Activation of R-Ras GTPase by GTPase-activating Proteins for Ras, Gap1m, and p120GAP

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The enzymatic properties of Gap1m were characterized using three Ras and R-Ras proteins as substrates and were compared with those of p120GAP. Gap1m stimulated the GTPase of Ras better than that of R-Ras, in contrast to p120GAP which promoted the GTPase of R-Ras better than that of Ras. The EC50 values of Gap1m for Ha-Ras and R-Ras were 0.48 ± 0.02 and 1.13 ± 0.12 μM, respectively, whereas the EC50 values of p120GAP for Ha-Ras and R-Ras were 23.1 ± 1.9 and 3.86 ± 0.38 μM, respectively. The affinities of Gap1m and p120GAP to the substrates determined by competitive inhibition by using Ha-Ras-GTP-γS (guanosine 5′-O-(3-thiotriphosphate)) or R-Ras-GTP-γS as a competitor agreed well with the substrate specificities of these GTPase-activating proteins. The Km values of Gap1m for Ha-Ras and R-Ras were 1.53 ± 0.27 and 3.38 ± 0.53 μM, respectively, which were lower than that of p120GAP for Ha-Ras (145 ± 11 μM) by almost 2 orders of magnitude. The high affinity of Gap1m to the substrates and its membrane localization suggest that Gap1m may act as a regulator of the basal activity of Ha-Ras and R-Ras.

Ras proteins (N-Ras, Ha-Ras, Ki-Ras) are three closely related members of the Ras family which act as a molecular switch for signal transduction pathways to control cell growth and differentiation (1). Like other guanine nucleotide-binding proteins, Ras cycles between an active GTP-bound form and an inactive GDP-bound form. The GDP-bound form is converted to the GTP-bound form through a GDP/GTP exchange reaction that is facilitated by guanine nucleotide-releasing factors (2). On the other hand, the GDP-bound form is converted to the GTP-bound form by the intrinsic GTPase activity, which is accelerated by GTPase-activating proteins (GAPs)1.(3).

R-Ras is a member of the Ras family proteins and is highly homologous to Ras (4). Despite a high sequence similarity between Ras and R-Ras, R-Ras does not transform Rat1 fibroblastic cells (5). However, recent results have demonstrated that the activated form of R-Ras transforms NIH 3T3 cells, and the transformant forms tumors in athymic nude mice (6, 7). Since R-Ras has an effector binding domain the amino acid sequence of which is very similar to that of Ras, R-Ras binds to and activates the c-Raf-1/mitogen-activated protein kinase cascade (6, 8). Besides its roles in the stimulation of cell prolifer-

atation, R-Ras may play other roles in different cell biological processes. It is reported that R-Ras binds to Bcl-2, which is a key molecule controlling the process of apoptosis; however, the binding to Bcl-2 is not GTP-dependent (9). It was also reported that R-Ras promotes apoptosis induced by growth factor deprivation by a mechanism that is suppressed by overexpression of Bcl-2 (10). Recently it has been described that R-Ras enhances cell adhesion to extracellular matrix substrates through the activation of integrins (11). However, the biochemical mechanisms by which R-Ras activity is regulated are still to be clarified.

Three mammalian GAPs for Ras have been identified so far. p120GAP, which was first described, is a prototype of this class of proteins (12). Besides a catalytic domain that stimulates Ras GTPase, p120GAP has two SH2 (Src homology 2) domains, one SH3 domain, one PH (plekstrin homology) domain, and one phospholipid binding domain (13). The second is neurofibromin (NF1), a product of the neurofibromatosis type I gene (14). Neurofibromin has a region that shows a sequence similarity to the catalytic domain of p120GAP and Ira proteins of Saccharomyces cerevisiae, and the domain was termed as a GAP-related domain. Indeed this region was shown to possess GAP activity for Ras and to suppress iro2 mutation (15). We have isolated the third Ras GAP (Gap1m) which is a mammalian homolog of the Drosophila Gap1 gene (16). In addition to the GAP-related domain, Gap1m has two putative phospholipid binding domains and a region similar to the domain unique to Btk tyrosine kinase (16).

