Cloning, sequence analysis and expression of ovine CD154 (CD40 ligand)

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Abstract

The CD154 (CD40 ligand) molecule is a member of the tumor necrosis factor (TNF) family and plays an important role in the interaction between antigen-specific lymphocytes and antigen-presenting cells. In this study, reverse transcription–PCR cloning was used to derive the sequence encoding ovine CD154. Sequence analysis of the cloned CD154 gene showed a similarity of 97, 89 and 88% with the bovine, porcine and human sequences, respectively, at the nucleic acid level. The deduced amino acid sequence for the ovine CD154 shared 97, 91, and 87% similarity with the CD154 protein of bovine, porcine and human. The cysteine residues characteristic of the TNF family and N-linked glycosylation sites are conserved although one of the cysteine residues (Cys^9) appeared only in ovine CD154. The isolated CD154 sequence was expressed as a mature protein in Chinese hamster ovary (CHO) cells. The analysis of

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expression of ovine CD154 in mammalian cells by Western Blot confirmed the cross reactivity with anti-CD154 antibody.

Keywords: CD154; CD40 ligand; sheep; DNA vaccine; adjuvant

1. Introduction

The CD154 (CD40 ligand) molecule is produced as a type II transmembrane protein with a molecular mass of 33 kDa (Zhai et al., 2004). It is a member of the tumor necrosis factor (TNF) family of cell surface signaling molecules, and is expressed on activated CD4+ T cells and other cell types (Ferrant et al., 2002; Armitage, 1994). CD154 stimulates B cells to differentiate into antibody secreting and memory cells, and also boosts cell-mediated immunity by activation of macrophages and dendritic cells and the generation of natural killer cells and cytotoxic T lymphocytes (Ferrant et al., 2002; Vogel and Noelle, 1998). CD154 has a role in regulating the function of both the humoral and cell-mediated immune response.

Progress towards successful DNA vaccines in large farm animals has been hampered by several factors including a relatively low response (Wang et al., 1998; Wang et al., 2001; Arulkanthan et al., 1999). Large numbers of plasmids containing cytokine sequences have been evaluated as DNA vaccine adjuvants (Scheerlinck, 2001). Plasmids have the advantage of being stable, simple to produce, and they can be genetically engineered to express any immunologic effectors or regulatory gene of interest (Barouch et al., 2004).

Plasmid encoded cytokine adjuvants offer the possibility of exerting specific and potent immunostimulatory effects. Recently, studies in sheep have shown that co-administration of plasmid-encoded bovine CD154, increased antibody responses to bovine herpesvirus 1 glycoprotein D (Manoj et al., 2003).

While previous studies in large animal species concluded that it is possible to enhance immune responses to DNA vaccination using various strategies, the role of cytokine molecules in enhancing immunity in domestic animals remains to be studied (Donnelly et al., 2003). It is well documented that cytokines may be involved not only in immunostimulatory role but also in development of undesirable pathological conditions (Buzoni-Gatel 2002).

Bovine CD154 was cloned by (Mertens et al.,1995) and high homology with sheep CD154 is to be expected. However, to study immunoregulation in sheep, the identical molecule needs to be used.

In this study we described the cloning, sequencing, sequence analysis and expression of ovine CD154 in Chinese hamster ovary (CHO) cells and characterization of the recombinant protein by Western Blot. Cloning and expression of this protein from ovine provides the basis for further investigation of role of CD154 in studying methods for enhancing immune responses to the proteins expressed by DNA vaccines. Recombinant cytokines are valuable tools for functional studies and analysis of the roles of different cytokines during immune responses.

2. Materials and methods

2.1 Molecular biology

All reagents for polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), DNA, RNA and plasmid isolation and gel extraction kits were purchased from Qiagen (Hilden, Germany).

2.2 Cells and tissues

Whole blood (WB) from healthy sheep was anticoagulated with EDTA and diluted 1 in 10 with F-12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), antibiotics (100 U/ml penicillin/100 µg/ml streptomycin, 2.5 µg/ml amphotericin B (Fungizone). Lymphocyte stimulation was done in triplicate wells in 96-well microculture plates with 15 ng/ml of concanavalin A (Con A; Sigma–Aldrich (St. Louis, MO, USA). Cells were incubated for 48 h at 37°C in a humidified 5% CO_2 air–water jacketed incubator.

