Mutational analysis of the SH2-kinase linker region of Bruton’s tyrosine kinase defines alternative modes of regulation for cytoplasmic tyrosine kinase families

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Abstract

Bruton’s tyrosine kinase (Btk) plays critical roles in B cell development and activation. Mutations of Btk cause X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency in mice. An Src homology domain 2-kinase linker region exists in all Src, Abl, ZAP70/Syk and Btk/Tec non-receptor tyrosine kinase families. Missense mutations in the Btk linker region can cause XLA, supporting an essential role for this protein segment. We investigated the regulatory role of the linker region in Btk function by mutational analysis. XLA-causing mutations L369F and R372G abolished Btk-mediated calcium response without affecting Btk protein stability and kinase activity significantly. Although mutation of a well-conserved tryptophan (W260A) in the linker region of the Src family kinase Hck has been shown to cause a hyperactive kinase, an analogous mutation in Btk (W395A) dramatically decreased Btk kinase activity. Tyrosine phosphorylation in the linker region was previously shown to regulate the function of Abl and ZAP70/Syk kinases. Even though tyrosine phosphorylation was detected on tyrosine 375 in the Btk linker region, no significant alteration was observed in Btk-signaling activity and biological function when this tyrosine was mutated in DT-40 cells or in Y375F knock-in mice. Our data and previous studies suggest that each cytoplasmic tyrosine kinase family has evolved a unique strategy to utilize the linker region to regulate the function of the enzyme.

Introduction

Tyrosine kinases are important regulators of cellular functions and inhibitors of these kinases have been shown to effectively treat hyperproliferative diseases (1, 2). Cytoplasmic tyrosine kinases include Src, Abl, ZAP70/Syk and Btk/Tec family kinases. Bruton’s tyrosine kinase (Btk), a member of the Btk/Tec family kinases, plays an indispensable role in B cell development and function. Mutations in Btk cause human X-linked agammaglobulinemia (XLA), an immunodeficiency syndrome characterized by a dramatic reduction in peripheral B cells (3, 4). A spontaneous point mutation in murine Btk (R28C) results in a milder condition in mice termed X-linked immunodeficiency (xid) (5, 6). Btk-deficient mice show a similar phenotype as the xid animals: peripheral B cells are reduced by 30–50% and these cells proliferate to a lesser extent than normal B cells when stimulated in vitro. Additionally, serum levels of IgM and IgG3 are lower in these mice. Other defects include the absence of a minor population of CD5+ B-1 cells in the peritoneum and the inability to respond to type II T-independent antigens (7–9).

Btk is an essential component of the B cell receptor (BCR) signalosome. Stimulation of the BCR activates phosphatidylinositol 3-kinase (PI 3-kinase) and the non-receptor tyrosine kinase Lyn. Btk is then recruited to the plasma membrane by phosphatidylinositol 3,4,5-trisphosphate and activated through phosphorylation by Lyn at Y551. These steps lead to the assembly of the BCR signalosome which includes other proteins such as BLNK, Syk and phospholipase Cγ2 (PLCγ2), resulting in calcium flux which activates downstream signals.
Phosphorylation has been shown to be a critical regulatory mechanism for controlling Btk function. In addition to phosphorylation at Y551 by Lyn, Y223 in the SH3 is an autophosphorylation site that potentially down-regulates Btk activity, phosphorylation at Y617 in the kinase domain is involved in the regulation of PLCγ2-mediated calcium response and S180 in the Tec homology domain negatively regulates Btk function when phosphorylated by protein kinase Cβ (12–15).

Btk shares with other cytoplasmic tyrosine kinases a similar domain structure: a kinase domain is linked to the Src homology domain 2 (SH2) through an SH2-kinase linker region (16, 17). The crystal structure of Src family kinases revealed that the SH2-kinase linker region is buried in the middle of the SH3 and the kinase lobes; the interactions between the linker region and these domains lock the kinase in an inactive state (18, 19). This has been supported by experiments in which mutations of conserved residues in the linker region resulted in increased kinase activity, demonstrating that the SH2-kinase linker region in Src family kinases participates in intramolecular interactions to down-regulate kinase activity (20–22).

