Missense mutations affecting a conserved cysteine pair in the TH domain of Btk

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Abstract Tec family protein tyrosine kinases have in their N-terminus two domains. The PH domain is followed by Tec homology (TH) domain, which consists of two motifs. The first pattern, Btk motif, is also present in some Ras GAP molecules. C-terminal half of the TH domain, a proline-rich region, has been shown to bind to SH3 domains. Mutations in Bruton's tyrosine kinase (Btk) belonging to the Tec family cause X-linked agammaglobulinemia (XLA) due to developmental arrest of B cells. Here we present the first missense mutations in the TH domain. The substitutions affect a conserved pair of cysteines, residues 154 and 155, involved in Zn^{2+} binding and thereby the mutations alter protein folding and stability.

Key words: Btk; Bruton's tyrosine kinase; Signal transduction; Cytoplasmic tyrosine kinase; XLA, X-linked agammaglobulinemia; Ras GAP

1. Introduction

The Tec family of cytoplasmic protein tyrosine kinases (PTKs) is formed of Btk [1,2], Itk/Tsk [3,4], Tec [5], and Bmx [6], which differ from other PTKs by having in the N-terminus two distinct regions called pleckstrin homology (PH) and Tec homology (TH) domains [7–9]. Tec family members are the only PH domain containing tyrosine kinases and Btk is the only protein where PH domain mutations are known to have a phenotype. Mutations in Btk cause X-linked agammaglobulinemia (XLA) in man (e.g. [1,2,10,11]) and X-linked immunodeficiency (Xid) in mice [12,13]. XLA is caused by a B cell development arrest leading to recurrent infections (for a review see [14–18]]. The patients have no or only very few mature B cells and consequently immunoglobulins are either missing or markedly reduced.

The TH domain of about 60–80 residues is located between the PH domain and SH3 domain in Tec family members. In the N-terminus there is a highly conserved pattern of 25 amino acids, called the Btk motif, which is followed by a proline-rich region (PRR) [7,8]. The full-length TH domain is present only in the Tec family PTKs except for Bmx. PH domain is accompanied by Btk motif also in some Ras GTPase activating proteins (GAPs) [8]. Ras has two forms [19] and several functions e.g. in proliferation and differentiation. The inactive GDP-bound form can be activated by converting GDP to GTP by intrinsic GTPase activity, a process regulated by GAPs [20]. The function of the TH domain is unclear, although Src family SH3 domains bind to PRR in vitro [21–24] and the Btk motif seems to be essential for interactions with G proteins [25,26].

The 10 amino acid PRR motifs in Btk interact with the SH3 domains of Fyn, Lyn and Hck in vitro [21–23], but it is not known whether the full-length Src family kinases can associate. Itk PRR is bound also by the same SH3 domains [21] and the corresponding region of Tec binds to Lyn [24]. These interactions and the effects of mutations have been discussed based on three dimensional structures [16]. Erythropoietin and IL3 stimulation induces the specific binding of Vav to Tec through the TH domain [56]. Mutations of the key residues both in PRR and SH3 domains have been shown to abolish binding to SH3 domain [21,23]. An unidentified 72-kDa protein binds to residues 186–192 in the Btk TH domain [21].

Although the Btk gene defect has been characterised from more than 400 XLA patients, no missense mutations, except for one double mutation containing also a non-sense mutation in the PH domain, have been reported from the TH domain [10,11,27,28]. Otherwise XLA mutations are relatively evenly scattered along the protein. Here, we describe XLA causing mutations affecting the conserved residues of the TH domain and discuss the function of the Btk and PRR motifs in interactions with cellular signaling partners and structural consequences of mutations.

