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, Elzbieta Janda1, Marco Sardiello3, Giuseppe Fiume1, Olga Fierro4, Marco Simonetta2, Notis Argiriou2, Raffaella Faranoio2, 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-TFI1-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 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.nlm.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) 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).
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 1x 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 (20 ng) was used to synthesize three double strand cDNAs with SuperScript One-Step RT–PCR with Platinum Taq (Invitrogen). The RT–PCR reactions were performed by using RACE System 2.0 kit (Invitrogen) to find two transcripts designed as: (i) 5'-GTC AGC CCT GTT GTG GAT-3' coupled with I24r (5'-GAG TGA GGA GGG GAA CT-3') specific for IBTKα and IBTKβ, and (ii) 5'-GTC AGC CCT GTT GTG GAT-3' coupled with I24r (5'-GAG TGA GGA GGG GAA CT-3') specific for IBTKα and IBTKβ and GSP5R primer (5'-GAG TCA AGT TTC 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-dC tail at the 3'-end of the primer. The first nested PCR round was performed with GSP2R primer (5'-GGT GAC CAG TCG CTA GAT GAA A-3'), specific for IBTKα and IBTKβ and GSP5R primer (5'-GAG TCA AGT TTC TGG GAT GTA ATA CTG-3') specific for the 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 ampiclons 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 TTT TGA TCC A-3'; for GAPDH probe: GAPDHf 5'-GAA GGT GAA GGT CGG AGT-3' and 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 TTC TGG GAT GTA ATA CTG-3') for IBTKγ. After adding an oligo-dC 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 CAG TCG CTA GAT GAA A-3'), specific for IBTKα and IBTKβ and GSP5R primer (5'-GAG TCA AGT TTC TGG GAT GTA ATA CTG-3') specific for the 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 ACG GGI IGG GII GIG-3') containing oligo-dC tail 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 AAT ATA-3') specific for IBTKα and IBTKβ and GSP4R primer (5'-GGG GCC TAA 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).

**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,
Cell transfection and luciferase reporter assays were performed as follows: 293T cells (3 × 10^5/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 cytomegalovirus promoter (Clontech, Mountain View, CA, USA). Each sample was transfected with luc reporter 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′-CCCTCCTGGTTGATCTC AGAAACT-3′) and RT25r (5′-GGG GTA CCA AAC GAG CAG TGT GTC AAG TTC CTT T-3′). The reverse primer was: 5′-GAA GAT CTG TCT GTC CAA TTC TTA GGG-3′. The forward primer was: 5′-GAA GAT CTC GGG AAC GGG GAT-3′. The following cell types: HeLa, DeFew, Jurkat cell line, 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, NFHEDDNQS, not shared by the G. gallus (shown in Figure S1). Two chickens were immunized with 31B peptide NFHEDDNQSC (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 boostings (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 (NHS-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 Ibk5(−/−) mouse by GST-IBtk immunization. The development of the Ibk5(−/−) mouse will be reported elsewhere. The cDNA fragment corresponding to the full-length IBtkγ was generated by RT–PCR (Invitrogen) of total RNA isolated from MC3 cells, by using the following primers: 5′-CCGGAATTCGGTGGTGGTCCGTTAATTGA-3′ and 5′-CC GCTCGAGGCATCTCAACTCCACAGTG-3′. The fragment was inserted in pGEX-4T-3 vector (GE Healthcare) at EcoRI and Xhol sites. GST-IBtkγ protein was expressed in BL21 host Escherichia coli strain. In soluble 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 a 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 IBtkγ 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 μM of penicillin and 100 μM 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 μM of penicillin, 100 μM of streptomycin and 1 μg/ml puromycin. HEK 293 LinX packaging cell line (Open Biosystem) was transfected with 10 μg of either empty plasmid, or with a plasmid expressing the IBTK-specific shRNA clone SH2407H3 (Open Biosystem). Twenty-four hours later, the culture medium was collected and filtered with 0.45 μm. Jurkat cells (+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 precleared 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. The fragment expressed in the full-length IBtk coding for a protein of 133.87 kDa (Figure 1E; the predicted amino acid sequence is shown in Figure S1B); this putative transcript spans over a 77.576 kb genomic region and includes 29 spliced exons (Figure 1A), with a predicted fully spliced transcript (nucleotide +4162–4183; shown in bold). The shRNA sequence is also specific for the IBTKy transcript (nucleotide +692–713); shown in Figure S6B.

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 1B) with an ORF coding for a protein of 1353 amino acids with a MW of 150.53 kDa (Figure 1E; the predicted 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 2328-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 bioinformatics 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 the 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′-GTCAGCCCTCCTGTTGTGAT-3′ and Ex25r 5′-TGCATTCACTGGTTTGGGGGC-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
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 IBtk\,γ isofrom (Figure S4). Pongo pygmaeus, C. familiaris and B. taurus also have the potential to encode an IBtk\,β isofrom (Figure S4). Conversely, all the potential start codons within the mouse Ibtk intron 24 are followed by stop codons; in this setting, IBtk\,γ isofrom 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 IBtk\,β protein isofrom 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 among all IBtk\,z protein (ECRs a-i) (Figure 3). A search of the PROSITE (42) and SMART (43) databases revealed that five ECRs fall within known protein domains:

(i) Ankyrin repeats, which are short, tandemly repeated modules usually involved in protein–protein interaction (44,45);
(ii) regulator of chromosome condensation 1 (RCC1), found in proteins that exert a role in the epigenetic modulation of gene expression (46,47) and
(iii) BTB/POZ, found in transcriptional regulators that control chromatin structure (48,49) (Figure 3B and C).

