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Expression of Bruton's tyrosine kinase in B lymphoblastoid cell lines from X-linked agammaglobulinaemia patients

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SUMMARY

X-linked agammaglobulinaemia (XLA) is an immunodeficiency caused by mutations in Bruton's tyrosine kinase (*Btk*) and is characterized by an almost complete arrest of B cell development. We analysed expression of *Btk* in B lymphoblastoid cell lines (BLCL) derived from four unrelated XLA patients. In one patient, with a 3·5 kb genomic deletion encompassing the first (untranslated) exon, mRNA levels and *in vitro* kinase activities were very low. The patient manifested a mild phenotype with a delayed onset of the disease. Another mutation, in which the intron 3 donor splice site is lost, was also associated with very low mRNA levels and an absence of detectable Btk protein. Patients with this mutation showed extensive heterogeneity of the immunological phenotype. In the BLCL of a third patient, with an Arg_{288} substitution in the SH2 domain, the mutation did not appear to affect the expression level, nor to abrogate *in vitro* phosphorylation activity. In the BLCL of the fourth patient, with an Arg_{28} mutation in the PH domain, tyrosine kinase activity in BTK precipitates appeared to be decreased compared with control BLCL.

Keywords Bruton's tyrosine kinase X-linked agammaglobulinaemia B lymphoblastoid cell lines X-linked immunodeficiency

INTRODUCTION

X-linked agammaglobulinaemia (XLA) is an immunodeficiency disease in man, caused by mutations in the gene coding for Bruton's tyrosine kinase (*Btk*) (reviewed in [1–4]). Patients suffer from protracted and recurrent bacterial infections due to very low serum levels of all immunoglobulin classes. XLA reflects an arrest in B cell differentiation at the transition from precursor B cell to later B cell stages. *Btk* is a cytoplasmic tyrosine kinase and is expressed throughout B cell differentiation except for plasma cells. Although recent observations implicate *Btk* in several receptor-coupled signal transduction pathways, the function of *Btk* in early B cell development or the molecular mechanisms that lead to the arrest of B cell differentiation in XLA are unknown.

Together with *Tec*, *Itk*, *Emt* and *TxK*, *Btk* forms a subfamily of *src*-related tyrosine kinases [1–4]. In addition to a catalytic domain and the *src*-homology SH2 and SH3 domains, *Btk* has a unique N-terminal region consisting of a pleckstrin homology (PH) and a Tec homology domain. The *Btk* gene mutations characterized so far—which include deletions, insertions and point mutations—are found in all domains. A database of *Btk* mutations of 189 unrelated families has been compiled [5]. From this collection of different mutations it is apparent that the phenotype in XLA cannot be predicted by the nature of the associated mutation. In contrast, the severity of XLA varies even among family members carrying the same mutation ([6], reviewed in [1]). In addition, it may prove difficult to relate the clinical and immunological manifestation of the disease to the character of the genomic mutation identified, as these may be associated with decreases in mRNA or protein instabilities. In this context, those few B lymphoblastoid cell lines (BLCL) from XLA patients that have been evaluated for the presence of Btk protein showed absent or reduced expression, even in the case of a single amino acid substitution [7–9].

Here we report the identification of two novel *Btk* mutations, one associated with a mild phenotype and one with extremely heterogeneous expression of the disease among family members. Both mutations resulted in an absence of detectable Btk protein in Western blotting. We also analysed Btk protein expression and kinase activity in BLCL established from two patients with mutations that have been described previously [10]. In particular, a BLCL from a patient with an Arg_{288} substitution in the SH2

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domain manifested normal Btk kinase activity, whereas decreased Btk kinase activity was found in a BLCL derived from a patient with an Arg_{28} mutation in the PH domain.

MATERIALS AND METHODS

Mutation analysis

BLCL were established by Epstein–Barr virus (EBV) transformation of peripheral blood mononuclear cell (PBMC) suspensions, using standard methods [11]. Isolation of total cellular RNA from the BLCL, first-strand cDNA synthesis, polymerase chain reaction (PCR) amplification of overlapping *Btk* fragments, cloning of amplified DNA fragments and sequencing were performed as described [10].

