

## Recombinant Expression of the Non-glycosylated Extracellular Domain of Human Transforming Growth Factor $\beta$ Type II Receptor Using the Baculovirus Expression System in Sf21 Insect Cells

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### ABSTRACT

Transforming growth factor beta (TGF $\beta$ 1,  $\beta$ 2 and  $\beta$ 3) are 25 kDa disulfide-linked homodimers that regulate many aspects of cellular functions, consist of proliferation, differentiation, adhesion and extracellular matrix formation. TGF $\beta$ s mediate their biological activities by binding of growth factor ligand to two related, functionally distinct, single-pass transmembrane receptor kinases, known as the TGF $\beta$  type I and type II receptors. The high affinity of type II TGF $\beta$  receptor to TGF $\beta$ 1 and TGF $\beta$ 3 makes it a potent and specific inhibitor for TGF $\beta$  related studies. In this study, we designed a recombinant construct including a part of the extracellular domain of type II TGF $\beta$  receptor (amino acids 38-159) which functionally was active according to previous crystallography studies. The recombinant protein was efficiently (30  $\mu$ g per 106 Sf21 cells) produced in Sf21 insect cells which were transfected by recombinant baculovirus. The recombinant protein was purified based on the presence of His-tag sequence using Ni-NTA agarose.

**Keywords:** Transforming growth factor beta, Type II TGF $\beta$  receptor, Sf21 Insect cell expression system, Baculovirus expression system

### INTRODUCTION

Transforming growth factor beta (TGF $\beta$ ) belongs to a superfamily of structurally related multifunctional proteins, which consists of three isoforms (TGF $\beta$ 1, 2, 3) in human [1]. TGF $\beta$ s are 25 kDa homodimeric polypeptides that are important in development, wound healing, immune responses and tumor cell growth and inhibition [2-5]. TGF $\beta$  signal transduction is mediated by binding to cell surface receptors. Two membrane proteins known type I and II (T $\beta$ R1, T $\beta$ R2) are required for most of TGF $\beta$  responses [6-10]. The T $\beta$ R1 and T $\beta$ R2 receptors are a group of transmembrane kinases containing a cysteine rich extracellular domain, a transmembrane region and a cytoplasmic segment consisting of a kinase domain with specificity for serine and threonine [11-13]. TGF $\beta$  signal transduction is initiated by binding of the growth factor ligand to the extracellular domain of T $\beta$ R2. After ligand binding, T $\beta$ R2 receptor phosphorylates the type I receptor and cause the signal propagation to downstream substrates

[2,14-15]. As TGF $\beta$  receptor type II keeps cells from growing and dividing in an uncontrolled way, it is important in suppressing the formation of tumors and is a potent and specific inhibitor to study the role of TGF $\beta$ s during diseases. The high affinity of T $\beta$ R2 to bind the TGF $\beta$  makes it a strong detector to investigate the folding of produced recombinant TGF $\beta$  proteins by protein-protein interaction analysis. Due to the involvement of T $\beta$ R2 in numerous biological processes, many attempts have been made to produce recombinant T $\beta$ R2 using heterologous systems (16-19). Recently, we successfully expressed the extracellular domain of T $\beta$ R2 in a bacterial expression system [20]. The obtained protein from bacterial system refolded in reduced and oxidized glutathione according to Glansbeek *et al.*, 1998 [17].

In this study, we successfully expressed and purified the extracellular domain of T $\beta$ R2 in Sf21 insect cell expression system. Due to the presence of a suitable cellular environment for post translation modification and proper folding of the obtained protein, this system has been considered to produce biologically active recombinant proteins.

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1 atggtcactg acaacgcagg tgcagtcgaag ttccacaac tgtgtaatt ttgtgatgtg agattttcca cctgtgacaa ccagaaatcc tgcagtcgca actgcagcat caactccatc tgtgagaagc cacaggaagt
taccagtgc tgttgctgac acgtcagttc aaagtggtt acacatttaa aacactacac tctaaaaggt ggacactggt ggtcttttagy acgtactcgt tgcagtcgta gtggaggtag acactcttcg gtgtcttca
>>.....TGF BR2.....>
m v t d n a g a v k f p q l c k f c d v r f s t c d n q k s c m s n c s i t s i c e k p q e
Asn 19 >>>
a

