STUDIA UNIVERSITATIS BABEŞ-BOLYAI, CHEMIA, LIV, Sp.Iss. 2, 2009

CONSTRUCTION OF AN EXPRESSION VECTOR FOR THE GITRL 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 expressed in T lymphocytes and in natural killer cells (NK). The receptor it is activated by its ligand, GITRL, which is a type II transmembrane protein. Human tumor cells express substantial levels of GITRL. GITRL attachment to its receptor blocks antitumoral effects of NK cells. As human tumor cells constitutively express GITRL, the presence of it can be used as a potential tumor marker. In this study the pETM50-hGITRL expression vector was constructed for heterologous expression of the ligand in a bacterial system.

Keywords: tumor necrosis factor, GITR, GITRL, expression vector, regulatory T cell

INTRODUCTION

The members of the tumor necrosis factor (TNF)/tumour necrosis factor receptor (TNFR) superfamily regulate diverse biological functions, including cell proliferation, differentiation and survival [1].

The glucocorticoid-induced TNF-related receptor (GITR) and its natural ligand (GITRL) belong to the TNF and TNF receptor superfamilies (TNFSF and TNFRSF) [2].

The GITR is a ~26-kDa type I transmembrane protein that displays 14–28% sequence identity to other members of the TNF receptor (TNFR) family and GITRL is a ~20-kDa type II transmembrane protein [3]. GITR is activated by its ligand [4].

In mouse GITR is expressed at low levels in resting CD4+ and CD8+ T cells and is rapidly up-regulated after activation [5]. GITR is constitutively expressed on CD4+CD25+ Tregs [6] and in particular has been implicated in

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inhibiting the function of CD4+CD25+ Treg cells that control immune effector cells. Treating Treg cells with GITR ligand (GITR-L) abrogates the suppressive function of Treg cells in vitro [7]. The GITR-mediated costimulation by anti-GITR mAb or GITRL may put CD4+CD25+ T cells in an active state, this may result in abrogation of the suppressive function of Treg cells [1].

In humans the Treg-mediated suppression of effector T cell function is not inhibited by GITR stimulation [8], so the GITR/GITRL signaling pathway plays different roles in mice and humans. In mice, GITR has implicated in the development of autoimmune diseases, graft versus host disease and in the immune response against infectious pathogens [9].

The hGITRL (AITRL - activation inducible tumor necrosis factor receptor ligand, TL6) gene mapped to chromosome 1q23, near the gene for the TNF homolog Fas/CD95 ligand [3]. The hGITRL gene codes for a type II transmembrane protein comprised of 177 amino acids, including a short cytoplasmic region, a hydrophobic transmembrane domain and an extracellular domain with two potential glycosylation sites in the C-terminal region [10, 11]. The putative mouse GITRL is 51% identical to AITRL [12].

In mouse tumors models GITR activation by a single dose of an anti-GITR agonist Ab (DTA-1) induces a delay in tumor progression [13], at the same time injection of adenovirus expressing recombinant GITRL (membrane-bound GITR-ligand and a truncated secreted form of GITR-L) into growing B16 melanoma tumors cells potently inhibited tumor growth [14].

The hGITR (AITR - activation inducible tumor necrosis factor receptor) is expressed on human natural killer (NK) cells, these cells substantially contribute to cancer immunosurveillance and GITR triggering may impair NK cell effector function [15, 16]. Human GITRL is constitutively expressed on tumor cells and GITRL expression by tumor cells diminishes NK cell cytotoxicity, thus the tumor cells survive [16].

As human tumor cells constitutively express GITRL, its presence can be used as a potential tumor marker. Our aim is the construction of an expression vector, which makes possible the heterologous expression of the human GITRL in a bacterial system, in order to study its biochemical properties.

RESULTS AND DISCUSSION

Isolation of the hGITRL coding sequence

The hGITRL mRNA expression was detected at highest level in small intestine, ovary, testis and kidney [3]. Human umbilical vein endothelial cells (HUVECs) constitutively express hGITRL, and its expression is up-regulated after stimulation with lipopolysaccharide. Human GITRL mRNA was also found in brain and pancreas as well [10]. In our study we cloned the coding

sequence of the extracellular region of hGITRL from human brain. We isolated total RNA from anatomically dissected human brain samples [17], first strand cDNA synthesis was performed with RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) using random hexamer primers. The first strand of cDNA was directly used as template to amplify the coding region corresponding to amino acids 44-177 by PCR as described in Experimental Section.

