Role of Prenylation in the Interaction of Rho-Family Small GTPases with GTPase Activating Proteins†

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ABSTRACT: The role of prenylation in the interaction of Rho-family small GTPases with their GTPase activating proteins (GAPs) was investigated. Prenylated and nonprenylated small GTPases were expressed in *Sf9* insect cells and *Escherichia coli*, respectively. Nucleotide binding to and hydrolysis by prenylated and nonprenylated proteins were identical, but three major differences were observed in their reactions with GAPs. (1) Membrane-associated GAPs accelerate GTP hydrolysis only on prenylated Rac1 and RhoA, but they are inactive on the nonprenylated form of these proteins. The difference is independent of the presence of detergents. In contrast to Rac1 and RhoA, nonprenylated Cdc42 is able to interact with membrane-localized GAPs. (2) Full-length p50RhoGAP and p190RhoGAP react less intensely with nonprenylated Rac1 than with the prenylated protein, whereas no difference was observed in the reaction of isolated GAP domains of either p50RhoGAP or Bcr with the different types of Rac1. (3) Fluoride exerts a significant inhibitory effect only on the interaction of prenylated Rac1 with the isolated GAP domains of p50RhoGAP or Bcr. The effect of fluoride is not influenced by addition or chelation of Al^{3+}. This is the first detailed study demonstrating that prenylation of the small GTPase is an important factor in determining its reaction with GAPs. It is suggested that both intramolecular interactions and membrane targeting of GAP proteins represent potential mechanisms regulating Rac signaling.

Small GTPases participate in the regulation of a wide variety of cellular processes (*1*, *2*). They function as timed molecular switches shuttling between the GTP-bound active and the GDP-bound inactive state. Timing is achieved by the endogenous GTP hydrolytic activity, on one hand, and by several regulatory proteins (GTPase activating proteins, guanine nucleotide exchange factors, guanine nucleotide dissociation inhibitors) and some target molecules, on the other hand (*3*). GTPase activating proteins (GAPs)[1] accelerate the hydrolysis of GTP by small GTPases, thereby promoting the downregulation or cessation of the regulated process.

The molecular mechanism of interaction of GAPs with different small GTPases of the Ras and Rho subfamily has been subject of intensive investigations (*4*−*9*). Both X-ray diffraction analysis of crystallized proteins (*4*−*6*) and formation of stable complexes between small GTPases and their relevant GAPs in the presence of aluminum fluoride (*7*−*9*) suggest that GAPs provide essential residues to the formation of the transition state of the GTPase. The situation seems to be analogous to the α-subunits of heterotrimeric G proteins, where an arginine from the helical domain participates in the formation of the catalytic site and stabilization of the active conformation in the presence of aluminum fluoride, beryllium fluoride, or magnesium fluoride (*10*−*13*). Indeed, structural and mutagenesis studies specified critical arginines in the different GAP proteins (*5*, *6*, *14*, *15*), and complex formation between GAP and small GTPase could be detected in the presence of both aluminum fluoride (*8*, *9*) and magnesium fluoride (*16*, *17*). However, mutation of the critical arginine in p50RhoGAP results in a dramatic decrease of the catalytic activity without a significant change in the high-affinity binding to Cdc42 and RhoA (*14*, *15*), suggesting that, in addition to the arginine critical for the formation of the transition state (and hence for enhancement of the catalytic activity), other parts of the molecules may be important in determining the interaction of small GTPases with their regulatory protein GAP.

Most of the above studies concerned the small GTPases Ras (*5*, *7*, *8*, *17*), Cdc42 (*6*, *9*, *14*), and RhoA (*15*−*17*) whereas critical experiments never have been published on Rac. In addition, in all experiments the investigated small GTPase has been expressed in *Escherichia coli*; thus the nonprenylated form of the proteins has been studied. The possible role of the prenyl moiety in the interaction with GAP has not been raised although it plays a critical role in the interaction with GDI (*18*), and inhibition of prenylation has drastic effects on cell growth and cytoskeletal organization (*19*, *20*).

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[1] Abbreviations: GAP, GTPase activating protein; GDI, guanine nucleotide dissociation inhibitor; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PMN, polymorphonuclear granulocyte; LB, Luria−Bertani medium; BSA, bovine serum albumin; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropyl fluorophosphate; PAGE, polyacrylamide gel electrophoresis.

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The aim of the present study was to elucidate the role of prenylation in the interaction of the small GTPases of the Rho family with different GAPs. We show that (1) both the membrane environment and protein sequences outside of the GAP domain of p50RhoGAP and p190RhoGAP exert a restricting effect on the Rac−GAP interaction that can be disrupted by prenylated Rac and (2) only the interaction of prenylated Rac with GAPs is significantly influenced by fluoride.

EXPERIMENTAL PROCEDURES

Materials. Glutathione, glutathione−agarose, ampicillin, components of LB medium, BSA, aprotinin, pepstatin, Chaps, DTT, PMSF, EGTA, EDTA, glycine, Trizma base, GTP, SDS, and DFP were purchased from Sigma, IPTG was from Promega, SF-900 medium was from Gibco BRL, desferoxamine (Desferal) was from CIBA, and Ficoll and nitrocellulose were from Pharmacia. Radioactive compounds ([γ-^{32}P]GTP and [^{35}S]GTPγS) were obtained from the Institute of Isotopes, Budapest, Hungary. All of the other reagents were of the highest available quality.

