rho GAP of 28 kDa (GAP2), but Not of 190 kDa (p190), Requires Asp
65 and Asp
67 of rho GTPase for Its Activation*

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Two distinct GTPase-activating proteins (GAPs), i.e. rho GAPs of 28 kDa (GAP2) and of 190 kDa (p190), stimulate the intrinsic GTPase activity of the rho protein. The rho GAP activity of p190 resides in its C-terminal domain (p190C). Neither GAP2 nor p190C activates the rho GTPase. We replaced Asp
65 and Asp
67 residues of rho GTPase with the corresponding ras residues and examined whether the domain containing them is involved in its activation by rho GAPs. Mutation of either Asp
65 to Glu or Asp
67 to Ser did not change the Kd value for GTPyS of the rho protein. The Ser
67 mutation reduced the intrinsic GTPase activity of the rho protein, while no change was observed with the Glu
65 mutation. Both mutations abolished activation of rho GTPase by GAP2. The GAP2-dependent activation of rho GTPase was inhibited by the addition of GTPyS-bound wild type rho but not by either GTPyS-bound Glu
65- or Ser
67-rho, indicating that both Asp
65 and Asp
67 are essential for interaction of rho protein with GAP2. On the contrary, p190C activated both Glu
65- and Ser
67-rho GTPases to the extent and in a dose dependence to those seen in the wild GTPase. These results suggest that GAP2 and p190 interact with different residues or domains of the rho GTPase for their activation.

There are at least four different GTPase-activating protein (GAP) families in mammalian cells, which selectively activate the corresponding GTPase families of monomeric G proteins. Ras GTPases (Ha-, Ki-, N-, and R-ras) are activated by two distinct GAPs (1-6), i.e. ras-GAP of 1,044 amino acids (GAP1) and NF1 of 2,818 amino acids. Rho GTPases (A, B, and C) are activated by two distinct GAPs (7-10), i.e. rho-GAP of 28 kDa (GAP2) and p190 of 1,512 amino acids. Rap1 GTPases (A and B) and Rac1 GTPase are activated by at least two distinct GAPs (11-13), i.e. cytoplasmic GAP3c of 55 kDa and membrane-bound GAP3m of 85 kDa. Rac GTPases (1 and 2) are activated by two distinct GAPs (14, 15), i.e. Bcr and n-chimaerin, which might be called GAP4b and GAP4n, respectively. Since these monomeric G proteins are GTP-activated signal transducers, the hydrolysis of GTP bound to each G protein to GDP by its GTPase activity leads to the attenuation of its signal-transducing activity (16). Thus, the corresponding GAP would serve as a potent signal attenuator of a given G protein. Interestingly, however, there is some evidence that while a specific domain of a given GAP serves as a signal attenuator, the remaining domain serves as an effector (or target) of the corresponding G protein (17-19). Thus, each GAP may act as a dual switch for the corresponding signal.

Since many ras mutants are oncogenic mainly due to reduction of their intrinsic GTPase activity or due to loss of its stimulation by GAP1 or NF1, an extensive mutational analysis of ras has been carried out to identify the key ras residues responsible for its oncogenicity and its sensitivity to GAP1/NF1-dependent GTPase activation. Taking advantage of the findings that (i) rapl shares 50% sequence identity with ras (20), (ii) unlike the ras mutants, the corresponding rap1 mutants are anti-oncogenic rather than oncogenic (21, 22), and (iii) its intrinsic GTPase activity is stimulated not by GAP1/NF1 but by the two GAP3s (11-13, 23, 24), we have analyzed ras/rap1 mutants systematically and identified several key residues of the ras and rap1 GTPases essential for their interaction with respective GAPs. These studies indicate that (i) residues 61-65 together with the effector domain are important in the ras interaction with GAP1/NF1 and the rap1 interaction with GAP3c (11, 23, 24), and (ii) the other GAP3, GAP3m, interacts with different residues or domains of rap1 for its activation (11). As shown in Fig. 1, the residues 61-65 of the ras and rap1 GTPases correspond to the 63-67 residues of the rho GTPase, and they are identical to the corresponding ras residues except Asp
65 and Asp
67. These observations raise the possibilities that (i) the residues 63-67 of rho GTPase might play a similar important role in its activation by rho-GAPs, and (ii) GAP2 and p190 might interact with different residues or domains to activate the rho GTPase. In this work, using the GAP2 purified from bovine adrenal gland and a recombinant rat p190C from Escherichia coli, we have demonstrated that both Glu
65 and Ser
67 mutations abolished the activation of rho GTPase by GAP2 but not by p190C. Our results suggest that both Asp
65 and Asp
67 of the rho GTPase are essential for its interaction with GAP2 and that GAP2 and p190 interact with different residues or domains of rho GTPase for activation.
**EXPERIMENTAL PROCEDURES**

