Autoinhibition of p50 Rho GTPase-activating Protein (GAP) Is Released by Prenylated Small GTPases*

Received for publication, November 8, 2004, and in revised form, December 9, 2004. Published, JBC Papers in Press, December 13, 2004, DOI 10.1074/jbc.M412563200

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Experimental Procedures

Small GTPases of the Rho subfamily (Rho, Rac, Cdc42) have a widespread role in the organization of numerous cell functions, such as polarity, motility, shape changes, membrane trafficking, gene expression, or production of toxic oxygen metabolites (1–3). Similarly to other GTP-binding proteins, they are active in the GTP-bound form and inactive in the GDP-bound state. The species of the bound nucleotide is regulated by three different types of proteins. Under physiological conditions, guanine nucleotide exchange factors promote the release of GDP and favor the binding of GTP, guanine nucleotide dissociation inhibitor maintains the small GTPase in its GDP-bound state, and GTPase-activating protein(s) accelerate the endogenous rate of GTP hydrolysis of the small GTPase (4).

According to recent data ~170 proteins are predicted to function as GAP for various small GTPases of the Ras superfamily, and 70 of these proteins contain a typical Rho/RacGAP domain (5, 6). Apparently, cells with specialized functions express different subsets of this “GAP repertoire” (7). Under in vitro conditions, many of the predicted Rho/RacGAPs interact with several members of the Rho subfamily (6). Acceleration of GTP hydrolysis is based on the participation of a critical arginine of the GAP in the stabilization of the transition state of the substrate binding site of the small GTPase (8). In addition to the GAP domain, Rho/RacGAPs contain a large variety of putative protein and lipid interaction domains, which could modify the localization, activity, or substrate specificity of the relevant GAP. However, our knowledge on the details of these interactions and on their functional consequences is scarce (9).

p50RhoGAP (also called Cdc42GAP) is a 50-kDa protein that is widely expressed in many tissues. The typical GAP domain is located near to the C-terminal end of the protein preceded by a short proline-rich sequence and by a domain that is homologous to Sec14p, a phospholipid transfer protein in Saccharomyces cerevisiae (Sec14 or BCh domain) (10). In a previous study we have shown that the isolated GAP domain reacted significantly better both with Rac and Rho than the full-length protein did. We suggested an intramolecular interaction that could limit the accessibility of the GAP domain (11). The aim of the present investigations was to gain insight into the details of the proposed interaction within the p50RhoGAP molecule. On the basis of two-hybrid interaction experiments, GAP assays, and tests of the activity of a Rac-regulated enzyme complex we show that two different parts of the p50RhoGAP participate in the shielding of the GAP domain. Interaction with the prenyl moiety of the small GTPase releases this restraint within the GAP molecule.
The constructs for the GST fusion protein of the GAP domain (198GAP) and the full-length p50RhoGAP, as well as Rac1, RhoA, and Cdc42 were generous gifts of Professor Alan Hall. The cDNA of the different fragments of p50RhoGAP (see Fig. 1B) was prepared by PCR using the GST-p50RhoGAP construct as template, and they were cloned in-frame into pGEX-4T2 vector. All plasmid constructs have been sequenced. The Escherichia coli clones producing p47phox and p67phox were a generous gift of Dr. F. Wientjes.

**Expression of Proteins**—The expression of GST fusion proteins in E. coli was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside and the proteins were purified as described in Ref. 13. Preynlated Rac1 was isolated from the membrane fraction of Sf9 cells and purified as described in Ref. 12. Cytochrome b558 was purified and relipidated as described in Ref. 14.

**Yeast Two-hybrid System**—The two-hybrid system described in Ref. 15 was used. Briefly, L40 yeast strain was transformed with two plasmids using classical LiAc protocol. Doubly transformed yeast cells were selected on a medium lacking Trp and Leu, and His and by monitoring β-galactosidase activity in a filter test, respectively (16). For a quantitative assay, the microplate test was used as described in Ref. 17. The expression level of all fusion proteins was controlled by appropriate antibodies obtained from Clontech.

