Activity of the Bcr GTPase-activating Domain Is Regulated through Direct Protein/Protein Interaction with the Rho Guanine Nucleotide Dissociation Inhibitor*

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The cycling of Rac GTPases, alternating between an active GTP- and an inactive GDP-bound state, is controlled by guanine nucleotide exchange factors, GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). Little is known about how these controlling activities are coordinated. Studies using null mutant mice have demonstrated that Bcr and Abr are two physiologically important GAPs for Rac. Here, we report that in the presence of RhoGDH, Bcr is unable to convert Rac–GTP to Rac–GDP because RhoGDI forms a direct protein complex with Bcr. Interestingly, RhoGDH binds to the GAP domain in Bcr and Abr, a domain that also binds to Rac–GTP and catalyzes conversion of the bound GTP to GDP on Rac. The presence of activated Rac diminished the Bcr/RhoGDH interaction. Moreover, a Bcr mutant that lacks the ability to promote hydrolysis of Rac–GTP bound to its GAP domain did not bind to RhoGDH in cell lysates, indicating that binding of RhoGDH and Rac–GTP to the Bcr GAP domain is mutually exclusive. Our results provide the first identification of a protein that regulates BcrGAP activity.

The small GTPase Rac functions as a molecular switch. It is active when it is GTP-bound and inactive in the GDP-bound conformation. Rac activity is tightly regulated. Cells contain activating guanine nucleotide exchange factors that catalyze the exchange of bound GDP for GTP, as well as inactivating GTPase-activating proteins (GAPs) that catalyze hydrolysis of the bound GTP to GDP (reviewed in Refs. 1 and 2). Mature Rac proteins are post-translationally modified at their C-terminal ends with a geranylgeranyl group. This modification makes them hydrophobic and therefore allows their association with membranes. To enable transport of Rac in the cytosol, a third class of Rac-regulating proteins exists: Rho family-specific guanine nucleotide dissociation inhibitors (GDIs) function as chaperones and are also able to insert into and extract Rac from membranes (3–9).

Although the designation of chaperone suggests a relatively passive role, GDIs are in fact dynamic transporters, the exact mechanism of which is still incompletely understood (Ref. 10; reviewed in Ref. 11). For example, studies have shown that tyrosine or serine phosphorylation of RhoGDI by Pak1, Src, and protein kinase C can affect its binding to Rac (12–15). Also, the binding of RhoGDI to other proteins, such as ERM (ezrin/radixin/moesin) and neurophin, at the membrane is known to cause release of the transported Rac (16, 17). It still remains unclear whether RhoGDI can bind to Rac in both its GTP- and GDP-bound conformations (9, 18, 19).

Bcr (breakpoint cluster region) and the highly related Abr protein are GAPs for Rac. Bcr and Abr have a Dbl/pleckstrin homology domain and a C-terminal GAP domain (20, 21). Bcr additionally has an N-terminal domain with serine/threonine kinase activity. Null mutant mice lacking Abr, Bcr, or both have been used to demonstrate that these two proteins are important negative regulators of activated Rac in cells of the innate immune system (22). In vivo, Bcr and Abr also regulate Rac functions in other organs, including the inner ear and cerebellum (23, 24).

The regulated Rac activation and deactivation cycle is a key for the proper function of cell motility, inflammatory responses, and endothelial cell barrier function, among others, but the coordination of steps that constitute this process is not precisely known. For example, GAPs such as Bcr and Abr are expected to transiently interact only with GTP-bound Rac, but how Bcr comes into contact with the substrate Rac–GTP or where the product (Rac–GDP) is delivered is not known. A previous study showed that the GAP activity of Bcr toward mature Rac–GTP is blocked in the presence of RhoGDI in vitro, but no exact mechanism was provided (25). Here, we investigated this mechanism in more detail and report an unanticipated direct binding of RhoGDH to the GAP domain of Bcr when it is not occupied with Rac–GTP, which prevents further GAP activity from taking place.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-1 cells were obtained from American Type Culture Collection (Manassas, VA). All tissue culture
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media and supplements were from Invitrogen. COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. Human brain microvascular endothelial cells (obtained from Monique Stins, The Johns Hopkins University) were cultured in RPMI 1640 medium containing 20% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 5 units/ml heparin.

**Plasmids and Antibodies**—Wild-type Bcr constructs and glutathione S-transferase (GST)-tagged full-length wild-type Bcr, p190, and p210 have been described previously (22, 24). GST-Abr was generated by ligation of a 0.4-kb 5′-BamHI/BstEII fragment with a 3′-BstEII/EcoRI fragment into the pLEF vector digested with BamHI/EcoRI. The human Abr cDNA used to make this construct is of the variant 2 type (NM_001092). The 5′-BamHI site in the 5′-untranslated region was introduced by PCR. The Bcr mutants with point mutations in the GAP domain of Bcr, respectively, as described (28).

