Short Communication

Construction of CTLA-4-Ig Fusion Gene in pBudCE4.1 Expression Vector

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Abstract

Background: CTLA-4 inhibitory signals prevent cell cycle progression and IL-2 production, leading to a halt on an ongoing immune response. CTLA4-Ig fusion proteins contain the extracellular domain of CTLA-4 and Fc fragment of human IgG antibody. In this study we aimed to fuse the *ctla-4* gene encoding the extracellular domain of CTLA-4 molecule with *igg1* gene encoding Fc region of human IgG.

Methods: After primer design, PCR reaction was performed using *pfu* polymerase enzyme and specific primers. PCR amplified fragment was ligated into the vector containing the human *igg1* gene. The resulting fusion fragment of *ctla-4* and human *igg1* genes was ligated to pBudCE4.1 expression vector.

Results: Extracellular domain of *ctla-4* gene was ligated to *igg1* gene and then *ctla4-ig* fragment was cloned into pBudCE4.1 vector. Construction of the expression vector was confirmed by restriction pattern analysis and sequencing.

Conclusion: By confirming the construct, in the next step, the recombinant DNA will be used to produce CTLA4-Ig recombinant protein for the clinical uses.

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Introduction

Following the T cell activation, cytotoxic T lymphocyte antigen-4 (CTLA-4), a negative regulatory molecule, will be expressed on T cells [1,2]. CTLA-4 is a homolog of CD28 but binds B-7 molecules with greater affinity. Inhibitory signals of this molecule inhibit cell cycle progression and IL-2 production, leading to a halt on an ongoing immune response [3-6].

Regarding the central role of CTLA-4 in down-regulation of the immune responses, co-stimulatory receptors became an important target for drug development. The best example is the CTLA4-Ig fusion protein, containing the extracellular domain of CTLA-4 and the constant region of human IgG antibody. This fusion protein can inhibits T cells dependent immune responses [5,7,8].

In this study we aimed to fuse extracellular domain of *ctla-4* gene to Fc region of human *igg1* gene. This recombinant DNA could be used to produce CTLA4-Ig protein and studding its function in future studies.

Materials and Methods

Enzymes and chemicals

All chemicals and antibiotics were purchased from Sigma, Merck (Germany) and Invitrogen (France), unless stated otherwise. DNA-modifying enzymes and restriction enzymes were obtained from Fermentas.

Vectors, microorganisms and growth conditions

Escherichia coli DH5α (CinnaGen, Iran) as a host and pBudCE4.1 as an expression vector were used. IgG1 containing vector was provided kindly by Dr. Rabbani (Avicenna Research Institute, Iran). *Escherichia coli (E. coli)* were cultured in LB medium at appropriate temperature (37°*C*) with shaking (150 *rpm*).

PCR amplification and CTLA4-Ig fragment construction

Extracellular domain of *ctla-4* gene was amplified using specific primers (CTLA4-FOR/CTLA4-fuse) and pUCCTLA4 vector as template. For subsequent cloning of the PCR-derived fragments, *Sal*I and *Bam*HI restriction sites were added to the 5'-end of these primers, respectively (Table 1). pUCCTLA-4 (synthetic construct) was used to amplify the *ctla-4* gene with *pfu* polymerase enzyme. PCR products were purified by High Pure PCR Product Purification Kit (Roche, Germany). The purified fragment was digested simultaneously with vector containing human *igg1* gene using *Sal*I/*BamH*I enzymes and then were ligated. *E. coli* DH5α cells were transformed using $CaCl_2$ method [9]. Recombinant colonies were confirmed by PCR using

Table 1. Oligonucleotides (primers) used in present study (restriction sites were showed in bold)

Primers	Sequence 5′to 3′	Orientation	5′ cloning site
A) Primers used for amplification of *ctla4-Ig*			
CTLA-4 FOR	5'-TT**GTCGAC**AGCCACCATGGCTTGCCTT-3	Sense	*Sal*I
CTLA-4 fuse	5'-TT**GGATCC**GTCAGAATCTGGGCA-3'	Anti-sense	*Bam*HI
B) Universal primers			
T7f	5' GTAAAACGACGGCCAGT	Sense	--
pBudCE4.1r	5' CAGGAAACAGCTATGAC	Anti-sense	--
M13f	5'-GTAAAACGACGGCCAGT-3'	Sense	--
M13r	5'-AACAGCTATGACCATG-3'	Anti-sense	--

specific primers. Plasmid DNA preparation was done using QIAGEN Mini Prep Kit (Germany).

Construction of CTLA4-Ig expression vector

Vector containing the *ctla4-ig* fragment digested by *Sal*I/*Xba*I enzymes and subcloned into pBudCE4.1 *Sal*I/*Xba*I cloning sites with the methods mentioned before. Recombinant colonies were confirmed by digestion with cloning enzymes and PCR pattern.

Sequence and computer analysis

Cloned DNA fragment in pBudCE4.1 (50-200 *ng/µl*) was sequenced by a Commercial Service (Bioneer, South Korea).

