Interaction of G protein Gβγ dimers with small GTP-binding proteins of the Rho family

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Abstract **Gβγ dimers of heterotrimeric G proteins have been shown to be important for the translocation of cytosolic proteins to membranes. The involvement of Gβγ in those signaling processes mediated by small GTP-binding proteins of the Rho family was studied using purified proteins. We showed specific binding of bovine brain Gβγ to immobilized GST-Rho fusion proteins. In addition, brain Gβγ, but not transducin Gβγ, was able to inhibit GTPγS binding to GST-Rho in a concentration-dependent manner. GTPγS binding to GST-Rac was also decreased by brain Gβγ whereas nucleotide binding to GST-Cdc42 was not changed. We conclude that Gβγ dimers may participate in the process of membrane attachment and/or other regulations of Rho and Rac.**

Key words: Small GTPase; Rho; Rac; G protein; Gβγ dimer

1. Introduction

Small GTP-binding proteins of the Rho family (Rho, Rac, Cdc42) have been characterized as important regulators of cell functions. Rho promotes the polymerization of actin stress fibers and controls the assembly of focal adhesion complexes [1]. Rac and Cdc42 trigger the formation of lamellipodia and filopodia, respectively [2,3]. Beside their function in actin filament organization, Rho family members play an important role as activators of mitogen-activating protein kinase (MAPK) cascades since Rac and Cdc42 have been shown to regulate the c-Jun-N-terminal kinase and the p38-MAPK pathways [4–6]. The activities of Rho-like GTPases are regulated by several accessory proteins modulating their GDP/GTP-bound state. Guanine nucleotide exchange factors (GEFs), including the oncogene products dbl, lbc, ost and Tiam [7–10], catalyze the conversion of Rho family members to the GTP-bound active state, whereas GTPase-activating proteins (GAPs), including p50 Rho-GAP and p190 [11,12], promote their return to an inactive state, and more regulators probably remain to be discovered. At present, only little is known about how the activities of these regulators are controlled.

Activation of Rho family members results in their translocation from the cytosol to the membrane, followed by an interaction with target and/or regulatory proteins [13]. However, it is not yet known whether Rho, Rac and Cdc42 directly interact with integral membrane proteins or membrane-associated proteins. A growing body of evidence indicates the importance of Gβγ dimers of heterotrimeric G proteins not only as regulators of ion channels, phospholipases or adenylyl cyclases [14] but also in translocation of cytosolic proteins to membranes. It has been shown that Gβγ dimers directly interact with the small GTP-binding protein ARF, suggesting a possible role of Gβγ in vesicular trafficking [15,16]. Moreover, Gβγ mediates translocation of cytosolic kinases, like Raf and the β-adrenergic receptor kinase (βARK), thereby stimulating Raf activation and βARK-induced receptor phosphorylation [17,18].

In order to study the possible involvement of Gβγ in signaling processes mediated by Rho family members, we investigated the interactions between purified proteins. We demonstrate the specific binding of Gβγ to immobilized GST-Rho fusion proteins. In addition, we show that Gβγ inhibits the rate of GTPγS binding to Rho and Rac in a concentration-dependent manner, whereas the GTPγS binding to Cdc42 is not affected by Gβγ.

2. Materials and methods

2.1. Expression of GST fusion proteins

The GST-RhoA, GST-Rac1 and GST-Cdc42Hs were expressed in *E. coli*. The constructs pGEX-2T-Rac1 and pGEX-2T-Cdc42Hs were kindly provided by Dr. A. Hall (London, UK). The expression plasmids for GST-RhoA and GST were obtained from Dr. P. Gierschik (Ulm, Germany). Expression and purification of GST fusion proteins were performed in accordance with published protocols [19]. The plasmids were introduced into the *E. coli* strain TG-1, and the expression of fusion proteins was induced by addition of isopropyl-β-D-thiogalactopyranoside (0.2 mM final concentration). Cells were harvested, lysed by sonication on ice and centrifuged at 10 000 rpm for 10 min. Supernatants were incubated with prewashed glutathione-Sepharose beads (Pharmacia) for 30 min at 4°C. The beads were washed six times with buffer A (50 mM Tris, pH 7.6, 50 mM NaCl and 5 mM $MgCl_2$). To elute GST fusion proteins, the beads were incubated for 2 min at 4°C with buffer B (50 mM Tris, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol and 5 mM glutathione). The beads were pelleted, and the supernatant was concentrated using Centricon 10 tubes (Amicon), diluted 10 times with a buffer containing 10 mM Tris, pH 7.6, 150 mM NaCl, 2 mM $MgCl_2$ and 0.1 mM dithiothreitol and again concentrated. The purified fusion proteins were aliquoted and stored at −80°C. Their concentrations were determined by comparison with bovine serum albumin standards using Coomassie blue stained gels.