Cloning of genomic Btk fragments

From a 340 kb YAC clone (9DA6 [12]), a cosmid mini-library was constructed by cloning of partial *Mbo*I digests in the PTCF cosmid vector [13]. *Btk*-positive cosmids were isolated and mapped by hybridization to the *Btk* cDNA and *Btk.123* probes (see below), and *Pst*I or *Eco*RI fragments were subcloned into pBlueScript (Stratagene, La Jolla, CA)).

Southern and Northern blotting, and reverse transcriptase-PCR

High molecular weight DNA was extracted and processed by Southern blotting using standard methods [14]. Northern blotting analysis was performed as described [15]. For reverse transcriptase (RT)-PCR, aliquots of 2–5 μg total cellular RNA were used for first-strand cDNA synthesis with an oligo-(dT) primer. PCR products were generated in 28–32 cycles of amplification, using primers specific for *Btk*, at positions 1300 and 1876 [16], and β-actin (TCGCCGCGCTCGTCGTCGAC and CCTCATGAAGATCCTCA). PCR conditions and Southern blotting of amplified products have been described previously [10]. Blots were analysed by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and hybridization signals were quantified using ImageQuant Version 4.1b.

The following probes were applied: a 2·8 kb *Btk* cDNA probe isolated from a pro-B cell cDNA library [10]; *Btk.123* (5′ end of the *Btk* cDNA; pos. 1–372), *Btk.prom2.5* (a genomic 2·5 kb PstI fragment (Fig. 1a), *Btk.2* (cDNA pos. 1094–2159 [16]) and β-actin [15].

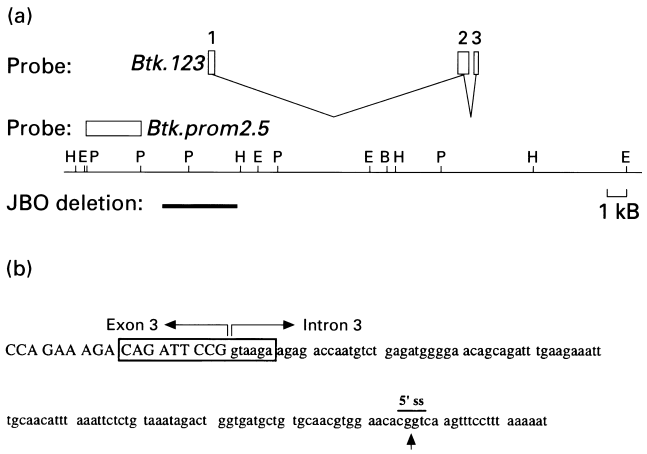

Fig. 1. (a) Restriction map of the 5′ part of the *Btk* gene including exon 1–3. Probes are indicated by open boxes. *Btk.123* probe is composed of exon 1–3. The deletion detected in patient JBO is indicated by a black bar. H, *Hin*dIII; E, *Eco*RI; P, *Pst*I; B, *Bam*HI. (b) Wild type genomic sequence of the 5′ end of exon 3 (capital letters) and part of intron 3 (lower case letters). Nucleotides deleted in patient RRA are boxed. The alternative splice site used in RRA is underlined, whereas the exact splice junction is indicated by an arrow. Information on the mutations and disease phenotypes are submitted to the Btk database [5].

Btk protein analysis

Preparation of cell lysates from BLCL, immunoprecipitations using a polyclonal serum, raised against a BTK/glutathione-S-transferase (GST) fusion protein, immunoblotting using an anti-Btk peptide (amino acids 69–88) serum, *in vitro* kinase assay and phosphoamino acid analysis were carried out as described [17]. A GST–Ig-α fusion protein (containing amino acids 165–227 of the Ig-α protein) was used as an exogenous substrate in the *in vitro* kinase assay.

To ensure equal loading, the amount of proteins in the lysates was determined. Densitometric analyses of *in vitro* kinase assays were performed using an LKB Ultroscan XL Enhanced Laser densitometer.

RESULTS

To be able to investigate mRNA and protein expression of mutated Btk, BLCL, which normally express Btk [15], were established by EBV transformation of PBMC from four unrelated XLA patients.

