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Identification of the binding site for Gqα on its effector Bruton's tyrosine kinase

(G protein/signal transduction)

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ABSTRACT **Heterotrimeric G proteins and tyrosine kinases are two major cellular signal transducers. Although G proteins are known to activate tyrosine kinases, the activation mechanism is not clear. Here, we demonstrate that G protein Gqα binds directly to the nonreceptor Bruton's tyrosine kinase (Btk) to a region composed of a Tec-homology (TH) domain and a sarcoma virus tyrosine kinase (Src)-homology 3 (SH3) domain both *in vitro* and *in vivo*. Only active GTP-bound Gqα, not inactive GDP-bound Gqα, can bind to Btk. Mutations of Btk that disrupt its ability to bind Gqα also eliminate Btk stimulation by Gqα, suggesting that this interaction is important for Btk activation. Remarkably, the structure of this TH (including a proline-rich sequence) -SH3 fragment of the Btk family of tyrosine kinases shows an intramolecular interaction. Furthermore, the crystal structure of the Src family of tyrosine kinases reveals that the intramolecular interaction of SH3 and its ligand is the major determining factor keeping the kinase inactive. Thus, we propose an activation model that entails binding of Gqα to the TH-SH3 region, thereby disrupting the TH-SH3 intramolecular interaction and activating Btk.**

Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) transduce signals from a variety of cell surface receptors to intracellular effectors such as enzymes and ion channels (1–3). G proteins function as molecular switches that cycle between the GDP-bound inactive state and the GTP-bound active state. Activation occurs when an extracellular ligand binds to its seven-helix transmembrane receptor, which catalyzes nucleotide exchange of G proteins. Once activated, G proteins can interact with effector molecules, which transmit the receptor signal to generate appropriate biological responses.

The molecular components directly regulated by activated G proteins have remained largely elusive. G proteins can be grouped into four families based on sequence and functional similarities of their α subunits: G_s, G_i, G_q, and G_{12} (3). Intensive efforts have shown that the α subunit of G_s family proteins can stimulate adenylyl cyclase (1, 4). While the α subunit of some members of G_i family proteins is able to inhibit certain types of adenylyl cyclases, the α subunit of transducin, which belongs to the G_i family, can stimulate cGMP-phosphodiesterase (1, 4). Phospholipase Cβ can be stimulated by the α subunit of G_q family proteins (5, 6). A direct target for the α subunit of G_{12} family proteins has not yet been identified. The $\beta\gamma$ subunits of G proteins are also signal transducers (7). The apparent impression that each α subunit has only one effector is indicative of our limited knowledge. Indeed, there are hints that more than one effector may exist for a given α subunit of G proteins (8–10).

We recently demonstrated that the α subunit of G_q protein can directly stimulate a new effector, Bruton's tyrosine kinase (Btk), which is a nonreceptor tyrosine kinase belonging to the Btk/Tec family (11). Defects in Btk are responsible for X-chromosome-linked agammaglobulinaemia (XLA) in humans and X-chromosome-linked immunodeficiency in mice (12–14). Similar to the regulation of adenylyl cyclase (such as type V), which can be stimulated by G_s protein and protein kinase C or inhibited by Ca^{2+} and G_i protein, the activity of Btk can be stimulated by G protein and Src family tyrosine kinases or inhibited by protein kinase C (11, 15–19). Although numerous previous reports have documented that G protein-coupled receptors/G proteins can activate tyrosine kinases and increase tyrosine phosphorylation of cellular proteins, little is known about the mechanism for direct activation of tyrosine kinases by G proteins.

In the present study, we demonstrate that G protein Gqα binds directly to Btk in a region composed of a Tec-homology (TH) domain and a Src-homology 3 (SH3) domain of Btk both *in vitro* and *in vivo*. The physiological significance of this interaction has been shown by the interference of the THSH3 fragment with the *in vitro* stimulation of Btk by Gqα, and by the abolition of *in vivo* activation of TH or SH3 mutated Btk by activated Gqα. Based on these data, a possible activation mechanism is proposed.

