Purification of a Novel ras GTPase-activating Protein from Rat Brain*

(Received for publication, March 1, 1993, and in revised form, May 18, 1993)

Midori Maekawa, Shun Nakamura, and Seisuke Hattori‡

From the Division of Biochemistry and Cellular Biology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ojima, Kodaira, Tokyo 187, Japan

GTPase-activating protein (GAP) and neurofibromin, a gene product of neurofibromatosis type I gene, have been identified as factors that stimulate GTPase activity of ras p21. We have previously suggested the presence of novel GAP activity that is immunologically distinguishable from GAP or neurofibromin in both the cytosolic and the particulate fractions of rat brain (Hattori, S., Maekawa, M., and Nakamura, S. (1992) Oncogene 7, 481-485). We have purified this novel GAP molecule from the cytosolic fraction of rat brain by more than 200,000-fold by five successive column chromatographies with a recovery of 6%. Apparent molecular mass of this molecule was estimated to be 100 kDa (p100GAP*). The same p100GAP* was purified from the particulate fraction after extraction with high salt. The activation of GTPase was observed with normal ras p21 but not with oncogenic ras p21, Rap1B, or Ram25K. The dissociation constant of p100GAP* toward ras p21 estimated by competitive inhibition using ras p21 in complex with nonhydrolyzable analog of GTP was two times higher than that of neurofibromin and was lower than that of GAP by 2 orders of magnitude. These results clearly indicate that p100GAP* is a novel ras GAP molecule.

GTP-binding proteins are involved in the regulation of various biological systems. Conformational change of the GTP-binding proteins induced by GTP/GDP conversion, originally noted for elongation factors for protein biosynthesis, may be a general mechanism that regulates the activity of the systems (1, 2). Ras gene product p21 is thought to function as a regulator of many kinds of signal transduction systems (3). Microinjection of neutralizing anti-ras monoclonal antibody inhibited the proliferation of fibroblastic cells (4), the differentiation of neuronal cells (5), and the maturating of Xenopus oocytes (6), which indicates the involvement of ras function in these processes.

It has been shown that only the GTP-bound form of ras p21 is biologically active (7, 8). A variety of extracellular stimuli including platelet-derived growth factor (9), epidermal growth factor (10), insulin (11), phorbol 12-myristate 13-acetate (12), interleukins 2 and 3, granulocyte macrophage colony-stimulating factor (13), and nerve growth factor (14) are shown to activate ras p21 by increasing the active GTP-bound form, suggesting that ras p21 mediates the signal transmission in these systems. Thus it is important to study the mechanism that regulates the level of ras p21-GTP complex.

Trahey and McCormick (7) described a cytoplasmic factor which stimulates GTPase activity of the normal but not the oncogenic form of ras p21 and termed the factor as GTPase-activating protein (GAP).1 GAP was purified from bovine brain as a monomeric protein having an apparent molecular mass of 116 kDa (p120GAP**) (15). Bovine and human GAP cDNA were subsequently isolated by Vogel et al. (16) and Trahey et al. (17), respectively, and the structural analyses of these cDNAs revealed a strikingly conserved structure beyond species difference. GAP has two SH2 (src homology 2) and one SH3 domains which are considered to function in protein-protein interactions (18). It has been shown that GAP is tyrosine-phosphorylated in cells stimulated by growth factors described above or in cells transformed by oncogenes having tyrosine kinase activity (19, 20).

Neurofibromin, a product of the neurofibromatosis type I (NF1) gene, has a region that shows a sequence similarity to catalytic domain of GAP and Ira proteins of Saccharomyces cerevisiae, and the region was termed GAP-related domain (GRD) (21). Indeed the NF1-GRD was shown to possess GAP activity toward ras p21 and to suppress ira mutation (22-24). Several groups including us identified neurofibromin as a 255-250-kDa protein (25-27). Multiple GAP molecules have also been identified for Krev-1/rap1 (28-30) or rho/rac p21 (31-34).

During the course of the identification of neurofibromin we found that both the soluble and the particulate fractions prepared from rat brain contained GAP activity that was not suppressed by anti-p120GAP or anti-NF1-GRD antiserum (27). This finding suggested the presence of a novel GAP molecule other than p120GAP and neurofibromin. In this paper we have purified this novel GAP from rat brain as a 100-kDa protein (p100GAP**). p100GAP** stimulated GTPase of normal ras p21 but not that of the oncogenic form of ras p21, Rap1, or Ram25K.

