Mutational and Kinetic Analyses of the GTPase-Activating Protein (GAP)-p21 Interaction: The C-Terminal Domain of GAP Is Not Sufficient for Full Activity

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The GTPase-activating protein (GAP) stimulates the GTPase reaction of p21 by 5 orders of magnitude such that the kcat of the reaction is increased to 19 s–1. Mutations of residues in loop L1 (Gly-12 and Gly-13), in loop L2 (Thr-35 and Asp-38), and in loop L4 (Gln-61 and Gln-63) influence the reaction in different ways, but all of these mutant p21 proteins still form complexes with GAP. The C-terminal domain of the human GAP gene product, GAP334, which comprises residues 714 to 1047, is 20 times less active than full-length GAP on a molar basis and has a fourfold lower affinity. This finding indicates that the N terminus of GAP containing the SH2 domains modifies the interaction between the catalytic domain and p21.

p21 proteins are the products of the N-, K-, and H-ras genes. They are small guanine nucleotide-binding proteins that are believed to function as signal switch molecules: in the active GTP-bound state, they are switched on; in the GDP-bound state, they are switched off. p21 has an intrinsic GTPase activity, the rate of which determines the lifetime of the active state (5, 10). Mutated forms of the ras genes have been identified in ca. 30% of human tumors. These ras oncogenes code for p21 proteins with point mutations in only three residues: Gly-12, Gly-13, and Gln-61. The mutated proteins have a reduced GTPase activity (5, 8, 9). The GTPase-activating protein (GAP) has been identified by its property of stimulating the conversion of normal p21-GTP to p21-GDP in Xenopus oocytes (32, 41). It has been cloned and sequenced and has a molecular mass of 120 kDa. The fact that it does not stimulate the GTPase activity of oncogenic mutants can explain the transforming properties of those mutants (18, 41, 42, 44).

The p21-GAP interaction has not been investigated in detail, and the stimulation of the GTPase by GAP was estimated to be 200-fold (41). By mutational analysis, it has been established that loop L2 of p21 is involved in the interaction with GAP (1, 13, 18, 32). From the three-dimensional structure, it would appear that loop L4 could also be involved in this interaction (33, 36, 37, 40). It has also been assumed that the C-terminal domain of GAP is sufficient for the GTPase stimulatory activity (44). We have reevaluated the properties of GAP and show that the maximal stimulation of the GTPase activity by GAP is 102-fold and that mutations in loops L1, L2, and L4 affect the p21-GAP interaction in different ways. We also show that the C-terminal domain of GAP is not sufficient for full activating activity and is apparently modulated by the SH2 (src homologous) domains of GAP.

MATERIALS AND METHODS

Protein preparations. p21 mutants were generated in M13 vectors by using the Eco-Pst fragment of ptcas (25, 43). After mutagenesis and sequencing of the mutated sequence, the Eco-Pst fragment was cloned back into the expression vector ptcas. p21 proteins were purified as described earlier (25, 43).

Spodoptera frugiperda sf9 insect cells containing the recombinant full-length GAP made by using the Autographa californica nuclear polyhedrosis virus baculovirus expression system (28) were a gift from F. McCormick. Preparation was done partly as described elsewhere (21). The sf9 insect cells were thawed and homogenized with a Dounce homogenizer in 20 mM sodium phosphate (pH 6.5) containing 1 mM EGTA (ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid), 1 mM dithiothreitol, 200 μM phenylmethylsulfonyl fluoride (PMSF), 2 μg of leupeptin per ml, and 1 μg of pepstatin A per ml. After centrifugation of the insoluble material at 100,000 × g for 30 min, the solution was applied to an S-Sepharose fast-flow column (60 ml) with a flow rate of 5 ml/min and eluted with a 2 × 250-ml linear gradient from 0 to 0.6 M NaCl in the same buffer. Active GAP fractions eluting between 150 and 200 mM NaCl were precipitated with 60% ammonium sulfate. The pellet was dissolved in buffer B (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, 200 μM PMSF, 1 μg of leupeptin per ml, 1 μg of pepstatin A per ml) and applied to a Sephadex G-150 superfine gel filtration column (1.6 by 90 cm) equilibrated with the same buffer. Active fractions were concentrated by ultrafiltration and dialyzed against buffer B without NaCl. Final purification was achieved on an FPLC Mono-Q column with a linear gradient of 0 to 600 mM NaCl. Purity at each step was controlled by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and by GAP assays (see below). The purified protein in buffer B containing 10% glycerol can be stored at 4°C for several months without loss of activity.