**Measurement of GTP Hydrolysis of Small G Proteins**—The nitrocellulose filter binding assay was applied as described in Ref. 18. The loading of the GTPase (1–4 µg of E. coli or Sf9 protein) was performed with high specific activity (3000 Ci/mmol) ([γ-32P]GTP in low magnesium buffer (16 mM Tris·Cl, pH 7.5, 20 mM NaCl, 0.1 mM dithiothreitol, 5 mM EDTA, and 100 mM [γ-32P]GTP (5 µCi)) for 5 min at room temperature and stopped by adding 20 mM MgCl2. The loaded G proteins were washed three times with 2 ml of ice-cold buffer consisting of 50 mM Tris·Cl, pH 7.7, and 5 mM MgCl2. The radioactivity was measured in a Labsystems iEMS Microplate Reader. In the fully purified system (198GAP) and the full-length p50RhoGAP, as well as Rac1, RhoA, and Cdc42 were generous gifts of Dr. F. Wientjes.

**Measurement of Superoxide Generation in the Cell-free System**—For a quantitative assay, the microplate test was used as described in Ref. 17. The expression level of all fusion proteins was controlled by appropriate antibodies obtained from Clontech.

**Demonstration of Intramolecular Interactions in p50RhoGAP**

**in a Yeast Two-hybrid System**—To investigate potential intramolecular interactions, we cloned the isolated GAP domain in pGAD vector and tested its reaction with various fragments of the protein cloned into pLex vector (Fig. 1A). As summarized in Table I, we found an interaction between the GAP domain and the critical domains are masked as the isolated GAP domain did not interact either with full-length p50RhoGAP or with the fragment lacking the first 48 amino acids (49GAP) (Table I). We did not find any reaction with wild-type Rac2 either (data not shown). Probably the interaction between the GDP form of this small GTPase and p50RhoGAP is not sufficiently stable to allow detection in the two-hybrid system. In contrast, the constitutively active mutant Q61LRac1 reacted with full-length p50RhoGAP and so did the T17N Rac1 mutant. Interestingly, both mutants were only able to interact with the full-length protein, the absence of the first 48 amino acids prevented the interaction. No reaction was detectable between wild-type or mutant Rac1 and any other fragment of p50RhoGAP either. Thus, the first 48 amino acids of p50RhoGAP have a decisive role in the stabilization of the p50GAP domain.

*Experiments carried out in our laboratories indicate that Rac1T17N is either present in the cell partially in GDP-bound form or exhibits a special conformation mimicking the GTP-bound state of wild-type Rac1. Detailed data will be published separately.*
interaction of this GAP with its substrate, Rac.

Next we asked whether the prenylation state of the small GTPase has a role in the interaction with p50RhoGAP. To this end we prepared C189GRac, Q61L, and T17N mutants, where the absence of the cysteine from the C-terminal CAAX box of Rac prevents its prenylation in the yeast cell. In a quantitative β-galactosidase assay, wild-type Rac1 did not show higher activity than the empty vector, and this feature was independent of the prenylation state of the small GTPase (Fig. 2). This finding is in accordance with our data obtained on the filter (Table II). In contrast, in the case of both the Q61L and the T17N mutants, a significant difference was observed whether the protein was prenylated or not. The activity measured with the non-prenylated proteins did not exceed the background value, whereas with pQ61LRac1 and pT17NRac1 a 2.2- and 1.7-fold increase was detected, respectively. Thus, the prenylation of the protein was prenylated or not. The activity measured with the non-prenylated proteins did not exceed the background value, whereas with pQ61LRac1 and pT17NRac1 a 2.2- and 1.7-fold increase was detected, respectively. Thus, the prenylation state of Rac prevents its prenylation in the yeast cell. In a quantitative β-galactosidase test was carried out as described under “Experimental Procedures.” The results are given in arbitrary β-galactosidase units. Means ± S.E. of three separate experiments are represented.

Effect of p50RhoGAP Fragments on GTP Hydrolysis by Rac—To test our results obtained in the two-hybrid system, we expressed the corresponding fragments of p50RhoGAP as GST fusion proteins (Fig. 1B) and studied their GTPase-activating effect in a classical radioactive GAP assay (Fig. 3). In accordance with our earlier finding (11), full-length p50RhoGAP had only a weak GAP effect on non-prenylated Rac1 prepared from bacteria. Removal of the first 48 amino acids improved its reaction, although it did not attain that of the isolated GAP domain (198GAP) (Fig. 3A). The difference in the reactivity of the three constructs of p50RhoGAP was not affected by the presence of geranylgeranyl pyrophosphate in equimolar ratio to non-prenylated Rac1 (data not shown). When GTP hydrolysis by prenylated Rac was investigated, the full-length protein and the two truncated forms proved to have similar efficiency (Fig. 3B).

