**G Protein βγ Subunits Act on the Catalytic Domain to Stimulate Bruton’s Agammaglobulinemia Tyrosine Kinase**

Received for publication, October 29, 2001
Published, JBC Papers in Press, November 6, 2001, DOI 10.1074/jbc.M110390200

*William E. Lowry and Xin-Yun Huang†*

From the Department of Physiology, Weill Medical College of Cornell University, New York, New York 10021

G proteins are critical cellular signal transducers for a variety of cell surface receptors. Both α and βγ subunits of G proteins are able to transduce receptor signals. Several direct effect molecules for Gβγ subunits have been reported; yet the biochemical mechanism by which Gβγ executes its modulatory role is not well understood. We have shown that Gβγ could directly increase the kinase activity of Bruton’s tyrosine kinase (Btk) whose defects are responsible for X chromosome-linked agammaglobulinemia in patients. The well-characterized interaction of Gβγ with the PH domain of Btk leading to increased kinase activity. Furthermore, we showed that the PH/TH module is required for Gβγ-induced membrane translocation of Btk. The membrane anchorage is also dependent on the interaction of Btk with phosphatidylinositol 3,4,5-trisphosphate, the product of phosphoinositide 3-kinase. These data support a dual role for Gβγ in the activation of Btk signaling function, namely membrane translocation and direct regulation of Btk catalytic activity.

Heterotrimeric (αβγ) GTP-binding regulatory proteins (G proteins) transduce signals from cell surface receptors across the membrane to the inside of cells (1). G proteins pass these extracellular signals to downstream effector molecules by directly interacting with these effectors. Both α and βγ subunits are able to interact with downstream effectors and actively participate in signal transduction (2). Gβγ subunits have been demonstrated to interact with several proteins in the yeast mating pathway (3), G protein-gated potassium channels (4), certain isotypes of adenyl cyclases (in the presence of Goα) (5), certain isotypes of phospholipase C-β (6–8), G protein-coupled receptor kinases (9), phosphoinositide 3-kinase-γ (10), and Btk1 (Bruton’s tyrosine kinase) (11). However, the biochemical mechanism by which Gβγ activates these effectors is not well understood.

We have previously shown that Gβγ could increase the kinase activity of Btk-family tyrosine kinases (11). Btk kinase was the first tyrosine kinase shown to be directly regulated by G proteins (11–13). The Btk family tyrosine kinases include Btk/Atk, Tec, Itk/Tsk, and Bmx/Etk (14). Defects in Btk are responsible for X chromosome-linked agammaglobulinemia in humans and X chromosome-linked immunodeficiency in mice. Gβγ subunits were shown to bind directly to the PH (pleckstrin homology) domain and its adjacent BM (Btk motif) domain (within the Tec-homology (TH) domain) of Btk (15, 16). This interaction was assumed to be responsible for the Gβγ activation of Btk. Here we show that Gβγ can stimulate a purified recombinant Btk lacking the PH/TH module as well as full-length Btk, demonstrating that the effect of Gβγ on Btk kinase activity is actually mediated by an alternate domain. In vitro binding studies further show that Gβγ can bind to both the PH/TH module and the catalytic domain, providing an activation mechanism for Btk. In addition, Gβγ could stimulate the membrane translocation of full-length Btk, but not BtkPH/TH. The membrane anchorage is also dependent on Btk interaction with phosphatidylinositol 3,4,5-trisphosphate since a Btk mutant defective in interaction with phosphatidylinositol 3,4,5-trisphosphate was not able to be translocated to the membrane. These data support a model whereby Gβγ can regulate Btk signaling by increasing its membrane localization and directly stimulating its catalytic activity.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—Sf9 cells were coinfected with baculoviruses containing cDNAs for Gβ1 and Gγ1 with a Hisα tag (17). Cells were harvested after 72 h and resuspended in buffer I (40 mM Tris, pH 8.0, 300 mM NaCl, 10 mM MgCl2, 10 mM 2-mercaptoethanol). Cells were lysed by sonication and Genapol was added to 0.1%. After addition of AlF4−, the lysates were gently agitated for 1 h at 4 °C. Ni-NTA beads were added and incubated for 4 h. After extensive washes in buffer I, Gβγ eluted three times in buffer I with 500 mM imidazole. After concentration and desalting into storage buffer (40 mM Tris, pH 8.0, 50 mM NaCl, 10 mM 2-mercaptoethanol, 10 mM MgCl2, 0.1% Genapol), Gβγ was flash frozen at −80 °C.

