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Structural basis for chromosome X-linked agammaglobulinemia: A tyrosine kinase disease

(Bruton tyrosine kinase/BTK/cytoplasmic tyrosine kinase/signal transduction)

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ABSTRACT **X-linked agammaglobulinemia (XLA) is a hereditary defect of B-cell differentiation in man caused by deficiency of Bruton tyrosine kinase (BTK). A three-dimensional model for the BTK kinase domain, based on the core structure of cAMP-dependent protein kinase, was used to interpret the structural basis for disease in eight independent point mutations in patients with XLA. As Arg-525 of BTK has been thought to functionally substitute for a critical lysine residue in protein-serine kinases, the mutation Arg-525 → Gln was studied and found to abrogate the tyrosine kinase activity of BTK. All of the eight mutations (Lys-430 → Glu, Arg-520 → Glu, Arg-525 → Gln, Arg-562 → Pro, Ala-582 → Val, Glu-589 → Gly, Gly-594 → Glu, and Gly-613 → Asp) were located on one face of the BTK kinase domain, indicating structural clustering of functionally important residues.**

X-linked agammaglobulinemia (XLA) is a human immunodeficiency disease characterized by a lymphocyte differentiation block that results in a deficiency of B cells and immunoglobulins, leading to increased susceptibility to infections (1). Recently, the gene mutated in XLA was shown to encode a cytoplasmic protein-tyrosine kinase (PTK) designated Bruton XLA tyrosine kinase (BTK; formerly ATK or BPK) (2, 3). XLA is thus the first Mendelian disease in which a cytoplasmic PTK was found to be mutated. BTK has a single kinase domain (also designated Src homology 1 [SH1] domain) and SH2 and SH3 domains, but lacks both an N-terminal consensus myristoylation sequence and a C-terminal regulatory region. Together with murine Tec and Itk/Tsk it forms a new family of cytoplasmic PTKs (4–7). The two point mutations reported to cause XLA have both been found in the kinase domain (2), whereas the murine X-linked immunodeficiency defect represents the first missense mutation (8, 9) in the recently identified pleckstrin homology region located in the N terminus of BTK (10–13).

The catalytic core of the large protein kinase family contains 9 invariant and 15 highly conserved residues involved in ATP binding and catalysis (14–16). The first crystal structure of a protein kinase was the binary complex of the catalytic subunit of cAMP-dependent protein kinase [cAPK; a protein-serine/threonine kinase (PSK)] with a specific protein kinase inhibitor, PKI(5-24) (17, 18), subsequently refined to 2.0 Å (19). The binary complex and the ternary complex with PKI(5-24) and MnATP (20) have a closed conformation, whereas the mammalian binary complex has an open conformation in which the upper domain is displaced 15° with respect to the lower domain (21). Structures of substrate and product peptides complexed with the enzyme (22) support a direct in-line mechanism (23).

The x-ray structures, including the independently solved ternary complex (24), provide a comprehensive description of the enzyme, which has been used in modeling of myosin light chain kinase (25), cell division cycle 2 (CDC2) protein kinase (26), and epidermal growth factor receptor PTK (27). The crystal structures of the human cyclin-dependent kinase 2 (CDK2) (28) and mitogen-activated protein (MAP) kinase ERK2 (extracellular signal-related kinase 2) (29) have the same overall fold as cAPK, suggesting that the kinase homology models are valid.

In addition to the previously known point mutations in XLA (Lys-430 → Glu and Arg-525 → Gln) (2), six new missense mutations were found in this disease: Arg-520 → Glu, Arg-562 → Pro, Ala-582 → Val, Glu-589 → Gly, Gly-594 → Glu, and Gly-613 → Asp. The functional implications of these mutants were investigated in structural terms by modeling the BTK structure to provide the first three-dimensional model of a cytoplasmic PTK.

MATERIALS AND METHODS

Molecular Modeling. The structure of the BTK core protein model was based on the ternary complex of cAPK with ATP and two Mn^{2+} ions at 2.2-Å resolution [protein data base (PDB) entry 1atp] and the structure of the enzyme–substrate complex before and after phosphorylation (20, 22). The sequence alignment was performed with Genetics Computer Group (GCG) (30) and MULTICOMP (31) program packages. Also the conservation of residues in the two kinase groups (14–16) was taken into account as well as knowledge about the function of residues gained in structural models of cAPK.