Both Syk and ZAP70 have relatively long SH2-kinase linker regions and three tyrosines in the linker region can be phosphorylated upon activation. These tyrosine phosphorylations enable the interaction with SH2-containing molecules. The binding of Cbl to the phosphotyrosines leads to down-regulation of kinase function via protein degradation. On the other hand, the interactions between phosphotyrosines in the linker region with PLC in Syk or PLC, Vav and Lck in ZAP70 activate downstream pathways such as calcium flux and nuclear factor of activated T cell (NF-AT) (23–25). This shows that tyrosine phosphorylation of the SH2-kinase linker region also modulates kinase function. However, recently it was suggested that these tyrosines may also play a critical structural role in the autoinhibition of ZAP70 kinase (26).

The SH2-kinase linker region of Abl kinase regulates kinase activity and function utilizing conserved residues as seen in Src family kinases and tyrosine phosphorylation as seen for ZAP70/Syk kinases. Mutations of residues in the Abl SH2-kinase linker region, which are conserved with Src kinase, increased the Abl kinase activity and the transforming ability of Abl kinase, which resulted from the interruption of intramolecular interactions (27). In addition, Abl also contains a tyrosine (Y245) in the SH2-kinase linker region and autophosphorylation of this site stimulates kinase activity, probably through disrupting intramolecular interactions as well (28).

Btk/Tec family kinases have a linker region that is relatively conserved within the family, but less conserved when compared with other non-receptor tyrosine kinase families (Fig. 1). Interestingly, Btk/Tec family kinase linker regions have the well-conserved tryptophan residue in the WEI motif seen in Src family kinases and two tyrosine residues as potential phosphorylation sites similar to Abl and ZAP70/Syk kinases. The crystal structure of the isolated Btk kinase domain showed typical kinase architecture with large and small lobes binding the catalytic site (29). Low-resolution X-ray synchrotron scattering indicated a linear structure for full-length Btk, with little or no inter-domain interactions (30). There is no definitive structural information about the role of the SH2-kinase linker region in regulating Btk/Tec kinases.

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**Fig. 1.** Domain structure and sequence alignment of the SH2-kinase linker region for cytoplasmic tyrosine kinases. Syk and ZAP70 have a longer SH2-kinase linker region as shown on the top. The sequences of the linker region from Abl, Src and Tec family kinases are shown at the bottom. XLA mutations are labeled in gray and the WEI motif in yellow. Conserved tyrosines are marked in magenta.
Missense mutations in Btk that lead to XLA have been found in the SH2-kinase linker region (31), indicating the biological significance of this region. We mutated the selected conserved residues and potential tyrosine phosphorylation sites in the linker region to investigate their role in Btk regulation in comparison to other cytoplasmic tyrosine kinase families. We found that XLA-causing mutations did not dramatically affect kinase activity, but abolished the ability of Btk to induce calcium flux upon BCR stimulation. While the W260A mutation resulted in a hyperactive Hck, mutation of the conserved tryptophan in Btk drastically decreased kinase activity. Unlike ZAP70/Syk and Abl family kinases, tyrosine phosphorylation in the SH2-kinase linker region of Btk, although detected, did not seem to play any significant role in B cell development and activation. Taken together, our data demonstrate that the SH2-kinase linker region contributes to the regulation of Btk function in a unique manner compared with other non-receptor tyrosine kinase families.

Methods

Cell culture, constructs and retrovirus production

Btk-deficient DT-40 cells are maintained in RPMI-1640 (GIBCO/BRL, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) and 1% chicken serum as previously described (32). HEK293T cells were cultured with Iscove’s modified Dulbecco’s medium with 10% FBS. 293T cells were transfected using a standard calcium phosphate-mediated method. Helper-free retroviruses were generated by transient co-transfection of 293T cells with retroviral constructs and an amphotropic packaging plasmid (33). Wild-type murine Btk and the mutants were cloned into the retroviral constructs and helper-free retroviruses were generated by transient co-transfection of 293T cells with retroviral constructs and a ψ-amphotropic packaging plasmid (33).

Calcium flux analysis

DT-40 cells were labeled with 1 µM Indo-1 AM (Molecular Probes) for 30–45 min at 37°C and re-suspended in HBSS (GIBCO/BRL) supplemented with 10 mM HEPES, pH 7.0. Calcium flux was measured using a fluorimeter (SLM 8000, BRL, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) and complete protease inhibitors (Roche, Indianapolis, IN, USA). Immunoprecipitation, western transfer and western blotting were performed using standard techniques. Quantification was made using software ImageJ (http://rsb.info.nih.gov/ij/).