**Kinase Assays**—Kinase assays were performed as previously described (12, 13). Purified Btk or BtkPH/TH kinase (from Sf9 cells) (20 nm) in Btk kinase buffer (30 mM Hepes, pH 7.4, 10 mM MgCl2) was combined with 70 μM Btk substrate peptide. 12 μCi of [γ-32P]ATP (3,000 Ci/mmol) was added, and the mixture (total 20 μl) incubated at 30 °C for 5 min. The reaction was stopped by adding Laemmli sample buffer. After 90 °C for 5 min, the substrate peptide was separated on 20% SDS-PAGE gel, dried, and autoradiographed.

**In Vitro Binding Assay**—GST pull-down assay was done as previously described (18). Two micrograms each of Gβγ and GST-Btk fusion proteins were combined as indicated in the figures in 300 μl of binding buffer (40 mM Tris, pH 8.0, 50 mM NaCl, 10 mM 2-mercaptoethanol, 10 μM MgCl2) and incubated at 4 °C for 2 h. Complexes were washed extensively in buffer with 100 mM NaCl and run on SDS-PAGE. The gel was transferred and Western blotted with antibody against Gβ1 (Santa Cruz Biotechnology).

**Immunocomplex Kinase Assay**—Btk immunocomplex kinase assay was done as described (19). Whole-cell extract of HEK-293 cells trans-
were assayed in the absence or presence of increasing concentrations of purified Gγ with or without Gβγ, were pre-cleared with 20 µl of protein A agarose beads, and Btk was immunoprecipitated with 1 µg of polyclonal anti-GFP antibody (Santa Cruz Biotechnology). After washing three times with IP buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 0.5% Nonidet P-40) and three times with kinase buffer (50 mM Hepes, pH 7.4, 10 mM MnCl2), 5 µg of peptide substrate and 10 µCi of [γ-32P]ATP were added, and the mixture was incubated at 30 °C for 30 min. After SDS/PAGE, the gel was autoradiographed and quantified. Fold of stimulation by Gβγ was normalized by the expressed Btk and mutant proteins detected by Western blot with anti-Btk antibody.

Fluorescence Microscopy—HEK-293 cells were plated onto coverslips coated with gelatin. These cells were transfected with plasmids carrying GFP, PHBM-GFP (20), Btk-GFP, Btk(R25C)-GFP, BtkPHPTH-GFP, Btk-GFP plus Gβγ, and Gγγ, BtkPHPTH plus Gβγ, and Gγγ. Btk(R25C)-GFP plus Gβγ and Gγγ, as indicated in the figures. Btk and Btk mutant cDNAs were subcloned into pEGFP-N1 (CLONTECH). The cells were either grown in serum-free media or serum containing media for 48 h and then fixed with 3.7% formaldehyde. The coverslips were then fixed onto slides and imaged using a Zeiss fluorescence microscope.

RESULTS

Gβγ Stimulates the Catalytic Activity of Both Btk and BtkPHPTH—We set out to study the biochemical mechanism by which Gβγ activates Btk. We previously showed that Gγγ subunits could stimulate Btk-family kinase activity (11). Also, there were two reports of the direct interaction of Gβγ with the PH domain and a portion of the adjacent sequences of the TH domain of Btk (15, 16). In light of these binding data, we investigated whether this interaction was responsible for stimulation of the kinase activity of Btk by Gβγ. Previously, utilizing purified recombinant Btk kinases, we have shown that deletion of the PH/TH module has little effect on the intrinsic catalytic activity of Btk on peptide substrates (21). This PH/TH module does, however, mediate protein substrate recognition, as purified Btk lacking this domain (BtkPHPTH) was unable to phosphorylate larger protein substrates but was able to phosphorylate peptide substrates (21).

If Gβγ binding to the PH/TH module of Btk was responsible for the increased kinase activity of Btk, this model would predict that Gβγ should increase the kinase activity of full-length Btk but not the PH/TH-truncated Btk (BtkPHPTH). As shown in Fig. 1, we found that Gβγ could increase the kinase activity of both Btk and BtkPHPTH. Purified Btk (lanes 1–6) or BtkPHPTH (lanes 8–13) were assayed in the absence or presence of increasing concentrations of Gβγ subunits. The kinase activity of Btk and BtkPHPTH was measured by the phosphorylation of a peptide substrate and autophosphorylation. Gβγ increased both the phosphorylation of the peptide substrate as well as the autophosphorylation of both Btk and BtkPHPTH with similar kinetics. These data indicate that the interaction of Gβγ with the PH/TH module is not essential for Gβγ activation of Btk and that, in addition to the PH/TH module, Gβγ must have other contact site(s) on Btk.

