Structural Basis for Pleckstrin Homology Domain Mutations in X-Linked Agammaglobulinemia†

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ABSTRACT: Deficiencies in a tyrosine kinase, designated Btk, cause X-linked agammaglobulinemia (XLA) in man, a hereditary defect of B-cell differentiation. Mutations in the newly found PH domain located at the N-terminus of Btk have been shown to be the direct cause of XLA, and here two new mutations, T33P and V64F, are presented. Btk is thus far the only protein in which mutations of the PH domain have been found to cause a disease. The three-dimensional structure of the Btk PH domain was modeled on the basis of the dynamin PH structure. Despite a relatively low sequence similarity the Btk PH domain seems to have the same two β-sheet structure observed in the known structures. The model was used to interpret the structural basis for disease in five independent point mutations and in an insertion in patients with XLA. The mutated residues F25, V64, and V113, and possibly residue(s) around Q103, could form a binding site, since these amino acids are located close to each other on the surface of the molecule.

Btk[1] (Bruton's agammaglobulinemia tyrosine kinase) is a 659 amino acid long protein, which consists of five domains: PH (pleckstrin homology), TH (Tec homology), SH3 (Src homology 3), SH2, and a kinase domain (Vetrie *et al.*, 1993; Smith *et al.*, 1994; Vihinen *et al.*, 1994a). Btk is crucial for B-cell maturation and mutations in the Btk gene cause X-linked agammaglobulinemia (XLA). XLA is a human immunodeficiency disease characterized by a lymphocyte differentiation block, resulting in a deficiency of B cells and immunoglobulins leading to an increased susceptibility to infections (Lederman & Winkelstein, 1985; Smith *et al.*, 1994). Point mutations in the kinase, SH2, and SH3 domains have previously been shown to cause XLA (Vetrie *et al.*, 1993; Bradley *et al.*, 1994; Conley *et al.*, 1994; Hagemann *et al.*, 1994; Ohta *et al.*, 1994; Saffran *et al.*, 1994; de Weers *et al.*, 1994; Vihinen *et al.*, 1994b; Zhu *et al.*, 1994a,b; Vořechovský *et al.*, 1995). Recently, a point mutation occurring in the PH domain of Btk has been shown to cause X-linked immunodeficiency (xid) in mice (R28C) (Thomas *et al.*, 1993; Rawlings *et al.*, 1993) and XLA in man (R28H) (de Weers *et al.*, 1994; Ohta *et al.*, 1994; this report). Two other mutations, F25S (Vořechovský *et al.*, 1995) and V113D (Conley *et al.*, 1994), have also been recently shown to cause XLA. These mutations and the newly discovered mutations T33P and V64F will be described here in detail with structural implications from modeling studies.

PH domains are small modular structures of approximately 100 residues. They have been found in a number of proteins, including protein kinases, GTPase-activating proteins, phospholipases, and cytoskeletal proteins (Haslam *et al.*, 1993; Mayer *et al.*, 1993; Shaw, 1993; Musacchio *et al.*, 1993; Gibson *et al.*, 1994). The Tec family, which includes Btk, is thus far the only tyrosine kinase family observed to have a PH domain. Sequence alignments have shown a wide amino acid diversity between the PH domains (Musacchio *et al.*, 1993; Gibson *et al.*, 1994). The function of the PH domain is not clear; however, different mechanisms have been discussed. There is some indication that the terminal helix and adjacent region interact with the $\beta\gamma$ subunits of heterotrimeric G-proteins (Koch *et al.*, 1993; Touhara *et al.*, 1994; Tsukada *et al.*, 1994). Binding to lipophilic molecules has been suggested (Yoon *et al.*, 1994), and recently, binding to phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] has been shown to occur at least in some PH domains (Harlan

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[1] Abbreviations: Btk, Bruton's agammaglobulinemia tyrosine kinase; ddF, dideoxy fingerprinting; FKBP, FK506 binding protein; PH, pleckstrin homology; SH, Src homology; TH, Tec homology; PtdIns-(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; xid, X-linked immunodeficiency; XLA, X-linked agammaglobulinemia.