Our preliminary results show that hGITRL is expressed in several different regions of brain, albeit at slightly different extent. The highest expression was observed in various region of the cortex. Figure 1 shows the results of the amplification of hGITRL coding sequence from 6 different human brain samples.

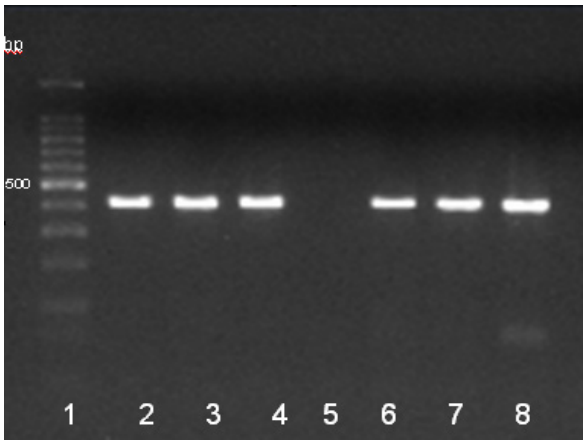

Figure 1. Results of the amplification of hGITRL coding sequence from human brain samples. Lane 1. 100 bp DNA ladder, Promega, Lanes 2,3,4. frontopolar cortex, Lanes 6,7,8. cerebellar cortex, 5. no template control

Construction of hGITRL expression plasmid

When expressed in *E. coli* proteins with disulfide bonds should be specifically directed to the periplasm because the cytoplasm is too reducing [18]. Periplasmic disulfide formation is regulated by members of the periplasmic disulfide isomerase (Dsb) protein system (DsbA, B, C, D) [19]. For this reason we choose pETM50 expression vector. The pETM50 plasmid was a generous gift of H.B. (EMBL Laboratories, Heidelberg). The multiple cloning site of pETM50 allows fusion of GITRL to the C terminus of DsbA protein containing a leader sequence for export. Using this vector the expressed protein accumulates in the periplasm, where the DsbA protein promotes the formation of correct disulfide bonds as well as the native structure of GITRL. To facilitate the purification of the recombinant proteins the vector contains a $(His)_6$-tag between DsbA and the fusion partner. Following purification $(His)_6$-tag and DsbA protein can be removed by tobacco etch virus (TEV) protease, since

there is a cleavage site for this protease preceding the multiple coding site. The expression vector pETM50 as we obtained already contained a fusion protein: receptor of advanced glycation end products (RAGE). This was replaced by GITRL utilizing the unique NcoI and BamHI sites. The scheme for the construction and the map of our expression vector pETM50-GITRL is outlined in Figure 2.

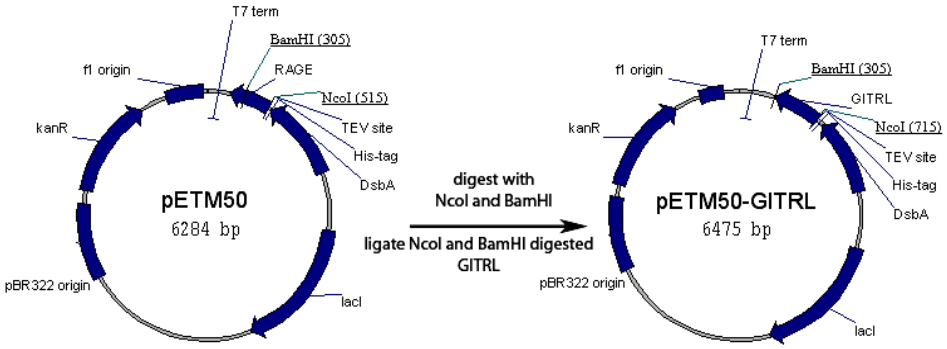

Figure 2. Construction of expression vector pETM50-GITRL

The GITRL coding gene was inserted in the pETM50 vector by T4 ligase. Figure 3 shows the results of the ligation reaction. The ligation mixture contains the digested GITRL coding sequence and the fragment of the expression vector digested by the same restriction enzymes (NcoI and BamHI).