Gβγ Directly Binds to Both the PH/TH Module and the Catalytic Domain of Btk—To identify the additional interacting site(s) of Gβγ on Btk, we purified GST fusion proteins of different domains of Btk: PH/TH, GST-SH3S2H2, and GST-CAT (for catalytic domain) (Fig. 2A). Purified Gβγ was incubated with these GST fusion proteins and GST alone (as a negative control). Bound Gβγ was precipitated with glutathione-agarose beads and Western blotted with an anti-Gβ antibody (Fig. 2B). While GST and GST-SH3S2H2 did not precipitate Gβ, GST-TH and GST-CAT did (Fig. 2B). Similar experiments using cell extracts expressing Gβγ as a source for G proteins yielded identical results (data not shown). These data demonstrate that Gβγ has two contact sites on Btk: the PH/TH module and the catalytic domain. Since Gβγ could increase the kinase activity of BtkPHPTH, the interaction with the catalytic domain is likely responsible for the Gβγ activation of Btk.

Gβγ Could Translocate Btk, but Not BtkPHPTH, to the Membrane—Btk is a cytoplasmic tyrosine kinase. It was proposed that membrane translocation is accompanied by activation (22–26). To investigate the activation mechanism further, we used fluorescence microscopy to analyze the subcellular distribution of Btk and BtkPHPTH after stimulation Gβγ (Fig. 3). We fused the GFP to Btk and BtkPHPTH to monitor the distribution of these proteins in living cells. GFP alone was used as negative control; with or without serum, GFP is uniformly distributed throughout the cells (Fig. 3A). The PH/TH domain of Btk alone (PHBM-GFP) was used as positive control (20): addition of serum, which contains a variety of growth factors (some that act through G protein-coupled receptors), caused PHBM-GFP to be redistributed from cytoplasm to plasma membrane (Fig. 3B). While Btk-GFP was uniformly distributed throughout cells in the absence of serum, co-expression of Gβγ or addition of serum led to predominantly plasma membrane localization (Fig. 3C). This indicates that after Gβγ stimulation, Btk is indeed translocated from cytosol to plasma membrane. On the other hand, Gβγ or serum had no effect on the subcellular distribution of BtkPHPTH-GFP, which is mainly in the cytosol (Fig. 3D). Thus, Gβγ could translocate Btk, but not BtkPHPTH, to the plasma membrane.

Gβγ Induced Btk Membrane Translocation in Cells through PI 3-Kinase—Since Gβγ could not translocate BtkPHPTH to...
the membrane, the PH/TH module must be essential for this translocation event. Because the PH/TH module can bind to Gβγ directly and Gβγ is membrane-associated, it is possible that this direct interaction is responsible for Btk membrane anchorage. Also, the PH domain of Btk can bind phosphatidylinositol 3,4,5-trisphosphate (PIP3), the product of phosphatidylinositol 3-kinase (PI3-kinase) (27). To test whether the membrane anchorage of Btk-GFP after Gβγ stimulation is due to the PH/TH module binding to Gβγ or binding to PIP3, we investigated the response of Btk-GFP after Gβγ stimulation (Fig. 4). Mutation of arginine to cysteine at residue 28 within the PH domain reduces the binding to PIP3, but not to Gβγ (16, 28). This mutation also causes X chromosome-linked agammaglobulinemia in patients (29). As shown in Fig. 4A, co-expressing Btk(R28C)-GFP and Gβγ did not lead to subcellular redistribution of Btk(R28C)-GFP, implying that the PIP3-PH interaction is essential for Btk membrane translocation. A prediction of this model would be that Gβγ-induced Btk membrane translocation is PI3-kinase-dependent. To test this, we examined the Btk-GFP subcellular distribution after Gβγ stimulation in the presence of a PI3-kinase inhibitor, LY294002. As shown in Fig. 4B, treatment with LY294002 blocked Gβγ-induced Btk-GFP membrane translocation. A role for PI3-kinase in Btk activation had been observed for other types of receptors (22–26). Therefore, Gβγ-induced Btk membrane translocation depends on PI3-kinase activation by Gβγ and the generation of PIP3.