2. Materials and methods

2.1. Immunoprecipitation and in vitro kinase assay

A synthetic peptide representing Btk amino acids 166–184 coupled to keyhole limpet hemocyanin via an N-terminally added cysteine residue was used to obtain a Btk antibody by immunisation of rabbits. Mononuclear cells (1–2.5×10^7 cells) were isolated from periph-

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Abbreviations: Btk, Bruton's tyrosine kinase; GAP, GTPase activating protein; PH, pleckstrin homology; PRR, proline-rich region; PTK, protein tyrosine kinase; SH, Src homology; TH, Tec homology; XLA, X-linked agammaglobulinemia

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eral blood of XLA patients. After PBS washes, the cells were solubilized in digitonin lysis buffer containing 1% digitonin, 50 mM HEPES (pH 7.4), 1 mM DTT, 2 mM EGTA, 2.5 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. After 20 min incubation on ice, detergent-insoluble material was removed by centrifugation. Cell lysates were precleared for 30 min at 4°C with protein A-Sepharose and then mixed with affinity purified polyclonal antibodies for 1 h at 4°C. Immune complexes were collected by adding Protein A-Sepharose and incubated for an additional 2 h at 4°C. The Sepharose-protein A-Btk beads were washed three times with lysis buffer free from digitonin, EDTA and EGTA. The beads were washed two times with kinase buffer containing 20 mM HEPES (pH 7.4), 10 mM $MnCl_2$, 5 mM $MgCl_2$ and 1 mM DTT, 1 mM Na_3VO_4, 500 μM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin, and finally, the immunoprecipited Btk was resuspended in the kinase buffer containing 100 μM ATP. Phosphorylation reactions were carried out at room temperature for 10 min in kinase buffer and terminated by addition of SDS-PAGE sample buffer.

Btk proteins were separated on SDS-PAGE gels and transferred to nitrocellulose or Hybond-C extra membrane (Amersham). The blots were blocked either in 5 or 10% skim milk or in 2% BSA. Filters were developed with secondary antibody conjugated to horseradish peroxidase using the ECL detection system (Amersham). Total RNA was isolated by the guanidium thiocyanate method [29]. First strand cDNA synthesis was done by using a cDNA synthesis kit according to the instructions of the manufacturer (Pharmacia).

2.2. Mutation analysis

Purified genomic DNA samples from XLA patients were amplified with PCR by using primers designed for each exon. The amplified DNA fragments were analysed on polyacrylamide gels. The mutation containing exons were analysed by sequencing. As another method dideoxy fingerprinting and sequencing of genome DNA was used as previously described [30].

2.3. PH-TH and TH expression and purification

Insert encoding the Btk amino acids 1–215 (PH-TH) was amplified by PCR with oligonucleotide primers containing restriction sites for *Nco*I and *Sal*I (5′ and 3′, respectively) for cloning into *Escherichia coli* expression vector, pBAT4 [31]. The overlap extension PCR technique [32] with VentR DNA polymerase (New England Biolabs), was used to create the mutants C155S and C155G on the PH-TH construct.

For TH domain expression an insert encoding Btk amino acids 137–218 (TH) was amplified by PCR. Oligonucleotide primers had restriction sites for *Nco*I and *Xho*I. The product was cloned into expression vector pGAT2 (Peränen et al., unpublished results) creating a His-tag with a thrombin digestion site at the N-terminus. The GST encoding region was digested with *Spe*I.

PH-TH constructs were expressed in *E. coli* BL21(DE3) strain. Protein expression was screened in 2×TY-medium with IPTG induction for 2 h at 37°C. Expression levels were analyzed using SDS-PAGE in the Phast System (Pharmacia). Large-scale expression in LB medium was done similarly except for induction at 30°C for 3 h. The cells were harvested and lysed by sonication in +4°C at PBS buffer containing 1% Triton X-100, 4 mM DTT, 0.1 μM aprotinin, 1 μM leupeptin, 1 mM EDTA, 1 mg/ml streptomycin sulphate and 0.2 mg/ml of lysozyme. The lysate was cleared by centrifugation and loaded on SP-Sepharose FF (Pharmacia) column. Elution was with 0.05 to 1.0 M salt-gradient. Fractions containing PH-TH were pooled and concentrated for gel filtration on Superdex 75 column (Pharmacia) equilibrated with 10 mM HEPES (pH 7.0), 100 mM NaCl and 2 mM DTT. The purity of the protein was analyzed with reversed-phase HPLC using Vydac C_4 column (4.6×150 mm) equilibrated in 2% acetonitrile and 0.1% TFA. Bound material was eluted using a linear acetonitrile gradient (1.45% per minute up to 60%).