141 ctgtgtgct gtagggagaa agaatgacga gaacataaca ctgagagacag ttgtccatga ccccaagctc cctaccatg actttattct ggaagatgct gcttctccaa agtgcattat gaaggaaaa aaaaagcctg
gacacaccga catacctctt tcttactgct cttgtattgt gatctctgtc aaacgttact ggggtctcag gggatggtac tgaataaga ccttctacga cgaagaggtt tcaactaata ctctctttt tttttggac
>.....TGF BR2.....>
v c v a v w r k n d e n i t l e t v c h d p k l p y h d f i l e d a a s p k c i m k e k k k p

281 gtgagacttt ctctcatggt tctgttagct ctgatgagtg caatgacaac atcatctctt cagaagaata taacaccagc aatctgacc accatcacca coactccatc cactga
cactctgaaa gaagtacaca aggacatcga gactactcac gttactgttg tagtagaaga gtctcttat attgtggtcg ttaggaactgg tggtagtgtt ggtagtggta gtgact
>.....TGF BR2.....>
g e t f f m c s c s e d e c n d n i i f s e e y n t s n p d
>>.....8x His-Tag.....>
h h h h h h h h

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**Fig. 1.** General scheme of nucleotide and amino acid sequence of recombinant TβR2-His construct. This construct contains the coding region for the extracellular ligand binding domain with asparagine to alanine substitution at position 41 and 8x His- tag sequence at the C- terminal of TβR2.

lysis buffer in two steps and eluted to (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 500 mM Imidazole, 2 mM TCEP, 0.1% Tween 20, pH 8.0) during running the program of BioSprint 15.

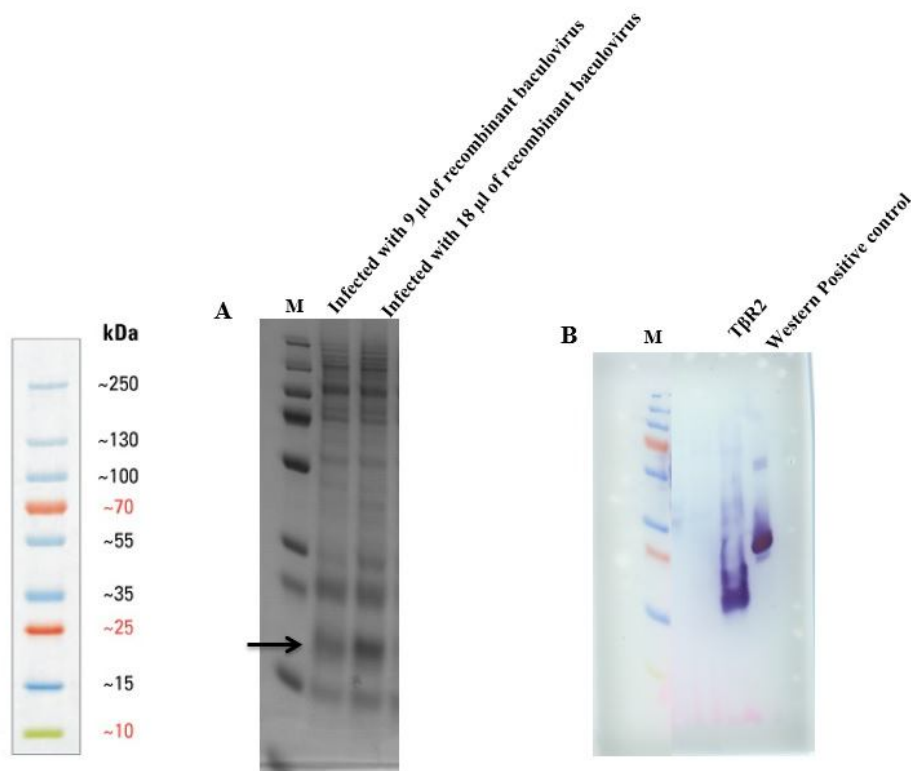
### SDS-PAGE and Western Blot Analysis

Protein samples were electrophorized on a 10% Tris-Glycin ReadyGel precast gels (ANAMED) under reducing conditions using the Mini-PROTEAN<sup>®</sup> Electrophoresis System (Bio Rad). Proteins were then stained directly with Instant Blue (Biozol) or transferred onto an Immobilon<sup>®</sup> PVDF membrane for Western blot analysis. The PVDF membranes were incubated 2 h in blocking buffer (50 mM TRIS base; 150 mM NaCl; 3% BSA) at room temperature and hybridized with 1:2500 dilution of a monoclonal Anti-polyHistidin-Peroxidase antibody (A7058, Sigma) for 2 h with gentle agitation. The membranes were washed 3 × 5 minutes with TBST (50 mM TRIS base; 150 mM NaCl; 1% Tween 20) with agitation to remove residual antibody. Immune detection of TβR2 protein was developed with 3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate system (Sigma).

