International Journal of Histology and Cytology ISSN 2447-9535 Vol. 6 (1), pp. 001-005, January, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Expression of complete rhoptry protein 2 (ROP2) gene of *Toxoplasma gondii* in eukaryotic cell

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Accepted 16 November, 2018

***Toxoplasma gondii* is the intracellular protozoan parasite responsible for animal and human toxoplasmosis. In immunodeficient patients, chronic infection with *T. gondii* can reactivate and produce encephalitis, which is often lethal. ROP2 (rhoptry protein of *T. gondii*) is one of the most important interferer in organelle and PVM blending. ROP2 protein is recognized by clone T-cell (Tcc32) in human body and also has epitope for B-cell. All of these characteristics of ROP2 makes it a candidate for cocktail vaccine and recombinant vaccine against toxoplasmosis. We described the expression of the gene which encodes the complete rhoptry protein 2 (ROP2) of *T. gondii* in CHO cells and confirmed it by SDS-PAGE and Western blot analysis. In the present work, genomic DNA of *T. gondii* was extracted and used for amplifying of ROP2 gene as a template. Then PCR product was cloned into pTZ57R/T vector, and plasmid containing ROP2 gene (pT-ROP2) was extracted from transformed bacteria and sequenced. We hope to use from this recombinant plasmid (pT-ROP2) to make DNA vaccine against toxoplasmosis.**

Key words: Cloning, sequencing, *Toxoplasma gondii*, ROP2.

INTRODUCTION

Toxoplasma gondii is the intracellular protozoan parasite responsible for animal and human toxoplasmosis. In immunocompetent individuals, infection with this parasite usually is clinically asymptomatic, but it may cause severe complications in immunodeficient individuals and in pregnant woman (Bhopale, 2003). In immunodefecient patients, chronic infection with *T. gondii* can reactivate and produce encephalitis, which is often lethal (McCabe and Remington, 1988). In fact, *T. gondii* is one of the major opportunistic pathogens in HIV-infected patients (Mc Cabe and Remington, 1988). Primary *T. gondii* infection of a mother during pregnancy can lead to abortion, neonatal malformations or other defects which appear during child development (Remington and Krahenbuhl, 1982; Wong and Remington, 1994). Natural infection with *T. gondii* generally leads to a state of long-lasting non-sterile protective immunity (Suzuki and Remington, 1988; Gazzinelli et al., 1991). This protection is T-cell-mediated and involves both CD 4^+ and CD 8^+ T-cells (Suzuki and Remington, 1988; Gazzinelli et al., 1991).

Therefore recombinant vaccines and protein vaccines against toxoplasmosis should be based on parasite antigens which induce this T-cell-mediated protective immunity. Like other unicellular organisms *T. gondii* is composed of various antigens, which are immunogenic and are structural components or metabolism products of parasite. The most important kinds of toxoplasma antigens are surface tachyzoite antigens and excreted/secreted antigens (Bhopale, 2003). Nowadays there are lots of interest in the somatic antigens also known as excreted/secreted antigens or exoantigens. *T. gondii* secretory proteins are potent antigens that can trigger strong immune responses. One of this kinds of antigens is rhoptry protein 2 (ROP2) that is secreted from rhoptry organelle. So far nine rhoptry proteins have been described. The timing of the release of these molecules, as well as their targeting to the host cell surface or parasitophorous vacuole, suggests a role in invasion

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(Perkins, 1992; Saffer et al., 1992). ROP2 is one of the most important interferer in organelle and PVM blending and causes lipid transfer from host cells mitochondria and ER to PVM (Perkins, 1992; Saffer et al., 1992). ROP2 protein is recognized by clone T-cell (Tcc 32) in human body and has also epitope for B-cell and produces IgA, IgM and IgG (Saavedra et al., 1991; Martin et al., 1998). All of these characteristics for ROP2 makes it a candidate for protein vaccine and recombinant vaccine against toxoplasmosis (Saavedra and Herion, 1991; Saavedra et al., 1991).

Expressed ROP2 is used as a candidate for evaluation of immune response against infection and is used as a candidate to make a diagnostic kit for toxoplasmosis (Martin et al., 2004; Leyva et al., 2001). ROP2 is a 64 kDa protein (was be coded by complete ROP2 gene) component of the rhoptries which is expressed in three stages of the parasite life cycle (Sadak et al., 1988; Beckers et al., 1994). Cleavage at ROP2 protein in the PVM creates much diversity for ROP2 molecular weight (Leriche and Dubremetz, 1991; Sadak et al., 1988).

