Physical and functional characterization of the genetic locus of IBtk, an inhibitor of Bruton’s tyrosine kinase: evidence for three protein isoforms of IBtk

Carmen Spatuzza1, Marco Schiavone1, Emanuela Di Salle2, Elzieta Janda1, Marco Sardiello3, Giuseppe Fiume1, Olga Fierro4, Marco Simonetta2, Notis Argiriou2, Raffaella Faraonio2, Rosanna Capparelli5, Ileana Quinto1,∗ and Giuseppe Scala1

1Department of Experimental and Clinical Medicine, University of Catanzaro ‘Magna Graecia’, 88100 Catanzaro, 2Department of Biochemistry and Medical Biotechnology, University of Naples ‘Federico II’, 3Telethon Institute of Genetics and Medicine, 80131 Naples, 4Institute of Food Sciences, CNR, Avellino and 5Department of Biotechnological Sciences, University of Naples ‘Federico II’, Naples, Italy

Received January 9, 2008; Revised June 12, 2008; Accepted June 13, 2008

ABSTRACT

Bruton’s tyrosine kinase (Btk) is required for B-cell development. Btk deficiency causes X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (Xid) in mice. Btk lacks a negative regulatory domain and may rely on cytoplasmic proteins to regulate its activity. Consistently, we identified an inhibitor of Btk, IBtk, which binds to the PH domain of Btk and down-regulates the Btk kinase activity. IBtk is an evolutionary conserved protein encoded by a single genomic sequence at 6q14.1 cytogenetic location, a region of recurrent chromosomal aberrations in lymphoproliferative disorders; however, the physical and functional organization of IBTK is unknown. Here, we report that the human IBTK locus includes three distinct mRNAs arising from complete intron splicing, an additional polyadenylation signal and a second transcription start site that utilizes a specific ATG for protein translation. By northern blot, 5′RACE and 3′RACE we identified three IBTKα, IBTKβ and IBTKγ mRNAs, whose transcription is driven by two distinct promoter regions; the corresponding IBtk proteins were detected in human cells and mouse tissues by specific antibodies. These results provide the first characterization of the human IBTK locus and may assist in understanding the in vivo function of IBtk.

INTRODUCTION

Bruton’s tyrosine kinase (Btk) is a member of the Tec family of nonreceptor protein tyrosine kinases that includes TECI and TECII, BMX, TXK, ITK and Dsrc28C (1–3). These kinases are characterized by the Src homology-1 (SH1) tyrosine kinase domain and by additional SH2 and SH3 regions, which function as protein–protein interaction sites (4). The structure of Btk includes a unique NH2-terminal region containing a Pleckstrin homology (PH) domain that regulates the Btk kinase activity; accordingly, mutations in several BTK domains lead to a severe X-linked agammaglobulinemia (XLA) in humans (5). Moreover, a specific mutation of the conserved Arg28 residue in the Btk-PH domain leads to a severe X-linked immunodeficiency (Xid) phenotype in mice (6,7). Individuals with XLA show a severe immunodeficiency as a consequence of a significant reduction of mature B cells and immunoglobulin levels (4). Accordingly, mice with Xid carry mutations in the BTK gene and show a decreased number of mature B cells that fail to proliferate properly upon B-cell receptor (BCR) cross-linking (4,8). Several signal pathways are induced upon Btk kinase activation. Evidence from Btk-deficient B cells (DT40) (9) indicates that Btk is required for a proper tyrosine phosphorylation of phospholipase C-gamma (PLC-γ), which in turn leads to inositol-3,4,5-triphosphate (IP3), a major mediator of [Ca2+]i mobilization, and to diacylglycerol, an activator of protein kinase C (PKC) (10,11). These pathways activate specific transcription factors, including nuclear...
factor-kappaB (NF-κB) and BAP135-TFII-I (12–15), which regulate the gene transcription program required for B-cell survival and cell-cycle progression. Btk activation is also induced upon a direct interaction between the Btk-PH domain and G-protein subunits (16). Further, Btk regulates some intracellular apoptotic pathways and plays a role in cell-cycle regulation and tumorigenesis of B cells (9,17,18). Indeed, Btk is a major regulator of B-cell apoptosis and cooperates with tumor suppressor genes, including SLP-65 (17–20).

Little is known of the regulation of Btk function. Unlike Src proteins, Btk lacks a negative regulatory domain and may rely on cytoplasmic Btk-binding proteins to regulate its kinase activity by trans-inhibitor mechanisms. Consistent with this possibility, we have studied a newly identified inhibitor of Btk (IBtk), which binds specifically to the PH domain of Btk and down-regulates its kinase activity. IBtk was isolated by screening a human B-lymphoblastoid cDNA library in the yeast two-hybrid system by using specific domains of Btk as a bait (21). The results were as follows: (i) IBtk is an evolutionary conserved protein encoded by a single genomic sequence at a 6q14.1 cytogenetic location, a region of recurrent chromosomal aberrations in lymphoproliferative disorders (22,23); (ii) IBtk specifically associates with Btk, as demonstrated by both in vitro and in vivo protein–protein interaction assays. Confocal microscopy revealed a submembrane co-localization of IBtk and Btk and (iii) upon binding to Btk, IBtk down-regulates the Btk kinase activity, as shown by using as a substrate both endogenous Btk and a peptide corresponding to the Btk-SH3 domain that includes the Tyr223 autophosphorylation site (21). Btk is essential for B-cell survival and cell-cycle progression following BCR triggering (4,24,25). In this setting, Btk regulates [Ca²⁺]i entry and mobilization from intracellular stores that ultimately lead to the activation of transcription factors, including NF-κB (12,14). Consistent with the above results, IBtk inhibited the [Ca²⁺]i fluxes in Indo-1-loaded DT40 cells upon anti-IgM stimulation and the NF-κB-driven transcription was observed upon anti-IgM stimulation; IBtk expression resulted in a dose-dependent inhibition of this activity (21). These results indicate that IBtk plays a crucial role in the in vivo regulation of Btk-mediated B-cell function; however, no reports have addressed the physical and functional characterization of the IBTK locus. In this study, we report a detailed description of the human IBTK locus and provide evidence for a complex genomic organization that gives rise to three distinct mRNAs, IBTKα, IBTKβ and IBTKγ, which are translated in the corresponding proteins IBtkα, IBtkβ and IBtkγ.

**MATERIALS AND METHODS**

**Genomic analysis of the IBTK genomic locus**

Canis familiaris, Bos taurus, Mus musculus, Gallus gallus, Xenopus tropicalis, Fugu rubripes and Tetraodon nigroviridis genomic sequences were searched for IBTK homologous genes with TBLASTN (http://www.ncbi.nih.gov/BLAST/) and BLAT (http://genome.ucsc.edu/) using the amino acid sequence of human IBtkα as a query. The retrieved genomic segments were aligned to the available cDNA/EST sequences to infer the gene architecture. For genes that lacked a transcript counterpart, a careful manual examination of candidate genomic sequences was performed, by looking for splicing donor and acceptor signals to define the gene structure (26,27).

**Evolutionary analysis of IBTK gene**

Amino acid sequence alignments were performed with MULTIALIN (28). Local evolution rates over the amino acid sequences of IBtkα proteins were estimated with the evolution–structure–function method (29). This analysis requires a preliminary reconstruction of the evolutionary relationships among all analyzed peptides. We built the phylogenetic tree of investigated IBtkα proteins using PHYLO-WIN (30) and by holding its branching pattern constant, we calculated the number of substitution per site in each 15-residue wide window over the entire amino acid alignment with CODEML (31). Finally, rate values were calculated by dividing the number of substitutions per site in each window by the average of all windows (providing the ‘relative rate’). The final values were then computed by smoothing the relative rates with a seven-position moving window arithmetic average. The evolutionary profile was plotted as a function of alignment position in a 2D array. A ranked list of local minima, which define the evolutionarily constrained regions (ECRs), was obtained by scanning the array from the bottom (minimum) to top (maximum). Pair-wise sequence identities were calculated at the European Bioinformatics Institute website (http://www.ebi.ac.uk/emboss/align/). Synonymous and nonsynonymous substitution ratios were calculated at the SNAP website [http://www.hiv.lanl.gov; (32)]. Analysis of canonical and noncanonical splice sites in mammalian genomes was performed as reported (33) by mining GenBank, EMBL (34), and UCSC Genome Browser Database (35).

