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Binding of βγ subunits of heterotrimeric G proteins to the PH domain of Bruton tyrosine kinase

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ABSTRACT **Bruton tyrosine kinase (Btk) has been implicated as the defective gene in both human and murine B-cell deficiencies. The identification of molecules that interact with Btk may shed light on critical processes in lymphocyte development. The N-terminal unique region of Btk contains a pleckstrin homology domain. This domain is found in a broad array of signaling molecules and implicated to function in protein–protein interactions. By using an *in vitro* binding assay and an *in vivo* competition assay, the pleckstrin homology domain of Btk was shown to interact with the βγ dimer of heterotrimeric guanine nucleotide-binding proteins (G proteins). A highly conserved tryptophan residue in subdomain 6 of the pleckstrin homology domain was shown to play a critical role in the binding. The interaction of Btk with βγ suggests the existence of a unique connection between cytoplasmic tyrosine kinases and G proteins in cellular signal transduction.**

Deficient expression or function of Bruton tyrosine kinase (Btk) has recently been identified as the genetic basis for human X chromosome-linked agammaglobulinemia (XLA) and murine X chromosome-linked immunodeficiency (XID) (1–4). Btk contains a single catalytic domain and two well-described protein interaction domains called SH2 and SH3. These features are also found in a large family of cytoplasmic tyrosine kinases closely related to the src gene. A defining feature of Btk structure and its close relatives Itk (5), Tsk (6), Tec (7), and *Drosophila* src (Dsrc28C) (8) is the presence of a highly basic N-terminal region of about 200 amino acids (aa) in length that lacks a myristoylation signal (1).

XLA is characterized by a block in pre-B-cell differentiation in the bone marrow, a generalized loss of functional B lymphocytes, and failure of immunoglobulin production. Patients experience serious bacterial infections that can be life threatening. Mutations that restrict expression, inactivate kinase activity, or alter potential protein–protein interactions by mutation of the SH2 or SH3 domain have been defined in different individuals (1, 2, 9–11). XID is characterized by a mild deficiency of total B-cell numbers and a selective loss of some immunoglobulin subclasses and B-cell subsets, lack of response to some T-cell-independent antigens, and failure to respond to certain cytokines, such as interleukin 5 or interleukin 10, and signals delivered through cell surface structures, including immunoglobulin and CD38 (refs. 12 and 13; M. Howard, personal communication). XID is caused by a mutation in the N-terminal unique region of Btk, which results in an arginine at position 28 of the wild-type enzyme being converted to a cysteine residue. A human mutation in a classical XLA patient has recently been defined as Arg-28 changed to histidine (10).

The N-terminal unique region of Btk contains a pleckstrin homology (PH) domain (14) that is found in numerous signaling proteins including several serine/threonine kinases, GTPases, GTP-activating proteins, nucleotide-releasing factors, and phospholipases (15, 16). Six weakly conserved sequence blocks define the PH domain. Within subdomain 6 a single tryptophan residue is conserved in all characterized sequences (14). The altered arginine residue in XID and XLA is located in subdomain 2 and is conserved in >60% of identified PH domains.

A significant insight to the potential function of PH domains comes from the demonstration that the β-adrenergic receptor kinase (βARK) and several phospholipases that contain PH domains can interact with the βγ subunit complex of heterotrimeric guanine nucleotide-binding proteins (G proteins) (17–21). This type of interaction could potentially recruit βARK or other PH domain-containing molecules to the cell membrane (22, 23). Although Btk is found in the cytoplasm (ref. 1; S.T. and O.N.W., unpublished data), a small fraction relocates to the membrane fraction in mast cells stimulated through the IgE receptor (24) that correlates with activation of Btk kinase activity and an increase in phosphotyrosine content. Although the overall degree of sequence identity between the Btk and βARK PH domains is limited (8% identical residues and 12% conserved residues, see Fig. 1), we wished to test the possibility that the PH domain of Btk could also mediate interaction with βγ subunits. Our data from *in vitro* binding assays and *in vivo* competition experiments strongly support this conclusion and implicate heterotrimeric G protein signaling pathways in the regulation of a tyrosine kinase signaling pathway critical for the development and function of B lymphocytes.