Preparation of Recombinant Proteins. Nonprenylated recombinant Rac1, RhoA, Cdc42, the GAP domain of Bcr (amino acids 871−1271), and full-length p50RhoGAP and its GAP domain (amino acids 198−439) were produced in the form of GST fusion proteins in *E. coli* bacteria and purified as described by Self and Hall (*21*). The GST domain was cleaved by thrombin. The protein-expressing clones were a gift of Professor Alan Hall. Prenylated Rac1 was isolated from the membrane fraction of *Sf9* cells by extraction with 1% Chaps and purified as described in ref *22*. The complex of prenylated RhoA and RhoGDI was expressed in *Sf9* cells and purified as described in ref *23*. Prenylated Rho was liberated from the complex by treatment with 1% Chaps (*23*). Whole-length p190RhoGAP protein was prepared as described in ref *24*. The purity of the produced proteins as determined by SDS−PAGE and Coomassie staining was over 95%.

Preparation of Human Neutrophils and Subcellular Fractions. Human neutrophils were prepared from buffy coats of healthy volunteers as described in Hjorth et al. (*25*). After suspension in PBS they were treated with 1 mM diisopropyl flluorophosphate (DFP) for 10 min at room temperature, washed with PBS, and resuspended in PBS containing 1 mM EGTA, 10 μg/mL aprotinin, 2 μM pepstatin, 10 μM leupeptin, and 0.1 mM PMSF. The cells were sonified (Branson 250), and membrane and cytosolic fractions were separated by ultracentrifugation as described in ref *26*. Membrane fractions were solubilized in PBS supplemented with 1% Triton X-100 nonionic detergent.

Measurement of GTP Hydrolysis of Small G Proteins. The nitrocellulose filter binding assay was applied as described by Self and Hall (*27*). Loading of the GTPase (1−4 μg of *E. coli* or *Sf9* protein) was performed with high specific activity (3000 Ci/mmol) [γ-^{32}P]GTP in low magnesium buffer [16 mM Tris-HCl, pH 7.5, 20 mM NaCl, 0.1 mM DTT, 5 mM EDTA, and 100 nM [γ-^{32}P]GTP (5 μCi)] for 5 min at room temperature and stopped by addition of 20 mM $MgCl_2$. In some experiments loading was carried out in the presence of 1−10 μM unlabeled GTP. The loaded G proteins were kept on ice to decrease nucleotide hydrolysis. The GTPase reaction was initiated by addition of 3 μL of loaded G protein to 27 μL of reaction mixture (20 °C) containing 16 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 1 mg/mL BSA, 1 mM unlabeled GTP, and the investigated protein with GAP activity. Aliquots of 5 μL were taken at regular intervals and filtered through nitrocellulose filters. The filters were washed three times with 2 mL of ice-cold buffer consisting of 50 mM Tris-HCl (pH 7.7) and 5 mM $MgCl_2$. The radioactivity was measured in distilled water on the basis of the Cerenkov effect in the Beckman LS 5000TD liquid scintillation spectrometer. Data are presented as the decrease in time of protein-bound radioactivity retained on the filters (Figures 1 and 2). In further calculations the endogenous hydrolysis rate of Rac is taken into account; therefore, the amount of [γ-^{32}P]GTP remaining protein-bound after 5 min in the presence of GAP is expressed as the percent of total available protein-bound [γ-^{32}P]GTP at the same time point (Figures 3 and 4, Tables 2 and 3).

Measurement of Nucleotide Exchange. The nucleotide exchange was measured similarly as described above for the GTP hydrolysis assay. The G proteins (1−4 μg) were loaded with [^{35}S]GTPγS (specific activity >1000 Ci/mmol; final concentration 2−10 μM), and the radioactivity was measured in a Triton−toluene-based scintillation cocktail.

Protein Concentrations. The protein content was determined as described by Bradford (*28*) using bovine serum albumin as the standard.

RESULTS

Effect of Membrane-Localized GAPs on Different Rho-Family Small GTPases. In our earlier studies we demonstrated the presence of intense Rac−GAP activity localized in the membrane fraction of human polymorphonuclear granulocytes (*29*, *30*). In these investigations we observed a striking difference in the reaction of prenylated and nonprenylated Rac. Rac protein prepared in *Sf9* insect cells (which are able to carry out the prenylation) and in *E. coli* cells (which lack the prenylating enzyme) exhibited an identical rate of endogenous GTPase activity. However, addition of the membrane fraction increased GTP hydrolysis only in the prenylated Rac protein and had hardly any effect on the nonprenylated protein (Figure 1A,B, Table 1). The Rac protein prepared from the membranes of the *Sf9* cells contained 1% Chaps as detergent that was diluted under our experimental conditions to less than 0.1%. We tested the effect of various detergents on the reaction of nonprenylated Rac with the PMN membranes. Neither Triton nor Tween or Chaps, up to the concentration of 1%, had any effect. In fact, the same difference in the reaction of prenylated and nonprenylated Rac could be observed when the PMN membrane was solubilized in Triton (Figure 1A,B, Table 1) or in octyl glucoside (data not shown). Thus, the observed difference is not the consequence of the presence of the detergent added with the prenylated Rac protein. No difference in the reactivity was observed whether the GST domain was cleaved or not from the Rac protein prepared in *E. coli*. In most experiments loading of the small GTPase was carried out with high specific activity of [γ-^{32}P]GTP. Similar data were however obtained when the specific activity of [γ-^{32}P]-GTP was decreased 10-fold. No difference was observed in the loading efficiency of prenylated and nonprenylated Rac1.