**Purification of Bacterially Expressed Protein**—Recombinant GST fusion proteins were purified from *Escherichia coli* DH5α as described previously (20). To remove GST, thrombin cleavage was performed (16, 15 units/mg) at 4 °C. Benzmidine-Sepharose 6B beads (Amersham Biosciences) were used according to the manufacturer’s instructions to remove the thrombin. Purified proteins were then concentrated in 1× Tris-buffered saline (10 mM Tris-HCl, pH 7.5, and 50 mM NaCl) using Centricron filters (M cutoff = 10,000; Millipore Corp.). The concentration of purified protein was determined using the BCA protein assay reagent (Pierce) and visually estimated by SDS-PAGE.

**Determination of Rac-GTP Levels and GAP Activity Assay**—A pulldown assay was performed to identify Rac-GTP levels in vivo by binding to the p21-binding domain (PBD) of Pak1. Rac-GTP levels were measured as described (22). GAP activity was determined using a RhoGAP assay kit (Cytoskeleton, Denver, CO) according to the manufacturer’s specifications. Briefly, 100 pmol of GST-BcrGAP fusion proteins was added to an assay mixture containing Rac1 and GTP or together with ≥25 pmol of RhoGDIα protein. After 20 min at 37 °C, developing reagent was added, and phosphate production was measured at 650 nm.

**In Vitro Affinity Binding Assay**—The in vitro binding assay was performed as described previously (31) with minor modifications. Using a 1:1 molar ratio of each protein, bacterially purified GST-BcrGAP (≥100 pmol) and RhoGDIα (≥100 pmol) were incubated for 1 h at 4 °C in binding buffer (1× Dulbecco’s phosphate-buffered saline containing 0.1% Igepal, 0.5 mM dithiothreitol, and 10% glycerol with phenylmethanesulfonyl fluoride, leupeptin, and aprotilin). Subsequently, 50 μl of a 50% slurry of glutathione beads was added, followed by incubation for an additional 1 h at 4 °C. Beads were washed three times with 50 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 100 mM NaCl, 10% glycerol, and 1% Igepal (Nonidet P-40, 0.5 mM dithiothreitol, and 10% glycerol with phenylmethanesulfonyl fluoride, leupeptin, and aprotilin); pelleted; and separated by SDS-PAGE. GTP loading of Rac was done as described previously (18) with minor modifications. Briefly, ≥25 pmol of Rac1 or V12Rac1 was incubated in 50 μl of GTP loading buffer (25 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 4.7 mM EDTA, 0.12 mM MgCl₂, and 100 μM GTP) for 5 min at 30 °C. Additional proteins in 450 μl of binding buffer were added, and complexes were allowed to form for 2 h in total. To evaluate the effect of V12Rac1 on the Bcr/RhoGDI interaction, BcrGAP and RhoGDI were incubated together for 1 h, after which incubation was continued for an additional 1 h in the presence of added V12Rac1. Bound RhoGDIα was analyzed by immunoblotting with anti-RhoGDI antibodies. For other experiments, GST-AbrGAP or GST-BcrGAP(R1090A) was also used instead of GST-BcrGAP.

**RESULTS**

**Bcr Forms a Complex with RhoGDIα in Mammalian Cells**—To investigate the mechanism by which RhoGDI inhibits the GAP activity of Bcr, we cotransfected COS-1 cells with constructs encoding full-length Bcr and RhoGDIα to test whether these proteins interact in vivo. As shown in Fig. 1A, in lysates of such cells, complexes that had been immunoprecipitated with
antibodies against RhoGDI\textsubscript{\textalpha} contained Bcr protein, and conversely, RhoGDI\textsubscript{\textalpha} was detected in immunoprecipitates generated with anti-Bcr antibodies. Notably, we were also able to co-immunoprecipitate endogenous Bcr with RhoGDI\textsubscript{\textalpha} in human endothelial cells, demonstrating that this interaction takes place when these proteins are present at normal concentrations (Fig. 1B). These results show that Bcr can physically and stably interact with RhoGDI\textsubscript{\textalpha} in mammalian cells \textit{in vivo}.