MATERIALS AND METHODS

Glutathione *S*-Transferase (GST) Fusion Proteins. To construct GST fusion proteins, DNA sequences corresponding to the indicated regions of human Btk cDNA were amplified by PCR and subcloned into pGEX-2T (Pharmacia). Each construct was confirmed by DNA sequencing. GST fusion proteins were expressed in BL21 cells and purified on glutathione-agarose beads (Sigma). One liter of bacterial culture was grown at 37°C until $OD_{600} \cong 2.0$. Then IPTG (Research Products International) was added to the final concentration of 0.1 mM to induce expression of fusion protein for 8 hr. Bacteria pellet was suspended into 50 ml of ice-cold 1X PBS and then was lysed by 60-sec mild sonication. After solubilization with 1% Triton X-100 at room temperature for 30 min, bacterial lysate was cleared of cellular debris by centrifugation and the cleared lysate was mixed with 1 ml of glutathione agarose beads at room temperature for 30 min with gentle agitation. After fusion proteins bound to the matrix, the complex was washed with 5 ml of 1X PBS three times to remove nonspecifically

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Abbreviations: Btk, Burton's tyrosine kinase; GST, glutathione *S*-transferase; HEK, human embryonic kidney; PH, pleckstrin homology; PR, proline-rich region; TH, Tec-homology; Src, sarcoma virus tyrosine kinase; SH2 or 3, Src-homology 2 or 3.
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bound proteins. In some experiments, beads together with the bound fusion proteins were used. In other experiments, the fusion proteins were eluted from the beads.

***In Vitro*-Binding Assays.** *In vitro*-binding assay was performed as described (20, 21). Human embryonic kidney (HEK)-293 cells were transfected with pCMV-Gqα(Q209L) cDNA. Cells were harvested, and whole-cell extracts were prepared (22–24) 24 to 36 hr later. Five micrograms of purified GST fusion proteins (together with the glutathione-agarose beads) were incubated with $\approx$1 mg of whole cell extract for 2 hr at 4°C and then washed three times with 1X PBS + 0.1% Triton X-100 to remove unbound proteins. Bound Gqα was resolved on 10% SDS/PAGE, electroblotted onto nitrocellulose membrane, and identified with anti-Gqα rabbit polyclonal antibody (C-19, Santa Cruz Biotechnology).

Affinity Chromatographic Binding Assay. *In vitro* affinity chromatographic binding assay was done as described (25, 26). Briefly, 1 μg of purified GST fusion protein (with the glutathione-agarose beads) and 0.05 μg of Gqα-GTPγS or Gqα-GDP (purified from recombinant baculovirus-infected Sf9 cells) (11) were incubated at room temperature for 5 min in 500 μl of the total volume of buffer 0 (20 mM Tris·HCl, pH 8.0/0.5 mM EDTA/20% glycerol/1 mM DTT/0.2% Nonidet P-40/0.5 mM phenylmethylsulfonyl fluoride). Reaction mixture was then loaded onto a glass-beads mini-column and washed three times with 1 ml of wash buffer (buffer 0 with 100 mM KCl). Bound Gqα was eluted with 100 μl of elution buffer (buffer 0 with 1 M KCl and 1% deoxycholate), resolved by SDS/PAGE, and analyzed by Western blot with anti-Gqα rabbit polyclonal antibody (C-19, Santa Cruz Biotechnology).

***In Vivo*-Binding Assay or Coimmunoprecipitation Assay.** Plasmid cDNA for Gqα(Q209L) was transfected in DT40 lymphoma cells with LipofectAMINE method (GIBCO/BRL), and whole cell extracts were made 48 hr later (22–24). One milliliter of whole cell extract ($\approx$1 mg of protein) in immunoprecipitation (IP) buffer was precleared with 20 μl of protein A agarose beads for 30 min at 4°C. Lysates were then incubated with polyclonal anti-Btk antibody (C-20, Santa Cruz Biotechnology) for 2 hr at 4°C. Then 40 μl of protein A agarose beads were added. After 2 hr incubation, the beads were washed three times with 500 μl of IP buffer and three times with wash buffer (22–24). To avoid interference from Ig proteins on Western blot analysis, coimmunoprecipitated proteins were eluted with buffer 0 with 1 M KCl on ice for 30 min. Samples were analyzed with 10% SDS/PAGE and Western blotted with anti-Gqα rabbit polyclonal antibody (C-19, Santa Cruz Biotechnology). For detection of the interaction of endogenous Btk and Gqα, DT40 lymphoma cells were treated with 100 μM carbachol for 5 min before the harvest of cells. In some experiments, atropine (10 μM) was added 5 min before the addition of carbachol.