2.2. Purification of Gβγ dimers

Membranes from bovine brains were prepared as described [20], and proteins were solubilized with 1% Na-cholate. Heterotrimeric G_i/G_o proteins were isolated by consecutive chromatographic steps on DEAE-Sepharose Fast Flow (Pharmacia), Ultrogel AcA-34 (Serva) and heptylamine-Sepharose [21]. These G_i/G_o proteins were activated with 50 μM $AlCl_3$, 10 mM NaF and 6 mM $MgCl_2$ and separated into Gα subunits and Gβγ dimers by an additional chromatography on heptylamine-Sepharose. Gβγ dimers were then subjected to further chromatography over hydroxylapatite and eluted

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Abbreviations: G protein, heterotrimeric regulatory guanine nucleotide-binding protein; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-(3-*O*-thio)-triphosphate; PAGE, polyacrylamide gel electrophoresis

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in buffer C containing 20 mM HEPES, pH 7.5, 50 mM NaCl, 1% cholate and 40 mM KH_2PO_4. Purified Gβγ was concentrated through an Amicon PM 10 filter (Danvers, MA), adjusted to a final concentration of 22 μM with buffer C and stored at −80°C. Protein concentrations were determined by the method of Lowry [22] with modifications [23].

2.3. Complex formation of GST-Rho with Gβγ

Freshly prepared glutathione-Sepharose beads loaded with GST-Rho (250 μl) were washed with buffer A and incubated for 60 min at 4°C with purified bovine brain Gβγ dimers (500 nM) under constant rotation. The beads were pelleted, and the supernatant was removed. Subsequently, the beads were washed six times with buffer A and eluted three times with buffer B. Eluate and wash fractions were collected and subjected to SDS-PAGE performed on 10% acrylamide gels according to Laemmli [24]. Blotting of proteins separated by SDS-PAGE and immunological detection of filter-bound proteins using antiserum AS 11 (anti-β_{common}) and the enhanced chemiluminescence Western blotting (ECL) system (Amersham) were carried out as described previously [20,25].

2.4. GTPγS binding assay

[^{35}S]GTPγS (1150–1400 Ci/mmol) was purchased from DuPont NEN (Bad Homburg). Samples were incubated with 25 mM Tris (pH 7.6), 1 mM dithiothreitol, 1.5 mM EDTA, 10 μM GTPγS and [^{35}S]GTPγS (5×10^5 cpm per sample) in a volume of 60 μl. Incubation was performed for 15 min (unless stated otherwise in the figure legends) at 26–27°C. Reactions were terminated by addition of 1 ml of ice-cold stopping buffer (25 mM Tris, pH 8, 30 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, 1 mM EDTA). The amount of bound [^{35}S]GTPγS was estimated by filtration and liquid scintillation counting. Nonspecific binding was measured by addition of GTP (final concentration 5 mM) to the reaction mixtures.

3. Results and discussion

In order to characterize the possible interactions between Gβγ dimers of heterotrimeric G proteins and small GTP-binding proteins of the Rho family, we performed an in vitro binding assay using purified proteins (Fig. 1A). Rho, Rac and Cdc42 were expressed as GST fusion proteins in *E. coli* and purified over glutathione-Sepharose beads, whereas Gβγ dimers were isolated from bovine brain membranes by different steps of column chromatography. GST fusion proteins immobilized on glutathione-Sepharose were incubated with Gβγ, washed to remove unbound proteins and eluted from the beads by competition with glutathione. Immunoblot analysis of the eluates with antiserum AS 11 revealed the binding of Gβγ to GST-Rho but not to GST (Fig. 1B). This result indicates a specific in vitro interaction between Gβγ and Rho, pointing to the possibility that Gβγ is involved in the process of membrane translocation of activated Rho.