GAP334 extending from amino acids 714 to 1047 was expressed in Escherichia coli, using the vector ptri99A containing the trc promoter (2). For purification, 10 g of E. coli}
coli cells was lysed with lysozyme and deoxycholate in 20 mM sodium phosphate buffer (pH 7.5)–0.5 mM PMSF–5 mM dithioerythritol (DTE)–1 mM EDTA. The cleared lysate was titrated with NaH₂PO₄ to pH 6.0 and applied to an S-Sepharose column (1.3 by 11.3 cm) equilibrated in 20 mM phosphate–1 mM EDTA–1 mM DTE (pH 6.0) and eluted with a linear gradient (2 × 200 ml) from 0 to 0.4 M NaCl. Pooled fractions containing GAP activity were brought to pH 7.4 with Na₂HPO₄ and applied to a RedA dye matrix (Amicon Corp.) column (1.3 by 7.3 cm). GAP334 was eluted with a linear gradient (2 × 150 ml) from 0 to 1 M NaCl in the same buffer. Appropriate fractions were pooled, dialyzed against 50 mM Tris-HCl–1 mM EDTA–5 mM DTE–10 mM NaCl (pH 8.0), and purified by Q-Sepharose (column, 0.7 by 18.6 cm) with a 2 × 100-ml linear gradient from 10 to 250 mM NaCl. The protein was stored in 10 mM Tris-HCl (pH 7.5)–5 mM DTE–1 mM EDTA.

GAP334 expressed from a recombinant baculovirus vector in insect cells was homogenized in sodium phosphate buffer (pH 6.0) and purified with S-Sepharose and RedA dye matrix as described for the GAP334 from E. coli. It was approximately 90% pure.

**GTPase and guanine nucleotide dissociation.** GTPase measurements were done as described previously (25) by loading the protein with [γ-³²P]GTP, incubating it at 37°C in standard buffers (64 mM Tris-HCl [pH 7.6], 5 mM dithiothreitol, 10 mM MgCl₂, 1 mM sodium azide), and monitoring the production of radiolabelled Pᵢ. The dissociation of guanine nucleotides was measured by loading the protein with ³H-labelled nucleotide, incubating it with a large excess of unlabelled nucleotide and monitoring the release of radiolabelled nucleotide on nitrocellulose filters as described previously (25, 43).

**GAP stimulation of p21 GTPase activity.** Complexes between p21 and [γ-³²P]GTP were formed by incubating the proteins with the radioactive nucleotide in the presence of 1 mM EDTA–64 mM Tris-HCl (pH 7.5)–1 mM DTE. Excess nucleotide was removed by gel filtration on a small G-25 commercial column (NAP-5; Pharmacia LKB). The protein was eluted from this column with GAP reaction buffer (20 mM N₂H₂-hydroxyethylpiperazine-N’-2-ethanesulfonic acid [HEPES]-NaOH [pH 7.5], 1 mM EDTA). The GTPase reaction was started by addition of the appropriate amount of recombinant human GAP in the presence of 2 mM MgCl₂. The GTPase reaction was measured as described previously (17, 18) by monitoring the decrease of [γ-³²P]GTP bound to p21 by filtration of the reaction mixture through nitrocellulose filters (0.45 µm). The decrease of radioactivity with time was fitted by a single-exponential decrease function, using the program Enzfit (Elsevier Biosoft). The initial linear portion of the curve was fitted as the initial rate of the reaction. The error of those measurements lies between 10 and 20%. For the determination of Kᵣ and kᵣ values, the initial GTPase rates measured with increasing concentrations of p21 were fitted directly to the Michaelis-Menten equation by using Enzfit.