Competition Assay. Kinase assay with purified Btk was performed as previously described (11). Briefly, purified Btk (1 ng) was combined with 2 μg of Btk substrate peptide and 50 nM Gqα-GTPγS. When indicated, the appropriate amount of purified GST fusion proteins was added to the reaction mixture. Then 10 μCi γ^{32}P-ATP was added, and the mixture was incubated at 30°C for 30 min. The reaction was stopped by adding 20 μl of 2X Laemmli sample buffer. After boiling for 5 min, the substrate peptide was separated on a 20% SDS/PAGE gel and analyzed by autoradiography. The corresponding bands were cut out of the gel and quantified with scintillation counter.

Immunocomplex Kinase Assay. Btk immunocomplex kinase assay was done as described (11, 24). Btk mutant constructs were generated through PCR and confirmed by DNA sequencing. Whole cell extract of HEK-293 cells transfected with wild-type Btk or mutated Btk, with or without Gqα(Q209L), with or without Lyn, were precleared with 20 μl of protein A agarose beads and Btk was immunoprecipitated with 1 μg of polyclonal anti-Btk antibody (C-20, Santa Cruz Biotechnology). After washing three times with IP buffer and three times with kinase buffer, 5 μg of substrate GST-CDB3 fusion protein and 10 μCi γ^{32}P-ATP were added and the mixture was incubated at 30°C for 30 min. After SDS/PAGE, the gel was analyzed with a phosphoimager.

RESULTS AND DISCUSSION

To understand the activation mechanism, we have identified the Gqα contact region on Btk both *in vitro* and *in vivo* and investigated the physiological consequence of this interaction on the activation of Btk by Gqα. The primary structure of Btk is organized into a series of domains: pleckstrin homology (PH) domain (residues 1–138), TH domain (139–215), SH3 domain (216–280), SH2 domain (281–377), and kinase domain (378–659) (12) (Fig. 1*a*). The carboxy-terminal portion of the TH domain contains a proline-rich region (PR) (residues 175–215) (27). These domains mediate protein–protein or protein–lipid interactions in cellular-signaling cascades (28, 29). PH domains bind to specific phospholipids and may be involved in recruitment of proteins to the membrane. SH3 domains are small, β-barrel modules that present a nonpolar groove complementary to peptides in a polyproline-II conformation. The PR segment within the TH domain serves as a ligand for binding to the SH3 domain in the Btk/Tec family tyrosine kinases (30). SH2 domains bind polypeptide segments that contain a phosphotyrosine. We produced GST-fusion proteins for all these individual domains (GST-PH, GST-TH, GST-PR, GST-SH3, GST-SH2, and GST-KIN) and tested each for its ability to bind Gqα (Fig. 1*b-d*). Only active Gqα (such as GTPγS-bound Gqα) can stimulate Btk; therefore *in vitro* binding was performed with whole-cell extracts made from activated Gqα [Gqα(Q209L)] transfected HEK293 cells (11). As shown in Fig. 1*d*, none of these individual separate domains bound Gqα. Given that binding of G protein G$\beta\gamma$ subunits to Btk requires sequences from both the carboxy-terminal portion of PH domain and the amino-terminal region of TH domain of Btk (20, 21), we made GST-fusion proteins with two adjacent domains: GST-PHTH (residues 1–215), GST-THSH3 (residues 139–280), and GST-SH3SH2 (residues 216–377) (Fig. 1 *b* and *c*). We found that only GST-THSH3 fragment could bind Gqα (Fig. 1*d*). The integrity of both TH and SH3 domains is required for the Gqα binding because truncations of either the TH domain (GST-PRSH3) (residues 175–280) or the SH3 domain (GST-THSH3ΔC) (residues 139–247) abolished binding (Fig. 1*d*). Thus, Gqα is capable of binding to a region on Btk containing both the TH and SH3 domains.

To further examine the direct interaction of the THSH3 fragment of Btk with Gqα, we tested purified Gqα and purified GST-THSH3 fusion protein for binding *in vitro*. We found that purified GTPγS-bound Gqα readily bound purified GST-THSH3 fusion protein, whereas GDP-bound Gqα did not (Fig. 2*a*). Binding of GTPγS-Gqα to control GST or GST-PH fusion proteins was negligible. This result further confirms the direct and specific interaction of the THSH3 region of Btk with Gqα.