To test whether constitutive membrane localization is sufficient to activate Btk, we examined the kinase activity of Btk-GFP and myr-BtkΔPHTH-GFP, in which the myristoylation signal sequence from c-Src (amino acid residues 1–15) was fused to the N terminus of BtkΔPHTH-GFP (Fig. 5A). The membrane localization of myr-BtkΔPHTH-GFP was verified by GFP localization on the cell membrane (Fig. 5B). Gβγ increased the kinase activity of Btk-GFP (by ~3- to 4-fold) (Fig. 5C). Btk-GFP had been shown to have similar level of kinase activi-
ity with Btk (26). More importantly, the basal kinase activity of myr-Btk/H9004PHTH-GFP (in the absence of G/H9252/H9253) was higher than that of Btk-GFP (Fig. 5C). Co-expression of G/H9252/H9253 further increased the kinase activity of myr-Btk/H9004PHTH-GFP moderately (by ~1.5-fold) (Fig. 5C). These data imply that constitutive membrane localization could partially activate Btk and that G/H9252/H9253 could further increase the kinase activity of membrane-localized Btk.

**DISCUSSION**

The data shown here reveal a possible activation mechanism by which G/H9252/H9253 regulates a tyrosine kinase. Here we show that G/H9252/H9253 can bind to not only the PH/TH module but also the catalytic domain of Btk. Given that G/H9252/H9253 could activate BtkΔPHTH, it is unlikely that the interaction of G/H9252/H9253 with the PH/TH module transmits allosteric information to the catalytic domain, leading to increased kinase activity of full-length Btk. Rather, the direct contact of G/H9252/H9253 with the catalytic domain is responsible for the activation. Based on our data, we proposed an activation model in which G/H9252/H9253 plays a dual role in both membrane translocation and direct activation of Btk (Fig. 6). Activation of G protein-coupled receptors, such as chemokine receptors in lymphocytes, leads to the release of G/H9252/H9253 subunits from heterotrimeric G proteins. G/H9252/H9253 then directly or indirectly activates a PI 3-kinase, which produces PIP_3. Btk is anchored to the membrane through binding to PIP_3 with its PH/TH module. G/H9252/H9253 then directly activates Btk by directly causing a conformational change in the catalytic domain of Btk, leading to increased kinase activity. Activated Btk could modulate various biological responses such as actin cytoskeletal reorganization, gene expression, calcium mobilization, apoptosis, and cell differentiation (14, 29–31) (Fig. 6). Since addition of the myristoylation signal sequence of c-Src to the N terminus of Btk/H9004PHTH increased myr-Btk/H9004PHTH kinase activity in cells in the absence of G/H9252/H9253, constitutive membrane localization can partially activate Btk in cells.

The direct interaction of G/H9252/H9253 with the catalytic domain of Btk and the subsequent activation of Btk kinase activity might have general implications for the activation mechanism of other G/H9252/H9253 effectors by G/H9252/H9253. G/H9252/H9253 can interact with the N-terminal PH domain of PLC-β2. It has recently been proposed that this binding might lead to the activation of PLC-β2 (32). However, it was also reported that G/H9252/H9253 could interact with the catalytic core of PLC-β2 (33). Although no direct biochemical data are available on a G/H9252/H9253 effect on a PH-deleted PLC-β2, it is...
possible that, similar to Btk, the interaction with the catalytic core rather than the PH domain is responsible for the activation of PLC-β by Gβγ. This model would be consistent with an observation that transfer of the PH domain of PLC-β2 by Gβγ subunits from heterotrimERIC G proteins. Gβγ activates a PI 3-kinase, which produces PI3,4,5P3, Btk is anchored or translocated to the membrane through binding to PI3,4,5P3 by its PT/TH module. Gβγ then activates Btk by directly causing conformational changes of the catalytic domain, leading to increased kinase activity.

Acknowledgments—We thank T. Balla for the PHBM-GFP plasmid DNA, T. Kozasa and A. Gilman for the Gβi and Gγ5 baculoviruses, and the National Cell Culture Center for growing the SF9 cells. We are grateful to Drs. R. Duvoisin, L. Levin, and T. Mauck for reading the manuscript.

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J. Biol. Chem. 2002, 277:1488-1492.
doi: 10.1074/jbc.M110390200 originally published online November 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110390200

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