**Materials**—The cDNA of rat p190 was kindly provided by J. Settleman and R. A. Weinberg (Massachusetts Institute of Technology, Boston). As a source of the native rhoA protein, we used adrenal gland membranes purified from bovine adrenal glands as described previously (25). Recombinant human rhoA (residues 1–193), c-Ha-ras (residues 1–189), and p190 (residues 1–193) were prepared as glutathione S-transferase fusion proteins in E. coli and affinity-purified on a GSH-agarose column as described previously (23, 24, 26). When intrinsic and GAP2-activated GTPase activities of the wild type rhoA fused to glutathione S-transferase were compared with those of the native rhoA purified from bovine adrenal gland, no significant difference was observed in either activity between the two (data not shown). Glutathione S-transferase molecule attached to the N terminus of rhoA was, therefore, presumed not to affect the GTPase activity. GAP2 was purified from bovine adrenal gland with 30 pmol, and previously purified 10-100 pmol. Because of the limited amount of the homogeneous preparation, a partially purified GAP2 preparation from the phenyl-Sepharose column was used. It showed no detectable p190 or ras-GAP activity.

**Assays**—The GAP2 activity was measured mainly by the filter paper method as described previously (8). Briefly, 1–2 pmol of each G protein was incubated with 50 pmol of [γ-32P]GTP (50 Ci/mmol) at 30 °C for 30 min in a total volume of 10 μl. The mixture was then added to 90 μl of a solution containing 20 mM Hepes, NaOH, pH 7.5, 1 mM MgCl₂, 1 mg/ml bovine serum albumin with or without GAP2. When the partially purified GAP2 preparation was used, 20 μM GTP-S or GDF was added to the GAP2 preparation to inhibit the spontaneous GTP binding activity present in the preparation as described previously (8). After incubation for the indicated times at 30 °C, the mixture was filtrated through a BA86 membrane under vacuum and rinsed five times with the washing buffer, and the radioactivity on the filter was measured in Triton-toluene scintillator on a Minaxi scintillation system as described previously (8).

**Effects of GAP2 on Asp₆⁵/Asp₆⁷ Double Mutant of Ha-ras GTPase**—To clarify whether these two residues (Asp₆⁵ and Asp₆⁷) of rho GTPase are sufficient for determining the GAP2 activity. This double mutation did not alter the intrinsic GTPase activity of the molecule (0.011 ± 0.002 and 0.011 ± 0.005/min in the wild type and the mutant, respectively, n = 3). As reported previously, GAP2 did not activate the GTPase of the wild type ras GTPase (Fig. 3A). When GAP2 was added to the mutant, no appreciable activation of GTP hydrolysis was observed (Fig. 3B). This result suggested that additional rho-specific residues other than the Asp₆⁵ and Asp₆⁷ are also required for activation by GAP2. Since the rho residues 59–67 are identical to the corresponding part of Asp₅/Asp₆ double mutant of ras GTPase (residues 57–65), the responsible rho-specific residues must be found within upstream of Asp₅ or downstream of Asp₆. The rho effector domain (residues 34–
rho GAP Interaction with Glu\textsuperscript{65} and Ser\textsuperscript{67} rho Mutants

FIG. 2. Effects of GAP2 on GTP hydrolysis by rhoA (A), Glu\textsuperscript{65}-rhoA (B), and Ser\textsuperscript{67}-rhoA (C). The \([\gamma-32P]\)GTP-bound form of each protein was incubated with (\(\bullet\)) or without (\(\circ\)) 0.5 \(\mu\)g of protein of the partially purified preparation of GAP2 for indicated periods at 30°C, and GTP hydrolysis was determined as described under "Experimental Procedures." The remaining [\(\gamma-32P\)]GTP bound to each protein is expressed as a percent of that measured at 0 min of incubation.

TABLE I
Inhibition of GAP2-activated GTP hydrolysis of rho GTPase by GTP\textsuperscript{yS}-bound rho and rho mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>Inhibition of GAP2-activated GTP hydrolysisa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GTP\textsuperscript{yS}-bound form</td>
</tr>
<tr>
<td>Wild type</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>Glu\textsuperscript{65}-rho</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Ser\textsuperscript{67}-rho</td>
<td>10 ± 2</td>
</tr>
</tbody>
</table>

aGAP2-activated GTP hydrolysis (GAP2 activity) was determined in the absence or presence of 0.8 \(\mu\)M either GTP\textsuperscript{yS}-bound or GDP-bound wild type rhoA or its mutants as described under "Experimental Procedures." Inhibition of GAP2 activity was calculated as follows: percent inhibition = 100 \times (GAP2 activity in the absence of rho or its mutants - GAP2 activity in the presence of rho or its mutants)/GAP2 activity in the absence of rho or its mutants.