MATERIALS AND METHODS

Purification of p100GAP* from the Cytosolic Fraction of Rat Brain—All procedures were carried out below 4 °C. Brains of Wistar rats (female, 3-6 months old, 60 g) were homogenized in 200 ml of a solution containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.2 mM sucrose, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 μM/ml antipain, and 1 μM/ml leupeptin using a motor-driven Teflon homogenizer. The homogenate was centrifuged

---

1 The abbreviations used are: GAP, GTPase-activating protein; PAGE, polyacrylamide gel electrophoresis; GTPyS, guanosine 5'-O-(3-thiotriphosphate); GRD, GAP-related domain; NF1, neurofibromatosis type I gene.
at 1000 × g for 15 min and then further centrifuged at 100,000 × g for 60 min, and the supernatant and the particulate fractions were saved. The supernatant was directly loaded onto a column of DEAE-Toyopearl 650 M (3.2 × 12 cm, Tosoh) which had been equilibrated with a solution containing 10 mM Tris-HCl (pH 8.0), 0.5 mM EGTA, 5% glycerol, 10 mM 2-mercaptoethanol, and 0.05% Brij 35 (Buffer A), and the flow-through fraction which contained the antiserasensitive GAP activity was saved.

The sample obtained from the previous step was applied to a column of hydroxylapatite (3 × 4.5 cm, Bio-Rad) which had been equilibrated with a solution containing 10 mM potassium phosphate (KPi) gradient (pH 7.4), 10 mM 2-mercaptoethanol, and 0.05% Brij 35 (Buffer B), and the column was developed with a linear 10–400 mM KPi gradient (150 ml each). The active fractions (eluted at around 290 mM KPi) were pooled, dialyzed overnight against Buffer A, and applied to TSKgel HA-1000 column (0.75 × 7.5 cm, Tosoh) preequilibrated with Buffer B. The column was developed at a flow rate of 0.45 ml/min with a linear 10–400 mM KPi (pH 7.4) gradient (20 ml each). The activity was eluted at 300 mM KPi, and then the active fractions were pooled and dialyzed against Buffer A without Brij 35 (Buffer C). The dialyzed material was subjected to column chromatography using TSKgel Heparin-5PW (0.75 × 7.5 cm, Tosoh) preequilibrated with Buffer C. Elution was performed with a linear 0–1.0 M NaCl gradient in Buffer C (18 ml each) at a flow rate of 0.3 ml/min, and 0.6-ml fractions were collected. The activity was eluted between 0.75 and 0.8 M NaCl. The active fractions were pooled and directly applied to a column of Sephacryl S-300 HR (1 × 46 cm, Pharmacia LKB Biotechnology Inc.) preequilibrated with a buffer containing 10 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 0.5 mM EGTA, 5% glycerol, and 10 mM 2-mercaptoethanol. Gel filtration was performed with the same buffer, and 1.2-ml fractions were collected.

Purification of p100GAP from the Particulate Fraction of Rat Brain—The particulate fraction obtained as described in the previous section was suspended at a protein concentration of 5–10 mg/ml in Buffer C containing 1 M NaCl. After stirring for 60 min on ice, the suspension was centrifuged at 100,000 × g for 60 min. The supernatant was dialyzed against Buffer C, and the dialyzed material was centrifuged at 100,000 × g for 60 min. Half of the antiserasensitive GAP activity in the particulate fraction was extracted by this high salt treatment and was purified by the same procedures as for the cytosolic fraction.

Antiseras—Anti-p120GAP and anti-NF1-GRD antisera were prepared as previously (27). Recombinant rat GAP (residual 40 to the carboxyl terminus (27)) and recombinant NF1-GRD (22) (expression plasmid was a generous gift of Dr. F. Tamanoi, University of Chicago) were used as antigens.

GAP Assay—GAP activity was measured as described previously (27, 35). The sample to be assayed was mixed with 100 ng of Ha-ras p21(−29−30)GTP (30 Ci/mmol) in a total volume of 50 μl and incubated for 3 min at 37 °C in the presence or absence of antiseras against GAP (1.2 μl) and NF1-GRD (0.8 μl), and the amount of remaining p21(−29−30)GTP was determined by filter binding assay. In the experiments presented in Fig. 3, [α-32P]GTP (30 Ci/mmol) was used instead of [γ-32P]GTP, and GTP hydrolysis was quantitated after immunoprecipitation of ras p21 followed by thin layer chromatography of p21-bound nucleotides (35).

