

Characterization of Bruton's Tyrosine Kinase Gene and Protein from Marine Sponge *Suberites domuncula*[†]

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Abstract. Bruton's tyrosine kinase (Btk) is a member of Tec family of protein tyrosine kinases. It is expressed in B cells and myeloid cells in humans and it is crucial for maturation of B-lineage lymphoid cells. Mutations in the gene encoding human Btk result in hereditary immunodeficiency X-linked agammaglobulinemia. Sponge proteins can probably reflect characteristics of the ancestral metazoan homologues before their diversifications and specializations in complex animals.

Gene coding for sponge Btk-like protein was fully characterized and its structure was compared with homologous genes from other organisms. Kinase activity of sponge protein was compared to human Btk activity.

We showed that sponge Btk-like gene is probably the most ancient member of Tec family while other members arose from the ancestral gene present in chordate ancestor. The ancestral-type protein was structurally and functionally similar to the highly sophisticated enzyme it is today, whose functions are usually associated with higher Metazoa. (doi: [10.5562/cca1862](https://doi.org/10.5562/cca1862))

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INTRODUCTION

The family of protein tyrosine kinases (PTKs) is a large group of proteins which have evolved together with multicellularity and are found in animals (Metazoa) and choanoflagellates.¹ PTKs have evolved in step with the increasing complexity of multicellular organisms and over a hundred PTKs are encoded in the human genome.² PTKs from the Tec family are non-receptor, cytoplasmic kinases. This family consists of five mammalian members: Btk, Bmx, Itk, Tec and Txk kinases. The evolution of PTKs from the Tec family has been described earlier.¹ The ancestral Tec PTK was present in unicellular eukaryotes prior to the appearance of metazoans. Tec family PTK is present in *Monosiga brevicollis*, a fully sequenced representative of choanoflagellates, which are the closest relatives to metazoans.¹ Tec PTKs are absent from the genome of *Caenorhabditis elegans*.³ Btk PTK contains five domains from the N-terminus: the Pleckstrin homology (PH) domain, followed by the Tec homology (TH) domain, which is further subdivided into the Zn²⁺-binding Btk motif and a proline-rich region, the Src homology 3 and 2 (SH2 and

SH3) and finally the tyrosine kinase (TK) catalytic domain.⁴ Human Btk is expressed in all hematopoietic cells except T-lymphocytes and plasma cells^{4,5} and has a well-defined role in B cell antigen receptor (BCR) signaling.⁶ Mutations in Btk gene cause X-linked agammaglobulinemia (XLA). XLA is a severe hereditary immune disease characterized by a lack of peripheral B cell and low levels of serum Ig.⁷ In mouse, *btk* mutations or deletion cause X-linked immunodeficiency (Xid), with a deficit of B2 B cells, the absence of B1 B cells and low levels of serum Ig.⁸

Sponges (Porifera) are excellent model organisms for molecular evolutionary studies. They represent the most basal metazoan phylum which existed prior to the Cambrian explosion and have changed little since,⁹ and can therefore be considered as living fossils. They do not have true tissues and organs, and lead a sessile lifestyle. In many aspects of their molecular biology, such as gene content, their functional repertoire and intron positions, sponges such as *Suberites domuncula* and *Amphimedon queenslandica* (with large transcriptome or genome datasets available) probably reflect the situation in metazoan ancestor (Urmetazoa). Systematic

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analysis of the sponge gene repertoire showed that the genomic complexity was already present at the very beginning of metazoan evolution, before the appearance of tissue or any other complex morphological feature found in extant animals.¹⁰ Previous analyses of phylogenetically conserved genes/proteins in sponges identified a number of very sophisticated multifunctional enzymes whose functions are usually associated with human diseases.^{3,11,12}

Btk-like 700-aa-long protein from the marine sponge *Suberites domuncula* (Demospongia) was described previously.³ Sponge Btk-like protein contains all of the characteristic domains for the Tec family of PTKs. The highest homology (38 % identity, 55 % overall similarity) was found with human Btk PTK and therefore the sponge homolog was named BtkSD. Our finding showed that Tec family gene/protein already existed in the common metazoan ancestor more than 600 millions years ago, *i.e.* before the separation of sponges from other animal lineages and remained highly conserved during animal evolution.³ Our goal was to determine the structure of the *btkSD* gene, compare it with its metazoan homologs and test whether the sponge BtkSD protein has a similar level of kinase activity as its human homolog.