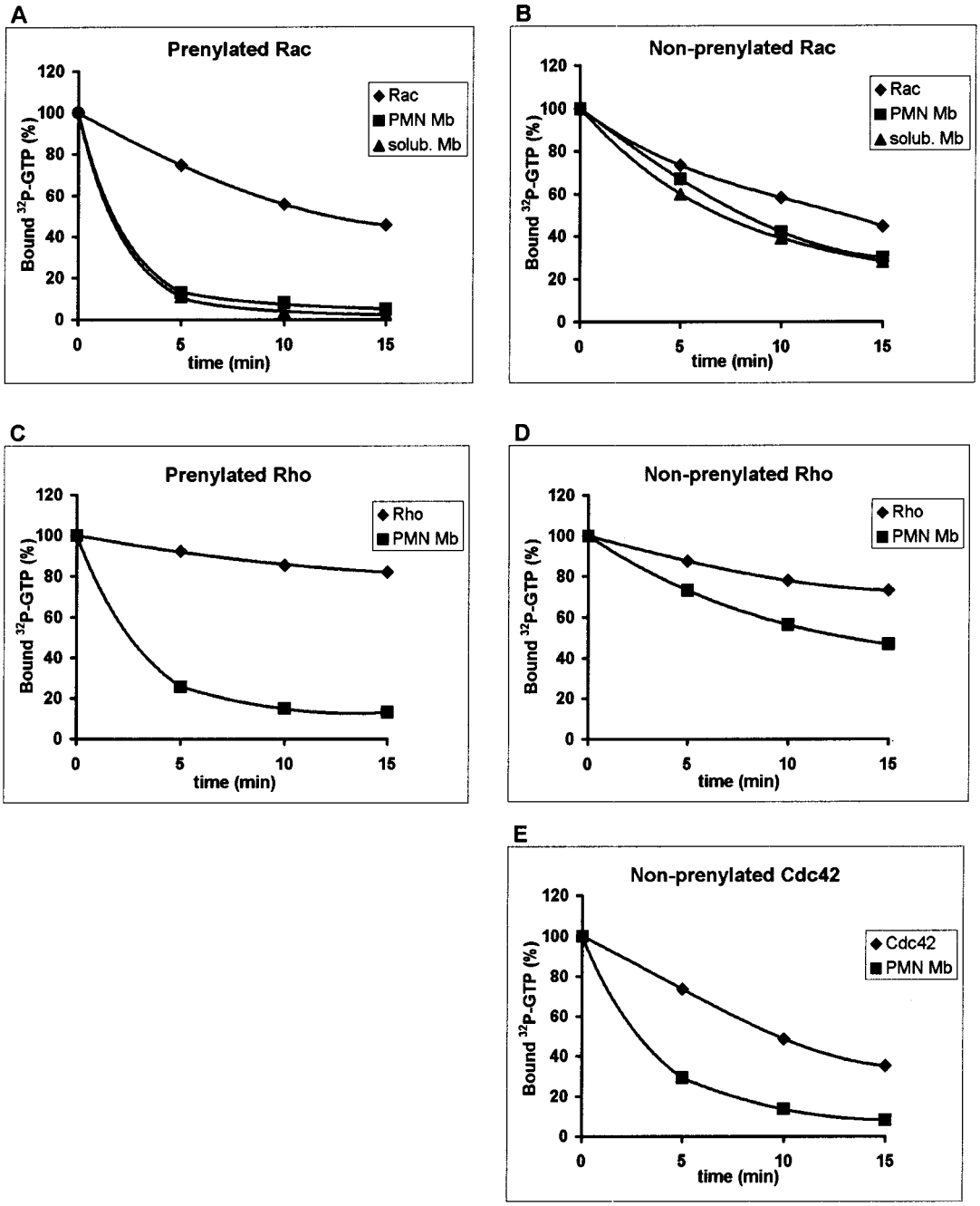

FIGURE 1: Effect of membrane-localized GAPs on prenylated (panels A and C) and nonprenylated (panels B, D, and E) small GTPases. Hydrolysis of [γ-^{32}P]GTP was followed either in the presence of the indicated small GTPase alone (◆) or in the presence of the small GTPase and 10 μg of the membrane fraction of human PMN (■) or in the presence of the small GTPase and 5 μg of PMN membrane solubilized in 1% Triton (▲). One representative out of 5−12 similar experiments.