MATERIALS AND METHODS

Materials. For fusion constructs of Btk and glutathione *S*-transferase (GST), fragments of the Btk N-terminal region were generated by PCR using the cDNA of murine Btk (1) as templates and cloned into pGEX vector (Pharmacia). Three constructs, GST-BTK(PH) (aa 1–196), GST-BTK(PH)N (aa 1–73), and GST-BTK(PH)C (aa 94–165), of the Btk unique region were made. GST-βARK contains 222 aa from the C-terminal residue of βARK1 fused to GST (17). GST-SH3 (aa 218–263, the SH3 domain), GST-SH2 (aa 264–382, the SH2 domain), and GST-BTK(Ct) (aa 589–659, a part of the kinase domain and its C-terminal flanking sequence) of murine Btk cDNA were made as control constructs. GST-ABL1 (aa 1044–1149) and GST-ABL2 (aa 731–825) contain the C-terminal unique region of Abl. The GST fusion proteins were purified as described (25). For transfection experiments, the

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Abbreviations: XLA, X chromosome-linked agammaglobulinemia; Btk, Bruton tyrosine kinase; XID, murine X chromosome-linked immunodeficiency; G protein, guanine nucleotide-binding protein; βARK, β-adrenergic receptor kinase; PH, pleckstrin homology; IP, inositol phosphate(s); GST, glutathione *S*-transferase; aa, amino acid(s); M2AChR, M2 acetylcholine receptor.

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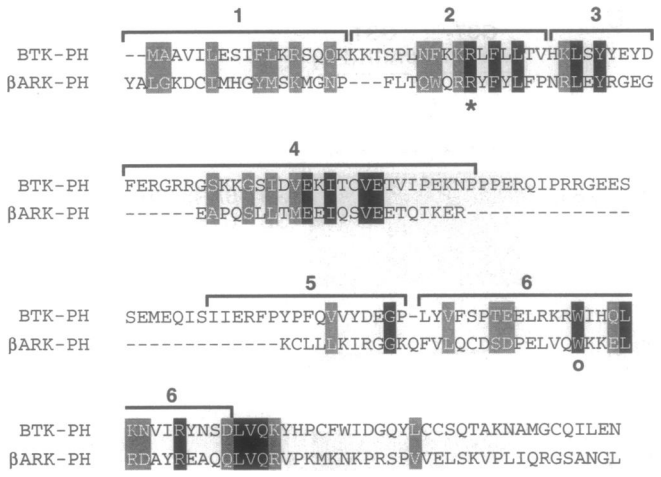

FIG. 1. Comparison of the PH domains of Btk and βARK1. The PH domain is divided into six subdomains and alignment was accomplished by the method outlined by Musacchio *et al.* (14). The N-terminal unique region of Btk contains 217 aa and the PH domain is composed of the first 137 aa. Subdomains 1 (aa 1–17), 2 (aa 18–34), 3 (aa 35–42), and 4 (aa 43–73) are adjacent to each other. There is an insert of 20 aa between subdomains 4 and 5 (aa 94–110) and subdomain 6 (aa 111–137) is adjacent to subdomain 5. The PH domain of βARK1 is composed of aa 559–652. The identical amino residues and the conserved amino residues between Btk and βARK1 are shown by black boxes and shaded boxes, respectively. The arginine that is altered in XID (*) and the conserved tryptophan in subdomain 6 (○) are shown.

Btk fragments were generated by PCR [aa 1–196 for BTK(PH), aa 1–123 for BTK(PH)1–123] and cloned between *Cla* I and *Not* I sites of pCIS expression vector (26). BTK(PH)W124G was made from two PCR fragments by introducing a *Bam*HI site via a silent mutation near Trp-124. The M2 acetylcholine receptor (M2AChR) cDNA and the bacterial β-galactosidase gene were both inserted into pCIS. Anti-β_1 antiserum was generated against the first 14 aa of β_1 protein and anti-γ_2 antiserum was generated against the first 15 aa of γ_2 protein. Anti-Btk antiserum preparation was as described (1).