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et al., 1994), indicating that PH domains could be important for membrane localization. Moreover, Btk PH domains have been shown to interact with protein kinase C (Yao *et al.*, 1994). PH domains have also been suggested to recognize phosphorylated serine/threonine residues (Gibson *et al.*, 1994), but there is no experimental evidence. The spectrin PH domain has been reported to have distant structural similarity to the binding site of FK506 binding protein (FKBP) (Macias *et al.*, 1994), pleckstrin and dynamin PH domains to the lipocalin family (Yoon *et al.*, 1994; Timm *et al.*, 1994; Ferguson *et al.*, 1994), and dynamin PH also to verotoxin-1 (Downing *et al.*, 1994).

Recently, NMR structures have been determined for the pleckstrin (Yoon *et al.*, 1994), spectrin (Macias *et al.*, 1994), and dynamin PH domains (Downing *et al.*, 1994) and X-ray structures for dynamin (Timm *et al.*, 1994; Ferguson *et al.*, 1994). All the PH domain structures have a similar fold, consisting of a β-barrel made up of two β-sheets, of three and four strands, respectively, and a C-terminal α-helix capping one end of the β-barrel.

From the sequence alignment and the structural information it is evident that there are preferred locations for insertions and/or deletions within the framework of the PH domain structure. We have previously studied structural reasons for XLA in the Btk SH3 domain (Zhu *et al.*, 1994a), SH2 domain (Vihinen *et al.*, 1994c), and kinase domain (Vihinen *et al.*, 1994b) mutations. In this study we have modeled the Btk PH domain on the basis of the three-dimensional structure of the dynamin domain to explain the effect of the XLA causing mutations within the PH domain.

MATERIALS AND METHODS

Mutation Analysis. The detection of mutations in the Btk cDNA was carried out by using reverse-transcribed PCR products from gradient-separated peripheral blood mononuclear cells. Two methods were employed. One was a modified dideoxy fingerprinting (ddF) method (Sarkar *et al.*, 1992) with direct cycle sequencing of reverse transcription PCR product. Briefly, total RNA was reverse-transcribed with oligo(dT) and amplified with two pairs of primers flanking the entire Btk coding region. For ddF, the PCR products were directly cycle-sequenced with ^{32}P end labeled primer according to the manufacturer's instructions except that only ddC was used and electrophoresis was performed throughout a 5.5% 1:48 polyacrylamide nondenaturing gel at 4 °C. The mutations detected by ddF were further confirmed by direct sequencing of both strands. In the second method PCR products encompassing overlapping Btk transcript fragments were generated with first-strand cDNAs as templates, using four sets of oligonucleotide primers. The PCR-amplified products were cloned into a TA vector kit (Invitrogen). DNA sequencing was performed by the dideoxy chain termination method, using the T7 sequencing kit (Pharmacia Biotech), with standard T7 and SP6 primers.

Molecular Modeling. The accuracy of modeled structures relies on the correct sequence alignment. The Btk PH domain was aligned with the dynamin sequence manually using information from multiple sequence alignments of PH domains (Shaw, 1993; Musacchio *et al.*, 1993) and secondary structural information from the three-dimensional structures. Modeling was performed using the Quanta (Molecular Simulations, Inc., Burlington, MA) and CHARMM packages (Brooks *et al.*, 1983). The NMR coordinates of the dynamin PH domain (Downing *et al.*, 1994) were used as a template for Btk. A side-chain rotamer library was used to model substitutions. Deletions were subjected to local minimization to bring the ends together and to alleviate local conformational strain. Insertions were modeled by searching a database of high-resolution (<2.0 Å) molecules. The search was performed for fragments of required length and end-point separation by using two residues at each end of the loop as anchor points. Fragments obtained were evaluated on three criteria: root mean square deviation from the anchor points, sequence similarity, and interference with the core region. There were four insertions within Btk with respect to dynamin. Three of these were no longer than 4 residues and thus easily and accurately modeled. One insertion, however, consisted of 28 residues. This loop could not be modeled by conventional means, and the final model gives only suggestions for the fold and location of the loop.

