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Expression and Purification of Homeodomain TGIFLY as a GST-Fusion Protein

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Abstract

Homeobox genes encode transcription factors which play important roles in the developmental processes of many multicellular organisms. *TGIFLX/Y* (*TGIFLX and TGIFLY*) are members of the homeobox superfamily of genes. Their expressions are specifically detected in the human adult testis but their functions are remained to be investigated. In this investigation we cloned full length of TGIFLY cDNA and produced recombinant GST-TGIFLY protein in bacterial system. Here we present production of GST-TGIFLY fusion protein as a soluble protein. The recombinant protein was confirmed by western blot analysis using anti-GST antibody. Through a single purification procedure using MagneGST Beads, approximately 20 mg of the recombinant protein was obtained per liter of bacterial culture. We suggest that GST-TGIFLY fusion protein could be utilized as a valuable molecular tool on investigation of TGIFLY target genes and identification of co-factors or partner proteins involved in TGIFLY function in normal and abnormal development.

Keywords: GST-TGIFLY; Western Blot; SDS-PAGE

Introduction

In humans there are about 230 Hox genes, a group of evolutionarily conserved genes encoding a large family of transcription factors that play important roles in various developmental processes [1-3]. They are characterized by the presence of a specific sequence motif known as the homeobox translated to the 60-amino-acid homeodomain, a helix-turn-helix DNA binding domain [1,4,5].

Three-amino acid loop extension (TALE) group of homeodomain proteins has a 3-amino-acid insertion between helices 1 and 2 of the homeodomain resulting in a 63 instead of the typical 60 amino acid Homeodomain [6]. Cooperative function among TALE family members is critical for transcription regulation [7] and it has been proved that they act as transcriptional activators and repressors [8].

Also, several members of TALE family function as essential contributors to Hox-mediated developmental

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programs [9-12]. *TGIF*s belong to the TALE superclass of homeobox-containing genes known to play critical roles in developmental process such as cell proliferation differentiation and cell fate [13,14]. *TGIFLX/Y* as a homeobox gene consists of two genes; *TGIFLX* (X-linked) and *TGIFLY* (Y-linked) are specifically expressed in human adult testis. *TGIFLX* originated from retrotransposition of *TGIF2*, located on long arm of chromosome 20 (q11.2-12), onto the X chromosome. The *TGIFLX* contains a 726 bp open reading frame that encodes a 26.675 KDa protein. The *TGIFLY* sequence has 2808 bps containing two exons and one intron. It contains a 558 bp open reading frame that encodes a 23 KDa protein. This protein exhibits a similarity to TGIFLX protein across the first 148 amino acids that include the homeodomain [15]. Previous studies have revealed that both TGIFLX and TGIFLY genes are specifically expressed in adult testis and involved in testis development and spermatogenesis [15,16]. However, the mechanisms by which these genes act are unknown.

In recent years the use of recombinant proteins has been greatly increased. Recombinant hybrids containing a polypeptide fusion partner, affinity tag, are widely used to facilitate the purification of the target polypeptides. Many different proteins, domains or peptides can be fused with the target protein. The advantages of using such fusion proteins in purification and detection of recombinant proteins are well-recognized [17,18]. Recently, we have produced Gluthatione-S-Transferase (GST)-TGIFLX recombinant protein, for studying the TGIFLX function [19]. In the present study we produced one of its recombinant hybrids called GST-TGIFLY.

Materials and Methods

TGIFLY Expression Constructs

For the construction of pGEX-6p-1/TGIFLY, the 558-bp human TGIFLY cDNA encoding full-length TGIFLY, was amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Promega, Madison, WI, USA) and the primers TGIFYF; 5'CGGGATCCA TGGAGGCCGCTGCAGACG-3 and TGIFLYR; 5'-CCGCTCGAGCTATTCCGGTGAGCTTCTGG-3'.
The underlined sequences represent the introduced *BamH*1 and *EcoR*1 restriction sites to facilitate cloning. Amplification was performed on Corbett DNA thermal as follows: 3 min at 97°C for initial denaturation step, 30 sec at 97°C, and 45 sec at 64°C with a 1°C decrease every second cycle down to 55°C then 55°C for the 14 cycles, 1 min at 72°C for extension, and finally 10 min at 72°C. After PCR, the purified PCR product was digested with BamH I and EcoR I and then inserted into the pGEX-6p-1 vector digested with BamH I and EcoR I. The correct DNA sequence and reading frame between the GST and TGIFLY DNA was confirmed by automated DNA sequencing using an ABI Prism dye terminator cycle sequencing kit (Gene Fanavaran, IRI).

