Mutation of the Pleckstrin Homology Domain of Bruton’s Tyrosine Kinase in Immunodeficiency

Impaired Inositol 1,3,4,5-Tetrakisphosphate Binding Capacity*

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Bruton’s tyrosine kinase (Btk), a cytoplasmic protein-tyrosine kinase, plays a pivotal role in B cell activation and development. Mutations in the pleckstrin homology (PH) domain of the Btk gene cause human X-linked agammaglobulinemia (XLA) and murine X-linked immunodeficiency (Xid). In this paper, we report that the PH domain of Btk functions as an inositol 1,3,4,5-tetrakisphosphate (IP4), inositol 1,3,4,5,6-pentakisphosphate, and inositol 1,2,3,4,5,6-hexakisphosphate (IP6) binding domain (Kₐ of approximately 40 nM for IP₄), and that all of the XLA (the XLA gene replaced by Ser at position 25 (F25S), R28H, T33P, V64F, and V113D) and Xid mutations in the PH domain result in dramatically reduced IP₄ binding activity. Furthermore, the rare alternative splicing variant, with 33 amino acids deleted in the PH domain corresponding to exon 3 of the Btk gene, also impaired IP₄ binding capacity. In contrast, a gain-of-function mutant called Btk*, which carries a E41K mutation in the PH domain, binds IP₄ with two times higher affinity than the wild type. Our data suggest that B cell differentiation is closely correlated with the IP₄ binding capacity of the PH domain of Btk.

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‡ This abbreviation was used: Btk, Bruton’s tyrosine kinase; PH, pleckstrin homology; TH, Tec homology; SH, Src homology; XLA, X-linked agammaglobulinemia; Xid, X-linked immunodeficiency; PKC, protein kinase C; IP₄, inositol 1,3,4,5-tetrakisphosphate; IP₅, inositol 1,3,4,5,6-pentakisphosphate; IP₆, inositol 1,2,3,4,5,6-hexakisphosphate; IP₄K, inositol 1,3,4,5,6-pentakisphosphate; IP₆K, inositol 1,2,3,4,5,6-hexakisphosphate; IP₄K, inositol 1,3,4,5,6-pentakisphosphate; IP₄K, inositol 1,2,3,4,5,6-hexakisphosphate; IP₄K, inositol 1,3,4,5,6-pentakisphosphate; IP₆K, inositol 1,2,3,4,5,6-hexakisphosphate; PCR, polymerase chain reaction; GST, glutathione S-transferase.

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Figure 1. Schematic representation of mouse Btk and characterization of the IP₄ binding properties of GST-Btk-PH. A, schematic representation of Btk: PH domain (shaded box), TH domain (black box), SH3, SH2 (hatched boxes), and SH1 (open box). The Btk homology domain consists of the PH domain and a N-terminal Cys-rich part of the TH domain (see Ref. 13). B, inhibition of specific [³H]IP₄ binding to GST-Btk-PH by various concentrations of inositol phosphates: IP₄ (open circles), IP₅ (squares), IP₆ (triangles), and IP₇ (closed circles). Specific binding is total binding minus nonspecific binding, which was determined in the presence of 5 μM nonradioactive IP₄. The data are means ± S.D. of three measurements.

Subcloned into the BamHI site of pGEX-2T (Pharmacia Biotech Inc.), three clones were selected and verified by DNA sequencing using a BcaBEST dideoxy sequencing kit (Takara Shuzo). One of them has a deletion of amino acids 48–80, which corresponds to exon 3 of the Btk gene (6).

Preparation of GST Fusion Proteins—cDNA encoding the PH domain of Btk was amplified by PCR with primers 3 and 4 (sense, amino acid residues 1–5; 5'-CCGATCCGAAGCTATGGCCGCAGTGAT-3'; antisense, amino acid residues 159–165; 5'-CGAATTCGAGCCGATCATTTTGG-3'), respectively. After digestion with BamHI and EcoRI, the cDNA inserts were subcloned into the BamHI-EcoRI site of pGEX-2T and verified by DNA sequencing. The PH domain of the mouse Btk was expressed as glutathione S-transferase (GST) fusion proteins in Escherichia coli JM109 and purified by glutathione-Sepharose 4B chromatography (Pharmacia) according to the manufacturer's recommendations. GST-Btk-PH coded for amino acids 1–165 of mouse Btk; GST-Btk-PH(V64F), and GST-Btk-PH(V113D) were produced by the same methods.