2.3 Cloning of ovine CD154

RNA was obtained from normal adult sheep blood following of in vitro ConA stimulation using a Qiagen RNeasy kit. RNA was resuspended in 30 µl of RNase-free water and quantified by spectrophotometry (Optical density; OD_{260}). RNA (3 mg) was used to synthesise single stranded complementary deoxyribonucleic acid (cDNA) with Qiagen Omniscript reverse transcriptase (5U/reaction), using anchored oligo-dT-primers . The cDNA-samples were stored at -30 °C.

CD154 gene (791 base pairs; bp) was amplified by PCR of ovine WB cDNA using two oligonucleotide primers adcdfor (5′- CAG CAT GAT MGA AAC

ATA CA -3′) and adcdrev (5′- TTC AGA GTT TGA GTA AGC CAA A -3′), which were designed on regions of high homology among the sequences of human and cattle CD154 (GenBank, accession numbers ***X67878*** and ***Z48469*** respectively). PCR was carried out in a total volume of 25µl reaction buffer containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.25 mM $MgCl_2$, 0.2 mM dNTPs, 5 U of Tag polymerase (Qiagen), 10 pmol each of the primers and 2 µl of cDNA. Cycling conditions for PCR were 10 cycles of 30 s at 95 °C, 30 s at 36 °C, 30 s at 72 °C and 30 cycles of 30 s at 95 °C, 30 s at 40 °C, 30 s at 72 °C, followed by the final extension for 7 min at 72 °C. The PCR product was cloned into pDrive cloning vector (Qiagen PCR Cloning Kit) and sequenced bidirectionally to confirm CD154 specificity (plasmid pDriveCD154). Based on this sequence, two specific primers: (5′- GCG GAA TTC TCT AGA ATG GAC AAG ATA GAA GA -3′) and (5′- GCT CTA GAG GTA CCT CAG AGT TTG AGT AAG CCA A -3′) were designed for the amplification of the mature CD154 which started from 51 amino acid (aa) without transmembrane sequence. The ovine CD154 PCR product (668 bp) and pVAX1(Invitrogen) DNA were digested with *Xba*I (New England BioLabs, Beverly MA). Product was gel-purified and DNA ligations performed using T4 ligase and transformed to the DH5α cells (Invitrogen) according to the manufacturer's description. Selected clones (referred to a pVAXCD154) were screened for recombinant protein CD154 by restriction enzyme analysis.

2.4 Transfection and production of recombinant proteins

To obtain recombinant protein CD154 for *in vitro* assays, CHO cells (American Type Culture Collection, ATCC) were cultured at a concentration of

$6X10^5$ cells/ml in 60 mm diameter Petri dishes (Nurc, Falcon?) in F-12 medium (cat no.) with 10% FBS, 100 U/ml penicillin/100 µg/ml streptomycin, 2.5 µg/ml amphoterica B (Fungizone). Subsequently the cells were transfected with pVAXCD154 using Escort reagent (Sigma-Aldrich) according to the manufacturer's instructions and cultured for 48 hr at 37 °C in 5% CO_2 humidified environment. The pVAX1 (Invitrogene) and pVAX1lac (Invitrogene) were used as a control of transfection and expression of the recombinant protein.

2.5 Western blot analysis

CHO cell supernatant containing CD154 recombinant protein was separated on 12% polyacrylamide gel under reducing condition and the proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The nitrocellulose transfer was made by using the Mini Trans-Blot Cell (Bio-Rad Laboratories) for 1 h at 200 mA. After treatment with 5% nonfat dry milk (Blotting Grade Blocker, Bio-Rad Laboratories) in Tris buffer containing 0.05% Tween 20, an for 1 h at room temperature (RT) under agitation with a human CD154-specific monoclonal antibody (1:1000) (Calbiochem, San Diego, CA, USA), 1:2000 final dilution, was performed. This was followed by a further incubation (1 h at room temperature) with anti-mouse IgG-horseradish peroxidase (Sigma-Aldrich 1:10,000 final dilution). Colorimetric detection was achieved by using 4-chloro-1-naphthol as substrate for horseradish peroxidase (Opti-4CN, Bio-Rad Laboratories) following the manufacturer's instructions.