EXPERIMENTAL

Materials

Live specimens of the sponge *Suberites domuncula* (Porifera, Demospongia, Tetractinomorpha, Hadromerida, Suberitidae) were collected in the northern Adriatic Sea near Rovinj, Croatia, and stored at -80°C .

Sponge *btkSD* Gene

For genomic DNA preparation, the specimens of *S. domuncula* were cut into pieces, frozen in liquid nitrogen, ground to fine powder from which total DNA was isolated using the Genomic DNA Purification kit (QIAGEN), according to the manufacturer's instructions. Sponge *btkSD* gene was amplified, using KOD XL polymerase (Novagen). Three sets of specific primers were used to amplify whole *btkSD* gene: (5'-TTT-CTGCTGGGAACAGGAT-3' and 5'-GTGGGGAGG-GTGCGAGGTT-3'); (5'-GAGGCAGTGTGAAAG-GTTATGTTG-3' and 5'-TTGCTCATCTCGTGCTCG-CC-3'); (5'-CCCCGAGCCCAAACCAT-3' and 5'-AAATGGGTTACATCCACACACA-3'). The amplified fragments were separated on a 0.8 % agarose gel, purified and sequenced (ABI PRISM[®] 3100-Avant Genetic Analyzer, Applied Biosystems, USA) using ABI PRISM BigDye Terminator v.3.1 Ready Reaction Cycle Sequencing Kit.

The reverse specific primer 5'-GGCTTGGTTTC-CTGGCATCTTATAGTGAG-3' was used in conjunc-

tion with the vector specific primer T3/T7 to amplify the 5'-promoter region of the *btkSD* gene on the genomic library from *S. domuncula*.¹³

Nucleotide sequences were analyzed using Lasergene sequence analysis software (DNASTar, Madison, WI). Homologous sequences from other organisms were identified by BLAST and retrieved from NCBI's GenBank. Multiple sequence alignments (MSA) and construction of the phylogenetic tree from the MSA was performed with ClustalX program.¹⁴ The exact position and the phase of each intron was verified by manual inspection.

The putative transcription factor binding sites in promoters of *btk* genes were identified using TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>), and there searching within TRANSFAC database.¹⁵ "Hamming Clustering Method for TATA signal prediction in Eukaryotic Genes" was used for TATA-box identification.¹⁶

A sample of 224 coding sequences of *S. domuncula* (including cDNA coding BtkSD) was collected for analysis of synonymous codon usage.¹⁷ CODONW program was used for synonymous codon usage analysis (<http://codonw.sourceforge.net/>) and calculated GC3S, Fgc and Fop parameters. GC3S is fraction of codons in gene which have either a guanine or cytosine at third codon position. Fgc is G + C content of gene. Fop is the frequency of optimal codons used in gene, the ratio of optimal codons to synonymous codons. This value is always between 0, which indicates no usage of optimal codon, and 1 which indicates that only optimal codons are used.

Plasmid Constructions

The cDNA for BtkSD³ was recloned using PCR into *Bam*HI (5'-GACAGGATCCCAGTGGTTAGTCATC-3') and *Nde*I (5'-CACTATCATATGCCAGGAAACCAAG-CC-3') restriction sites of pET15b expression vector (Novagen) downstream from the thrombin cleavage site.