**Inhibition of p21-GAP interaction.** Complexes of p21 with the noncleavable triphosphate analog GppNHp were prepared as described above for p21-[γ-³²P]GTP. GTPase activity was measured as described above in the presence of increasing concentrations of the inhibitor. The data were plotted as fractional activity, which is V₀₋V₀/V₀ max–V₀ versus inhibitor concentration. V₀ max is the maximal GAP-stimulated GTPase activity under these conditions, and V₀ is the GTPase activity in the absence of GAP. From these curves, the Kᵣ values were obtained as concentrations of p21-GppNHp at which 50% of the GAP-stimulated GTPase is inhibited.

**Biological activity of p21 in PC12 cells.** The pheochromocytoma cell line PC12 was grown under standard conditions with Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum, 5% fetal calf serum, and antibiotics. Approximately 5 × 10⁴ cells were collected by centrifugation in an Eppendorf tube; 40 µl of a protein solution containing 3 mg of p21 per ml in standard buffer was added to the cells. The protein was introduced into the cytoplasm by pushing the cells through a yellow pipette tip held at the bottom of the tube. This creates enough pressure to slightly rupture the cell membrane so that the protein can enter the cell. The cells were incubated again in standard medium in a petri dish, and neurite outgrowth was then monitored under the microscope approximately 50 to 70 h after loading of the cells.

**RESULTS**

**Purification and catalytic properties of GAP.** Recombinant full-length GAP was expressed in s9 insect cells harboring a recombinant baculovirus (21) and was purified by using a three-column procedure similar to that described previously (21) (Fig. 1; Table 1). With the standardized assay that we employ, which uses 1 µM p21-[γ-³²P]GTP and an at least 100-fold-lower concentration of GAP, and by analyzing only the initial, linear part of the hydrolysis reaction, we obtain a specific activity for purified GAP of 1,800 U/mg of protein. One unit is the amount of protein that catalyzes the GTPase activity of 1 nmol of GTP bound to p21 in 1 min at 25°C. Under these conditions, the analysis is linear for the amount of p21 and GAP. The specific activity of recombinant human GAP from insect cells has been reported previously to be 20 U/mg, using 18 nM p21 (21). Assuming a linear relationship, this corresponds to 1,100 U/mg, which is comparable to our specific activity.

It has been reported that the in vitro GTPase activity of wild-type p21, which proceeds at a rate of 0.028 min⁻¹ at 37°C (25), is accelerated 100- to 200-fold by GAP, as measured under non saturating conditions (41). To determine the maximally stimulated GTPase activity, we saturated a fixed amount of GAP (2.5 nM) with increasing concentrations of...
p21-GTP and measured initial rates of GTPase activity. The data were treated as for an enzyme-substrate reaction (although the catalytic center of the reaction is on p21, and not on GAP), which gives the $V_{\text{max}}$ or $k_{\text{cat}} (= V_{\text{max}}/E_0)$ and $K_m$ for the GAP-stimulated GTPase reaction. Figure 2A shows the results of this titration, from which we obtain a $K_m$ value of 9.7 μM and a $k_{\text{cat}}$ of 19 s⁻¹ at 25°C. Since the rate constant of the intrinsic GTPase is $1.2 \times 10^{-4}$ s⁻¹ under these conditions, GAP accelerates the GTPase activity of p21 by more than 5 orders of magnitude. The specificity factor $k_{\text{cat}}/K_m$ is $1.9 \times 10^6$ mol⁻¹ s⁻¹, a typical value for an enzyme-catalyzed reaction. Since the Michaelis constant $K_m$ is equal to the affinity between enzyme and substrate only in the case of a fast equilibrium between substrate and enzyme, we have compared it with the dissociation constants between GAP and the p21-GppNHp (4.8 μM) and the p21-GppCH2p (2.3 μM) complexes as obtained by inhibiting the GAP-stimulated GTPase activity with those complexes. The p21-triphosphate analog complexes were assumed to be true competitive inhibitors of p21-GTP, but this has not been proven rigorously.