Modeling of the 28-Residue Insertion. The structure, minus the insertion, was subjected to global minimization using the CHARMM program. The 28 residue long insertion was built in the minimized structure using a database search, which resulted in a structure containing an antiparallel β-sheet consisting of two strands. The insertion has a number of prolines, two of which are at the ends of the insertion. These prolines could direct the loop to fold back onto the structure. Using the prolines as a guideline, the loop was folded manually to one side of the β-sheet to extend the sheet. Once a number of preferential folds were established, the insertion was subjected to 100 ps of molecular dynamics at 1000 K. The whole protein was also simulated for comparison. The final structure of the Btk PH domain was checked for correctness using programs Poldiag (Baumann *et al.*, 1989) and Verify_3d (Lüthy *et al.*, 1992).

RESULTS AND DISCUSSION

XLA-Causing Mutations on the Btk PH Domain. Mutation of nucleotide 215 from G to A in two brothers changes PH domain residue R28 to histidine (Figure 1A, patient JN), and in two related individuals a transition of nucleotide 229 A to C causes substitution T33P (patient SG in Figure 1A). A single point mutation changing nucleotide 322 from G to T causing substitution V64F was found in two brothers (Figure 1B). All these patients had hardly any (<1%) circulating mature B lymphocytes. The two brothers with the V64F mutation were investigated for pre-B cells and found to have normal numbers in their bone marrow as frequently seen in classical XLA (Campana *et al.*, 1990). All patients had low IgG and low, or undetectable, IgM and IgA in serum. Patients with the R28H and T33P mutations were investigated for immune response to bacteriophage $\phi X174$ (Ochs *et al.*, 1971). A weak (R28H) or absent (T33P) antibody response was found. While there was some heterogeneity based on these observations, the disease phenotype of all patients can be classified as classical XLA.

Modeling of the Btk PH Domain Structure. The Btk PH domain was modeled on the basis of the dynamin structure, which is the most related among the structures determined so far. All the three known PH domain structures (Downing *et al.*, 1994; Ferguson *et al.*, 1994; Macias *et al.*, 1994; Timm *et al.*, 1994; Yoon *et al.*, 1994) have the same scaffolding,