Finally, using [^{35}S]GTPγS, we checked the rate of nucleotide exchange on the investigated small GTPases. The exchange rate was similar on prenylated and nonprenylated Rac, and it was not influenced by the addition of isolated GAP proteins or fluoride. PMN membrane contained weak exchange activity that amounted to less than 10% of the GAP activity, but it was not detectable after solubilization of the membrane either in Triton or in octyl glucoside. Thus, the difference in Rac-bound [γ-^{32}P]GTP retained on the nitrocellulose filters represents mainly the differences in the rate of hydrolysis of protein-bound [γ-^{32}P]GTP.

Other members of the Rho-family small GTPases, RhoA and Cdc42, have also been investigated. In agreement with previous findings, the endogenous GTP hydrolysis rate of Rho was significantly lower than that of Rac. The endogenous rate of hydrolysis did not differ significantly in the case of the prenylated and nonprenylated protein. The reaction of prenylated and nonprenylated RhoA with PMN membranes showed similar behavior as described for Rac (Figure 1C,D, Table 1). In contrast to this, nonprenylated Cdc42 interacted with membrane-associated GAPs significantly better than Rho or Rac did (Figure 1E, Table 1).

Effect of Purified GAPs on Prenylated and Nonprenylated Rac and Rho. The membrane fraction of PMN is abundant in p50RhoGAP and contains low levels of p190RhoGAP, too (*30*). Therefore, in the next experiments we compared the effect of these proteins on prenylated and nonprenylated small GTPases. The GAP domain of p50RhoGAP was

Table 1: Effect of Membrane-Associated GAPs, Recombinant Full-Length p50RhoGAP, p190RhoGAP, and the Isolated GAP Domain of p50RhoGAP on Prenylated and Nonprenylated Small GTPases[a]

proteins present	% [γ-^{32}P]GTP remaining bound to prenylated GTPase after 5 min	% [γ-^{32}P]GTP remaining bound to nonprenylated GTPase after 5 min
Rac1	75 ± 2	74 ± 2
+PMN membrane	17 ± 4	64 ± 2
+solubilized membrane	21 ± 3	61
+full-length p50RhoGAP	19 ± 6	53 ± 14
+GAP domain of p50	17 ± 6	20 ± 7
p190RhoGAP	23 ± 2	52 ± 3
RhoA	91 ± 2	87 ± 3
+PMN membrane	30 ± 2	67 ± 8
Cdc42		74 ± 5
+PMN membrane		26 ± 2

[a] Hydrolysis of [γ-^{32}P]GTP was followed in the presence of the indicated proteins, and the amount of [γ-^{32}P]GTP remaining protein-bound after the first 5 min is expressed in a percentage of the initial (0 min) amount of protein-bound [γ-^{32}P]GTP. Data are expressed as the mean ± SEM of 3−15 measurements.

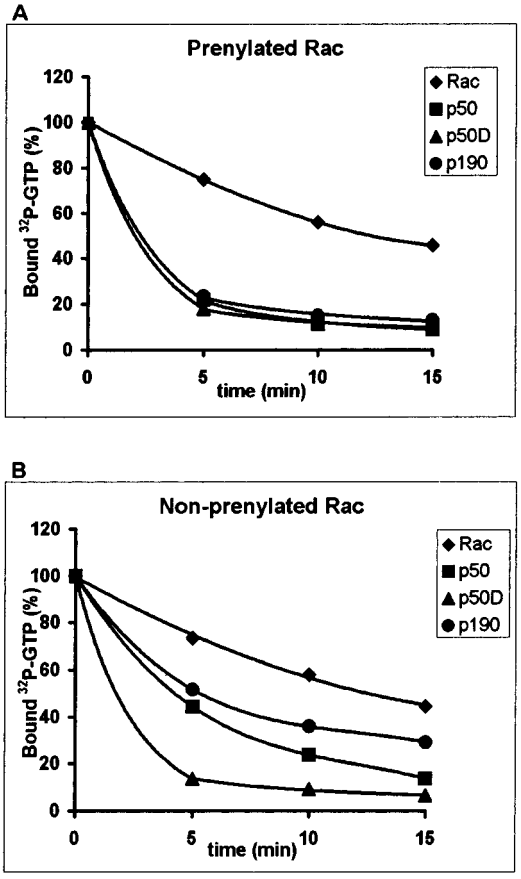

FIGURE 2: Effect of recombinant GAPs on prenylated (panel A) and nonprenylated (panel B) Rac1. Hydrolysis of [γ-^{32}P]GTP was followed either in the presence of the indicated small GTPase alone (◆) or in the presence of the small GTPase and full-length p50RhoGAP (■) or in the presence of the small GTPase and the GAP domain of p50RhoGAP (▲) or full-length p190RhoGAP (●). One representative out of five similar experiments.

equally effective on prenylated and nonprenylated Rac (Figure 2, Table 1). Similar results were obtained with the GAP domain of the Bcr protein (data not shown). However, the full-length p50RhoGAP clearly distinguished between

