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Stability and peptide binding specificity of Btk SH2 domain: Molecular basis for X-linked agammaglobulinemia

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Abstract

X-linked agammaglobulinemia (XLA) is caused by mutations in the Bruton's tyrosine kinase (Btk). The absence of functional Btk leads to failure of B-cell development that incapacitates antibody production in XLA patients leading to recurrent bacterial infections. Btk SH2 domain is essential for phospholipase C-γ phosphorylation, and mutations in this domain were shown to cause XLA. Recently, the B-cell linker protein (BLNK) was found to interact with the SH2 domain of Btk, and this association is required for the activation of phospholipase C-γ. However, the molecular basis for the interaction between the Btk SH2 domain and BLNK and the cause of XLA remain unclear. To understand the role of Btk in B-cell development, we have determined the stability and peptide binding affinity of the Btk SH2 domain. Our results indicate that both the structure and stability of Btk SH2 domain closely resemble with other SH2 domains, and it binds with phosphopeptides in the order pYEEI > pYDEP > pYMEM > pYLDL > pYIIP. We expressed the R288Q, R288W, L295P, R307G, R307T, Y334S, Y361C, L369F, and I370M mutants of the Btk SH2 domain identified from XLA patients and measured their binding affinity with the phosphopeptides. Our studies revealed that mutation of R288 and R307 located in the phosphotyrosine binding site resulted in a more than 200-fold decrease in the peptide binding compared to L295, Y334, Y361, L369, and I370 mutations in the pY + 3 hydrophobic binding pocket (~3- to 17-folds). Furthermore, mutation of the Tyr residue at the βD5 position reverses the binding order of Btk SH2 domain to pYIIP > pYLDL > pYDEP > pYMEM > pYEEI. This altered binding behavior of mutant Btk SH2 domain likely leads to XLA.

Keywords: Btk; CD; phosphopeptide binding; SH2 domain; SPR; XLA

X-linked agammaglobulinemia (XLA) is characterized by markedly reduced or absence of serum immunoglobulins of all isotypes. The patients fail to produce antigen-specific antibodies and, consequently, often suffer from pyrognic bacterial infection and enteroviral diseases (Zhu et al., 1994). This gene defect is intrinsic to B-cell lineage because T-cell dependent immunity is normal in XLA patients (Tsukada et al., 1993). The number of pre-B cells in the patient's bone marrow is normal; the defect thus resides in the development pathway of B-cells (Tsukada et al., 1993; Smith et al., 1994a, 1994b). The gene responsible for this disease encodes a cytoplasmic tyrosine kinase, Bruton's tyrosine kinase (Btk) (Vetrie et al., 1993). This kinase is expressed in early and mature human B-cell lines but is absent in terminally differentiated plasma cell lines. This distribution indicates that Btk, like other nonreceptor tyrosine kinases, is required for normal B-cell differentiation. The kinase is expressed in most haematopoietic cells, but is selectively down regulated in plasma cells and T-lymphocytes. This explains the normal T-cell immunity in XLA patients. Mutations or dele-

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tions in the Btk gene were detected in unrelated XLA patients (Hammarstrom et al., 1993; Smith et al., 1994b), which strongly suggest that the kinase is directly involved in the disease and, therefore, in the process of B-cell development.

Btk, along with Tec, Itk, and Bmx, belongs to a small family of tyrosine kinases (the Tec family) that share common structural features (Vetrie et al., 1993; Pawson, 1995; Shokat, 1995). In vivo and in vitro studies have shown that Btk can bind to the B-cell linker protein, the protein product of the *c-cbl* protooncogene, the $\beta\gamma$ subunits of heterotrimeric G proteins, Fyn, Lyn, Hck, and protein kinase C (Cheng et al., 1994; Tsukada et al., 1994; Yao et al., 1994; Cory et al., 1995; Langhans-Rajasekaran et al., 1995; Hashimoto et al., 1999). Like many other cytoplasmic tyrosine kinases involved in signaling pathways, Btk contains an N-terminal pleckstrin homology (PH) domain, an proline-rich Tec homology (TH) domain, an Src homology 3 (SH3) domain, an Src homology 2 (SH2) domain, and a catalytic tyrosine kinase domain (Yu & Schreiber, 1994; Cohen et al., 1995; Pawson, 1995; Shokat, 1995). The exact function of the PH domain in signaling proteins is still unknown, but it is thought to effect protein–protein interactions and/or membrane localization (Cohen et al., 1995; Pawson, 1995; Shokat, 1995). The SH3 and SH2 domains are small protein modules that mediate protein–protein interactions and occur in many proteins involved in intracellular signal transduction (Yu & Schreiber, 1994; Cohen et al., 1995; Pawson, 1995; Shokat, 1995). It has been reported that SH3 domains bind to proline-rich sequences and SH2 domains bind to phosphotyrosine sequences of the receptor protein tyrosine kinases (Kuriyan & Cowburn, 1993; Pawson & Schlessinger, 1993; Songyang et al., 1993; Sudol, 1998).