***In Vitro* Binding of βγ and GST Fusion Proteins.** 293T cells (4×10^6 cells per 10-cm dish) were transfected with 5 μg of β_1 and/or γ_2 cDNA by the calcium phosphate method. After 60–72 hr, cells were harvested, washed twice with phosphate-buffered saline (PBS), and lysed with lysis buffer (0.5% lubrol in PBS, PH 7.0/1 mM phenylmethyl sulfonyl fluoride/1 mM EDTA/10 μg of aprotinin per ml/10 μg of leupeptin per ml). Cells were incubated with the lysis buffer for 20 min on ice and centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatant was dialyzed against 0.01% lubrol/PBS overnight and used as the source of β_1 or γ_2 proteins in subsequent experiments. In typical experiments, 5 μg of GST protein immobilized on glutathione *S*-Sepharose beads (Pharmacia) was incubated with the cell lysate for 2 hr at 4°C and washed four times with 0.01% lubrol/PBS. SDS gel loading buffer was added to the beads and the samples were resolved on a 15% SDS/polyacrylamide gel and electroblotted on a nitrocellulose filter (0.45-μm pore size filter for β_1 blotting, 0.2-μm pore size filter for γ_2 blotting) (Schleicher & Schuell). The blots were probed with anti-β_1 or anti-γ_2 antiserum and processed using the enhanced chemiluminescence detection system (Amersham).

Inhibition Experiments of βγ-Mediated Phospholipase C β Isoform Activation by Btk Constructs. COS-7 cells (1×10^5 cells per well) were seeded in 12-well plates 1 day before transfection. The total amount of DNA in all transfections was 0.9 μg per well. Nine-tenth microgram of DNA mixed with 5 μl of LipofectAmine (Bethesda Research Laboratories) in 0.5 ml of Opti-MEM (Bethesda Research Laboratories) was added to each well and 5 hr later 0.5 ml of 20% fetal calf serum (FCS) in Dulbecco's modified Eagle medium (DMEM) was added. One day posttransfection, the medium was removed and the cells were washed with PBS and incubated in 0.4 ml of inositol-free DMEM with 10% dialyzed FCS containing 10 μCi of *myo*-[2-^{3}H]inositol per ml (NEN; 1 Ci = 37 GBq). Twenty-four hours later, the cells were washed with PBS and 200 μl of inositol-free medium containing 10 mM LiCl was added. The cells were incubated for 10 min at 37°C and then incubated with 100 μM carbachol for 25 min at 37°C (baselines were obtained without carbachol). Each reaction was stopped by adding 200 μl of cold 10% perchloric acid and 20 μl of phytic acid (20 mg/ml) and incubated on ice for 10 min. Then 200 μl of the supernatant was transferred to a microcentrifuge tube and neutralized with 2 M KOH. After centrifugation, the supernatant was loaded on a 0.5-ml AG1-X8 anion-exchange column (200–400 mesh, formate form, Bio-Rad). The separation was done as described (27), 0.5 ml of the elution was mixed with 10 ml of scintillation cocktail BCS (Amersham), and ^{3}H-labeled inositol phosphate [^{3}H]IP levels were counted.

RESULTS

***In Vitro* Binding of the Btk PH Domain to the βγ Dimer of Heterotrimeric G Proteins.** We utilized an immobilized ligand strategy similar to that employed by Koch *et al.* (20) in their analysis of the interaction of the PH domain of βARK to βγ dimers. GST fusion constructs of the entire PH domain of βARK and Btk, as well as an N-terminal fragment (residues 1–73) and a C-terminal fragment (residues 94–165) of the Btk PH domain were prepared (Fig. 2*A* and *Materials and Methods*). The N-terminal fragment contained subdomains 1–4 of the PH domain, while the C-terminal region contained subdomains 5 and 6. Each fusion protein was expressed in bacteria and used following extraction and affinity chromatography on glutathione *S*-Sepharose. Preparations were prechecked for amount of expression of the desired protein and intactness prior to use.

