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Enhanced Expression of Growth Factor-β Type II Receptor

Sara Kharazmi, Amin Baghizadeh, and Masoud Torkzadeh-Mahani

ABSTRACT

Transforming growth factor-beta (TGF-β) is a growth suppressor or stimulator for many different cell types whose functions are mediated by binding to cell surface receptors, TβRI and TβRII. TβRII is a transmembrane glycoprotein used as a specific inhibitor for TGF-β. To date, the many attempts to produce recombinant TβRII using different expression systems have failed to express the protein (without the fused partner) in soluble form and with a satisfactory yield. The authors in this study developed an efficient bacterial expression system for large-scale production of soluble, homogenous extracellular binding domain of human TβRII (TβRII ECD) in three strains of *Escherichia coli* (*E. coli*) using a recombinant expression vector harboring a strong T7 promoter and TβRII ECD coding sequence. Truncated TβRII ECD was successfully expressed at high level in BL21-CodonPlus (DE3)-RIL and BL21 (DE3) pLysS strains, resulting in the formation of insoluble inclusion bodies, which were further refolded to the soluble forms. The expression of TβRII ECD construct in Rosettagami pLysS resulted in the secretion of a soluble protein, which was purified based on the presence of His-tag with more than 95% purity and a yield of 70 mg/L. Expression of homogenous recombinant TβRII ECD with at least five-fold higher yield, in comparison with previously reported data, provided a promising platform for the efficient production of this protein, which is amenable to structural studies. In addition, this protein can be used as a potential vaccine to inhibit TGFβ activities, if the protein is proven to be functional and biologically active.

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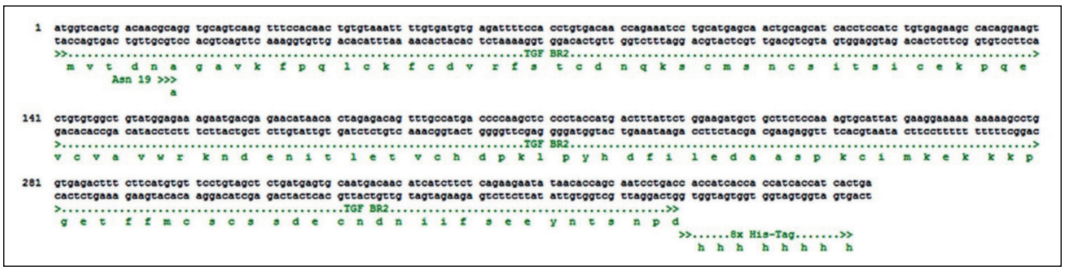
Transforming growth factor-beta (TGF-β) is a multifunctional protein involved in several biological processes, such as cell proliferation and differentiation, immune responses, tumor cell growth, and inhibition (1–4). The action of TGF-β is initiated by signaling, through two homologous functionally distinct transmembrane receptors, designated as type I and type II receptors (TβRI and TβRII, respectively) (4). In the sequential model for TGF-β signaling, growth factor ligand first binds to the small extracellular domain (ECD) of TβRII. TβRI is subsequently associated with the TβRII ligand complex and activated by the constitutive kinase activity of TβRII. Then, TβRI activates the downstream signaling pathway (5–7). The high affinity of TβRII for binding to the TGF-β makes it a potent and specific TGF-β inhibitor (8). The TβRII is secreted as a polypeptide, including a cysteine-rich extracellular domain (23–166 amino acids), a single transmembrane domain (167–187), and a cytoplasmic domain (188–567), following the cleavage of

Figure 1. General scheme of nucleotide and amino acid sequences of recombinant extracellular binding domain of TβRII construct. This construct contains the coding region for the extracellular ligand binding domain, with asparagine to alanine substitution at position 19 and 8x His-tag sequence at the C-terminus.



a 23-amino-acid N-terminal signal peptide (9). The extracellular domain of TβRII (TβRII ECD) contains three potential N-glycosylation sites and 12 cysteine residues. Because of the important role of TβRII in TGF-β related studies as an inhibitor, high-level protein expression is required. Although many attempts have been made to produce recombinant protein from different coding regions of TβRII in different heterologous expression systems, the yield of the expressed proteins is still far from being satisfactory (8,10,11,12).