Although the importance of the PH and SH3 domains of Btk has been documented on the basis of their structural and functional roles in the B-cell development (Cheng et al., 1994; Tsukada et al., 1994; Yao et al., 1994; Zhu et al., 1994; Cory et al., 1995; Vihinen et al., 1995; Chen et al., 1996; Hyvonen & Saraste, 1997; Patel et al., 1997; Hansson et al., 1998; Baraldi et al., 1999; Morrogh et al., 1999; Nore et al., 2000; Tzeng et al., 2000), little is known about its SH2 domain. It was shown that the Btk SH2 domain is essential for phospholipase C-γ phosphorylation (Takata & Kurosaki, 1996; Fluckiger et al., 1998; Scharenberg et al., 1998) and mutations in this domain lead to XLA (Hammarstrom et al., 1993; Smith et al., 1994b). Recently, the B-cell linker protein (BLNK, also called SLP-65) was found to interact with the SH2 domain of Btk, and this association is required for the activation of phospholipase C-γ (Hashimoto et al., 1999; Su et al., 1999). However, the molecular basis for the interaction between the Btk SH2 domain and BLNK and the exact cause of XLA remain unclear.

Further study of the association of Btk SH2 domain with ligands can help to define consensus motifs and their modes of binding. In addition, it would also provide additional insights into the control of the signaling processes. The binding specificity of SH2 domains is provided by the phosphotyrosine, and by the residues carboxy-terminal to it (Songyang et al., 1993). To determine the preferred binding sequences for the Btk SH2 domain, we synthesized several phosphotyrosine-containing peptides based on the specific recognition sequences for Src, Nck, p85 N- and C-terminal, PLC-γ1 N- and C-terminal SH2 domains (Songyang et al., 1993) and quantitated their affinities with Btk SH2 domain using surface plasmon resonance (SPR). Besides, little is known about which residues in the Btk SH2 domain contribute to substrate recognition. Therefore we expressed the Btk SH2 domain with point mutations identified from XLA patients (Hammarstrom et al., 1993; Smith et al., 1994b) and measured their stability and binding affinity with the phosphotyrosine-containing peptides. Our results provide a molecular basis for understanding the role of the Btk SH2 domain in XLA.

Results

Stability of Btk SH2 domain

The CD spectrum for the Btk SH2 domain showed a broad minimum between 208 and 220 nm (Fig. 1), which is consistent with the characteristic α-helix and β-sheet mixture of SH2 domain (Shoelson et al., 1993). Thermal denaturation of the Btk SH2 domain was monitored by changes in ellipticity at 222 nm in PBS buffer (pH 7.4), as shown in Figure 2. Using a two-state model (Chen et al., 1996), the melting temperature (T_m) of the Btk SH2 domain was found to be 56 °C. This melting temperature is comparable to that of the PI-3 kinase p85 SH2 domain (57.2 °C) (Williams & Shoelson, 1993) but higher than that of the STAT3 SH2 domain (43 °C) (Haan et al., 1999). The guanidine hydrochloride (Gdn·HCl) induced unfolding process of the Btk SH2 domain was also monitored by changes in ellipticity near 222 nm at 25 °C, pH 7.4 (Fig. 3A). Midpoint concentration (C_m) of Gdn·HCl was found to be 2.2 M, which is also higher than that of the STAT3 SH2 domain (1.7 M) (Haan et al., 1999). The data points in the transition region of the curve in Figure 3A were used to calculate Gibbs free-energy change for the unfolding process. When this free-energy change of unfolding process is plotted against the Gdn·HCl concentration, a linear curve is obtained, which can be extrapolated back to zero molar Gdn·HCl (Fig. 3B) (Chen et al., 1996). The intrinsic free energy change ΔG_{H_2O} thus obtained was 2.95 kcal/mol.