Next we wanted to gain information on which part of the Sec14 domain could be responsible for the restriction of the interaction of 49GAP with non-prenylated Rac. In deciding on the site of further truncations, we were led by two considerations. 1) The sequences participating in lipid binding of the Saccharomyces protein Sec14p correspond to the stretches 96–100, 105–115, 140–145, and 170–180 of p50RhoGAP (19), and 2) the sequence between amino acids 155–170 shows weak homology to an immunoglobulin domain and in some proteins (e.g. Rho guanine nucleotide dissociation inhibitor, Trio) an Ig domain was shown to interact with the prenyl group (20, 21). Thus, we prepared GST fusion proteins starting at amino acid 86, 121, or 169 (Fig. 1B). In the GAP assay all of these fragments behaved similarly to 49GAP (Table III). The fact that they were less effective than fragment 198GAP in stimulating GTP hydrolysis on non-prenylated Rac, indicates that amino acids 169–197 play a role in the interaction of p50RhoGAP with prenylated small GTPases. On the other hand, truncation of the molecule beyond the fragment 198GAP did not improve its reaction with non-prenylated Rac1 (data not shown). Thus, our GAP assays provided the information that two different parts of the p50RhoGAP molecule, namely the N-terminal 48 amino acids and the stretch between amino acids 169 and 197 at the C-terminal end of the Sec14 domain, are involved in the structural changes induced by prenylated Rac1. It should be remarked, that the above fragments of p50RhoGAP gave identical reactions with non-prenylated RhoA and Cdc42 as detailed for Rac1 (data not shown).

Effect of p50RhoGAP Fragments on the Activation of NADPH Oxidase—We wanted to substantiate the results obtained in the GAP assay in a functional test where participation of the GTP-bound form of Rac is decisive. The NADPH oxidase of phagocytic cells is a multisubunit enzyme (for review see Ref. 22), the assembly and catalytic activity of which depends on the prevalence of Rac in the GTP-bound active form (23). We applied the fully purified version of the cell-free activation system of this enzyme, consisting of purified and repurified cytochrome b558 and recombinant p47phox, p67phox, and GAP-domain prenylated or non-prenylated Rac (Fig. 4). In our earlier experiments (7), we showed that this system does not contain any GAP activity, allowing a high rate of O2− production in the presence of GTP (Fig. 4, control) that is independent of the prenylation state of Rac (Fig. 4, compare A with B). The addition of full-length p50RhoGAP to the activation system reduced the rate of O2− production, indicating the GAPase-activating
effect on Rac. When non-prenylated Rac was used, 49GAP was more effective than the full-length p50RhoGAP, and the isolated GAP domain was more effective than 49GAP (Fig. 4A). In contrast, in the case of prenylated Rac, all the different fragments of p50RhoGAP had the same efficiency (Fig. 4B). Thus, the results indicating the activity of RacGTP in a complex enzymatic system fully support the data obtained in the GAP assay.

**DISCUSSION**

The crystal structure of the GAP domain of p50RhoGAP has been resolved both in uncomplexed state (24) and in complex with Cdc42Hs-GMPPNP, representing the ground state of the small GTPase (25) and with Rho-GDPAlF₄, representing the transition state during hydrolysis of GTP (26). These studies clearly showed the decisive role of Arg-85 of the GAP domain as in the transition state it participates in the active site of the small GTPase. However, these studies were carried out with the isolated GAP domain (fragment 198–439) and with small GTPases expressed as GST fusion proteins in *E. coli*, thus, without the posttranslational modifications typical for expression in eukaryotic cells, isoprenylation, truncation, and methylation (4). The crystal structure of the *S. cerevisiae* phosphatidylinositol transfer protein (Sec14) is also available (27), and this protein shows sequence homology with the N-terminal part of p50RhoGAP. However, at present there is no structural information either on the relation of the two parts of the p50RhoGAP molecule or on the possible role of the prenyl group in the interaction with its substrate, although in the cellular milieu the true interacting partners are full-length p50RhoGAP and prenylated small GTPases. To our knowledge this is the first study trying to decipher on a functional basis the possible intramolecular interactions within p50RhoGAP and its relation to the prenylated small GTPase.

**FIG. 4.** Effect of full-length and truncated p50RhoGAP on the activity of the NADPH oxidase enzyme complex. Phagocytic NADPH oxidase was activated in the fully purified system as described under "Experimental Procedures." In A non-prenylated RacGTP was applied, and in B prenylated RacGTP was applied. Control samples did not contain any GAP, the other samples were supplemented with the indicated fragment of p50RhoGAP. Means ± S.E. of five separate experiments are represented.