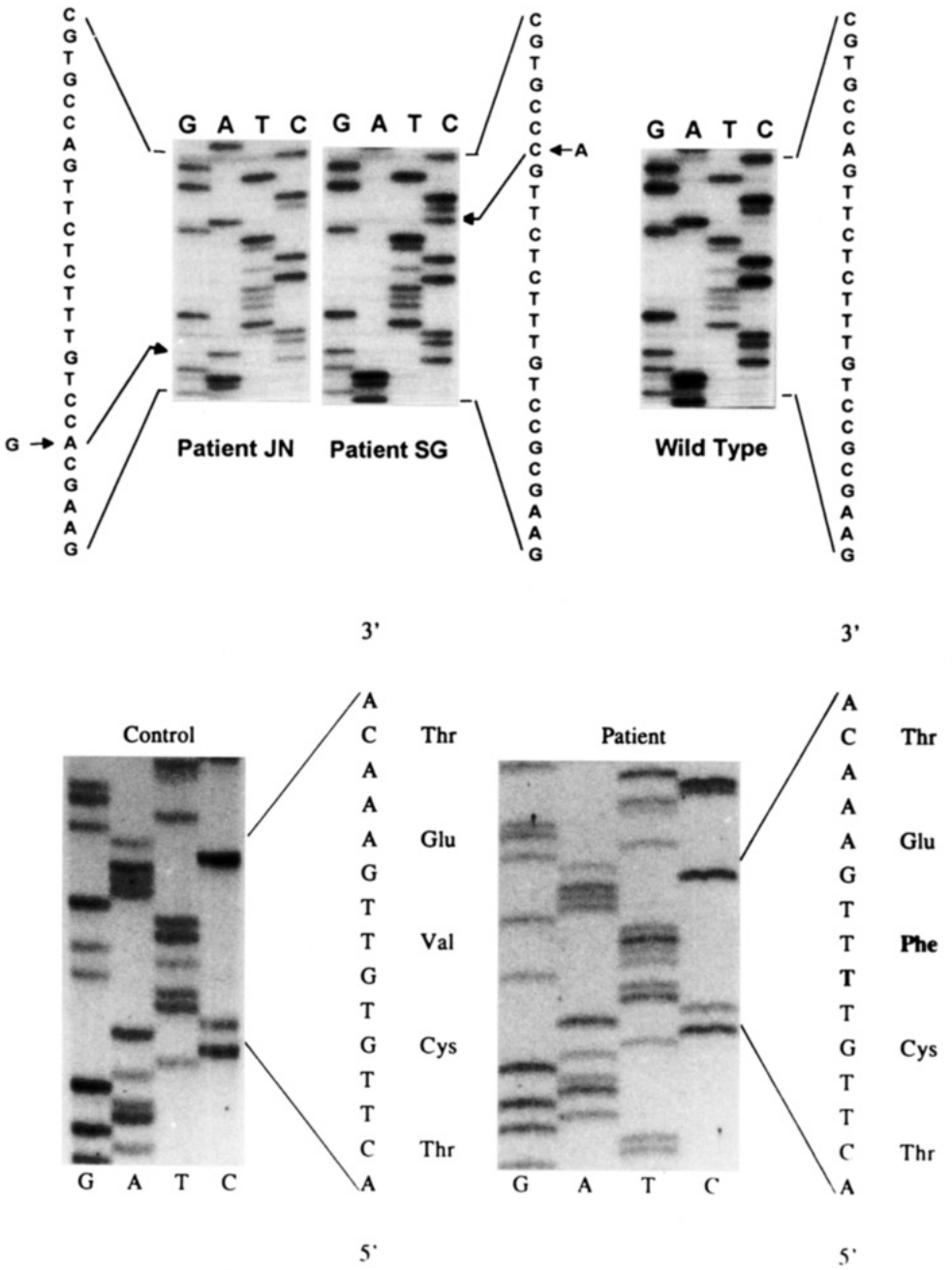

FIGURE 1: PCR sequence analysis of cDNA of Btk of the affected males. (A, top) Location of the G to A transition at nucleotide 215 causing mutation R28H (patient JN) and transition of nucleotide 229 from A to C causing substitution T33P in patient SG. The normal sequence is shown to the right. (B, bottom) Direct sequencing of the PCR product showing change in nucleotide 322 from G to T resulting in mutation V64F. The normal sequence is shown to the left for comparison.

where the major differences appear on surface loops. The secondary structures are well conserved in all three PH domains although the sequence similarities are very low (Figure 2). Btk PH alignment with the dynamin sequence demonstrated six insertions/deletions which all are located in the loops connecting the secondary structural elements (Figure 2). Except for the large insertion of 28 residues they were easy to model. The final model was tested and found to have a typical globular structure according to the inverse folding test (Lüthy *et al.*, 1992). The program Poldiag was used to study the distribution of polar/unpolar, charged/neutral residues both inside and on the surface of the structures. The model passed all six tests.

The Btk PH domain has presumably the same two β-sheet fold as the known PH domain structures (Figure 3). Also the α-helix is conserved. The model contains two deletions of one and eight residues. The larger deletion between the sixth and seventh strand is on a variable region, where Btk is more related to the shorter loops of spectrin and pleckstrin (Figure 2). Otherwise, dynamin was more related to Btk PH, and thus the best starting point for modeling.

There were four insertions of 2, 3, 4, and 28 residues. The shorter ones extended the connecting loops between the β-strands. The largest loop was also modeled, despite its length, by using molecular dynamics simulation to search for energetically favorable conformations. The loop folded during the simulation so that it had interactions with the core of the domain and it might even extend the existing β-sheet. When the whole structure was simulated without constraints for comparison at 1000 K, the C-terminal α-helix unfolded. However, it seems unlikely that this sort of process could appear *in vivo* since the PH domain structure is presumably