Results

ctla-4 fragment was amplified using specific primers and *pfu* polymerase. A specific band about 483 *bp* showed the expected size (Figure 1). Extracellular domain of *ctla-4* gene was inserted into the *Sal*I and *Bam*HI pGEMIgG vector and designated as pGEMCTLA4-Ig. The new construct was confirmed by restriction pattern using *Sal*I/*BamH*I and *Sal*I/*Xba*I enzymes and PCR product pattern. According to the size of *ctla-4* gene external domain (483 *bp*) and *igg1* gene (993 *bp*), the resulting fragment (1476 *bp*) confirmed the fusion of extracellular domain of *ctla-4* to human *igg1* gene (Figure 2).

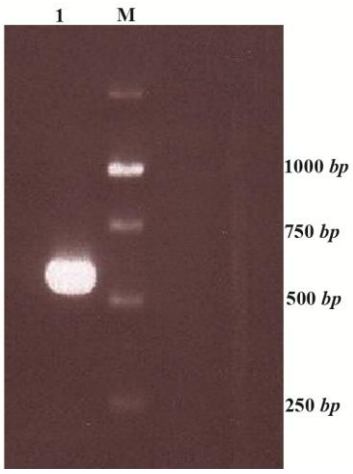

Figure 1. *ctla-4* gene PCR product with *Pfu* polymerase enzyme
M: 1 *kb* ladder
1: PCR product using specific primers (CTLA4- FOR / CTLA4-fuse).

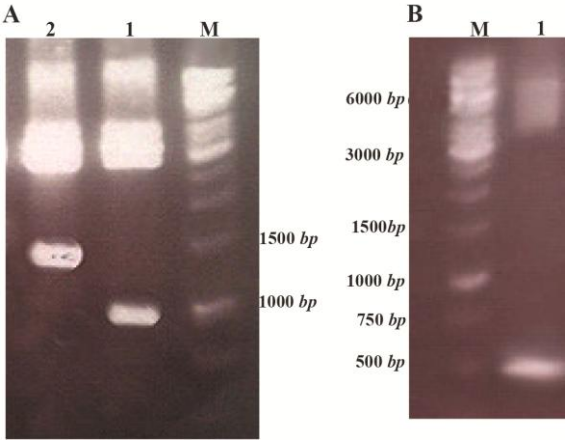

Figure 2. Confirmation of new construct with digestion pattern
A) 1: Digestion of vector containing *igg1* gene with *Sal*I/*Xba*I enzymes
2. Digestion of new construct with *Sal*I/*Xba*I enzymes
B) 1: Digestion of new construct with *Sal*I/*Bam*HI enzymes.

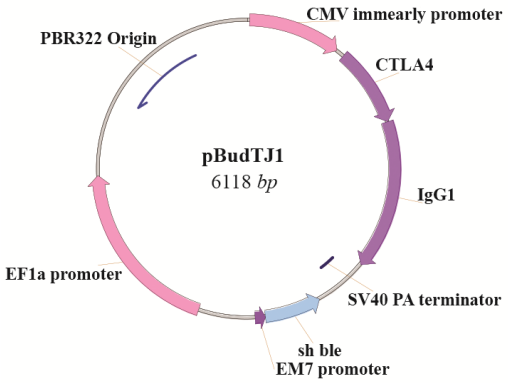

Figure 3. pBudTJ1 schematic view.

To clone *ctla4-ig* fragment in pBudCE4.1 expression vector, pGEMCTLA4-Ig construct was digested and gel purified fragment was cloned into pBudCE4.1 vector. Construction of the expression vector was confirmed by restriction pattern analysis using *Sal*I and *Xba*I. The cloned fragment was sequenced by T7f/pBudCE4.1r universal primers. DNA sequencing showed an open reading frame, 1476 *bp* in length, encoding a 492 amino acid polypeptide. The new construct designated pBudTJ1 is shown in figure 3.

Discussion

Co-stimulatory molecules play a critical role in controlling the immune response. The central role of CD28 family, especially the CTLA-4, makes it a useful tool for immunotherapy in autoimmune disease and transplant rejection [6,10].

Two approaches have been selected in respect to the potential clinical applications of CTLA-4 in immunotherapy, anti CTLA-4 antibody and CTLA4-Ig, respectively [7,11]. CTLA4-Ig is a fusion protein containing the extracellular domain of CTLA-4 and the Fc portion of human IgG1. This protein is capable of preventing the stimulatory effect of CD28 through competing and binding to B-7s on APCs [12-14].

In this study, we have fused the extracellular domain of CTLA-4 to the Fc fragment of the human IgG1 antibody. The resulting construct was ligated to pBudCE4.1 expression vector and confirmed by sequencing. Analysis of the fusion sequence revealed an open reading frame encoding a protein of 445 amino acids with predicted molecular mass of about 50 *kDa* without glycosylation (ExPASy). In subsequent study, this recombinant DNA can be used to produce CTLA4-Ig recombinant protein.

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