2.6 Sequence analysis

Plasmid pDriveCD154 was sequenced at Waikato DNA Sequencing Facility (Hamilton, New Zealand). Sequence data analyses were performed using the BLAST search of the National Center for Biotechnology Information. The protein prediction was performed using software at the ExPASy Molecular Biology Server (http://expasy.pku.edu.cn). Levels of similarity and identity nucleotide and amino acid sequence were determined wit the CLUSTALX and the GeneDoc programs. The sequence data have been submitted to GenBank and assigned accession number ***DQ054533*** for CD154 cDNA. Phylogenetic analysis was performed using the CLUSTALX program and TREEVIEW version 1.6.6 was used for visualization of the tree.

3. Results

3.1 cDNA cloning and sequence analysis of sheep CD154

Total cDNA was made from total cellular RNA isolated from activated WB from healthy sheep and used as a template for the synthesis of CD154 cDNA. A PCR product was amplified using degenerate primers adcdfor and adcdrev, based on the DNA alignment of CD154 sequences from human and cattle. The identity of the product was confirmed by DNA sequencing (Fig.1) and aligned to known CD154 sequences from GenBank.

The full-length CD154 mRNA is 787 nucleotides (GenBank accession no. ***DQ054533***). The nucleotide sequence of ovine CD154 (oCD154) shared 97%

similarity to cattle, 89% to pig CD154 and 88% to human and cat sequences (Tab1.).

3.2. Amino acid sequence analysis of oCD154

Comparison of the deduced amino acid sequence of the CD154 from sheep with the sequence of cattle, pig, cat, dog, mouse and human (Tab.1) indicated that the deduced protein had a higher degree of similarity to cattle (97% of amino acid identity) than to pig (89%), cat and human sequence (88% of homology) (Fig.2). The similarities results were further confirmed by the phylogenetic analysis (Fig. 3). The oCD154 showed close relationship between sheep and cattle and pig CD154.

Search for conserved domain motifs revealed the presence (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) of a Tumor Necrosis Factor (TNF) domain in the C-terminal region of CD154 (Fig.1). In the TNF domain, the ovine sequence was 97%, 92%, and 90% identical to the TNF region of cattle, pig and human CD154, respectively. The lowest homology of the TNF sequence with ovine sequence was found for dog (84%) and mouse (75%).

The analysis of the oCD154 sequence by the NetNGlyc 2.0 software (http://www.expasy.org) revealed that the sheep homologue of CD154 contained one potential *N* -glycosylation site located at the TFN domain. This N residue was conserved in the aligned sequenced from cattle, pig, cat, dog, mouse and human CD154. The 261 amino-acid sequence of oCD154 contain six cysteine (Cys)

residues whereof one of them is characteristic only for sheep Cys^{9} and one Cys^{194} is present in all compared sequence excepting mouse.

The molecular weight of the predicted CD154 is 29.271,77 Da, with the calculated PI being 8.83.

3.3. Expression of CD154 gene in mammalian cells

For the expression of oCD154 protein in a eukaryotic expression system the recombinant plasmid was constructed. The mature fragment of oCD154 without transmembrane sequence was amplified by PCR. The 668 bp PCR product of oCD154 was cloned into the mammalian expression vector pVAX1 behind the CMV promoter. The resultant construct named pVAXCD154 was then used to transfect CHO cells. As a control of the expression we used commercially available plasmid pVAXlac. For protein characterization of the expressed oCD154, Western Blot analysis was performed with the human CD154 specific monoclonal antibody. The Western Blot analysis with indicated a molecular weight of 23 kDa for the ovine CD154 without its signal anchor sequence was performed. Fig. 4.

