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Pleckstrin Homology Domains of Tec Family Protein Kinases

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Pleckstrin homology (PH) domains have been shown to be involved in different interactions, including binding to inositol compounds, protein kinase C isoforms, and heterotrimeric G proteins. In some cases, the most important function of PH domains is transient localisation of proteins to membranes, where they can interact with their partners. Tec family protein tyrosine kinases contain a PH domain. In Btk, also PH domain mutations lead into an immunodeficiency, X-linked agammaglobulinemia (XLA). A new disease-causing mutation was identified in the PH domain. The structures for the PH domains of Bmx, Itk, and Tec were modelled based on Btk structure. The domains seem to have similar scaffolding and electrostatic polarisation but to have some differences in the binding regions. The models provide new insight into the specificity, function, and regulation of Tec family kinases.

Pleckstrin homology (PH) domain was initially discovered as an internal repeat in pleckstrin and later found from more than 150 different proteins having diverse cellular signalling and cytoskeletal functions, including protein kinases, both protein tyrosine kinases (PTKs) and protein serine/threonine kinases (PSKs), and their substrates, phospholipase C (PLC) isoforms, GTPases, GTPase activating proteins, guanine nucleotide releasing factors, and adaptor proteins (1–3). The function of the PH domain is still somewhat unclear, but in many cases it acts as a membrane localisation domain. Certain signalling molecules such as Src family PTKs are constitutively membrane-bound by N-terminal myristylation (4). The N-terminal half of at least certain PH domains binds inositol compounds thereby being possibly important for membrane localisation for proteins that have to be at least transiently close to membrane (5–9).

Many PH domains have been shown to interact with subunits of heterotrimeric G proteins (10–16). Certain PH domains can bind also to protein kinase C (PKC) isoforms (17–20). Btk PH domain interacts also with BAP-135 protein of unknown function (21).

The three dimensional structure has been determined for a number of PH domains including Btk (22, 23), dynamin (24–27), PLC δ (8), pleckstrin (28), SOS (29, 30), spectrin (7, 31), and β-ARK1 (32). Although PH domains share very limited sequence identity, they have the same fold consisting of a β-barrel formed of two β-sheets and a C-terminal α-helix that caps one end of the β-barrel.

The Tec family of cytoplasmic PTKs is formed of Btk (33, 34), Itk/Tsk (35–37), Tec (38), and Bmx (39). In addition to the Src homology 2 (SH2) and SH3 and the kinase domain, Tec group kinases have in their N terminus a PH domain followed by a Tec homology (TH) domain (40–42). The Tec family is thus far the only PTK family known to contain a PH domain. TH domain contains two parts. N terminal Btk motif is followed by a proline rich region (41, 42). The Btk motif contains HC_3 pattern (one histidine and three cysteines) which binds stabilising Zn^{2+} ion (22, 42). The proline rich region in Btk binds to certain SH3 domains (43–45), possibly also intramolecularly to the adjacent SH3 domain (Okoh and Vihinen, in preparation) as in Itk (46), thereby regulating the function of these kinases.

Btk is crucial for B cell development and mutations lead into an immunodeficiency, X-linked agammaglobulinemia (XLA) (33, 34). XLA is the best known signal transduction related disease with over 300 different mutations (47). Btk is expressed in hematopoietic lineage, except for T cells and plasma cells (40). IL-2 inducible T-cell kinase (Itk) (35) is expressed in thymus, NK cells and T cells. Tec appears in several alternatively spliced forms in which the termini are differently processed. It is expressed in T and myeloid lineages. Bmx has wider expression pattern including endothelial cells. These proteins are involved in many signalling events, but the signalling pathways are still largely unknown.

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To study the relationships of the Tec family members, the structures of their PH domains were modelled and compared to understand different inositol compound, protein kinase C, and G protein binding. BAP-135 binding was not studied because of lack of structural information. The domains were found to fold similarly, but the differences in ligand binding could be explained in structural terms. The model and X-ray structure of the Btk PH domain have previously been used to interpret Btk PH domain function (7, 48). The effects of the XLA-causing mutations in the other family members were studied based on the models.