Similar assays were performed using Ha-ras p21 having valine at the 12th residue (Ha-ras p21Val12), Rap1B and Ram25K. Rap1B and Ram25K were purified as described (36) and kindly donated by Drs. K. Nagata and Y. Nozawa (Gifu University School of Medicine). Recombinant GAP and NF-GRD were expressed in and purified by the method of Bradford (37) using bovine serum albumin as a standard. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed according to the method of Laemmli (28). Proteins were visualized with Coomassie Brilliant Blue R-250 for silver staining using a kit supplied by Daiichi Pure Chemicals. Dillute protein solution was concentrated by precipitation with trichloroacetic acid in the presence of 0.033% sodium deoxycholate (39).

RESULTS

We recently suggested that there is a novel GAP molecule in both the cytosolic and the particulate fractions of rat brain because 30–40% of total GAP activity of these fractions was suppressed by neither anti-GAP nor anti-NF1 GRD antisera (27). We have purified this novel GAP molecule from both fractions by the procedures described under “Materials and Methods.”

In the first column chromatograph of the cytosolic fraction on DEAE-Toyopearl, the specific activity of antiseras-insensitive GAP increased by 8.3-fold; however, the active fractions still contained the antisera-sensitive GAP activities. In the next column chromatograph, hydroxylapatite, three peaks of GAP activity were observed. From their sensitivity to the specific antisera, the first and the second peaks were identified as neurofibromin and p120GAP, respectively. The antisera-insensitive GAP was eluted as the third peak. Because the residual p120GAP activity was completely separated by TSKgel HA-1000 column chromatography, GAP activity after this step was measured in the absence of the antisera.

The specific activity greatly increased through TSKgel Heparin-5PW column chromatography. We analyzed the proteins in the active fractions and compared their elution pattern with that of the activity (Fig. 1). The activity was coeluted with the protein having an apparent molecular mass of 100 kDa (fractions 52–55). In the final step, gel filtration on Sephacryl S-300 HR, the activity was eluted into two peaks, a major peak (fractions 24–25) followed by a minor peak (fractions 26 and 27) (Fig. 2A). The major peak contained a protein having an apparent molecular mass of 100 kDa as a sole detectable protein (Fig. 2B). The same protein was also observed in the latter minor peak. GAP activity of these fractions was closely proportional to the intensity of this 100-kDa protein band. The split of the activity may be due to the interaction of a novel GAP molecule with the resin because all of the GAP activity was eluted at the position corresponding to the minor peak when NaCl concentration of the elution buffer was reduced from 0.3 M to below 30 mM (data not shown).

Half of the antisera-insensitive GAP activity in the particulate fraction was extracted by high salt treatment and then purified by the same procedure. GAP activity was eluted from
purification of a novel ras GAP

FIG. 2. Gel filtration on Sephacryl S-300 HR. Active fractions from TSKgel Heparin-5PW were pooled and directly applied to Sephacryl S-300 HR column (1 x 46 cm), and 1.2-ml fractions were collected. The results with the soluble (A and B) and the particulate (C) fractions are shown. A, GAP activity in each fraction was measured using 8-μl aliquots (C). Elution positions of the standard proteins are indicated by the arrows. B and C, proteins of each fraction were analyzed by 10% SDS-PAGE, and the proteins were visualized by silver staining. Molecular masses of standard proteins in kDa are shown on the left. B, aliquots of 900 μl were precipitated by trichloroacetic acid in the presence of 0.033% sodium deoxycholate and subjected to 10% SDS-PAGE. C, 65-μl aliquots were directly applied to 10% SDS-PAGE.

Sephacryl S-300 HR at the same position where antisera-insensitive GAP purified from the cytosolic fraction was eluted (data not shown). The active fractions contained the same 100-kDa protein as a major constituent whose concentration was proportional to the activity (Fig. 2C).

These findings taken together clearly indicate that this 100-kDa protein is responsible for the activity. The elution position of this 100-kDa GAP (p100GAP) was slightly ahead of bovine serum albumin, suggesting that this GAP molecule may be monomeric. The purification procedure of 100-kDa GAP from cytosol is summarized in Table I. The increase in the specific activity through the five column chromatographies was more than 200,000-fold with a recovery of 6%.

GAP activity was also observed when the assay was carried out using p21-[α-32P]GTP as the substrate (Fig. 3). The summation of the amounts of p21-[α-32P]GTP and p21-[α-32P]GDP did not change during the incubation, which shows that p100GAP has no effect on the nucleotide exchange reaction between p21-bound and exogenous nonradioactive GTP. In addition, p100GAP itself had no detectable GTPase activity (data not shown).