The model was built with the program INSIGHTII (Biosym Technologies, San Diego). The insertions and deletions were modeled by searching loops from a data base, which contained either most of the PDB structures or an unbiased selection of the PDB (32). The model was refined by energy minimization with the programs CHARMM (version 22) (33) using the all-hydrogen parameter set 22 and with X-PLOR (version 3.1) (34) using parameter sets PARAM19 (33) and the corresponding nucleic acid parameters (35) in a stepwise manner whereby the conserved regions were constrained and the largest deviations were on the insertions/deletions. First

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Abbreviations: BTK, Bruton tyrosine kinase; XLA, X-linked agammaglobulinemia; PTK, protein-tyrosine kinase; PSK, protein-serine/threonine kinase; cAPK, cAMP-dependent protein kinase; PKI, protein kinase inhibitor; PDB, protein data base; 3-D, three dimensional; CMM, chemical cleavage and mismatch method.

the new loops were minimized. After 1000 cycles, the insertion/deletion regions were subjected to simulated annealing from 2000 to 300 K, followed by minimization for 500 cycles. Then the C_α atoms of the conserved regions, ATP, and Mg atoms were harmonically constrained, and the structure was minimized until the rms gradient was below 0.0001 kcal/(mol·Å). The tyrosine was positioned manually in the catalytic site based on the location of the oxygen atom of the serine in the cAPK substrate complex.

The structure was evaluated by testing the distribution of polar and nonpolar residues (36). The model passed five of the six tests except for external polar fraction. This might be because we have modeled only part of the protein, and the missing parts were not taken into account in the calculations. The model was tested also by the 3-D verification technique (37) and found to have the score of a typical globular protein.

***In Vitro* Kinase Analysis.** BTK was immunoprecipitated from peripheral blood mononuclear cells and subjected to *in vitro* kinase analysis or identified in immunoblotting (Western blotting) with anti-BTK peptide-specific rabbit antisera (38). Briefly, lysates of peripheral blood mononuclear cells from healthy controls or patients with the Arg-525 → Gln mutation were precipitated with anti-BTK antisera. The precipitates were suspended in kinase buffer (20 mM Tris, pH 7.5/10 mM $MgCl_2$/0.1% Triton X-100) to which 1 mCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP (3000 Ci/mM) was added. The reaction was terminated after a 10-min incubation at room temperature by adding SDS-containing sample buffer. Autophosphorylated proteins were separated by SDS/PAGE and visualized by autoradiography.

Mutation Analysis. Detection of XLA point mutations in the BTK kinase domain was carried out by using the chemical cleavage and mismatch method (CMM) with direct sequencing of PCR products (39, 40) or by using direct sequencing of PCR products of BTK cDNA without CMM.

FIG. 1. Sequence alignment of BTK with the catalytic subunit of cAPK. △, Conserved residues in PSKs; ▽, conserved residues in PTKs; ◇, conserved residues in both groups according to ref. 16. Red indicates α-helices, and blue indicates β-strands according to cAPK structure. The two regions distinguishing PTKs from PSKs are in green in the BTK sequence.

RESULTS AND DISCUSSION

BTK and cAPK sequences have 22% residue identity (Fig. 1). All of the insertions and deletions appeared on the surface of the molecule and outside secondary structures except for the second α-helix (αB), which was deleted (Fig. 1). BTK homology modeling (Fig. 2) was based on the cAPK core structure and the sequence alignment. The upper domain responsible for ATP binding is most conserved, with only two changes in the length of the peptide chain: a single residue deletion between β strands 2 and 3 and a four-residue deletion disrupting the αB helix (Fig. 2). This helix is also missing from the crystal structure of CDK2 (28), demonstrating that αB might not be necessary for the kinase fold. The glycine-rich loop and the other residues in the upper domain having contact with the ATP are conserved (Fig. 3 *Upper*). Of 11 residues involved in binding ATP and Mg^{2+} in cAPK, 6 are conserved in BTK, and backbone atoms of residues 477, 525, and 538 are required. Thus, corresponding residues are missing only for Glu-127 and Lys-168 of cAPK (Cys-481 and Ala-523 in BTK). Furthermore, in PTKs the side chain corresponding to Arg-525 of BTK has been thought to functionally substitute for Lys-168 in cAPK (27). The structural similarity of the ATP and Mg^{2+} binding indicates functional similarity and suggests that BTK, and PTKs in general, have the direct in-line reaction mechanism of PSKs (22, 23).