Peptide binding affinity of Btk SH2 domain

Songyang et al. used a phosphopeptide library to determine the peptide binding specificity of SH2 domains (Songyang et al., 1993;

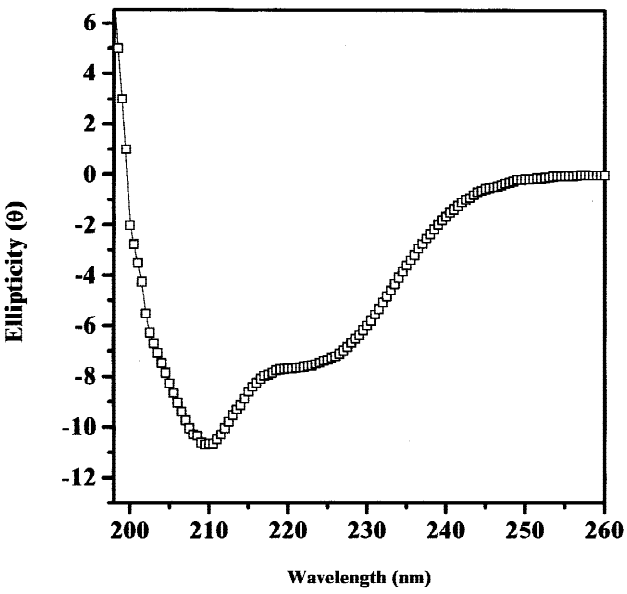

Fig. 1. CD spectrum of Btk SH2 domain recorded at 25 °C in PBS buffer, pH 7.4.

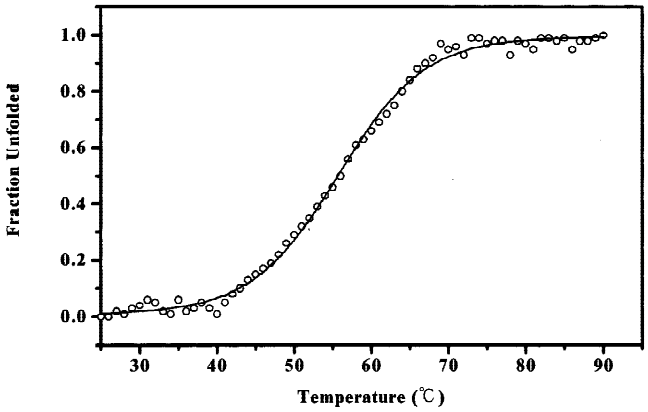

Fig. 2. Thermal denaturation curve for the Btk SH2 domain in PBS buffer, pH 7.4. Fraction of protein in unfolded conformation is plotted against temperature. Datum points were collected by monitoring change in ellipticity at 222 nm.

Songyang & Cantley, 1995). They have found that one group of the SH2 domains (Src, Fyn, Lck, Abl, Nck, etc.) binds preferably with the general motif pTyr-hydrophilic-hydrophilic-hydrophobic while the other group (PLC-γ1, p85, Shc, and SHPTP2) selects the pTyr-hydrophobic-X-hydrophobic motif. We synthesized several phosphotyrosine-containing peptides according to the specific recognition sequences for Src, Nck, p85 N-, and C-terminal, PLC-γ1 N- and C-terminal SH2 domains (Songyang et al., 1993). The exact sequences of these phosphopeptides were: pYEEI (GDGpYEEISPLLL), pYDEP (GDGp YDEPSPLLL), pYMEM (GDGpYMEMSPLLL), pYIIP (GDGp YIIPSPLLL), pYLDL (GDGpYLDLSPLLL). We examined the association and dissociation of the above-mentioned phosphopeptides with the Btk SH2 domain using surface plasmon resonance (Table 1). The analysis consisted of two phases (Fig. 4). In the association phase, the phosphopeptide was passed over the sensor chip matrix. The rising slope of the resonance unit (RU) during the injection of phosphopeptide indicates that phosphopeptide binds to the immobilized Btk SH2 domain. In the dissociation phase, the phosphopeptide was removed from the running buffer and the RU decreased, indicating the reduction of the amount of phosphopeptide bound to the sensor chip matrix. When no protein was immobilized on the sensor chip, there was no detectable binding of the phosphopeptide. Interestingly, the dissociation rate constants (k_{diss}) of these phosphopeptides were very similar but the association rate constants (k_{assoc}) were markedly different (Table 1). Similar results were also reported for the SH2 domain of PLC-γ (Ji et al., 1999). The dissociation constants (K_D) obtained were in the low μM range in consistent with other studies (Sawyer, 1998). However, it can be found from Table 1 that the Btk SH2 domain binds with the phosphopeptides in the order pYEEI > pYDEP > pYMEM > pYLDL > pYIIP.

Point mutations in the Btk SH2 domain resulted in loss of binding

Mutations in the Btk SH2 domain were found in several patient families with XLA (Bradley et al., 1994; de Weers et al., 1994; Hagemann et al., 1994; Saffran et al., 1994; Vorechovsky et al., 1995; Schuster et al., 1996; Vihinen et al., 1996; Conly et al., 1998). We expressed several Btk SH2 domains with point mutations identified from XLA patients and measured their binding with the pYEEI peptide (Table 2). Apparently, the R288 and

Table 1. *Association rates (k_{ass}), dissociation rates (k_{diss}), and dissociation constants (K_D) of the binding of phosphopeptides by the BTK SH2 domain*[a]

Ligand type	k_{ass} (10^4 $M^{-1}s^{-1}$)	k_{diss} (s^{-1})	K_D (μM)	Relative affinity
pYEEI	6.25	0.121	1.92	1
pYDEP	5.28	0.112	2.08	0.923
pYLDL	1.60	0.141	8.78	0.219
pYMEM	2.52	0.133	5.27	0.364
pYIIP	0.77	0.165	21.3	0.090

[a]Errors are less than 10% from five independent experiments.