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FACS analysis

Single-cell suspensions from spleen were depleted of red blood cells and stained with the following antibodies: IgM–PE (Pharmingen, San Diego, CA, USA), IgD–biotin (Southern Biotechnologies, Santa Cruz, CA, USA) and Upstate Biotechnology (Waltham, MA, USA), respectively, and mouse anti-chicken IgM M4 was obtained from Southern Biotechnology (Birmingham, AL, USA). Cells were lysed in a buffer containing 1% Triton X-100, 50 mM Tris pH 8.0, 150 mM NaCl and complete protease inhibitors (Roche, Indianapolis, IN, USA). Immunoprecipitation, western transfer and western blotting were performed using standard techniques. Quantification was made using software ImageJ (http://rsb.info.nih.gov/ij/).

Immunization and ELISA

Trinitrophenol (TNP)-specific Igs were measured as previously described (36). Briefly, mice were immunized with 100 µg of TNP-Ficoll (Biosearch Technologies, Novato, CA, USA) and serum was collected after 6 days.

Generation of Btk Y375F knock-in mice

The Btk Y375F knock-in knock-in vector was constructed from the 7.1-kb NheI fragment genomic DNA. A new Apol restriction site was introduced with Y375F mutation by site-directed phosphopeptide-specific antibody generation

Phosphopeptide (residues 369–381 with a phosphate on Y375) was prepared by Chris Turk (Howard Hughes Medical Institute, University of California, San Francisco), conjugated to keyhole limpet hemocyanin and used to immunize Balb/c mice. Immune sera were tested by ELISA and immunoblot assays. Splenocyte fusion with murine myeloma cells and hybridoma subcloning were performed by Susan Ou (California Institute of Technology) using standard techniques. Hybridoma culture supernatants were screened by ELISA and immunoblot assay for recognition of Btk phosphorylation sites. Hybridoma supernatants were purified by protein A Sepharose (34).

Immunoprecipitation and western blot analysis

The following antibodies were used in this study: Btk N-terminal antibody, pY551 antibody and pY223 antibody were produced as previously described (4, 35); PLCγ2 antibody and 4G10 antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) and Upstate Biotechnology (Waltham, MA, USA), respectively, and mouse anti-chicken IgM M4 was obtained from Southern Biotechnology (Birmingham, AL, USA). Cells were lysed in a buffer containing 1% Triton X-100, 50 mM Tris pH 8.0, 150 mM NaCl and complete protease inhibitors (Roche, Indianapolis, IN, USA). Immunoprecipitation, western transfer and western blotting were performed using standard techniques. Quantification was made using software ImageJ (http://rsb.info.nih.gov/ij/).

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The Btk Y375F knock-in knock-in vector was constructed from the 7.1-kb NheI fragment genomic DNA. A new Apol restriction site was introduced with Y375F mutation by site-directed nitrocellulose. The membrane was used for both autoradiography and western blot with anti-Btk antibody.
mutagenesis with primers 5’-CTGAAATCCCTGTCTACAAACAAACACGCCGCAAGTCCGACACAAG-3’ and 5’-CACAGGGAATTTGAGCCTGATATGAGGCCTGAAACAG-3’. A PGK-neo LoxP fragment was inserted into the NdeI site in intron 13. The targeting vector was electroporated into 129SvJ ES cells. Then Cre was introduced into the positive clones to screen for the loss of PGK-neo fragment by PCR and Southern blot analysis. ES cells from these positive clones were injected into blastocysts harvested from C57BL/6 mice (UCLA Transgenic Facility). Chimeric males were crossed with Btk−/− 129 females. Germ line transmission of the Btk Y375F knock-in allele was detected by PCR with primers 5’-GGATCAATTTCCCATGATCC-3’ and 5’-GATGATGGGCATGTGCAAG-3’ and digestion with Apol of tail DNA from F1 offsprings. All mice were bred and maintained according to the guidelines of the Department of Laboratory and Animal Medicine at the University of California, Los Angeles.

**Results**

The SH2-kinase linker region is conserved in Btk/Tec family kinases

Sequence alignment of the linker region of non-receptor tyrosine kinases showed that the linker regions are relatively conserved within the Btk/Tec family, while diversified from other non-receptor tyrosine kinase families (Fig. 1). The 30-residue linker sequence for Btk showed about 50% identity and >60% similarity to the other four members (Tec, Itk, Txk and Bmx) in the Btk/Tec family.

We introduced mutations to three types of residues in the Btk SH2-kinase linker region: (i) XLA mutations L369F and R372G; (ii) WEI motif mutations W395A and E396A and (iii) for tyrosines Y375 and Y392, we used a glutamic acid replacement to mimic the phosphorylated protein and a phenylalanine replacement to block tyrosine phosphorylation.

