicating that the factor is a protein. On sodium dodecylsulfate polyacrylamide gel electrophoresis, the peak fractions contained one major protein band at a molecular mass of 37 kDa (details of purification will be published elsewhere). The effect of the partially purified 100 kDa factor on the release of [^{3}H]GDP from Gly12 and Val12 p21 was examined in the presence of excess cold GTP. The partially purified factor, like crude brain membrane extracts, dramatically enhanced dissociation of [^{3}H]GDP from Gly12 p21, as well as Val12 p21 in a dose-dependent manner (fig.3a). We have also estimated the nucleotide exchange rate of p21. While the p21 · GDP complex is relatively stable at a physiological concentration of Mg^{2+} (1 mM approx.), the off rate for GDP increases

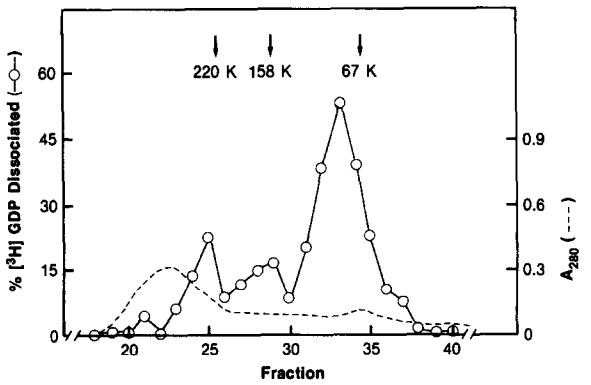

Fig.2. FPLC gel filtration chromatography of p21 guanine nucleotide exchange factor contained in bovine brain membrane extract. The membrane extract (15 mg) was passed through a Mono Q HR 5/5 column, and the peak fractions containing the activity to stimulate the guanine nucleotide exchange reaction of p21 were pooled. Then the sample was applied to a Superose 12 column, and fractions (0.4 ml) were collected. 20 μl of each fraction were assayed for the factor activity using Gly12 p21-[^{3}H]GDP (200 ng), as described in section 2. Activity is given as a percentage of bound [^{3}H]GDP dissociated. Approximately 8000 cpm of [^{3}H]GDP was bound to the p21 incubated without extracts. Molecular masses were determined by passing molecular mass markers through the column.

Table 1

Effect of the Y13-259 antibody on the p21-guanine nucleotide exchange reaction

Additions	[^{3}H]GDP bound (% control)	
	Gly12 p21	Val12 p21
None	100% (8700 cpm)	100% (9500 cpm)
Extract[a]	19	21
Rat IgG	76	88
Y13-259	95	96
Rat IgG + extract	21	24
Y13-259 + extract	89	82

Gly12 or Val12 p21 (170 ng) preincubated with 2 μM [^{3}H]GDP, as described in fig.1, was mixed with 4 μg of anti-*ras* Y13-259 monoclonal antibody or non-immune rat IgG for 30 min at 4°C, and the mixtures were incubated with the partially purified membrane extract (8 μg) and unlabeled GTP (0.1 mM) for 10 min at 37°C. The inhibitory effect of the Y13-259 antibody was saturated by this amount under the conditions used. The residual radioactive nucleotide-bound p21 was determined by the nitrocellulose filtration method as described in fig.2. Data represent means of two separate experiments.

[a] Extract partially purified from bovine brain cell membranes as described in fig.2.

10-fold in low Mg^{2+} (0.5 μM), suggesting the possibility that the effect of Mg^{2+} depletion on the p21-nucleotide exchange reaction may mimic that of receptor stimulation in vivo [8–10]. Consistent with earlier reports [8], our results indicate that the nucleotide exchange reaction with normal Gly12 p21 was 3-fold faster than that with T24 Val12 p21 in the presence of 2 mM $MgCl_2$; the half-life of the p21 · GDP complex was approximately 20 min for Gly12 p21 and 60 min for Val12 p21. When the factor was added to [^{3}H]GDP · Gly12 p21, the rate of nucleotide exchange was stimulated up to 25-fold (fig.3b). Although Val12 p21 alone slowly exchanged GDP for GTP as compared to Gly12 p21, the GDP dissociation rate of Val12 p21 was enhanced to the same level as that of Gly12 p21 by the addition of the factor (fig.3C). The factor was free from the activity to hydrolyze [^{3}H]GDP bound to p21, as determined by thin layer chromatography of reaction products, and did not have any detectable GDP/GTP binding activity.