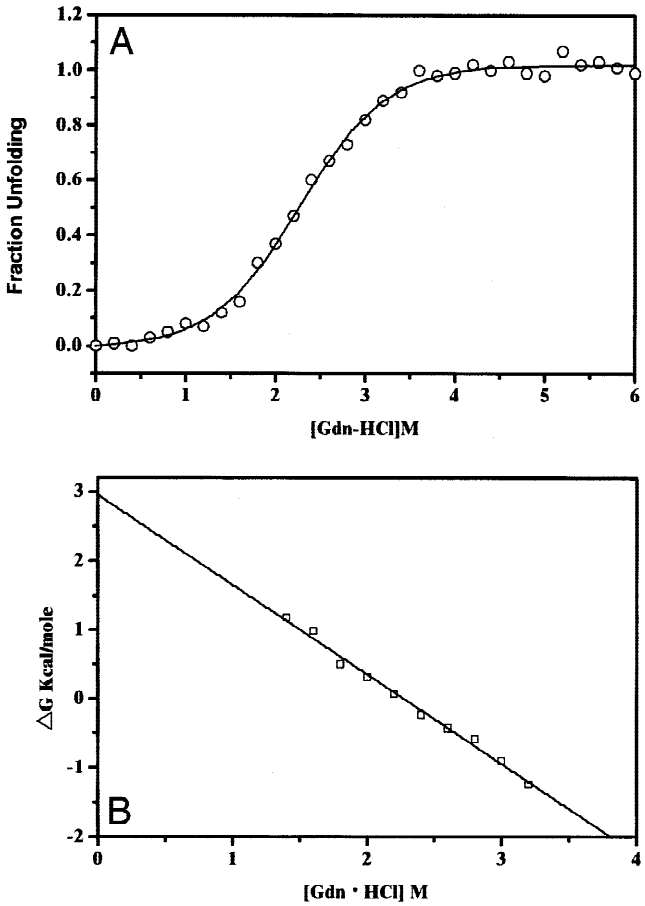

Fig. 3. A: Denaturation of the Btk SH2 domain as a function of Gdn·HCl concentration. CD datum points were obtained by monitoring changes in ellipticity at 222 nm at 25 °C in PBS buffer, pH 7.4. Midpoint concentration (C_m) is 2.2 M of Gdn·HCl. **B:** Plot of Gibbs free energy of unfolding (ΔG) of the Btk SH2 domain. Only the datum points corresponding to transition region in the denaturation curve were used to calculate ΔG. The linear curve was extrapolated to obtain the value of ΔG at zero denaturant.

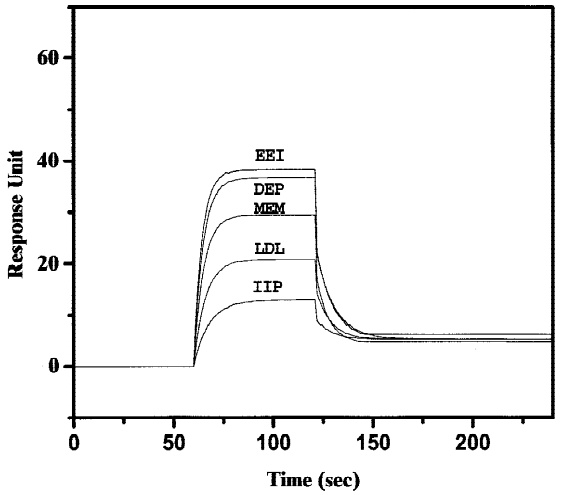

Fig. 4. SPR sensorgrams of the pYEEI, pYDEP, pYMEM, pYLDL, and pYIIP peptides binding to the Btk SH2 domain.

R307 mutations cause severe loss of the peptide binding affinity (~200 folds) than mutation of the L295, Y334, Y361, L369, and I370 residues (~3–17 folds). Based on the result that the Btk SH2 domain binds with the phosphopeptides in the order pYEEI > pYDEP > pYMEM > pYLDL > pYIIP and its sequence homology with the Src SH2 domain (Fig. 5), we have constructed a structural model of pYEEI complexed with the Btk SH2 domain from X-ray structural studies of the Src SH2 domain (Fig. 6) (Waksman et al., 1993; Sawyer, 1998). This structural model clearly depicts that residues R288 and R307 are involved in the phosphotyrosine binding site and residues Y334, Y361, L369, and I370 are involved in the hydrophobic binding pocket at the pY + 3 position (Bradshaw et al., 1998, 1999; Bradshaw & Waksman, 1999). Residue L295, on the other hand, may play only a structural role to stabilize the αA helix and thus maintaining its structural integrity.