Protein Expression, Purification and Western Analysis

BtkSD was overproduced in *Escherichia coli* strain BL21 tagged with six histidine residues at the *N*-terminus and purified to homogeneity from bacterial lysates using cobalt affinity chromatography (TALON). Soluble protein visible after SDS-PAGE was obtained under modified growth conditions: *E. coli* strain BL21 harboring the plasmid construct was grown to OD₆₀₀ 1.5 and induced with 0.05 mM IPTG for 3 h at 18 °C. Cells were incubated 30 minutes on ice in lysis buffer (50 mM Tris HCl, 300 mM NaCl, 10 mM imidazole, and 1 mg/mL lysozyme) and sonicated 8 × 30 sec (50 % of the full power). After centrifugation (12 000 rpm) for 25 min at 4 °C, the supernatant was applied onto

cobalt-charged agarose column (TALON). Histidine tagged proteins were eluted with 500 mM imidazole. The samples were loaded on SDS-PAGE and electrotransferred to a PVDF Hybond-P membrane (Amersham Biosciences). The membranes were incubated with anti-His antibody (Amersham Biosciences) for detection of 6His-BtkSD. Protein bands were visualized using chemiluminescence detection (Amersham ECL Plus, GE Healthcare).

BtkSD protein was applied to a PD-10 desalting gel filtration column (Sephadex, GE Healthcare) equilibrated and eluted with 20 mM Tris HCl, pH = 9 (start buffer). Ion-exchange chromatography (monoQ 5/50 GL column) using the fast protein liquid chromatography (FPLC) system was employed for protein purification. The column was equilibrated with start buffer and eluted with linear NaCl gradient up to 1.0 mol dm⁻³. Amicom Ultra-15 was used for desalting and buffer exchange (25 mM HEPES, pH = 7.5, 25 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 2 mM DTT, 0.008 % triton X-100).

Kinase Assay

Kinase activity was measured using a coupled pyruvate kinase-lactate dehydrogenase assay. Reaction kinetics was followed by connecting the turnover of ATP to the turnover of NADH to NAD. NADH concentrations were measured by spectrophotometry. Five hundred μ L of reaction mixtures were incubated in quartz cuvettes at room temperature in the presence of ATP as phosphate donor and poly EY (4:1) MW 20 000–50 000 as substrate. The final concentrations were as follows: 25 mM HEPES, pH = 7.5, 25 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 2 mM phosphoenolpyruvate, 0.25mM NADH, 1 mM ATP, 2 mM DTT, 2 units of pyruvate kinase, 3 units of lactate dehydrogenase, 0.008 % triton X-100 and 0.2 mg/mL poly EY. Reactions were initiated by the addition of 25 nM of BtkSD enzyme and activity was monitored in an Ultrospec[®] 2100 *pro* (Amersham Pharmacia Biotech, USA) measuring the decrease in absorbance at $\lambda = 340$ nm. The control reactions omitting BtkSD produced minor rates but were subtracted from the BtkSD values. Reactions were done in triplicate.

RESULTS AND DISCUSSION

Phylogenetic Analysis

It is already known that Tec family PTK is present in the unicellular choanoflagellate *Monosiga brevicollis* which is the closest known relative to metazoans.¹ In invertebrate and some vertebrate (amphibian) genomes, a single gene from the Tec family PTK was found which showed the highest similarity to Btk.¹ Interestingly, we found two different Tec family PTKs from *Monosiga ovata* (BAG55524; BAG55489) which share