Measurement of [³H]Ins1,3,4,5-P₄ Binding to GST Fusion Proteins—GST fusion proteins (1 μg or 200 μg) were incubated with 9.6 nM [³H]IP₄, (DuPont NEN) in 50 μl of 50 mM HEPES-KOH, pH 7.2, for 10 min at 4 °C. The sample was then mixed with 1 μl of 50 mg/ml γ-globulins and 51 μl of a solution containing 30% PEG6000 and 50 mM HEPES-KOH, pH 7.2, and placed on ice for 5 min. The precipitate obtained by centrifugation at 10,000 × g for 5 min was solubilized in 500 μl of Solvable (Packard Instrument Co.) and radioactivity was measured in Aquasol 2 (Packard) with a liquid scintillation counter (16). Inhibition of specific [³H]IP₄ binding to GST fusion proteins was also performed in the above reaction mixture containing various inositol phosphates.

Figure 2. Mutational analysis of the IP₄ binding domain of Btk. A, sequence similarity between the Btk homology domains (or PH domains) of human Gap1 (hsGap1H, residues 569–704; GenBank™ accession number X93999) (see Ref. 23), rat brain Gap1™ (rnGap1m, residues 600–736; GenBank™ accession number D30734) (see Ref. 13), Dro sophila Gap1 (dmGap1, residues 757–894; GenBank™ accession number M86655) (see Ref. 24), mouse TecII (Tec, residues 1–143; GenBank™ accession number J00215) (see Ref. 21), mouse Itk (Itk, residues 1–149; GenBank™ accession number L06019) (see Ref. 22), and mouse Btk (Btk, residues 1–165; GenBank™ accession number L08967). Residues that are identical in at least four sequences are boxed. XLA and Xid mutations are indicated by arrowheads, and the Btkmutation (see text) is indicated by an asterisk. The Roman numerals at the top indicate the subdomain of PH (see Ref. 7). The underlined sequence in Btk corresponds to exon 3 of the Btk gene, which was spliced out in BtkΔE3. B, effect of XLA and Xid mutations on IP₄ binding to the PH domain of Btk. GST fusion proteins (200 ng or 1 μg) were analyzed by [³H]IP₄ binding assay as described under “Materials and Methods.”

RESULTS

IP₄ Binding Properties of the PH Domain of Btk—To determine whether the PH domain of Btk serves as an IP₄ binding site, the PH domain was expressed as a GST fusion protein (GST-Btk-PH), and IP₄ binding activity was tested. Consistent with the results of our previous study of the PH domain of Gap1™ (12), GST-Btk-PH bound IP₄ with a Kₘ value of approximately 40 nM. The Bₘₐₓ value was calculated at 0.27 pmol/pmol of protein by GraphPad-Prism (version 2.0), indicating substoichiometric binding of IP₄ to GST-Btk-PH. Such substoichiometric binding was also observed in the case of synaptonatgin II (15). This may result from instability of the IP₄-sensitive form of GST-Btk-PH. Inositol phosphate binding specificity was also analyzed in competition experiments (Fig. 1B). Competitive potencies in decreasing order were IP₄ > IP₅ ≥ IP₆ > IP₇. IP₅ and IP₆ were less effective than IP₄, and IP₇ was almost completely inactive. Thus, the PH domain of Btk seems to function as an IP₄, IP₅, and IP₆ binding site.

Effect of XLA and Xid Mutations in the PH Domain on IP₄ Binding Capacity—To examine the effect of XLA and Xid mutations on IP₄ binding capacity, six mutants of GST-Btk-PH carrying XLA and Xid mutations were produced (see “Materials and Methods”). PH domains were divided into six subdomains (7), and these mutations were distributed in subdomains II (F25S, R28C, R28H, and T33P), IV (V64F), and VI (V113D) (Fig. 2A). The IP₄ binding activity of GST-Btk-PH(F25S), GST-Btk-PH(R28C), and GST-Btk-PH(R28H) was reduced to less than 15% of that of GST-Btk-PH and GST-Btk-PH(T33P), GST-Btk-PH(V64F), and GST-Btk-PH(V113D) displayed almost no significant IP₄ binding (Fig. 2B).

