GDP Dissociation Inhibitor Prevents Intrinsic and GTPase Activating Protein-stimulated GTP Hydrolysis by the Rac GTP-binding Protein*

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The majority of the GTP-binding proteins of the Ras superfamily hydrolyze GTP to GDP very slowly. A notable exception to this are the Rac proteins, which have intrinsic GTPase rates at least 50-fold those of Ras or Rho. A protein (or proteins) capable of inhibiting this GTPase activity exists in human neutrophils as a cytosolic protein complexed to (Rho)GDI, we examined the ability of (Rho)GDI to inhibit GTP hydrolysis by Rac.

(Rho)GDI produced a concentration-dependent inhibition of GTP hydrolysis by Rac1 that paralleled its ability to inhibit GDP dissociation from the Rac protein. Maximal inhibition occurred at or near equimolar concentrations of the GDI and the Rac substrate. The ability of two molecules exhibiting GTPase activating protein (GAP) activity toward Rac to stimulate GTP hydrolysis was also inhibited by the presence of (Rho)GDI. The inhibitory effect of the GDI could be overcome by increasing the GAP concentration to levels equal to that of the GDI. (Rho)GDI weakly, but consistently, inhibited GTPγS (guanosine 5'-3-O-(thio)triphosphate) dissociation from Rac1, confirming an interaction of (Rho)GDI with the GTP-bound form of the protein. These data describe an additional activity of (Rho)GDI and suggest a mechanism by which Rac might be maintained in an active form in vivo in the presence of regulatory GAPS.

There is a superfamily of low molecular weight GTP-binding proteins involved in the regulation of a wide variety of cellular functions (1-3). The exact mechanisms of action of these proteins and the macromolecular interactions which they undergo are only now beginning to be defined. The GTP-binding and GTP hydrolytic activities of the low molecular weight GTP-binding proteins are essential elements of their function and regulation (2, 3). There are three identified classes of protein that can regulate these activities (4). GTPase activating proteins (GAPs) stimulate GTP hydrolysis by the ras superfamily members and may participate in the transfer of downstream signals (5, 6). GDP dissociation and GDP/GTP exchange are enhanced by GTP/GDP dissociation stimulators (GDSs) and inhibited by GDP dissociation inhibitors (GDI:s). The latter two regulatory molecules are also able to modulate the interaction of low molecular weight GTP-binding proteins with membranes (4).

The Rac proteins are members of the rho family of GTP-binding proteins, which also consists of the various forms of Rho and CDC42Hs (2). The Rac1 and Rac2 proteins have been shown to regulate the activity of the NADPH oxidase system of phagocytes (7, 8). This complex, multi-component enzyme is crucial for bacterial killing by phagocytic cells, generating superoxide anion (O2), which is converted into a variety of microbicidal products (9). Rac1 has also been reported to modulate the assembly of cytoskeletal elements associated with membrane ruffling (10).

Both functions of Rac require the GTP-bound form of the protein for activity (11). A number of molecules that could potentially regulate Rac activity in vivo have been described. bcr, the product of the gene that is rearranged in Philadelphia chromosome-positive chronic myelogenous leukemias and several chronic lymphocytic leukemias, has been shown to act as a GAP for Rac, as has the related brain protein n-chimaerin (12). The p190 protein, which forms a complex with Ras-GAP subsequent to cell activation by growth factors or transforming tyrosine kinases, can also stimulate Rac GTP hydrolysis (13), as can a GAP originally isolated as a CDC42Hs GAP (Ref. 14; see also “Results”). A GDI protein, which was originally described as an inhibitor of GDP dissociation specific for Rho, has been reported to act on Rac1 as well (15), and a similar or identical GDI active on CDC42Hs has been purified (16).