MATERIALS AND METHODS

Mutation analysis. Genomic DNA was prepared from the whole blood of patient using QIAamp blood midi kit (Qiagen, Germany). All the 19 exons of the Btk gene were amplified using the improved primers as described by Vořechovský *et al.* (49). PCR was performed in 25 μl volume containing a 5′-terminal primer labelled with 5 pmoles of γ-^{32}P-dATP (3000 Ci/mmol), which was incorporated using T4 polynucleotide kinase. The amplification reactions had 100 ng DNA, 2 μl of BESS dNTP (Epicentre Technologies, Madison, Wisconsin), 25 pmoles of downstream primer, and 2 U DyNAzyme DNA polymerase (Finnzymes, Finland). The amplified products were excised using BESS excision enzyme (Epicentre Technologies), and subjected to 8% polyacrylamide sequencing gel. The exon revealing differences after scanning with T-Scan and G-Tracker was reamplified without radiolabel. The products were resolved in an agarose gel stained with ethidium bromide. Band of interest was excised and the DNA purified using QIAquick PCR purification kit (Qiagen). The DNA sequencing was carried in both directions.

Molecular modelling. The sequences for Bmx (accession number P51813), Btk (Q06187), Itk (Q08881), and Tec (P42680) were taken from databases. The Btk PH domain structure with the Btk motif (750). The final alignment was obtained by manual adjustment based on the multiple sequence analysis and knowledge about the three dimensional structure of PH domains.

Modelling was carried out using InsightII and Discover (Molecular Simulations, Inc., San Diego, CA) programs. Deletions and insertions were modelled by searching loops from a database, which contained a representative selection of the PDB files. The models were refined with energy minimisation in a stepwise manner by using Amber force field. First, all hydrogens within the molecule were freed, then the side chains of the built loops followed by the backbone of the loops. The C_α atoms of the conserved regions were constrained in the last step. The final models were tested and found to have a typical globular structure according to several tests (51). The Ins(1,3,4,5)P_4 was docked to the models based on the Btk co-crystal structure (23). The electrostatics of the PH domains was calculated with program GRASP (52).

RESULTS AND DISCUSSION

A novel PH domain mutation leads to a classical XLA. A new classical XLA-causing mutation, insertion of t at 442, was identified in exon 5 with BESS mutation detection method and direct sequencing. This unique mutation (BTKbase PIN V105X122(1), Accession number, A0541) causes a frame shift and stop codon at position 122. The truncated protein is not functional.

Structure of Tec family PH domains. The sequence alignment of the Tec family kinases shows considerable sequence similarity and identity of over 60 and 50 percent, respectively (Fig. 1). The sequence identity is higher than between PH domains in general. Btk PH domain was the template for the other family members (Fig. 2). The gaps in the alignment are similarly located in all the proteins. The gaps lead only into deletions on surface loops connecting secondary structural elements. The four sequences have 50 invariant residues, and 32 conserved sites, which form a substantial part of the protein core, secondary structures and certain regions of protein surface (Fig. 2). The sequences are more variable in their N-terminal half and there are no insertions or deletions in the C-terminal half including the Btk motif. The Zn^{2+} binding residues are invariant and residues interacting with the other ligands are rather conserved.