To analyze the effect of these mutations on Btk kinase activity and signaling function, we introduced wild-type Btk and Btk mutants via retrovirus infection into the chicken B cell line DT-40 cells which have been rendered deficient in endogenous Btk (37). The BCR-signaling pathway in DT-40 cells is similar to murine and human B cells. In addition, these cells have a robust calcium response upon BCR stimulation, which is dependent on Btk (32).

**Fig. 2.** XLA mutations cause defective calcium response in DT-40 cells. (A) Btk L369F and R372G mutants cannot flux calcium. DT-40 cells containing WT Btk, Btk L369F or Btk R372G were loaded with Indo-1 AM and stimulated with M4 antibody (2 µg ml⁻¹). Calcium flux was recorded with a fluorimeter. (B) Btk L369F and R372G mutations do not affect protein stability. 1x10⁶ DT-40 cells were lysed and the total cell lysates were separated by SDS-PAGE. After transferring to a nitrocellulose membrane, immunoblot was carried out with anti-Btk antibody. (C) Btk L369F and R372G mutants retain Btk kinase activity. Btk protein was immunoprecipitated from DT-40 cell lysates and incubated with 32P-γ-ATP. The reaction was separated by SDS-PAGE. After transferring to a nitrocellulose membrane, the membrane was used for radioautography and western blot analysis with anti-Btk antibody. (D) PLCγ2 activation was defective in cells containing Btk L369F or R372G mutation. DT-40 cells were stimulated with M4 (10 µg ml⁻¹) for the indicated time and PLCγ2 was immunoprecipitated and analyzed with phosphor-PLCγ2 and PLCγ2 antibodies.
if the loss of calcium response reflected a reduced kinase activity, an *in vitro* kinase activity assay was carried out. No significant difference in autophosphorylation (Fig. 2C) and transphosphorylation of an exogenous substrate enolase (data not shown) was observed, suggesting that both mutants retained 80–90% of the wild-type kinase activity. In addition, the cellular tyrosine phosphorylation pattern was quite similar in DT-40 cells expressing wild-type Btk, Btk L369F or Btk R372G mutant after BCR stimulation. The only discernable difference was the absence of a band at around 140 kDa in the cells containing the Btk L369F or R372G mutant (data not shown). Based on the molecular weight, this band could correspond to PLCγ2, one of the key substrates for Btk kinase activity and a critical mediator of calcium signals. We therefore examined the phosphorylation of PLCγ2 in activated DT-40 cells. The phosphorylation of PLCγ2 was significantly decreased in cells containing the Btk L369F or R372G mutant (Fig. 2D), suggesting that these residues are critical for PLCγ2 activation. Importantly, these two mutants do not act as dominant-negative alleles when introduced into wild-type DT-40 cells in Btk-mediated calcium response (data not shown).

These findings suggest that signal transduction from Btk to PLCγ2 is blocked by these mutations.

**Mutations of W395 and E396 decrease Btk kinase activity**

Tryptophan 260 in the WEI motif in Src family kinases interacts with the amino-terminal lobe of the kinase domain to hold the kinase in a tight inactive state (19). Since this motif is conserved in all the Src, Tec and Abl family kinases, we hypothesized that mutations of this motif (W392A and E396A) would result in enhanced kinase activity in Btk, as previously seen of W260A in Hck. Surprisingly, DT-40 cells containing the Btk W395A mutant had a dramatically decreased calcium response compared with cells with a wild-type Btk (Fig. 3A). Protein analysis of these cells showed that the mutant Btk was expressed at levels comparable to the wild type (Fig. 3B). The kinase activity in Btk W395A-expressing cells was analyzed by an *in vitro* autophosphorylation assay. As shown in Fig. 3(C), in contrast to the Hck W260A mutant, the Btk W395A mutant showed very low kinase activity compared with the wild type. Similar reduction in kinase activity toward enolase was also observed (data not shown). We also analyzed the activation status of PLCγ2. As shown in Fig. 3(D), PLCγ2 phosphorylation is slightly decreased in cells with the Btk W395A mutant (~15% decrease), which is consistent with decreased Btk kinase activity and impaired calcium response. The Btk E396A mutant showed a similar pattern as the W395A mutant, but was even more defective in calcium response and kinase activity. Although the WEI motif is conserved in a number of kinase families, this motif in Btk contributes to the positive regulation of kinase activity, in contrast to the negative regulation in Hck.