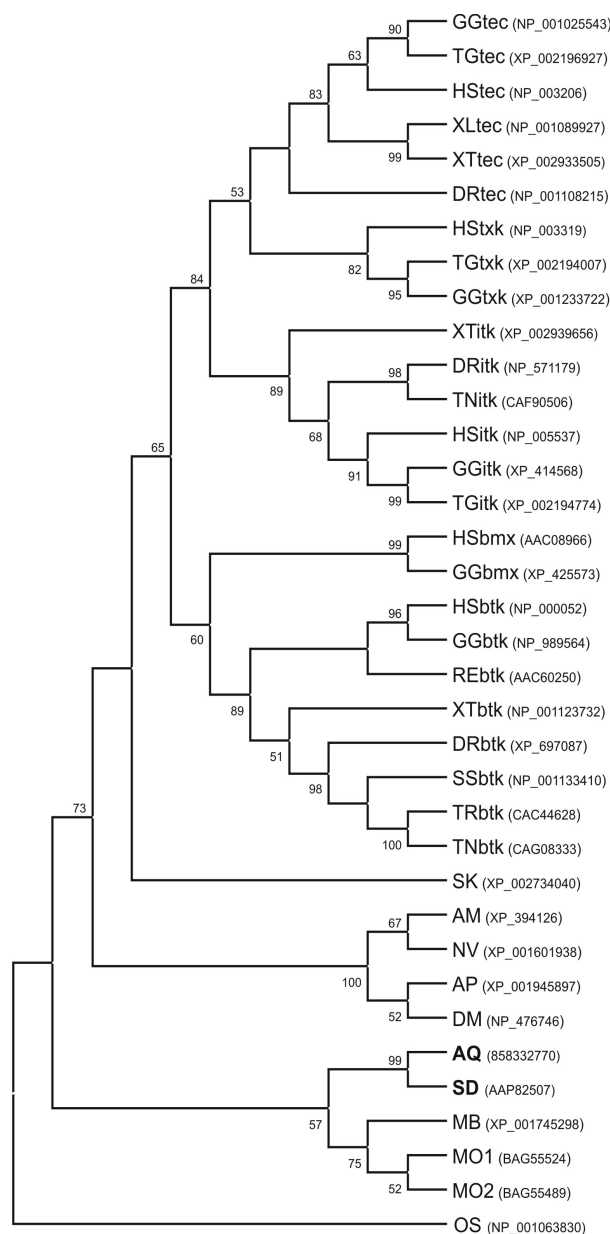


Figure 1. Maximum parsimony phylogenetic tree of Tec family of PTKs. Proteins from the following species were analysed: *Suberites domuncula* (SD), *Amphimedon queenslandica* (AQ), *Monosiga brevicollis* (MB), *Monosiga ovata* (MO), *Drosophila melanogaster* (DM), *Acyrtosiphon pisum* (AP), *Nasonia vitripennis* (NV), *Apis mellifera* (AM), *Saccoglossus kowalevskii* (SK), *Tetraodon nigroviridis* (TN), *Takifugu rubripes* (TR), *Salmo salar* (SS), *Danio rerio* (DR), *Xenopus (Silurana) tropicalis* (XT), *Gallus gallus* (GG), *Homo sapiens* (HS), *Xenopus laevis* (XL), *Taeniopygia guttata* (TG), *Oryza sativa* (OS).

65 % similarity. Both of them showed the highest similarity with Btk Tec family member which is indicative for independent duplication not related to the appearance of recent Tec family members. Furthermore, we found three different Tec family PTKs from

Xenopus tropicalis (XP_002933505, XP_002939656, NP_001123732), whereas there is only one present in *Xenopus laevis*. This is probably due to the lack of complete genome sequence of *X. laevis* rather than difference in number of Tec family members in those two closely related species. Phylogenetic analysis of TK domains PTKs from the Tec family was presented in Figure 1. The Btk-like homologs from sponges clearly group together with those from choanoflagellates. Sponge Btk-like protein reflects the characteristics of the protein ancestral to Tec family, before the duplications and diversifications within the metazoan lineage.

Analysis of *btkSD* Gene

The *btkSD* gene from *S. domuncula* encompasses one intron in 5' untranslated region (5'UTR; 222 bp) and 15 introns in the coding region (~2500 bp, 522 bp, 324 bp, 246 bp, 432 bp, 72 bp, 79 bp, 229 bp, 223 bp, 164 bp, 71 bp, 283 bp, 187 bp, 253 bp and 255 bp) with the average length of 378 bp. Introns in human *btk* gene are much longer ranging between 179 and 10747 bp with the average length of 1898 bp. Sponge introns are relatively short with the exception of the first intron in coding region. The same was found for introns in several other sponge genes.^{18,19} While intron lengths drastically differ between these two organisms, exon lengths are pretty much similar. Human coding exons are ranging from 63 to 217 bp in size with the average length of 110 bp. Sponge coding exons range from 55 to 389 bp in size with the average length of 131 bp.