\textit{Bcr Binds to RhoGDI\textsubscript{\textalpha} through Its GAP Domain—}Bcr was originally identified as part of a Bcr-Abl fusion protein, which is responsible for the development of chronic myelogenous leukemia (32). The two most common types of Bcr-Abl fusion proteins, p190 and p210, differ in the exact Bcr moiety, with p190 including only the domain of Bcr that encodes the serine/threonine kinase. The domain structures of p190, p210, and the highly related Abr protein in relation to that of Bcr is shown in Fig. 2A. To investigate whether RhoGDI\textsubscript{\textalpha} can bind to these three Bcr-related proteins, we cotransfected GST-tagged p190, p210, or Abr with RhoGDI\textsubscript{\textalpha}. As shown in Fig. 2B, neither p190 nor p210 was present in RhoGDI\textsubscript{\textalpha} immunoprecipitates. However, the GST-tagged full-length Abr protein did form a complex with RhoGDI\textsubscript{\textalpha} (Fig. 2B).

Bcr contains a domain encoding a serine/threonine kinase and its N-terminal oligomerization domain that mediates the formation of complexes with Bcr-Abl, a deregulated tyrosine kinase. RhoGDI\textsubscript{\textalpha} contains multiple serine/threonine residues as well as tyrosine residues, and phosphorylation of these residues is known to be important for its regulation. We therefore considered the possibility that the interaction between Bcr and RhoGDI\textsubscript{\textalpha} leads to phosphorylation of RhoGDI\textsubscript{\textalpha}. We performed immunocomplex kinase assays with precipitated Bcr, Bcr-Abl, or Bcr plus Bcr-Abl in the presence of GST or GST-RhoGDI\textsubscript{\textalpha}. However, although Bcr and Bcr-Abl clearly autophosphorylated under these conditions, no phosphorylation of RhoGDI\textsubscript{\textalpha} was detected (supplemental Fig. 1).

As the Bcr-Abl proteins (containing the N-terminal end of Bcr) did not show binding to RhoGDI\textsubscript{\textalpha}, we assayed a Bcr construct including only the C-terminal GAP domain for RhoGDI\textsubscript{\textalpha} binding. As shown in Fig. 3A, the GAP domain was readily co-immunoprecipitated with RhoGDI\textsubscript{\textalpha}, and conversely, immunoprecipitation of the Bcr GAP domain also brought down RhoGDI\textsubscript{\textalpha}. We investigated whether the interaction between these proteins is direct or indirect using an \textit{in vitro}
binding assay with bacterially expressed GST-BcrGAP and RhoGDIα. As shown in Fig. 3B, the GST-BcrGAP fusion protein specifically bound with bacterially expressed RhoGDIα in vitro, showing that this complex can form directly without involvement of other molecules. A similar result was obtained with the Abr GAP domain (Fig. 3C).

Bcr binds to the C-terminal end of RhoGDIα—RhoGDIα consists of two structurally distinct regions. The N-terminal region binds to the switch regions of the small GTPase, whereas the C-terminal region provides a binding site for its isoprenylated tail (3, 5). To investigate whether Bcr binds to one or both of the domains, an N-terminal fragment including residues 2–67 (Xpress-RhoGDI-NT) and a C-terminal fragment (Xpress-RhoGDI-CT; residues 68–204) were cotransfected with BcrGAP. As shown in Fig. 3D, the C-terminal fragment of RhoGDIα showed a strong interaction with Bcr, whereas the N-terminal fragment of RhoGDIα exhibited no significant binding. Thus, the C-terminal end of RhoGDIα is sufficient to mediate the binding to Bcr.

RhoGDIα inhibits the GAP activity of Bcr in vivo and in vitro—Hancock and Hall (25) reported that, in vitro, Bcr is unable to act as a GAP toward prenylated Rac-GTP when RhoGDI is present. They suggested that under their experimental conditions, RhoGDI and fully processed Rac-GTP form a stable complex that precludes GAP-stimulated GTP hydrolysis. To examine this in vivo, we investigated the level of active Rac1 (Rac1-GTP) in COS-1 cells transfected with RhoGDIα and BcrGAP. As shown in Fig. 4A, the levels of activated Rac1 were decreased by transfection of the Bcr GAP domain compared with a vector control. The presence of RhoGDIα significantly blocked its GAP activity in vivo.

Hancock and Hall (25) reported that when they loaded Rac protein extracted from the aqueous fraction of a cell lysate with GTP, RhoGDI was unable to block BcrGAP-promoted GTP hydrolysis in vitro. Unmodified, bacterially expressed Rac protein shows very weak affinity for RhoGDI (33), but lack of prenylation does not impair the binding of Rac to the Bcr GAP domain (34). Therefore, we used a bacterially expressed Rac protein and tested BcrGAP activity in the absence or presence of RhoGDI. As shown in Fig. 4B, Bcr was active as a GAP on GTP-loaded, bacterially expressed Rac1 in vitro. However, we found that RhoGDIα specifically inhibited this activity. A similar result was obtained with RhoGDIβ (data not shown). The inhibition of BcrGAP activity was RhoGDI dose-dependent (Fig. 4B).