**Potential tyrosine phosphorylation in the linker region**

YW375 and Y392 in the linker region are conserved in all Tec family kinases except Bmx, yet the phosphorylation status of these residues during Btk activation was not known. Phosphopeptide-specific antibodies were made to specifically recognize the linker peptide with phosphorylation at either residue 375 or 392, named pY375 antibody and pY392 antibody, respectively (Fig. 4A). The pY392 antibody, while able to detect the Y392 phosphorylated linker peptide, failed to detect Y392 phosphorylation in the context of the Btk protein (data not shown). When Btk is over-expressed in 293T cells, the pY375 antibody detected a strong signal on wild-type Btk. Mutation of Y375 to phenylalanine abolished this signal (Fig. 4B). Co-expression of Btk with a constitutively active upstream kinase Lyn (Lyn with Y508F mutation, LynF) increased Y375 phosphorylation slightly, suggesting that Y375 is not likely a direct substrate for Lyn. When co-expressed with activated PI 3-kinase (myristylated, constitutively active, p110*), Y375 phosphorylation was reduced, indicating a potential negative regulation of this post-translational modification by the PI 3-kinase pathway.

It has been shown that the autophosphorylation on Btk Y223 is accentuated by transphosphorylation at Y551 by Lyn (14). To determine if Y375 represents an autophosphorylation site and if its phosphorylation status depends on other tyrosine phosphorylations, we co-expressed Btk with activated Lyn. Mutations in Y223 and Y392 did not affect Y375 phosphorylation under these conditions. In contrast, Y375 phosphorylation was greatly reduced by the Y551 mutation (Fig. 4C). This
indicates that the activation of Btk kinase activity is important for Y375 phosphorylation. To support this, the kinase-dead Btk mutant (K430R) also showed low phosphorylation on Y375. These data demonstrate that Y375 can be phosphorylated under these conditions and most likely represents an autophosphorylation site.

To investigate the effect of tyrosine phosphorylation on the function of Btk, we introduced Y375E/F and Y392E/F mutants into DT-40 cells. When these cells were activated by BCR cross-linking, all the mutants showed a normal level of calcium response, similar to the wild-type protein (Fig. 4D). These tyrosine mutations did not affect Btk protein expression level or kinase activity as measured by autophosphorylation. Moreover, PLCγ2 activation in cells with either of the Btk tyrosine mutations was not affected (data not shown). Even if both of the tyrosines were replaced by phenylalanine (Btk Y375FY392F double mutant), no difference was detected in kinase activity and calcium response compared with the wild-type Btk (data not shown). These data suggest that, although Y375 phosphorylation is detected by the phosphopeptide-specific antibody when over-expressed, it is not required in the BCR-induced calcium response in DT-40 cells.

Y375 phosphorylation is not required for Btk function in B cell development and activation

Although the Y375F mutation did not affect the Btk function in BCR-induced calcium response in DT-40 cells, we hypothesized that phosphorylation of this residue might be required only during B cell development. Alternatively, the effect of the Y375F mutation in BCR activation could be overcome by the high-level expression of Btk in these DT-40 cells (about 5- to 10-fold higher than endogenous Btk, data not shown). To study the roles of Y375 phosphorylation during B cell development and at physiological levels of expression, we introduced the Y375F mutation in the Btk genomic locus, using a knock-in strategy.

As depicted in Fig. 5(A), we isolated a Btk genomic DNA fragment, including exon 13 which encodes the SH2 linker region. Y375 was mutated to phenylalanine by site-directed mutagenesis. To facilitate selection for homologous recombination, a loxp-flanked PGK-neo marker was inserted into the NdeI site in the intron following exon 13. After the first round of transfection, ES cell clones were selected for homologous recombination. Then Cre was introduced into the positive ES cells and the deletion of PGK-neo was screened to get the Btk
Y375F knock-in clones for blastocyst injection. In this way, the Btk locus was minimally manipulated to ensure that the expression of the knock-in allele would not be affected. Mice carrying the Btk Y375F knock-in allele were bred with Btk knockout mice (7) to study the effects of this mutation in the absence of the wild-type allele. In splenic B cells, the expression of the knock-in protein was comparable to the wild-type protein (Fig. 5B), indicating that the manipulation of the genomic DNA did not alter transcription and translation of Btk. However, when tested for the immune response against the T-independent type II immunogen TNP-Ficoll, the knock-in mice produced as much anti-TNP IgM antibody as the wild-type mice (Fig. 5C and data not shown). Splenic B cells were able to up-regulate IgD expression and down-regulate IgM expression in the knock-in mice as well as the wild type (Fig. 5D). Peritoneal CD5+ B-1 cells develop normally in the knock-in mice by cell-surface marker analysis (data not shown). When stimulated by cell-surface IgM cross-linking in vitro, splenic B cells proliferated as normal as the wild type under the conditions tested (data not shown). In summary, Btk Y375F knock-in mice showed normal B cell development and activation and we have not been able to detect any significant difference between these mice and wild-type mice.