Multiple sequence alignment of *btkSD* gene homologues was produced and used to compare intron positions (Figure 2). The sponge *btkSD* gene has three introns in the PH domain, one intron at the end of TH domain (prolin rich region), one intron in the SH3 domain, one intron in front of the SH2 domain, two introns in SH2 domain and one in front of the TK domain.

Within the TK domain of *btkSD* gene there are six introns: one intron is in the following subdomains I, III, V, VIb, IX and XI. The human homolog has 17 introns, *D. melanogaster* homolog has 11 introns and *M. brevicollis* homolog 10 introns. The *btkSD* gene from sponge has 9 introns in the same positions and the same phases as the human homolog and three more introns found in the same phases but on slightly different positions within the unconserved regions, probably of the same origin. On the other hand, *btkSD* gene from sponge has only three introns in the same positions and the same phases as the fruit fly homolog and two more introns probably of the same origin (in the same phases but slightly different positions). *D. melanogaster* probably lost seven ancestral introns and gained six new ones which indicate accelerated evolution and shows high intron dynamics in *Drosophila* lineage. As seen from Figure 2 only one intron (phase 2 intron in front of the SH2 domain) is common to all analyzed organisms. The position of the interrupted amino acid is always the same, just in front of WY/F, the conserved N-terminus of the SH2 domain. Interestingly, intron at the identical position is also present, not only in some other non-receptor PTK genes like *csk*, *src* and *frk*,¹⁸ but also in many SH2 domains found in other unrelated genes.²⁰ The SH2 domains in all these different, multidomain proteins must have originated from the same ancestral proto-SH2 domain and the common evolutionary heritage is still visible in their gene structures. Shuffling of the ancient, SH2 encoding gene to many unrelated genes happened very early in the evolution of animal multicellularity.¹⁸

The ancestral metazoan *btkSD* gene was intron-rich. Position of introns in sponge *btkSD* gene probably reflects the archetype of the metazoan Tec family gene. From 15 introns within coding region most of them, 47 % were in phase 0. Overabundance of phase 0 introns was observed in many species²¹ and this preference was probably present also in metazoan ancestor.

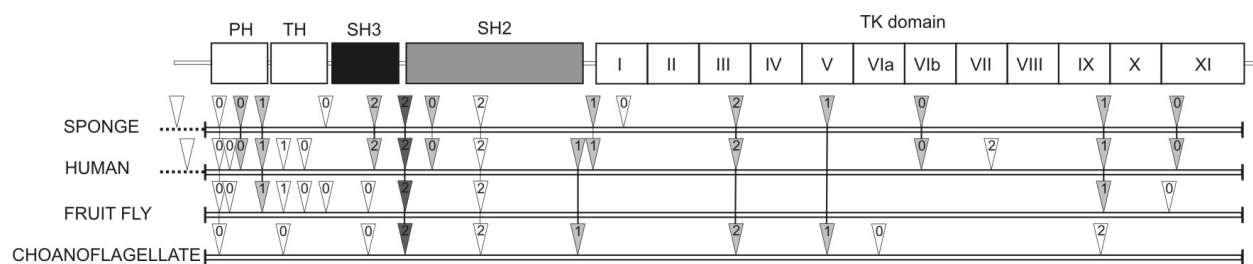


Figure 2. Schematic presentation of intron positions in *btk* PTK gene from sponge *S. domuncula*, human, fruit fly and choanoflagellate. Regions encoding PH, TH, SH3, SH2 and TK domains (divided in subdomains I-XI) are indicated. The positions of introns are marked by arrowheads and phases by numbers in arrowheads. Intron in front of SH2 domain (phase 2) is conserved in all genes and is shown with dark grey arrowhead. Introns shown with grey arrowheads are found at the same positions in at least 2 organisms and those shown with white arrowheads are gene-specific. Arrowheads connected with black line represent introns which are present at the same positions and phases. Arrowheads connected with thin line show introns which are in the same phase, but at slightly different positions (shifted for several aa). Sequences of genes (with indicated intron positions) were taken from the corresponding genomic databases.