Truncated p21. The three-dimensional structure of truncated p21 (residues 1 to 166) (33, 36, 37, 40) has been solved, but no crystals suitable for X-ray crystallography could be obtained from the full-length p21 protein. We tested whether the removal of 23 amino acids from the C terminus affects the interaction with GAP. Since p21C- (=p21[1-166]) does not bind to nitrocellulose filters, we are not able to measure quantitatively the GAP-stimulated GTPase (25). Instead, we measured the affinity between GAP and p21C- by the inhibition assay described above. The affinity of p21C-GppNHp was found to be 6.5 μM, very similar to the value for normal p21. This finding supports our earlier notion that none of the biochemical properties of p21 is affected by the C-terminal truncation. Similar results have been obtained for smg p21B, the product of the rap1B gene, for which the tryptic removal of the C terminus does not affect the interaction with the smg p21 GTPase-activating protein (rap1-GAP) (22).

Loop L1 mutants. We also determined the affinities of several oncogenic versions of p21 with mutations of Gly-12 and Gly-13. As shown in Table 2, we find that G12V and G12R have lower affinities than does wild-type p21. The relative affinity of G12V but not the absolute value is in line with earlier observations (44).

Mutations of Gly-13 to Val or Asp were first found in human acute myeloid leukemia (9), and many more ras oncogenes with various Gly-13 mutations have since been found in various tumors. It has been shown that the mutation of Gly-13 to Val but not Ser renders p21 oncogenic (15). The biochemical properties of Gly-13 mutants have not been investigated, and it would be interesting to determine whether the interaction with GAP can be correlated with their transforming ability. Table 3 shows the nucleotide dissociation and GTPase rates of p21(G13V) and p21(G13S). It is obvious that the G13V mutation has a more pronounced affect on the properties of p21.

We find that GAP activates the GTPase of p21(G13S) but does not activate p21(G13V). The $K_m$ (21.6 μM) and $k_{\text{cat}}$ (0.5 s⁻¹) values for the interaction between p21(G13S) and GAP, measured under saturating conditions as described above for the wild type, are higher and lower, respectively, than those for wild-type p21. The GTPase stimulation is probably efficient enough to significantly reduce the amount of p21(G13S)-GTP in the steady state, since p21(G13S) is found to not be transforming (5). This seems to support the dogma that the concentration of p21-GTP in the cell is a measure of its transforming ability.

Mutants of the effector region. It has been reported that mutations in the effector region of p21 consisting of loop L2

### TABLE 1. Preparation of human recombinant GAP and GAP334

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length GAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>1,240</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>S-Sepharose</td>
<td>35</td>
<td>16,345</td>
<td>467</td>
</tr>
<tr>
<td>G-150</td>
<td>9</td>
<td>10,998</td>
<td>1,222</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>3</td>
<td>5,499</td>
<td>1,833</td>
</tr>
<tr>
<td>GAP334</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>589</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>S-Sepharose</td>
<td>67</td>
<td>2,010</td>
<td>300</td>
</tr>
<tr>
<td>Red A dye</td>
<td>64</td>
<td>1,700</td>
<td>276</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>45</td>
<td>1,490</td>
<td>332</td>
</tr>
</tbody>
</table>

* One unit of activity is defined as the amount of GAP that catalyzes the GTPase reaction of 1 nmol of p21-GTP per min under the conditions used (1 μM p21-GTP, 25°C; see Materials and Methods).

* ND, the GAP activity in crude extracts cannot be reliably determined.