Complete ROP2 gene encodes a 561 aa poly peptide (without cleavage). Complete ROP2 gene is about 2235 bp and its coding parts is about 1686 bp which is located between 440 and 2125 of the whole gene (Beckers et al., 1994). In this paper we describe the expression of *T. gondii* complete rhoptry protein 2 gene by expression plasmid in CHO cells and confirm it by SDS-PAGE and Western blot analyses. In the future we hope to use this expressed ROP2 to make protein vaccine and recombinant vaccine against toxoplasmosis as well as a diagnostic kit for toxoplasmosis.

MATERIALS AND METHODS

Production of *T.* gondii tachyzoites

A high virulent strain of *T. gondii* (presented in experimental laboratory of Parasitology Department of Medical Sciences Faculty of Tarbiat Modarres University, known as RH strain), maintained in BALB/c mice by serial intraperitoneal inoculation of about tachyzoites, was used for production of tachyzoites.

Genomic DNA extraction and PCR

About 5×10^7 *T. gondii* tachyzoites (100 µl) were concentrated by centrifugation, washed with phosphate buffer saline (PBS), then lysed in 900 µl lysis buffer (0.1 M Tris-HCl pH 8.0 containing 1% sodium dodecyl sulphate, 0.1 M NaCl and 10 mM EDTA) and then treated with 10 µl proteinase K (100 µg/ml) at 55°C for 2 h (Kimbita et al., 2001). The lysate was then added to an equal volume of phenol/chloroform (25:25) to remove proteins. This mixture was centrifuged at 13000 rpm for 15 min and an equal volume of chloroform was added to the supernatant was mixed with 1/10 volume of 3 M sodium acetate and two volumes of 100% ethanol to precipitate DNA by centrifugation at 13000 rpm for 10 min. The DNA pellet was washed with 70% ethanol, dissolved in sterile distilled water and stored at -20°C until use (Sambrook et al., 1989). DNA extraction products were detected in 0.8% agarose gel and photographed.

Genomic DNA isolated from tachyzoites was used as a template to amplify the ROP2 gene by PCR performed in 25 µl of solution containing 3 µl of template DNA, 0.5 µl Taq DNA polymerase, 2.5 µl 10X PCR buffer, 0.75 µl $MgCl_2$, 15.75 µl distilled water and 1 µl each of primers (Forward primer, 32 nt: introduced Hind III recognition site, underlined: 5' -ATT AAG CTT ATG GAA AAC TGT GCG TCG GTC AG- 3. Reverse primer, 29 nt; introduced EcoRI recognition site, underlined: 5'-ATT GAA TTC CGG TTC TCC ATC AG- 3'). Under the following conditions: After an initial 5 min denaturation at 94°C, each cycle consisted of 60 s at 94°C, 30 s at 62°C and 60 s at 72°C at the end of the 32 cycles of amplification, a final extension was continued for 30 min at 72°C.

The PCR products analyzed by electrophoresis on a 1% agarose gel and photographed. The size markers used to estimate PCR products were the 1 kbp DNA ladders (Fermentas). The DNA sequence of gene encoding the ROP2 of *T. gondii* was obtained from the Gene Bank database (http://www.ncbi.com) with accession No. Z36906 and 1686 bp. The forward and reverse primers were designed according to the nucleotide sequence in Gene Bank database and GenRuner Software.

Cloning of ROP2

PCR products were purified using a DNA Extraction Kit from agarose gel (Fermentas) following the manufacturer's recommendations. The purified PCR products were ligated to pTZ57R/T cloning vector (InsT/Aclone^{TM} PCR product cloning kit, Fermentas), following the manufacturer's protocol. The recombinant plasmid (pT-ROP2) was used to transform competent *Escherichia coli* TG1 strain. The cloned ROP2 was confirmed by PCR, restriction enzymes (HindIII and EcoRI) digestion and nucleotide sequencing.

Expression plasmid constructions and *In vitro* transient transfection of CHO cells

The coding region for the *T. gondii* ROP2 was subcloned from pT-ROP2 into the HindIII and EcoRI sites of the pcDNA3 (Invitrogen, USA) to produce recombinant eukaryotic expression vector pcROP2. These were used to transfected CHO cells. CHO cells were grown to 60 - 70% confluence at 37°C and 5% CO_2 in 35 mm wells in Dulbecco's modified Eagle's medium (DMEM, Gibco) each containing 100 mL^{-1} penicillin and streptomycin and 10% fetal calf serum (FCS). Cells were washed in a serum-free medium and the transfection was performed with a transfection kit (Genejuice Transfection Kit, Novagene, USA) according to instructions of the manufacturer.