Expression and Purification of Recombinant TGIFLY

Recombinant expression vectors pGEX-6P-1/TGIFLY was transformed into *Escherichia coli* strain ER2566 and then cultured overnight in 2×YT medium containing 10 mg/ ml ampicillin. The cultures were then diluted 1:100 with fresh pre-warmed 2×YT and incubated at 37°C with shaking. When the OD_{600} reached a value of 0.45, fusion gene expression was induced by adding IPTG with the final concentration of 0.1 mM. Uninduced cultures were also included as control to compare bacterial protein expression. The culture was incubated at 37°C for an additional 70 min and then cells were harvested by centrifugation 14000g, 1 min 4°C. Cell pellets were resuspended in 40 µl of phosphate-buffered saline PBS per ml of bacterial culture. The bacterial proteins present in the supernatant, were analyzed by SDS-PAGE board using molecular weight standards.

Western Blotting

Western blotting was performed as describe by Heidari et al. [18] 10 µl bacterial supernatant was used in electrophoresis through 10% SDS-PAGE gels and then transferred to a membrane. The membranes were blocked for 1 h with blocking buffer and then incubated with a 1/1000 dilution of a polyclonal rabbit anti-TGFLY antiserum. The membranes were incubated with a HRP-conjugated anti-rabbit IgG secondary antibody and incubated on shaker for 1 h. When the specific band was observed, subsequently the gel scanned for further analysis.

Results

Cloning of TGIFLY Coding Region

In the present study, the full length of wild type *TGIFLY* reading frame (558bp) was obtained by PCR amplification of human male's DNA using specific primers corresponding to TGIFLY sequence, namely TGIFLYF and TGIFLYR (Fig. 1). PCR product of TGIFLY was successfully cloned as N-terminal fusion into the *EcoR* I and *BamH* I sites of the pGEX-6P-1

expression vector (Fig. 2). The recombinant plasmid was produced by transformation into *E.coli* (ER2566). The presence of cDNA inserts was verified by digestion of recombinant plasmids using restriction enzymes analysis (*EcoR* I and *BamH* I) (Fig. 3). The correct DNA sequence and reading frame between GST and TGIFLY DNA was confirmed by automated DNA sequencing. BLAST of GenBank database was used to check for correct nucleotide sequences which showed a complete match with those previously reported [15].

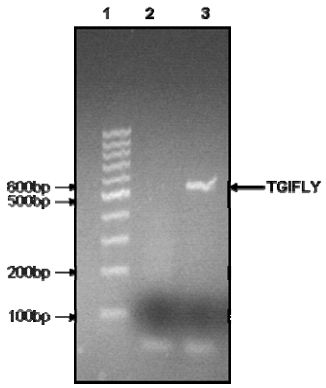
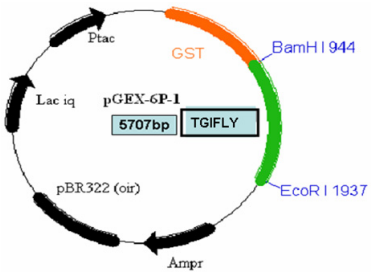


Figure 1. PCR amplification of human TGIFLY gene. Ethidum bromide stained agarose gel (1.2%) showing amplification of 558bp human. Lane 1, molecular weight marker (100bp); lane 2 and Lane 3, Negative control (H_2O) and TGIFLY encoding full length of TGIFLY CDS from a human male, respectively. Arrow shows the interested band corresponding to size of TGIFLY (558bp).