FIG. 2. Michaelis-Menten kinetics of the p21-GAP interaction. GTPase initial reaction rates were measured with a constant amount of either full-length human GAP (A) or GAP334, the C-terminal truncated version (B), and increasing concentrations of p21-GTP. By nonlinear fitting, we obtain $K_m$ and $k_{\text{cat}}$ values as indicated on the corresponding axes.
whether these mutants physically interact with GAP. We measured the affinity of p21(D38E) and p21(D38A) by inhibiting the GAP-mediated GTPase activity of wild-type p21. Table 2 shows that these two p21 mutants have almost wild-type affinity to GAP, but their GTPases are not activated. p21 with a Thr-35→Ala mutation has recently been found not to be activated, and we find the affinity to GAP to be several times lower than that of wild-type p21 (100 μM); however, these results may reflect a rearrangement of the protein structure around the Mg2+ binding site, since Thr-35 is a ligand of Mg2+. Many more mutants of the effector regions have not been shown to not be activated by GAP. However, their affinities to GAP have not been measured, and it is not unlikely that some of these mutants also bind to it. From the experiments with the Asp-38 mutants, we may conclude that the effector region is more involved in the stimulation of the GTPase activity than in the binding of p21 to GAP.

Loop L4 mutants. Recently, we have postulated that Gln-61 in the high-resolution three-dimensional structure of p21 is directly involved in GTP hydrolysis by activating a water molecule that is responsible for nucleophilic displacement (37). We have also shown that when Glu-61 is in a conformation to activate the nucleophilic water, the Glu-63 side chain is close enough to the Gln-61 side chain for a possible assistance in the catalytic reaction. The involvement of loop L4 has also been proposed on the basis of deletion mutants of p21 (27). Residues 61 to 63 were mutated in order to investigate their involvement in the p21-GAP interaction, and their interactions with nucleotides and GAP are summarized in Tables 2 and 3. It appears that all mutations of Glu-61 increase the affinity between p21-GTP and GAP, as shown in Table 2, although p21(Q61L) has by far the highest affinity. This relatively high affinity of the Q61L mutation has been noted before (44), but p21(Q61H) also has a high affinity. The GTPase of the Q61L mutant is assumed to be not activated because Glu-61 is necessary for the catalytic reaction. With Q61H, we find a slight activation of the GTPase activity by GAP, which confirms a recent observation (6). This finding supports the notion that His-84 in E. coli EF-Tu has a function similar to that of Gln-61 for p21.

The E62H mutation changes the dissociation rate constant of GTP but not that of GDP, and it also decreases its intrinsic

### TABLE 2. Characteristics of the interaction of wild-type and mutant p21 with GAP

<table>
<thead>
<tr>
<th>p21 protein</th>
<th>GAP activation</th>
<th>GAP affinity (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt*</td>
<td>++ +</td>
<td>4.8</td>
</tr>
<tr>
<td>wt(1–166)</td>
<td>++ +</td>
<td>6.5</td>
</tr>
<tr>
<td>G12V</td>
<td>–</td>
<td>20.0</td>
</tr>
<tr>
<td>G61H</td>
<td>+</td>
<td>35.0</td>
</tr>
<tr>
<td>G61L</td>
<td>–</td>
<td>5.0</td>
</tr>
<tr>
<td>E62H</td>
<td>++ +</td>
<td>0.5</td>
</tr>
<tr>
<td>E62H</td>
<td>++ +</td>
<td>1.0</td>
</tr>
<tr>
<td>G13V</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>G13S</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>D38E</td>
<td>–</td>
<td>7.5</td>
</tr>
<tr>
<td>D38A</td>
<td>–</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* wt, wild type.

* ND, not determined.

### TABLE 3. Kinetic constants of new p21 mutants at 37°C, 10 mM Mg2+

<table>
<thead>
<tr>
<th>Protein</th>
<th>k_{diss,GDP}</th>
<th>k_{diss,GTP}</th>
<th>GTPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21c</td>
<td>0.0079</td>
<td>0.005</td>
<td>0.028</td>
</tr>
<tr>
<td>E62H</td>
<td>0.009</td>
<td>0.005</td>
<td>0.011</td>
</tr>
<tr>
<td>E63H</td>
<td>0.004</td>
<td>0.004</td>
<td>0.001</td>
</tr>
<tr>
<td>G13V</td>
<td>0.013</td>
<td>0.03</td>
<td>0.013</td>
</tr>
<tr>
<td>G13S</td>
<td>0.023</td>
<td>0.005</td>
<td>0.032</td>
</tr>
</tbody>
</table>

* k_{diss}, dissociation rate constant.