β_1, γ_2, or a combination of both proteins was prepared by transfection of 293T cells as described (*Materials and Methods*). Total cell extracts were prepared and analyzed for binding to control beads containing GST alone, the GST-βARK(PH) fusion protein, or the GST-BTK(PH) fusion protein (Fig. 2*B*). Following extensive washing, bound proteins were released by boiling in SDS-containing buffer, separated by gel electrophoresis, transferred to nitrocellulose membranes, and analyzed by immunoblotting with either β_1-specific or γ_2-specific antibodies. Both GST-βARK(PH) and GST-BTK(PH) could efficiently bind a complex of $\beta_1\gamma_2$ but neither could bind the singly expressed β_1 or γ_2 forms. A small amount of β_1 recovered in the single transfection protocol could represent complexing with endogenous γ proteins in 293T cells. The specificity of these interactions was further tested by evaluating a panel of additional GST fusions with protein fragments including the Btk SH2, SH3 domains and several irrelevant protein fragments derived from the c-*Abl* oncogene. No detectable binding to β_1, γ_2, or a combination of $\beta_1\gamma_2$ was observed (data not shown).

To further detail the relative efficiency of binding of $\beta_1\gamma_2$ to the Btk and βARK PH domains, a dilution series to vary the amount of each GST fusion protein was compared (Fig. 2*C*, top panel). To maintain a constant amount of total protein on the beads for each reaction, an amount of GST protein was added to compensate for dilution of the specific PH domain fusions. Extracts from 293T cells coexpressing β_1 and γ_2 were tested as described above. Immunoblotting to detect the amount of β_1 or γ_2 bound shows that the interaction of the PH domain of Btk with $\beta_1\gamma_2$ is as strong as the PH domain of