Table 2. *Binding constants (K_D) of pYEEI to the wild-type and mutated BTK SH2 domains*[a]

SH2 domain type	K_D (μM)	Relative affinity
Wild-type	1.92	1
R288Q	>1,000	<0.002
R288W	57.1	0.034
L295P	8.75	0.219
R307G	312	0.006
R307T	512	0.004
Y334S	8.12	0.236
Y361C	27.3	0.070
L369F	9.75	0.197
I370M	5.18	0.371

[a]Errors are less than 10% from three independent experiments.

Evaluation of phosphopeptides binding with wild-type and mutant Btk SH2 domains

To further investigate the affinities of specific phosphopeptides for the wild-type and the R307G-, Y334S-, and L369F-mutated Btk SH2 domains, the IC_{50} values were determined for peptide inhibition of the wild-type Btk SH2 domain and its variants (Table 3). Not surprisingly, the wild-type Btk SH2 domain shows a preference in the order of pYEEI > pYDEP > pYMEM > pYLDL > pYIIP (Table 3), which correlate well with our kinetic analysis (Table 1). For R307G, where the mutation is involved in the phosphotyrosine binding site (Fig. 6) (Bradshaw et al., 1999), no binding was observed up to a peptide concentration of 1,000 μM. Interestingly, the Y334S mutant, where the Y334 at the βD5 position was mutated to a serine residue, prefers binding with the pYIIP and pYLDL peptides over the pYDEP, pYMEM, and pYEEI peptides (Table 3, see Discussion). In comparison with the wild-type Btk SH2 domain, the L369F mutant, where the mutation is involved in the hydrophobic binding pocket (Fig. 6), showed reduced affinities for phosphopeptides (with the exception of pYIIP).

Discussion

Bruton's tyrosine kinase resides largely in cytoplasm and is crucial for the development of B-cells. Dysfunction in this protein's activity causes a inheritable genetic disorder (Tsukada et al., 1993; Smith et al., 1994a, 1994b). It has been reported that the Btk SH2 domain is essential for phospholipase C-γ phosphorylation (Takata & Kurosaki, 1996; Fluckiger et al., 1998; Scharenberg et al., 1998). Recently, the B-cell linker protein (BLNK) was found to interact with the SH2 domain of Btk and this association is required for the activation of phospholipase C-γ (Hashimoto et al., 1999; Su et al., 1999). However, in spite of the importance of Btk in signaling in normal cells and in XLA, the molecular basis for its function remains unknown. To investigate the role of the Btk SH2 domain in XLA, we have successfully expressed the protein in *Escherichia coli* and measured its thermal and chemical stability using circular dichroism (CD).

An oriented degenerate phosphopeptide library was used to investigate the specificity of several SH2 domains toward the phosphotyrosine-containing peptides with various residues at +1, +2, and +3 carboxyterminal to the pTyr (Songyang et al., 1993). The reported X-ray and NMR structures of SH2 domain-ligand complexes also provide us additional insights to the molecular basis of specificity (Sawyer, 1998). CD studies showed that the structure and stability of the Btk SH2 domain closely resembles those of the other SH2 domains (Panayotou et al., 1992; Shoelson et al., 1993; Williams & Shoelson, 1993). We synthesized several

Table 3. *IC_{50} (μM) of various phosphopeptides to the wild-type and mutated BTK SH2 domains*

Peptide	Wild-type	Y334S	L369F
pYEEI	3.12	163	44.1
pYDEP	4.54	106	20.4
pYLDL	14.1	88.0	78.6
pYMEM	10.7	158	47.3
pYIIP	85.6	78.4	61.0