Most of the proteins which were found to bind in vitro to immobilized GST-Rho, GST-Rac or GST-Cdc42 have been characterized as GEFs or GAPs for this small GTP-binding protein. On the other hand, the oncogene product ect2 was shown to bind specifically to immobilized GST-Rho and GST-Rac but failed to have any catalytic activity as an exchange factor for these proteins, suggesting a possibly modulating effect of ect2 protein [26]. In order to characterize the functional significance of the interaction between GST-Rho and brain Gβγ dimers, we tested the effect of Gβγ on guanine nucleotide exchange of Rho family proteins. Gβγ at a final concentration of 100 nM decreased the binding of [^{35}S]GTPγS to GST-Rho (Fig. 2). Moreover, Gβγ was able to reduce GTPγS binding to GST-Rac. In contrast, the GTPγS binding to GST-Cdc42 was not changed by Gβγ, indicating that Gβγ failed to interact with all members of the Rho family.

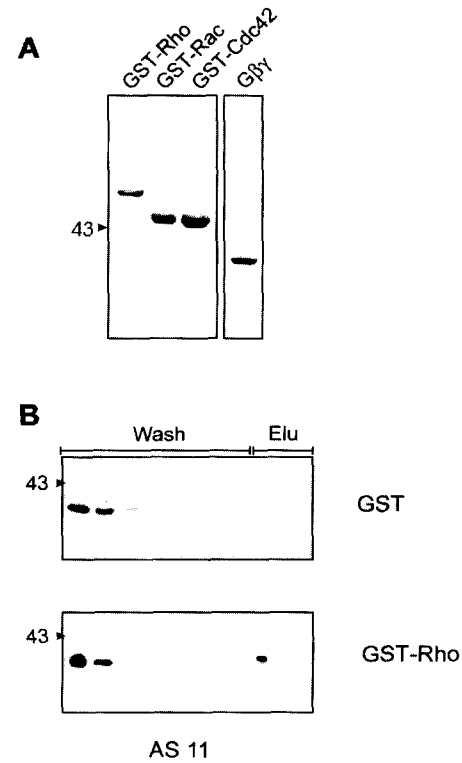

Fig. 1. Interaction of immobilized GST-Rho with purified brain Gβγ. A: GST fusion proteins of Rho, Rac and Cdc42 and bovine brain Gβγ were purified as described in Section 2. Proteins were acetone-precipitated, separated by SDS-PAGE and stained with Coomassie blue (GST-Rho, GST-Rac, GST-Cdc42) or silver (Gβγ). B: Purified brain Gβγ dimers (500 nM) were incubated with GST or GST-Rho immobilized on glutathione-Sepharose beads (250 μl) for 60 min at 4°C. Beads were washed and eluted as described in Section 2. Aliquots (250 μl) of washes (Wash) and eluates (Elu) were supplemented with 10 μg of bovine serum albumine, precipitated with acetone and subjected to SDS-PAGE. Proteins were immunoblotted and probed with anti-β_{common} antiserum AS 11 (dilution of 1:300). The ECL system was used for detection of filter-bound antibodies. The position of a 43 kDa marker protein is indicated.

To compare the effects of different Gβγ combinations on GTPγS binding to Rho family members, we used purified Gβγ dimers from transducin ($G_t\beta\gamma$, equivalent to $G\beta_1\gamma_1$). These proteins had no inhibitory effects on GTPγS binding to GST-Rho, GST-Rac and GST-Cdc42 even at high $G_t\beta\gamma$ concentrations (1.6 μM), indicating marked differences in efficacy between brain and retinal Gβγ (data not shown). Similar differences between these Gβγ combinations have been described for the Gβγ-induced enhancement of βARK-mediated phosphorylation of rhodopsin and β_2-adrenergic receptors [27]. In addition, both Gβγ preparations were shown to vary in their ability to inhibit Ca^{2+}/calmodulin-stimulated type I adenylylcyclase, i.e. retinal Gβγ was approximately 20 times less potent than brain Gβγ [28]. From our results it can be concluded that Rho and Rac have lower affinities to $G\beta_1\gamma_1$ dimers than to Gβγ combinations occurring in non-sensory systems.