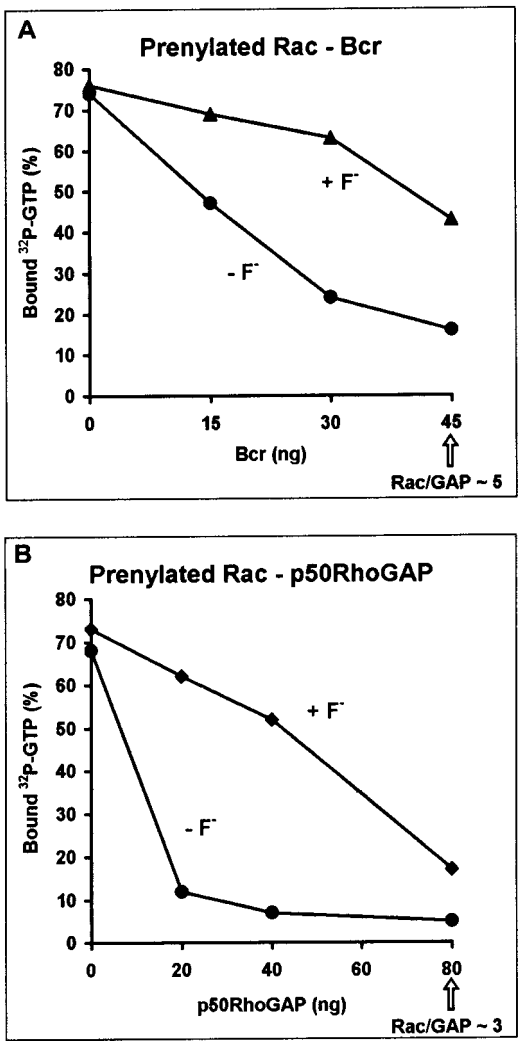

FIGURE 3: Effect of fluoride on the action of the GAP domain of Bcr (panel A) or p50RhoGAP (panel B) upon prenylated Rac. Dependence on the concentration of the applied GAP. Where present, fluoride was added at a concentration of 30 mM. The amount of [γ-^{32}P]GTP remaining protein-bound after 5 min is indicated on the *y*-axis. The value obtained in the absence of GAPs (70−75%) is due to the endogenous hydrolytic activity of Rac. One representative out of four similar experiments.

the prenylated and nonprenylated form of the small GTPase. Using identical amounts of Rac and p50RhoGAP proteins, the amount of [γ-^{32}P]GTP bound to prenylated and nonprenylated Rac was reduced in the first 5 min to 19 ± 6% and 53 ± 14%, respectively (Table 1). A similar difference was observed with full-length p190RhoGAP (Figure 2). Both the GAP domain and the full-length p50RhoGAP protein have been expressed in *E. coli* in the form of GST fusion proteins whereas p190RhoGAP was expressed in the insect cells. Thus, the mode of expression of the full-length GAPs does not seem to be decisive in their reactions with the different forms of the small GTPases.

Effect of Fluoride on the Interaction of GAPs with Different Forms of Rac. Several members of the small GTPase superfamily (e.g., Ras, Rap2, Rho, Cdc42) have been shown to form stable complexes with their relevant GAP proteins in the presence of fluoride ions and either aluminum or magnesium (*8*, *9*, *16*). For Ras, Rap2, and Cdc42, complex formation was shown to occur in the GDP-bound state (*8*, *9*) whereas in the interaction of RhoA with p190RhoGAP