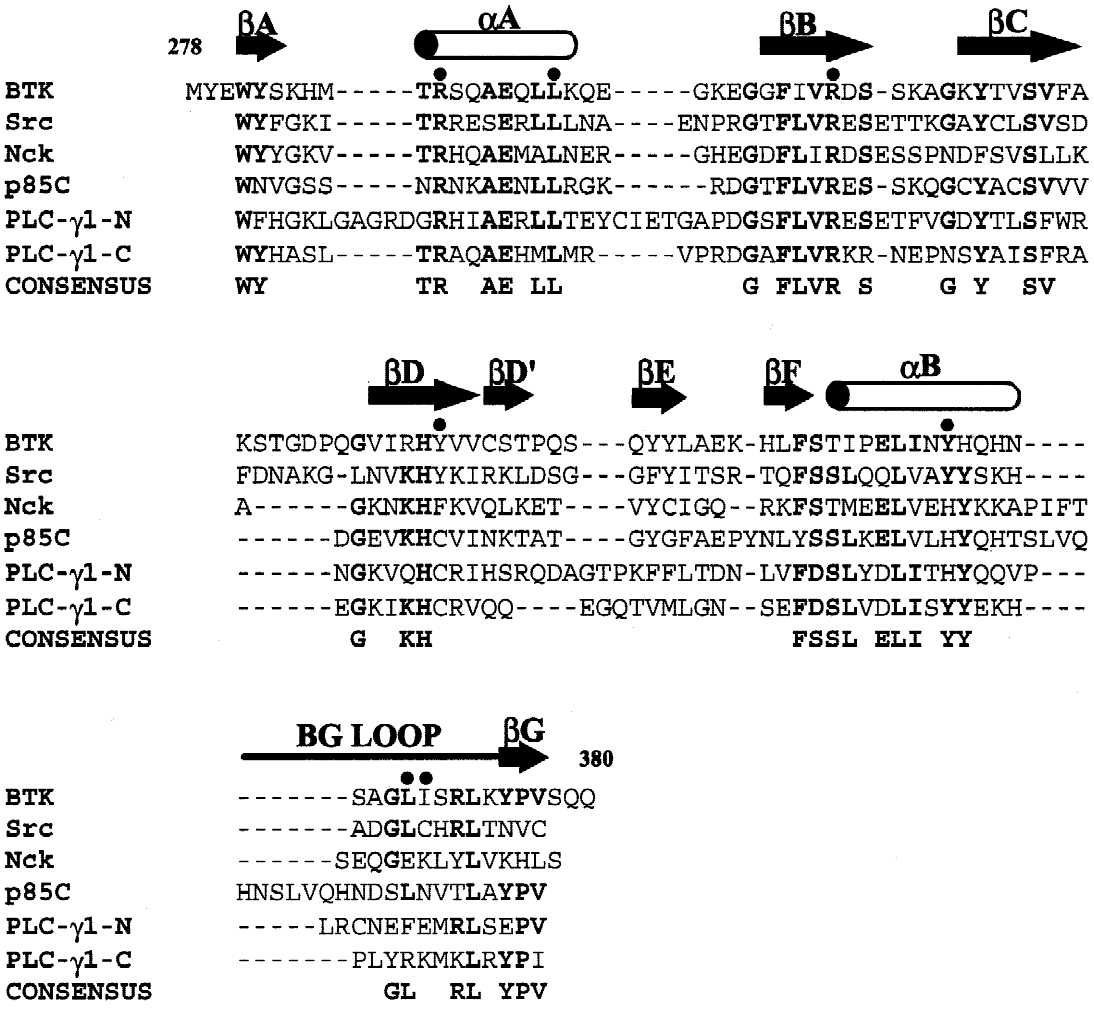

Fig. 5. A structure-based multiple sequence alignment of Btk, Src, Nck, p85N, PLCγ1-N, and PLCγ1-C SH2 domains. The secondary structure is shown above the alignment (arrows for β-sheets and cylinders for α-helices). Notation for the secondary structure is defined by Waksman et al. (1993). Mutations that found from XLA patients in this domain are represented by filled dots.

phosphotyrosine-containing peptides based on the specific recognition sequences observed for Src, Nck, p85 N- and C-terminal, PLC-γ1 N- and C-terminal SH2 domains (Songyang et al., 1993) and quantitated their affinities with the Btk SH2 domain using surface plasmon resonance. Our results showed that the Btk SH2 domain binds preferably in the order of pYEEI > pYDEP > pYMEM > pYLDL > pYIIP (Fig. 4).

Based on these results and the amino acid sequences of BLNK (Fig. 7), the most possible Btk SH2 domain binding sites in BLNK are predicted to be pYENP (Y72) and pYEPP (Y96). Recently, the Itk SH2 domain (also a Tec family kinase) binding sites in SLP-76 were mapped to be pYESP (Y113), pYESP (Y128), and pYEPP (Y145) using oriented peptide library screening (Bunnell et al., 2000). These possible Itk SH2 domain binding sites in SLP-76 are in accordance with the pYENP and pYEPP sites in BLNK. However, our present results show that K_D values for binding of the pYEEI, pYDEP, pYMEM, pYLDL, and pYIIP peptides to Btk SH2 domain are within approximately an order of magnitude that is consistent with a recent review that binding specificity of SH2