An alternative splicing form of Btk, in which 33 amino acids within the PH domain had been deleted (amino acid residues...
Btk homology domain was amplified by reverse transcriptase PCR with primers 3 and 4 (see "Materials and Methods") in the presence of 0.7 μCi of [α-32P]ATP (Amersham). The cycling conditions are denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 3 min for 27 cycles, in which PCR products are increased exponentially (data not shown). The amplified PCR products were electrophoresed on a 3.5% polyacrylamide gel and visualized by autoradiography. The proportion of Btk-ϕAPE3 to Btk-ϕH was estimated by Fuji Bioimage Analyzer (Fuji Photo Film Co.) using imaging plate. The size of the molecular weight markers (λ/Sty1) is shown as base pairs at the left of the panel. The PCR products (arrows) were purified by Gene clean kit II, digested with BamHI and EcoRI, subcloned into the BamHI-EcoRI site of pGEX-2T vector, and then verified by DNA sequencing.

48–80), was isolated in the course of cloning mouse Btk cDNA. This form of Btk (BtkΔE3) was expressed at a much lower level in the adult mouse spleen (3–5% of normal Btk-ϕH; Fig. 3). The effect of deletion of subdomain IV (Fig. 2A, underlined) on IP4 binding capacity was also examined. GST-Btk-ϕAPE3 did not exhibit any significant IP4 binding activity (data not shown).

Recently, Li et al. (14) isolated a constitutive activated form of BtK, designated Btk*, that contains a point mutation (E41K) in the PH domain (asterisk in Fig. 2A). Btk* induces fibroblast transformation by enhancing transphosphorylation of Tyr-551 by endogenous Src family tyrosine kinases and autophosphorylation of Tyr-223 in the SH3 domain (17, 18). As shown in Fig. 4, GST-Btk-ϕH(E41K) bound IP4 and IP5 with the same affinities as those of GST-Btk-ϕH, but displayed about two times higher affinity for IP6 (K of approximately 60 nM) than wild type. Furthermore, the inositol phosphate binding specificity of GST-Btk-ϕH(E41K) was slightly different from that of the wild type. The competitive potencies were IP4 ≥ IP5 ≥ IP6, ≥ IP3.

**DISCUSSION**

In our preceding paper we showed that the PH domain of Gap1 binds IP4, IP5, and IP6 (12). Although the PH domains are generally divergent, the PH domain of Gap1 is much more homologous to that of Btk than other PH domains and designated Btk homology domain (4, 13). In this study we have shown that Btk is an IP4-, IP5-, and IP6-binding protein, and that mutations in the PH domain observed in XLA and Xid, or deletion within the PH domain by alternative splicing, result in a dramatic reduction in IP4 binding capacity. We have also shown that the gain-of-function mutant of Btk, Btk*, binds IP6 with higher affinity than that of wild type Btk. These findings suggest that B cell differentiation is closely correlated with the IP4, IP5, and IP6 binding capacity of the PH domain of Btk.

By analogy with the recent x-ray crystal structure of the high affinity complex between the PH domain from phospholipase C-δ1 and IP4 (19), Phe-25 and Arg-28 may directly link to the 1-phosphate and 5-phosphate of IP4, respectively, via hydrogen bonds. Thus, mutation of these two residues decreases IP4 binding capacity. In addition, substitution by Lys at position Glu-41 enhanced IP6 but not IP4 or IP5 binding capacity, probably as a result of interaction of Lys with the 2-phosphate of IP4.

Other mutations (T33P, V64F, and V113D), or deletion of subdomain IV, may distort the IP4 recognition site or the PH domain structure itself, because they displayed almost no significant IP4 binding activity.

What is the function of IP4, IP5, and IP6 binding to the PH domain of Btk? IP4 is known to increase during hematopoietic cell differentiation and accumulates at higher concentrations than IP4 and IP3 (20). These observations raised the possibility that IP4 is a physiological ligand and activates Btk function in vitro, although the activation mechanism remains unknown. One possibility is that IP4 disrupts the interaction between the PH domain of Btk and certain PKC isoforms and overcomes the negative regulation by PKC (11).

In this study, we showed that all of the XLA and Xid mutations in the PH domain impaired IP4 binding capacity. Since Tec (21) and Itk (22), other members of Btk/Tec/Itk protein-tyrosine kinase family expressed in hematopoietic cells, also have the similar PH domain and bind IP4, IP5, and IP6, we propose that these inositol polyphosphates may be involved in hematopoietic cell differentiation by activating the Btk/Tec/Itk family.

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*IP₄ Binding Properties of PH Domain from Btk*