RhoGDIα and Rac1 share a common binding region in the Bcr GAP domain—Because both Bcr and RhoGDIα can bind Rac1 (5, 22), we investigated whether the presence of Rac1 affects the interaction of Bcr with RhoGDIα. We made use of was incubated with bacterially expressed, glutathione-agarose-linked GST or GST-BcrGAP protein. The interaction was analyzed by immunoblotting using anti-RhoGDI antibodies (upper panel). The recombinant proteins were visualized by Coomassie Brilliant Blue (CBB) staining of the membrane. C, the conditions were similar to those described for B but with GST-AbrGAP protein. D, an Xpress (Xpr)-tagged RhoGDIα- or C-terminal fragment was expressed alone or together with BcrGAP in COS-1 cells. The Bcr GAP domain was immunoprecipitated using anti-Bcr antibodies (C20). The interactions were analyzed by immunoblotting with anti-Xpress antibodies. These results are representative of three independent experiments. WB, Western blot.

FIGURE 3. Interacting domains of Bcr and RhoGDI. A, COS-1 cells were transfected with the indicated constructs. Antibodies used for immunoprecipitation (IP) are indicated below the panels. B, birexially purified RhoGDIα...
wild-type Rac1 as well as N17Rac1, which is locked in a GDP-bound conformation, and V12Rac1, which is permanently in a GTP-bound conformation. Interestingly, the interaction between BcrGAP and RhoGDIα was decreased in the presence of V12Rac1 (Rac1-GTP) (Fig. 5A). In the presence of N17Rac1 or wild-type Rac1, the interaction of Bcr and RhoGDIα was similar to that of cells transfected only with Bcr and RhoGDIα (Fig. 5A), indicating that Rac in its GDP-bound state neither stimulates nor impedes the BcrGAP-RhoGDIα binding.

We have previously described Bcr derivatives that contain point mutations in the GAP domain (22). Of these, the Bcr(R1090A) mutant (with a mutation in the catalytic Arg residue of the Arg finger region) binds to Rac-GTP but has no GAP activity, whereas the Bcr(R1090A/N1202A) mutant retains partial activity (~30% of the wild-type) to hydrolyze the GTP bound to Rac (22). Interestingly, Bcr(R1090A) was completely unable to interact with RhoGDIα in cell lysates, whereas Bcr(R1090A/N1202A) bound to RhoGDIα equally well as the wild-type Bcr GAP domain (Fig. 5B).

Inhibition of Bcr/RhoGDI Interaction by Bcr/Rac-GTP Interaction—These results suggest that when Bcr is bound to Rac-GTP, the interaction of Bcr with RhoGDIα is inhibited. To
examine such interactions in the absence or presence of Rac, we made use of purified proteins. As shown in Fig. 6A, the complex formation between BcrGAP and RhoGDIα was decreased in a dose-dependent manner upon addition of increasing amounts of GTP-loaded Rac1. We also preincubated BcrGAP with RhoGDIα and then added V12Rac1. The amount of RhoGDIα pulled down with BcrGAP was decreased by ~50% compared with the interaction of BcrGAP with RhoGDIα in the absence of V12Rac1 (Fig. 6B). When BcrGAP was first incubated with V12Rac1 and then RhoGDIα was subsequently added, the amount of RhoGDIα interacting with BcrGAP was even further reduced (Fig. 6B).

We also purified bacterially expressed BcrGAP(R1090A) protein. Although this mutant did not bind to RhoGDIα in vivo (Fig. 5B), in vitro (when not bound to Rac-GTP), it was able to form a complex with RhoGDI (Fig. 6C, first lane). The interaction between these two proteins was reduced by addition of Rac-GTP (Fig. 6C, fourth lane).