Among the expression systems used today, the bacterial expression system still remains the preferred method of choice for large-scale production of recombinant proteins. Despite high protein yields, however, an important limitation of the bacterial system is the production of inactive proteins, requiring *in-vitro* refolding to regain their functions. The lack of post-translational modifications in bacteria results in the formation of non-functional proteins. However, it allows for the production of homogenous samples, suitable for crystallization (10, 11, 13). Herein, the authors reported the high-level expression of homogeneously recombinant TβRII extracellular domain in Rosettagami pLysS, BL21-CodonPlus (DE3)-RIL and BL21 (DE3) pLysS strains of *Escherichia coli* (*E. coli*), transformed with a recombinant expression vector harboring the strong T7 promoter and a truncated TβRII ECD coding region.

MATERIAL AND METHODS

Construction of recombinant TβRII ECD expression vector. To express the N-terminal truncated TβRII, a recombinant construct (see **Figure 1**) was designed by insertion of the human extracellular TβRII coding region (residues 15–159), downstream of the strong T7 promoter. An 8x-His tag sequence was added to the C-terminal domain of the TβRII ECD to facilitate the detection and purification of recombinant protein. The coding region for the first 14 residues of TβRII ECD was eliminated in this construct, and the asparagine at position 19 was substituted with alanine to prevent problems associated with deamidation (14). Amplification of this recombinant construct (396 bp) was performed by Phusion Flash PCR, using the following two oligonucleotides: 5´-TTACAATCAAAGGAGATATACCATGGTCACTGACAACGCAGGTGCAG TC-3´ and 5´-CGGCCGCAAGCTTCGTCATCAGTGATGGTGATGGTGGTGATGGTGGTCAGGATTGCTGGTGTTATATTCTTCTGAGAAG-3´. PCM5B-TGFβ receptor II (addgene) was used as a template for the polymerase chain reaction (PCR). The PCR product was inserted into the BseRI and NcoI sites of a modified pTriEx-4 expression vector (Crelux), using the sequence and ligation independent cloning (SLIC) technique. This expression vector was the pTriEx-4 vector, in which the DNA sequence (1016–1919 nucleotides), including the cytomegalovirus (CMV) promoter and CMV enhancer, was deleted using the following excision oligonucleotides: 5´- GGCCATTCTAGACGGACCGAAATTAATACGACTCACTATAGGG-3´ and 5´-CCGGTATCTAGAGGCCATCAGCAACTATATATTGATAGAC-3´. Then, the recombinant expression vector was transformed into NEB Turbo competent *E. coli*. Positive colonies were recovered on Luria-Bertani (LB) agar containing ampicillin, and the presence of the target DNA sequence was verified by sequencing (GATC Biotech).

Expression of recombinant TβRII ECD in *E. coli*

The recombinant TβRII ECD expression vector was chemically transformed into three strains of *E. coli*, including Rosettagami

pLysS (Novagen), BL21-CodonPlus(DE3)-RIL (Agilent Technologies), and BL21(DE3) pLysS (Stratagene). Transformants were selected on LB agar plates containing the appropriate antibiotics. For BL21-CodonPlus (DE3)-RIL and BL21 (DE3) pLysS, the LB agar plates contained 100 µg/mL ampicillin and 30 µg/mL chloramphenicol. For Rosettagami pLysS, the plates contained 100 µg/mL ampicillin, 30 µg/mL chloramphenicol, 50 µg/mL kanamycin, and 5 µg/mL tetracycline. Then, LB mediums containing the proper antibiotics were inoculated with a single recombinant colony and incubated approximately 16 h at 37 ºC while shaking at 250 rpm. The primary cultures were added into new Terrific Broth (TB) medium containing the proper antibiotics and grown at 37 ºC until the cell density reached an optical density measurement (absorption) at 600 nm (OD600) of 1–1.2. In this step, expression of recombinant protein was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, and the cultures were grown for an additional 5 h at 37 ºC. The harvested cells were used for further experiments.