TH construct was expressed as the PH-TH-constructs, except for induction in large-scale fermentation that was at 30°C for 6 h. The lysate was cleared by centrifugation at 18000×*g* for 45 min. The lysate was loaded on Ni^{2+}-NTA-agarose (Qiagen, Germany) column, which was washed with 10 volumes of PBS buffer. The protein was eluted with PBS after over night thrombin digestion. Fractions containing TH domain were pooled and concentrated for gel filtration on Superdex 75 column equilibrated with a buffer containing 20 mM phosphate-buffer, pH 6.5 and 50 mM NaCl.

2.4. PH-TH-SH3-SH2 production and analysis

The Btk PH-TH-SH3-SH2 fragment encoding amino acids 1–383 was constructed with PCR similar to the other constructs. The insert was cloned in the pBAT4/PHTH$^{1-215}$ vector using *Bgl*II and *Xho*I cloning sites. The large-scale protein expression was as with PH-TH-constructs, except for induction over night at 16°C in the presence of 3% of ethanol. The clarified lysate was loaded on SP-Sepharose FF (Pharmacia) column and eluted with 0.05 to 1.0 M salt-gradient. SH2 domain specific affinity chromatography with L-phosphotyrosine coupled Sepharose 6B CL was used as the second purification step. Concentrated fractions were gel filtrated on Superdex 75 column.

The metal analysis was done using inductively coupled plasma mass spectroscopy at the Åbo Akademi University (Turku, Finland). The lyophilised sample (24.5 mg) was dissolved in 1.5 ml nitric acid, and diluted to 20 ml. The analysis was performed using a quantitative program. Protein concentration was determined spectrophotometrically in 6 M GndHCl using calculated molar absorption coefficient 56970 M^{-1} [33].

2.5. Computer analysis

The amino acid sequences were taken from sequence databases. The sequence databases TrEMBL, SWISSPROT and PIR were analyzed with the program Profilesearch from the GCG program package [34]. The multiple sequence alignment was obtained with the GCG software.

3. Results and discussion

The TH domain of Tec family members consists of a conserved Btk motif and a proline-rich region [8]. The Btk motif has previously been found also in a GAP1 protein of *Drosophila melanogaster* [35] and in a human interferon-γ binding protein [36] adjacent to the PH domain (Table 1). Sequence analysis indicated that Ras GAP molecules from human (entry g1060909), bovine (R-RasGAP) [37], rat [38] and mouse (g972944) share the sequence similarity of PH domain and Btk motif. In addition, also human PtdIns(1,3,4,5)P_4-binding protein belonging to the GAP1 family [39] is related to Tec family N-terminus. The Ras GAP activity of this protein is specifically stimulated by PtdIns(1,3,4,5)P_4 and inhibited by phospholipids in vitro. Recently the mouse GAP1^m has been shown to bind PtdIns(1,3,4,5)P_4 in a PH and TH domain dependent manner [40]. Murine GAPIII also contains a Btk motif, which is required for GTPase activity [41]. In all these Ras GAP molecules the PH domain and Btk motif are located in the C-terminus and the sequence similarity to the Tec family is restricted to the PH domain and the Btk motif. No homologs were found from other organisms analysed. Although the complete genome of yeast has been sequenced [42] and found to contain numerous kinases [43] tyrosine kinases are missing as well as TH domains.