(A)



(B)

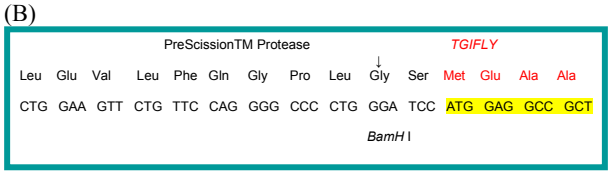


Figure 2. Schematic representation of pGEX-6P-1/TGIFLY expression constructs which encodes the GST-TGIFLY fusion protein. A. Features of the expression vector and restriction sites used are shown. B. Sequence of pGEX-6P-1/5'-TGIFLY junction showing the reading frame.

Expression and Purification of GST-TGIFLY Fusion Protein

GST-TGIFLY fusion protein expression was induced by adding IPTG into overnight cultures of transformed *E. coli* (*ER2566*) cells with pGEX-6p-1/TGIFLY vector. In order to optimize expression of GST-TGIFLY fusion protein a range of cell densities, different IPTG concentrations and various time of incubation were applied (data not shown). Uninduced cultures were also included to compare bacterial protein expression. To establish that the GST-TGIFLY fusion protein has been expressed in a soluble form in *E. coli*, expressing cells with OD600=0.9 were lysed and separated into supernatant and pellet fractions. Figure 4 represents supernatant fractions analyzed by SDS-PAGE after IPTG induction which revealed that the recombinant proteins were present mainly in the supernatant. Thus, GST-TGIFLY which has apparent molecular weight of 49 KDa was expressed as soluble proteins in *E. coli.*

The expression of GST-TGIFLY fusion protein allowed for its convenient purification from other soluble bacterial proteins by using MagneGST Beads. Supernatant samples were purified at room temperature and the recombinant protein eluted from the magnet beads was then checked on a SDS-PAGE gel stained with coomassie brilliant blue R-250 (Fig. 5). When the GST-TGIFLY was tested with an anti-GST antibody the expected band of 49kDa gave a strongly positive result (Fig. 6). This confirmed the identity of soluble 49kDa protein as correctly expressed TGIFLY fusion protein.

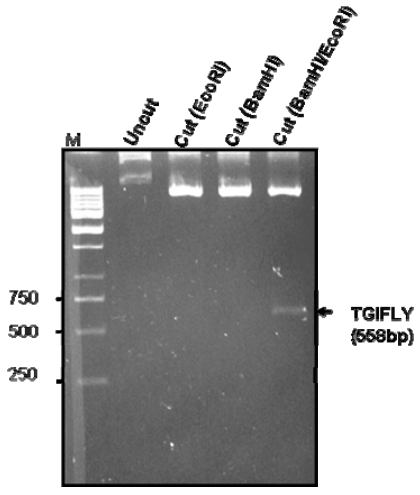


Figure 3. Restriction enzyme analysis of recombinant pGEX-6p-1/TGIFLY plasmids. Uncut, cut (*EcoR*I), cut (*BamH* I) and cut (*EcoR* I/*BamH* I). M, DNA marker (1kb). Arrow shows the insert corresponding to size o TGIFLY (558bp).

We observed that intact GST-TGIFLY could be produced with high level of purity, and at a final yield of approximately 20 mg/L of bacterial cell culture.

Discussion

Our results indicated that induction at high cell densities of bacterial cells containing pGEX-6p-1/TGIFLY led to production of extremely unstable recombinant protein (data not shown). This result was consistent with the nature of some homeodomain fusion proteins [18]. It is because some studies have indicated that the stability of recombinant HOX proteins differ through homeodomain proteins and seems to be related to their natures [18,20]. In contrast with our previous results that homeodomain GST-HOX11 protein was extremely stable in induction of high cell densities; GST-TGIFLY was unstable under these conditions. The GST-TGIFLY was produced with high level of purity, at a final yield of approximately 20 mg/L of bacterial culture. When the GST-TGIFLY and empty vector were tested with an anti-GST antibody, the expected bands of 49 and 26 kDa gave a strongly positive result, respectively (Fig. 6).