Purification and refolding of recombinant TβRII ECD

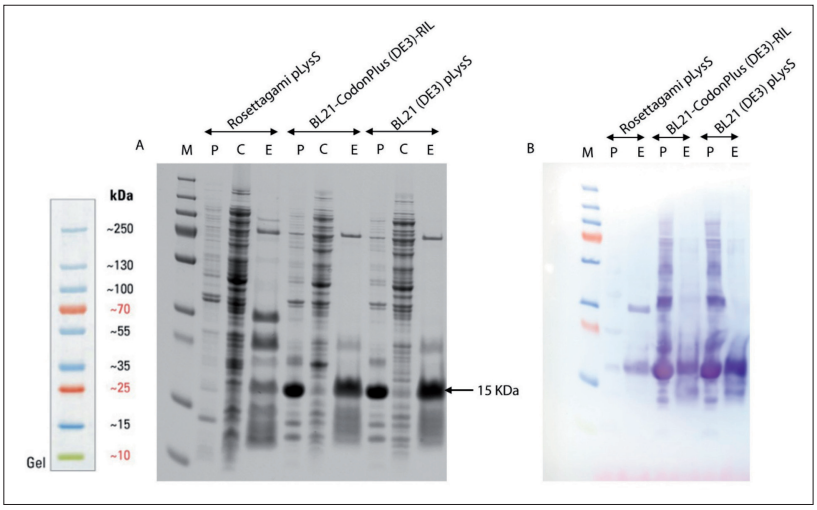
Purification of recombinant TβRII ECD was performed according to the following methods: first, to test the expression of recombinant TβRII ECD in three strains of *E. coli*, small amounts (10 mL) of induced cultures were centrifuged and the cell pellets applied for testing protein purification using BioSprint 15 (Qiagen). To perform the purification, cell pellets were resuspended in 900 µL lysis buffer (50 mM NaH2PO4, 300 mM sodium chloride (NaCl), 20 mM imidazole, 2 mM tris (2-carboxyethyl) phosphine (TCEP), and 0.1 % Tween 20 at pH 8.0) and frozen in liquid nitrogen. After thawing the cells, 6 µL of Benzonase was added into the samples. Cells were then sonicated for five minutes at 4–8 ºC, and the harvested supernatants were employed for purification by Ni-NTA magnetic beads using BioSprint 15. The Ni-NTA magnetic beads were washed with the lysis buffer in two steps and eluted with a final volume of 100 ºL using the following elution buffer: 50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole, 2 mM TCEP, and 0.1 % Tween 20 at pH 8.0. Finally, the pellet, cytosolic, and elution fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

In an alternative method, purification and refolding were performed using Ni-NTA resin, according to the method previously reported by Glansbeek *et al.* (11). Briefly, a 5-gm cell pellet from the 3-L culture medium, inoculated with the transformed BL21-CodonPlus (DE3)-RIL strain, was solubilized in 240 mL of lysis buffer (0.1 M NaH2PO4, 0.01 M Tris, one tablet of cOmplete EDTA-free (Roche)/100 mL lysis buffer, 8 M urea at pH 8.0). After homogenization, the lysate was centrifuged at room temperature (RT), 12,000 X g for 30 min, and sonicated. The harvested suspension was incubated with 4 mL of Ni-NTA resin for 1 h at 4 ºC. After centrifugation, the collected resin was washed three times with washing buffer (50 mM Tris/hydrochloric acid [HCL], 8 M urea, pH 8.0), resuspended in 8-mL washing buffer, and dialyzed with 100-fold sample buffer volume overnight at 4 ºC against the refolding buffer (0.1 M urea, 50 mM Tris/HCL, 2 mM reduced glutathione, 0.02 mM oxidized glutathione, 0.5 M L-arginine, pH 8.0). Then, the Ni-NTA resin was eluted with 4 mL elution buffer (refolding buffer + 300 mM imidazole), and the recombinant protein separated from the Ni-NTA resin by centrifugation. The harvested supernatant was subsequently dialyzed against 50 mM Na-phosphate, 150 mM NaCl, at pH 7.8.

SDS–PAGE and western blot analysis

Protein samples were loaded on a 10 % Tris-Glycin Ready Gel precast gels (Anamed) and electrophoresed 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 polyvinylidene difluoride (PVDF) membrane for western blot analysis. The PVDF membrane was incubated 2 h in blocking buffer (50 mM Tris base; 150 mM NaCl; 3% bovine serum albumin [BSA]) at RT and hybridized with 1:2500 dilution of a monoclonal anti-polyHistidin-Peroxidase antibody (A7058, Sigma) for 2 h with gentle agitation. The membrane was washed for 5 min three times with tris buffered saline with Tween 20 (TBST) (50 mM Tris base; 150 mM NaCl; 1% Tween 20) while agitating to remove any residual antibody. Immune detection of TβR2 protein was developed using 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma).

Figure 2. Analyses of extracellular binding domain of TβRII expression, 5 h after induction in three *E. coli* strains by 10 % Tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (A) and western blot (B). M is protein molecular weight marker; P is cell lysate from pellet; C is cytosolic fraction; E is elution after purification with Ni-beads using BioSprint15.



