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Recombinant Expression of the Non-glycosylated Extracellular Domain of Human Transforming Growth Factorβ Type II Receptor Using the Baculovirus Expression System in Sf21 Insect Cells

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ABSTRACT

Transforming growth factor beta (TGFβ1, β2 and β3) are 25 kDa disulfide-linked homodimers that regulate many aspects of cellular functions, consist of proliferation, differentiation, adhesion and extracellular matrix formation. TGFβs mediate their biological activities by binding of growth factor ligand to two related, functionally distinct, single-pass transmembrane receptor kinases, known as the TGFβ type I and type II receptors. The high affinity of type II TGFβ receptor to TGFβ1 and TGFβ3 makes it a potent and specific inhibitor for TGFβ related studies. In this study, we designed a recombinant construct including a part of the extracellular domain of type II TGFβ receptor (amino acids 38-159) which functionally was active according to previous crystallography studies. The recombinant protein was efficiently (30 µg 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 TGFβ receptor, Sf21 Insect cell expression system, Baculovirus expression system

INTRODUCTION

Transforming growth factor beta (TGFβ) belongs to a superfamily of structurally related multifunctional proteins, which consists of three isoforms (TGFβ1, 2, 3) in human [1]. TGFβs are 25 kDa homodimeric polypeptides that are important in development, wound healing, immune responses and tumor cell growth and inhibition [2-5]. TGFβ signal transduction is mediated by binding to cell surface receptors. Two membrane proteins known type I and II (TβR1, TβR2) are required for most of TGFβ responses [6-10]. The TβR1 and TβR2 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]. TGFβ signal transduction is initiated by binding of the growth factor ligand to the extracellular domain of TβR2. After ligand binding, TβR2 receptor phosphorylates the type I receptor and cause the signal propagation to downstream substrates [2,14-15]. As TGFβ 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 TGFβs during diseases. The high affinity of TβR2 to bind the TGFβ makes it a strong detector to investigate the folding of produced recombinant TGFβ proteins by protein-protein interaction analysis. Due to the involvement of TβR2 in numerous biological processes, many attempts have been made to produce recombinant TβR2 using heterologous systems (16-19). Recently, we successfully expressed the extracellular domain of TβR2 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 TβR2 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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MATERIAL AND METHODS

Construction of Recombinant TβR2 Transfer Vector

The coding region of the extracellular domain of human TβR2 (amino acids 38-159) was inserted into a modified pTriEx-4 transfer vector (Crelux-GmbH) by Sequence and Ligation Independent Cloning (SLIC) technique. In this construct, as deamidation of asparagine can naturally occur at position 41, asparagine substituted to alanine at this position [20]. To facilitate the detection and purification step of recombinant protein, an 8x His-tag sequence was added at the C-terminal end of TβR2 (Fig. 1). To generate the recombinant TβR2-His construct, 396 bp fragment was produced by phusion flash PCR using two oligonucleotides:5'TTACAATCAAAGGAGATATACCAT GGTCACTGACAACGCAGGTGCAGTC3',5'CGGCCGC AAGCTTCGTCATCAGTGATGGTGATGGTGGTGATG GTGGTCAGGATTGCTGGTGTTATATTCTTCTGAGAA G3'. pCM5B-TGFβ receptor II (addgene) was used as a template for TβR2 amplification. This PCR product was introduced into the *BseRI* and *NcoI* sites of modified pTriEx-4 transfer vector following the T4 polymerase reaction to generate overlapping regions and annealing reaction to bind the overlapping regions together. The recombinant plasmid was introduced in NEB Turbo competent *E. coli* (NEW ENGLAND BioLabs Inc) by chemical transformation. The sequence of the open reading frame was confirmed by sequencing (GATC Biotech, Germany).

Cell Cultures and Transfection

Sf21 cells delivered from *spodoptera frugiperda* ovarian cells (Oxford expression technologies, England) were grown in Lonza Insect-Xpress medium (Bio Whittaker) at 27 °C and used for transfection and virus amplification. Briefly, transfection was done in 24 well block by adding the transfection mix (300 µl TC-100 insect medium, 6 µl baculoaFECTIN, 2.5 µl flashBACULTRA DNA, 500 ng recombinant plasmid which incubated 30 min at room temperature) into each well including Lonza Insect-Xpress medium, Gentamycin and 1 × 10^6 cells/ml Sf21 cells. The block was incubated at 27 °C for 7 days. The well content was used to infect 200 ml of Lonza Insect-Xpress medium including 2 × 10^6 cells/ml Sf21 cells and incubated at 27 °C with gentle rocking in an orbital shaker for 3 to 6 days till the cell density reached 2-5× 10^6 cells/ml and cell viability was lower than 15% . This cell suspension was centrifuged and supernatant containing the virus along with 10% FBS was stored at 4 °C for subsequent steps.