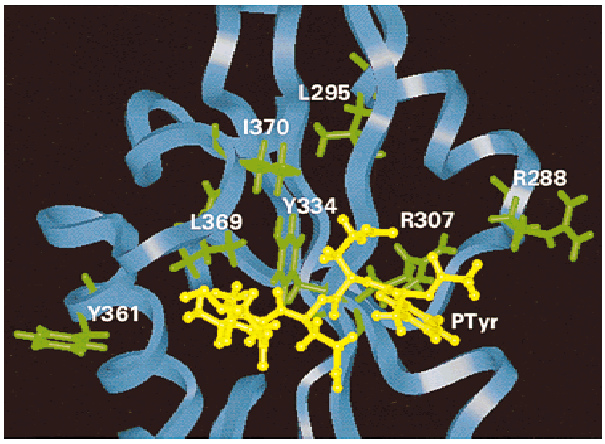

Fig. 6. A structural model of the Btk SH2 domain (blue ribbon) complexed with the pYEEI peptide (yellow) based on the published X-ray structures of the Src SH2 domain (Waksman et al., 1992, 1993). Point-mutations found in XLA patients are shown in green.

Fig. 7. Sequence alignment of BLNK and SLP-76. The possible binding sites for Btk (Y72 and Y96) and Itk (Y113 and Y145) SH2 domains are boxed. The putative SH3 domain binding site in both BLNK (residues 138–149) and SLP-76 (residues 184–195), which is adjacent to the possible Btk and Itk SH2 domain binding site, is also boxed.

domains is limited (Ladbury & Arold, 2000). Thus, the binding specificity between Btk and BLNK must not only come from the Btk SH2 domain alone. Interestingly, there is also a putative SH3 domain binding site in both BLNK (residues 138–149) and SLP-76 (residues 184–195) that is adjacent to the possible Itk and Btk SH2 domain binding site, pY145 in SLP-76 and pY96 in BLNK. The presence of adjacent binding sites for Btk SH3 and SH2 domains suggested that BLNK could bind synergistically to the Btk SH3 and SH2 domains and give a higher level of specificity due to the favorable free energies of binding and the relative positions of the domains (Bunnell et al., 2000).

To understand more about how the mutation in the Btk SH2 domain effects the binding, which can possibly lead to XLA, we prepared several mutant Btk SH2 domains found in patient families having XLA. We cloned and expressed the R288Q, R288W, L295P, R307G, R307T, Y334S, Y361C, L369F, and I370M mutants of the Btk SH2 domain found in XLA patients (Vihinen et al., 1996). It was observed that mutations of R307G and R307T resulted in more than 200-fold decrease in the binding affinity, which clearly indicates that the Arg βB5 (R307) residue of the Btk SH2 domain plays a crucial role in pTyr recognition (Fig. 6). Similarly, the binding affinity was either totally lost (R288Q) or very poor (R288W) for R288 mutant (Table 2), suggesting that Arg αA2 is equally important in pTyr recognition. These results are in agreement with studies recently reported for the Src SH2 domain (Bradshaw et al., 1999). In addition to the pTyr binding site, mutations of other residues, i.e., Y334S, Y361C, L369F, and I370M, located in the pY+3 hydrophobic binding pocket (Fig. 6) also resulted in decrease of the binding affinity. This poor binding affinity correlates with its decreased selectivity for the residues at +2 and +3 positions of pTyr. The substitution of L295 with proline may prevent the αA helix elongation and hence reduce the pTyr binding, which was also observed from its decrease in α-helicity from 19 to 16% (data not shown).

The residue at βD5 was shown to be predictive of the type of phosphopeptide that will associate with the SH2 domain. Accordingly, the SH2 domain has been classified into four distinct groups (Songyang & Cantley, 1995). Group I SH2 domains, having either Tyr or Phe at βD5, select peptides with the general motif pTyr-hydrophilic-hydrophilic-hydrophobic. In contrast, group III SH2 domains having Cys or Ile at the βD5 position select the peptide with sequence pTyr-hydrophobic-Xxx-hydrophobic. Songyang et al. (1995) reported that a single-point mutation (Ile at βD5 to Tyr) switches the specificity of PI-3 kinase p85 N-terminal SH2 domain (group III) to that of a group I SH2 domain. Surprisingly, on comparing the binding of the mutated Btk SH2 domain (Y334S) with the wild-type showed that mutation of Y334 residue at βD5 position with serine resulted in the reversal of binding preference,

i.e., it preferred to bind more with pYIIP than pYEEI. However, it does not really improve binding to the pYIIP peptide but rather spares the weak binding to this peptide while dramatically reducing binding to peptides with hydrophilic residues at +1. This change in binding probably arises from the loss of hydrogen bonding as well as hydrophobic interaction between the aromatic ring of the tyrosine residue with aliphatic side chains of the peptide. The exact behavior of this mutation will be revealed from the structural studies of this Btk SH2 domain with these phosphopeptides, which is in progress in our laboratory. To the best of our knowledge, this is the first reported case wherein the mutation of a single residue switches the selectivity of phosphopeptides from a group I SH2 domain to a group III SH2 domain.