The scaffolding of known PH domains consists of a seven-stranded β-sheet, with a flanking C-terminal α-helix (7–8, 22–31). There are three deletions of one, two and 20 residues in the alignments of Bmx and Itk, whereas Tec has only two deletions. The long loop between β5 and β6 is typical only for Btk and it has very low density in Btk crystal structure (22). The two residue deletions between β3 and β4 are in a region, which has variable length in many PH domains. Overall, the structures are rather similar (Fig. 2), but there are differences in the binding sites. The Btk motif of the Tec family members is conserved and the Zn ligands are in identical positions. The polarisation of the Tec family PH domains is clearly evident in Fig. 3. PH domains are strongly electrostatically polarised molecules (53, 54). In addition to structural similarities, electrostatic polarisation suggests Tec family PH domains to have related, but not identical properties and functions. The positively charged region coincides with the Ins(1,2,3,4)P_4 binding region in Btk. In Btk, many of the XLA-causing mutations either reduce or reverse the electrostatics of the domain, especially in the inositol interaction region (Fig. 3).

Binding to inositol compounds. The N-terminal half of certain PH domains has been shown to bind phosphoinositides. Residues implicated in this binding have been localised in Btk (23), PLC δ1 (8), and β-spectrin (7, 55) structures.

The Ins(1,3,4,5)P_4 molecule was docked to the models according to the binding in Btk. The positively charged surface of the PH domain binds to the negatively charged phosphate groups of the inositol molecules, which can play a role in membrane recruitment. The Ins(1,3,4,5)P_4 has several ligands in the Btk PH domain, in its N-terminal part including residues K12,

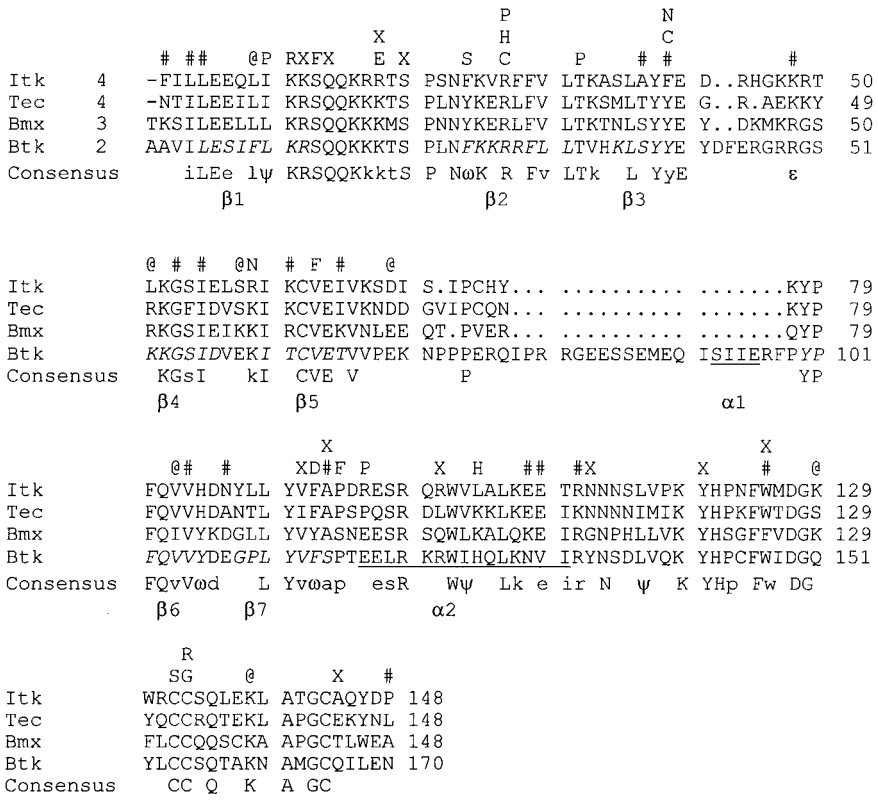

FIG. 1. Sequence alignment of the PH domain and Btk motif of Tec family members. The secondary structures taken from the PDB (entry 1btk) are shown with underlining for α-helix in italics for β-strands. The invariant residues are shown in capital letters and conserved residues in lower case letters in the bottom. Conserved aromatic residues are indicated with ω, basic residues with ε, and hydrophobic residues in ψ. Mutations causing XLA are indicated above the sequence, X indicating nonsense mutations, # deletions, and @ insertions.