3.1. TH domain sequences

The sequence alignment of TH domains (Fig. 1) indicates high conservation, even so that there are no gaps in any of the Btk motifs. Of the 25 residues in the Btk motif six are invariant and one almost invariant as previously noted [8]. The first conserved residue is either Y or F in all the sequences. The following residues are HP except for in Bmx which carries HS. Then there are five variable intervening residues before an invariant G. Amino acids 14 and 15, CC, are invariant. The motif ends in the invariant GC pair (residues 24 and 25). In addition to these overall similarities, residue six is F in the Tec family and A in almost all of the GAP sequences. Also residues 1 (lysine), 9 (aspartate), 17 (glutamine) and 19 (lysine) are invariant in the Tec family. Thus, the Btk motif has both

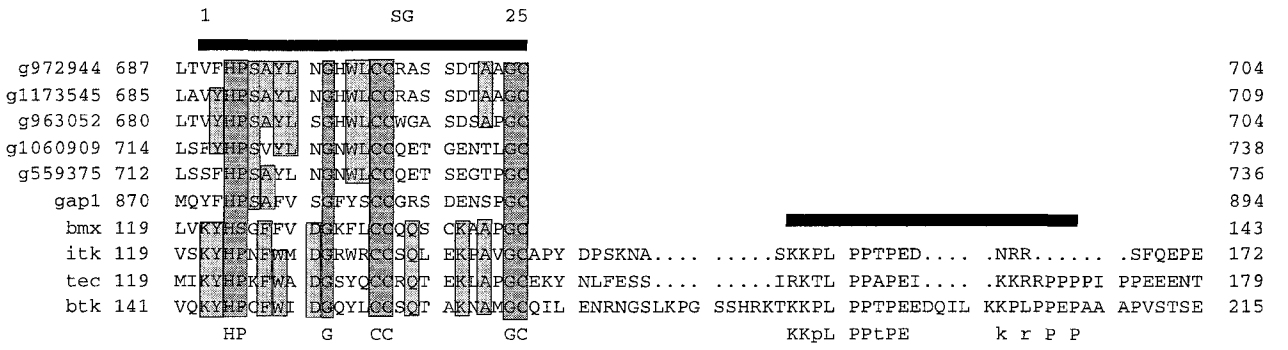

Fig. 1. Alignment of proteins related to Btk family PH and TH domains. The TH domain contains two distinct motifs, the Btk motif and a proline-rich region (indicated with bars above the sequence). The numbering of the Btk motif is above the sequence as well as XLA causing mutations. The almost invariable residues are indicated by dark shading and as consensus sequence on the last line. The less conserved residues and group specific conservation is shown with light shading. The sequences are taken from databanks as follows: Btk, entry x58957 [1,2], Bmx (hsbmxgene) [6]; Itk (l05631) [3,4]; Tec (ju0215) (Mano et al., unpublished); bovine R-Ras GAP (g117345) [37]; *Drosophila melanogaster* GAP1 (gap1_drome) [35]; human GAP (g1060909) (M. Kobayashi et al., unpublished); human GAP1^{IP4BP} (g963052) [39]; mouse GAPIII (g972944) [41]; rat GAP1^{m} (g559375) [38]; interferon-γ binding protein (a09787) [36].

general as well as family related similarities as also residues 12 and 13 (WL) are almost invariant in GAP proteins.

The TH module can be expressed and purified as a domain from *E. coli* (data not shown). The Btk motif contains a conserved histidine and three cysteines, residues that are conserved also in zinc finger proteins. Elemental analysis of the purified Btk PH-TH-SH3-SH2 construct indicated the protein to contain Zn^{2+} ion almost in equimolar ratio (1:0.94). The Btk PH domain with Btk motif has been shown to bind Zn^{2+} [44]. The metal can be released with dialysis giving a molar ratio of 1:0.12. Btk motif represents a new type of Zn^{2+} binding domain, since the organisation of the histidine and cysteine residues differs from those previously described [45].