## RESULTS

### Construction of a TβR2 Transfer Vector

Crystal structure of the TβR2 extracellular domain (amino acids 38-159 from coding region for TβR2) showed that this region is in complex with dimeric TGFβ3 [21]. Also, it has been shown that the presence of asparagine naturally at position 41 results in some problem associated with deamidation [20]. According to previous studies, this part of the coding region of the extracellular domain of human TβR2 was inserted in downstream of a p10 promoter in the modified pTriEx-4 transfer vector. To streamline purification, we also inserted an 8x His-tag sequence immediately after the coding region of the extracellular domain of human TβR2. In this construct, asparagine substituted to alanine at position 41 (Fig. 1). The 366 bp PCR product of designed sequence was introduced by SLIC into the double *BseRI* and *NcoI* digested vector. The sequence and right direction of the construct were confirmed by sequencing. This recombinant transfer vector was used to make recombinant baculovirus base on the homologous recombination phenomenon.



**Fig. 2.** Analysis of TβR2 expression 66 h after transfection of Sf21 insect cells by different volume of recombinant baculovirus using SDS-PAGE (A) and western blot analysis (B). M: protein molecular weight marker; E: fraction of elution after purification with Ni-beads by BioSprint15.

### Expression and Purification of TβR2 Protein

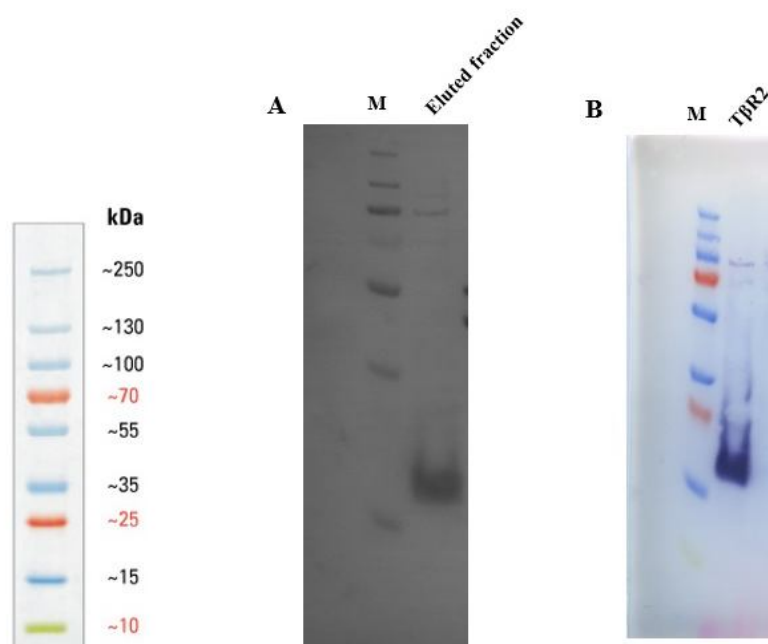
In order to check the ability of Sf21 insect cells for intracellular expression of the TβR2 protein, firstly 5 ml Lonza Insect-Xpress medium containing the proper amount of Sf21 insect cells transfected by recombinant baculovirus and approximately 3 days after transfection, protein extraction from cells was carried out. Test purification was done by Ni-NTA magnetic beads with BioSprint 15, as described in the previous section. Obtained protein from the last step of purification procedure was evaluated by SDS-PAGE gel and western blot analysis. As indicated in fig. 2A, TβR2 appeared as a recombinant protein with a molecular mass of ~15 kDa. Also, in order to be sure that observed band is related to TβR2 protein, western blot analysis with Anti-polyHistidin-Peroxidase antibody was performed and confirmed the SDS-PAGE result (Fig. 2B).