TβR2 Expression and Purification

For the expression of the TβR2 protein, 750 ml Lonza Insect-Xpress medium including Gentamycin (25 µg ml^{-1}) and 2 × 10^6 cells/750 ml Sf21 cells were infected with the proper volume of virus. The cells were harvested 66 hours after infection and used for purification. Purification was performed using Ni-NTA resin. At first, the cell pellet was solubilized in lysis buffer (20 mM HEPES, 500 mM NaCl and 20 mM Imidazole, 1x tab Complete EDTA-free (Roche)/100 ml lysis buffer, pH 7.5) and sonicated. Then, the lysate was centrifuged at 500 g, 4 °C for 5 min and suspension was incubated with 4 ml Ni-NTA resin for 1 h at 4 °C on a rotating wheel. Ni-NTA agarose was equilibrated with lysis buffer. The Ni-NTA agarose was harvested by centrifugation at 500 g, 4 °C for 5 min and the harvested Ni-NTA agarose was applied in gravity column. The column was then washed with 20 ml of lysis buffer and the protein was eluted by an elution buffer 5 × 3 ml containing 20 mM HEPES, 500 mM NaCl and 300 mM Imidazole at pH 7.5. Eluted protein fractions were pooled and subjected to SDS-PAGE.

Optimization of Protein Purification Using BioSprint 15

To test the expression and purification of TβR2 before performing the production, transfection was done in 24 wells block including 5 ml Lonza Insect-Xpress medium, Gentamycin (25 µg ml^{-1}) and 2 × 10^6 cells/ml Sf21 cells. Block was shacked at 107 rpm, 27 °C on mini-shaker. The cells was harvested 66 h after infection and used for purification by BioSprint 15 (Qiagen). First of all , the cell pellet was resuspended in 300 µl lysis buffer including; 570 µl (50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM Imidazole, 2 mM TCEP, 0.1% Tween 20, pH 8.0), 30 µl Insect Pop Culture Reagent (Novagen) and 6 µl of Benzonase. Then cells were incubated 30 min, 1100 rpm at room temprature. Subsequently, 100 µl Ni-NTA magnetic beads were added to the first well of BioSprint's block and washed with the

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1   atggtcactg acaacgcagg tgcagtcaag tttccacaac tgtgtaaatt ttgtgatgtg agattttcca cctgtgacaa ccagaaatcc tgcatgagca actgcagcat cacctccatc tgtgagaagc cacaggaagt
    taccagtgac tgttgcgtcc acgtcagttc aaaggtgttg acacatttaa aacactacac tctaaaaggt ggacactgtt ggtctttagg acgtactcgt tgacgtcgta gtggaggtag acactcttcg gtgtccttca
    >>..............................................................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 ctgtgtggct gtatggagaa agaatgacga gaacataaca ctagagacag tttgccatga ccccaagctc ccctaccatg actttattct ggaagatgct gcttctccaa agtgcattat gaaggaaaaa aaaaagcctg
    gacacaccga catacctctt tcttactgct cttgtattgt gatctctgtc aaacggtact ggggttcgag gggatggtac tgaaataaga ccttctacga cgaagaggtt tcacgtaata cttccttttt tttttcggac
    >...............................................................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 cttcatgtgt tcctgtagct ctgatgagtg caatgacaac atcatcttct cagaagaata taacaccagc aatcctgacc accatcacca ccatcaccat cactga
    cactctgaaa gaagtacaca aggacatcga gactactcac gttactgttg tagtagaaga gtcttcttat attgtggtcg ttaggactgg tggtagtggt ggtagtggta gtgact
    >........................................TGF BR2.......................................>>
     g  e  t   f  f  m  c   s  c  s   s  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_2PO_4, 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® Electrophoresis System (Bio Rad). Proteins were then stained directly with Instant Blue (Biozol) or transferred onto an Immobilon® 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.