The other insertions and deletions were on the surface in the lower domain and outside secondary structures (Fig. 2). The largest difference compared with cAPK is the insertion of residues 556–559 preceding one of the two regions distinguishing PTKs from PSKs (Fig. 1). The insertion is too close to the ATP and to the glycine-rich loop for the substrate-binding mode of cAPK to be preserved. For catalysis to occur, it is reasonable to assume that the phosphorylation site has to be in a similar position relative to the ATP in BTK as in cAPK. To model this, the tyrosine substrate was placed on BTK so that its hydroxyl group coincided with the hydroxyl

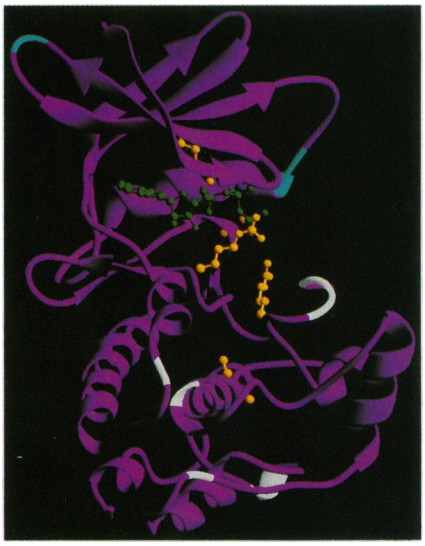

FIG. 2. Ribbon representation of BTK kinase domain. The model was based on the cAPK structure (17, 19). Residues preceding deletions in cAPK are blue, residues inserted are white, ATP is green, and Mg^{2+} ions are the two single orange spheres just above Arg-562. The residues changed by mutations causing the disease XLA are yellow and from the top are Lys-430, Arg-525, Arg-562, Ala-582, and Gly-594.

