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Heterologous expression of Translocated promoter region protein, Tpr, identified as a transcription factor from *Rattus norvegicus*

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ABSTRACT

Our earlier studies have demonstrated that the 35 kDa isoform of Translocated promoter region protein (Tpr) of *Rattus norvegicus* was able to augment *c-jun* transcription efficiently. Identification of direct targets that may in part downregulate *c-jun* transcription might prove to be an ideal target to curtail the proliferation of normal cells under pathophysiological conditions. In order to evaluate its potential as a pharmaceutical target, the protein must be produced and purified in sufficiently high yields. In the present study, we report the high level expression of Tpr protein of *R. norvegicus* employing heterologous host, *Escherichia coli*, to permit its structural characterization in great detail. We here demonstrate that the Tpr protein was expressed in soluble form and approximately 90 mg/L of the purified protein at the shake flask level could be achieved to near homogeneity using single step-metal chelate affinity chromatography. The amino acid sequence of the protein was confirmed by mass spectroscopic analysis. The highly unstable and disordered Tpr protein was imparted structural and functional stability by the addition of glycerol and it has been shown that the natively unfolded Tpr protein retains DNA binding ability under these conditions only. Thus, the present study emphasizes the significance of an efficient prokaryotic system, which results in a high level soluble expression of a DNA binding protein of eukaryotic origin. Thus, the present strategy employed for purification of the *R. novergicus* Tpr protein bypasses the need for the tedious expression strategies associated with the eukaryotic expression systems.

Introduction

The *c-Jun*, a product of an early response proto-oncogene and a member of the AP-1 family of transcription factor(s), is involved in a variety of biological processes that influence cell differentiation, proliferation, growth and apoptosis [1–4]. Regulation of *c-jun* expression under different physiological conditions is achieved by interaction of protein factors with the regulatory sequences present in the upstream region of the *c-jun.* Earlier studies from our laboratory have resulted in the identification of distinct *cis*-acting elements and *trans*-acting factor(s) involved in its positive regulation [5,6]. We have demonstrated the presence of a *cis*-acting regulatory element spanning −538 to −514 region of *c-jun* which plays a positive role in *c-jun* transcription [7]. The protein that interacts with the −538 to −514 region of the *c-jun* and augments transcription from the *c-jun* promoter is identified as the Translocated promoter region protein (Tpr[1]). Since the potential of designing therapeutic drugs targeting transcription factors is well known [8,9], knowledge about the proteins that interact with the identified *cis*-acting element and modulate *c-jun* expression can be exploited for designing small molecule inhibitors to curtail *c-jun* expression during pathophysiological conditions. The first report on the identification of Tpr (involving ≈140–230 NH_2 terminal residues) in fusion with several proto-oncogenes (*ras*, *raf*, *met*, *trc*) and the potential of this chimera to induce neoplastic transformation provided evidence to the importance of chromosomal translocations in rendering normal cells tumorigenic [10,11]. Until now, Tpr has been characterized as a nucleocytoplasmic protein and no other function has been assigned to this protein [12]. The nuclear pore complex (NPC) links the inner and outer membranes of the nuclear envelope. These pore complexes at the nuclear periphery facilitate the passive diffusion of ions, metabolites and small molecules across the membrane efficiently [13]. Nuclear pore complex proteins are also known to govern the mRNA export post transcription to the cytoplasm for their subsequent translation. The signal mediated transport of macromolecules across the nuclear membrane via the NPC is a highly regulated process and thus points to an important mechanism for control of gene expression [14,15]. Several nucleoporins contain typical conserved motifs that provide evidence to their probable functions. Unlike Tpr, Nup153, a nucleoporin contains four zinc finger motifs through which it binds to DNA in a zinc-dependent fashion *in vitro* [16–18]. This is consistent with the proposal that nucleoporins play an

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[1] *Abbreviations used:* Tpr, translocated promoter region; EMSA, electrophoretic mobility shift assay; RT, room temperature.

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important role in the chromatin organization and nucleocytoplasmic transport. Similar to the Tpr, Nup107 also possesses leucine zippers at its C-terminus, suggesting that either dimerization or the formation of homo-or heteromers is a requisite for its functioning [19]. However, the actual function of NPC-associated Tpr, occurring ubiquitously in all the cells, remained unknown. Normally by itself Tpr does not possess oncogenic/transforming properties and its role in modulating *c-jun* transcription remains enigmatic. Therefore, discovery of such genes and their fusion products can be exploited as potential targets for cancer therapy. To gain an insight into the diverse functional and structural aspects of Tpr, the present study was aimed to produce the Tpr protein in sufficient yields and quantity to attain high-resolution structure and for making DNA–protein interaction studies feasible.

Materials and methods

Materials

Escherichia coli DH5α and M15 strains were obtained from Gibco-BRL, USA and BL21 (λDE3) strain was procured from Novagen, USA. The plasmid harboring the cDNA encoding Tpr in pCMVsport 6.ccdb vector (IMAGE clone ID: 7929511) was obtained from the American Type Culture Collection (ATCC), USA. All the chemicals required for DNA manipulation (restriction enzymes and chemicals) were purchased from New England Biolabs, USA and Promega, USA. The radioactive nucleotide α-^{32}P [dCTP], used in the study was procured from BRIT, India. His-3 polyclonal antibody conjugated with the alkaline phosphatase was procured from Sigma Co., USA. All other chemicals of analytical grade were procured from Sigma Co., USA, unless otherwise stated. DNA manipulations were carried out using standard protocols unless otherwise stated [20]. The oligonucleotides and primers used in the study were synthesized by Sigma Co., USA.

