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PURIFICATION AND CLEAVING OF THE HUMAN GITRL EXPRESSED AS FUSION PROTEIN

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ABSTRACT. The glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR) is a member of the TNF receptor superfamily. GITR is activated by its ligand, GITRL. GITRL is a type II transmembrane protein. Human tumor cells express high levels of the GITRL; the presence of it thus can be used as a potential tumor marker. In the present study, the constructed pETM52-hGITRL recombinant vector was transformed into the chemically competent *Escherichia coli* BL21 Star (DE3) strain. After induction with 0.5 mM isopropyl β-D-thiogalactoside (IPTG), the expression was maintained at 37^{o}C for an additional 4h. The protein was obtained in sufficient quantity for refolding studies, after which it was purified by affinity chromatography then cleaved from the fusion partner by tobacco etch virus (TEV) protease treatment**.**

Keywords: *GITR, human GITRL, tumor marker, affinity chromatography, TEV protease*

INTRODUCTION

Tumor necrosis factor receptor superfamily member 18 (TNFRSF18) also known as glucocorticoid-induced tumor necrosis factor receptor (TNFR) family-related protein (GITR) in mouse or activation-inducible TNFR family receptor (AITR) in human is a type I transmembrane protein [1, 2]. The GITR was initially identified as a dexamethasone-inducible molecule on a murine T cell hybridoma [1]. Human GITR and its ligand, GITRL were identified independently by two groups in 1999 [2, 3]. In human tumor models triggering of GITR on NK cells by cell surface-expressed GITRL diminishes effector functions of NK cells [4, 5]. Neutralization of soluble GITRL present in tumor cell supernatants

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and patient sera using a GITR-Ig fusion protein restored NK cell reactivity [6], but in mouse tumor models injection of anti-GITR-mAb into tumors provoked potent tumor-specific immunity [7]. This finding suggests that GITR plays a different role in mice and in humans [8], as described more detailed previously [9].

Our aim is the construction of a bacterial expression system, which makes possible the heterologous expression of the human GITRL in soluble form [9]. In this study, we expressed the extracellular region of the human GITRL as a fusion protein, the fusion partner being the molecular chaperone protein DsbA. The solubilized protein was renatured and purified by affinity chromatography, followed by cleavage of the hGITRL from the fusion partner by TEV (tobacco etch virus) protease treatment.

RESULTS AND DISCUSSION

Expression of the human GITRL as a fusion protein

Expression of GITRL in bacterial system reported by previous studies [10, 11, 12] resulted in formation of insoluble inclusion bodies. In our construct, pETM52-hGITRL the putative chaperone action of the fusion partner DsbA might promote the correct folding of hGITRL.

The coding sequence of the hGITRL gene was isolated from human brain cDNA, as described before [9]. The isolated gene was cloned in the pETM52 expression vector. The multiple cloning site of pETM52 allows fusion of GITRL to the C terminus of a leaderless DsbA (disulfide-bond A oxidoreductase) protein sequence. DsbA protein is believed to promote formation the native structure of its fusion partners [13].

The recombinant plasmid was transformed into chemically competent *Escherichia coli* BL21 Star (DE3) cells. A starter culture from one single colony was grown overnight at 37^{o}C in Luria-Bertani (LB) medium containing 20 µg/ml kanamycine and 1% glucose. The starter culture was added to inoculate 250 ml of LB supplemented with 20 µg/ml kanamycine. The expression culture was grown at 37°C to an $OD_{600\ nm}$ of 0.8, then induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The induced culture was grown for an additional 4h.

The result of the fusion protein expression is presented in Figure 1. As identified by SDS-PAGE the expressed fusion protein consisted of about 60% of total bacterial protein. Contrary to our expectation, the majority of the fusion protein was found in inclusion bodies and a small fraction of the protein was found in soluble form. As this soluble protein precipitated after purification by affinity chromatography, refolding of the insoluble fraction of the protein was carried out in order to obtain hGITRL in sufficient quantity in native form for further studies.