SDS-PAGE and Western blot analysis

The cells (transfected and non-transfected control cells) were harvested for 48 h following the transfection and lysed in sample buffer. After sonication, the cells were concentrated by centrifugation and their protein profile was resolved in 12.5% reducing SDS-PAGE according to the method of Laemmli (1970) and transferred to nitrocellulose membrane. Membrane strips were blocked with 1% BSA-PBST20 overnight and sequentially probed with toxoplasma antibody-positive human sera (high titers, IgM4+, were found by IgM-ELISA) (Martin et al. 1998) and a peroxidase-conjugated anti-human IgM (DAKO, Denmark) diluted in 1% BSA-PBST20 (1/200 and 1/2000, respectively). Specific binding was revealed with diamino benzidine (DAB) (DAKO, Denmark). The Western-blotting analysis showed that the mature proteins produced *in vitro* in CHO cells upon transfection with pcROP2/P64 plasmid is of the expected molecular mass and recognized by specific polyclonal antibodies, whereas no *T. gondii* proteins were

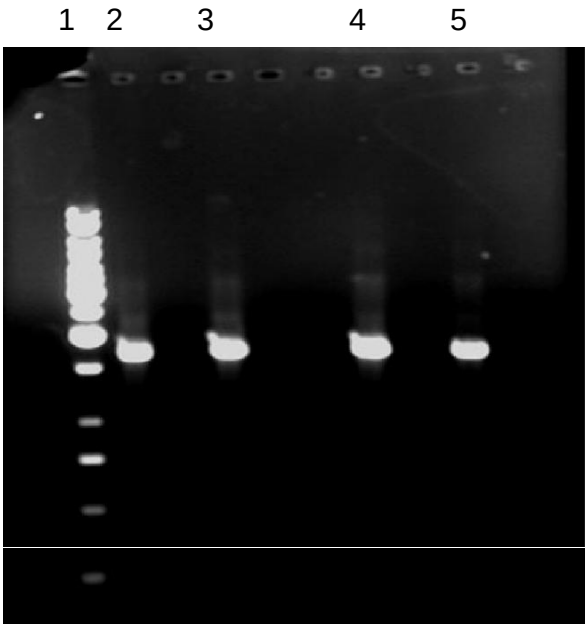

Figure 1. ROP2 PCR amplification and gel electrophoresis. Lanes 2, 3, 4 and 5, PCR product (approximately 1686 bp); Lane 1, 1 Kbp DNA ladder.

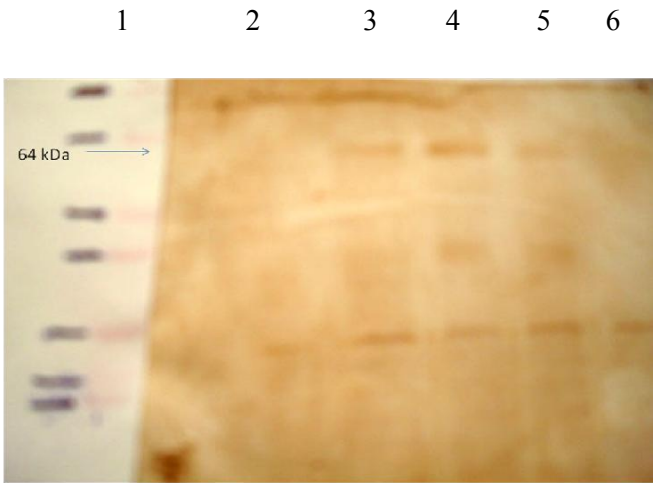

Figure 2. Western blot analysis of expressed gene product. Western blotting showed human *T. gondii* positive sera recognizing ROP2 protein from transfected CHO cells. It was not detected in non-transfected control cells. Lane 1, protein molecular weight marker (top to down 116, 66.2, 45, 35, 25, 18.4, 14.4 kDa). Lane 2, CHO cells (negative control). Lanes 3, 4 and 5, transfected cells containing Pc-ROP2 plasmid . Lane 6, transfected cell containing Pc-DNA 3 (negative control).

detected in non-transfected control cells.

RESULTS

PCR amplification and cloning

Figure 1 shows that DNA fragment PCR amplified was about 1686 pb and similar to the expected *T. gondii* ROP2 gene size. Nucleotide sequence analysis of the ROP2 cloned in pTZ57R/T vector revealed high homology (98%) with RH strain Gene Bank Accession No. Z36906.1 and Gene Bank Accession No. S54994.1 (not shown).

The ROP2 gene was cloned into pcDNA3 vector by using the restriction enzymes HindIII and EcoRI. The resulting vector consisted of a cytomegalovirus immediate-early promoter that can drive the transcription. The sequence analysis proved that no PCR-induced mutation occurred and the resulting vector consisted of 1686 base pairs (positions 1 to 1686 according to Gene Bank, Z36906.1) from ROP2 gene.