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                        βA                                  βB       βC
Pleckstrin    1 MEPKRIREGYLVKKGSV.........FNT...WKPMWVVLLEDGIEFYKKK  39
Spectrin      1       MEGFLNRKHEWEAHNKKASSRS...WHNVYCVINNQEMGFYKDA  41
Dynamin     516 DEILVIRKGWLTINNIG......IMKGG...SKEYWFVLTAENLSWYKDD 558
Btk           1 MAAVILESIFLKRSQQ.......KKKTSPLNFKKRLFLLTVHKLSYYEYD  43

                            βD     βE
Pleckstrin   40 SDNSP......KGMIPLKGSTLTSPCQDFG.....................  63
Spectrin     42 KSAASGIPYHSEVPVSLKEAICEVALD.YK.....................  70
Dynamin     559 EEK......EKKYMLSVDNLKLRDVEKGFM..................... 580
Btk          44 FERGRR..GSKKGSIDVE..KITCVETVVPEKNPPPERQIPRRGEESSEM  89

                            βF                  βG          *  αA
Pleckstrin   64 .......KRMFVFKITTTKQ.......QDHFFQAAFIEERDAWVRDINKA  99
Spectrin     71 .......KKKHVFKLRLSDG.......NEYLFQAKDDEEMNTWIQAISSA 106
Dynamin     581 .......SSKHIFALFNTEQRNVYKDYRQLELACETQEEVDSWKASFLRA 623
Btk          90 EQISIIERFPYPFQVVYDEGP........LYVFSPTEELRKRWIHQLKNV 131
                                ↑
               αA
Pleckstrin  100 IKCIEG   105
Spectrin    107 ISSDKH   112
Dynamin     624 GVYPER   629
Btk         132 IRYNSD   137
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FIGURE 2: Sequence alignment of pleckstrin, spectrin, dynamin, and Btk PH domains. The residues forming the first β-sheet are in red and those forming the other in blue. The helix-forming residues are in cyan. The underlining in the pleckstrin sequence indicates the residues reported to be involved in PtdIns(4,5)P_2 binding (Harlan *et al.*, 1994). The residues in spectrin corresponding to those in FK506 binding in FKBP are shown by underlining. The invariant tryptophan in PH domains is indicated with an asterisk. The sites where mutations in Btk have been observed to cause XLA are underlined. Residue 103 preceding XLA causing insertion is shown by an arrow.

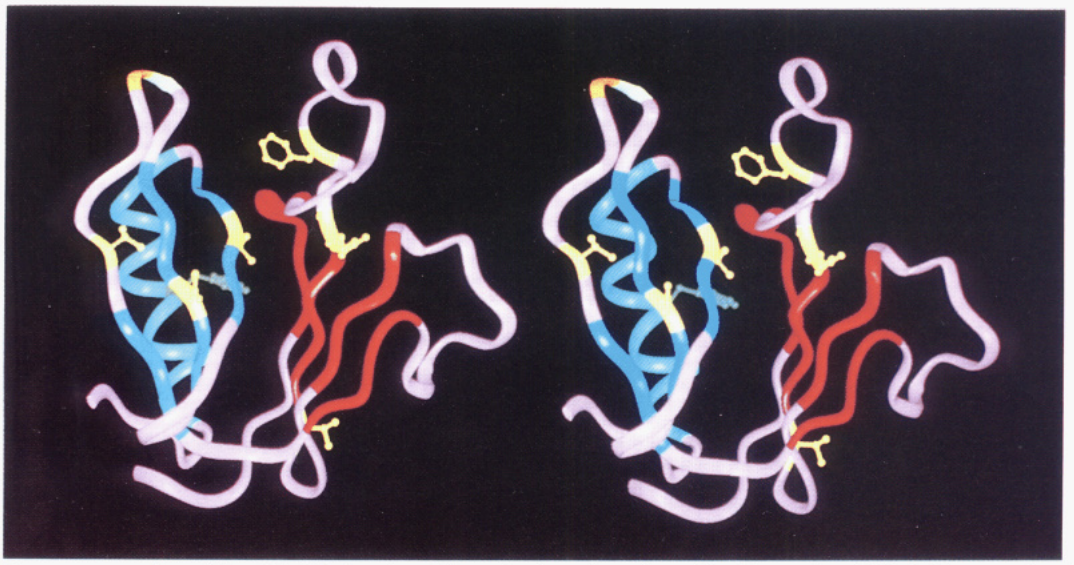

FIGURE 3: Ribbon representation of the Btk PH domain without the large insertion. The β-strands and the helix are colored as in Figure 2. The residues preceding and following the large insertion (not shown) are in orange and white, respectively. The residues causing XLA when mutated are in yellow: F25 (top) and from left to right V64, Q103, V113, and R28. T33 is down. The W124 is in green.