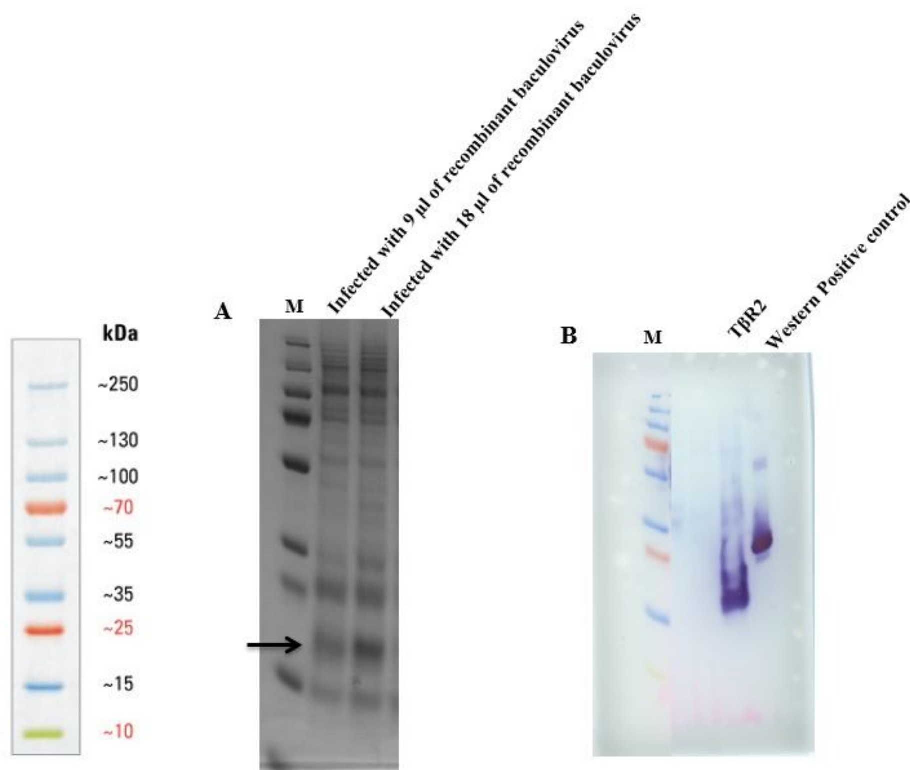

Fig. 2. Analysis of TβR2 expression 66 h after transfection of Sf21 insect cells by different volume of recombinant baculovirus using SDS-PAGE (A) and western blot analysis (B). M: protein molecular weight marker; E: fraction of elution after purification with Ni-beads by BioSprint15.

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 seprated 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

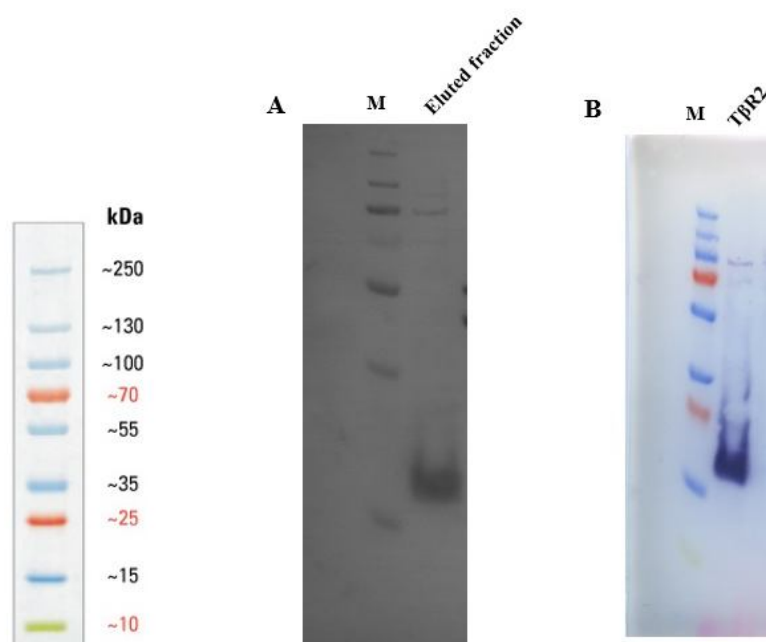

Fig. 3. The eluted fraction from Ni-NTA agarose loaded onto 10% Tris-Glycin SDS-PAGE (A) and western blot analysis (B).

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 TGFβ inhibitors is necessary to study the role of TGFβs in pathological processes and to maintain cellular hemostasis. TβR2 appeared to be a potent inhibitor for TGFβ1 and TGFβ3 [16-27]. According to what mentioned above, TβR2 has been considered suitable target to produce a recombinant protein in heterologous systems. TβR2 gene encodes a protein with 567 amino acids (aa) which 22 aa from its N-terminal is related to the TβR2 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 TβR2 with high affinity bind to the TGFβs [16]. Also, the crystal structure of the TβR2 extracellular domain (amino acids 38-159) in complex with dimeric TGFβ3 was reported [21].

In this study relying on the crystal structure of TβR2 extracellular domain and previous studies, we designated a recombinant construct including TβR2 extracellular domain (amino acids 38-159 without the signal sequence) to investigate the intracellular expression of TβR2 in Sf21 insect cells. In previous studies, Ventura *et al*., in 1994 [28] produced the full length of TβR2 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 TβR2 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 TβR2 gene. Interestingly, we observed just a predicted molecular mass of ~15 kDa from the expression of the TβR2 extracellular domain in Sf21 cells with a high yield of approximately 30 μg per 10^6 Sf21 cells. This result can be due to the deletion of native signal sequence from the TβR2 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 overexeperession 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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