ROP2 expressed *in vitro* by transfected CHO cells

ROP2 was synthesized in a eukaryotic system. The CHO cells (transfected and non-transfected control cells) were harvested for 48 h following the transfection and lysed in sample buffer. The protein extracts were then analyzed by SDS-PAGE and Western blotting. A band at about 64 kDa was recognized by toxoplasma antibody-positive human sera in protein extracts of cells transfected with pcROP2. ROP2 protein was not detected in non-transfected control cells. Thus the functionality of the vector, in terms of *in vitro* production of the ROP2 protein, was confirmed (Figure 2).

DISCUSSION

It is known that chronically infected individuals and animals develop lifelong immune protection against reinfection. The effective immune response is mediated by CD 4^+ and CD 8^+ T cells and is associated with the production of gamma interferon (IFNγ). So a live vaccine based on an attenuated strain of *T. gondii* is being used in farm animals. However, such a vaccine is not suitable for human use due to reactivation to the pathogenic form. For this reason the use of recombinant technology and expression of useful genes arises as a powerful and interesting tool for the development of a vaccine for humans (Martin et al., 2004).

In attempting to develop a recombinant vaccine, we have focused on the expression of complete rhoptry protein 2 (ROP2) gene in eukaryotic cells (CHO), because previous studies for preventing toxoplasmosis have shown that immunization with ROP2 peptides, proteins or as recombinant vaccine can elicit a broad range of immune responses that are capable of decreasing mortality of animals acutely infected with *T. gondii* and reducing the level of tissue cysts in the brain of infected animals; but it is still necessary to increase their efficacy. We describe the expression of the complete

gene which encodes ROP2 of *T. gondii* in CHO cells and confirm it by SDS-PAGE and Western blot analysis. Results showed that ROP2 is 64 kDa and can express it from genomic DNA of three stages. Cleavage at ROP2 protein (64 kDa) in the PVM will create much diversity for ROP2 molecular weight. Complete ROP2 protein contains an 561 aa polypeptide (without cleavage).

Sadak et al*.* (1988), Saavedra et al*.* (1991) and Herion et al*.* (1993) also showed that ROP2 antigen is a protein component of the rhoptries which is expressed in three stage of the parasite life cycle. Also, it is one of the most important ROP2 family proteins in toxoplasma, and this antigen is specially observed in toxoplasma and is not seen in other apicomplexes. Perkins (1992), Saffer et al. (1992), Sinai et al*.* (1997) and Beckers et al*.* (1994) also observe that ROP2's coding parts is about 1686 bp without introns and ROP2 protein is about 64 kDa. Wei et al*.* (2006) and Leyva et al*.* (2001) cloned the complete ROP2 gene into a eukaryotic expression plasmid pcDNA3 and used it for immunizing mice. After three immunizations with the plasmid, mice developed antibodies that could be detected by ELISA using a recombinant truncated form of ROP2 and these antibodies also recognized the natural ROP2 protein by Western blot. In Leyva's work a ROP2 fragment was expressed in *E .coli* as a fusion protein using the pRSET vector. The recombinant protein (rROP2) contained the sequence included between residues D-186 and A-561 of ROP2 fused to a 38-residue N-terminal peptide derived from the expression vector.

Fachado et al. (2003) and Martin et al*.* (2004) used incomplete ROP2 gene (residues 196 to 561) to make DNA vaccine cocktail and recombinant ROP2 protein against toxoplasmosis in mice. An important difference between our work and those of Fachado et al. (2003) and Martin et al*.* (2004) is that we used the complete ROP2 gene cloned in pcDNA3 plasmid (in which the ROP2 protein is expressed naturally with its signal peptide, while they deleted the sequence coding for the ROP2 signal peptide and cloned the gene in a plasmid in frame with the PA signal peptide, which enables that ROP2 protein to be secreted). Manipulation of a gene and cloning in a plasmid that leads to the secretion of the protein can modify the type and strength of the immune response. Thus, our recombinant expression plasmid (that includes a complete ROP2 gene) will be useful to make vaccine against toxoplasmosis. Another important difference is that in our work plasmid expression was analyzed in transfected CHO cells but in their work plasmid expression was analyzed in transfected COS7 cells.

The result of this study showed that we successfully cloned the complete ROP2 gene into expression plasmid pcDNA3 and expressed it by eukaryotic cells. We will use from this expressed ROP2 as a candidate to make recombinant vaccines and also to produce a diagnostic kit for evaluation of immune response against toxoplasmosis.

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