For propagation of the recombinant vector with the correct conformation, the ligation reaction was transformed in chemically competent *Escherichia coli* TOP10F' cells and was selected on the basis of kanamycin resistance. The plate *lig2* and *lig3* contains the control reaction mix. Ligation mix in *lig2* contained only the linearized plasmid (large fragment), *lig3* only the GITRL coding sequence (small fragment).

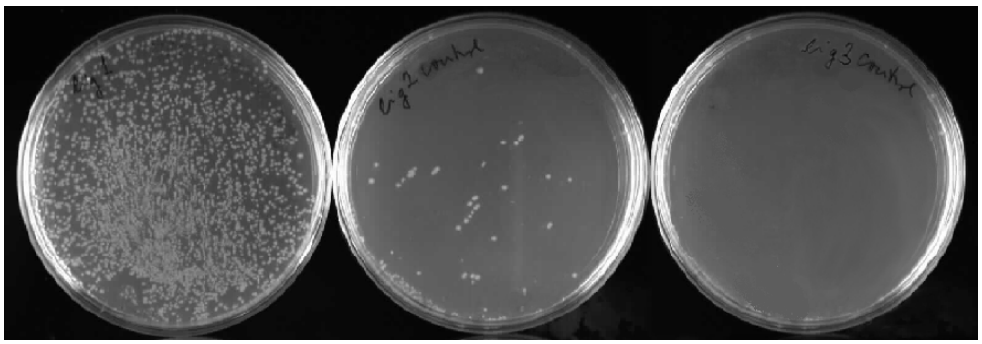

Figure 3. Results of the ligation
Ligation mix in *lig1* contained both large and small fragment while in *lig2* contained only the large fragment, *lig3* only the small fragment

Some transformed colonies from the plate *lig1* were picked up and grown in the presence of kanamycin to propagate the recombinant plasmid. After that plasmid DNA was isolated. One isolated plasmid DNA was analyzed on a 1% agarose gel, as presented in Figure 4. Insertion of the fragment of interest into the recombinant plasmid was controlled by double digestion with NcoI and BamHI.

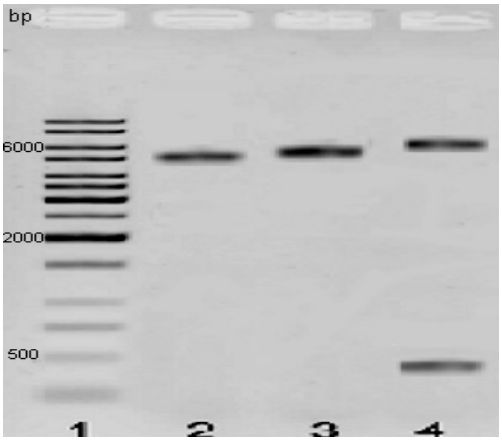

Figure 4. Restriction analysis of the obtained recombinant pETM50-hGITRL vector. Lane 1. 1kb DNA ladder, Fermentas, Lane 2. original pETM50 plasmid, Lane 3. pETM50-hGITRL, Lane 4. pETM50-hGITRL isolates digested with NcoI and BamHI restriction enzymes

The calculated size of the original pETM50 plasmid is 6284 bp while that of pETM50-hGITRL is 6475. Table 1. shows the obtained value derived from Figure 4. Due to their superhelical nature undigested plasmids (Lane 2 and Lane 3) display significantly lower size than the calculated one. On the other hand we got excellent agreement analyzing the digestion mixture. In Lane 4, the pETM50-hGITRL vector digested with NcoI-BamHI is shown; the double digestion resulted in two fragments of 6070 and 406 bp. The fragment appearing at 406 bp corresponds to the hGITRL coding sequence. The fragment appearing at 6070 bp corresponds to the NcoI/BamHI-digested pETM50.

Table 1. Resulted fragments of the pETM50-hGITRL vector

vector	*obtained, bp*	*Calculated values of fragments, bp*
pETM50	5100	6284
hGITRL coding sequence	406	410
pETM50-hGITR	5900	6475
NcoI/BamHI-digested pETM50	6070	6065

Besides restriction analysis the success of cloning was verified by automated dideoxy sequencing (ABI Prism) using the Big Dye Terminator 3.0 Kit utilizing the T7 termination sequence in the vector (Fig. 2). The obtained sequence was identical to the published one (NCBI Reference Sequence: NM_005092.3), except at position 372 where a T→C mutation was found. This mutation has already been described as an allelic variant.