Patient 276

Patient 276 belongs to a large Dutch pedigree *66.8* [18] containing eight patients diagnosed as having XLA. Serum immunoglobulin levels of four of the patients are given in Table 1. The pedigree exhibited heterogeneity in the clinical and immunological phenotype, e.g. 276 mainly had recurrent respiratory infections, whereas 1803 suffered from eye infections, sinusitis, arthritis, urinary tract infections and fevers of unknown origin. In all patients B cell numbers were severely decreased. The mutation in this pedigree has been described previously [10] and is a replacement of Arg_{228} in the SH2 domain by Trp.

Patient M76

Patient M76 is from the *M-3* pedigree, in which four brothers exhibited classical XLA phenotypes and have been described previously (Table 1; [10,18]). The mutation in this pedigree (Arg_{28} to His) affects the same codon as is altered in immunodeficient *xid* mice [10].

Patient RRA

Pedigree *F* [11] has four XLA patients (RRA, SF, PF and JF; Table 1). All patients suffered from serious frequent and recurrent infections, some of which were not typical for XLA: respiratory infections, arthritis, hepatitis, mastoiditis and neurological abnormalities (peroneus pareses). Three of the patients showed granulocyte deficiencies, such as defective intracellular killing of *Staphylococcus aureus* or transient severe granulocytopenia, sometimes during septicaemia periods. In all patients, B cell numbers were severely decreased: not detectable in SF and JF and 0–1% in PF, although in RRA up to 3% immunoglobulin-positive cells were found in the peripheral blood. RRA showed proliferative responses to *S. aureus*, which were 25% of normal. SF

Table 1. Serum immunoglobulin levels and mutations identified in X-linked agammaglobulinaemia (XLA) patients

Pedigree	Patient	Age	Immunoglobulin levels (mg/ml) IgG	IgM	IgA	Mutation	Domain	Disease phenotype
66.8	276	12 years	2·75	ND	ND	$C_{993}\rightarrow$ T	SH2	Heterogeneous
	274	14 years	1·2	ND	ND			
	182	20 years	8·5	<0·1	<0·1			
	1803	22 years	5·53	0·05	0·16			
M-3	M76	20 years	1·2	0·1	ND	$G_{215}\rightarrow$ A	PH	Classical
F	RRA	1 year	0·21	0·91	0·15	15 bp genomic deletion	PH	Heterogeneous
	SF	4 years	6·3	3·4	5·3			
		6 years	3·2	0·24	2·9			
	PF	8 years	2·46	0·29	0·28			
	JF	5 months	1·05	0·13	ND			
		3 years	0·14	0·18	0·04			
BO	JBO	10 years	2·2	0·16	0·07	3·5 kb genomic deletion	Promotor region	Mild

ND, Not detectable.

and PF developed low but detectable antibody responses to tetanus and influenza on vaccination. SF manifested normal serum immunoglobulin levels in the first year of his life. In subsequent years, IgM was normal or decreased, IgG was decreased and IgA was significantly elevated (Table 1). Also, patient PF showed normal immunoglobulin levels in his early childhood (3 years), which decreased later on.

BLCL *Btk* RT-PCR products were cloned and sequenced from RRA, and a 9-bp deletion immediately followed by a 100-bp insertion at cDNA pos. 363 was identified. The inserted segment was found to be of intronic origin, when compared with the published genomic sequence of the human *Btk* gene [16]. Sequence analysis of RRA genomic DNA revealed a 15-bp genomic deletion encompassing the last 9 bp of exon 3 and the first 6 bp of intron 3 in RRA (Fig. 1b). An alternative donor splice site was used, located 106 bp downstream, which fulfilled the 5′ splice consensus sequence [43] (Fig. 1b). The 15-bp genomic deletion contains a short palindrome (CCGG) and is flanked by a 5-bp inverted repeat (AGACA//TGTCT) and a 5-bp symmetric element (CCAGA// AGACC). These kinds of elements are often associated with deletional mutagenesis [19].