To assess the physiological relevance of this interaction, we investigated the interaction between Gqα and Btk *in vivo*. Endogenous Btk from lymphoma DT40 cells transfected with activated Gqα(Q209L) was immunoprecipitated by antibodies directed against the carboxy terminus of Btk. Immunoblot analysis showed that Gqα was detected in the immunoprecipitate with an anti-Btk antibody but not in the immunoprecipitate obtained with a control antiserum (anti-MEK) (Fig. 2*b*). Approximately 10% of the expressed Gqα was coimmunoprecipitated with Btk. To detect the interaction of endogenous Btk and endogenous Gqα proteins, we expressed the Gq-coupled m1 muscarinic acetylcholine receptors in DT40 cells and examined the association of Btk and Gqα after stimulation

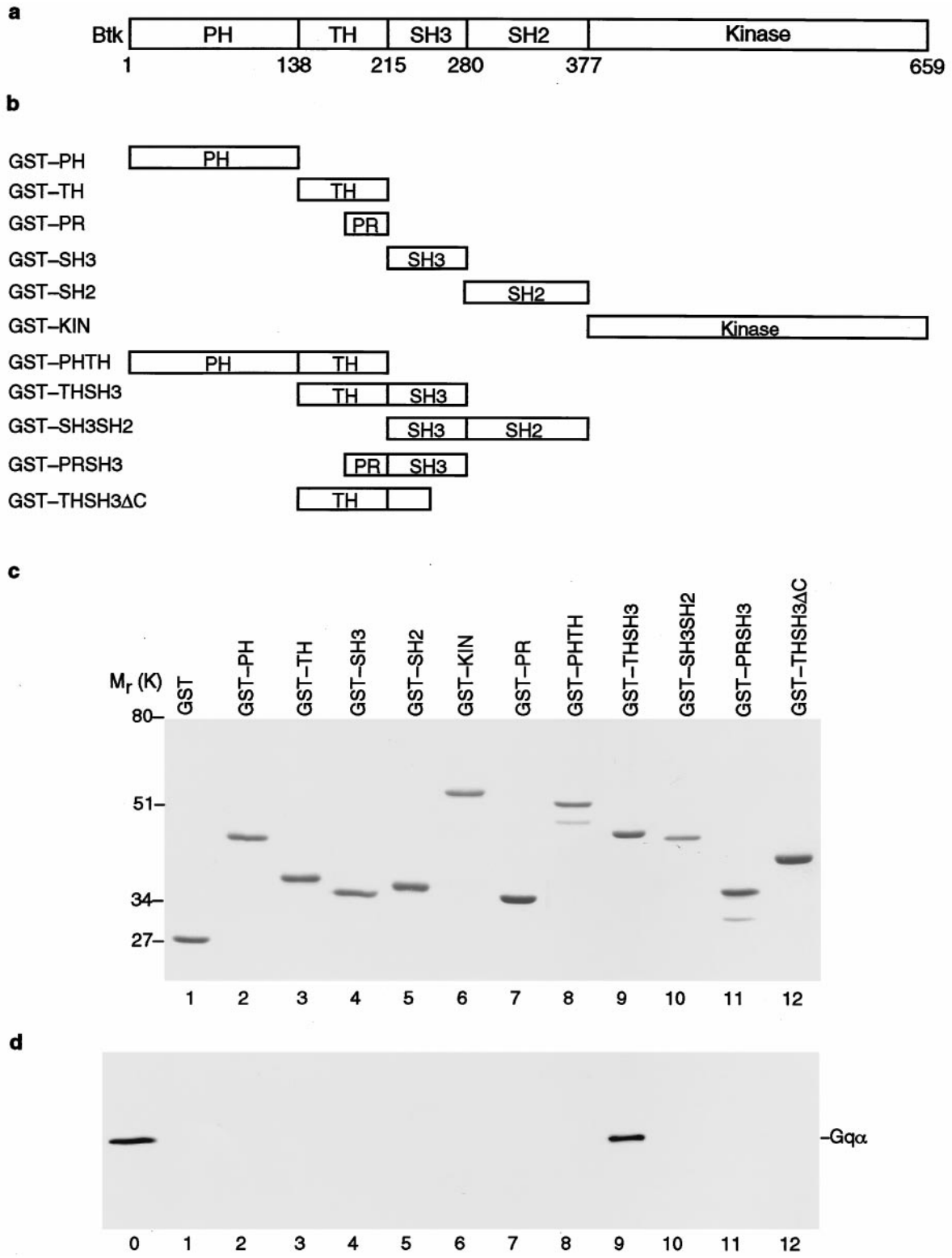

FIG. 1. Gqα association with Btk. (*a*) Schematic representation of Btk and its structural domains. Kin, kinase domain. The numbers at the bottom indicate positions of amino acid residues. (*b*) Representation of GST-fusion protein constructs. (*c*) Coomassie staining of SDS/PAGE of purified GST-fusion proteins. (*d*) Binding of Gqα to the THSH3 region of Btk. GST-fusion proteins were incubated with whole-cell extracts made from Gqα(Q209L)-transfected HEK293 cells. After SDS/PAGE, bound Gqα was detected with anti-Gqα antibody. Lane 0 was loaded with 40 μl of whole-cell extract as control. Lanes 1–12 are loaded with GST-fusion proteins as indicated in *c*.