Like the majority of the low molecular weight GTP-binding proteins, the Rho proteins exhibit a very low rate of intrinsic GTP hydrolysis of about 0.002-0.005 mol/min at 30 °C (17). CDC42Hs has a significantly higher rate of GTP hydrolysis of ~0.015 mol/min even at room temperature (14). In contrast, Rac1 (~ 0.390 mol/min at 37 °C; Ref. 18) and Rac2 (~ 0.198 mol/min at 30 °C; Ref. 19) have rates that are 50-fold greater than that of the Rho proteins at 30 °C. Such high rates of GTP hydrolysis for the Rac proteins suggest that, even in the absence of additional GAPS, Rac1 and Rac2 would rapidly have their active state terminated by the intrinsic conversion

1The abbreviations used are: GAP, GTPase activating protein; GDI, GDP dissociation inhibitor, where (Rho)GDI is meant to describe that GDI which was originally described as a GDI for Rho, but which is also active on Rac1 and Rac2; CDC42Hs, the human homolog of the S. cerevisiae CDC42 GTP-binding protein, previously known as G25K; bcr, breakpoint cluster region; GTPγS, guanosine 5'-3-O-(thio)triphosphate; DTT, dithiothreitol; GDS, GDP/GDP dissociation inhibitor.


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of GTP to GDP. Since GAPs active on Rac do exist, the existence of mechanisms which might decrease the hydrolysis of GTP by Rac1 and Rac2 in vivo is hypothesized. In this communication, we report that human (Rho)GDI is able to inhibit GTP hydrolysis catalyzed by Rac. In addition, we find that GDI also prevents the catalytic action of several GAPs that are active in stimulating GTP hydrolysis by the Rac proteins. These findings establish inhibition of Rac GTP hydrolysis as an additional activity of (Rho)GDI and suggest a mechanism by which Rac might be maintained in a GTP-bound active state in vivo.

EXPERIMENTAL PROCEDURES

Materials—Recombinant unprocessed Rac1 and Rac2 proteins were purified from overexpressing Escherichia coli as described (19). Isoprenyl-modified Rac1 and Rac2 proteins were purified from S. frugiperda membranes after expression using a baculovirus system. C6C424Hs GAP and p190 were gifts from Dr. R. A. Cerione (Cornell) and Drs. R. A. Weinberg and Jeffrey Settleman (Whitehead Institute for Biomedical Research), respectively. BA85 nitrocellulose filters were obtained from Schleicher & Schuell. [35S]GTPyS, [3H]GDP, and [γ-32P]GTP were purchased from Du Pont-New England Nuclear. All other reagents were of the highest grade commercially available.

Expression and Purification of Recombinant Human (Rho)GDI—Human (Rho)GDI was cloned from a Agt1 cDNA library constructed from M5SO-differentiated HL60 cells using polymerase chain reaction amplification (Perkin-Elmer Cetus). Two oligonucleotides were designed as primers based on the Rho GDI genomic DNA sequence, except that Neod and BamHI restriction sites were added to the NH2- and COOH-terminal primers, respectively, to facilitate cloning. After 28 cycles of PCR amplification (55 °C for 1 min, 72 °C for 2 min, 94 °C for 1 min), the generated product was subcloned into a T7 promoter-controlled expression vector, PET-11d (Novagen). Double-strand sequencing was performed by a standard protocol (U. S. Biomedical). E. coli strain BL21(DE3) was transformed with the constructed vector, and production of recombinant GDI was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. For purification of GDI, the cells were harvested by centrifugation, suspended in solution A (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT) plus 1 mM phenylmethylsulfonyl fluoride, and homogenized by sonication. After centrifugation, proteins in the supernatant were precipitated with ammonium sulfate and the fraction from 40-60% saturation containing GDI was collected and dialyzed to remove the ammonium sulfate. The dialyzed sample was applied to a 30-ml DEAE-Sephacel column equilibrated with solution A and eluted in solution B (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 µg/ml bovine serum albumin, and 10 µM GTP (6 x 10^4 cpm/pmol) at 30 °C for 4 min.

To determine the time course of the GDI effect on GTP hydrolysis by Rac proteins, 30 nM human unfractionated Rac complex was incubated with or without 180 nM GDI at room temperature for 5 min. Hydrolysis of GTP was initiated by addition of MgCl2 and GTP to final concentrations of 19 mM and 190 nM, respectively. At the indicated times, samples were taken and the reaction terminated with 2 ml of ice-cold stop solution.