Patient JBO

JBO is an isolated case (Table 1). He did not suffer from any serious infections until he was 9 years old, when he was found to be hypogammaglobulinaemic after otitis and fevers of unknown origin. His B cell numbers were severely decreased: surface immunoglobulin-positive cells in peripheral blood were <1%. No IgM/IgG or IgA production was detected in *in vitro* ELISA spot assays. The bone marrow contained 14% TdT-positive cells, pre-B cell numbers were moderately decreased, B cells were severely decreased and IgG-expressing cells and plasma cells were absent. Some plasma cells were present in the jejunum. He had low but detectable specific antibody responses to diphtheria, tetanus and poliomyelitis antigens. Since gammaglobulin substitution therapy was started, he suffered only from minor dermal infections. The patient had a growth retardation (below the 3rd percentile at 6 years old) which appeared to be constitutionally determined and not due to a growth hormone deficiency, as growth hormone levels after testosterone priming were normal.

In Southern blot analysis of JBO genomic DNA, the first *Btk* exon was found to be deleted, using a partial *Btk* cDNA probe specific for the first three exons (Fig. 1a). Fragments hybridizing to exon 2 and 3 were still present in JBO. Since *Bam*HI, *Eco*RI and *Hin*dIII fragments detected by the *Btk.prom2.5* probe were 3·5 kb smaller in JBO compared with a healthy control, we concluded that a 3·5-kb genomic DNA segment, containing exon 1, is deleted in JBO.

Characterization of Btk transcripts in BLCL from XLA patients

BLCL were analysed by Northern blotting using a *Btk* cDNA probe. BLCL from patients 276 and M76 contained *Btk* transcripts of the same size and intensity as BLCL from healthy controls. Under the same Northern blotting conditions, BLCL from patients RRA and JBO did not appear to express *Btk* transcripts. Only after prolonged exposure were faint hybridizing bands observed (Fig. 2). In RT-PCR experiments, using primers at positions 1300 and 1876, the RRA and JBO BLCL showed low but detectable *Btk* expression, with levels similar to those found in phytohaemagglutinin (PHA)/IL-2-stimulated T cell lines (0·5–2·5% of control BLCL; data not shown).

Identification of Btk protein in BLCL of XLA patients

By immunoblotting analysis using a polyclonal rabbit anti-Btk peptide serum, the 77-kD Btk protein was identified in BLCL total cell lysates from patient 276, from patient M76, and from two healthy controls (Fig. 3a). As the intensities of the observed 77-kD bands were similar, the mutations in patients 276 and M76 did not appear to affect the expression level of the Btk protein in BLCL. Apart from the Btk band, other specific bands were visible, which were also present in cells that do not express BTK, such as T cells (not shown). In BLCL from patients RRA and JBO, no 77-kD Btk protein, nor any other proteins specifically reacting with the anti-Btk serum, could be detected (Fig. 3a).

Kinase activity of Btk in BLCL from XLA patients

Using the polyclonal anti-BTK/GST antibody, Btk was immunoprecipitated from BLCL lysates and subsequently tested for *in vitro* autokinase activity and for the ability to phosphorylate an exogenous substrate, a GST–Ig-α fusion protein (Fig. 3b).

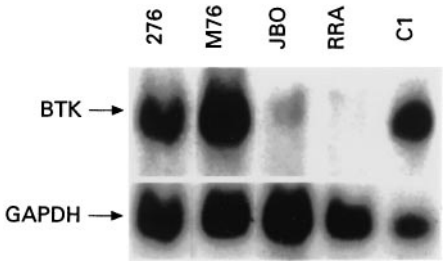

Fig. 2. Northern blot analysis of *Btk* transcription in B lymphoblastoid cell lines (BLCL) of the four X-linked agammaglobulinaemia (XLA) patients and a healthy control (C1). A 2·8-kb *Btk* cDNA probe was used. GAPDH was used as a control for equal loading.