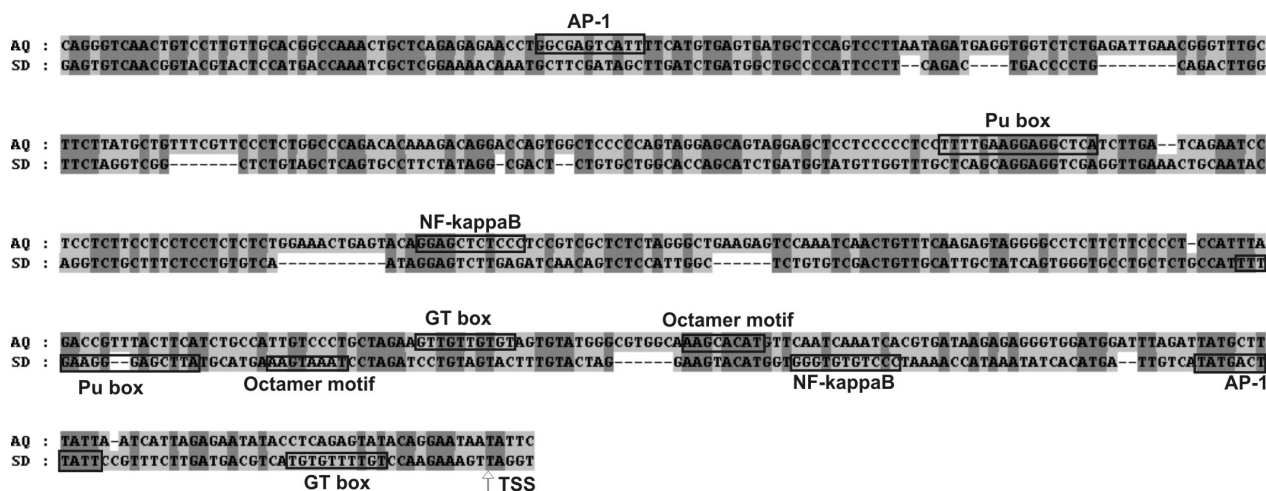


Figure 3. The structure of *btk* promoters from two sponges (*S. domuncula* and *A. queenslandica*). Putative motifs for binding of transcription factors and coactivators which have been demonstrated to bind to the human *btk* promoter were found and designated (Pu box, GT box, Octamer motif and NF-kappa B) in both sponges. Motif AP-1 was found in sponges only. TSS indicates transcription start site.

The promoter regions of *S. domuncula* and *A. queenslandica* *btk* genes were analyzed. Putative motifs for binding of transcription factors were found in both promoters. Pu box, GT box, Octamer motif and NF-kappa B were identified in promoters of both sponges when non-stringent cut-offs were used (Figure 3). These transcription factors and coactivators have been demonstrated to bind to the human *btk* promoter.²² The structure of sponge *btk* promoter indicates similar gene regulation as in the human homolog. However, in both

sponges other common motifs were identified, not found in human, like AP-1 binding site (Figure 3). Interestingly, all of these motifs are not located at same positions in these two sponges which indicate fast evolving nature of sponge promoters. In accordance with this is observance that sponge *S. domuncula* lacks strong TATA-box signal in -60 bp region, while in *A. queenslandica* two TATA-box like motifs were identified (from -32 to -23 bp AATATACCTC and from -20 to -11 bp AGTATACAGG).