The molecular weight of p100GAP is clearly different from that of p120GAP or neurofibromin. Furthermore, p100GAP was antigenically different from p120GAP or neurofibromin, because antisera against p120GAP and NF1-GRD did not inhibit the activity of p100GAP under the conditions where activities of p120GAP and NF1-GRD were completely suppressed (Fig. 4). Because recombinant p120GAP and NF1-GRD, which were used to make the antisera, contained their catalytic domains (GAP contains the full length except for 40 amino-terminal residues; NF1-GRD is a catalytic domain itself), the antisera should inhibit the activity of any proteolytic fragments derived from them if they exist. Moreover, these antisera did not react with p100GAP in immunoblot analysis (data not shown). Thus it is unlikely that p100GAP is a proteolytic fragment derived from p120GAP or neurofibromin.

Substrate specificity of p100GAP was examined using normal c-Ha-ras p21, Ha-ras p21Val12, rap1B p21, or Ram p25 (Fig. 5). Like p120GAP and NF1-GRD, p100GAP stimulated GTPase of only normal Ha-ras p21 and not that of the oncogenic form of ras p21, Rap1B, or Ram25K. This specificity was the same with those of p120GAP and neurofibromin. We thus termed the novel GAP as p100GAP*.

It has been reported that the affinity toward ras p21 of neurofibromin is much higher than that of p120GAP (23). These results were obtained by a competition GAP assay using ras p21 bound to a nonhydrolyzable analog of GTP as a competitor. GAP activity of p100GAP* was measured in the presence of various concentrations of ras p21 bound to guanosine 5′-O-(3-thiotriphosphate) (GTPyS). The apparent affinity of p100GAP* for p21-GTPyS, determined as a concentration at which 50% of GAP activity was inhibited, was 620 nM (Fig. 6). This value was 2 times higher than that of neurofibromin (300 nM) and was lower than that of p120GAP (>20 μM) by 2 orders of magnitude. This finding may also be supporting evidence that p100GAP* is a novel GAP molecule.

DISCUSSION

In this paper we report the purification of p100GAP, a novel ras GAP molecule, from rat brain by five successive column chromatographies. To date p120GAP and neurofibromin have been identified as GAP molecules for ras p21. Our present findings indicate that p100GAP is a third GAP for ras.

p120GAP was purified by Gibbs et al. (15) from the cytosolic fraction of bovine brain. They employed a DEAE-Sephacel column for the first step of purification and reported that GAP activity was eluted with 70–90 mM NaCl. We tried the same condition of column chromatography to purify p100GAP; however, the activity was eluted rather broadly with 50–200 mM NaCl. This might be the reason why p100GAP activity was overlooked and left unidentified to date.

A truncated form of p120GAP, 95-kDa GAP, which may be produced by alternative splicing, is reported to be present in placenta but not in other tissues (40). This 95-kDa GAP is recognized by anti-p120GAP, and its activity is neutralized by the antibody. In contrast, p100GAP is not detected by anti-p120GAP, and its activity is not suppressed by the antisera, indicating that p100GAP and 95-kDa GAP are different molecules.

The antisera-insensitive GAP activity was also detected in the particulate fraction. We tried various conditions to extract this antisera-insensitive GAP activity, including 1 M NaCl, 0.1 M ethanalamine (pH 10.5), 4 M urea, 0.6 M KI, 1% Triton X-100, 1% octyl glucoside, 1% heptlythioglicol, and 5 mM EDTA. Among them, 1 M NaCl gave the best result,
TABLE I

<table>
<thead>
<tr>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>1,780 mg</td>
<td>5,518 unit</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>500</td>
<td>12,800 unit/µg</td>
<td>8.3</td>
<td>232</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>17</td>
<td>8,738 unit/µg</td>
<td>166</td>
<td>158</td>
</tr>
<tr>
<td>TSKgel HA-1000</td>
<td>1.9</td>
<td>6,042 unit/µg</td>
<td>9.4</td>
<td>109</td>
</tr>
<tr>
<td>TSKgel Heparin-5PW</td>
<td>0.0013</td>
<td>1,327 unit/µg</td>
<td>106,000</td>
<td>8.1</td>
</tr>
<tr>
<td>Sephacryl S-300 HR</td>
<td>0.00035</td>
<td>960</td>
<td>291,000</td>
<td>5.8</td>
</tr>
</tbody>
</table>

a GAP activity was measured in the presence of 1.2 µl of anti-p120GAP and 0.8 µl of anti-NF1-GRD antisera.

b One unit of GAP activity is defined as the amount of GAP that stimulates the hydrolysis of 50% of the [γ-32P]GTP bound to ras p21.

c Forty rat brains were used as a starting material.

d GAP activity after TSKgel HA-1000 was measured both in the presence and in the absence of the antisera. After this step, the activity was measured without antisera. Since rabbit serum (even the preimmune serum) was inhibitory to GAP activity, the specific activity and the yield after this step were calculated taking the values obtained with the sample from HA-1000 as 9.4 units/µg and 109%, respectively.

e Protein concentration was densitometrically determined using bovine serum albumin as a standard.