5. Discussion

The CD154 is known to play an important role in cellular and humoral immune response (van Kooten and Banchereau, 2000). In this study we report the cloning, sequencing, nucleotide and amino acid sequences analysis and expression of ovine CD154 in mammalian cells. We designed our PCR primers

based on regions of high homology among the sequences of CD154 genes from cattle and human and we analyzed obtained nucleotide and deduced amino acid sequence of oCD154 gene.

DNA vaccines have a number of additional potential advantages compared to the conventional vaccines in terms of economy and simplicity of vaccine production. Numerous studies have confirmed that plasmid based DNA vaccine can induce protective immune responses in mice, but similar vaccine efficiency has not been observed for large animals species (Drew et al., 2001, van Drunen Littel-van den Hurk et al., 2000). Wang et al., 2001, Arulkanthan et al., 1992) Improving the immunogenicity of DNA vaccines especially in livestock animals (e.g. swine, cattle and sheep remains a major challenge (Krishnan and Rajendra, 2000). Some studies have showed a variety of ways to boost immune responses by changing the delivery parameters (van Rooij et al., 1998) and improving the transfection efficiency (Braun et al., 1999) or by adding immune modulatory molecules such as: cytokine (e.g. CD154) and costimulatory molecules (van Drunen Littel-van den Hurk et al., 2004).

The nucleotide sequence of the oCD154 coding region has higher homology to cattle (97%) than to porcine (89%), human (88%), cat (88%), dog (86%) and mouse (80%).

Amino acid sequence of oCD154 encodes 261 amino acid polypeptides which contain 24 amino acid long transmembrane domain, and 22 amino acid long, N-terminal intracellular domain and 140 amino acid TNF domain. Deduced

amino acid sequence of oCD154 showed a high degree of homology to respective CD154 from cattle (97%), pig (91%) though relatively lower homology to human (87%), cat (86%), dog (83%) and mouse (77%). Therefore, these results revealed a close phylogenetic relationship between ovine and cattle and pig. The ovine CD154 gene high homology was expected, since other TNF superfamily members like: TNF-alpha, lymphotoxin-alpha, Apo2L, Fas ligand, and osteoprotegerin ligand genes (Bodmer et al., 2002), shares high identity in TNF domain, which determines 54% of amino acid sequence of oCD154. In the TNF domain the ovine sequence was 97%, 92% and 90% identical to the TFN region of cattle, pig and human CD154 respectively.

The level of homology suggested that the oCD154 would be active on another B cells of the heterologous species especially on cattle and pig which shown the higher homology in CD154 sequence than other species. Bovine and ovine CD40 are 95% homologous at the amino acid level (Manoj et al., 2003).

Murine CD154 has been shown that to mediate B-cells proliferation as well as IgE production in human B cells (Armitage et al., 1992a, Armitage et al., 1992b) and human CD154 is active on both murine and human B cells (Spriggs et al., 1992). Analysis of the similarity of amino acid sequence of the murine CD40 (mCD40) and human CD40 (hCD40) which shared 62% identity suggested that the binding of both mCD154 and hCD154 to either form of CD40 would be possible (Torres and Clark, 1992, Spriggs et al., 1992).

The observed crossreactivity in our experiment between a mouse monoclonal anti-human CD154 antibody and the ovine CD154 in the Western Blot analysis support the described homology of the protein sequence. The similar immunology crossreactivity was confirmed for the porcine, mouse and human CD154 (Wienhold et al., 2002, Gauchat et al., 1993, Maliszewski et al., 1993, Fanslow et al., 1994)

CD154 is expressed on T cells following activation (Castle et al., 1993) and has a critical role in the generation on of both cells mediated and humoral immune responses, as well as regulation of antigen presenting cell activity (van Kooten and Banchereau, 2000). Several studies on the interactions between CD40 and CD154 have contributed extensively to our understanding of how both humoral and cellular immune responses are regulated (Grewal and Flavell., 1998). This raises the possibility that response to a DNA vaccine may be enhanced by using CD154 as an adjuvant.