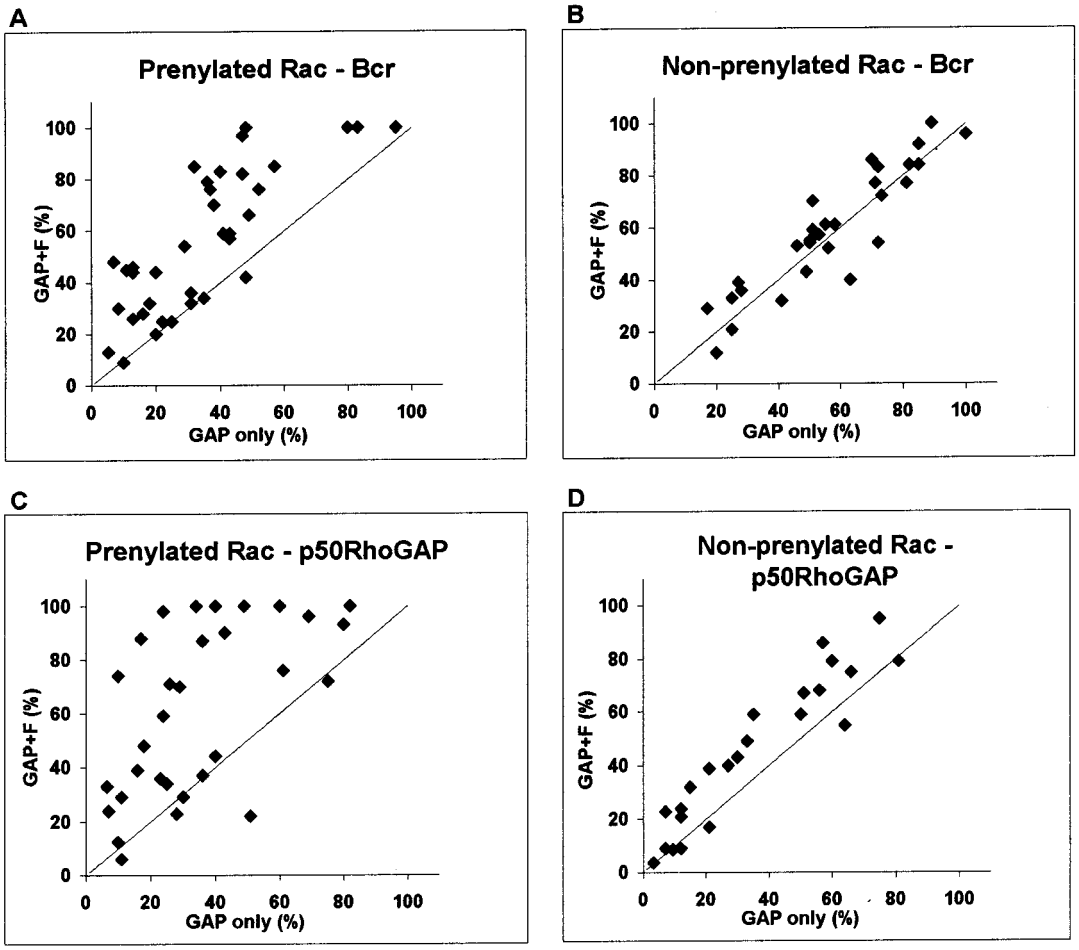

FIGURE 4: Effect of fluoride on the GTP hydrolysis by prenylated (panels A and C) and nonprenylated Rac (panels B and D) enhanced by the GAP domain of Bcr (panels A and B) or the GAP domain of p50RhoGAP (panels C and D). The amount of [γ-^{32}P]GTP remaining protein-bound after 5 min in the presence of the indicated GAP is expressed as the percent of total available protein-bound [γ-^{32}P]GTP at the same time point (detected in the absence of any GAP). The values obtained in the absence or presence of 30 mM fluoride are represented on the *x*- and *y*-axis, respectively. All the data obtained in 26−42 measurements are represented.

the species of the nucleotide did not seem to be critical (*16*). In all of these cases the investigated small GTPase exhibited high affinity toward its GAP, the K_d being 60 nM or around 2 μM (*7*, *31*). In contrast, the K_d of the interaction of Rac with p50RhoGAP or p190RhoGAP is more than 10 times higher than the K_d of the interaction of Cdc42 with p50RhoGAP or that of RhoA with p190RhoGAP (investigated in refs *9* and *16*, respectively) (*31*) and 50 to 100 times higher than the K_d of the Ras−neurofibromin interaction (*8*). The low affinity of Rac for its GAPs may explain the fact that complex formation between Rac and a GAP has only been shown in one single publication, and this complex seemed to be very weak (*16*).

However, complex formation between Rac and GAP should affect GAP function; hence it should be detectable in the GTPase assay. In the presence of an excess of the small GTPase, participation of a fraction of the GAP in a complex is expected to decrease the effective concentration of GAP, and thus, the rate of GTP hydrolysis is also expected to be decreased. In fact, in the presence of the specific GAP protein, a decrease of phosphate release from GTP-loaded Ras, Rap2, and Ran has been observed upon addition of the GDP-bound form of the respective small GTPase (*8*). Following this rationale, we investigated the effect of fluoride on the hydrolytic activity of Rac. The endogenous rate of GTP hydrolysis was not influenced by fluoride (either in the presence or absence of Al^{3+}). This finding is in agreement with earlier reports showing that there was no interaction between the small GTPase and fluoride (*7*−*9*, *17*). In contrast to this, fluoride did influence the accelerating effect of the GAP domain of p50RhoGAP and Bcr on hydrolysis of Rac-bound [γ-^{32}P]GTP. As shown in Figure 3, the effect of fluoride depended on the ratio of Rac to GAP present in the reaction. Clear inhibition of the hydrolysis of Rac-bound [γ-^{32}P]GTP occurred in the presence of fluoride when the concentration of the small GTPase was 10−15 times higher than that of the GAP domain whereas the inhibitory effect gradually disappeared as the concentration of GAP was increased. Similar results were obtained with the GAP domains of both Bcr and p50RhoGAP. Augmentation of the amount of RacGDP present in the reaction enhanced the inhibitory effect of fluoride (data not shown).

The inhibitory action of fluoride was also compared on prenylated and nonprenylated Rac proteins. The activity of the different preparations of the GAP domains of p50RhoGAP and Bcr showed significant variations; therefore, the effect of fluoride was not always consistent. Figure 4 summarizes the results of 30−40 experiments carried out with several Rac and GAP preparations. The proportion of [γ-^{32}P]GTP remaining Rac-bound after 5 min in the presence and absence

Table 2: Effect of Fluoride on the GTPase Accelerating Effect of the GAP Domain of p50RhoGAP and Bcr[a]

proteins	Rac−[γ-^{32}P]GTP without fluoride (%)	Rac−[γ-^{32}P]GTP with fluoride (%)	ratio +F/−F
p50 + pRac	35 ± 4 (n = 36)	64 ± 5	1.8
p50 + cRac	35 ± 4 (n = 26)	43 ± 5	1.2
Bcr + pRac	35 ± 3 (n = 42)	57 ± 3	1.6
Bcr + cRac	56 ± 4 (n = 35)	58 ± 4	1.0

[a] The amount of [γ-^{32}P]GTP remaining protein-bound after 5 min in the presence of the indicated GAP is expressed as the percent of total available protein-bound [γ-^{32}P]GTP (detected in the absence of any GAP). When present, fluoride was applied at a concentration of 30 mM. pRac and cRac indicate prenylated Rac obtained from *Sf9* cells and nonprenylated Rac obtained from *E. coli*, respectively. Data are expressed as the mean ± SEM of the indicated number (n) of measurements.