The other half of the TH domain, PRR, is not present in the Ras GAP proteins (Fig. 1). This pattern contains tandem repeat in Btk and Tec. PRRs are commonly involved in protein-protein interactions with SH3 and WW domains [46,47]. The proline residues and hydrophobic amino acids are located at critical sites capable of forming polyproline type II (PPII) helix of RLP class [48]. The first half of the Btk PRR is identical with the single proline-rich stretch in Itk. The lysine residues in the beginning of both patterns govern the orientation of binding. Interestingly, the PRR of Itk has recently been shown to bind intramolecularly to its SH3 domain, thereby possibly regulating enzyme activity [49], as previously hypothesized for Btk [7].

3.2. *The first TH domain missense mutations*

Although the gene defect has been determined for more than 400 XLA patients, the BTKbase mutation registry [27,28] does not contain missense mutations in the TH domain. Here we describe the first two TH domain mutations associated with XLA. These alterations affect the invariant CC pair in the middle of the Btk motif. C154S mutation (PIN C154S(1), accession number A0292) alters the invariant cysteine number 154 in the Btk motif (Fig. 2). The mutation is

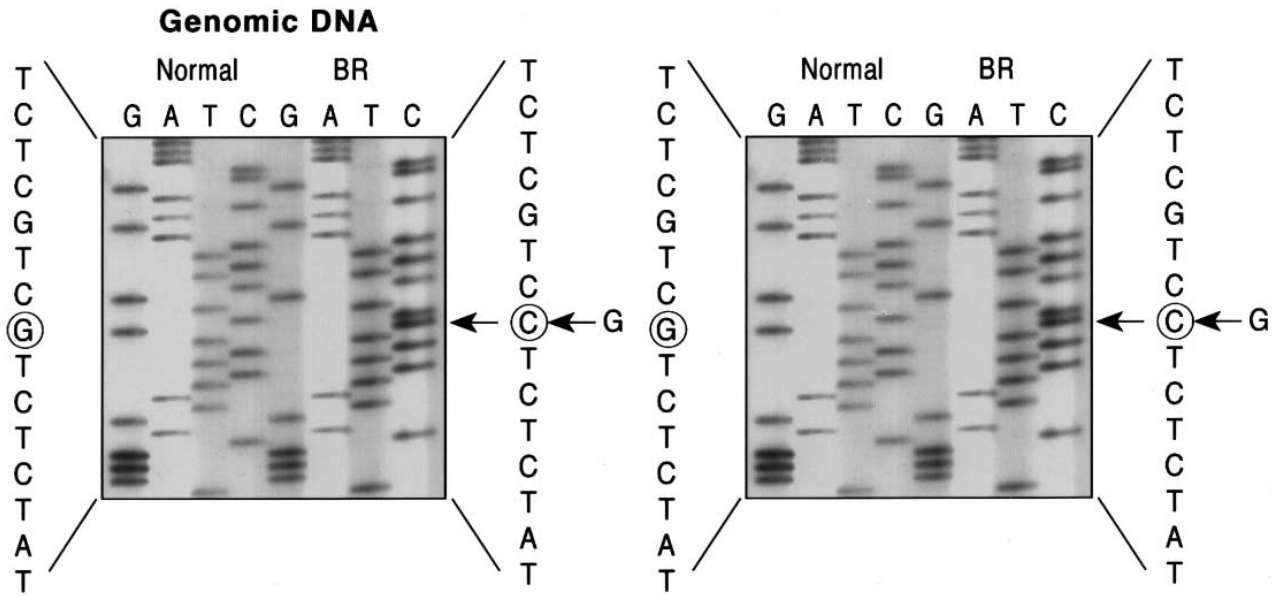

Fig. 2. Sequence analysis of genomic DNA of the BTK gene form patient BR, demonstrating a G to C transversion affecting nucleotide 593 with the TH domain, resulting in mutation C154S.