with the muscarinic receptor agonist carbachol. As shown in Fig. 2*c*, carbachol stimulation induced the coimmunoprecipitation of endogenous Btk with endogenous Gqα. This coimmunoprecipitation was decreased by pretreatment of DT40 cells with the muscarinic receptor antagonist atropine (Fig. 2*c*). Taken with previous data, these results demonstrate that Gqα can interact with Btk *in vivo* as well as *in vitro*.

The functional consequence of this interaction between Btk and Gqα is the stimulation of Btk kinase activity (11). To further establish the importance of the interaction of Gqα and

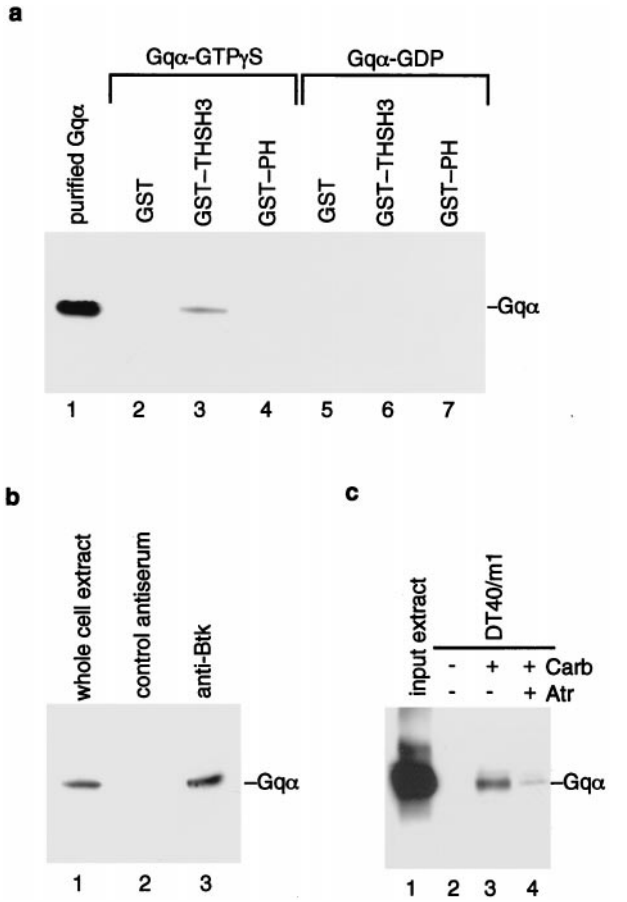

FIG. 2. Gqα interacts with Btk both *in vitro* and *in vivo*. (*a*) Direct interaction of purified Gqα-GTPγS with purified GST-THSH3 fusion protein *in vitro*. Purified Gqα-GTPγS or Gqα-GDP was incubated with glutathione agarose beads containing GST alone, GST-THSH3, or PH. After binding, beads were loaded onto a column and washed. Western blot analysis revealed that Gqα-GTPγS, not Gqα-GDP, bound to the THSH3 fragment. Lane 1 was loaded with purified Gqα as control. (*b*) *In vivo* association of Gqα with Btk. Whole cell lysates were subjected to immunoprecipitation with anti-Btk antibody (lane 3), and immunoprecipitated proteins were analyzed by immunoblotting with anti-Gqα antibody. Precipitation with anti-MEK (lane 2) was included as control antiserum. Also, lane 1 was loaded with whole cell extract as control. (*c*) Coimmunoprecipitation of endogenous Gqα with endogenous Btk. Carbachol (Carb) stimulation induced the coimmunoprecipitation of endogenous Btk with endogenous Gqα (lanes 2 and 3). This coimmunoprecipitation was decreased by pretreatment of DT40 cells with atropine (Atr) (lane 4). Lane 1 was loaded with whole cell extract as control.