Table 3: Effect of Aluminum and the Chelator Desferoxamine on the Inhibitory Action of Fluoride[a]

small GTPase	percent			
	GAP alone	GAP + F	GAP + F + Al	GAP + F + Des
pRac	21 ± 4	65 ± 9	65 ± 7	
	58 ± 12	88 ± 6		90 ± 8
cRac	36 ± 5	43 ± 8	44 ± 3	

[a] The amount of [γ-^{32}P]GTP remaining protein-bound after 5 min in the presence of the GAP domain of p50RhoGAP is expressed as the percent of total available protein-bound [γ-^{32}P]GTP (detected in the absence of GAP). When present, fluoride was applied at a concentration of 30 mM, $AlCl_3$ at 2 mM, and desferoxamine (Des) at 0.1 mM. pRac and cRac indicate prenylated Rac obtained from *Sf9* cells and nonprenylated Rac obtained from *E. coli*, respectively. Data are expressed as the mean ± SEM of 6−10 measurements.

of fluoride is shown on the *y*- and *x*-axis, respectively. The straight line represents the case when fluoride has no effect at all. The data points of Figure 4A and Figure 4C fall mostly in the upper segment, indicating an increase in the proportion of Rac-bound [γ-^{32}P]GTP in the presence of fluoride. Thus, in most experiments fluoride inhibited the action of the GAP domain of both Bcr and p50RhoGAP on prenylated Rac. In contrast to this, in Figure 4B, where the interaction of the GAP domain of Bcr with nonprenylated Rac is demonstrated, the data points are scattered around the straight line, indicating no inhibition by fluoride. In Figure 4D the data points show a slight upward tendency, indicating a weak inhibition by fluoride of the effect of the GAP domain of p50RhoGAP on nonprocessed Rac.

Table 2 provides the statistical analysis of the data presented in Figure 4. On the average of approximately 40 measurements, using the catalytic domains of p50RhoGAP or Bcr, 1.8 and 1.6 times more radioactivity remains bound to prenylated Rac in the presence of fluoride than in its absence. In the experiments where nonprenylated Rac was used, the corresponding values are 1.2 and 1.0. Thus, the difference in the inhibitory action of fluoride on the interactions of prenylated and nonprenylated Rac with the GAP domain of both proteins is clearly detectable. A similar difference was observed when full-length p50RhoGAP was used instead of the catalytic domain (data not shown).

The GTPase assay contained 2 mM Mg^{2+}. In agreement with our previous findings (*29*), under our conditions the inhibitory effect of fluoride was independent of aluminum. As summarized in Table 3, neither the addition of $AlCl_3$ nor the chelation of potential impurities of Al^{3+} by desferoxamine had any effect on the inhibition of the interaction of prenylated Rac with the GAP domain of p50RhoGAP brought about by fluoride. In the case of nonprenylated Rac, fluoride remained ineffective even in the presence of Al^{3+}. Both Al^{3+} and desferoxamine were without any effect when fluoride inhibition of the Rac−Bcr interaction was investigated (data not shown). These results suggest that similarly to the Rho−p190RhoGAP (*16*) and Rho−p50RhoGAP (*17*) interactions, also Rac is able to form a MgF_x complex with the GAP domain of p50RhoGAP or Bcr; however, this ability is restricted to prenylated Rac.

DISCUSSION

Our experiments revealed clear differences in the interaction of prenylated and nonprenylated Rac with various GAP proteins. These differences were evident at three different levels.

Action of Fluoride on Isolated GAP Domains. The endogenous rate of GTP hydrolysis by prenylated or nonprenylated Rac was identical, and isolated GAP domains of p50RhoGAP and Bcr were equally effective in acceleration of GTP hydrolysis by both forms of Rac (Figure 2). However, only the effect of the GAP domains upon prenylated Rac protein was inhibited significantly by fluoride. On the basis of previous reports (*8*) and our experiments shown in Figure 3, we interpret fluoride inhibition as an indication of complex formation between the small GTPase and GAP in the presence of magnesium fluoride. Thus, the data summarized in Figure 4 and Table 2 suggest that prenylation affects the ability of Rac to form MgF_x complex with the GAP domain of various GAPs. Previous investigations have revealed that the amino acids involved in the binding of the Rho-family small GTPases to their respective GAPs and those responsible for the catalytic activity of GAP are not identical (*14*, *15*, *32*). It is thus conceivable that prenylation alters the position of a (few) amino acid(s) of Rac which is not reflected either in the endogenous or in the GAP-accelerated rate of GTP hydrolysis but has an influence on the complex formation in the presence of magnesium fluoride.