In summary, we have determined the stability and peptide binding affinity of the Btk SH2 domain. We have also found that mutants of the Btk SH2 domain identified from XLA patients exhibit weaker affinities for the phosphopeptides. It is likely that the point-mutated Btk SH2 domains fail to present to the ligand the crucial residue in the correct context, thus leading to a weaker binding. The altered binding behavior likely renders the kinase abnormal and possibly causes XLA. In addition, the data presented here also indicate the potential to modulate the binding of the Btk SH2 domain by mutations at sites predicted to bind the side chains of the phosphopeptides.

Materials and methods

Protein expression and purification

The DNA fragments encoding residues 272–380 in the human Btk SH2 domain (Fig. 5) were amplified by polymerase chain reaction (PCR). The amplified PCR products were subcloned into pET-15b vector. The plasmids encoding Btk SH2 domain were introduced into *E. coli* strains BL21(DE3). The bacteria cells were grown at 37 °C to $OD_{600} \sim 1.5$, and then the proteins were induced by adding 2 mM isopropyl-β-thiogalactoside at 16 °C. After 16 h, the cells were pelleted and kept frozen at −20 °C overnight. The pellets were then defrosted and suspended into 50 mL of ice cold lysis buffer (50 mM sodium acetate, 0.01% Triton X-100, pH 4.8), and the mixture was sonicated until the lysis was completed. The lysates were centrifuged at 16,000 rpm for 30 min at 4 °C, and the supernatant was applied to an ionic SP column (Pharmacia, Uppsala, Sweden). The fractions containing Btk-SH2 were pooled, concentrated, and added to a 1.6 × 60.0 cm Sephacryl S-100 column (Pharmacia). Finally the protein was eluted with the PBS buffer. Point mutations of the Btk SH2 domain were constructed using the megaprimer method (Karreman, 1998). Mutant Btk SH2 domains were expressed and purified in the same manner as the wild-type Btk SH2 domain.

Peptide synthesis

The phosphotyrosine-containing peptides were synthesized by standard solid phase Fmoc method using Fmoc-Tyr(OP(OMe)$_2$. After synthesis, the peptides were deprotected and cleaved from the resin by treating with a mixture of trifluoroacetic acid and water containing phenol/1,2-ethandithiol/thioanisole (reagent "K") (Cheng et al., 1996). Further purification on a reversed-phase high-performance liquid chromatography (HPLC) preparative column (Vydac, RP-18 column) afforded pure peptides. The purity of these synthetic peptides was confirmed by HPLC and mass spectroscopy.

Optical biosensor

The BIAcore X instrument (Pharmacia) was used to investigate the binding in real time of various phosphotyrosine-containing peptides with the Btk SH2 domain. Each chip used was divided into two flow channels, one of which was left uncoupled for a controlled measurement. To avoid erroneous analysis generated by fusion proteins (Ladbury et al., 1995), we expressed the Btk SH2 domain in the native form and diluted the protein sample to 1 μM in PBS buffer. The surface density was controlled by using the manual inject command. Finally, about 4,800 RU of Btk-SH2 was immobilized on the CM5 surface, and phosphotyrosine-containing peptides in PBS buffer were then injected with a flow rate of 20 μL/min. Binding information was obtained by analyzing the binding curves. Inhibition experiments were performed to measure IC_{50} of the phospho-tyrosine containing peptides. In the inhibition experiments, pYEEI was immobilized onto a chip. The Btk-SH2 domain and its variants were incubated for at least 2 h with various concentrations of the phosphotyrosine-containing peptides prior to injection. Data analysis was performed with the BIA evaluation software (Pharmacia).

Circular dichroism

CD spectra were recorded on an AVIV 62DS spectropolarimeter after calibration with *d*-l0-camphorsulfonic acid. All measurements were carried out using an l-mm pathlength cuvette at a protein concentration of 50 μM in PBS buffer of pH 7.4. Far-UV spectra were collected in the range of 195–260 nm using a 0.5 nm stepsize and 1 s averaging time. Thermally induced unfolding of the protein was monitored by measuring ellipticity at 222 nm from 25 to 90 °C. Measurements were performed at a temperature increment of 1 °C with 2 min equilibrium time and 60 s averaging time for each increment using a water-jacketed cell. Thermal denaturation was presented using fraction folded vs. temperature to facilitate comparison (Chen et al., 1996). Guanidine hydrochloride (Gdn·HCl) induced chemical denaturation was monitored by the decrease of the ellipticity in the CD spectra of protein at 222 nm, 25 °C.

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