To determine effects of GDI concentration on GTP hydrolysis by Rac protein, GDI at the indicated concentrations was incubated with 80 nM [γ-32P]GTP-Rac complex for 5 min at room temperature. GTP hydrolysis was initiated and terminated at 10 min at room temperature. Binding of [γ-32P]GTP to Rac protein was quantitated by filtration on BA-85 nitrocellulose filters and liquid scintillation counting (19).

RESULTS

Purified human neutrophil Rac2 and recombinant Rac1 and Rac2 were found to have very high rates of GTP hydrolysis (18, 19); however, these rates could still be increased by several GAPs active on Rac (Fig. 3). During attempts to detect Rac2 GAP activity in human neutrophil cytosol, we observed that hydrolysis of [γ-32P]GTP was not enhanced, but occurred at even a slower rate when cytosol was added to the GAP assay. This suggested that a protein existed in neutrophil cytosol which could inhibit GTPrase activity of Rac2. We had shown (19) that Rac2 appears to exist in neutrophil cytosol as a complex with (Rho)GDI, and an assay of neutrophil cytosol revealed the existence of a large amount of GDI activity, as determined by inhibition of [γ-35S]GDP dissociation from Rac1 or Rac2 (not shown). We therefore examined whether (Rho)GDI might inhibit GTP hydrolysis by Rac proteins.

The human form of (Rho)GDI was cloned from a differentiated HL60 library, expressed in E. coli, and purified as described under “Experimental Procedures.” This recombinant protein inhibited the dissociation of [35S]GDP from both Rac1 (Fig. 1A) and Rac2 (not shown), which had been expressed and purified from the membrane fraction of S. frugiperda cells. The human (Rho)GDI was able to almost totally suppress [35S]GDP dissociation from Rac at a molar ratio of nearly 1 to 1 (Fig. 2), consistent with (Rho)GDI forming a stoichiometric complex with Rac. Human (Rho)GDI also inhibited the hydrolysis of [γ-32P]GTP by S. frugiperda-expressed Rac (Fig. 1B). The inhibition of GTP hydrolysis by (Rho)GDI appears to be an effect on the hydrolysis reac-

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GDI Inhibits Rac GTP Hydrolysis

FIG. 1. Inhibitory effects of GDI toward Rac. The inhibitory effects of (Rho)GDI upon the dissociation rate of [3H]GDP (panel A) and hydrolysis of [γ-32P]GTP (panel B) were determined as described under “Experimental Procedures.” The concentration of Rac1 was 80 nM (A) and 30 nM (B), and of (Rho)GDI was 240 nM (A) and 180 nM (B). Data shown are representative of at least 3 experiments. P, post-translationally modified; UP, unprocessed.

FIG. 2. Concentrations of GDI required for inhibition of GDP dissociation and GTP hydrolysis. The concentration of (Rho)GDI was varied as indicated in the presence of 80 nM of Rac1. [3H]GDP dissociation and [γ-32P]GTP hydrolysis were determined as described under “Experimental Procedures.” Data shown are means ± S.E. of values from 3 experiments.

FIG. 3. Inhibition of GAP activity by (Rho)GDI. Panel A, hydrolysis of [γ-32P]GTP by 30 nM Rac1 was determined with (+) or without (−) 100 nM (Rho)GDI, 5 nM CDC42Hs GAP (GAP), or 10 nM p190 GAP (p190). Panel B, hydrolysis of [γ-32P]GTP by 30 nM Rac1 was determined without (open bars) or with (shaded bars) 100 nM (Rho)GDI, and the concentration of p190 GAP was varied as indicated. Results shown are means ± S.E. of values from 3 experiments. Relative amount of [γ-32P]GTP bound (y-axis) is given as percent of control in the absence of GDI or p190 GAP.