stabilized also by interactions with other parts of the Btk molecule.

Structural Description of XLA-Causing Mutations. Mutation at position 28 in Btk PH causes xid in mice (Thomas *et al.*, 1993; Rawlings *et al.*, 1993). Mutation R28H is found to cause typical XLA in three unrelated families (de Weers *et al.*, 1994; Ohta *et al.*, 1994; this report). Thus, four of the ten reported Btk PH substitutions affect this residue. R28 is surrounded by charged residues R13, Q16, K17, K18, K26, E41, and K53. The pleckstrin PH domain has six clustered lysines (13, 14, 22, 37, 38, and 45), three of which are conserved in this region (Figure 2). These residues form a highly charged patch on the surface of the molecule. It has been anticipated that this cluster of basic residues might form a binding site at the lip of the β-barrel (Downing *et al.*, 1994; Timm *et al.*, 1994; Yoon *et al.*, 1994). Some of the pleckstrin PH domain residues, having chemical shift changes when PtdIns(4,5)P_2 is bound (Harlan *et al.*, 1994), are conserved also in the Btk PH domain (Figure 2). The spectrin PH domain has been suggested to resemble the binding site of FKBP (Macias *et al.*, 1994). Some of these amino acids correspond to PtdIns(4,5)P_2 binding residues in pleckstrin (Figure 2). Thus, although the nature of the bound compound may not be clear, these results seem to indicate the existence of a binding site close to R28 in Btk PH. Therefore, mutations R28C and R28H presumably cause electrostatic changes, which might be critical for binding. R28 is not completely conserved in the known PH domains, but the basic residues arginine and lysine are frequently found. Cysteine appears in this position only in human Bcr (Lifshitz *et al.*, 1988), which, however, has hardly any sequence similarity to other PH domains in this region.

The mutation T33P appears on a tight β-turn between strands B and C (Figures 3 and 4A). A proline residue could