Recently R-Ras GAP, the entire structure of which is closely related to Gap1m, was isolated (17). The identity of the amino acid sequences of R-Ras GAP and Gap1m is 60%. Despite the high sequence similarity to Gap1m, R-Ras GAP stimulates the GTPase of R-Ras better than that of Ras (17). A previous study described that p120GAP stimulates the GTPase of R-Ras as efficiently as Ha-Ras (18). We showed that Gap1m stimulates the GTPase of the wild type of Ras but not that of the activated form of Ras, Rap1, or GAP-binding proteins of other families (19). Hence we investigated in this study the enzymatic properties of Gap1m and compared them with those of p120GAP using Ras and R-Ras proteins as substrates. The results indicate that both Gap1m and p120GAP promote the GTPase of R-Ras and that Gap1m stimulates the GTPase of Ras better than that of R-Ras, in contrast to p120GAP the activity of which is higher with R-Ras as the substrate.

EXPERIMENTAL PROCEDURES

Preparation and Purification of Ras Family Proteins—The pGEX expression vectors for the Ras family proteins (17) were generously provided by Dr. K. Kaibuchi. The N-Ras expression system was a kind gift from Dr. A. Wittinghofer. Glutathione S-transferase (GST)-Ras fusion proteins were induced in Escherichia coli and purified using glutathione-Sephose 4B (Pharmacia Biotech Inc.) as described by Smith and Johnson (20). Fusion proteins were dialyzed overnight against 100 volumes of buffer A (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% (v/v)

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Enzymatic Properties of Gap1m

**Fig. 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Gap1m and p120GAP. Purified Gap1m and p120GAP (200 ng each) were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel was stained with SYPRO Orange stain kit. Molecular masses of standard proteins (M) in kDa are shown on the left of the gel. Lane 1, Gap1m; lane 2, p120GAP.

**Fig. 2.** Dose-dependent effect of Gap1m and p120GAP on the GTPase activity of Ras family proteins. [γ-32P]GTP-loaded Ras family proteins (100 nM each) were incubated at 30 °C for 10 min with indicated amounts of Gap1m (panel A) and p120GAP (panel B). The value of control sample without added GAP which was kept on ice was taken as 100%. The data were plotted as relative values to the control sample. The substrates used were N-Ras (△), Ha-Ras (●), Ki-Ras (▲), and R-Ras (○).

Ha-Ras-GTPyS, Ha-Ras-GDP, R-Ras-GTPyS, or R-Ras-GDP at 30 °C for 10 min. The GAP activity observed with a control sample without added competitor was taken as 100%, and the GAP activity of each sample was expressed as a percentage of the control sample.

**RESULTS**

**Catalytic Activity of Gap1m and p120GAP toward Ras Family Proteins**—We identified and isolated Gap1m as the third GAP for Ras (16, 19). Since the entire structure of Gap1m is not similar to that of p120GAP or neurofibromin, we deduced that Gap1m may have distinct enzymatic properties. To investigate the substrate specificities of Gap1m and p120GAP, various concentrations of either Gap1m or p120GAP were incubated with each of the four Ras family proteins, and the enzyme concentration at which 50% of the maximal stimulation was achieved (EC50) was determined (Fig. 2). Gap1m showed a similar effect on the GTPase activities of three Ras proteins (EC50 values for N-Ras, Ha-Ras, and Ki-Ras were 0.56 ± 0.09, 0.48 ± 0.02, and 0.46 ± 0.04 nM, respectively). However, Gap1m was less effective in stimulation of the GTPase of R-Ras (EC50 1.13 ± 0.12 nM). In contrast, p120GAP exhibited higher activity in stimulation of the GTPase of R-Ras (EC50 3.86 ± 0.38 nM) than those of three Ras proteins (EC50 values for Ki-Ras, N-Ras, and Ha-Ras were 12.5 ± 0.9, 23.1 ± 1.9, and 22.7 ± 1.5 nM, respectively). A similar tendency of substrate specificities of Gap1m and p120GAP was also observed when time course experiments were carried out using the four Ras family proteins as substrates (Fig. 3). These data demonstrated that Gap1m stimulated GTPase activities of both Ras and R-Ras where Ras was activated higher than R-Ras. In contrast, p120GAP showed much higher activity toward R-Ras than Ras proteins under the same experimental conditions.