To obtain the high level of purified TβR2, transfected

Sf21 cells with a proper volume of recombinant baculovirus (2.7 ml of recombinant baculovirus in 750 ml of Lonza Insect-Xpress medium) were harvested and subjected to Ni-NTA agarose column, as described in material and method section. Due to the high and specific affinity of His-tagged protein for Ni-NTA, the desired protein was bonded onto Ni-NTA agarose and separated during the elution steps. The eluted fractions were pooled and loaded onto the SDS-PAGE. As can be seen in Fig. 3A, the purification procedure resulted in more than 90% pure protein. Also, Western blot analysis showed a ~15 kDa protein band related to purified TβR2 (Fig. 3B).

### DISCUSSION

TGFβ is the prototype of a large family of structurally related cytokines that is involved in a widespread biological



**Fig. 3.** The eluted fraction from Ni-NTA agarose loaded onto 10% Tris-Glycin SDS-PAGE (A) and western blot analysis (B).

activities like; stimulation or inhibition of cell proliferation and differentiation, angiogenesis, extracellular matrix production, tissue repair, modification and inhibition of inflammatory and immune responses [22-26]. Therefore, the existence of potent and specific TGF $\beta$  inhibitors is necessary to study the role of TGF $\beta$ s in pathological processes and to maintain cellular hemostasis. T $\beta$ R2 appeared to be a potent inhibitor for TGF $\beta$ 1 and TGF $\beta$ 3 [16-27]. According to what mentioned above, T $\beta$ R2 has been considered suitable target to produce a recombinant protein in heterologous systems. T $\beta$ R2 gene encodes a protein with 567 amino acids (aa) which 22 aa from its N-terminal is related to the T $\beta$ R2 signal peptide. The rest of encoded gene is a transmembrane protein that has a cysteine-rich extracellular domain (23-166 aa), a single transmembrane region (167-187 aa) and a cytoplasmic region (188-567 aa). It has shown that the extracellular domain of T $\beta$ R2 with high affinity bind to the TGF $\beta$ s [16]. Also, the crystal structure of the T $\beta$ R2 extracellular domain (amino acids 38-159) in complex with dimeric TGF $\beta$ 3 was reported [21].

In this study relying on the crystal structure of T $\beta$ R2 extracellular domain and previous studies, we designated a

recombinant construct including T $\beta$ R2 extracellular domain (amino acids 38-159 without the signal sequence) to investigate the intracellular expression of T $\beta$ R2 in Sf21 insect cells. In previous studies, Ventura *et al.*, in 1994 [28] produced the full length of T $\beta$ R2 protein in Sf9 insect cells. However, their production resulted in several forms, ranging from 65-75 kDa with the yield of 25 ng per  $10^6$  Sf9 cells. In addition, in 1995, Tsnag *et al.* [16], produced a recombinant soluble T $\beta$ R2 protein corresponding to the 159 amino acids extracellular domain (including the signal sequence) in Sf9 insect cells, without reported production yield. They visualized multiple bands with molecular weights greater than predicted one by silver staining [16]. These observed heterogeneity in Sf9 insect cells at above mentioned studies likely resulted in varying amounts of glycosylation on the potential N-glycosylation sites of the T $\beta$ R2 gene. Interestingly, we observed just a predicted molecular mass of ~15 kDa from the expression of the T $\beta$ R2 extracellular domain in Sf21 cells with a high yield of approximately 30  $\mu$ g per  $10^6$  Sf21 cells. This result can be due to the deletion of native signal sequence from the T $\beta$ R2 sequence. It has been shown that the insect cells are able to recognize mammalian signal sequence and causes rapid translocation

across the membrane of the rough endoplasmic reticulum, where co-translational N-glycosylation take place [29]. Probably, the lack of signal peptide in our construct, prevent to send the expressed protein into the secretory pathway and addition of variable oligosaccharide. Although, the expression of different parts of TβR2 in Sf9 cells at previous studies resulted in biologically active proteins, also it is necessary to check the activity of obtained protein from the expression of the TβR2 extracellular domain without the signal peptide in Sf21 cells.

It should be mentioned that the existence of His-tag at the C-terminus of TβR2 protein which facilitates the purification procedure, do not disturb the receptor functional properties [13,30].

In summary, TβR2 can be efficiently expressed intracellularly in Sf21 insect cell transfected by recombinant baculovirus. This expression system can be considered as a powerful overexpression system in producing of biologically active mammalian proteins because of its ability for post translational modification. Also, based on obtained results from other studies regarding the extracellularly expression of TβR2 in Sf9 insect cells, the extracellularly expression of this protein in Sf21 cells by using the signal sequences in recombinant construct sounds be hopeful.

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