Construction of 6×His-tagged Tpr clones

The full length gene encoding Tpr of *Rattus norvegicus* was PCR amplified using gene specific primers, [Forward: 5′-CCG**GGATCC**ATGGCGGCGGTGTTGCAGCAAGTGC-3′; Reverse: 5′-CCG**GGTACC**TTATTCCATAAGGTCTTTAGATTCTTCC-3′ containing *Bam*H I and *Kpn* I (bold and underlined), respectively, for the ease of cloning the gene] and plasmid pCMVsport 6.ccdb as the template. The amplified *tpr* fragment digested with *Bam*H I and *Kpn* I was ligated to pQE-30 and pRSET-A prokaryotic expression vectors digested with the same enzymes. Putative recombinants were screened using restriction enzyme digestion analysis and further integrity of the tpr gene in both the constructs was confirmed by automated dideoxy DNA sequencing (Applied Biosystem Model 393A). The recombinant constructs were designated as pQE-30.*tpr* and pRSETA.*tpr*.

Expression and purification of rTpr in E. coli

The *E. coli* BL21 (λDE3) cells harboring the pRSETA.*tpr* and M15 cells harboring the pQE-30.*tpr* were induced with 1 mM IPTG and the expression of the recombinant proteins was analyzed 8 h post-induction. The localization analysis for the expressed recombinant protein was performed in the induced cell lysates obtained from both the *E. coli* hosts, essentially as described earlier [21].

All protein purification steps were performed at 4 °C. The recombinant Tpr protein (rTpr) from both the constructs was purified from the soluble fraction under native conditions [21]. The expressed Tpr protein from both the constructs harboring the hexa-histidine tag at the N-terminus was subsequently purified using Ni^{2+}-NTA affinity chromatography. The purified protein fractions were pooled, dialyzed in 20 mM Tris–HCl, pH 8.0 with and without glycerol (10%), unless otherwise stated and stored at 4 °C for immediate use or −20 °C in 50% glycerol for long term use or until further use.

The authenticity of the purified recombinant Tpr protein was confirmed by Western blotting analysis by resolving the purified fraction on 12% SDS–PAG and transferring onto nitrocellulose (NC) membrane. The blot was blocked with 2% BSA in 1 × PBS containing 0.05% Tween 20 (PBST) for 2 h at RT followed by three subsequent washes with PBST for 10 min each at RT. The membrane was then incubated with the anti-6×-His tag antibody conjugated with alkaline phosphatase. The immunoreactive bands were visualized by the Western blue stabilized substrate solution (Promega, USA). Protein concentration was determined by the method of Lowry et al., [22] using bovine serum albumin as a standard.

Antibody generation against the rTpr

BALB/c mice (4–6 weeks) were used for immunization experiments to raise primary antibodies against recombinant protein. The guidelines prescribed by the Institutional Animal Ethics Committee, JNU, New Delhi, India were followed while handling the animals and the animals used for the project had the approval of the Institutional Animal Ethics Committee (IAEC-JNU Project Code No. 01/2005).

Mass spectrometry

The specific band corresponding to the Tpr protein was excised from the polyacrylamide gel with a sterile scalpel and digested with trypsin. The digested samples were analyzed by an ultraflex MALDI-TOF–TOF instrument (Bruker, Germany). Peptide mass fingerprinting (PMF) was performed by comparing the masses of identified peptides to NCBI protein database using the MASCOT search engine (http://www.matrixscience.com) as described [21].

DNA binding ability of the rTpr using EMSA

Electrophoretic mobility shift assay (EMSA) was carried out using 1 μg of the purified rTpr (dialyzed in buffer with and without glycerol) and 5 ng of the α-^{32}P[dCTP] labeled Jun-25_{SA} (oligonucleotide spanning −538 to −514 region of *c-jun*, ≈40,000 cpm) in a final reaction volume of 40 μl at 25 °C for 30 min (unless otherwise mentioned) as described [7]. The complex was loaded onto a pre-electrophoresed 5% non-denaturing PAG and the products were analyzed by autoradiography. Supershift assay was performed by preincubating the rTpr (1 μg) with 1 μg of the anti-rTpr antibody prior to the addition of the labeled Jun-25_{SA}. The complex was resolved on a pre-electrophoresed 5% non-denaturing PAG, and the products were analyzed by autoradiography.

Results and discussion

Cloning, expression and purification of the R. norvegicus recombinant 6×His-Tpr

The versatile functions associated with Tpr, its localization in various cellular compartments and existence of several isoforms/homologs has been reported [10–15]. However, the mechanisms involved therein are far from clearly understood. In order to elucidate these functions, pure and functional Tpr is required. Earlier studies demonstrating different functions of Tpr have used purified cDNAs to produce full length Tpr protein using an *in vitro* coupled transcription and translation system for both human and rat Tpr proteins [23,24]. Earlier attempts to purify the full length human