**TABLE III**

| Control | Full-length GAP | 49GAP | 86GAP | 121GAP | 169GAP | 198GAP |
|---------|----------------|-------|-------|--------|--------|--------|
| npRac   | 81.6 ± 1.4     | 58.5 ± 3.1 | 44.4 ± 1.6 | 44.6 ± 2.4 | 44.2 ± 3.8 | 41.8 ± 2.7 | 13.1 ± 1.5 |
| pRac    | 69.2 ± 4.7     | 24.0 ± 2.4 | 26.6 ± 4.3 | 25 ± 2.9 | 27.5 ± 5.2 | 29.5 ± 5.1 | 24.1 ± 0.9 |

Data obtained in the yeast two-hybrid system revealed an important role of the N-terminal 48 amino acids of p50RhoGAP both in establishing intramolecular interaction with the C-terminal GAP domain (Table I) and in the stable interaction with Rac (Table II). Furthermore, the two-hybrid experiments indicated that the positive interaction between Rac and p50RhoGAP depends also on the prenyl moiety of the small GTPase (Fig. 2). Based on these data we carried out a direct investigation of the role of the N-terminal 48 amino acids in GAP assays, which fully supported the results of the two-hybrid study. The removal of the N-terminal 48 amino acids increases the GTPase-activating action of p50RhoGAP on non-prenylated Rac (and Rho and Cdc42), whereas it does not influence the effect upon prenylated Rac (Fig. 3). The same observation was made in the activation of NADPH oxidase, a complex enzymatic reaction that depends on the prevalence of Rac in the GTP-bound form (Fig. 4). Thus, the first 48 amino acids of p50RhoGAP play a critical role in recognition of the prenyl moiety and increasing the accessibility of the GAP domain to prenylated small GTPase.

However, some findings suggest that the removal of the
N-terminal 48 amino acids does not interrupt the intramolecular interactions within the p50RhoGAP molecule completely. 1) In the two-hybrid system, positive interaction with the isolated GAP domain required also the contribution of amino acids on the C-terminal end of the Sec14 domain (Table I). 2) Both the GAP assay and the NADPH oxidase test indicated that the removal of amino acids 169–197 resulted in a further increase of the GAP activity of the relevant fragment on the non-prenylated GTPases whereas no change was observed in the reaction with prenylated Rac (Figs. 3 and 4). Apparently, this stretch of amino acids is also involved in recognition of the prenyl moiety.

On the basis of our experimental data we propose the model presented in Fig. 5. In the basic state p50RhoGAP is in a closed conformation, where the N-terminal 48 amino acids and the stretch 169–197 at the C-terminal end of the Sec14 domain are involved in the intramolecular interaction restricting the accessibility of the GAP domain. The prenylated small GTPase is able to release the intramolecular restraint and to bring the protein into a more open conformation. In this state the essential amino acid(s) of the GAP domain have free access to the active site of the small GTPase. The following experimental findings indicate that the prenyl group by itself is not sufficient for releasing the autoinhibited state. 1) Soluble geranylgeranyl pyrophosphate is not able to replace the prenyl tail covalently attached to the small GTPase, and 2) prenylated wild-type Rac1 or Rac2 is not able to interact with p50RhoGAP in the two-hybrid system. The proposed model provides another example that the prenyl moiety of the small GTPase plays a significant role not only in the localization of the molecule to hydrophobic surfaces, but also in the interaction with certain proteins (20, 21, 28–30).

Suggesting an intramolecular interaction as means of regulation of the activity of a GAP protein is not unprecedented. In the case of p120RasGAP, the N-terminal PH domain was suggested to bind to the C-terminal catalytic domain and inhibit the GAP activity of the latter (31). Also for the Rho/Rac/Cdc42GAP oligophrenin-1 it has been recently shown that the N-terminal part of the molecule inhibits its RhoGAP activity in fibroblasts (32). In in vitro studies, we had similar indications for p190RhoGAP (11). Autoinhibition released by the small GTPase is a common phenomenon among the targets of different Rho family proteins (33, 34). Recent data also indicate that multiple domains of the effector protein and regions of the GTPase outside of the classical switch I and II are involved in the interaction of Rho family GTPases with their target proteins (35). Further studies have to decide the similarities in the reaction of small GTPases with their target versus regulatory proteins and also, how widespread autoinhibition is among GAs for different subfamilies of the small GTPases. Our studies call attention to the prenyl moiety as a possible additional region of the interaction between small GTPases and their relevant GAs.

Acknowledgments—We thank Marie-Claire Joseph, Laurence Macari, and K. Schmitz for devoted and expert technical assistance.

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