![FIG. 3. GAP activity of p100GAP measured by using p21-[α-32P]GTP. The indicated amounts of sample obtained after Heparin-5PW column were used in GAP assay using p21-[α-32P]GTP. Lane 1, buffer control kept on ice; lane 2, buffer control after incubation for 10 min at 37 °C; lane 3 and 4, with 0.67 and 1.1 ng of the active fraction, respectively.](image)

![FIG. 4. The effect of anti-NF1-GRD (left) or anti-p120GAP (right) antisera on the activity of p100GAP. GAP activity was measured in the presence of the indicated amount of antisera. Preimmune serum was added to make the total volume of the serum in each sample the same. The activity of the control sample, which received only the preimmune serum, was expressed as 100%, and GAP activity of each sample was expressed as the percentage to the activity of the control sample. Sample obtained after TSKgel Heparin-5PW (2 ng) was used ( ). Recombinant NFI-GRD (0.5 ng) ( ), and recombinant p120GAP (7 ng) ( ) were used in the left and right panels, respectively. In the absence of antisera, p120GAP, NFI-GRD, or p100GAP stimulated hydrolysis of 50-60% of input ras-bound [γ-32P]GTP. Which suggests that p100GAP has an ionic interaction with membranous or cytoskeletal structure. Because only a half of the antisera-insensitive GAP activity was extracted with 1 M NaCl, we could not exclude the possibility that another GAP molecule might be present.](image)

![FIG. 5. Specificity of the p100GAP. GAP activity was measured using normal Ha-ras p21 (A), rasV12 p21 (B), RapiB (C), or Ram25K (D) as the substrates. Samples were incubated at 37 °C for 10 min in A, B, and D and at 30 °C for 3 min in C. The amount of low molecular weight GTP-binding proteins in complex with [γ-32P]GTP was shown as the percentage, taking the value obtained with the control sample kept on ice as 100% (2-3 pmol). Column 1, buffer control kept at 0 °C; column 2, buffer control after incubation; column 3, active fraction of TSKgel Heparin-5PW (1.4 ng); column 4, recombinant p120GAP (7.5 ng); column 5, recombinant NF1-GRD (0.12 ng).](image)
that the predicted structure contains a region with bcr/n-"chimaerin similarity (43). p190 also shows ras GAP activity (34). Thus it seems general that each low molecular weight GTP-binding protein has multiple GAP molecules.

It is noted that p120GAP and neurofibromin are present in the same cells (44), and at least three forms of ras GAPS are present in the same tissue, brain. Clarifying the specific roles of these GAP molecules may be the next important subject. GAP activity is higher in the growing cells than in the quiescent cells (35), which may function in the down-regulation of ras. It should be interesting to study which GAP molecule is under control of the cell density.

Treatment of cells with growth factors induces tyrosine phosphorylation of p120GAP (19, 20), which may function in the translocation of p120GAP from cytosol to growth factor receptors (45). In contrast, neurofibromin is predominantly located in the particulate fraction (25, 27, 46). p100GAP is present in both the cytosolic and the particulate fractions. It also seems intriguing to study whether the activity or the localization of p100GAP might be controlled by the extracellular signals or whether p100GAP might undergo any post-translational modification.

It has been reported that p120GAP also acts as a mediator of downstream signaling of ras. GAP prevents the coupling of the muscarinic receptor to the potassium channel (47), and the SH2-SH3 region of GAP stimulates the proliferation of fibroblasts (48), both by ras-dependent manner. GAP is associated with p190 and p62 upon stimulation by growth factors. p190 has GTPase motif, transcriptional repressor motif, and rho/rac GAP domain (43), and p62 shows RNA binding activity (49). Consistent with their structural features, portions of both p62 and p190 are located within nuclei (43, 49). These findings raise the possibility that p62 and p190 mediate downstream signaling of ras through p120GAP. Because p190 shows ras GAP activity, GAP-GAP interaction might regulate the cross-talking of the different biological systems. Structural analysis of a novel ras GAP, p100GAP, may shed light on a further understanding of ras-signaling pathways.

Acknowledgments.—We thank Drs. K. Nagata and Y. Nosewae for Rap1B and Rap25K and K. Hattori for critical reading of the manuscript. The initial phase of this project was carried out by N. Ohmi. We also thank Knorou Okuda for her excellent secretarial work.