**IBTK intron 24 amplification from Pongo pygmaeus and Pan troglodytes**

Genomic DNA of *P. pygmaeus* and *P. troglodytes* were used to test the evolutionary conservation of IBTK intron 24 among the primate nonhuman species. Genomic DNA (250 ng) was used to perform a PCR reaction. Specific primers were used to amplify the IBTK intron 24: primer ex24f (5’-GTC AGC CCT CCT GTT GTG GAT-3’) and primer ex25r (5’-TGC ATT CAC TGG TTT GGG GGC-3’). The amplified DNA was eluted from the agarose gel and analyzed by sequencing with an ABI PRISM DNA sequencing system (Applied Biosystems, Foster City, CA, USA).

**Cell cultures**

MC3 and DeFew B cells (36), NB4 and Jurkat T cells were grown in RPMI 1640 (Cambrex, East Rutherford, NJ, USA) supplemented with 10% fetal bovine serum (FBS) (Cambrex), 2mM l-glutamine, 100 U/ml of penicillin and 100 U/ml of streptomycin (Biowhittaker, Walkersville, MD, USA). 293T (human embryonic kidney cells) and HeLa (human epithelial cells from cervical carcinoma)
cells were grown in DMEM (Cambrex) containing 10% FBS, 2mM L-glutamine, 100 U/ml of penicillin and 100 U/ml of streptomycin. Cell lines were incubated at 37°C with 100% humidity in 5% CO₂. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats by using Ficoll Paque gradient (GE Healthcare Europe, Munich, Germany). Briefly, donor blood was diluted 1:10 in PBS 1× and stratified on Ficoll solution with a 3:1 v/v ratio. After a 30 min centrifugation at 2200 r.p.m., PBMC were recovered and re-suspended in RPMI-1640 medium supplemented with 10% FCS.

RNA extraction and RT–PCR

Total RNA was extracted by using the TRIzol reagent (Invitrogen, Karlsruhe, Germany). PolyA mRNA was isolated by using Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany). MC3 total RNA (200 ng) and polyA mRNA (20 ng) was used to synthesize three double strand cDNAs with SuperScript One-Step RT–PCR with Platinum Taq (Invitrogen). Briefly, the RT–PCR reactions were performed in a single step using three pairs of primers: (i) ex24f (5'-GTC AGC CCT GTT GTG GAT-3') coupled with I24r (5'-GAG TGA GGA GGG GAA CT-3') specific primers: (ii) ex24f (5'-GTC AGC CCT GTT GTG GAT-3') coupled with I24r (5'-GAG TGA GGA GGG GAA CT-3') specific primers: (iii) I24f (5'-AAA ATG CTC ACA AGT T-3') coupled with ex25r (5'-GGT GAC CAG TCG CTA GAT GAA A-3') specific for IBTKα and IBTKβ and GSP5R primer (5'-GAG TCA AGT TCG TGG GAT GTA ATA CTG-3') specific for the IBTKγ, coupled with an Abridged Anchor Primer (AAP) (5'-GGC CAC GGC TCG ACT AGT ACG GGI IGG GII GIG-3') containing oligo-di-dG at the 3'-end of the primer. The second nested PCR round was performed with GSP3R primer (5'-GGT GGA TTC CGC AGG GTC CAC ATA-3') specific for IBTKα and IBTKβ and GSP4R primer (5'-GGC GGG GAA ATT GCC CAG AAA AGG-3') specific for IBTKγ. The resulting DNA fragments were eluted from agarose gel and were analyzed by sequencing with an ABI PRISM DNA sequencing system (Applied Biosystems).

Northern blot

MC3, Jurkat and HeLa polyA mRNA (1 μg) was fractionated by electrophoresis on a formaldehyde–1% agarose gel and transferred on Hybond N + nylon strips (GE Healthcare). Filters were alternatively hybridized with two 32P-labeled human IBTK fragments: ex12 probe and int24 probe. A 32P-labeled human GAPDH fragment was used as equal loading control probe. The following primers were used to generate DNA amplicons as probes: for IBTK (α and β) ex12 probe: ex12f 5'-GCA ATA GAC TCT TCC CTG CAC-3' and ex12r 5'-GGG AGA AGA GCA AAC CTA AAT-3'; for IBTKγ int24 probe: I24f 5'-TGG GCA ATT TAG CCT CCA TA-3' and I24r 5'-AAC TTG TGA GCA ATT TAG CCT A-3'; for GAPDH probe: GAPDHf 5'-GAA GGT GAA GGT CGG AGT-3', GAPDHR 5'-GAA GAT GGT GAT GGG ATT TC-3'.

5'-rapid amplification of cDNA end

PolyA mRNA was isolated from MC3 cells by using the TRIzol reagent method (Invitrogen) and Oligotex mRNA mini kit (Qiagen). Two 5'-rapid amplification of cDNA end (5'-RACE) reactions were performed by using 5'-RACE System 2.0 kit (Invitrogen) to find two transcription start sites (TSS): one for IBTKα and IBTKβ, and a distinct one for IBTKγ. The 5'-RACE was performed in three reactions: one RT–PCR to synthesize the first strand cDNA followed by two nested PCR. Briefly, the first strand cDNAs were synthesized from 1 μg of mRNA by SuperScriptII RNA polymerase reaction using the specific primers GSP1R (5'-GAG TGA GGA GGG GAA CT-3') for IBTKα and IBTKβ, and GSP6R (5'-GAG TCA AGT TCG TGG GAT GTA ATA CTG-3') for IBTKγ. After adding an oligo-di-c tail to the 3'-ends of cDNAs, two nested PCR rounds were performed using more internal primers. The first nested PCR round was performed with GSP2R primer (5'-GGT GAC CAC TCG CTA GAT GAA A-3'), specific for IBTKα and IBTKβ and GSP5R primer (5'-GAG TCA AGT TCG TGG GAT GTA ATA CTG-3') specific for the IBTKγ, coupled with an Abridged Anchor Primer (AAP) (5'-GGC CAC GGC TCG ACT AGT ACG GGI IGG GII GIG-3') containing oligo-di-dG at the 3'-end of the primer. The second nested PCR round was performed with GSP3R primer (5'-GGT GGA TTC CGC AGG GTC CAC ATA-3') specific for IBTKα and IBTKβ and GSP4R primer (5'-GGC GGG GAA ATT GCC CAG AAA AGG-3') specific for IBTKγ. The resulting DNA fragments were eluted from agarose gel and were analyzed by sequencing with an ABI PRISM DNA sequencing system (Applied Biosystems).

3'-rapid amplification of cDNA end

To map the 3'-end of the three transcripts IBTKα, IBTKβ and IBTKγ, we performed two 3'-rapid amplification of cDNA end (3'-RACE) reactions. The first strand cDNAs were synthesized from 1 μg of MC3 PolyA mRNA by reverse transcriptase using 3'-RACE System (Invitrogen) with a 3'-RACE primer displaying an adaptor sequence at the 5'-end of the oligo-dT (5'-GGC CAC GGC TCG ACT AGT ACT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT-3'). Three PCR-rounds were performed: in the first one, cDNA was amplified with the adaptor primer and specific primers, GSP4L (5'-CTT TCT TTT CGC AGG AAT TTAG GCC TCC CAT A-3') for IBTKγ and GSP7L (5'-GCG TGG GAG ACA CAT AAG CAA T-3') for IBTKα and IBTKγ. The first nested PCR round was performed by using adaptor primer and other more internal specific primers, GSP5L (5'-CAG TAC ATC ATC CCA TA-3') for IBTKγ and GSP8L (5'-GAAG CTT GGG AGA CTT GGG ACC ACC CAT A-3') for IBTKα and IBTKγ. The last nested PCR round was performed by using adaptor primer and specific primers GSP6L (5'-TGG ATA TGT TGG AGG GCT CAT C-3') for IBTKβ and GSP9L (5'-CAG AGG TGA AGG CTT GGG ACC ACC CAT A-3') for IBTKα and IBTKγ. Following electrophoresis in 2% agarose gel, the final PCR product was eluted (QIAEXII Gel Extraction kit—Qiagen). DNA sequence was determined with an ABI PRISM DNA sequencing system (Applied Biosystems).