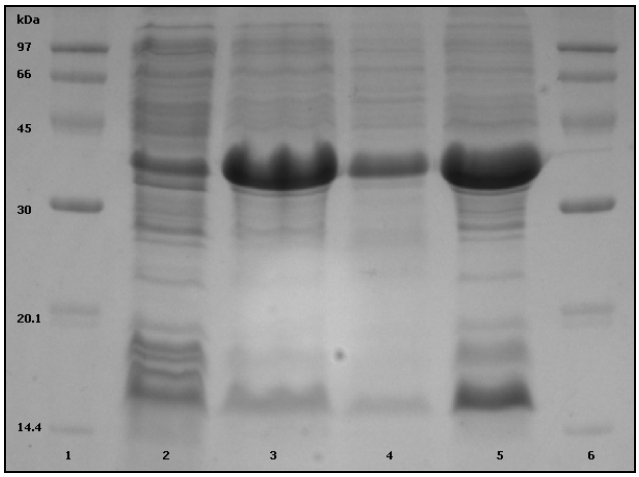

Figure 1. Illustration of hGITRL-DsbA expressed in *E. coli*. Lanes 1, 6. protein molecular weight marker, Amersham; Lane 2. proteins from the expression culture before induction; Lane 3. cellular proteins after 4 hours of expression; Lanes 4. soluble proteins; Lane 5. insoluble proteins;

Solubilization of inclusion bodies was performed by guanidine hydrochloride. The refolding of the solubilized inclusion body was achieved by rapid dilution in an oxidized and reduced glutathione system followed by dialysis in Tris-HCl buffer. Figure 2. shows that efficiency of the protein refolding from insoluble fractions was about 95%.

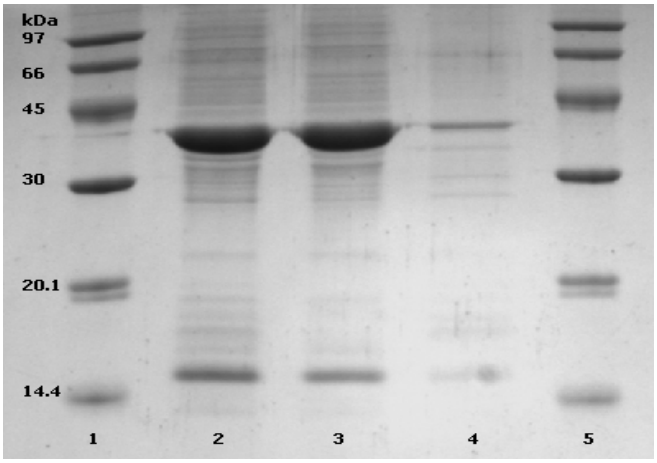

Figure 2. Renaturated hGITRL-DsbA analyzed by 16.5% SDS-PAGE. Lane 1. protein molecular weight marker, Amersham; Lane 2. total refolded protein after dialysis. Lane 3. soluble protein after dialysis. Lane 4. insoluble protein after dialysis.

Purification of the hGITRL by affinity chromatography

The obtained fusion protein, containing a 6xHistidine tag between DsbA and the GITRL sequence, was purified on the principle of affinity chromatography as the $(His)_6$tag is forming a chelate complex with Ni^{2+} ions. The fusion protein was eluted from the affinity matrix with 250 mM imidazole (Figure 3).

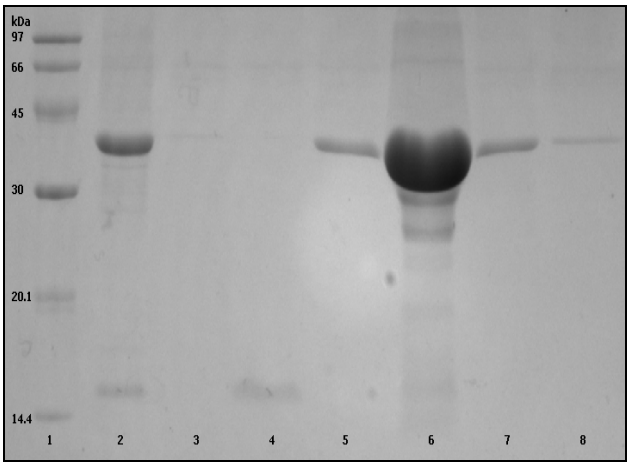

Figure 3. Purification of the obtained protein by affinity chromatography. Lane 1. protein molecular weight marker, Amersham; Lane 2. soluble proteins after renaturation; Lane 3. unbound proteins; Lane 4. protein fraction collected after washing; Lanes 5,6,7,8. elution fractions with 250 mM imidazole.