FIG. 4. Effect of GDI on [35S]GTPγS dissociation rate. Rac1 was prebound with [35S]GTPγS and dissociation of the radiolabel assayed as described under “Experimental Procedures.” Concentration of Rac1 and (Rho)GDI were 65 nM and 200 nM, respectively. Data are means ± S.E. of values from 3 experiments.

increasing the p190 concentration to that of (Rho)GDI allowed the inhibitory effect to be reversed (Fig. 3B).

In order to determine whether the interaction of GDI with the GTP-bound form of Rac affected the rate of dissociation of GTP, we utilized Rac1 protein prelabeled with [35S]GTPγS. The use of this non-hydrolyzable GTP analog allowed us to

examine dissociation rates in the absence of GTP conversion to GDP. As shown in Fig. 4, GDI produced a small but consistent inhibition of $^{[35S]}$GTPyS dissociation from Rac1.

**DISCUSSION**

We have demonstrated that (Rho)GDI is able to inhibit both intrinsic and GAP-stimulated GTP hydrolysis by Rac proteins. This appears to be due to competition between GDI and GAP for common or overlapping binding sites on Rac. Rho GDI has been shown to bind at least in part to the carboxyl terminus of Rho, as indicated by a number of criteria, including the necessity for post-translational modification of Rho for interaction with GDI (4, 21). In contrast, the binding of GAPs is thought to involve portions of low molecular weight GTP-binding proteins that are nearer to the amino terminus, including the "effector" domain (amino acids 32-40 or Loop 2) and perhaps the conformationally active region of amino acids 59-65 (Loop 4), based upon studies of Ras and Rap GAPS (3, 23, 24). This would be consistent with the ability of both p190 and CDC42Hs GAP to stimulate GTP hydrolysis by unprocessed (E. coli) Rac1 and Rac2. The exact orientation of the carboxyl terminus with regard to Loops 2 and 4 has not yet been resolved in any of the three-dimensional analyses of Ras structure obtained to date (2, 24).

CDC42Hs GAP and p190 have structural and biochemical similarities which place them in a recently recognized family of bcr-related GAPs (25). This family includes bcr, $\eta$-chimaerin, Rho-Gap and/or CDC42Hs GAP, and p85, a subunit of phosphatidylinositol 3-kinase (25, 26). These proteins appear to utilize Rho, Rac, or CDC42Hs as substrates to varying degrees and may bind to these low molecular weight GTP-binding protein substrates at similar, if not identical, sites. It has recently been shown by Hart et al. (27) that a CDC42Hs GDI isolated from bovine brain can inhibit GTP hydrolysis by CDC42Hs and can block the stimulatory effects of both platelet CDC42Hs-GAP and bcr protein on GTP hydrolysis by CDC42Hs. In conjunction with the results presented here, this suggests that inhibition of GTP hydrolysis by GDI may be an important function of this regulatory protein that is common to its interaction with members of the Rho family. It is possible, however, that Rho may differ in this characteristic, as it has a very low intrinsic GTP hydrolysis rate and Rho GDI has been reported not to interact with the GTP-bound form of Rho (4, 20).

The ability of GDI to inhibit GTP hydrolysis by Rac indicates that GDI is able to interact with the GTP-bound form of Rac. Our data are consistent with the view that this interaction would stabilize the GTP-bound form of Rac and protect it from the inactivating effects of various GAPs. We have observed that GAPs active on Rac can inhibit NADPH oxidase activation in a cell-free system. We have also found, as reported by others (11), that the addition of (Rho)GDI to the NADPH oxidase prior to stimulation can block activation of the system due to its ability to inhibit GDP exchange on Rac. It is of interest that a third NADPH oxidase cytotoxic factor referred to as Sigma 1 was isolated by Pick and associates (8) and shown to consist of a complex of Rac1 and RhoGDI. Although Sigma 1 as isolated was capable of enhancing NADPH oxidase activity in a cell-free assay, recombining Rac1 in the same study stimulated oxidase activity only after pre-binding of GTPyS (8). We suggest that Sigma 1 may actually exist as a Rac-GTP/GDI complex. The possibility of an active Rac-GTP/GDI complex as a regulatory component of the human neutrophil NADPH oxidase system is currently being evaluated in our laboratory.

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