IBTK-luciferase reporter assays

Distinct fragments of IBTKα, IBTKβ and IBTKγ promoter regions were obtained by PCR amplification of human genomic DNA and inserted in pGL3-basic vector (Promega, Madison, WI, USA) after digestion with KpnI and BglII. For IBTKα and IBTKβ promoter, we analyzed four fragments designed as: −754/+22, −159/+22,
−82/+22 and −67/+22. The following forward primers were used: for −754/+22, 5′-GGG GTA CCA GCC ATT CAG CAG CAG TGT G-3′; for −159/+22, 5′-GGG GTA CCC GCC GAG GTG AAC TCCCATGAGAAAC-3′; for −67/+22, 5′-GGG GTA CCC GCC GAG GTG AAC TCCCATGAGAAAC-3′. The reverse primer was: 5′-GAA GAT CTC GGG AAC GGG GAT GTA GA-3′. In the case of the IBTKγ promoter, we analyzed two fragments designed as: −691/+5 and −155/+5. The following forward primers were used: for −691/+5, 5′-GGG GTA CCG GCT GTA GTG CAG TGG TAT GAT-3′; for −155/+5, 5′-GGG GTA CCT CCT GTG TAT TAT-3′. The reverse primer was: 5′-GAA GAT CTG TCT GTC CAA TTC TTA GGG TAT G-3′.

Cell transfection and luciferase reporter assays were performed as follows: 293T cells (3 × 10⁴/well) were seeded in 6-well tissue culture plates 24 h before transfection. Cells were transfected by using the calcium phosphate method (Invitrogen) according to the manufacturer’s instruction. The luciferase gene expression driven by an empty pGL3 Basic vector was used as negative control. To normalize the transfection efficiency, cells were co-transfected with pCMV β-gal vector (0.2 μg), which expresses the β-galactosidase gene from the cytophaga-galovirus promoter (Clontech, Mountain View, CA, USA). Each sample was transfected with luc report plasmids (0.5 μg). Twenty-four hours posttransfection, cells were washed twice with PBS and harvested. Cell lysates and luciferase reporter assay were performed using Dual Light System (Applied Biosystem). Results were normalized according to the luciferase to β-galactosidase activity ratio and normalized for the protein concentration. A minimum of four independent experiments were performed; arithmetic mean and SEM values were used for graphic representation.

Real-time PCR analysis

Real-time quantitative RT–PCR on IBTK cDNAs was carried out with the iQ™ SYBR® Green Super mix using the iCycler iQ real-time detection system (Bio-Rad, Munich, Germany) with the following conditions: 95°C, 1 min; (94°C, 10 s; 60°C, 30 s) × 40. The oligonucleotides for PCR were RT24f (5′-CCTCCTGTGATGATCCT AGAACAT-3′) and RT25r (5′-GAGAAGATTTAAC TCCATGGAAG-3′) for IBTKα; RT24f and RT24Ar (5′-AGGGCAGAATACATCAAGTAATG-3′) for IBTKβ; RT24ff (5′-CCGTAACCTTTATTTAAGC GAAATTATGATT-3′) and RT25rII (5′-CATGCAATCTTGGTTTGGGC-3′) for IBTKγ. The reactions were performed in triplicate using as templates cDNA preparations from 15 different human tissues [Human multiple tissue cDNA (MTC) Panel I and II, Clontech] and the following cell types: HeLa, DeFew, Jurkat cell lines and primary PBMC. Expression levels were calculated relative to GAPDH mRNA levels as endogenous control. Relative expression was calculated as 2^(-ΔΔCt) (37). Oligo efficiencies were tested using as a template serial dilutions of α, β and γ ampiclons cloned in pTA cloning vector plasmid (Invitrogen).

IBTK antibodies production and western blot analysis

Antibody against IBTkz and IBTkβ was raised in chicken by taking advantage of a unique peptide sequence, NFHEDDKQKS, not shared by the G. gallus (shown in Figure S1). Two chickens were immunized with 31B peptide NFHEDDKQSC (aa 672–681 of IBTkz and IBTkβ) conjugated with KLH (keyhole limpet hemocyanin) (Gallus Immunotech Inc., Fergus, ON, Canada). One primary immunization (0.75 mg of 31B peptide in Freund’s complete adjuvant) and three boosts (0.25 mg of 31B peptide in Freund’s incomplete adjuvant) were performed. IgY anti-31B specific were purified by affinity chromatography using 31B-conjugated sepharose (NSH-activated Sepharose 4 Fast Flow, GE Healthcare). In addition, to overcome the constraint of amino acid sequence homology of IBtk over multiple species, including M. musculus, polyclonal antibodies against IBTkz and IBTkγ were raised in “/−/−” mouse by GST-IBtkγ immunization. The production of the IBtk−/− mice will be reported elsewhere. The cDNA fragment corresponding to the full-length IBtk was generated by RT–PCR (Invitrogen) of total RNA isolated from MC3 cells, by using the following primers: 5′-CGGAGTTCCGGTGTTGGTTCGTTAAGTAG TATAATTTCAGCAAATGTTTCCTC-3′ and 5′-CC GCTCGAGGCATCTCAACTCCACAGTG-3′. The fragment was inserted in pGEX-4T-3 vector (GE Healthcare) at EcoRI and XhoI sites. GST-IBtkγ protein was expressed in BL21 host Escherichia coli strain. Insoluble GST-IBtkγ was recovered from inclusion bodies by using a denaturing solution (8 M urea, 1% Triton X-100, 5 mM DTT) and purified by gel electrophoresis. Mice were primed with 0.2 mg of GST-IBtkγ in Freund’s complete adjuvant and boosted with three distinct inoculations of GST-IBtkγ (0.1 mg) in incomplete Freund’s adjuvant. The titers and specificity of anti-IBtk sera were assessed by ELISA. Endogenous IBtk proteins were detected by western blot as follows: mouse (adult C57BL) and human cells were lysed in RIPA buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and protease inhibitors) and subjected to SDS–polyacrylamide 8% or 12% gel electrophoresis followed by transfer to a PVDF membrane (GE Healthcare). IBtkz and IBtkβ were detected by using the chicken 31B IgY antibody (10 μg/ml) followed by incubation with an anti-chicken-HRP (Gallus Immunotech Inc.). The binding specificity was assessed by preincubating the primary antibody with the 31B peptide (molar ratio IgY/peptide = 1/1000). IBtkz and IBtkβ proteins were detected by using an anti-GST-IBtkγ serum (dilution 1:500) followed by incubation with anti-mouse-HRP (GE Healthcare). In the case of DeFew cells, the primary antibody was preincubated with either GST (60 nmoles/ml), or GST-IBtkγ (60 nmoles/ml). Cellular γ-tubulin was detected by immunoblotting with a specific antibody (Sigma-Aldrich, Buchs, SG, Switzerland) followed by an anti-mouse-HRP (GE Healthcare). Antibody–protein bindings were visualized by enhanced chemiluminescence (ECL and ECL Plus, GE Healthcare).
Regulation of IBTK transcripts by an IBTK-specific shRNA
HEK 293 LinX packaging cell line (Open Biosystem, Huntsville, AL, USA) was cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin and 100 U/ml of streptomycin 100 μg/ml of hygromycin. Jurkat cells expressing either a stably control shRNA, or the IBTK-shRNA SH2407H3, were cultured in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, 100 U/ml of streptomycin and 1 μg/ml puromycin. HEK 293 LinX packaging cell line (Open Biosysstem) was transfected with 10 μg of either empty plasmid, or with a plasmid expressing the IBTK-specific shRNA clone SH2407H3 (Open Biosystem). Forty-eight hours posttransfection, the cell supernatants were collected and filtered with 0.45 μm. Jurkat cells (4 x 10⁶) were infected with 1 ml of filtered supernatant by spinoculation (38); 24 h later, the culture medium was supplemented with 1 μg/ml puromycin. The sequence of IBTK shRNA SH2407H3 was 5'-TGCTGGTGACAG TGGCAGACGGGATTCTTCTACTGAGAAGATGTG AGGCCACAGATGTTACTTCTTCTAGTAAAGAAATCCC GGTCGCTACTCTGCCTCGGA-3' with annealing sequence to exon 26 of the IBTKα transcript (nucleotide +4162–4183; shown in bold). The shRNA sequence is also specific for the IBTKγ transcript (nucleotide +692–713); shown in Figure S6B.