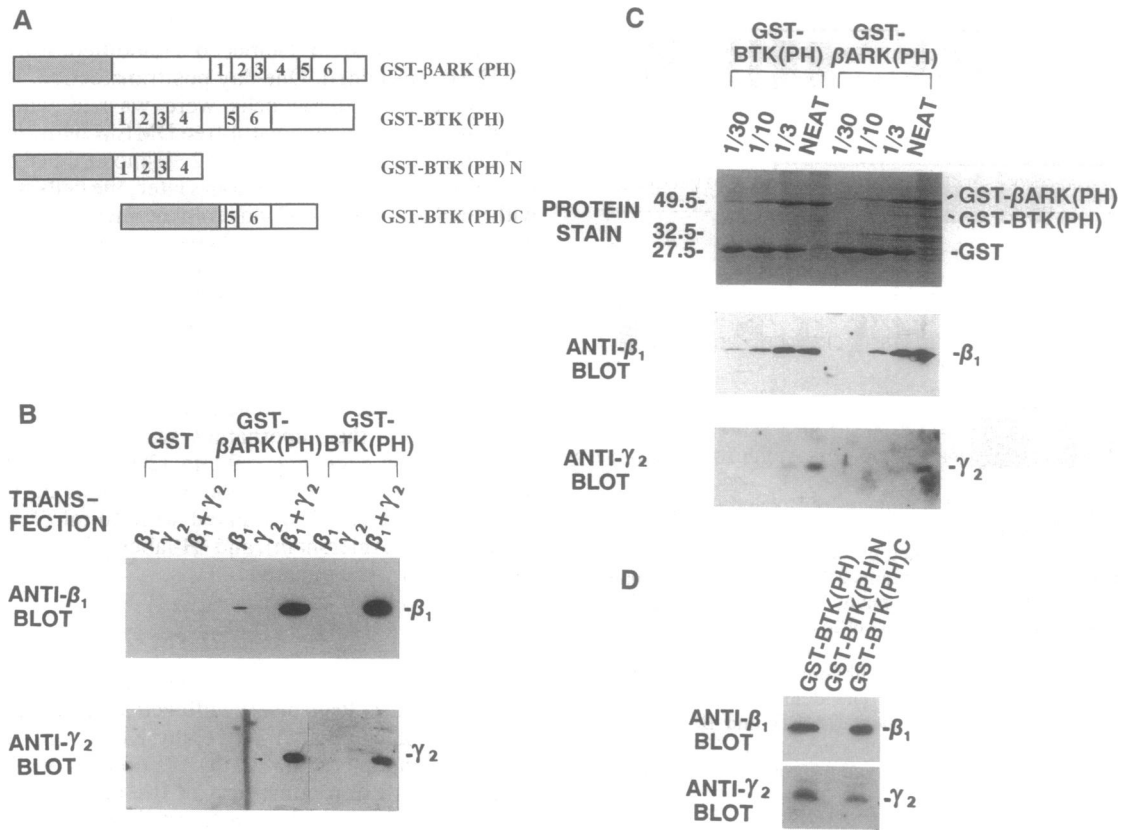

FIG. 2. Detection of binding of $\beta\gamma$ to GST fusion proteins *in vitro*. (*A*) PH domain constructs used in *in vitro* binding assay. GST-βARK(PH) contains 222 aa of the C-terminal region of βARK1. GST-BTK(PH) contains all of the PH domain and its C-terminal flanking sequence (aa 1–196). GST-BTK(PH)N contains subdomains 1–4 and GST-BTK(PH)C contains subdomains 5, 6, and its C-terminal flanking sequence. (*B*) Five micrograms of GST, GST-βARK, or GST-BTK(PH) immobilized on glutathione *S*-Sepharose beads was incubated with β_1 protein, γ_2 protein, or a mixture of β_1 and γ_2 proteins. After incubation, beads were washed and β_1 or γ_2 protein bound on beads was detected by anti-β_1 or anti-γ_2 blot (see text). (*C*) Comparison of the binding affinities of GST-BTK(PH) and GST-βARK(PH) to $\beta\gamma$. The amounts of fusion proteins were sequentially decreased while the total amounts of GST proteins were kept constant at 5 μg with neat GST. GST proteins were incubated with a mixture of β_1 and γ_2, and the binding affinities were compared. (*D*) Binding affinities of truncated PH domain constructs to $\beta_1\gamma_2$.

βARK under these conditions (Fig. 2*C*, middle and bottom panels).

The family of proteins containing PH domains is quite diverse and various signaling pathways could be affected by PH domain interactions. There is no reason to assume that the sequence homology region defined as the PH domain would only mediate a single type of protein–protein interaction. The SH2 domain, which is about 100 aa in length, can mediate both phosphotyrosine-dependent and phosphoserine-dependent interactions with different proteins (28, 29). The PH domain in Btk is organized with subdomains 1–4 over the first 73 residues, followed by a region of no homology of 21 residues and then subdomains 5 and 6 in a run of 43 residues. We wished to test if the interaction with $\beta\gamma$ dimers required the entire Btk PH region or could be mediated by shorter fragments. Fig. 2*D* demonstrates that $\beta_1\gamma_2$ dimers bind as efficiently to a small C-terminal BTK(PH) fragment containing only subdomains 5 and 6 as they do to the entire BTK(PH) domain. No detectable binding to a fragment containing subdomains 1–4 was seen.

***In Vivo* Interaction of Btk PH Domain with $\beta\gamma$ Dimers.** The combined *in vitro* binding studies strongly support the interaction of the Btk PH region with $\beta\gamma$ dimers as a potential mediator of the effects of stimulation of heterotrimeric G-protein-coupled receptors and/or the regulation of a tyrosine kinase-dependent pathway.

To test if $\beta\gamma$ subunits can associate with the Btk unique region *in vivo*, an indirect assay system was employed. It has been shown that cells transfected with M2AChR display a pertussis toxin-sensitive, carbachol-induced hydrolysis of inositol phospholipids. In this pathway, $\beta\gamma$ is dissociated from the activated G-protein trimer and then activates the β isoform of phospholipase C (30). Activated PLC hydrolyses inositol phospholipids, releasing IP. The indirect assay system is based on the concept that this ligand-induced pathway requires free $\beta\gamma$ dimer as the active component. Any coexpressed protein that binds to $\beta\gamma$ would inhibit the accumulation of IP. Fig. 3*A* shows the structures of the potential competitor proteins analyzed for their ability to block heterotrimeric G protein receptor-mediated IP release.

Fig. 3*B* shows the level of IP released in COS-7 cells transfected with the M2AchR upon addition of 100 μM carbachol. The control cells transfected with a vector expressing the lacZ protein display a significant level of IP released. Cells transfected with a plasmid expressing the complete BTK(PH) domain inhibit carbachol induction by 70–75%, indicating that BTK(PH) binds the free $\beta\gamma$ dimer and neutralizes its activity. The inability of BTK(PH) 1–123 to inhibit the carbachol induction suggests that the N-terminal region of the BTK(PH) domain cannot bind $\beta\gamma$ or binds it very weakly.