**Inhibition of GAP Activity by Ras Family Proteins Bound to GTPyS or GDP**—We next investigated whether there may be a relationship between the substrate preferences of Gap1m and p120GAP and their affinities to the substrates (Figs. 4 and 5). We measured the ability of Ha-Ras or R-Ras bound to either GTPyS (a nonhydrolyzable analog of GTP) or GDP to inhibit competitively the GAP activity of Gap1m (Fig. 4). The inhibition constant (Ki) is obtained as a concentration of the inhibitor at which 50% of GAP activity is inhibited (19). Ha-Ras-GTPyS exhibited more inhibitory activity on the inhibition of Gap1m (Ki = 0.83 ± 0.12 μM) than Ha-Ras-GDP (Ki = 3.27 ± 0.67 μM). R-Ras-GTPyS was also more effective in the inhibition of Gap1m activity (Ki = 2.00 ± 0.21 μM) than R-Ras-GDP (Ki = 4.31 ± 1.02 μM). In this analysis, the Ki of R-Ras-GTPyS for Gap1m activity was 2.5 times higher than that of Ha-Ras-GTPyS. This higher affinity of Gap1m to Ha-Ras-GTPyS...
was in good agreement with the substrate preference of Gap1m; the EC$_{50}$ of Gap1m for Ha-Ras was 2.5 times lower than that for R-Ras. The $K_i$ value of Ha-Ras-GDP was also lower than that of R-Ras-GDP.

A similar experiment was carried out using p120GAP (Fig. 5). The concentrations of the inhibitors were not enough for full inhibition of the activity. R-Ras-GTP$\gamma$S was most effective in the inhibition of p120GAP activity; however, the $K_i$ of R-Ras-GTP$\gamma$S was still over 10 $\mu$M. The effect of Ha-Ras-GTP$\gamma$S on the substrate preference of p120GAP was weaker than R-Ras-GTP$\gamma$S, indicating that R-Ras-GTP$\gamma$S bound to p120GAP more tightly than Ha-Ras-GTP$\gamma$S did. Neither Ha-Ras-GDP nor R-Ras-GDP was inhibitory on p120GAP activity at the concentrations tested. Although we could not determine the $K_i$ of Ha-Ras-GTP$\gamma$S, Vogel et al. (22) reported that the $K_i$ of Ha-Ras-GTP is 110 $\mu$M. Thus, the relative affinities of p120GAP to R-Ras and Ha-Ras again correlated well with the substrate preference of p120GAP. These results suggested that the substrate specificities of both Gap1m and p120GAP may be determined by their affinities to the substrates.

Kinetics of Interaction between GAPs and Their Substrates—To investigate further the enzymatic properties of Gap1m and p120GAP, we determined the kinetics of the GTPase of Ha-Ras and R-Ras stimulated by GAPs. A fixed amount of Gap1m or p120GAP was incubated with various concentrations of either Ha-Ras-$\gamma^{32}$P[GTP] or R-Ras-$\gamma^{32}$P[GTP], and the reaction rate of GTPase was determined (Fig. 6). Gap1m was much more active than p120GAP in the stimulation of the GTPase activity of Ha-Ras. In contrast, activation of the R-Ras GTPase by Gap1m was similar to that by p120GAP at low concentrations of R-Ras, and p120GAP was more active at high concentrations of R-Ras. Since the activity of p120GAP was not saturable under the experimental conditions, the activity of p120GAP for Ha-Ras might also be higher than Gap1m at very high concentrations.