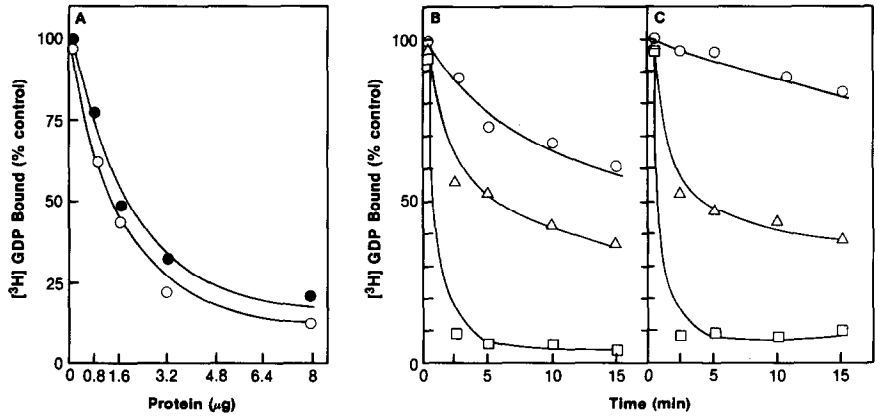

Fig.3. Effect of partially purified extract on the p21 nucleotide exchange reaction. (A) The dependence of [^{3}H]GDP release from p21 on the concentration of extracts. The peak fractions from a Superose column were 10-fold concentrated, and the indicated amounts of the concentrate were incubated with Gly12 (—○—) or Val12 (—●—) p21 · [^{3}H]GDP (200 ng) in the presence of 0.1 mM GTP and 2 mM $MgCl_2$ at 37°C for 10 min. Radioactivity remaining bound is given as percent relative to the value for the sample incubated without extract. These control values were 8500 cpm for Gly12 p21 and 9500 cpm for Val12 p21. (B) and (C) Effect of the extract on the nucleotide dissociation rate. Gly12 (B) or Val12 (C) (200 ng) p21 · [^{3}H]GDP was prepared as described in fig.1, and the formed binary complex was incubated with either 0 μg (○), 1.6 μg (△), or 12 μg (□) of the partially purified membrane extract in the presence of 0.1 mM GTP and 2 mM $MgCl_2$ for the indicated time intervals at 37°C. Radioactivity remaining bound is given as percent relative to the value for the samples harvested at 0 min. These control values were 8500, 8300, and 8000 cpm for 0, 1.6, and 12 μg of extract in (B) and 9500, 9200, and 8900 cpm for 0, 1.6, and 12 μg of extract in (C).

It is known that anti-*ras* Y13-259 monoclonal antibody neutralizes the ability of p21 to induce cell proliferation [11] and specifically blocks the exchange reaction between p21-bound [^{3}H]GDP and exogenous GTP, possibly by stearic hindrance due to the bulky immunoglobulin molecule [12]. GAP activity is also inhibited by the antibody [3]. To analyze whether the antibody influences the activity of the partially purified 100 kDa factor, [^{3}H]GDP · p21 binary complexes were incubated with the antibody and then reacted with the factor in the presence of cold GTP. Y13-259 antibody blocked the stimulation of the p21-nucleotide exchange reaction by the factor, while non-immune rat IgG had no effect (table 1). The neutralizing effect of the antibody may, in part, be explained by its suppressive effect on the factor-stimulated nucleotide exchange reaction.

4. DISCUSSION

In summary, we have partially purified a factor with a molecular mass of 100 kDa, which markedly stimulated the guanine nucleotide exchange reaction between p21-bound GDP and exogenous GTP. This factor was predominantly located in the membrane fraction. In an attempt to determine whether the factor has similar effects on the GDP/GTP exchange reaction of other GTP binding proteins, we tested both transducin Tα and *E. coli* translation elongation factor Tu. No stimulation of nucleotide exchange activity by the factor was observed with these proteins (unpublished data). Moreover, the estimated molecular mass of the factor was different from that of any known guanine nucleotide exchange factors. We will refer to this factor as rGEF (*ras* guanine nucleotide exchange factor). It is assumed that in *ras*-mediated signal transduction, GTP-bound p21 interacts with GAP, and that GAP rapidly shuts off signals as it converts the active GTP · p21 to the inactive GDP · p21 by the hydrolysis of bound GTP [13]. Then GDP · p21 must be reactivated by a certain active transmembrane signal through a GDP/GTP exchange event. We postulate that rGEF may be involved in this rate-limiting step. The association of rGEF with normal Gly12 p21 would lead to a conformational change in the p21, increasing the GDP/GTP exchange activity. In contrast, the oncogenic p21 protein fails to undergo down-regulation through GTPase activity stimulated by GAP and remains GTP-bound. Since the concentration of GTP is much higher than that of GDP in the intracellular nucleotide pool, the probability of exchange of GTP bound to the oncogenic p21 with GDP would be very low. Thus, the oncogenic p21 protein remains persistently in an active state. There is indirect evidence that receptor-mediated phopholipid turnover is influenced by p21 proteins [14,15], and that growth factors, such as EGF, might change the affinity of guanine nucleotides to p21 [16]. It is known that in the yeast *S. cerevisiae* cells, the *CDC* 25 gene, either directly or indirectly, regulates guanine nucleotide exchange of yeast *ras*2 protein coupled with adenylate cyclase [17]. Exchange of guanine nucleotides bound to eukaryotic translation intitiation factor (eIF-2), eukaryotic elongation factor (EF-1), and bacterial elongation factor (EF-Tu) are also recycled by specific guanine nucleotide exchange factors [18]. Thus, there must be some common regulation mechanism for the activity between *ras* p21 proteins and some GTP binding proteins which are structurally related to p21 within the GTP binding domain. Further study will be necessary before we make any conclusion on whether rGEF is really a physiological target for p21 in vivo. Purification of rGEF and cloning of the gene will facilitate the biochemical study on the *ras*-rGEF interaction and the determination of the biological role of rGEF in the *ras*-controlled signalling pathway.

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