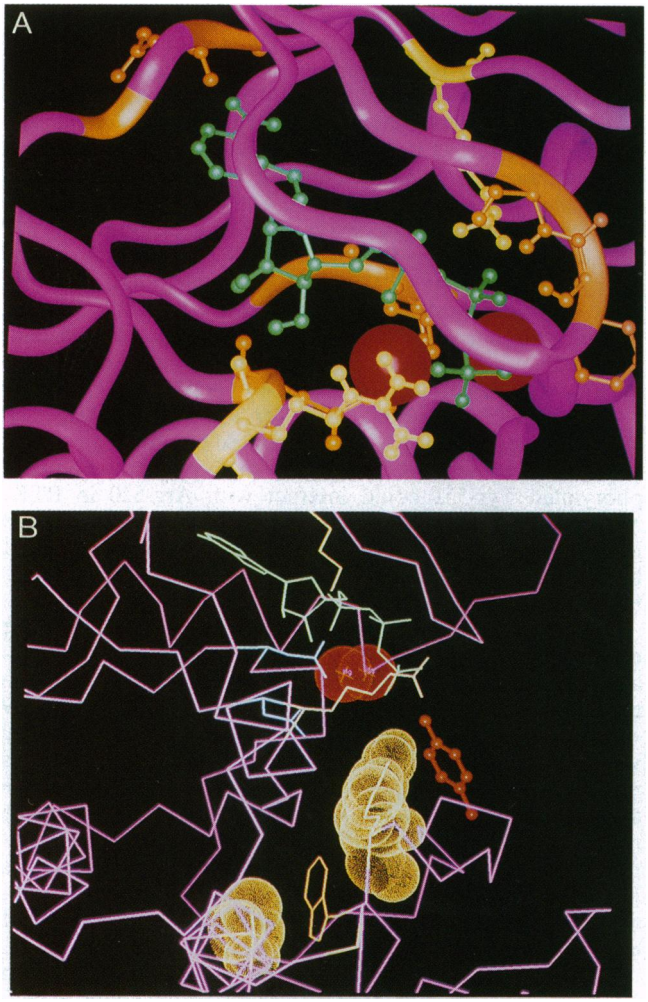

FIG. 3. (*Upper*) ATP and Mg^{2+} binding in BTK. Of the 11 residues involved in binding in cAPK, 9 are conserved in BTK as follows: 6 conserved residues and 3 with backbone interactions, suggesting similar binding. Conserved residues are orange. Side chains are shown only when they are involved in binding. Lys-430 (at the top) and Arg-525 (at the bottom) are yellow, Mg^{2+} ions are red, and ATP is green. (*Lower*) Catalytic site of BTK. ATP is green, and Mg^{2+} ions are red spheres. The tyrosine (orange ball and stick presentation) was positioned according to the hydroxyl group of serine in the substrate complex of cAPK. Conserved blue residues are Asn-526 (lower) and Asp-539 (upper). The yellow stick residues are Lys-430 (at the top) and Arg-525 (in the middle). The conserved Tyr-563 (orange) is sandwiched between the yellow-dot surfaces of Arg-562 (right) and Ala-582 (left).

of the serine residue in the cAPK–substrate complex (22) (Fig. 3 *Lower*). If the backbone atoms of the serine substrate in cAPK had been used, there would not have been sufficient space for the bulkier tyrosine side chain. The substrate backbone thus cannot be bound in exactly the same way in BTK as in cAPK. The different binding mode may be accommodated by the four-residue insertion after Gly-200 (556–559 in BTK) and the four-residue deletion at 82–85 (after Glu-439 in BTK) acting in a concerted way to give a different binding cleft from cAPK.

XLA can be caused by various genetic abnormalities involving BTK, including point mutations. The previously described point mutations in the human BTK gene resulting in Lys-430 → Glu and Arg-525 → Gln changes (2) are readily explained with the BTK model. Lys-430 on β3 is one of the invariant ATP-binding residues, and replacement by glutamate changes the charge and eliminates the interactions with the α and β phosphates of ATP. Furthermore, this mutant destroys the invariant interaction with Glu-445. All known mutations of this residue in both PSKs and PTKs have caused inactivity (41–48). Recently, the exactly corresponding lysine-to-glutamate mutation in cytoplasmic PTK FES was found to inactivate kinase function (49).

The other previously described missense mutation resulting in a Arg-525 → Gln change is (2) located between β6 and β7 on the conserved catalytic region that distinguishes PTKs from PSKs (Fig. 1). PTKs have a highly conserved sequence Ala-Ala-Arg, whereas in PSKs the most frequent corresponding sequence is Lys-Pro-Glu, where the lysine interacts with the γ phosphate of ATP and the phosphate of the product peptide. In PTKs, an arginine in position 170 in cAPK (525 in BTK) has been thought to replace the lysine at 168 in cAPK (27). This functional replacement is facilitated in BTK by the two alanine residues preceding Arg-525 that impose no structural constraints for the arginine to interact with the phosphate in a similar way as the Lys-168 of cAPK (22). The functional similarity of Arg-525 in BTK and Lys-168 in PSKs is further emphasized by the activity of the Lys-168 → Ala mutation in TPK1, which is reduced to 0.3% (48). The Arg-525 → Gln change cannot provide the necessary interaction with the ATP, possibly making it unsuited for reaction by changing the γ-phosphate orientation.

To study the functional consequences of the Arg-525 → Gln mutation, the mutant protein was analyzed *in vitro*. The abnormal protein was present in patients in amounts similar to the amounts of wild-type protein in healthy subjects, but it was not phosphorylated (Fig. 4). As the disease phenotype in two males carrying this mutation is characteristic of typical XLA with virtual absence of B lymphocytes (0.2% in both patients; normal value, 2.1–16%) and low immunoglobulin levels, these findings are compatible with a catalytic function for Arg-525.

Conserved Arg-166 interacts with the phosphate of the stable phospho-enzyme intermediate in alkaline phosphatase (50, 51). The bidentate arginine forms two hydrogen bonds with the oxygens of the phosphoserine. Many protein phosphatases have been shown to operate via stable phospho-enzyme intermediates (52). Recently Arg-174 has been observed to contact the γ phosphate of guanosine 5′-[α-thio]triphosphate in transducin-α (53). Furthermore, missense mutations of the cognate arginine in other α subunits may cause human endocrine tumors (54–57). Thus, although the binding of GTP to GTPases differs from the contacts between ATP and protein kinases in several respects (58), it demonstrates frequent interaction between the guanidinium group of an arginine residue and the γ phosphate in nucleotide-binding enzymes.