As shown in Fig. 4A, more than 90% of total GTP bound of rho GTPase by C3 exoenzyme occurs in its effector domain at Asn\textsuperscript{41} (29, 30) but does not affect the GAP2-dependent activation of rho GTPase (8), although it abolishes the signal transduction of rho protein in vivo (31-34).

Activation by p190C of Glu\textsuperscript{65} and Ser\textsuperscript{67} Mutants of rho GTPase—A recombinant C-terminal 55-kDa fragment of rat p190 from E. coli has been shown previously to activate both rho and rac GTPases but not ras GTPases (10). To clarify whether the rho-GAP domain of p190 also requires both Asp\textsuperscript{65} and Asp\textsuperscript{67} residues to activate the rho GTPase, we have prepared a similar C-terminal fragment (p190C, residues 1167-1512) of the p190 as a GST fusion protein in E. coli and compared its GAP activity with that of GAP2. As shown in Fig. 4A, GAP2 enhanced GTP hydrolysis by the mutant proteins only slightly even with the saturating amount for the wild type protein. On the contrary, the p190C activated both Glu\textsuperscript{65} and Ser\textsuperscript{67} mutants of the rho GTPase as much as the normal rho GTPase (Fig. 4B). These observations indicate that, unlike GAP2, p190 does not require either Asp\textsuperscript{65} or Asp\textsuperscript{67} to activate the rho GTPase. Thus, it is likely that these two distinct rho GAPS, i.e. GAP2 and p190, interact with different rho residues or domains for the GTPase activation. We are currently identifying the rho residues required for p190C-dependent GTPase activation.

A difference similar to that found in GAP2 and p190 was also observed between two GAP3s for rap1 GTPases. We recently found that Thr\textsuperscript{65} is essential for rap1 GTPase activation by GAP3c (11), while it can be replaced by Ser without affecting its activation by GAP3m, indicating that these two distinct GAP3s interact with different residues or domains to activate rap1 GTPases.

As shown in Fig. 4A, more than 90% of total GTP bound...
**Fig. 4.** Dose-activation curves for GAP2 (A) and p190C (B) on GTP hydrolysis by rhoA, Glu69-rhoA, and Ser77-rhoA. The GTP hydrolysis by the wild-type (○), Glu69 mutant (△), and Ser77 mutant (□) of rhoA-GST protein was measured in the presence of indicated amounts of the partially purified GAP2 preparation (A) or p190C (B). On the basis of its activity and previous purification of GAP2 (28), 1 µl of the partially purified GAP2 preparation used in this study (0.5 mg protein/ml) was presumed to contain 0.5 ng of GAP2. In this study, the second reaction in experiment A was carried out at 25°C in a total volume of 100 µl in the same buffer as in experiment B. Hydrolyzed GTP is expressed as a percent of total GTP bound to each protein.

to rhoA GTPase was hydrolyzed in the presence of 1 µl/tube of the partially purified GAP2 preparation. Because this preparation contained 0.1% of pure GAP2 (8), this activation was achieved by 5 ng/ml (178 pM) of GAP2, while equivalent activation required more than 20 µg/ml (370 nM) of p190C. Thus, the GAP2 appears to activate the rho GTAPase at least three orders of magnitudes more efficiently than the recombinant p190C under the present assay conditions, although we do not know if such a difference in the catalytic activity is present between the native two GAPs in the cell.

We did not detect GAP activity for Ser77-rhoA mutant in a homogenate of mouse fibroblasts (data not shown), suggesting that p190 is not the major GAP in the homogenate. Because p190 interact with other signaling molecules such as ras-GAP (9), it is likely that its GAP activity is enhanced by these molecules. McGlade et al. (35) recently reported that p190 makes a complex with the amino-terminal fragment of ras-GAP and that overexpression of this fragment in Rat-2 fibroblasts induces a phenotypic change, which could be explained by increased cellular rho-GAP activity. It, therefore, remains an interesting issue in what physiological context: GAP2 and p190 work distinctively in the cell, and Glu69 and Ser77 mutants of rhoA could serve as specific tools to examine these conditions.

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**REFERENCES**