**Figure 6. Binding of Rac-GTP to Bcr inhibits Bcr/RhoGDIα interaction.**

A, GST-BcrGAP and RhoGDIα (100 pmol each) were incubated alone or with increasing amounts of GTP-loaded Rac1 as indicated, and complex formation was measured by glutathione-agarose pulldown (upper panels) and densitometric scanning (lower panel). Error bars indicate the means of scans of two independently performed experiments. B, GST-tagged BcrGAP (GST-B) protein was incubated with RhoGDIα (Rh; second lane) or first with RhoGDIα and then subsequently with V12Rac1 (V12; third lane) or first with V12Rac1 and then subsequently with RhoGDIα (fourth lane). The amount of RhoGDIα pulled down with Bcr was visualized by immunoblotting (upper panels) and quantitated by densitometric scanning (lower panel). C, GST-tagged wild-type (WT) BcrGAP or the BcrGAP(R1090A) mutant (RA) was incubated with RhoGDIα, and complex formation between RhoGDIα and BcrGAP was analyzed by immunoblotting. In the fourth lane, GDP-bound Rac1 was added to the incubation. The results shown are representative of two independent experiments. D, GST-BcrGAP and RhoGDIα were incubated alone or in the presence of GTP-loaded Rac1. RhoGDIα bound to Bcr was pulled down with glutathione-agarose, and the presence of Rac-GDP bound to RhoGDIα was analyzed by Western blotting (WB) for Rac1 (upper panel). CBB, Coomassie Brilliant Blue.

**DISCUSSION**

Hancock and Hall (25) reported that, in vitro, RhoGDI blocks the GAP activity of Bcr toward Rac. Interestingly, this effect was seen only when prenylated Rac isolated from the membrane fraction was used and not with cytoplasmic (presumed unprocessed) Rac. Chuang et al. (18) demonstrated that RhoGDI also blocks CDC42HsGAP- and p190RhoGAP-stimulated Rac1-GTP hydrolysis. Hart et al. (8) similarly showed that bovine brain GDI inhibits the Cdc42GAP and...
BcrGAP activities on Cdc42, and this was also reported by Sasaki et al. (9) for RhoGDI and RhoGAP activities on Rac1. The proposed mechanism for these effects was a competition between RhoGDI and the GAP for the overlapping binding site on Rac1 or Cdc42: RhoGDI has multiple contact points with mature Rac proteins, including the switch I and II regions as well as the isoprenylated C-terminal end (35), and GAPs also contact the switch I and II regions (36). If the model suggested by Hancock and Hall were correct, bacterially expressed (non-isoprenylated) Rac-GTP would lack affinity for RhoGDI and would be efficiently converted to Rac-GDP by Bcr. In contrast to their results, we found that RhoGDI was able to prevent non-isoprenylated Rac-GTP conversion to Rac-GDP by the Bcr GAP domain. The discrepancy between their and our results could possibly be explained by the different source of Rac: Hancock and Hall used Rac extracted from the aqueous phase of COS-1 cell lysates, the exact modifications of which are unclear, whereas we used bacterially expressed Rac, which is known to be non-processed.

Interestingly, Sasaki et al. (9) reported that RhoGDI binds 10 times more weakly to Rac-GTP than to Rac-GDP. Based on this and our own results, we therefore considered an alternative mechanism for the inhibitory effect of RhoGDI on BcrGAP activity. Because we found that RhoGDI can directly bind to the GAP domain of Bcr both in vitro and in vivo, we propose that the interaction serves to physically block the entry of Rac-GTP into the Bcr GAP domain, thus preventing GAP activity.

Our combined results strongly support a model in which RhoGDIα can bind only to the Bcr GAP domain when that domain is not occupied by GTP-bound Rac. Based on this, it is possible that one of the functions of the Bcr/RhoGDI interaction is to deliver newly converted Rac-GDP from its binding site in the Bcr GAP domain to RhoGDI (supplemental Fig. 3). Because the conversion of Rac-GTP to Rac-GDP is expected to significantly diminish the affinity of the GAP domain for Rac, the binding of Rac-GDP to Bcr is expected to be extremely transient and difficult to detect. Indeed, we have not been able to immunoprecipitate Bcr with N17Rac1.5 However, the finding of a trimolecular complex consisting of BcrGAP, RhoGDIα, and Rac-GDP supports this model.

A second function of the binding of RhoGDIα to the Bcr GAP domain is to inhibit further uptake of Rac-GTP by the Bcr GAP domain. RhoGDIα thus represents the first protein identified that directly regulates the GAP activity of Bcr. A similar mechanism is likely to apply to the regulation of Abr because its GAP domain shares 80% amino acid sequence identity with the Bcr GAP domain, and we showed that it also binds to RhoGDI in cell lysates or as purified protein. We also observed that RhoGDIβ, a RhoGDI that is expressed mainly in hematopoietic cells (4, 37), binds to BcrGAP in transfected COS-1 cells. This result suggests that the GAP activity of Bcr and Abr may be regulated in many cell types by RhoGDI proteins. It will be of interest to see whether this mechanism applies to other RhoGAP proteins.

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