Q15, Q16, K17, K18, S21, N24, K26, R28, and Y39 (23). These residues are invariant in the Tec family (Fig. 1) except for K18, which is replaced by a conservative substitution by arginine in Itk.

The results of the specificity and affinity studies of inositol compounds are often contradictory possibly due to different assay methods and conditions. Btk PH domain has been shown to bind Ins(1,3,4,5)P_4 with K_d of about 40 nM (56). On the other hand PtdIns(3,4,5)P_3 was shown to be specific for Btk PH domain (57). In one study, Btk PH domain had higher affinity to inositol phosphate than the soluble analogue of phosphoinositide (58), but in another study phosphoinositides had a higher affinity than the corresponding inositol phosphates (59).

The binding affinities to Ins(1,3,4,5)P_4 and Ins(1,2,3,4,5,6)P_6 have been measured for all the Tec family PH domains (58). Btk mutations reduced binding to Ins(1,2,3,4)P_4 (56). R28C mutation reduced the selectivity of binding dramatically (57, 59) presumably because the substitution removes the basic arginine from the ligand binding cleft and affects the strength of the electrostatic polarisation (Fig. 3). Btk and Tec had similar affinity for both Ins(1,3,4,5)P_4 and Ins(1,2,3,4,5,6)P_6, whereas Bmx binds only weakly to Ins(1,3,4,5)P_4 and Itk to neither of them (58).

The binding regions are quite similar in the four PTKs (Fig. 3). The explanation for Itk not binding to the inositol compounds may be that the loop containing a number of binding residues may be slightly differently flipped compared to the other residues. The loop formed by residues 15–23 in Btk is very conserved. Itk has S at position 25 while all the others have either L or N. Very small differences and shifts in the loop will affect the shape and electrostatics of the binding region, and thereby affect specificity and affinity. Similar explanation might apply also the Bmx, which has at position 21 a methionine while the others have threonine. The loop undergoes a conformational change upon inositol compound binding (23, 55), which is important for binding specificity and amino acid differences account for different affinity and specificity.

A gain of function mutation, Btk*, E41K (60) binds more strongly to Ins(1,3,4,5)P_4 than the wild type domain. The same mutation in the other Tec family members will most likely increase their polarisation and membrane localisation. E41 locates in the loop between β3 and β4 next to a deletion of two residues compared to Btk (Fig. 1).