By using a systematic screening approach for identifying mutations in XLA families, six additional missense mutations causing XLA disease have been found (Fig. 5). The mutations were found to result in the following amino acid changes:

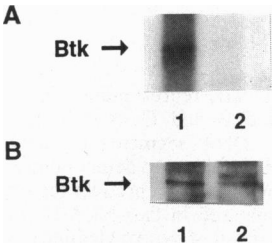

FIG. 4. Analysis of BTK protein and its ability to autophosphorylate. Lanes: 1, material from a healthy subject; 2, material from a patient with the Arg-525 → Gln mutation. Similar results were obtained with material from his cousin carrying the same mutation. Both patients have been shown to make normal amounts of BTK mRNA (38). (*A*) *In vitro* kinase assay. (*B*) Western blotting.

Arg-562 → Pro, from a G-to-C mutation at position 1817; Ala-582 → Val, from a C-to-T mutation at position 1877; Arg-520 → Glu, from a G-to-A mutation at position 1691; Glu-589 → Gly, from an A-to-G mutation at position 1898; and Gly-613 → Asp, from a G-to-A mutation at position 1970. Only Arg-562 of these sites could be involved in the catalytic activity of BTK. Arg-562 is in the second region typical for PTKs and is located immediately after the four-residue insertion in the loop between β9 and αF (Fig. 1). All PTKs have arginine or lysine at this position except for Jak and Tyk families and Dpyk1 (16). Arg-562 is close to the active site, but the model cannot predict whether it is involved in catalysis or substrate binding, although the model would allow the flexible arginine side chain to reach out and interact with Asp-521, a homologue for catalytic Asp-166 of cAPK. Arg-562 also keeps the invariant Trp-563 of PTKs in place between the backbones close to the two regions typical for PTKs. The function of the Trp-563 might be to keep the integrity of the PTK-specific regions, and the mutation Arg-562 → Pro would disturb that. The bulkier side chain of the proline could change the local conformation because of its limited torsion angles.

The mutation Ala-582 → Val in αF provides another indication of the importance of Trp-563. The side chain of Trp-563 is sandwiched between Arg-562 and Ala-582, and mutation of either of the surrounding residues could thus interfere with it sterically. The corresponding residue for Ala-582 is either alanine or serine in all PTKs except Dpyk2 (16). The differences in Dpyks have been suggested to arise from their hybrid nature between PSKs and PTKs (59). Serine does not have the bidentate structure of valine, and its OH group could be pointing away from Trp-563. These three

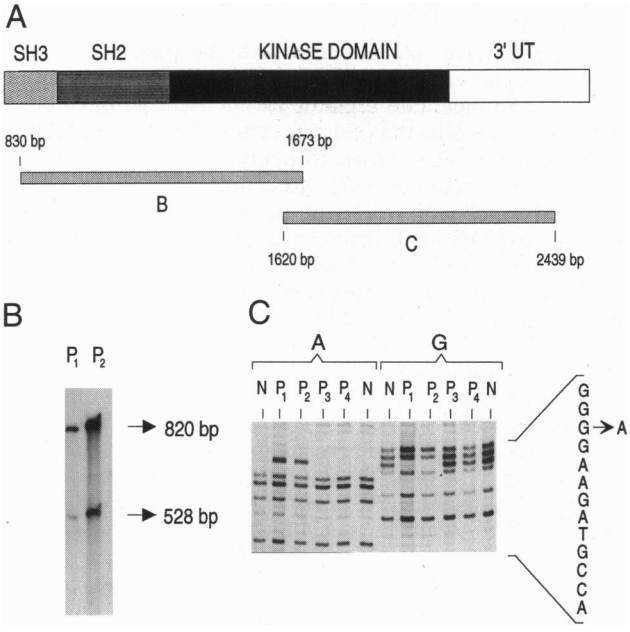

FIG. 5. (*A*) Schematic representation of the two PCR-amplified fragments designated B and C used in the chemical mismatch analysis. (*B*) Both cDNA segments for each XLA patient were subjected to the CMM method to detect point mutations. In addition to the normal 820-bp full-length product for segment C, the Gly-594 → Glu mutation appeared in two XLA brothers (P_1 and P_2) as a 528-bp product as a result of hydroxylamine modification. (*C*) Direct sequencing of the PCR products of segment C for patients P_1 and P_2 showed a G-to-A mutation at nucleotide position 1913 resulting in a Gly-594 → Glu change. Shown in the figure are only the G and A sequencing tracks for the two affected brothers as well as two unaffected relatives (lanes P_3 and P_4) and the normal sequence (lanes N). The normal sequence around the this mutation is shown to the right.