In BLCL 276 the Btk kinase activity was found to be similar or slightly enhanced compared with BLCL from healthy controls (Fig. 3b). In BLCL from patient M76 the Btk kinase signals were decreased to 12–20% of control BLCL (as determined by densitometric analyses of five independent experiments). The GST–Ig-α transphosphorylation signal in M76 was decreased to $\approx$ 10% of the values of control BLCL. Consistent with the absence of detectable Btk protein (Fig. 3a), no specific *in vitro* kinase activity was detected in BLCL from patient RRA (Fig. 3b). BLCL from patient JBO showed very low Btk autophosphorylation and transphosphorylation activities.

Phosphoamino acid analysis of in vitro *phosphorylated BTK*

The *in vitro* phosphorylated 77-kD proteins (Fig. 3b) were subjected to phosphoamino acid analysis. The control BLCL C1 and C2 showed a primary specificity for tyrosine residues, but some serine/threonine kinase activity was also detected (Fig. 3c). BLCL 276 showed a relative increase in tyrosine *versus* serine/threonine phosphorylation. In the M76 BLCL the phosphorylation of tyrosine residues was found to be significantly decreased, whereas phosphorylation of the serine and threonine residues was unaltered compared with control BLCL. The extremely low levels of phosporylated 77-kD proteins in BLCL from JBO and RRA precluded phosphoamino acid analysis.

DISCUSSION

We analysed expression of *Btk* in BLCL from four unrelated XLA patients. In two patients, RRA and JBO, novel genomic deletions were identified, both leading to very low or undetectable levels of Btk mRNA and protein. In patient RRA the inclusion of 100 bp intronic DNA did not only lead to the introduction of a premature stop codon, but also appeared to result in mRNA instability. Decreased mRNA steady state levels have also been reported for premature stop codon mutations in 5′ regions of several other genes [20–22]. In patient JBO, the 3·5-kb genomic deletion encompasses the *Btk* promoter region [23,24] and exon 1. Although over 148 unique molecular mutation events have been described in the Btk mutation database [5], this deletion is the first mutation identified that affects only the first *Btk* exon, leaving the translation start and the open reading frame intact. The deletion clearly results in a severe reduction in *Btk* transcription, protein expression and kinase activity. These findings imply that in the absence of the regular transcription initiation site (located within the 3·5-kb DNA segment deleted in JBO), *Btk* transcription is still occurring, albeit at a very low level.

In the two pedigrees, the (almost) complete absence of Btk protein resulted in different clinical and immunological presentations. In pedigree *F*, the immunological abnormalities were very diverse: SF and PF had normal immunoglobulin levels in their early childhood, but RRA and JF manifested low immunoglobulin levels from a very early age. Although variable expression in kindreds with XLA has been previously described, only in one report of discordant phenotypes was the mutation involved identified [6]. Interestingly, the described mutation ($Met_1 \rightarrow Thr$) also leads to an absence of detectable Btk kinase activity, as in the *F* pedigree. A further detailed analysis of clinical and immunological findings in genotyped pedigrees with multiple affected males is clearly required to investigate whether certain types of *Btk* mutations, e.g. null-mutations, are particularly prone to a variable disease phenotype. The observation of elevated IgA serum levels (5·3 and 2·9 mg/ml) in patient SF indicated that significant numbers of B cells in this patient can differentiate into late stages of B cell development. In an evaluation of serum IgA levels in the Btk mutation database [5], IgA levels were < 0·1 mg/ml or not detectable in 91 cases, and 0·1–0·5 mg/ml in eight cases. Only in one pedigree with classical XLA was IgA 1 mg/ml in one