Protein–protein interaction
PBMC extracts were performed in RIPA-Buffer. Antibodies (5 μg) were preincubated with (20 μl) of protein G-Agarose (GammaBind Plus Sepharose GE Healthcare) in 200 μl of immunoprecipitation RIPA buffer overnight at 4°C on a rocking platform. The cell extracts were precooled on protein G. The protein G-agarose-coupled antibodies were incubated with cell extract (1 mg) in 500 μl of immunoprecipitation RIPA buffer overnight at 4°C on a rocking platform. The immunocomplexes were collected by centrifugation at 2200 r.p.m. for 3 min at 4°C, washed five times in 900 μl of RIPA buffer and resuspended in SDS gel loading buffer. The proteins were separated on either 8% or 12% SDS–polyacrylamide gel, transferred to PVDF membrane and analyzed by immunoblotting. IBtkα and IBtkβ were detected by using the chicken 31B IgY antibody (10 μg/ml) followed by incubation with a anti-chicken-HRP (Gallus Immunotech Inc.). IBtkγ was detected by using an anti-GST-IBtkγ serum (dilution 1:500) followed by incubation with anti-mouse-HRP (GE Healthcare). The following antibodies were used for immunoprecipitation and for control immunoblotting: anti-Btk (E-9), anti-Emt (Itk) (2F12), anti-PLCγ1 (E-12) and anti-Akt1 (B-1), normal mouse IgG1 (Santa Cruz, Santa Cruz, CA, USA).

Confocal analysis
Intracellular localization of IBtk isoforms in B lymphoma cells (DeFew) was assessed by confocal microscopy as previously described (21,39). The cDNA fragments corresponding to the full-length IBtkα, IBtkβ and IBtkγ were generated by RT–PCR (Invitrogen) of total RNA isolated from MC3 cells, by using the following primers: for IBtkα 5'-CCGAAGCCCTTCCTGACTGCATCAAAGGT GTCGATC-3' and 5'-CGGGATCCCTAGCATGGTCGATC-3'; for IBtkβ 5'-CCGAAGCTTCTGCTGACATAAAGTGTCGATC-3' and 5'-CGGGATCCCAAGTTCAGTGGATGAATAAGTTACTG-3'; for IBtkγ 5'-TTAATTGATAATTTCTAGGGAAATGTGTCGATC-3' and 5'-CGGGATCCCTGCATGGTCGATC-3'. The fragment was inserted in p3XFLAG-CMV-7.1 vector (Sigma) at HindIII and BamHI sites. DeFew cells were transiently transfected with plasmids coding for the three isoforms of IBtk (α, β and γ) fused to FLAG tag. Twenty-four hours later the cells were fixed and stained with FITC-conjugated anti-FLAG Ab (Green) and anti-Btk Ab, followed by Alexa-568 secondary Ab (Red, Molecular Probes, Eugene, OR, USA) and DAPI to detect nuclear DNA. DeFew B cells were seeded on poly-L-lysine-treated glass coverslips, fixed and permeabilized using Cytofix/Cytoperm kit (BD Biosciences Pharmingen, San Diego, CA, USA). Coverslips were mounted on glass slides by using ProLong Antifade Kit (P7481, Molecular Probes). Images were collected on a Leica TCS-SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) with a 63× Apo PL A oil immersion objective (NA 1.4) and 60 μm aperture. Z stacks of images were collected using a step increment of 0.2 μm between planes. In each panel, a single plane confocal image shows the center of a transfected cell (Figure 7).

RESULTS
Bioinformatic analysis of the IBTK genomic locus
The IBTK locus was mapped at the 6q14.1 cytogenetic location (21). We took advantage of the annotated sequence of chromosome 6 (40) to compare the cDNA coding for IBTK with the putative gene present in region 6q14.1. This region reports a gene, termed IBTK according to the sequence accession number AL050333, which spans over a 77,576 kb genomic region and includes 29 putative exons (Figure 1A), with a predicted fully spliced mRNA of 5798 nucleotides (Figure 1C). This region includes an ATG translation start codon and a polyadenylation signal (AAUAAA) within exon 24, which would give rise to a 4437 nucleotide mRNA (Figure 1C). This region includes an ATG translation start codon and a TAA stop codon with an ORF coding for a protein of 1353 amino acids with a MW of 150.53 kDa (Figure 1E; the amino acid sequence is shown in Figure S1A); this genomic sequence will be hereafter referred to as IBTKα (Figure 1B). A sequence analysis of the full-length IBTK sequence revealed a putative TSS within exon 1 and a polyadenylation signal (AAUAAA) within intron 24, which would give rise to a 4437 nucleotide mRNA (Figure 1C). This region includes an ATG translation start codon and a TAA stop codon with an ORF coding for a protein of 1196 amino acids with a MW of 133.87 kDa (Figure 1E; the predicted amino acid sequence is shown in Figure S1B); this putative transcript will be hereafter referred to as IBTKβ (Figure 1C).

The previously reported IBTK cDNA (21) (accession number: AF235049) is 2382-bp long and includes an ORF coding for a protein of 240 aa with a MW of 26.31 kDa. This cDNA shows a perfect match with fully spliced exons 25–29 of the IBTK gene and includes an additional nucleotide sequence located within intron...
24 of the IBTKα sequence (Figure 1D), which codes for 31 amino acids of the amino terminus of the protein, hereafter referred to as IBtkγ (Figure 1E; the amino acid sequence is reported in Figure S1C).

Analysis of IBTK transcripts

According to bioinformatic analysis, northern blot of total RNA from HeLa, Jurkat and MC3 cells using specific probes was consistent with the occurrence of three distinct spliced RNAs leading to IBTK transcripts of about 6.0, 4.5 and 2.2 kb (Figure 2A).

To verify the occurrence of additional nucleotide sequences from intron 24 in IBTK transcripts, mRNA was isolated from MC3 cells followed by RT–PCR amplification and nucleotide sequencing. Primer pairs were selected according to the predicted IBTK transcripts (reported under Materials and methods section). Figure 2B shows a schematic representation of the region covering exons 24–25; this region includes intron 24, which hosts a putative TSS and a polyadenylation signal. The results of the RT–PCR analysis were as follows: (i) a fully spliced IBTKα RNA transcript, including the splicing of intron 24 (Figure 2C); (ii) a second IBTKβ transcript that originates from an RNA splicing of introns 1–23 and is terminated at a polyadenylation signal within intron 24 of the IBTK gene sequence (Figure 2D);
(iii) a third IBTKγ transcript that originates from a TSS located within intron 24 (Figure 2E). The PCR-amplified transcripts are shown in Figure 2F.

Next, we identified the TSSs of the putative IBTK transcripts by using the 5′RACE. Both the IBTKα and IBTKβ transcripts originated from a +1 nucleotide (CTTCCTC) within exon 1, which was followed by an ATG translational start codon at nucleotide +551 (Figure S2A, left panel). In the case of the IBTKγ transcript, the TSS (CAGACT) was identified within intron 24 (Figure S2A, left panel). The 5′RACE products are shown in Figure S2A, right panel. Further, we performed a 3′RACE analysis of polyA mRNA to define the 3′-end three IBTK transcripts. The 3′RACE followed by PCR analysis and nucleotide sequencing confirmed the presence of one polyadenylation site shared by IBTKα and IBTKγ transcripts (Figure S2B, left panel) and a distinct polyadenylation site of the IBTKβ transcript within intron 24 (Figure 3B, left panel). The 3′RACE amplicons are shown in Figure S2B, right panel.

As summarized in Figure S2C, these results confirmed the occurrence of three distinct IBTK transcripts: (i) IBTKα mRNA (5798 nt) that arises from splicing of the complete set of 28 introns and represents the full-length IBTKα mRNA (sequence submission: DQ005633); (ii) IBTKβ mRNA (4437 nt) that is contributed by exons 1–24 and includes an additional region of 51 amino acids located within intron 24; this transcript results from the usage of a polyadenylation signal (AAUAAA) within intron 24 (sequence submission DQ005634). In this context, intron 24 is not spliced and functions as an extension of exon 24; (iii) IBTKγ mRNA (2328 nt) that includes a cap region within intron 24, which contributes a short exon that hosts a +1 TSS and codes for 31 amino acids of the amino terminus of IBtk protein (sequence submission: DQ005635).