important residues (Arg-562, Trp-563, and Ala-582) are conserved in BTK, Itk/Tsk and Tec as well as in the Src family, where the alanine is replaced by serine. The Arg-562 → Pro and Ala-582 → Val mutations were found in XLA patients having <1% B lymphocytes and very low immunoglobulin levels. However, as the Arg-562 → Pro mutation was found in a sporadic case, and as phenotypic heterogeneity has been reported in some families with XLA (60), the implications of this phenotype should be interpreted with caution.

The conserved Arg-520 located at the N terminus of the catalytic loop is structurally and functionally vital. In cAPK the corresponding Arg-165 interacts with the stable Thr-197 phosphorylation site (17). Both of these residues are conserved also in BTK, although the corresponding phosphorylated residue in PTKs is a tyrosine (Tyr-551 in BTK). This residue precedes the homologue for Thr-197 in cAPK (61). Many kinases are known to be activated by phosphorylation at either of these sites. According to the model, the phosphorylated Tyr-551 could interact with Arg-520 in BTK. Mutation Arg-520 → Glu causes XLA without detectable B lymphocytes, presumably because of the lack of contact between the catalytic loop and the regulatory phosphorylation site.

The conserved Glu-589 of PTKs locates in the αF in a cleft corresponding to the substrate-binding region in cAPK. Inactivity of the Glu-589 → Gly change is presumably due to the absence of ionic interaction with the substrate. The Glu-589 → Gly mutation was found in three patients from one family with very low levels of immunoglobulins and B-cell numbers ≤ 1%. Another possible substrate binding residue is the almost invariable Gly-594 that lies on the surface of the protein between αF and αG. BTK has a similar cleft as is present in cAPK, but there are numerous charged residues including arginine residues at positions 487, 490, 492, 600, and 618. Introduction of glutamate at position 594 might electrostatically change the entire region.

The Gly-594 → Glu mutation was found in a family with XLA and concomitant growth hormone deficiency, where the abnormality seems to be confined to this alteration, since no evidence for aberrations in genes flanking BTK was found (62). The XLA phenotype was mild with detectable B lymphocytes in the two investigated patients [B-lymphocyte levels of 1% and 2% and IgG of 1.3 and 2.3 g/liter, respectively (normal, ≥7.0 g/liter), in two affected brothers]. The location of residue 594 outside the catalytic region is compatible with the milder disease phenotype observed in this XLA family.

The conserved Gly-613 of PTKs follows helix G and lies on the bottom of the lower domain (Fig. 2). Mutation to Glu-613 causes XLA with a milder form (detectable levels of IgM, IgG, and IgA, with IgG > 4 g/liter in two affected individuals). The mutation might prevent interaction with other domains of BTK or with the substrate.

Recently several new mutations causing XLA have been published (63, 64). Among the 13 mutations, only one missense mutation, Ala-607 → Asp, was found in the BTK kinase domain. Ala-607 locates on the αG on surface of the molecule outside the catalytic cleft. It presumably is not needed for substrate binding.

In summary, point mutations causing XLA have been interpreted in structural terms with the aid of a 3-D BTK model. The catalytic-core domains of PSKs and PTKs seem to fold in a similar way, and they are thought to have a related in-line reaction mechanism. Residues involved in ATP and Mg^{2+} binding were highly conserved, whereas substrate binding is different. The disease-causing mutations are thought to disturb ATP binding (Lys-430 → Glu and Arg-525 → Gln), prevent activation due to missing contact between the catalytic loop and the regulatory phosphorylated residue (Arg-520 → Glu), interfere with substrate binding (Glu-589 →

Gly and Gly-594 → Glu) and/or domain interactions (Gly-613 → Glu), or clash with the conserved Trp-563 (Arg-562 → Pro and Ala-582 → Val). All of the eight mutations were located on one face of the BTK kinase domain, indicating structural clustering of functionally important residues.

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