Discussion

It has been shown that the SH2-kinase linker region regulates the function of Src, ZAP70/Syk and Abl family kinases through different mechanisms. By mutating a number of conserved residues and potential tyrosine phosphorylation sites in the linker region in Btk, we found that the Btk linker region regulates Btk function in a unique mode compared with other tyrosine kinase families as summarized in Table 1. Although the WEI motif is highly conserved in non-receptor tyrosine kinases (Fig. 1), mutation of the well-conserved tryptophan residue (W260A) in Hck caused a hyperactive kinase (20), while an analogous mutation (W395A) dramatically decreased the Btk kinase activity in vitro (Fig. 3). W260 in Hck is believed to interact with the N-terminal lobe of the kinase domain and...
Our kinase-inactive Btk exhibited only subtle defect in B cell function. However, transfection of a phosphotyrosine-specific antibody when Btk is overexpressed, phosphorylation on either residue does not seem to be required for proper Btk function. First, mutations of these residues do not affect the Btk activity or signaling function in DT-40 cells. Second, although Y375 phosphorylation is detected by a phosphotyrosine-specific antibody when Btk is overexpressed, phosphorylation has not been detected from endogenous Btk in B cell (data not shown). Third, a knock-in allele of Btk carrying the Y375F mutation does not affect murine B cell development and activation. However, transgenic mice expressing Btk autophosphorylation site mutant Y223F showed a minimal phenotype and mice expressing kinase-inactive Btk exhibited only subtle defect in B cell development, indicating that Btk may function partially as a scaffold protein in B cell development (39). Our in vitro and in vivo data together suggest that tyrosine phosphorylation at Y375 in the linker region does not play an essential role in the regulation of Btk function.

XLA mutations have been found in each domain of the protein as listed by BTKbase (http://bioinf.uta.fi/BTKbase). This suggests that Btk is an integrated machinery in which all the domains, including the SH2-kinase linker region, are important. In addition to insertion, deletion, frameshift and nonsense mutations, missense mutations may impair Btk function through a number of mechanisms, including decreased protein stability, reduced kinase activity and defects in protein–protein interactions. Interestingly, Y375, Y392 and previously identified tyrosine phosphorylation sites Y551, Y223 and Y617 are not among the missense mutation sites reported so far. To date, 180 unique missense mutations have been identified in XLA families and they cover all the Btk domains except the SH3 (31). However, little is known about the effect of these mutations on Btk protein expression, kinase activity and protein function. Interestingly, mutations L369F and R372G in the linker region did not significantly affect kinase activity or protein stability. However, downstream calcium signaling was completely abolished in DT-40 cells (Fig. 2). Btk is required for a robust calcium response, and this is accomplished through the assembly of BCR signalosome at the plasma membrane. The signalosome is composed of a number of signaling molecules, including PI 3-kinase, phosphatidylinositol-4-phosphate 5-kinase, Lyn, Syk, Btk, PLCγ2 and adaptor proteins such as B-cell linker protein (BLNK), B cell adaptor for PI3-kinase (BCAP), linker for activation of B cells (LAB) and Bam32 (10, 11, 40). Although the interaction of Btk L369F mutant or R372G mutant with BLNK is not affected (data not shown), some other interacting proteins may not be able to bind to these Btk mutants and as a result these mutants cannot fit properly into the BCR signalosome to function. These XLA mutations may be useful tools for the isolation of novel interacting proteins involved in Btk–calcium signaling pathway.

It is interesting that the SH2-kinase linker region in cytoplasmic tyrosine kinases not only connects two well-defined functional domains but also regulates the kinase function through multiple mechanisms. Previous studies and our data suggest that different tyrosine kinase families may utilize distinct modes of regulation for this linker region. Further studies of the interacting partners of this linker region will shed more light on the details of the regulation. The crystal structure of a full-length Btk will be beneficial for our understanding of the placement and function of the SH2-kinase linker region.

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### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<td>Btk</td>
<td>Bruton's tyrosine kinase</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>PI 3-kinase</td>
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