CONCLUSION

The isolation of hGITRL coding sequence from brain and construction of the recombinant pETM50-hGITRL vector was completed successfully. The restriction analysis of the isolated vector gave results that confirm the calculated lengths of the restriction fragments.

Although in previous studies different expression vectors were constructed for the GITRL protein [6, 20, 21], but employment of these plasmids for the expression of GITRL in bacterial system resulted in formation of insoluble inclusion bodies. Our construct allows periplasmic expression of hGITRL. The periplasmic secretion and the putative chaperone action of the fusion partner DsbA might promote the correct folding of hGITRL.

Our aim for the future is realization of the protein expression in *E. coli* BL21(DE3) Star cells and purification of the protein. During this study it is important to find the optimal conditions which provide the expression of the protein in appropriate quantity and quality. Once purified the protein, an antibody will be prepared which can be used for tumor detection.

EXPERIMENTAL SECTION

Isolation of the hGITRL coding sequence

Isolation of total RNA from human brain and the synthesis of cDNA is described elsewhere [17]. The putative extracellular portion of hGITRL (amino acid positions 44-177) was amplified by the polymerase chain reaction (PCR) using the Corbett Research Thermocycler (Cornett CG1-96) system. The primers were designed with OligoExplorer 1.2 program and were obtained from Eurogentec. An NcoI site was added to the 5' end of the forward primer and a BamHI site was added tot the 3' end of the reverse primer. Correspondingly, the sequence of the forward primer was 5'-cggccATGGCTAAGTTTGGACCATTACC-3', and that of the reverse primer was 5'-gcggatcCTACATGTGCTGAAGGGAATGAGG-3'.

The amplification procedure for PCR was as follows: an initial denaturation at 95^{o}C for 2 minutes, followed by 30 cycles of amplification (95^{o}C for 30 sec; 55^{o}C for 45 sec; 74^{o}C for 2 min), a final extension at 72^{o}C

for 5 min and a subsequent incubation at 4°C. The amplified product (excepted size 406 bp) for hGITRL was separated by 1% agarose gel electrophoresis in TAE buffer with ethidium bromide staining and visualization by GelDoc System from BioRad.

Construction of hGITRL expression plasmid

For cloning of hGITRL gene was used the pETM50 plasmid.

The PCR product was digested by NcoI and BamHI restriction enzymes and the digestion mixture was separated on a preparative 1% agarose gel, and the 406 bp fragment containing the coding sequence was excised and isolated by the MiniElute Gel Extraction Kit (Qiagen), according to the instructions the manufacturer. The isolated fragment was obtained in 50 µl of TE buffer.

For the preparation of the large fragment, the pETM50 plasmid was digested by 10 U NcoI and 10 U of BamHI, in 1x Red Buffer (Fermentas) in a total volume of 60 µl for 4 hours at 37°C; the large fragment was obtained as described above in 30 µl of TE buffer. The purifity of large and small fragment isolates was verified by gel electrophoresis.

The ligation reaction was set up 20 µl total volume, containing 14 µl of NcoI/BamHI-digested PCR product small fragment, 2 µl of pETM50 large fragment and 2 µl T4 ligase (Invitrogen) in 1x Ligation Buffer (Invitrogen) at 25°C for 1 hour, together with two control ligations, one without the large fragment and one without the small fragment.

The ligation and the control mixtures were transformed into 100 µl of chemically competent Escherichia coli Top10F' cells (Invitrogen) and plated on LB medium with kanamycin. 4 transformed colonies were picked up and grown overnight (12 hours) in 5 ml LB with kanamycin, at 37°C and 250 rpm. Plasmid DNA was isolated and purified by the Plasmid Miniprep Kit (Quiagen), according to the instructions of the manufacturer.

DNA sequencing reactions were performed in 20 µl containing 4 µl Ready Reaction Premix (BigDye® Terminator v3.1), 2 µl BigDye Sequencing Buffer, 6 pmole T7 ter primer and 300 ng plasmid. Thermal cycling was performed according to the instruction of the manufacturer. Capillary electrophoresis of the samples was performed by Biomi Ltd. (Gödöllő, Hungary).

ACKNOWLEDGMENTS

This study was supported by the Department of Technical Sciences, University of Sapientia and the Department of Biochemistry, Eötvös Loránd University.

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