Evolutionary analysis of the IBTK gene

To investigate whether similar IBTKβ and IBTKγ transcripts were present in other organisms related to humans, we performed a comparative analysis of human IBTK intron 24 with the corresponding sequences from close and distant mammalian species. To this end, we amplified genomic DNA from chimp P. troglodytes and orang P. pygmaeus by PCR using primers flanking human IBTK intron 24 (Ex24f 5′-GTCAGCCCTGTTGTGAG-3′ and Ex25r 5′-TGCATTCACTGGTTTGGCGC-3′). The PCR products were purified from agarose gels and sequenced (Figure S3). This analysis indicated that the distinct IBtk isoforms are conserved across different organisms. Indeed, we observed a 99% similarity across the analyzed species and a predicted production of the three IBtk protein isoforms (Figure S3). Further, we searched the mouse, cow and dog genomes for IBTK orthologs in BLAST analysis at the University of

Figure 2. Analysis of IBTK transcripts. (A) Northern blot analysis of the distinct IBTK mRNAs in HeLa, Jurkat and MC3 cells as follows: (i) IBTKα, 5798 nt; (ii) IBTKβ, 4437 nt and (iii) IBTKγ, 2328 nt. (B) Schematic representation of the IBTK genomic region covering exons 24 and 25. The region of intron 24 reports the +1 nucleotide of IBTKγ transcript and the polyadenylation signal of IBTKβ transcript. (C) The splicing of intron 24 was assessed by RT–PCR analysis and nucleotide sequencing of the region of exons 24 and 25. The bold bar shows the length of the PCR product. (D) Schematic representation of the IBTKβ transcript that includes exon 24 and intron 24, as assessed by RT-PCR analysis of the IBTK region. The bold bar shows the length of the PCR product. The polyadenylation site identified within intron 24 is shown. (E) The IBTKγ transcript was identified by using specific primers as shown. The bold bar shows the length of the PCR product. The picture also includes the +1 nucleotide TSS as identified by 5′-RACE. (F) RT-PCR products of IBTK transcripts using the primers shown in (C-E).
California at Santa Cruz (UCSC) genome browser by using the human sequence as a probe. Each retrieved sequence was carefully compared to the human IBTK genomic sequence using pair-wise TBLAST and TBLASTX, looking for splicing donor and acceptor sites to infer the gene architecture. The results showed that each investigated species has only one gene homologous to human IBTK. All genes share a common intron/exon structure (data not shown) and are therefore putative orthologs. The comparison of human IBTK intron 24 with its orthologous sequences showed that P. pygmaeus, C. familiaris and B. taurus have translation start codons ORFs that are contiguous to the downstream exons, as is the case of the human gene, indicating that their genomes can code for IBTkγ isoform (Figure S4). Pongo pygmaeus, C. familiaris and B. taurus also have the potential to encode an IBTkβ isoform (Figure S4). Conversely, all the potential start codons within the mouse Ibtk intron 24 are followed by stop codons; in this setting, IBTkγ isoform begins from an ATG codon located within exon 25 (Figure S4). In addition, the sequence analysis of mouse intron 24 showed a lack of the polyadenylation signal, suggesting that the IBTkβ protein isoform is not expressed in mice (Figure S4) (41). An investigation of rat Ibtk showed an analogous sequence configuration (data not shown).

The degree of evolutionary conservation for protein-coding regions and the rates of synonymous or nonsynonymous substitutions for the four investigated mammalian species (H. sapiens, M. musculus, B. taurus and C. familiaris) were calculated, and reported as Supplementary Figure S5. Sequence comparisons among the putative IBTK coding sequences in four species (H. sapiens, M. musculus, B. taurus and C. familiaris) showed that the IBTKα, IBTKβ, and IBTKγ isoforms do not differ significantly in terms of sequence conservation or evolutionary pressure (Figure S5).

Within a protein, sequences of functional relevance are subject to evolutionary constraints that force them to evolve with a significantly lower rate than other protein sites. Therefore, assessing ECRs can help to infer important functional sites along the protein sequence (29). To perform an ECR analysis of Ibtkz, we retrieved additional homologous sequences from representative vertebrates (Chicken, Xenopus, Fugu and Tetraodon) at the UCSC genome browser (Figure 3). ECR analysis identified nine regions with strong conservation along the Ibtka sequence. ANK, ankyrin repeats; RCC1, regulator of chromosome condensation; BTB, BTB/POZ domain. (A) A list of the detected ECRs is shown, which were ranked by their evolutionary relative rates. The correspondence between ECRs and known protein domains is shown. (D) Phylogenetic analysis of Ibtk proteins. The enrooted maximum parsimony tree was used as an input for ECR analysis.
constrained region of IBtk (Figure 3A) and is shared by IBtkα and IBtkα isoforms. Of interest, the ECRi corresponds to the IBtkγ region that binds to the PH domain of Btk (21), suggesting that the ECRi region may function as an evolutionary conserved domain that mediates the physical interactions of IBtk with proteins that carry a PH domain.

Identification and functional characterization of IBTK regulatory regions

Next, we undertook a physical and functional analysis of the unknown regulatory regions of the distinct IBTK transcripts. To this end, the genomic region spanning from –754 to +22 bp of the IBTKα/β genes upstream of the TSS were PCR amplified and cloned 5′ to the luciferase reporter gene in the pGL3B plasmid, as previously reported (50). This region includes cis-regulatory sequences, including CCAAT (nucleotides –75/–70), SP1 (nucleotides –87/–81), NFAT (nucleotides –635/–627) and YY1 (nucleotides –662/–656). In parallel, 5′ deletion mutants were also generated (Figure 4A). Upon transient expression of the luciferase reporter gene, indicating that the CCAAT box functions as a major enhancer (Figure 4A). The nucleotide sequence 5′ to the CCAAT box includes additional cis-binding sequences for SP1, NFAT and YY1 transcriptional factors; however, these regions did not increase the basal promoter activity of the –82/+22 region (Figure 4A). These results point to CCAAT-binding proteins as major regulators of the IBTKα/β basal gene transcription.

To identify the regulatory regions responsible for the transcription of IBTKγ, a region of 691 bp corresponding to nucleotide –691 to +5 respective to +1 TSS of IBTKγ mRNA was PCR amplified and cloned 5′ to the luciferase gene in pGL3B plasmid. A sequence analysis of this region (PROSITE) (42) identified an array of potential regulatory cis-sequences, such as binding sites for C/EBPα (nucleotides –5/+3; –45/-37), SP1 (nucleotide –210/–200), AP1 (nucleotide –291/–284), C/EBPβ (nucleotides –416/–406), NFAT (nucleotides –577/–571), IRF (nucleotides –586/576). As shown in Figure 4B, a significant luciferase activity was observed in 293T cells transfected with the luciferase reporter plasmid carrying –691 to +5 nucleotide, while the deletion from –691 to –156 nucleotide abrogated the expression of the luciferase reporter gene. These results indicate that the cis elements contained within the sequence –691/–156 were required for promoter activity.
In vivo examined cells and tissues, followed by IBTK was achieved by using IBtk isoform-specific antibodies. In vivo detection of IBtk protein isoforms (shown in Figure 6A; peptide amino acid sequence is divergent in *G. gallus* and is shared by both IBtkα and IBtkβ isoforms (shown in Figure 6A; peptide amino acid sequence is underlined in Figure S1, panels A and B). To overcome the high degree of amino acid sequence homology of the IBTK locus among several species, we developed *ibtk<sup>−/−</sup>* mice by gene targeting (C. Spatuzza et al., manuscript in preparation), which were immunized with GST-IBtkγ protein. As expected from the *IBtk* gene organization, the mouse anti-GST-IBtkγ serum was specific for IBtkα and IBtkγ proteins, while the anti-31B chicken serum recognized specifically IBtkβ and IBtkβ (Figure 6A and B). The three IBtk isoforms were detected in DeFew cells (Figure 6A and B). This analysis was extended in a panel of 293T, HeLa and lymphoid cells (DeFew, Jurkat, MC3 and NB4) (Figure 6C). Further, the expression of IBtk proteins was detected in an array of mouse tissues (Figure 6D); in particular, western blot analysis detected IBtkα and IBtkγ selectively in liver, spleen, thymus and lung; IBtkγ was also expressed in kidney. Both proteins were undetected in brain, cerebellum and heart. Of interest, IBtkγ was expressed at higher levels in spleen and thymus, suggesting a major role in immune regulation (Figure 6D). According to the nucleotide sequence of the mouse *IBtk* locus, the IBtkβ isoform was undetected in the mice tissues (Figure 6D).