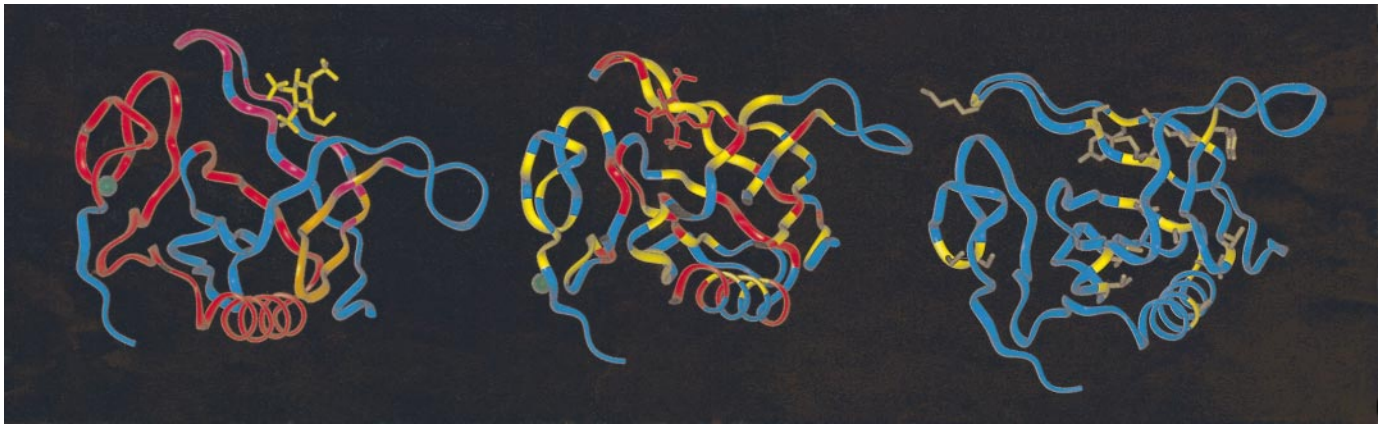

FIG. 2. Model of the Itk PH domain and Btk motif (left). The regions involved in ligand binding are color coded as: inositol compound binding residues in magenta, the minimal PKC binding region in orange, and G protein interaction surface in red. Zn is in green and Ins(1,2,3,4)P_4 in yellow. (Center) Sequence conservation in the family indicated in the model of Tec PH domain. The invariant residues are in yellow and the conserved ones in red. The Ins(1,2,3,4)P_4 is in magenta stick model and the Zn as a magenta ball. On right, distribution of the XLA-causing point mutations is indicated in yellow in the Bmx PH domain model. Ins(1,3,4,5)P_4 is in red and Zn in green.

Binding to protein kinase C. Btk and Itk PH domains have been shown to interact with different PKC isoforms *in vitro* (17). PKC is a PSK that phosphorylates also Btk and downregulates its activity (17). Btk was shown to physically associate with PKC in mast cells and possibly also in other hematopoietic cells. Also Itk binds with high affinity. The minimal binding site in Btk PH domain was located to region β2 to β3