Conversely, no known protein domains could be assigned to some ECRs, including ECRi, which is the most

---

**Figure 3.** Evolutionary analysis of IBtk. (A) The diagram shows a plot of local evolutionary rates across vertebrate IBtk proteins from H. sapiens, C. familiaris, M. musculus, G. gallus, X. tropicalis, F. rubripes and T. nigroviridis. The values plotted on the y-axis are a measure of the average number of amino acid substitutions per site for residues at the corresponding x-axis position of the protein alignment. The red circles mark the nine highest scoring ECRs detected (ECRs A to I from N- to C-terminus). (B) Schematic of known protein domains found along the IBtk sequence. ANK, ankyrin repeats; RCC1, regulator of chromosome condensation; BTB, BTB/POZ domain. (C) 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 IBtkβ isoforms. Of interest, the ECRi corresponds to the IBtkα 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 in 293T cells, we identified a minimal region of –82 + 22 of the IBTKα gene that regulates the transcription of IBTKα and IBTKβ transcripts; this region includes a CCAAT cis sequence whose deletion abrogates the 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 examination of cells and tissues, followed by IBTK was achieved by using IBtk isoform-specific antibodies. The detection of IBtk in vivo was performed by real-time PCR (Figure 5A). The expression of the IBTK was described and evaluated by primer-optimized real-time PCR (Figure 5A). The expression of the three distinct IBTK isoforms, with the general trend showing IBTKα as the highest expressed isoform in all the examined cells and tissues, followed by IBTKβ and IBTKγ (Figure 5A and B).

**In vivo detection of IBtk protein isoforms**

In vivo detection of IBtkα, IBtkβ, and IBtkγ proteins was achieved by using IBtk isoform-specific antibodies. In particular, IBtkα and IBtkβ were detected in cell lysates by using the 31B antibody raised in chicken by immunization with a peptide whose 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 IBtkα 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 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 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**

In particular, IBtkα and IBtkβ were detected in cell lysates by using the 31B antibody raised in chicken by immunization with a peptide whose 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 IBtkα 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 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 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**

In particular, IBtkα and IBtkβ were detected in cell lysates by using the 31B antibody raised in chicken by immunization with a peptide whose 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 IBtkα 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 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 unaffected (shown in Figure S6B).
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

Figure 6. Detection of IBtkα, IBtkβ and IBtkγ endogenous proteins. (A) IBtkα (150.53 kDa) and IBtkβ (133.87 kDa) were detected by western blot analysis of DeFew cell extracts (150 μg) by using the anti 31B IgY antibody (10 μg/ml) raised in chickens by using the 31B peptide, which was shared by IBtkα and IBtkβ isoforms. The antibody specificity was assessed by using either the competitor 31B peptide (60 nmoles/ml, lane 2), or a 31B scrambled peptide (60 nmoles/ml, lane 1) in competition experiments. Input proteins were equalized by detecting the endogeneous γ-tubulin. (B) IBtkα (150.53 kDa) and IBtkγ (26.31 kDa) were detected by western blot analysis of DeFew cell extracts (100 μg) by using an anti-GST-IBtkγ serum (1:500). The antibody specificities were assessed by using GST (60 nmoles/ml, lane 2), or GST-IBtkγ (60 nmoles/ml, lane 3) proteins in competition experiments. The preimmune serum was unreactive (lane 4). Input proteins were equalized by detecting the endogeneous γ-tubulin. (C) Expression of IBtk isoforms. Protein extracts from 293T, HeLa and lymphoblastoid cells (DeFew, Jurkat, MC3, NB4) (50 μg to detect IBtkα and IBtkβ, 100 μg to detect IBtkγ) were tested in western blots by using the indicated antibodies. Input proteins were equalized by detecting the endogeneous γ-tubulin. (D) Expression of IBtkα and IBtkγ in mouse tissues. Western blot analysis was performed by testing 50 μg of protein extracts from mouse tissues. To detect IBtkα, we used anti GST-IBtkγ serum as primary antibody. IBtkα was detected by the anti-31B IgY. Consistent with the gene organization of mouse Btk, the mouse IBtkβ isoform was not detected. ECL Plus was performed to detect human IBtkγ.
in vivo with the tested proteins (Figure 8, upper panel). The anti-GST-IBtkγ antibody detected the IBtkγ association with the Btk, Itk and Akt kinases (middle panel). Moreover, IBtkγ did not bind to PLCγ1, while IBtkα showed a strong binding with Btk and a weak interaction with PLCγ1, 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 (α, βI and βII) and Ca^{2+}-independent isoforms (ε and ζ) 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 Tyr253 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+]i 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 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 Iktk 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 IBtk in 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 IBtkz, and IBtkγ protein isoforms in human and mouse 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 Iktk−/− 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 IBtkz, 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 IBtkz 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 IBtkz and IBtkγ with Btk; consistent with the lack of a PH-binding domain, IBtkβ was not associated with Btk. These results indicate that IBtkz 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 Iβtk isoforms were tested for in vivo binding to Btk, Itk (51), Akt (52) and PLCγ1 (53) proteins. As shown in Figure 8, Iβtkz and Iβtk γ isoforms showed a distinct association with the tested proteins; as expected from the amino acid sequence of Iβtkδ, 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 Iβtkδ.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We are grateful to Dr P. Schwartzberg, NHGRI-NIH for helping in generating the Iβtk–/– mice. Dr N. Zambrano for the Iβtk siRNA clone. We thank Dr M. Rocchi, Institute of Biomedical Biotechnologies-CNR, 70100 Bari, Italy, for providing the genomic DNA of 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