The rate of GAP-stimulated GTPase was plotted as a function of substrate concentration according to the Michaelis-Menten equation which gave $k_{cat}$ ($V_{max}/E_0$) and $K_m$ for the reactions (Fig. 7). The $K_m$ values of Gap1m for Ha-Ras and R-Ras were 1.53 ± 0.27 and 3.38 ± 0.53 $\mu$M, respectively (Fig. 7A). This result indicated that Gap1m had a higher binding affinity to Ha-Ras than R-Ras which agreed well with the results presented in Fig. 4. At saturating concentrations of the substrates, Gap1m showed somewhat higher activity for R-Ras than for Ha-Ras ($k_{cat}$ values for Ha-Ras and R-Ras were 3.96 ± 0.31 and 4.96 ± 0.74 s$^{-1}$, respectively).

p120GAP shows 7.5 times higher activity for R-Ras than Ha-Ras at any of the substrate concentrations examined (Fig. 6). By fitting the data to a double reciprocal plot, the $K_m$ and the $k_{cat}$ values of p120GAP for Ha-Ras were determined to be 145 ± 11 $\mu$M and 23.0 ± 3.4 s$^{-1}$, respectively (Fig. 7B). Accurate values of the $K_m$ and $k_{cat}$ of p120GAP for R-Ras could not be determined because the specific activity was completely proportional to the substrate concentration. These results are summarized in Table I.

**DISCUSSION**

In this study we examined the enzymatic properties and substrate specificity of Gap1m and compared them with those of p120GAP. Previously we demonstrated that Gap1m does not stimulate the GTPase of Rap1, Rho, and Ral25K (19). Therefore, in this study we used three Ras proteins and R-Ras as the substrates.

Both Gap1m and p120GAP stimulated the GTPase of Ras and R-Ras. EC$_{50}$ values of Gap1m for Ras proteins were two times lower than that for R-Ras (Fig. 1 and Table I), thus Gap1m stimulates Ras GTPase better than that of R-Ras ($p < 0.01$). In contrast, the EC$_{50}$ of p120GAP for R-Ras was much lower than those for Ras proteins. Thus p120GAP activates the GTPase of R-Ras better than that of Ras. A previous report described that the catalytic domain of p120GAP (GAPette) stimulates the GTPase of both Ras and R-Ras with almost equal EC$_{50}$ values (18). However, it was described that domains of p120GAP outside of the catalytic domain are necessary for the full activity (23, 24). Since the p120GAP used in this study...
Enzymatic Properties of Gap1m

GAPs and Substrates $k_{cat}$ $K_m$ $K_{cat}/K_m$ $K_i$

|        | $s^{-1}$ | $\mu M$ | $nM$ | $\mu M$ |
|--------|----------|---------|------|---------|
| Gap1m  |          |         |      |         |
| Ha-Ras | 3.96 ± 0.31 | 1.53 ± 0.27 | 0.48 ± 0.02 | 0.83 ± 0.12 |
| R-Ras  | 4.96 ± 0.74 | 3.38 ± 0.53 | 1.13 ± 0.12 | 2.00 ± 0.21 |
| p120GAP|          |         |      |         |
| Ha-Ras | 23.0 ± 3.4 | 145 ± 11 | 23.1 ± 1.9 | 110^a |
| R-Ras  | ND       | ND      | 3.86 ± 0.38 | >10^a |

^a Determined by using Ha-Ras·GTP·S or R-Ras·GTP·S as a competitor.
^b Taken from Ref. 22.
^c Not determined.

Recently another Gap1m family termed R-Ras GAP, whose entire domain structure is very similar to Gap1m, was described (17). The overall identity of the amino acid sequence is 60%. R-Ras GAP also stimulates the GTPase of Ras, but the stimulation is lower than that observed with R-Ras. In contrast, a GAP-related domain of neurofibromin stimulates GTPase of Ras stronger than R-Ras GTPase (17). Thus, all the known Ras GAPs and R-Ras GAP activate the GTPase of both Ras and R-Ras, with some different substrate preferences. GAP1^HRAP (26) and GAP1^III (27) seem to be human and mouse homologs of R-Ras GAP, respectively, since both of them show higher sequence similarity to R-Ras GAP than to Gap1m. Residues in the switch I region of Ras which are critical to the stimulation by p120GAP have been extensively characterized (12). Within this region, the amino acid sequence of Ras from residue 32 to 40 is completely preserved in R-Ras and Rap1. However, although Rap1 binds to p120GAP, the GTPase of Rap1 is not stimulated by p120GAP (28). Whereas both position 31 of Ras (glutamic acid) and the equivalent position of R-Ras (aspartic acid) are acidic residues, Rap1 has lysine at the corresponding position. Substitution of the 31st glutamic acid of Ras by lysine renders the mutated Ras a phenotype like Rap1 with concomitant loss of susceptibility to p120GAP (29). These findings suggest that residue 31 of Ras or residues at the corresponding positions of R-Ras and Rap1 may be critical to their susceptibility to GAPs.