Tryptophan is the only amino acid conserved in subdomain 6 of PH in all characterized sequences (14). To examine the importance of this conserved tryptophan in $\beta\gamma$ binding, BTK(PH)W124G was constructed in which the conserved tryptophan (Trp-124) was replaced with a glycine. Coexpression of this mutated protein together with the M2AChR led to only a 20–25% inhibition of IP release (Fig. 3*B*). Since this mutated protein is expressed at a higher level than the

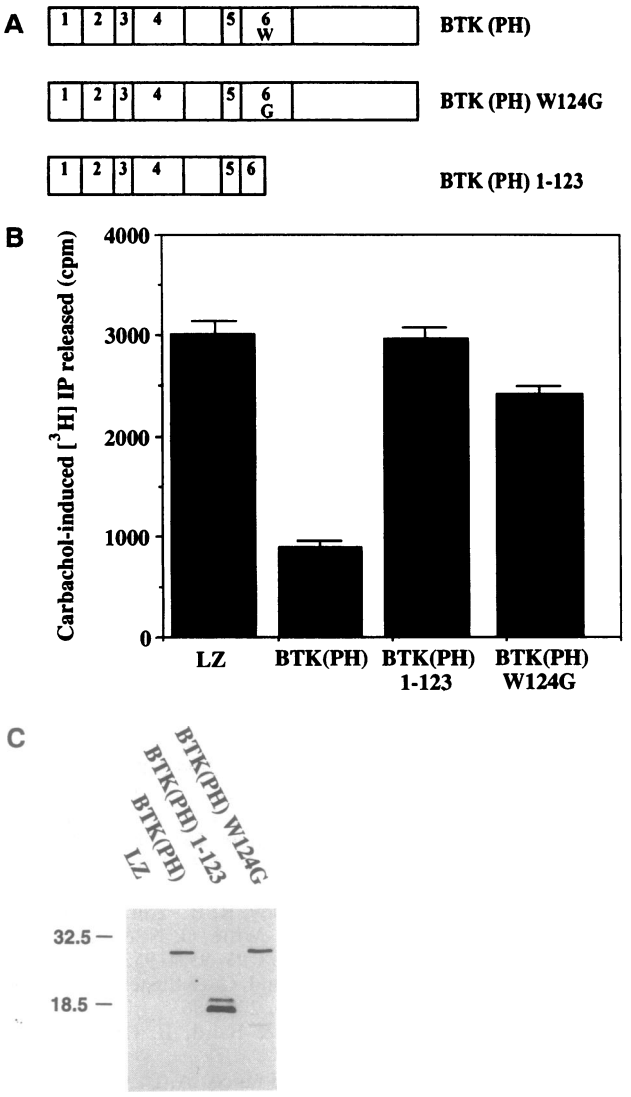

FIG. 3. Effect of coexpression of Btk N-terminal regions on M2AChR-mediated PI hydrolysis. (*A*) PH domain constructs used in this study. The Btk unique region fragment in BTK(PH) is the same as that of GST-BTK(PH) in Fig. 2*A*, while in BTK(PH)W124G the conserved Trp-124 is mutagenized to glycane. In BTK(PH)1–123, subdomain 6 is disrupted at aa 123. (*B*) Carbachol-induced accumulation of [^{3}H]IP in transfected COS-7 cells. COS-7 cells were transfected with M2AChR cDNA (0.1 μg per well) together with one of the following cDNAs (total of 0.8 μg per well): β-galactosidase (indicated by LZ), BTK(PH), BTK(PH)1–123, and BTK(PH)W124G. The cDNAs transfected are indicated in each panel. Cells were stimulated with 100 μM carbachol for 25 min and [^{3}H]IP released was determined. Transfection, labeling, and analysis of [^{3}H]IP are described in the text. Basal level values of [^{3}H]IP released were similar and were in the range of 1200 ± 200 cpm. Data presented are mean values of duplicate determinations in a single experiment and each error bar shows the range of the determinations. Three additional experiments gave similar results. (*C*) Immunoblot analysis of the expression of the transfected Btk constructs. Molecular masses are indicated in kDa.

wild-type BTK(PH) (Fig. 3*C*), the low inhibition is quite likely due to reduced affinity toward $\beta\gamma$.