The decrease in GTPγS binding to GST-Rho and GST-Rac induced by bovine brain Gβγ was studied in more detail. Fig. 3 shows the effects of different incubation times and of different Gβγ concentrations on the GTPγS binding to Rho family members. The inhibition of GTPγS binding occurred at all incubation times studied and increased with rising concentra-

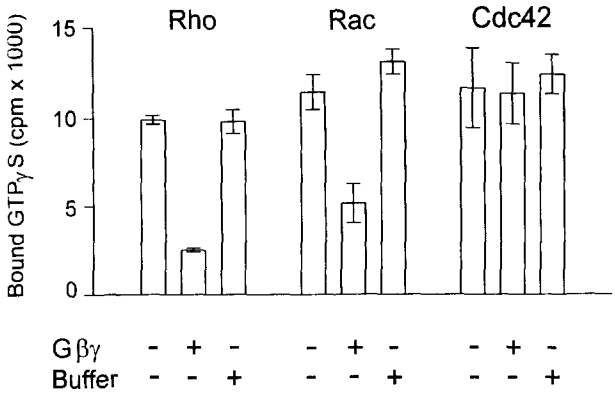

Fig. 2. Effects of purified brain Gβγ on GTPγS binding to Rho family proteins. Binding was performed at 26–27°C in 25 mM Tris (pH 7.6), 1 mM dithiothreitol, 1.5 mM EDTA, 10 μM GTPγS and [^{35}S]GTPγS (5×10^5 cpm per sample). Assays contained 1.5 μg of GST-Rho, 0.9 μg of GST-Rac or 0.8 μg of GST-Cdc42. As indicated, brain Gβγ was added to a final concentration of 100 nM. Gβγ storage solution (buffer) containing 20 mM HEPES (pH 7.5), 50 mM NaCl, 1% cholate and 40 mM KH_2PO_4 (see Section 2) was used as control. After incubation for 15 min samples were filtered, and radioactivity bound to the filter was counted. For determination of nonspecific binding 5 mM GTP was added. The data shown are the average of triplicate determinations (±S.D.) from a single experiment that is representative of three separate experiments.

tions of Gβγ. As shown in Fig. 3B, the amount of GTPγS bound to GST-Rho was reduced by 100 nM of Gβγ to less than 50%, whereas Gβγ at 20 nM showed no inhibitory effect. The inhibition of GTPγS binding to GST-Rac induced by brain Gβγ was also concentration-dependent, with the efficacy of Gβγ in the same range as found for GST-Rho (see Fig. 3C). Thus Rho and Rac appear to have similar affinities for brain Gβγ.

The results presented here provide the first evidence for a direct interaction between Gβγ dimers of heterotrimeric G proteins and the small GTP-binding proteins Rho and Rac. The concentrations of Gβγ found to be effective in inhibiting GTPγS binding to GST-Rho and GST-Rac exceed concentrations reported for Gβγ-induced inhibition of adenylylcyclase type I or for stimulation of adenylylcyclase type II, but they are in the same range as the Gβγ concentrations required for the enhancement of G protein-coupled receptor kinase-catalyzed phosphorylation of rhodopsin, β_2-adrenergic and m_2-muscarinic receptors [27–29].