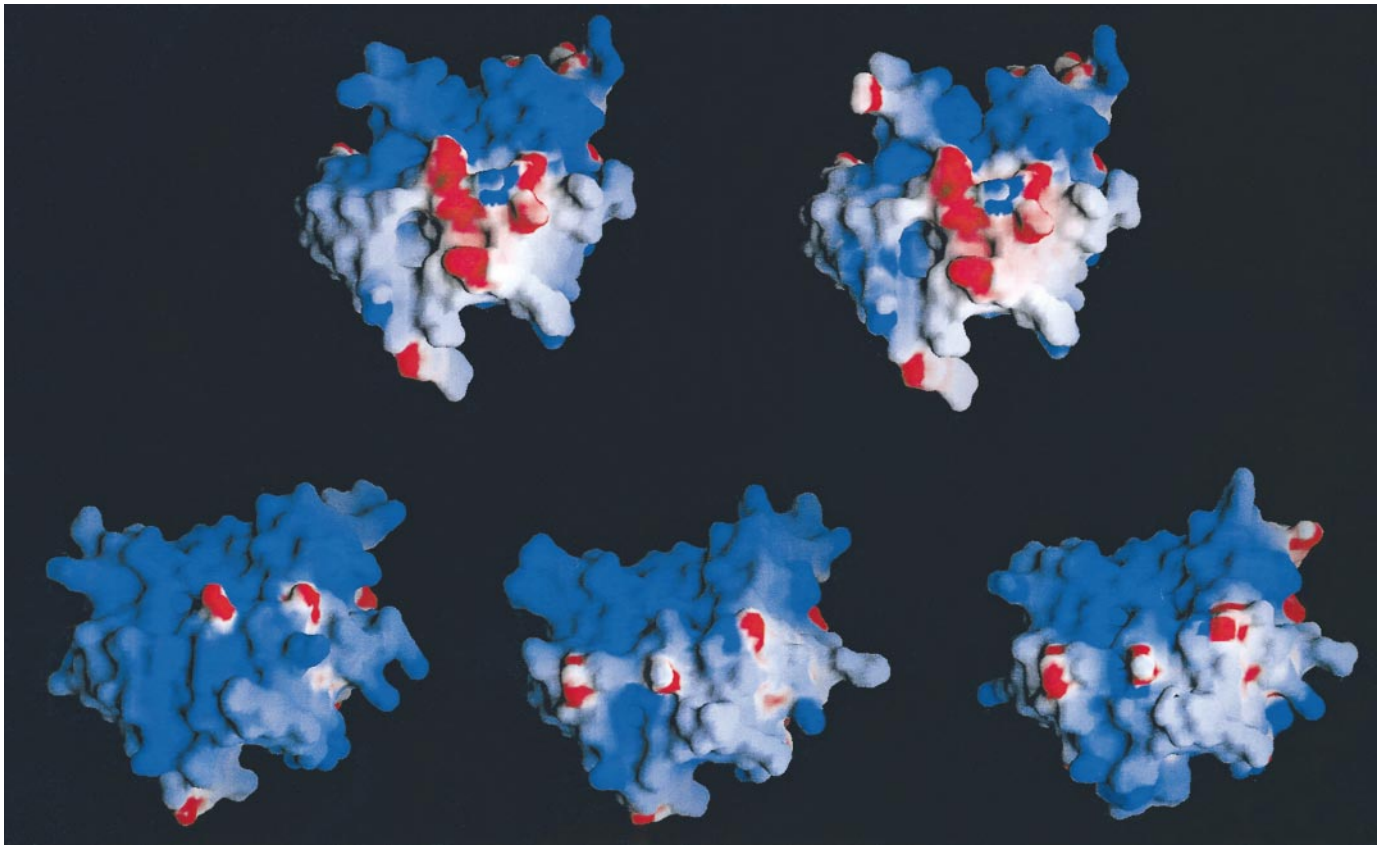

FIG. 3. Electrostatics of Tec family PH domains. From top, wild type Btk (left) and Btk PH domain with XLA-causing mutations (right). At bottom, from left: Bmx, Itk, and Tec PH domains. The inositol compound binding cleft is pointing upwards.

from residue 28 to 45 (18). Also some inositol compound binding residues locate in this region. This region is formed of an antiparallel β-sheet which could have stabilised the produced peptide and thereby increased its affinity.

The physiological relevance of PKC interaction needs to be further studied. There are several possible action mechanisms including Btk targeting to membranes, and signalling downstream of Btk. Interestingly, the phenotype of PKC β knock-out mice is very similar to Btk knock-out *xid* mice (61). The PKC binding competes with PtdIns(4,5)P_2 binding, suggesting that the binding sites overlap or at least locate in near vicinity to each other (18).

The Ins(1,3,4,5)P_4 in Btk is bound to residues from β1–3 as well as from the loop between β1 and β2. The minimal PKC binding region contains of these residues R28 and Y39. The β1–β3 region bears several essential amino acids that impair Btk function when mutated (Fig. 1) including residues 28, 33, and 40. Mutation R28C, which causes the mouse *xid* phenotype (62, 63), has been found also in man. Further, the gain-of-function E41K mutation (60) is located in this critical region. The minimal binding region is part of the positively charged end of the PH domain. The Tec family members are related in this region up to residue 42 in Btk. According to the models, the Tec family PH domains all form an antiparallel two-stranded β-sheet on the protein surface. In addition to Btk and Itk, the whole family is predicted to have affinity to PKC isoforms because the binding surfaces have similar conformation and electrostatic properties.

Binding to heterotrimeric G proteins. Many receptors transmit signals via G proteins by catalysing the exchange of GTP for GDP. Both α and $\beta\gamma$ subunits have been shown to interact with PH domains (10–12, 14). Both Btk (11, 12, 64) and Itk (11) bind to $\beta\gamma$ subunits and they both are activated by the interaction (64). Also Gqα (14) and Gα12 (16) subunits bind to and activate Btk. The binding sites for both Gα (15) and $\beta\gamma$ subunits (11, 12) overlap and locate in the C-terminal part of the PH domain and in the Btk motif of the TH domain, residues 108-162 in Btk.