GTase (Table 3). The GAP-stimulated GTPase is not affected by the Glu-62→His mutation. This is surprising because E62 is totally conserved in all small guanine nucleotide-binding proteins, and no functional or structural role has yet been assigned to this residue. For E63H, we find that the nonstimulated as well as the GAP-stimulated GTP hydrolysis is affected. The nonstimulated GTPase is reduced by a factor of 2.6. For a fixed GAP concentration, the GTPase stimulation of the mutant is threefold lower than for wild-type p21. The nucleotide binding properties (Table 3) are also slightly changed, which is surprising considering that Glu-62 and Glu-63 do not contribute to nucleotide binding in the three-dimensional structure of p21 in the GDP and the GTP conformations (33, 36, 37, 40).

Since E62H and E63H have different reactivities toward GAP, it was of interest to measure their biological properties. This was done by pressure loading the proteins into PC12 cells. Figure 3 shows that p21(E63H) but not p21(E62H) induces neurite outgrowth in PC12 cells. The potency in this assay (3% of cells show neurite outgrowth) is lower than that of p21(G12V) (5%). Wild-type protein and p21(E62H) show no induction of differentiation under these conditions. This means that p21(E63H) is also transforming, since the induction of neurite outgrowth and fibroblast transformation are apparently correlated (38).

The C-terminal domain of GAP, GAP334. It has been reported that the C-terminal domain of GAP displays full GTPase-activating activity toward p21, suggesting that the SH2 domain and the GTPase-activating domain act independently of each other (30). This analysis has been done by using lacZ-fusion constructs with various fragments of bovine GAP. We have obtained the nonfusion catalytic subunit of human GAP, GAP334, representing amino acids 714 to 1047, as a soluble protein from E. coli containing an expression system based on the ptc99A vector (2). The protein is purified from the soluble extract by using a three-column procedure as outlined in Table 1 and Fig. 1. The purified protein has a specific activity of 330 U/mg under the conditions outlined above. This is sixfold lower than the activity of full-length GAP on a milligram basis and 20-fold lower on a molar basis. Figure 2B shows that the decreased activity is due in part to a different $K_m$ value (19 μM) which is twofold lower for GAP334 and in part to a different $k_{cat}$ (4.2 s⁻¹).

We also investigated whether the dissociation constant between GAP334 and p21-triphosphate complexes is reduced similarly to the enzymatic properties. The affinity between GAP334 and p21-GppNHp is 21 μM. The dissociation constant of the most tightly bound mutant p21(061L) is also increased to approximately 5 μM. This finding shows that the characteristics of interactions between p21 and GAP are only qualitatively preserved.

Since the full-length GAP was prepared from insect cells
mechanism for the uncatalyzed GTP hydrolysis reaction of p21 has been proposed: the nucleophilic water which attacks the \( \gamma \)-phosphate is activated by the main chain carbonyl of Thr-35 and the side chain of Gln-61 (37). Two mechanisms have been proposed for the role of GAP in stimulating the GTPase reaction (11). In one, p21 has the catalytic machinery for a fast GTP hydrolysis, and the role of GAP would be to put or keep p21 in a GTPase-competent conformation. In the other mechanism, GAP would change the catalytic center of p21 by supplying additional components necessary for a fast cleavage reaction. Our results do not favor either mechanism, and additional structural and kinetic work is necessary to explain the very fast reaction of guanine nucleotide-binding proteins in the presence of effectors.