Absorption spectrum of the purified fusion protein was measured by Beckman DU-650 Spectrophotometer, the molar extinction coefficient of the fusion protein for calculations being determined by ProtPARAM TOOL based on the amino acid sequence. The yield of the purified hGITRL-DsbA was about 50-60 mg/liter culture.

Cleaving of the human GITRL from DsbA by TEV protease

The fusion protein contains a TEV protease cleavage site between DsbA and hGITRL, so the fusion partner can be removed by TEV protease treatment. The purified fusion protein was dialyzed in Tris-HCl buffer, and then the protein of interest (hGITRL) was cleaved from DsbA by TEV protease treatment, as presented in Figure 4. During the TEV protease treatment of the fusion protein, probes were retained in different timepoints for electrophoresis. The quantity of the cleaved protein showed an increase in function of time of the TEV treatment.

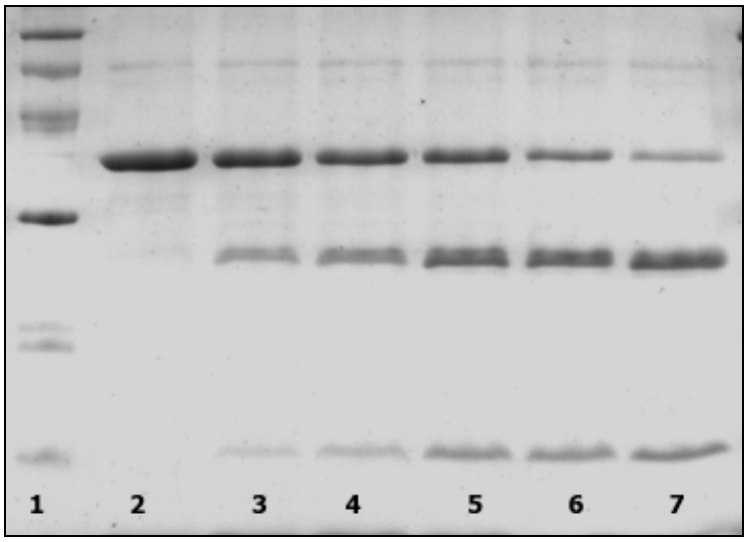

Figure 4. Results of the cleavage by TEV protease treatment of the fusion protein (hGITRL-DsbA). Lane 1. Low Protein Marker, Amershan; Lane 2. purified fusion protein; Lane 3. cleaved fusion protein by TEV protease after 1 hour; Lane 4. after 2 hours; Lane 5. after 4 hours; Lane 6. after 8 hours; Lane 7. after 24 hours.

CONCLUSION

Production of the hGITRL-DsbA fusion protein in *Escherichia coli* was realized successfully. The pETM52 expression vector, containing the DsbA sequence as fusion partner for the protein of interest, was used for the first time to obtain hGITRL gene expression. Previous studies reported that expression of GITRL in *Escherichia coli* resulted in formation of insoluble inclusion bodies [10, 11, 12], but do not contain information about conditions and efficiency of protein refolding. In our study, although the protein was mainly produced as inclusion body, the efficiency of the refolding was about 95%, so we can state that an effective refolding method was elaborated. This unusually high yield is considered to be a result of the chaperon function of DsbA. The fusion protein was purified by affinity chromatography, after which the native state hGITRL was cleaved from the fusion partner from by TEV protease treatment.

Our aim for the future is to separate the cleaved fusion protein by gel filtration, testing the hGITRL activity and the association of recombinant GITRL with different human T cell lines or primary lymphocytes. The study of GITRL would also open alternative pathways for tumor detection and for monitoring of efficacy of various cancer therapies.