The binding region is large and the actual interactions have not been characterised. The essential region is divided into two halves. The C-terminal half folds close to the inositol compound interaction surface (Fig. 2). The three dimensional structure is similar in this part of all the Tec proteins including also the Zn binding Btk motif. There are 17 invariant residues and 15 conserved sites and no gaps. Although the actual interacting residues are unknown, it could be likely that all the four proteins could bind to G protein subunits, although possibly with different specificities and affinities due to amino acid substitutions. Metal binding may not be essential for the interaction because many of the proteins shown to bind to G proteins (11) do not include the Btk motif.

It is not clear if the proteins have direct interaction with Tec family members *in vivo* or if there are mediating factors such as PI3-kinase, which has been shown to bind directly to $\beta\gamma$ subunits via its p110 subunit (65). Whether there is direct interaction or not, the interaction surface contains a number of XLA-causing mutations in Btk.

XLA-causing mutations. Several Btk PH domain mutations have been described in patients as well as in a mice model, including missense and nonsense mutations, insertions and deletions (33, 34, 47). Btk is the only protein where PH domain mutations are known to cause a disease. A mutation has been found from an Alzheimer disease patient having R105H substitution in the PLC γ1 PH domain (66), but it is unclear if the mutation is responsible for the disease although it decreases affinity to Ins(1,4,5)P_3 and hydrolysis of PtdIns(4,5)P_2 *in vitro.*

The majority of the mutated residues locate in the inositol compound binding region and in its vicinity. The electrostatic properties of the domain change due to many of the mutations (Fig. 3). L11 is situated in the strand β1, where substitution to proline most likely destroys the secondary structure leading to structural alterations. Similar consequences are likely also in the other family members. K12R substitution affects an invariant ligand-binding residue. The replacement of the invariant S14 by phenylalanine causes a sterical clash with the ligand. Substitution of an almost invariant K19 by E close to the ligand alters the electrostatic properties of the domain. Residue at position 25 is aromatic, either tyrosine or phenylalanine, in all the four proteins. The mutated protein most likely forms a stable fold (22), but the ligand interaction may be altered.

K27R mutation is found from a patient having a number of mutations. It is not known if the substitution is responsible for the phenotype. K27 is in the area where the diacyl glycerol part of phosphatidylinositols is located and could therefore be disease causing. R28 binds to the Ins(1,3,4,5)P_4.

Invariant residue T33 is not in the ligand recognition region. Introduction of a proline in the tight turn between strands β2 and β3 will have structural effects. Y40 is an invariant residue in β3 and next to the ligand binding Y39. Mutation either to C or N will alter the electrostatics of the domain. I61 and V64 are conserved in the Tec family and located in the core of the domain. At least V64F mutation affects the protein structure and the domain could not be produced in soluble form (22).

Several of the G protein-binding residues are mutated. Amino acid corresponding to number 113 in Btk is either valine or isoleucine in the Tec family. Substitution V113D in the middle of the β7 will have structural effects. S115, T117, and Q127 appear only in Btk

and they are exceptions to invariant residues among XLA-causing mutations. S115 is in the protein core where phenylalanine could not be fitted. T117P mutation in the short turn between sheet $\beta 7$ and helix $\alpha 2$ affects the structure of the domain due to the special properties of proline in protein backbone. Q127H alteration might prevent G protein interactions. Invariant Zn^{2+} ligands C154 and C155 lead into XLA due to structural alterations because of missing metal ion interactions.

In summary, the Tec family PH domains and Btk motifs share similar three-dimensional structure. Despite rather similar structures the Tec family PH domains have somewhat different ligand specificities, which were discussed based on the models. Differences in inositol compound interaction arise from subtle variation in the phosphate recognition sites and their surroundings, as well as the shape of the binding sites and the electrostatics of the domains. All the family members are likely to interact with PKC proteins, but there may be differences, because the binding region overlaps with that for phosphoinositides. The region binding to G proteins is large and contains many conserved features of the Btk motif.

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