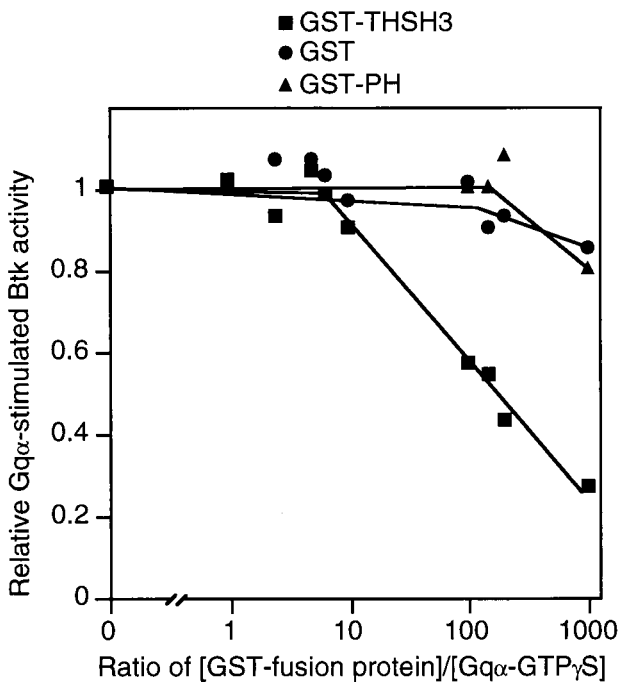

FIG. 3. Inhibition of Gqα stimulation of Btk kinase activity by GST-THSH3. Peptide substrate phosphorylation by Gqα-GTPγS-stimulated Btk (11) was measured in the absence or presence of increasing concentrations of GST (●), GST-THSH3 (■), or GST-PH (▲). Although preincubation of Gqα with GST-THSH3 reduced the ability of Gqα to stimulate Btk, preincubation with GST or GST-PH had no significant effect. Data are from one of three similar experiments.

the THSH3 region of Btk, we tested the ability of GST-THSH3 fusion protein to interfere with the stimulation of Btk kinase activity by Gqα (31) As shown in Fig. 3, increasing concentrations of GST-THSH3 fusion protein suppressed the activation of Btk by active GTPγS-bound Gqα, presumably due to the sequestration of Gqα by the THSH3 fragment. No significant inhibitory effects were observed with GST or GST-PH fusion proteins (Fig. 3).

To further confirm the *in vivo* functional interaction, we examined the *in vivo* stimulation of wild-type Btk and Btk mutants by Gqα (Fig. 4). Because truncation of the N-terminal portion (residues 139–174) of the TH domain (construct GST-PRSH3 in Fig. 1*b*, with intact PR and SH3 domains) abolished Gqα binding as shown in Fig. 1*d*, we made a mutant Btk with residues 139–174 deleted from the full-length Btk [Btk(ΔTHN)] (Fig. 4). This Btk(ΔTHN) mutant has similar basal kinase activity as Btk (Fig. 4). Cotransfection of wild-type Btk and activated Gqα(Q209L) leads to increased kinase activity of Btk (Fig. 4) (11). However, cotransfection of Btk(ΔTHN) mutant with Gqα(Q209L) did not increase Btk kinase activity (Fig. 4), a phenomenon that is probably due to the defective binding of Gqα to the mutant Btk. As a control, this Btk(ΔTHN) mutant could still be stimulated by overexpression of another tyrosine kinase Lyn. Similarly, a SH3 deletion mutant Btk(ΔSH3) (deleting residues 216–280) could not be stimulated by Gqα (Q209L) (Fig. 4). The stimulation of this Btk(ΔSH3) mutant by Lyn also was reduced. It is interesting to point out that deletion of the SH3 domain of Btk did not result in an increase of Btk activity, suggesting that Btk is likely to be regulated at multiple sites or by alternative ways in the absence of the SH3 domain. A third Btk mutant with deletion of the PH domain (removing residues 2–138) could be stimulated by Gqα(Q209L) as well as by Lyn, but less effectively. Therefore, the THSH3 region of Btk is important for the activation of Btk by Gqα.