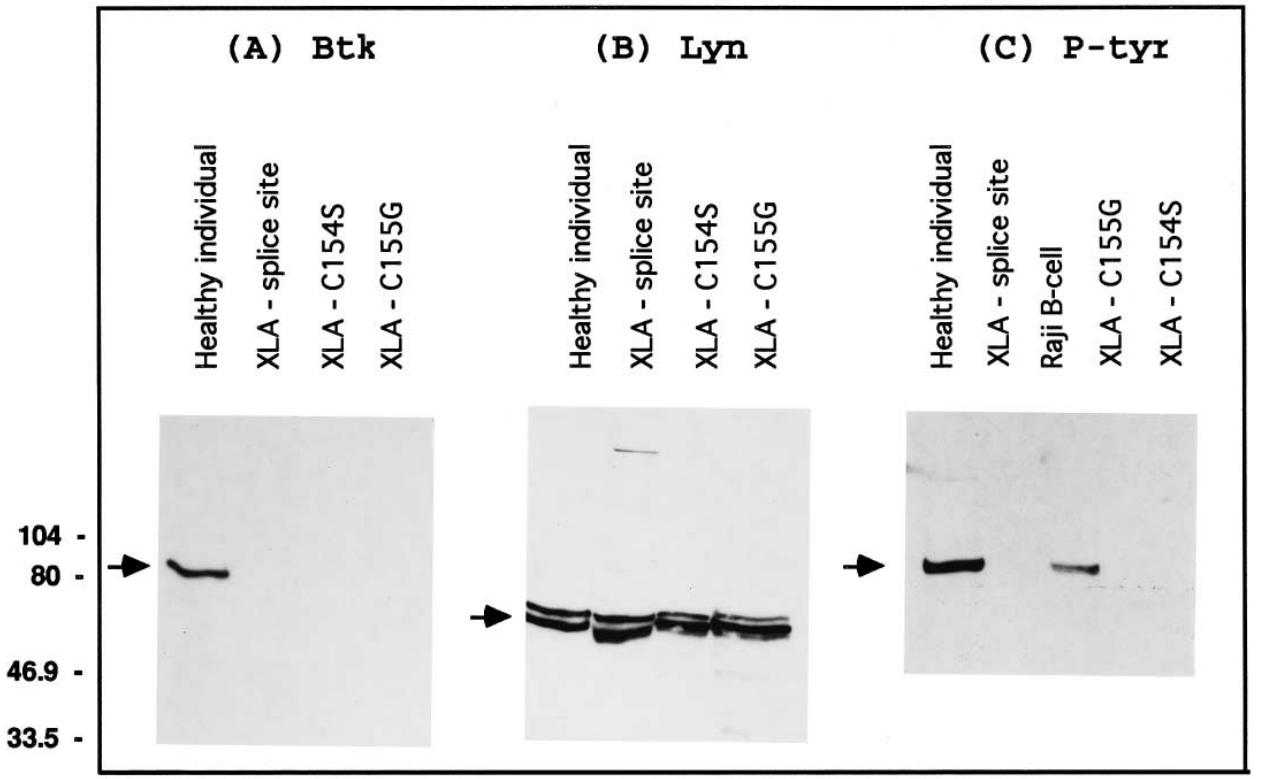

Fig. 3. (A) Btk expression from XLA patients with mutations C154S and C155G. Btk expression was analysed also from a healthy individual as well as from a patient with a splice site mutation. (B) Lyn expression by probing the same filter as in A. (C) Immune complex kinase assay of phosphotyrosine containing Btk by immunoblotting with PY20H (from Signal Transduction Laboratories). Human Raji B-cell line was used as a positive control.

due to G593 to C alteration changing TGC codon to TCC. The patient (BR) with moderately severe XLA was diagnosed at age of 8 years, but had had recurrent upper respiratory infections since the age of one year. The patient has no family history. Both B cells (1%) and immunoglobulins (IgG 1.66, IgM 0.18, and IgA 0.03 mg/ml) were markedly reduced. He failed to produce antibodies to diphtheria and tetanus and to polysaccharides. Following two immunizations with bacteriophage ϕX174 [50], he cleared the antigen normally, but made very little antibody (peak Kv during primary response was 0.034, less than 1% of normal, and 2.18 following the secondary immunization, again less than 1% of normal).