High-level production of GST-TGIFLY fusion protein could be a prerequisite for investigation on biological functions of this transcription factor. Recently we used GST-HOX11 fusion protein for detection of HOX11 binding target sequences and its protein partners [18]. Therefore, the successful production of recombinant TGIFLY protein now permits a range of investigations including the whole-genome PCR (WG-PCR) technique to study target DNA sequences that directly bind to TGIFLY. This recombinant protein can also be utilized to study its potential interacting protein partners. Such studies should ultimately help to define the role of this transcription factor in both normal and abnormal development which remains to be investigated in future studies.

In summary we have described a method to produce large amounts of intact GST-TGIFLY fusion protein.

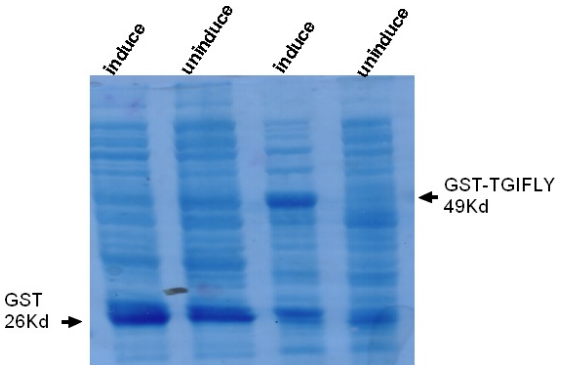


Figure 4. Expression of soluble GST-TGIFLY fusion protein under optimized conditions. *E. coli* strain *ER2566* was transformed with pGEX-6p-1, pGEX-6p-1/TGIFLY and after cell lysis, a 15µl aliquot of the supernatants representing about 300µl cell culture was analysed on 12.5% SDS-PAGE gel stained with Coomassie blue. IPTG induction resulted in the expression of GST (26kDa), GST-TGIFLY (49 kDa), respectively (arrows).

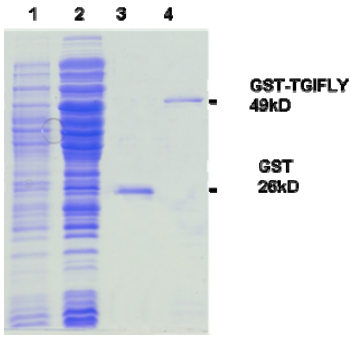


Figure 5. Purification of GST-TGIFLY and GST proteins using MagneGST Beads kit Protein purifications were performed at room temperature. 15 µl of uninduced supernatant (Lane 1), induced bacterial supernatant with OD_{600} =0.9 (lane 2), purified GST protein, (Lane 3) and purified GST-TGIFLY (Lane 4) loaded onto 12.5% SDS PAGE. The gel was stained with Coomassie Blue. Arrows indicate bands corresponding to molecular weights of GST-TGIFLY (49kDa) and GST (26 kDa) proteins.

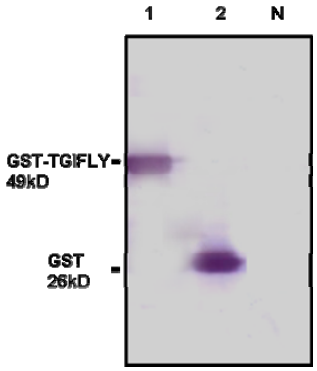


Figure 6. Western blot analysis of GST-TGIFLY and GST proteins. 10µg of the purified GST and GST-TGIFLY fusion proteins were separated by 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The membrane was probed with an antibody directed against the GST protein, which revealed the presence of GST-TGIFLY (lane 1), GST (lane 2) and negative control lane3). Arrows indicate the interested bands corresponding to GST (26kDa) and GST-TGIFLY fusion protein (49kDa).

The details that were particularly critical to the success of our procedure were: 1) induction of TGIFLY expression at low cell densities and 2) purification of GST-TGIFLY fusion proteins at RT. Using this protocol, soluble TGIFLY protein can be produced at a high purity and consequently used in protein-protein interaction or protein-DNA interactions which may provide significant understanding of TGIFLY partner proteins and target sequences involved in gene regulation. This in turn will help to define the role of this transcription factor in both normal and abnormal developments.

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