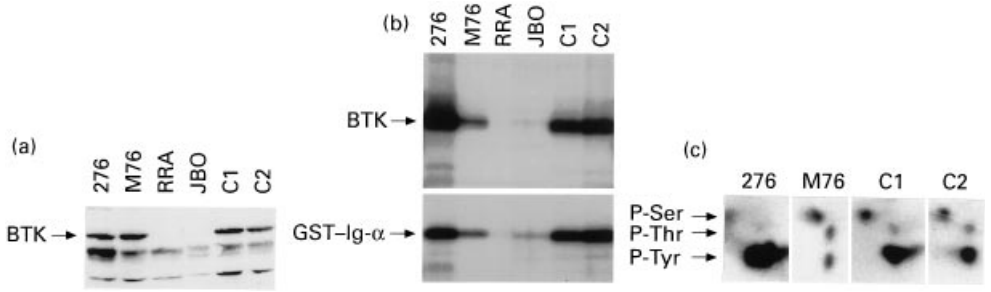

Fig. 3. (a) Western blot analysis of Btk protein expression in B lymphoblastoid cell lines (BLCL) of four X-linked agammaglobulinaemia (XLA) patients and two healthy controls (C1 and C2). Btk was detected in total cell lysates by immunoblotting with an anti-Btk peptide serum. (b) *In vitro* kinase assay of Btk immunoprecipitates isolated from BLCL total cell lysates of the four XLA patients and the two healthy controls. A glutathione S-transferase (GST)–Ig-α fusion protein was added as an exogenous substrate. After SDS–PAGE, samples were transferred to nitrocellulose and exposed to x-ray film. (c) Phosphoamino acid analysis of *in vitro* phosphorylated Btk protein. The positions of phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) are indicated.

patient, but not detectable in his two affected brothers. Hence, we conclude that elevated serum IgA is very rare in XLA. In spite of the heterogeneity of the immunological phenotypes in the *F* pedigree, all patients had multiple episodes of serious infections.

In contrast, the phenotype of patient JBO could be classified as mild, as he did not suffer from severe infections and was only diagnosed as having hypogammaglobulinaemia when he was 9 years old. So far, several XLA patients with a mild presentation of the disease have been reported ([9,25]; reviewed in [5]). BLCL of two of such patients also did not contain detectable levels of *Btk* transcripts [25], whereas in one patient an unstable Btk protein was expressed [9]. In other cases, the identified mutations were located at the C-terminal end of the protein [5], and the mildness of the XLA phenotype might be explained by the limited effect of these mutations on *Btk* function. As protein expression was not evaluated, it cannot be excluded that Btk protein was absent or unstable. In any case, the findings by Conley *et al.* [25] and in patient JBO support the conclusion that an absence of Btk protein does not necessarily lead to a severe phenotype. Moreover, the presence of specific responses (JBO, SF and PF) and high IgA levels (SF), suggest that in the absence of Btk protein, B cell development can, to some extent, proceed to late differentiation stages. This would imply that Btk function is then (partially) taken over by other signalling molecules or pathways. These compensatory mechanisms may not be effective if significant amounts of mutated Btk molecules are expressed.

In patient JBO, some *Btk* transcription might be initiated outside the 3·5-kb genomic DNA segment that is deleted. Since the ORF is not altered by the deletion, such transcripts might give rise to very low levels of intact Btk protein. These small quantities of functional Btk protein could well explain the mildness and late onset of the disease in this patient.

In patients M76 and 276 with point mutations leading to amino acid substitutions, the levels of *Btk* mRNA or protein expression were not affected. The effect of the mutations on the enzymatic function of the Btk protein was analysed in BLCL, which showed constitutive Btk activity [6,25]. Replacement of the highly conserved Arg_{288} by tryptophan in patient 276, which would abrogate the interaction of the BTK-SH2 domain with tyrosine phosphorylated proteins [22], apparently has no negative effect on this constitutive kinase activity, but even appeared to result in a somewhat higher activity.

The EBV-transformed BLCL from patient M76 manifested a significant reduction of *in vitro* autophosphorylation and transphosphorylation activities. This finding may reflect an intrinsic defect of the Btk enzyme due to the presence of the Arg_{28} substitution in the PH domain. This conclusion is supported by a similar reduction in kinase activity found in an $Arg_{28} \rightarrow$ Cys mutation in a human Btk gene transfected in chicken DT-40 cells [26]. However, in the *xid* CBA/N mouse, the replacement of the Arg_{28} by Cys does not affect the *in vitro* Btk kinase activity in splenic B cells [33,34]. This discrepancy may reflect differences of Btk enzyme activity or kinetics in murine splenic B cells and human EBV-transformed BLCL or transfected DT-40 cells. As the Arg_{28} residue is located within a potential ligand binding site [49,50], the Arg_{28} mutation might affect the interaction of the PH domain with such a ligand required for the constitutive Btk kinase activity in human EBV-transformed BLCL.

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