DISCUSSION

Heterotrimeric G proteins are present in all eukaryotic cells and control metabolic and developmental functions by connecting cell surface receptors to various effectors (31). Receptor activation of a G protein induces a dissociation of the GTP-bound α subunit from the $\beta\gamma$ dimer and results in the generation of two potential regulatory molecules. The regulatory roles of $\beta\gamma$ dimers in several signaling components have recently been reported. These include phospholipase C β_2 (19, 30, 32) and β_3 (33), phospholipase A2 (34), several G-protein-coupled receptor kinases (17, 18, 20), cardiac potassium channel (35), adenyl cyclase (36), phosphoinositol 3-kinase (37) mitogen-activated protein and kinase (38). These observations support the involvement of heterotrimeric G proteins in a broad array of cellular signaling pathways. Our results suggest a possible connection of tyrosine kinases like Btk with heterotrimeric G proteins.

Btk can be activated and tyrosine-phosphorylated upon cross-linking of cell surface immunoglobulins on B cells (39) or of Fc_ε receptors on mast cells (24). No direct binding of Btk to either immunoglobulin or $Fc_\varepsilon RI$ has been detected. Btk is generally found in the soluble cell fraction and is not known to be lipid modified. While the involvement of heterotrimeric G proteins in B-cell receptor or Fc_ε receptor signaling is at present controversial (40–44), it is possible that $\beta\gamma$ dimers could recruit Btk to the membrane following receptor activation.

The C-terminal half of the Btk PH domain plays the major role in the binding of $\beta\gamma$. This is consistent with the observation that the C-terminal half of the PH domain in βARK is critical for the binding of $\beta\gamma$ (20). The C-terminal half of the PH domain of phospholipase C γ, which contains a split PH domain, is sufficient for $\beta\gamma$ binding *in vitro* (21). Two recent reports utilized NMR to define solution structures for the PH domains of pleckstrin (45) and β-spectrin (46). Despite the low amino acid identity among PH domains, these studies suggest that PH domains may all possess a similar three-dimensional structure (seven antiparallel β sheets and a C-terminal α-helix), which supports a common function for many PH domains. The α-helix structure of subdomain 6 is highly conserved (45, 46). The conservation of specific amino acids within the analogous regions of βARK and Btk is quite limited (Fig. 1), but the general structure of the exposed α-helix would be conserved.

The tryptophan in subdomain 6 of the PH domain is unique in being highly conserved. Peptide fragments of the βARK PH domain containing this residue could competitively inhibit the interaction of βARK with $\beta\gamma$ dimers (20). The inhibitory effect of the Btk PH domain for $\beta\gamma$-induced phospholipase C β activation was reduced by replacing this tryptophan with glycine. Touhara *et al.* (21) recently reported that fusion proteins of GST with various PH domains including Btk could bind to $\beta\gamma$ *in vitro*. Thus, despite a relatively weak conservation at the primary sequence level, there appears to be a common protein–protein interaction for many PH domains.

One XLA patient is reported to have an insertion of 7 aa between aa 103 and 104 (9), which disrupts subdomain 5 of the Btk PH domain. The severe XLA phenotype of this patient (undetectable B cells and immunoglobulin levels) suggests that this domain is critical for the biological function of Btk. Phe-102 and Val-104 in subdomain 5 of the Btk PH domain are well conserved in many PH domains and these two residues are implicated to form a hydrophobic core that stabilized the three-dimensional structure of the PH domain (46). The 7-aa insertion between these two residues may destabilize the structure of the Btk in this XLA patient. It will be interesting to determine if such insertions have an inhibitory effect on the binding activity of the Btk PH domain to $\beta\gamma$ dimers. In XID mice, the altered residue is located within the N-terminal region of the PH domain at residue 28 within subdomain 2 (3, 4). The N-terminal portion of the Btk PH domain is not necessary for $\beta\gamma$ binding but could determine interactions with other molecules or regulate the avidity of interaction with different $\beta\gamma$ dimers in the cell.

Many proteins involved in diverse functions, including the β subunit of G protein, have a repetitive 40-aa sequence motif, within which several amino acids and their spatial relationships are highly conserved (WD-40 motif) (31, 47–51). While βARK most likely binds to $\beta\gamma$ of the heterotrimeric G protein, it cannot be ruled out that other PH domain-containing proteins may bind different WD-40 proteins.

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