EXPERIMENTAL SECTION

Expression of the human GITRL as fusion protein

Isolation of total RNA from human brain and the synthesis of cDNA are described previously [14]. The gene of human GITRL (amino acids position 56-177) was isolated from human brain cDNA using forward primer, G1: 5'-CCATGGAGCCCTGTATGGCTAAGTTTGGACC-3' and reverse primer, GITRL: 5-GCGGATCCTACATGTGCTGAAGGGAATGAGG-3' [20]. This gene was inserted in pETM52 expression vector. The pETM52 plasmid was a generous gift of H.B. (EMBL Laboratories, Heidelberg). The construction of the pETM52 vector is described prevoiusly [10]. Construction of the recombinant vector was obtained following a modified protocol as described elsewhere [9]. 1 µl recombinant plasmid was transformed into 100 µl chemically competent *Escherichia coli* BL21 Star (DE3) cells. Protein expression was carried out based on a modified protocol as described before [15]. To produce hGITRL, 250 ml LB medium supplemented with 20 µg/ml kanamycine was inoculated with the starter culture (a single colony was grown overnight at 37°C in 10 ml LB medium containing 20 µg/ml kanamycine and 1% glucose) and was grown at 37°C until OD_{600}=0.8. Induction of transcription of the hGITRL gene was realized by IPTG (Sigma) in a final concentration of 0.5 mM and culture was maintained at 37°C in a shaking incubator at 250 rpm for an additional 4 h. After the derepression period cells were harvested by centrifugation (15 minutes at 5000 rpm, 4°C) and lyzed by sonication. The inclusion body was washed three times with 30 ml of 10 mM Na-phosphate buffer pH 7.0, 0.1 M NaCl and 0,1% β-mercaptoethanol.

The inclusion body was solubilized by 10 ml 6 M guanidine hydrochloride (Gu-HCl, Sigma) treatment, supplemented with 50 mM Tris-HCl buffer pH 8.8 and 1 mM dithiothreitol (DTT, Serva) and was reduced overnight at room temperature. The solubilized material was refolded by the fast dilution method in an oxidized/reduced glutathione (Sigma) system. 2 ml of the solubilized material was diluted into 60 ml ice-cold refolding buffer (0.8 M Gu-HCl, 0.1 M Tris-HCl pH 8.0, 5 mM $CaCl_2$, 0.5 mM EDTA, 5 mM reduced glutathione and 0.5 mM oxidized glutathione). The solution was stirred by magnetic stirring at 4°C for 8 h. The refolded protein was dialyzed in 50 mM NaCl and 10 mM Tris-HCl buffer pH 8.0.

Purification of the obtained protein by affinity chromatography

Affinity purification of the His-tagged protein was carried out by adsorption on a Ni-charged polymer matrix (Porfinity IMAC Ni-charged Resin, BioRad). Soluble proteins resulted from 60 ml of diluted protein were adsorbed on 3 ml of Ni-NTA Sepharose resin, after washing of the resin with 20 ml Binding Buffer (50 mM sodium phosphate buffer pH 8.0 supplemented with 0.3 M NaCl) for

equilibration. After the adsorbtion of the soluble proteins the beads were washed again with Washing Buffer (50 mM sodium phosphate buffer supplemented with 0.5 M NaCl). Elution of the protein from the adsorber matrix was realized by four-step elution with a 250 mM concentration of elution agent imidazole (Sigma), which has increased binding affinity to the Ni-charged matrix with respect to the 6xHis-tagged proteins. The supernatant containing the purified protein was stored at 4°C; from each fraction electrophoresis probes of 20 µl were retained.

Cleaving of human GITRL from DsbA by TEV protease

The eluted fraction was dialyzed in 20 mM Na-phosphate buffer, pH 8.0 supplemented with 100 mM NaCl. The dialysis buffer was changed two times, after which the protein solution was centrifuged. The cleavage with a tobacco etch virus (TEV protease recombinant, Invitrogen) protease was performed in 1mM DTT at room temperature.

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