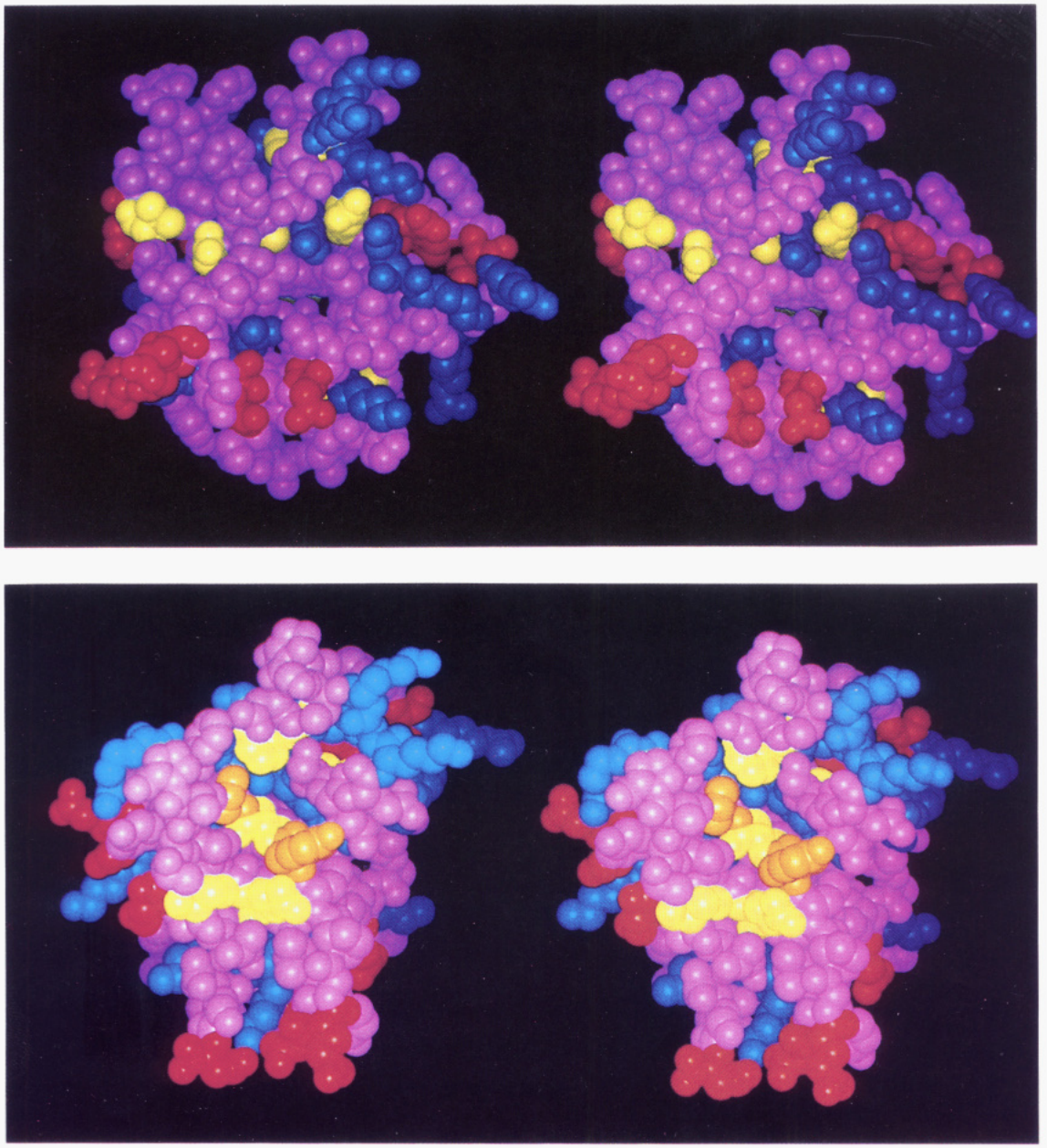

FIGURE 4: (A, top) The putative binding site of the Btk PH domain. The surface is shown in a space-filling model, where yellow residues are from left to right V64, Q103, V113, and R28, with F25 above and T33 below. Other arginine and lysine residues are in blue, and aspartate and glutamate residues are in red. The invariant tryptophan, visible mainly from the reverse face, is in green. The large insertion is not included. (B, bottom) The putative binding site with the Tec family almost invariant FYF motif: F25 (yellow, top), Y112 (gold, right), and F114 (gold, left). The coloring for the other residues is as in panel A.

disturb the structure so that interactions between the β-sheet-forming strands are weakened. Another explanation for XLA might be prevention of interaction either with other domains of Btk or with other molecule(s). This mutation is on the opposite side of the molecule compared to the other five mutations. Residue 33 located immediately after the β-strand B is not conserved although it is generally hydrophobic or aromatic. It is interesting that the related Tec kinase has a proline at this position (Mano *et al.*, 1993). The only other known sequence with proline is human Bcr.

Mutation V64F is on the same face of the molecule as residue 28 (Figure 4A) at a site that is not conserved in the known PH domain sequences, but is usually hydrophobic, in spite of being exposed on the surface. It is surrounded by several other hydrophobic residues which might be involved in forming a recognition site. It might also interact with the large Btk-specific insertion. Since this residue is on the surface, and the mutation presumably does not distort the structure, this alteration is compatible with the prevention of vital interactions resulting in XLA.

A seven-residue insertion at position 103 causes XLA (Bradley *et al.*, 1994). This residue lies in the middle of sheet F, where the insertion might destroy the fold or impair the putative binding site. On the basis of the model residue 103 locates between amino acids 28 and 64 on the same face of the molecule. The surface model of the Btk PH domain (Figure 4) indicates another possible binding region that could employ the mainly hydrophobic amino acids surrounding V64 and Q103.