To evaluate the specificity of the IBtk antibodies toward the endogenous proteins, we tested the inhibitory activity of a shRNA (clone SH2631H3, Open Biosystems) that targets specifically the *IBTKα* and *IBTKγ* transcripts (Figure S6A). As expected, expression of the SH2631H3 shRNA in Jurkat cells resulted in a drastic reduction of IBtkα and IBtkγ protein isoforms, while the IBtkβ isoform was unaffected (shown in Figure S6B).

**Sub-cellular localization of the IBtk isoforms**

The localization of the single IBtk isoforms and their *in vivo* association with Btk was analyzed by confocal microscopy as reported (21). In these experiments, the distinct IBtk isoforms were expressed in DeFew B cells and tested for co-localization with endogenous Btk. As shown in Figure 7, the α and γ isoforms showed a mostly cytoplasmic localization and co-localized with Btk at discrete sub-membrane and cytoplasmic regions (shown in Figure 7, upper and lower panels), consistent with the previous evidence showing that the PH-binding domain of IBtk mediates the physical interaction with Btk (21). Accordingly, the IBtkβ, which showed a nuclear localization, did not colocalize with Btk (Figure 7, middle panels). 

**In vivo association of IBtk with proteins expressing the PH domain**

The amino acid sequence analysis of the three IBtk isoforms shows that the α and γ isoforms share a domain that mediates a binding with the PH domain of Btk; this domain is missed in the IBtkβ isoform (21); shown in Figure 3A and S1A–C). This evidence suggests that the
α and β IBtk isoforms may bind to the PH domain of multiple proteins (http://scop.mrc-lmb.cam.ac.uk/scop-1.69/data/scop.b.c.ib.b.b.html). To test this possibility, a panel of antibodies specific for Btk, Itk (51), Akt1 (52) and PLCγ1 (53), which harbor a PH domain, were used to immune precipitate endogenous IBtk isoforms from primary PBMCs. As reported in Figure 8, IBtkα was detected by western blot by using the specific 31B antibody; in the same setting, the 31B antibody failed to detect the IBtkβ isoform, indicating the IBtkβ does not interact
in vivo with the tested proteins (Figure 8, upper panel). The anti-GST-IBtk\(\gamma\) antibody detected the IBtk\(\gamma\) association with the Btk, Itk and Akt kinases (middle panel). Moreover, IBtk\(\gamma\) did not bind to PLC\(\gamma\)1, while IBtk\(\alpha\) showed a strong binding with Btk and a weak interaction with PLC\(\gamma\)1, indicating differential binding affinities for the tested proteins. The lower panel shows the cell expression of the tested proteins, as detected by western blots of the single strips from the upper and middle panels, by using the protein-specific antibodies.

**DISCUSSION**

The role of Btk in B-cell lymphopoiesis is highlighted by the clinical outcomes of XLA patients and xid mice, which have impaired Btk kinase function (1,54). In fact, XLA patients and xid mice show a defective development of pre-B cells into later B-cell stages and incomplete differentiation of B-cell precursors to pre-B cells (25). Patients with XLA show heterogeneity in BTK gene mutations, including deletions, insertions and substitutions (1,4,6). Mutations occurring in the kinase domain as well as the PH, SH3 or SH2 domains, can induce variable degrees of immunodeficiency (5,24). Thus, distinct regions of Btk are critical for its function in B cells, suggesting that Btk interacts physically with multiple cellular proteins. In fact, the Btk-PH domain binds to both the Ca\(^{2+}\)-dependent (\(\alpha, \beta I\) and \(\beta II\)) and Ca\(^{2+}\)-independent isoforms (\(\zeta\) and \(\zeta\)) of PKC.
in mouse mast cells and B cells (55,56); to IP3 (57,58); to βγ as well as Gz12 subunits of heterotrimeric G proteins and to BAP-135 in B cells. Both G12 and BAP-135 function as positive regulators of Btk activity and do not account for the negative regulatory mechanisms required for proper Btk function. In this setting, we have previously identified IBtk as a Btk-PHBM-binding protein that effectively downregulates the Btk tyrosine kinase activity (21). Indeed, IBtkγ binds to the Btk PH domain and promotes a conformational change in Btk, leading to inefficient phosphorylation of Tyr223 and reduction of Btk kinase activity by trans-inhibitory mechanisms (21,59). Upon binding to Btk, IBtkγ regulates early events in B-cell activation, such as [Ca2+] fluxes, and ultimately regulates the gene transcription program mediated by Btk upon BCR cross-linking. IBtk could play a crucial role in the in vivo regulation of Btk-mediated B-cell function. In this regard, further study of regulators of Btk, such as IBtk, may lead to a better understanding of the clinical heterogeneity in XLA syndromes characterized by genetic mutations in the Btk-PH domain. To this end, we undertook a comprehensive analysis of the genomic organization of the human IBTK locus. Here, we provide the first detailed characterization of IBTK as follows: (i) human IBTK spans over 77.58 kb and include 29 exons that give raise to three distinct IBTK mRNAs; (ii) a fully spliced IBTKα mRNA of 5798 nt coding for a protein of 1353 amino acids with a predicted MW of 150.53 kDa; (iii) a second IBTKβ mRNA of 4437 nt that ends at a polyadenylation site within intron 24. The IBTKβ mRNA includes an ORF coding for a protein of 1196 amino acids with an expected MW of 133.87 kDa. Of interest, the mouse Ik bitcoin ortholog does not show a polyadenylation signal within intron 24, indicating that IBtkβ protein is not produced in mice. In this regard, a phylogenetic comparative analysis of the human IBTK locus with dog, mouse, chicken, Xenopus, Fugu and Tetradon orthologs indicates that IBtkβ is recently evolved in the human genome, suggesting a distinct role for the IBtkβ isoform (41); (iv) a third human IBTKγ mRNA results from a TSS within the intron 24 of the IBTK gene and extends over 2328 nt with an ORF coding for a protein of 240 amino acids with an expected 26.31 kDa MW. Of interest, the mouse Ikbitcoin ortholog utilizes an ATG TSS within exon 25, with a predicted mouse IBtkγ isoform of 193 amino acids with a MW of 20.95 kDa.

The three IBTK transcripts are regulated by two distinct promoter regions that utilize specific cis-regulatory sequences. Indeed, the upstream promoter region (nucleotide −754 to +22 from the +1 TSS) regulates the expression of IBTKα and IBTKβ transcripts by using the CCAAT box (Figure 4A), which binds preferentially to CAAT-enhancer binding proteins that are shared by most tissues and epithelial cell types (60,61). The transcription expression of IBTKγ is regulated by a complex array of IRF-1, NFAT (62,63), CEBPα, AP1 and SP1 transcription factors that binds to the cognate cis sequences located at a region spanning from nucleotides −691 to +5 from the +1 nucleotide of the IBTKγ (Figure 4B). This complex promoter organization is expected to be restricted to few cell types, such as immune regulatory cells that require a fine-tuned regulation of gene transcription (62). Consistent with the IBTK promoter organization, the real-time PCR analysis of an array of human cell lines and tissues, underscored a differential expression of the three IBTK transcripts, with IBTKα transcripts showing the highest expression in most of the examined cells and tissues (Figure 5).