Our results also show that the affinity between p21 and GAP is very sensitive to the sequence of amino acids in positions 61 and 63 in loop L4. The exchange of Gln-61 for Leu causes the most drastic increase in affinity. The dissociation constant of the Q61L mutant (0.1 \( \mu \text{M} \)) is similar to that for the complex between GAP and the product of the \( \text{rap}1A \) gene (50 nM), also called Krev-1 (26). This protein has been shown to be an inhibitor of the GAP-stimulated GTPase of p21 and to inhibit cellular transformation induced by oncogenic p21 (17, 26). The fact that a terminally truncated RAS1 (the homolog of p21 in yeast cells) with a Q68L mutation can inhibit the mammalian oncogenic p21(G12V)-induced germinal vesicle breakdown in oocytes thus supports the idea that the Krev-1-induced inhibition of transformation functions via the inhibition of GAP (19). This explanation assumes that GAP is the downstream effector molecule of p21. If it is not, one could assume that Krev-1 inhibits the interaction with the real effector of p21.

It has been reported that the C terminus of GAP, which is homologous to regions of the yeast GAP-like proteins IRA1, IRA2, and Gap1 and the neurofibromatosis gene product NF1 (4, 12, 23, 31, 45, 46), is sufficient for the GTPase-activating activity of the protein (30). Using purified full-length and truncated GAP protein, we have shown here that the C-terminal domain is 20-fold less active than the full-length protein and that the affinity is 3- to 4-fold lower. Our results are apparently in contradiction to those reported recently by Skinner et al. (39), who find activities for truncated GAP to be similar to those reported for full-length GAP (21). Correcting their specific activity for the same amount of p21 used in our assay, one finds that their specific activity, determined under somewhat different conditions, is qualitatively similar to that found for GAP334. The various results for the activity of GAP stress the importance of using similar conditions because it is known that the GAP-stimulated GTPase reaction is very sensitive to buffer conditions.

Our results imply that the N-terminal part of GAP, which consists of two so-called SH2 domains interrupted by a so-called SH3 domain, modulates the enzymatic activity of GAP. This also implies that the interactions of GAP with various other proteins such as hormone receptor tyrosine kinases or Src, which take place via the SH2 domain (3, 34, 35), should be modulated by p21. We can thus postulate that the N-terminal part of the protein somehow senses the presence of p21, as depicted in the structural model shown in Fig. 4. The model shows schematically the interaction between loops L1, L2, and L4 of p21 and the C terminus of GAP, which represents an independent structural domain as shown by protease digestion experiments (27a). Since the SH2 domains as Trp-fusion proteins retain the ability to mediate the association of GAP with activated growth factor receptors, the N terminus of GAP could also represent one
or more structural domains (34). The proposed interaction between the two (or more) domains of GAP and p21 supports the notion that the interaction of tyrosine kinases and GAP and the phosphorylation may somehow modify the p21-GAP interaction.

Two recent observations support the notion that the N-terminal region regulates the GAP catalytic domain. It has been reported that GAP inhibits the coupling of muscarinic receptors to atrial K+ channels and that the effect of GAP is dependent on the interaction with p21 (47). In further experiments, it has recently been shown that the SH2 and SH3 domains of GAP alone have the same inhibitory effect, which, however, is no longer dependent on the interaction with p21 (32a). The transformation of NIH 3T3 cells by the activated c-src<sup>257P</sup> was shown to be inhibited by overexpression of GAP. In that same assay, the C terminus (residues 685 through 1047) of GAP was found to be more active than the full-length protein (14). Since it is postulated that p21 functions downstream of src and that overexpression of GAP reverses transformation due to the down-regulation of p21-GTP, those results imply that the N terminus of GAP modulates the activity of the GTPase-activating domain. At least the latter experiment shows an inhibitory effect of the SH2/SH3 domain(s) on the GTPase-activating domain of GAP, which is opposite to its effect on the GTPase-activating reaction reported here. How these contradictory observations on the mutual effects of the domains of GAP can be correlated must be clarified by further experiments.

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REFERENCES


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