The binding site model is further supported by two new XLA-causing Btk PH domain mutations. Residue F25 close to R28 is on the border of the putative binding site (Figure

4). Mutation F25S (Vořechovský *et al.*, 1995) removes the aromatic ring. Since this mutation causes classical XLA and does not presumably distort the PH domain structure, the phenotype is likely to be secondary to an impaired function. Mutation of another nearby residue V113 to aspartate (Conley *et al.*, 1994) introduces an opposite charge to the charged patch surrounding R28 (Figure 4). In addition, the last β-strand has to be transferred since D113 would have a steric clash with R13.

The space-filling representations (Figure 4) clearly indicate the presence of a putative binding site consisting of residues F25, V64, Q103, and V113 as well as the hydrophobic amino acid surrounding them. A channel leads from residues 64, 103, and 113 to F25 with walls formed by residues Y112 and F114. Thus although only the backbone atoms of V113 are required, a mutation to aspartate moves the strand and is likely to destroy the putative binding site. The three aromatic residues, tentatively designated the FYF motif (F25, Y112, and F114), are almost invariant in the Tec family although not conserved in other PH domains, which might indicate binding specificity. It seems highly likely that mutations also at positions 112 and 114 will cause XLA. It remains to be seen if the two putative binding regions, one formed by charged residues including R28 and the other composed mainly by hydrophobic amino acids including residues 25, 64, 103, and 112−114, are connected to recognize the very same molecule.

In Tec family members the region following the PH domain has been designated the TH domain (Smith *et al.*, 1994; Vihinen *et al.*, 1994a). These proteins contain a highly conserved region of 27 residues, tentatively called the Btk motif, that is found also in a putative GAP of *Drosophila melanogaster* and man (Maekawa *et al.*, 1994) and a human interferon binding protein (Vihinen *et al.*, 1994a). The PH domain and Btk motif are present also in the new Tec family member, human Bmx (Tamagnone *et al.*, 1994). Functional significance has been suggested for the C-terminal helix of the PH domain and the Btk motif in $\beta\gamma$ binding (Touhara *et al.*, 1994; Tsukada *et al.*, 1994). The precise putative binding site of the $\beta\gamma$ dimer is not known, since the peptide used contained also a stretch following the PH domain (Touhara *et al.*, 1994). The coiled-coil interaction with $\beta\gamma$ has been claimed to be unlikely for several PH domains (Ferguson *et al.*, 1994; Downing *et al.*, 1994; Timm *et al.*, 1994). Thus, the Btk motif might be responsible for the binding in Btk. It remains to be seen if this interaction appears also in cells. The Btk PH domain without the Btk motif has been shown to interact with protein kinase C, which downregulates Btk kinase activity by phosphorylation (Yao *et al.*, 1994).

The terminal α-helix is probably the most conserved region in PH domains and contains the only invariant residue, W124 in Btk. The tryptophan is partly on the surface of the molecule surrounded by other side chains. It seems likely that its function is structural, presumably by stabilizing the folded conformation. The substitution of this residue has so far been studied only in Btk, where a site-directed mutation W124G impaired binding of the helical region and the Btk motif to a $\beta\gamma$ dimer (Tsukada *et al.*, 1994). However, *functional* evidence for $\beta\gamma$ interactions in the Btk signaling pathway is still lacking.

To conclude, the Btk PH domain might be thought to have a twofold function. Analysis of XLA-causing mutations in the model suggests that a major part of the protein could be involved in forming one or two binding sites presumably containing residues 25, 64, 103, 112, 113, and 114 and the other consisting of charged residues close to R28. In lack of experimental evidence and due to several hypotheses considering lipids, proteins, and phosphorylated serine and threonine residues, the nature of the recognized molecules is unclear. Another function has been assumed for the terminal helix and the adjacent Btk motif in $\beta\gamma$ binding. This functional dualism is tentatively supported structurally by the split PH domain appearing in PLCγ and syntrophins (Gibson *et al.*, 1994).

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