What may be the biological implication deduced from the enzymatic properties of Ras GAPs? The weak binding affinity of p120GAP to the substrates suggests that factors that help the association of p120GAP with the substrate may increase the activity of p120GAP toward the substrates. Yao and Cooper (30) reported that p120GAP with a membrane targeting signal showed higher specific activity in intact cells than p120GAP without the targeting signal. Since p120GAP binds to auto-phosphorylated receptors (31), such binding may help the local contact of p120GAP to its substrates located on the membranous structure. As pointed out by Bernarda (32), this implies that p120GAP may act as a quencher of growth factor-activated signals rather than a regulator of basal activity of Ras and R-Ras. In contrast, the affinity of Gap1m or neurofibromin with their substrates is rather high such that they may be able to stimulate the GTPase of the substrates efficiently by themselves. Some portion of Gap1m and neurofibromin resides in the membranous fraction (19, 33). Such membrane localization of neurofibromin and Gap1m may facilitate the contact of these GAPs with their substrates. Thus Gap1m may regulate the basal activity of Ras and R-Ras as does neurofibromin (32).

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FIG. 6. Michaelis-Menten kinetics of Gap1m and p120GAP. Gap1m (circles, 1.5 nM for Ha-Ras and 1.5 nM for R-Ras) or p120GAP (squares, 20.0 nM for Ha-Ras and 3.0 nM for R-Ras) was incubated with various concentrations of Ha-Ras·[γ-32P]GTP or R-Ras·[γ-32P]GTP for 10 min at 30 °C. The reaction rate at each substrate concentration is shown.

FIG. 7. Double-reciprocal plot of GAP activity of Gap1m and p120GAP. The data presented in Fig. 6 are fitted to a double-reciprocal plot of reaction rate and substrate concentration. Panel A, Gap1m activity toward Ha-Ras (C) and R-Ras (●). Panel B, p120GAP with Ha-Ras (□) and R-Ras (●).

lacks only 38 amino acid residues at the amino terminus, it seems to be an intrinsic property of p120GAP that it is more active for R-Ras than for Ras.

We also measured the inhibition constant ($K_i$) of Ras and R-Ras bound to GTP·S. The $K_i$ of Ras·GTP·S for Gap1m was 2.5 times lower than that of R-Ras·GTP·S, which agrees well with the ratio of $K_m$ values of Gap1m for Ras and R-Ras and also with the substrate specificity in that Gap1m stimulates GTPase of Ras better than that of R-Ras. Similarly, p120GAP promotes the GTPase of R-Ras better than that of Ras, and p120GAP binds more tightly to R-Ras than Ras. Hence, there may be a good correlation between the affinity of the GAPs to the substrates and their substrate preference.

The $K_m$ values of p120GAP and neurofibromin for Ras have been reported to be 9.7 and 0.3 μM, respectively (24, 25). Under our experimental conditions the $K_m$ of p120GAP for Ras was 145 μM. This difference in the $K_m$ values of these two p120GAP may be the result of different experimental conditions, i.e., temperatures, substrates, and the sources of p120GAP. The $K_m$ values of the two preparations are similar (19 s$^{-1}$ (24) and 23.4 s$^{-1}$ (this study)). A similar high $K_m$ value of full-length p120GAP for Ras has also been reported (23). The $K_m$ of Gap1m for Ras was 1.53 μM (Table I). Vogel et al. (22) described that Ras·GTP complex with nonradioactive GTP competes in the p120GAP-catalyzed reaction with the $K_m$ value of 110 μM. The $K_m$ of Ras·GTP·S for recombinant Gap1m is 0.83 μM (Table I), which is similar to the value obtained using authentic Gap1m (19). The difference between these $K_m$ values of the two GAPs agrees well with that between the $K_m$ values of p120GAP and Gap1m. Thus the affinity of p120GAP to the substrates is much weaker than that of Gap1m or neurofibromin by almost 2 orders of magnitude.
Enzymatic Properties of Gap1™

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