Western blot analysis detected the distinct IBtkα, and IBtkγ protein isoforms in human and mice cells. According to the bioinformatics analysis and as confirmed by nucleotide sequencing, the IBtkβ isoform was detected in human cells and undetectable in mice tissues (Figure 6). Of interest, the IBtkγ isoform was highly expressed in human lymphoid cells and in mouse spleen cells pointing to a major role of IBtkγ in immune regulation; this possibility is further supported by a first analysis of Ibk−/− mice that show abnormal proliferation and activation and lymphoid B cells (C. Spatuzza et al., manuscript in preparation). The specificity of the anti-IBtk antibodies was assessed by transducing Jurkat cells either with empty retroviral particles, or with retroviral particles expressing a shRNA sequence specific for the IBTKα and IBTKγ transcripts. Consistent with the IBTK locus organization, a significant decrease of the α and γ IBtk isoforms was observed in shRNA transduced cells; as expected, the expression of IBtkβ protein was unaffected by the shRNA expression (shown in Figure S6).

The resulting IBtkα, IBtkβ and IBtkγ protein isoforms are evolutionarily conserved across vertebrates and include ECR including (i) regulator of chromosome condensation 1 (RCC1) (46,47,64) and BTB/POZ domains that plays a role in chromatin organization and epigenetic regulation of gene expression (48,49,65); (ii) and ankyrin repeats, which mediate protein–protein interactions (44,45). In addition, the computational analysis has shown a conserved domain (aa 1286–1320 of IBtk, aa 173–207 of IBtkγ) with the highest relative score located at the carboxyl terminus; this region mediates the IBtkγ interaction with the PH domain of Btk (66).

To address the issue of the cell colocalization of the distinct IBtk isoforms, DeFew B lymphoma cells were transduced with plasmid expressing either the α, β or the γ IBtk isoform as FLAG-tag proteins. Cells were stained with anti-FLAG and anti-Btk antibodies and analyzed by confocal microscopy (Figure 7). This analysis underscored a restricted cytoplasmic localization of the IBtkγ isoform; the IBtkα isoform showed a strong cytoplasmic localization and detectable levels of nuclear expression, while IBtkβ showed a restricted nuclear localization. The merging of the IBtk and Btk stainings revealed a colocalization of IBtkα and IBtkγ with Btk; consistent with the lack of a PH-binding domain, IBtkβ was not associated with Btk. These results indicate that IBtkα and IBtkβ, which harbor the RCC1 and BTB/POZ domains, have diverged for the γ isoforms to exert distinct functions as nuclear proteins.

As PH domains are shared by several proteins involved in intracellular signal transductions (67,68), IBtk isoforms may bind to additional proteins and may play a regulatory role beyond the Btk-mediated signal transduction.
This possibility was tested by protein–protein interaction experiments in primary PBMC, where the three distinct IBtk isoforms were tested for 

in vivo binding to Btk, Itk (51), Akt (52) and PLCγ1 (53) proteins. As shown in Figure 8, IBtkz and IBtk γ isoforms showed a distinct association with the tested proteins; as expected from the amino acid sequence of IBtkβ, which lacks the PH-binding domain (Figures 3 and S1B), no binding with the PH domain of the tested proteins was observed in the case of IBtkβ.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

**ACKNOWLEDGEMENTS**

We are grateful to Dr P. Schwartzberg. NHGRI-NIH for helping in generating the IBtk−/− mice. Dr N. Zambrano for the IBTK siRNA clone. We thank Dr M. Rocchi, Institute of Biomedical Biotechnologies-CNIR, 70100 Bari, Italy, for providing the genomic DNA of Pongo pygmaeus and Pan troglodytes. This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC), MIUR-PRIN, MIUR-FIRB, ISS to G.S. Funding to pay the Open Access publication charges for this article was provided by Department of Experimental and Clinical Medicine, University of Catanzaro, Italy.

Conflict of interest statement. None declared.

**REFERENCES**

1. Tsukada, S., Rawlings, D.J. and Witte, O.N. (1994) Role of Bruton’s tyrosine kinase in immunodeficiency. *Curr. Opin. Immunol.* 6, 622–630.

2. Berg, L.J., Finkelstein, L.D., Lucas, J.A. and Schwartzberg, P.L. (2005) Tec family kinases in T lymphocyte development and function. *Annu. Rev. Immunol.* 23, 549–600.

3. Broussard, C., Fleischacker, C., Horai, R., Chetana, M., Venegas, A.M., Sharp, L.L., Hedrick, S.M., Fowlkes, B.J. and Schwartzberg, P.L. (2006) Altered development of CD8+ T cell lineages in mice deficient for the Tec kinases Itk and Rlk. *Immunity*, 25, 93–104.

4. Tsukada, S., Salfran, D.C., Rawlings, D.J., Parolini, O., Allen, R.C., Kliaš, L., Sparkes, R.S., Kubagawa, H., Hondtser, T., Jan, S. et al. (1993) Deficient expression of B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell*, 72, 279–290.

5. Lindvall, J.M., Blomberg, K.E., Valija, J.O., Vargas, L., Heinonen, J.E., Berglof, A., Mohamed, A.J., Nore, B.F., Vihinen, M. and Smith, C.I. (2005) Bruton’s tyrosine kinase: cell biology, sequence conservation, mutation spectrum, siRNA modifications, and expression profiling. *Immunol. Rev.*, 203, 200–215.

6. de Weers, M., Mensink, R.G., Kraakman, M.E., Schuurman, R.K. and Hendriks, R.W. (1994) Mutation analysis of the Bruton’s tyrosine kinase gene in X-linked agammaglobulinemia: identification of a mutation which affects the same codon as is altered in immunodeficient xid mice. *Hum. Mol. Genet.*, 3, 161–166.

7. Lee, S.H., Kim, T., Jeong, D., Kim, N. and Choi, Y. (2008) The Tec family tyrosine kinase Btk regulates RANKL-induced osteoclast maturation. *J. Biol. Chem.*, 283, 11526–11534.

8. Thomas, J.D., Siders, P., Smith, C.I., Vorechovsky, K., Chapman, V. and Paul, W.E. (1993) Co-localization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. *Science*, 261, 355–358.

9. Uckun, F.M., Waddick, K.G., Mahajan, S., Jun, X., Takata, M., Bolen, J. and Kurosaki, T. (1996) BTK as a mediator of radiation-induced apoptosis in DT-40 lymphoma B cells. *Science*, 273, 1096–1100.

10. Scharenberg, A.M. and Kinet, J.P. (1998) PtdIns-3,4,5-P3: a regulatory nexus between tyrosine kinases and sustained calcium signals. *Cell*, 94, 5–8.

11. Carpenter, C.L. (2004) Btk-dependent regulation of phosphoinositide synthesis. *Biochem. Soc. Trans.*, 32, 326–329.

12. Petrov, J.B., Rahman, S.M., Ballard, D.W. and Khan, W.N. (2000) Bruton’s tyrosine kinase is required for activation of IkappaB kinase and nuclear factor kappaB in response to B cell receptor engagement. *J. Exp. Med.*, 191, 1745–1754.

13. Novina, C.D., Kumar, S., Bajpai, U., Cheryath, V., Zhang, K., Pillai, S., Wortis, H.H. and Roy, A.L. (1999) Regulation of nuclear localization and transcriptional activity of TFII-I by Bruton’s tyrosine kinase. *Mol. Cell Biol.*, 19, 5014–5024.

14. Shinner, N.P., Carlesso, G., Castro, L., Hook, K.L., Corn, R.A., Woodland, R.T., Scott, M.L., Wang, D. and Khan, W.N. (2007) Bruton’s tyrosine kinase mediates NF-kappaB activation and B cell survival by B cell-activating factor receptor of the TNF-F family. *J. Immunol.*, 179, 3872–3880.

15. Yu, L., Mohamed, A.J., Simonson, O.E., Vargas, L., Blomberg, K.E., Bjorkstrand, B., Arteaga, H., Nore, B.F. and Smith, C.I. (2008) Proteasome dependent auto-regulation of Bruton’s tyrosine kinase (Btk) promoter via NF-κB. *Blood*, 111, 4617–4626.

16. Mao, J., Xie, W., Yuan, H., Simon, M.I., Han, H. and Wu, D. (1998) Tec/Bmx non-receptor tyrosine kinases are involved in regulation of Rho and serum response factor by Galpha12/13. *EMBO J.*, 17, 5638–5646.