The physiological significance of an interaction between Rho, Rac and Gβγ remains to be clarified. One possibility is that Gβγ is directly involved in the regulation of the activity state of these small GTP binding proteins, i.e. Gβγ may have a functionally relevant GAP activity for Rho and Rac. It is more likely, however, that Gβγ facilitates or even mediates the association of activated Rho and Rac to the membrane and thereby amplifies the interaction of these small GTP-binding proteins with other proteins. The latter may be proteins acting as regulatory factors for or as substrates of Rho and Rac, like Rho GAPs or several enzymes including phospholipase D, phosphoinositide 3-kinase or phosphoinositide 5-kinase [30–32]. Furthermore, our results suggest that Gβγ dimers are involved in the regulation of distinct subgroups of MAP kinases. Beside the activation of extracellular signal-regulated kinases (ERKs) mediated via the Ras-Raf pathway, which was recently shown to be due to Gβγ-dependent tyrosine phosphorylation of the protein Shc [33], the interaction of Gβγ with the Rho family member Rac may also influence the activation of c-Jun-N-terminal kinases, since Rac and Cdc42 were shown to play an important role in controlling this pathway [4,5].

In conclusion, we show a novel interaction between small GTP-binding proteins belonging to the Rho family and the Gβγ dimers of heterotrimeric G proteins. Whether this inter-

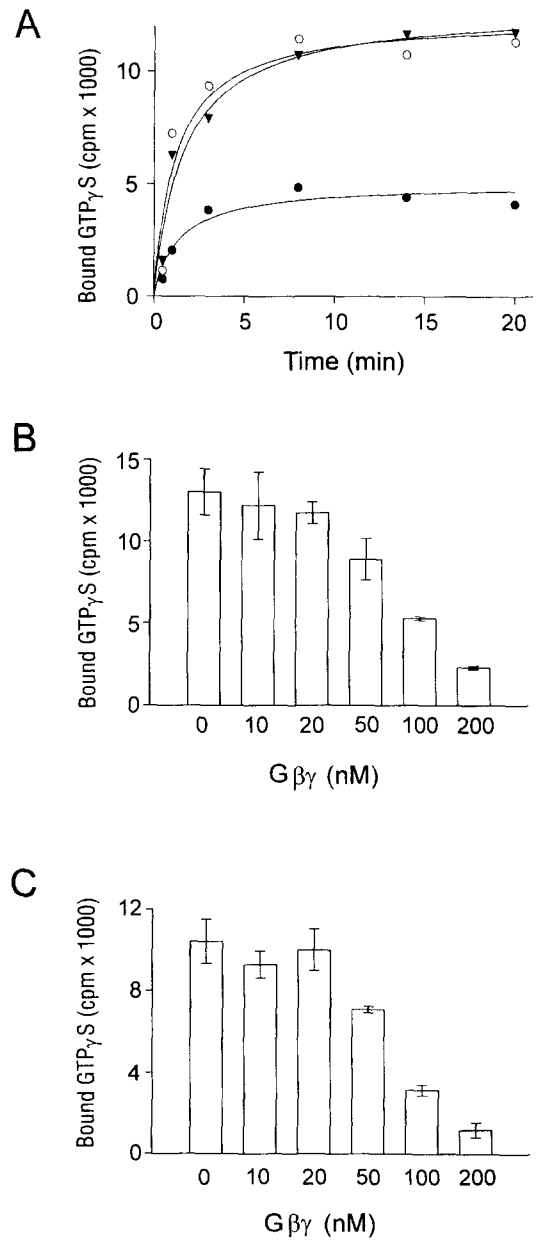

Fig. 3. Time and concentration dependence of Gβγ-induced inhibition of GTPγS binding to GST-Rho and GST-Rac. Binding assay was carried out as described in Secton 2. A: GST-Rho (1.6 μg) was incubated for different times with 10 μM GTPγS and [^{35}S]GTPγS (5×10^5 cpm per sample). Incubation was performed without (open circles) or with addition of Gβγ (100 nM final concentration, closed circles) or an equal amount of Gβγ storage solution (closed triangles). B: GST-Rho (1.8 μg) was incubated in binding buffer with GTPγS and [^{35}S]GTPγS for 15 min in the presence of Gβγ at the indicated concentrations. C: GST-Rac (0.9 μg per tube) was incubated with GTPγS in the presence of increasing Gβγ concentrations. Presented data are means ± S.D. of triplicate determinations from a representative experiment.

action contributes to downstream signalling from G protein-coupled receptors remains to be elucidated.

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