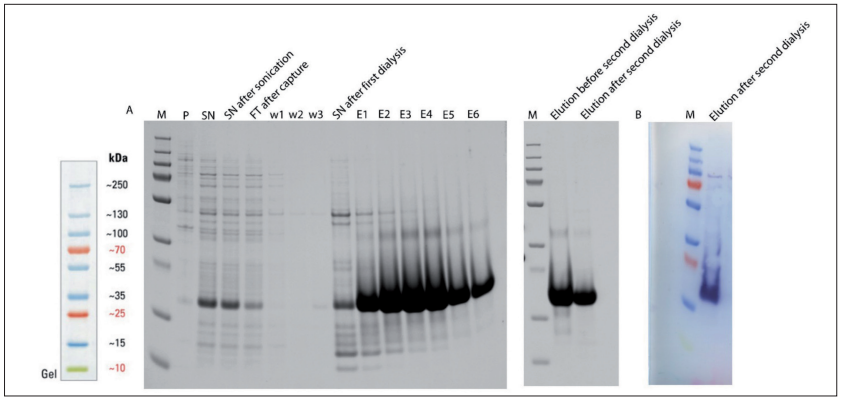
RESULTS

According to the structural information of binding, and by taking into consideration the transforming growth factor-TβRII interactions, the recombinant construct was engineered to encode the truncated region of the extracellular domain of human TβRII (residues 15–159) preceded by one initiator methionine residue. Eight histidine residues were placed in the C-terminus. This construct lacked the coding region for the TβRII signal peptide (23 residues) and the coding region for the first 14 residues of TβRII ECD. To prevent the deamidation reaction usually associated with protein degradation, asparagine 19 was substituted with alanine at this position. This recombinant construct was designed as previously described (14). Designed TβRII ECD construct was then inserted into an expression vector improved by Crelux. The main characterization of this expression vector is its strong, inducible T7 promoter.

Rosettagami pLysS, BL21-CodonPlus(DE3)-RIL, and BL21(DE3) pLysS strains of *E. coli* were transformed with the recombinant expression vector, and the production of recombinant TβRII ECD in the designed expression system was induced by adding IPTG. The presence of the desired recombinant protein was assayed in cell lysates from pellet, cytosolic, and elution fractions by SDS–PAGE and western blot analysis. The results showed that the recombinant TβRII ECD, efficiently expressed and accumulated in the cytoplasmic compartment of bacterial cells, appeared on the SDS–PAGE gel as a protein band with the approximate molecular weight of 15.4 kDa (see **Figure 2**). The presence of the desired band in cell lysate from pellet samples, obtained from BL21-CodonPlus (DE3)-RIL and BL21 (DE3) pLysS strains, demonstrated that the overexpression of TβRII ECD in the aforementioned bacterial strains results in the formation of insoluble inclusion bodies. However, the lack of a TβRII ECD-related band in the cell lysate from the pellet of Rosettagami pLysS suggests that the TβRII ECD expresses as a soluble, homogeneously non-glycosylated protein in this strain. The presence of an extra band with a molecular weight greater than the predicted band for TβRII ECD monomer in western blot analysis of purified protein from Rosettagami pLysS strain is presumably associated with the dimeric form of TβRII ECD (see **Figure 2B**). This result might be an indication of properly folded TβRII ECD in Rosettagami pLysS.

Further, the production of recombinant TβRII ECD was performed on a larger scale, in 3-L culture medium inoculated with the transformed BL21-CodonPlus (DE3)-RIL strain. Recombinant TβRII ECD was successfully recovered and refolded from 5 gm of the harvested cells. The purification and refolding processes were carried out, according to

Figure 3. Analyses of recombinant extracellular binding domain of TβRII (TβRII ECD), isolated from insoluble inclusion bodies after each step of purification and refolding procedures, on 10 % Tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (A). Western blot analysis was performed for purified TβRII ECD protein (B). M is protein molecular weight marker; P is cell lysate from pellet; SN is supernatant; FT is passed flow through the column; W is washing steps; E is elution steps.



the procedure described previously (11). To prevent the formation of misfolded aggregates, TβRII ECD was refolded while it was immobilized on Ni-NTA resin. After only one-step purification and refolding, 210 mg of 95% pure soluble TβRII ECD was obtained from the 3-L culture medium (see **Figure 3A**). Identification of the purified recombinant TβRII ECD was further confirmed by western blot analysis (see **Figure 3B**).