In addition, recent research has shown that murine CD154 can function as an adjuvant for DNA vaccines by co-administering plasmids that individually express a vaccine antigen and CD154. The expression of CD154 appeared to enhance both immune responses and disease protection (Mendoza et al., 1997, Gurunathan et al., 1998, Tripp et al., 2000). Some studies have shown that co administration of plasmid encoded bovine CD154 enhanced antibody responses to bovine herpesvirus I glycoprotein D in sheep (Manoj et al., 2003). Bovine CD154 was used to enhance sheep humoral immune response induced by DNA

vaccine. Plasmid encoding CD154 was used as an effective genetic adjuvant together with plasmid encoding GM-CSF gene. That combination of plasmids gives strong humoral immune responses against a plasmid encoding antigen (Burger et al., 2001).

In conclusion co-expression of CD154 can be used to enhance humoral immune responses induced by a DNA vaccine. However, when using this approach for vaccination, it may be important to carefully consider the biological activity of both the vaccine antigen and the CD154 molecule to ensure that the both proteins does not have competing receptors binding activities as pointed out by Manoj et al. 2003.

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atgatcgaaacatacagtcaaccttgtccccgctctgtggccactggcacacctgtcagt

M I E T Y S Q P C P R S V A T G T P V S

atgaaaatttttatgtatttacttactgtttttcttatcacccagatgattgggtcagcg

M K *I F M Y L L T V F L I T Q M I G S A*

ctttttgctgtgtatcttcacagaagattggacaagatagaagatgaaaggaatcttcac

L F A V Y L H R R L D K I E D E R N L H

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E D F V F M K T I Q R C N K G E G S L S

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L L N C E E I R S R F E D L V K D I M Q

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A P K G Y Y T L S S N L V T L E N G K Q

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L A V K R Q G F Y Y I Y T Q V T F C S N

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R E A L N Q A P F I A S L C L K S P S G

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S E R I L L R A A N T H S S S K P C G Q

caatccattcacttgggaggagtctttgaattgcaacagggtgcttcggtgtttgtcaat

Q S I H L G G V F E L Q Q G A S V F V N

gtgactgatccaagtcaggtgagccacgggactggcttcacatcatttggcttactcaaa

V T D P S Q V S H G T G F T S F G L L K

ctctga

L -

Fig.1. The nucleotide (GenBank accession numbers: ***DQ054533***) and deduced amino acid sequences for ovine CD154. Italicized amino acid residues indicate the transmembran sequence while the underlined fragment represents the predicted *N* -glycosylated sites. Bold amino acid is CD154 conserved domain Tumor Necrosis Factor (TNF).