Investigation of the preferred codons in marine sponge *S. domuncula* revealed that highly expressed genes preferentially use C- and G-ending codons.¹⁷ Sponges as the oldest metazoan lineage probably provide the best possible insight into the characteristics of the genome of the metazoan ancestor. The preference for C- and G-ending codons has previously been documented in other metazoans.²³⁻²⁶ Therefore, it is likely that the codon usage bias was present in the metazoan ancestor. Fgc, GC3S and Fop for cDNA encoding BtkSD protein, were calculated using CODONW program. Fop parameters were based on results of correspondence analysis of 224 *S. domuncula* genes.¹⁷ Fgc for BtkSD was 46 %, Fop 0.287 and G + C content at silent codon position 38.2 %. Fop varies from 13.7 to 60.6 %. Potentially highly and lowly expressed genes are distinguished in Figure 4 according to their Fop and G + C content (Fgc). Conspicuously, ribosomal proteins and contigs are clustered more to the right, which indicates higher optimal codon usage. Codon usage in gene *btkSD* indicates that this gene is relatively lowly expressed in *S. domuncula* (Figure 4). This is further corroborated with the finding that there is only one BTK transcript in the *S. domuncula* EST library with over 13 000 random sequences.

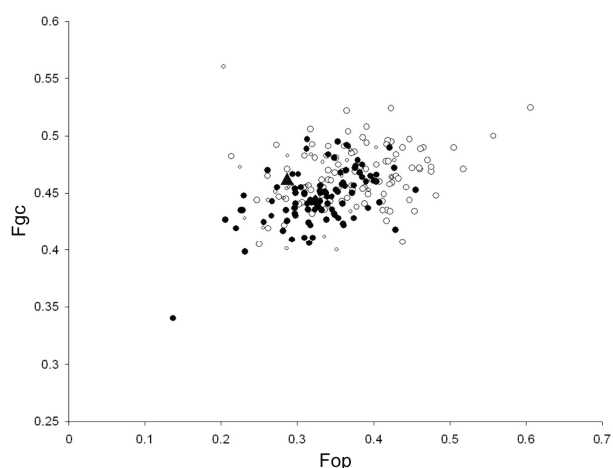


Figure 4. Fop plot of 224 *S. domuncula* genes (frequency of optimal codons are plotted against G + C content – Fgc). Contigs and ESTs coding for ribosomal proteins are marked with ○, singlets and cDNAs codings for the Ras family proteins are marked with ●. Remaining sequences are marked with ○. Black triangle (▲) indicates the position of BtkSD. Codon usage of gene *btkSD* indicates that this gene is relatively lowly expressed in sponge.

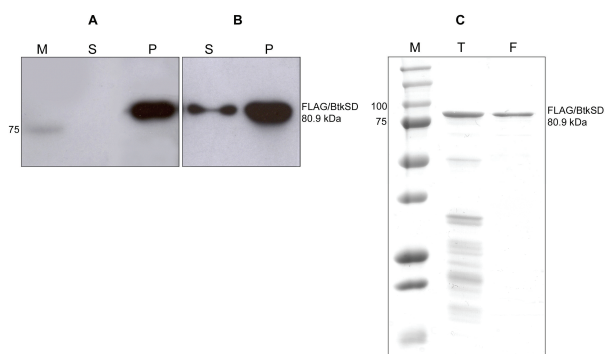


Figure 5. Purification of full-length BtkSD and Western blot analysis with anti-His antibodies. Under standard growth conditions (A) BtkSD could be detected in pellet (P), but not in supernatant (S). Soluble recombinant protein was obtained under modified growth conditions (B): OD₆₀₀ 1.5 and decreased amount of IPTG (0.05 mM) and then purified (C) with TALON (T) and ion-exchange chromatography (F).

Protein BtkSD from Marine Sponge *S. domuncula*

Recombinant protein is present only in pellet fraction under standard growth conditions (Figure 5A). Soluble recombinant protein was obtained under modified growth conditions as described in materials and methods section (Figure 5B). Coomassie Blue staining showed that after cobalt-charged agarose column (TALON) protein was ~80 % pure, and after Mono Q was > 90 % pure (Figure 5C). Kinase activity of purified BtkSD was tested as described in materials and methods section. Reaction was done in triplicate (Figure 6). As shown in Figure 6 the lag phase ~ 120 s was pronounced as in human less phosphorylated Btk Mono Q fraction.²⁷ Human Btk sample reached observed V_{\max} of 174 min⁻¹.²⁷ Our average observed V_{\max} of 131 min⁻¹ indicates very similar kinase activity of sponge ancestral Tec family member.