DISCUSSION

TGF-β is the prototype of a large family of structurally related cytokines, considered as an essential component in the regulation of the immune system, by stimulation and inhibition of cell growth (2,5,15). To maintain the cellular homeostasis, this protein triggers the cellular responses mediated by TGF-β-TβRII ligand (16). To study the role of TGF-β protein in modulating immune responses and pathological processes, *in-vitro* production of TβRII has been considered as a potent TGF-β inhibitor. To date, many attempts have been made to produce the recombinant TβRII by expression of DNA sequences encoding different part of the extracellular binding domain in eukaryotic and prokaryotic systems (8 ,9, 10, 11, 14). Although they have succeeded in producing biologically active protein, in some cases, the yield and homogeneity of the expressed proteins were not satisfactory.

In this study, the authors were interested in producing a large-scale and homogenous form of the TβRII extracellular binding domain by expressing this protein in *E. coli*. Due to the lack of post-translational glycosylation in the bacterial expression system, it often results in higher yields of homogenous protein samples compared to other expression systems (10). Although there are three potential N-glycosylation sites in TβRII ECD structure, it has been shown that the binding affinity of the non-glycosylated form of TβRII ECD is in agreement with the affinity of commercial glycosylated TβRII (12). Therefore, the inability of *E. coli* to carry out post-translational glycosylation does not affect the inhibitory function of expressed TβRII ECD.

The crystal structure of human TβRII extracellular ligand-binding domain, in combination with human TGF β3, was reported by Hart *et al*. (17). Based on the coding sequence of TβRII ECD, used in Hart's experiment, the authors of this study designed a recombinant construct containing the coding sequence of 15–159 residues. The coding sequence was inserted into the modified pTriEx-4 expression plasmid, and, subsequently, the three strains of *E. coli* were transformed with the plasmid. The expression of recombinant TβRII ECD in Rosettagami pLysS resulted in a soluble, homogenously non-glycosylated protein, while the expression in BL21-CodonPlus (DE3)-RIL and BL21 (DE3) pLysS strains resulted in insoluble inclusion bodies, which were further subjected to solubilization and refolding processes for obtaining soluble, homogenously non-glycosylated protein samples.

Hinck *et al.* (14) also produced the functional extracellular domain of TβRII (15-159 residue) in *E. coli* strain BL21 (DE3) using pET32a expression vector, but the yield of expressed recombinant protein has not been reported (14). In addition, in 1997 Glansbeek *et al.* (11) expressed the extracellular domain of TβRII (aa3-aa114) in *E. coli* using p-QE16 expression vector and obtained 15 mg/L of insoluble recombinant protein. They also used several refolding procedures and determined the efficient method for isolating and refolding of the TβRII ECD from insoluble inclusion bodies by testing the ability of the refolded protein to neutralize the TGF-β protein. They surprisingly observed that the refolded TβRII ECD produced in *E. coli* was a stronger inhibitor, compared to the TβRII ECD produced in yeast (11).

In another study, Boesen *et al.* (10) attempted to produce the soluble form of TβRII in a bacterial expression system by designing several recombinant constructs. However, they only obtained the soluble TβRII (0.1 mg/10 L), when they used glutathione S-transferase as a fusion partner (10). In addition, attempts to produce recombinant TβRII in mammalian and insect cells resulted in the lower level of expression, compared to the reported amounts of bacterial expression systems (8,11).

Finally, to verify the inhibitory effect of the recombinant protein in this study, an affinity binding assay would need to be carried out. The authors of this study, however, anticipate that, because the designed recombinant construct in this study is the same as the construct reported by Hinck *et al.* (14) and the refolding buffer used in the experiments in this study does not differ greatly from the one used in the Hinck study, the protein obtained in this study would demonstrate the expected inhibitory function. Further validation is, nonetheless, necessary.

CONCLUSION

The designed expression system for the production of recombinant TβRII ECD has several advantages over previously reported ones. First, the yield of purified recombinant protein obtained from BL21-CodonPlus (DE3)-RIL strain was 70 mg/L, which is approximately a five-fold increase compared to the maximum yield previously reported (11). Moreover, the Rosettagami pLysS strain was able to produce the soluble extracellular domain of TβRII, heedless of the fusion partner. Furthermore, the ability of this system to produce the homogenous TβRII ECD protein with high purity on a larger scale can provide an efficient bacterial expression system for obtaining the recombinant TβRII ECD for further structural studies.

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