17. Kersseboom, R., Middendorp, S., Dingjan, G.M., Dahlernorg, K., Reith, M., Jumaa, H. and Hendriks, R.W. (2003) Bruton’s tyrosine kinase cooperates with the B cell linker protein SLP-65 as a tumor suppressor in Pre-B cells. *J. Exp. Med.*, 198, 91–98.

18. Jumaa, H., Bossaller, L., Portugal, K., Storch, B., Lotz, M., Fleming, M., Schrappe, M., Postila, V., Riikonen, P., Pelkonen, J. et al. (2005) Deficiency of the adaptor SLP-65 in pre-B-cell acute lymphoblastic leukemia. *Nature*, 423, 452–456.

19. Hendriks, R.W. and Kersseboom, R. (2006) Involvement of SLP-65 in Btk suppression and malignant transformation of pre-B cells. *Semin. Immunol.*, 18, 67–76.

20. Halcomb, K.E., Contreras, C.M., Himran, R.M., Coursey, T.G., Wright, H.L. and Satterthwaite, A.B. (2007) Btk and phospholipase C gamma 2 can function independently during B cell development. *Eur. J. Immunol.*, 37, 1033–1042.

21. Liu, W., Quinto, L., Chen, X., Palmieri, C., Rabin, R.L., Schwartz, O.M., Nelson, D.L. and Scala, G. (2001) Direct inhibition of Bruton’s tyrosine kinase by Btk, a Btk-binding protein. *Nat. Immunol.*, 2, 939–946.

22. Mitelman, F., Mertens, F. and Johansson, B. (1997) A breakpoint map of recurrent chromosomal rearrangements in human neoplasia. *Nat. Genet.*, 15, 417–474.

23. Inoue, A., Maru, A., Zettl, A., Streb, P., Muller-Hermelink, H.K. and Starostik, P. (2002) Chromosome 6 suffers frequent and multiple aberrations in thymoma. *Am. J. Pathol.*, 161, 1507–1513.

24. Dinh, M., Grunberger, D., Ho, H., Tsing, S.Y., Shaw, D., Lee, S., Barnett, J., Hill, R.J., Swinney, D.C. and Bradshaw, J.M. (2007) Activation mechanism and steady state kinetics of Bruton’s tyrosine kinase. *J. Biol. Chem.*, 282, 8768–8776.

25. Contreras, C.M., Halcomb, K.E., Randl, L., Himran, R.M., Gutierrez, T., Clarke, S.H. and Satterthwaite, A.B. (2007) Btk regulates multiple stages in the development and survival of B1 cells. *Mol. Immunol.*, 44, 2719–2728.

26. Sardelli, M., Annunziato, L., Roma, G. and Ballabio, A. (2005) Sulfatases and sulfatase modifying factors: an exclusive and promiscuous relationship. *Hum. Mol. Genet.*, 14, 3203–3217.

27. Sardelli, M., Lisciuilli, F., Catalano, D., Attimonelli, M. and Caggese, C. (2003) MitoDrome: a database of Drosophila melanogaster nuclear genes encoding proteins targeted to the mitochondrion. *Nucleic Acids Res.*, 31, 322–324.

28. Cornpet, F. (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.*, 16, 10881–10890.

29. Simon, A.L., Stone, E.A. and Sidow, A. (2002) Inference of functional regions in proteins by quantification of evolutionary constraints. *Proc. Natl Acad. Sci. USA.*, 99, 2912–2917.

30. Galtier, N., Gouy, M. and Gautier, C. (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. *Comput. Appl. Biosci.*, 12, 543–548.
et al. (2002) Physical and functional interaction of HIV-1 Tat with E2F-4, a transcriptional regulator of mammalian cell cycle. *J. Biol. Chem.*, 277, 31448–31458.

51. Kawakami, Y., Yao, L., Tashiro, M., Gibson, S., Mills, G.B. and Kawakami, T. (1995) Activation and interaction with protein kinase C of a cytoplasmic tyrosine kinase, Itk/Tsk/Emt, on Fe epsilon RI cross-linking on mast cells. *J. Immunol.*, 155, 3556–3562.

52. Wang, S. and Basson, M.D. (2008) Identification of functional domains in AKT responsible for distinct roles of AKT isoforms in pressure-stimulated cancer cell adhesion. *Exp. Cell Res.*, 314, 286–296.

53. Suzuki, T., Seth, A. and Rao, R. (2008) Role of phospholipase Cgamma-induced activation of protein kinase Cepson (PKCepson) and PKCbeta1 in epidermal growth factor-mediated protection of tight junctions from acetaldehyde in Caco-2 cell monolayers. *J. Biol. Chem.*, 283, 3574–3583.

54. Takada, H., Kanegane, H., Nomura, A., Yamamoto, K., Ibara, K., Takahashi, Y., Tsukada, S., Miyawaki, T. and Hará, T. (2004) Female agammaglobulinemia due to the Bruton tyrosine kinase deficiency caused by extremely skewed X-chromosome inactivation. *Blood*, 103, 185–187.

55. Yao, L., Suzuki, H., Ozawa, K., Deng, J., Lehčel, F., Fukamachi, H., Anderson, W.B., Kawakami, Y. and Kawakami, T. (1997) Interactions between protein kinase Cε and pleckstrin homology domains. Inhibition by phosphatidylinositol 4,5-bisphosphate and phospholipase Cδ-m1-ristate 13-acetate. *J. Biol. Chem.*, 272, 13033–13039.

56. Venkataraman, C., Chen, X.C., Na, S., Lee, L., Neote, K. and Tan, S.L. (2006) Selective role of PKC beta enzymatic function in regulating cell survival mediated by B cell antigen receptor cross-linking. *Immunol. Lett.*, 105, 83–89.

57. Salim, K., Bottomley, M.J., Querfurth, E., Zvelebil, M.J., Gout, I., Shibuya, K., O'Hagan, D. and McMichael, A.J. (1994) Differential expression of the human Sp1 gene promoter by the specificity protein (Sp) family members. *EMBO J.*, 13, 6241–6250.

58. Saito, K., Tobis, K.F., Sack, A., Koon, H.B., Humphries, L.A., Scharenberg, A., Rawlings, D.J., Kinet, J.P. and Carpenter, C.L. (2003) BTK regulates PtdIns-4,5-P2 synthesis: importance for calcium signaling and PI3K activity. *Immunity*, 19, 669–678.

59. Starr, R., Willson, T.A., Viney, E.M., Murray, L.J., Rayner, J.R., Jenkins, B.J., Gonda, T.J., Alexander, W.S., Metcalf, D., Nicola, N.A. et al. (1997) A family of cytokine-inducible inhibitors of signalling. *Nature*, 387, 917–921.

60. Nicolais, M., Noe, V. and Ciudad, C.J. (2003) Transcriptional regulation of the human Sp1 gene promoter by the specificity protein (5p) family members nuclear factor Y (NF-Y) and E2F. *Biochem. J.*, 371, 265–275.

61. Gronostajski, R.M. (2000) Roles of the NF1/CTF gene family in transcription and development. *Gene*, 249, 31–45.

62. Macian, F. (2005) NFAT proteins: key regulators of T-cell development and function. *Nat. Rev. Immunol.*, 5, 472–483.

63. de Gorter, D.J., Vos, J.C., Pals, S.T. and Spaargaren, M. (2007) The B cell antigen receptor controls AP-1 and NFAT activity through Ras-mediated activation of Raf. *J. Immunol.*, 178, 1405–1414.

64. Dasso, M. (1993) RCC1 in the cell cycle: the regulator of chromosome condensation takes on new roles. *Trends Biochem. Sci.*, 18, 96–101.

65. Bardwell, V.J. and Treisman, R. (1994) The POZ domain: a conserved protein-protein interaction motif. *Genes Dev.*, 8, 1664–1677.

66. DiNitto, J.P. and Lambright, D.G. (2006) Membrane and juxta-membrane targeting by PH and PTB domains. *Biochem. Biophys. Acta*, 1761, 850–867.

67. Yao, L., Kawakami, Y. and Kawakami, T. (1994) The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C. *Proc. Natl Acad. Sci. USA*, 91, 9175–9179.

68. Kawakami, Y., Yao, L., Han, W. and Kawakami, T. (1996) Tec family protein-tyrosine kinases and pleckstrin homology domains in mast cells. *Immunol. Lett.*, 54, 113–117.