In summary, we have demonstrated that Gqα binds Btk through a region containing the TH and SH3 domains of Btk both *in vitro* and *in vivo*. The physiological significance of this interaction has been shown by the interference of the THSH3 fragment with the *in vitro* stimulation of Btk by Gqα and by the abolition of *in vivo* activation of mutated Btk by activated Gqα. Mutations in the TH and SH3 domains have been found in X-chromosome-linked agammaglobulinaemia (XLA) patients (12), suggesting that interaction with one or more crucial TH and/or SH3 binding proteins is required for Btk signaling. The identification of the Gqα-contacting site of Btk to the THSH3 fragment immediately suggests a possible activation mechanism of Btk by Gqα.

Regulation of tyrosine kinases has been demonstrated to occur through modular protein–protein interactions (32, 33). The enzymatic activity of tyrosine kinases is maintained at a basal level by intramolecular interactions. The crystal structures of Src-family tyrosine kinases (Src and its close relative,

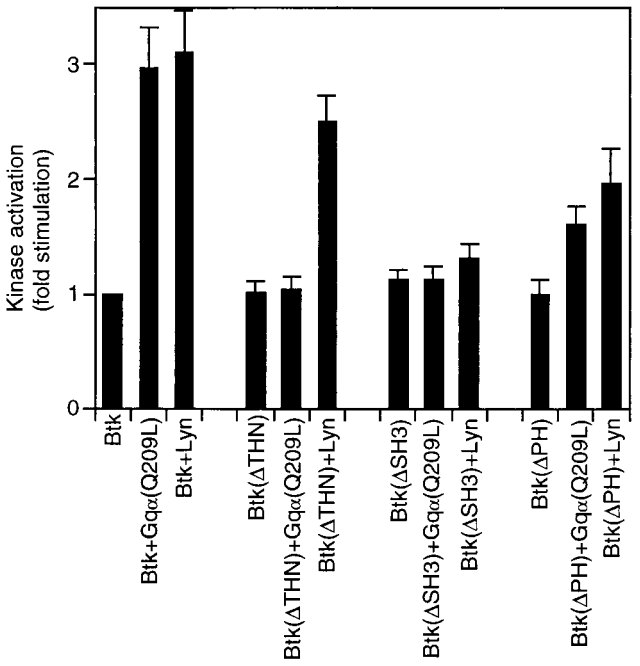

FIG. 4. Mutations in the TH-SH3 region abolished the ability of Gqα to activate the mutated Btk *in vivo*. Whole cell extracts from transfected HEK293 cells were subjected to immunoprecipitation with anti-Btk antibody. Immuno-complex kinase assays were performed with GST-CDB3 fusion protein as substrate (24). GST-CDB3 phosphorylation was quantified by phosphoimaging and relative stimulation fold of kinase activity was shown. Mutant Btk(ΔTHN) has a deletion of the N-terminal portion (residues 139–174) of the TH domain from the full-length Btk. Mutant Btk(ΔSH3) is with deletion of residues 216–280. Mutant Btk(ΔPH) is without residues 2–138. Values shown represent means ± standard deviation of three experiments.

Hck) reveal two intramolecular interactions. The SH2 domain forges an intramolecular interaction with the phosphorylated tyrosine residue in the carboxy-terminal tail (34, 35). The SH3 domain binds the peptide sequence that links the SH2 and kinase domains. These intramolecular interactions lock the kinase in a closed, inactive state (34, 35). Meanwhile, inactive Src kinases have a more closely apposed ATP-binding lobe and a peptide-binding lobe in the catalytic domain, leaving the active site in the cleft disabled. Activation of the kinase presumably involves dissociation of the SH2 and/or SH3 domains from their intramolecular ligands, removing the constraint on the catalytic domain and producing an open, active kinase.

Members of the Btk family are not regulated by carboxy-terminal phosphorylation. However, structural analysis has shown that the SH3 domain does maintain an intramolecular interaction with the PR segment of the TH domain (30). This intramolecular interaction could keep the kinase domain inactive. Therefore, disrupting this intramolecular interaction would be an activating mechanism by allowing the catalytic residues to be realigned. Thus, with the identification of the site of Gqα interaction to the THSH3 region of Btk, we propose a similar activation mechanism that entails binding of Gqα to the THSH3 region leading to the disruption of the intramolecular SH3-PR interaction and activating Btk.

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