The second mutation identified alters T595 to G changing TGC to GGC resulting in C155 to G mutation (PIN C155G(1), accession number A0357). The mutation destroys a *Tse*I restriction site. This patient has clinically a mild disease although no B cells could be detected. The diagnosis was made at the age of 35 years. In this family there are six affected males in two generations.

Total cell lysates of mononuclear cells isolated from the XLA patients with mutations C154S and C155G, respectively, were analysed for Btk and Lyn expression and Btk kinase activity (Fig. 3). Btk expression was absent in both patients as well as in a patient with a splice site mutation. Normal quantity of Btk was found in the cells of a healthy control suggesting that the mutations affect the structure of the protein. As an internal control, the presence of Lyn was confirmed (Fig. 3B) in all cell lysates. No traces of Btk were found even after immunoprecipitation of total cell lysates of patients with mutations C154S and C155G (Fig. 3C).

The two mutations identified in the Btk TH domain appear in the conserved Zn^{2+} binding cysteines thus destroying the binding site and affecting both folding and stability. The 3D structure indicates that the cysteines are crucial for metal binding [44] and a substitution in any of them would affect folding. This is evident also from expression studies, since the mutated proteins that are efficiently expressed in *E. coli* could not be purified although the proteins were mainly soluble after lysis (Fig. 4). Similarly, some PH domain mutations affect the structure of the expressed proteins [44].

A previously reported missense mutation located in the TH domain [51] affected the first proline-rich repeat in the PRR and could cause XLA due to preventing SH3 domain binding. However, this patient has also another mutation (PIN #G54X56/E205D(1)) and it is not possible to define which of these alterations occurred first. The substitution could be a previous pathological change in the family, which might have gained later another frameshift mutation in the gene encoding already non-functional protein. The mutations in the Zn^{2+} binding residues affect the structure and stability of Btk. It is highly likely that substitutions in the PRR will also cause XLA, although no examples are known to date.

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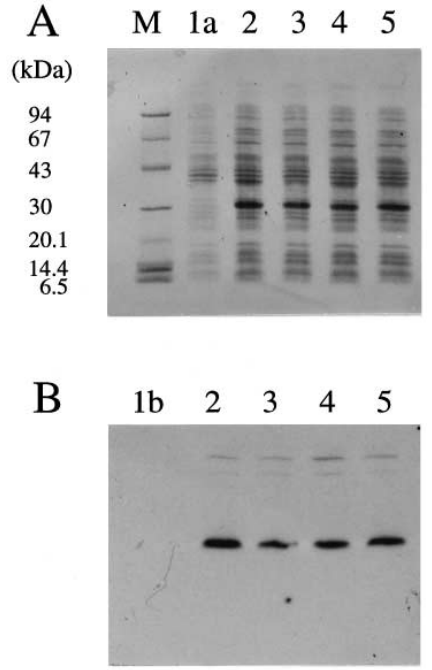

Fig. 4. (A) The expression level of the native and mutation-containing PH-TH proteins in *E. coli* analysed with SDS-PAGE. Lanes: 1a, non-induced whole cell preparate; 2, induced whole cell preparate of native PH-TH; 3, supernatant of lysate of native PH-TH; 4, supernatant of lysate of C154S; 5, supernatant of lysate of C155G. Molecular weight markers (M) contained aprotinin. (B) Identification of human Btk PH-TH constructs by Western blotting. Lanes: 1b, whole cell preparate of *E. coli* BL21 (DE3) containing pBAT4; 2–5 as above. The immunoblot was developed using ECL.

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