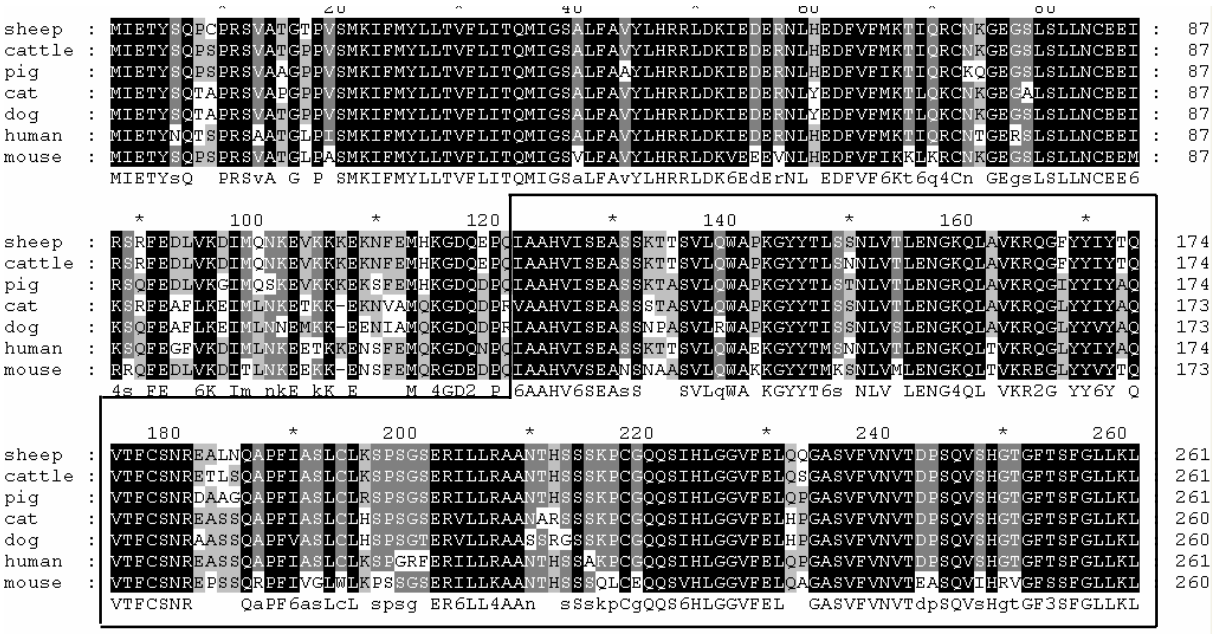

Fig.2. Alignment of deduced amino acid sequence of sheep (GenBank accession no. ***DQ05453***), cattle (GenBank accession no. ***Z48469***), pig (GenBank accession no. ***AB04044***), human (GenBank accession no. ***X6787***), cat (GenBank accession no. ***AF07910***), dog (GenBank accession no. ***AF08671***) and mouse CD154 (GenBank accession no. ***AY41606***). Boxed sequence is CD154 conserved domain TNF.

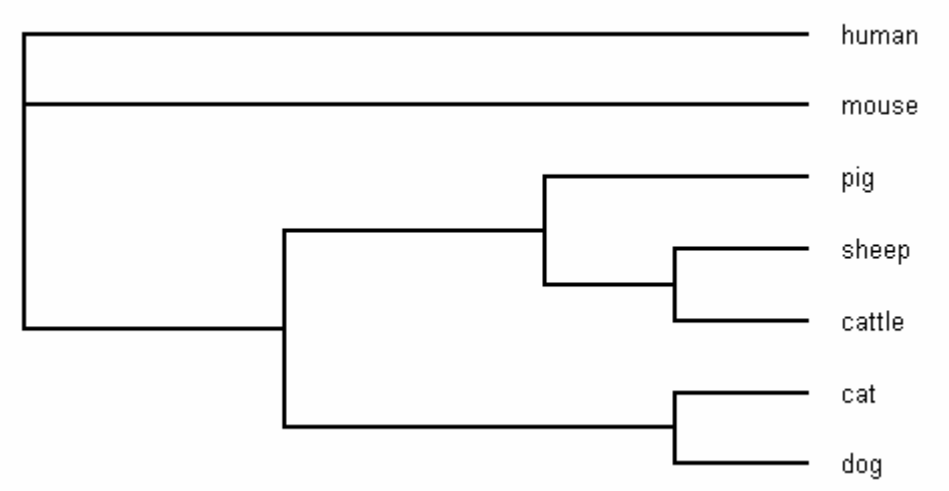

Fig.3. Phylogenetic relationship between sheep and other mammalian CD154. The sequence alignment was performed and the tree wee drawn as described in Materials and Methods.

Fig. 4. Analysis of ovine CD154 by Western blot.

Species	Sheep DQ054533	Cattle Z48469	Pig AB040443	Cat AF079105	Dog AF086711	Human X67878	Mouse AY416063	Nucleotide Sequence Identity (%)
Sheep	**----**	**97**	**89**	**88**	**86**	**88**	**80**	
Cattle	97	**----**	**89**	**88**	**85**	**88**	**80**	
Pig	91	91	**----**	**87**	**85**	**86**	**79**	
Cat	86	86	84	**----**	**90**	**87**	**79**	
Dog	83	83	81	92	**----**	**85**	**78**	
Human	87	88	85	84	82	**----**	**83**	
Mouse	77	78	75	73	73	77	**----**	

Amino Acid Sequence Identity (%)

Tab.1. The nucleic and amino acid sequences % similarities of CD154. Bold section: percentage of nucleic acid.