Our results indicate that this ancestral-type protein is probably structurally and functionally similar to the highly sophisticated multifunctional enzyme it is today, whose functions are usually associated with higher Metazoans. The biological role of Btk-like protein in the simple sponge organisms, lacking any organized tissue, is yet unknown, but it is possible that the basic role of Btk-like protein in sponges is associated with actin remodeling. In mammals, Btk is translocated in regions of membrane, lamellipodia, which are formed by the polymerization of actin.²⁸ It has been shown that Btk-like protein from *Drosophila* is required for controlling contraction of the actin-myosin microfilament ring during embryonic cellularization.²⁹ Sponges are able to control the water current through their bodies by lowering the pressure in one region to draw water from another. Myocytes in sponges surround each osculum,

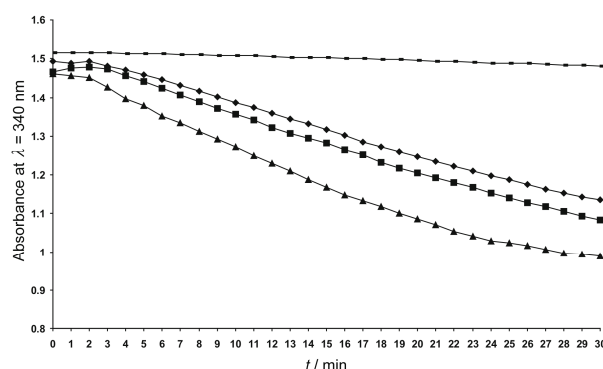


Figure 6. Kinase activity of BtkSD was determined by measuring the oxidation of NADH in a coupled pyruvate kinase-lactate dehydrogenase enzyme assay system. Reactions were initiated by the addition of 25 nM of BtkSD enzyme in 25 mM HEPES pH = 7.5, 25 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 2 mM phosphoenolpyruvate, 0.25 mM NADH, 1 mM ATP, 2 mM DTT, 2 units of pyruvate kinase, 3 units of lactate dehydrogenase, 0.008 % triton X-100 and 0.2 mg/mL poly EY. Activity was monitored measuring the decrease in absorbance at $\lambda = 340$ nm for 30 minutes. Top line represents control reaction. Lower curves represent reaction in triplicate.

have actin and myosin and can contract. Changes in osculum pore size can also prevent the entry of unwanted material.³⁰ Modulation of actin polymerization by Tec family kinases may have important role even in processes of cell proliferation, cell shape, motility and cell adhesion.²⁸ The other possible function of Tec family ancestral-type protein in sponge is regulation of apoptosis. Apoptosis is general metazoan process for controlling tissue homeostasis in all developmental stages. Overexpression of Btk in HeLa cells led to apoptosis, but endogenous levels of Btk protected cells from apoptosis.³¹ Sponges possess homologs of genes involved in apoptotic pathways in human cells.^{32,33} However, molecular mechanism of apoptosis in sponges is yet unknown and Btk may play important role in deciding the fate of the cell depending on the biological response specific for the cell type.

CONCLUSION

In conclusion, this paper emphasizes four major points: (i) the sponge *btkSD* gene reflects the structural characteristics of the metazoan ancestral Tec family gene which was intron-rich; (ii) the structure of sponge promoter indicates similar gene regulation as in the human homolog; (iii) based on codon usage analysis and EST library search, *btkSD* gene is probably relatively lowly expressed in *S. domuncula*; (iv) the kinase activities of the sponge BtkSD enzyme and human homolog are similar.

Therefore, we presume that even at the evolutionary stage of first metazoans this ancestral-type protein was structurally and functionally similar to the multi-functional enzyme it is today.

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