Action of Full-Length GAPs. The second indication for differences in the behavior of prenylated and nonprenylated Rac was revealed when the catalytic effects of the GAP domain and the full-length p50RhoGAP were compared. In the same experiment, under identical conditions, the full-length protein proved to be about half as effective on the nonprenylated than on the prenylated form of Rac (Figure 2). Full-length p190RhoGAP also behaved differently on prenylated and nonprenylated Rac. Thus, we have to suggest that amino acid sequences outside of the GAP domain influence the accessibility and/or reactivity of the catalytic domain in both proteins. This steric hindrance could be released by interaction of the prenyl moiety of Rac with the GAP protein, explaining the limited effectivity on the nonprenylated form. A similar observation has been recently described for the interaction of the yeast small GTPase Ras2p with the exchange protein Cdc25 (*33*). In this case the isolated GEF domain reacted with both the farnesylated and nonfarnesylated form of the small GTPase, whereas the full-length protein induced nucleotide exchange only on farne-

sylated Ras2p. A regulatory function of the N-terminal domain was proposed upon the C-terminal catalytic domain limiting the interaction with the nonprocessed form of the small GTPase. Intramolecular regulation was also suggested in the case of p120RasGAP, when it was demonstrated that the N-terminal pleckstrin homology domain of p120 is able to bind to the catalytic domain and thereby influence the Ras signaling pathway in transfected NIH3T3 cells (*34*).

Prenylation was shown to be critical for the interaction of Rho-family small GTPases with the regulatory protein RhoGDI (*35*). The crystal structure of the Cdc42−RhoGDI complex clearly indicates the hydrophobic pocket on the RhoGDI, which accepts the geranylgeranyl moiety of the prenylated Cdc42 protein (*18*, *36*). Immunoglobulin-like domains have a major role in the formation of the hydrophobic pocket (*18*). More recently, the interaction of RhoA with one of its target proteins, Trio, was shown to depend also on the prenylated state of the small GTPase, and an immunoglobulin-like domain was proposed to be responsible for recognition of the prenyl moiety (*37*). The amino acid sequence does not indicate any Ig-like domain in either the case of p50RhoGAP or the case of p190RhoGAP. However, the Ig-like folds detected in the crystal structure of RhoGDI do not show up in the amino acid sequence. Thus, the potential contribution of Ig-like domains in these two GAPs can be finally decided only when the structure of the crystallized proteins will be resolved. On the other hand, the N-terminal part of p50RhoGAP contains a Sec14p-like domain indicating the possibility of lipid regulation (*38*), and the N-terminal part of p190RhoGAP contains a sequence homologous to small GTPases. The possible involvement of these regions in the modulation of the interaction of the RhoGAP domain has to be elucidated in further experiments. A GAP protein reacting with Rab3A was also found to act only on the lipid-modified small GTPase (*39*), but to our knowledge, this is the first time that the decisive role of prenylation in the interaction of Rho-family GTPase with its respective GAP is demonstrated.

Action of Membrane-Localized GAPs. Finally, the membrane environment seems to have an additional effect on the Rac−GAP interaction. In our experiments, membrane-associated or membrane-extracted GAPs were unable to accelerate GTP hydrolysis on nonprenylated Rac (Figure 1A,B), although this Rac protein had the same endogenous GTPase activity as its prenylated counterpart and was fully sensitive to isolated GAP domains (Figure 2). It should be noted that in previous experiments, using the Rac−[γ-^{32}P]-GTP overlay technique to identify GAPs after separation by SDS−PAGE, transfer on nitrocellulose, and partial renaturation, nonprenylated Rac was able to interact with several GAPs of membrane origin (*30*). Apparently, the lipid components and/or protein−protein interactions resisting solubilization significantly modify the reactions of membrane-associated GAP proteins. Earlier studies demonstrated the inhibitory effect of various lipid compounds on the activity of both RasGAPs and RhoGAPs, though the potential role of prenylation has not been raised in those reports (*40*−*43*). Whether only the lipid components of the membrane are responsible for restriction of the accessibility of membrane-associated GAPs for not-modified Rac or also protein−protein interactions are involved has to be the subject of further investigations.

It has been reported recently for the Rho-family members Cdc42 and Rac that dimerization of the protein resulted in a significant increase of the catalytic activity. This GAP-like effect was dependent on a critical arginine present in the C-terminal polybasic region of Cdc42 and Rac but absent in RhoA (*44*−*46*). Theoretically, the acceleration of GTP hydrolysis detected in our experiments could also be the consequence of induction of the dimerization of the small GTPase. However, under our conditions the concentration of the small GTPase was significantly lower than the range where dimerization has been observed, and dimerization was shown to be independent of the prenylation state of the small GTPase (*44*). Thus, we consider that our data showing variations of the GTPase activity of prenylated and nonprenylated Rac reflect variations of the interaction of the GTP-binding protein with its GAP.

Taken together, our results suggest that prenylation is an important factor determining the interaction of Rac (and possibly other small GTPases) with GAP proteins, as prenylated Rac has better access both to isolated full-length and to membrane-localized GAPs. It is however conceivable that the prenyl moiety is not the only factor modifying the accessibility of GAPs of different intracellular localizations. Thus, both the suggested intramolecular interactions and the consequences of membrane targeting of GAP proteins represent potential sites and mechanisms for modulation of Rac−GAP activity and Rac signaling in the cell.

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