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# Molecular cloning, characterization and tissue expression of CD4 in Chinese goose

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ABSTRACT

CD4 protein is a single chain transmembrane glycoprotein belonging to the immunoglobulin superfamily that recognizes the major histocompatibility complex (MHC) class II molecules. It plays an important role in cell-mediated immunity. Here, the full-length cDNA of CD4 in Chinese goose (*Anser cygnoides*) was cloned and identified. The goose CD4 is 1940 bp in length and encodes a single open-reading frame of 480 amino acids. The putative amino acid sequence of goose CD4 consisted of a signal peptide, four potential *N*-glycosylation sites, a transmembrane region and a cytoplasmic tail. The multiple sequence alignment and phylogenetic analysis suggested that goose CD4 shared a higher similarity with avian than other vertebrates. Semi-quantitative RT-PCR analysis showed that the highest level of CD4 mRNA transcripts was presented in the thymus, and relatively lower in the spleen, small intestine, brain and trachea. Low expression was seen in the bursa of fabricius, cecal tonsil, cecum, skin, lung, kidney and liver. In gosling, the CD4 transcript was expressed with high abundance in the thymus, and relatively lower in the spleen, cecal tonsil and small intestine. However, in adult goose, high expression was seen in the thymus, spleen and cecum, and relatively lower in the cecal tonsil and small intestine. During the course of NGVEV infection, the obvious increase in CD4 gene expression was observed in the spleen, bursa of fabricius and harderian gland. Interestingly, a notable decrease in CD4 mRNA expression in the small intestine at 5 d PI and followed by an increase of that at 19 d PI were shown.



## 1. Introduction

The keystone of adaptive immunity is the interaction between antigen presenting cells (APC) and T lymphocyte. T cells populating the peripheral blood or secondary lymphoid organs were distinguished early on by the mutually exclusive expression of either CD4 or CD8 (Benoist and Mathis, 1999). Expression of CD4 and CD8 is critical for cell-mediated immune (CMI) defense and T-cell development in the thymus (Cammarota et al., 1992; Salter et al., 1990). $T_H$ cells and $T_C$ cells are major subsets that carry CD4 and CD8, respectively (Germain, 2002). These molecules are responsible for recognition of antigens bound to MHC class II and class I molecules respectively (König and Germain, 1992). The T cell antigen receptor (TCR) recognizes the MHC–Ag complex which can cause T cell activation (Wange, 1996). Mature T cells have an essential role in CMI defense.

CD4 is a single chain transmembrane glycoprotein, expressed on the surface of immune cells such as T helper cells, thymocytes, monocytes, macrophages, and dendritic cells (Kazazi et al., 1989; Parnes, 1989; Rezzani et al., 2008). It contains four Ig-like domains, a transmembrane region, and a cytoplasmic tail that associates with a tyrosine protein kinase, p56lck, involved in T-cell activation (Veillette et al., 1988a, 1988b). The primary structure of CD4 protein has been identified in several mammalian and avian species (Classon et al., 1986; Dumont-Drieux et al., 1992; Fomsgaard et al., 1992; Gustafsson et al., 1993; Hague et al., 1992; Koskinen et al., 1999; Maddon et al., 1985; Milde et al., 1993; Norimine et al., 1992; Omatsu et al., 2006; Romano et al., 1999). The molecule has also been identified in fish which is composed of 12 exons, differing from other CD4 genes, but showing conserved synteny and many conserved sequence motifs in the promoter region (Buonocore et al., 2008; Dijkstra et al., 2006; Patel et al., 2009; Suetake et al., 2004). Chicken CD4 consists of 1990 nucleotides encoding an open reading frame of 402 amino acids including four Ig-like domains, a 24-residue hydrophobic transmembrane region, and a 33-residue cytoplasmic tail, which is 24% identical with both human and mouse sequences (Koskinen et al., 1999). With similar functions to mammalian CD4, chicken CD4 therefore

*Abbreviations:* MHC, major histocompatibility complex; cDNA, complementary to RNA; NGVEV, new type gosling viral enteritis virus; RT-PCR, reverse transcription-polymerase chain reaction; PI, post infection; APC, antigen presenting cells; CMI, cell-mediated immune; TCR, T cell antigen receptor; RACE, rapid amplification of cDNA ends; GSP, gene specific primers; AP, adapter primer; AAP, Abridged Anchor Primer; AUAP, Abridged Universal Amplification Primer; ORF, open reading frame; BLAST, basic local alignment search tool; UTR, untranslated region; MIS, mucosal immune system; CIS, cutaneous immune system.

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plays a crucial role in the immune response (Chan et al., 1988; Luhtala et al., 1993). Interestingly, there is no cDNA sequence or amino acid of goose CD4 has been reported.

Comparatively little is known about the immune system of waterfowl at the molecular and cellular levels other than of chicken. Interest in the goose immune system has sparked not only from its importance as a food animal species but also from its role as a natural reservoir of many avian viruses. CD4+ T-cell subsets play a critical role in the regulation of viral infection (Whitmire, 2011). Virus infection expands populations of antigen-specific CD4+ T-cells. These virus-specific CD4+ T-cells are extremely important in controlling viral infections by inducing protective memory CD8+ T-cell and promoting CD8+ T-cell function and survival as well as humoral immunity (Phares et al., 2012; Whitmire, 2011).

Here, for the first time, we described the molecular cloning of the first *Anser cygnoides* CD4 cDNA from the Sichuan White Goose (Chinese domestic goose). Furthermore, the cDNA and amino acid sequence, the structural and phylogenetic analysis of goose CD4, as well as the tissue distribution of CD4 in adult goose and gosling were studied. New type gosling viral enteritis virus (NGVEV), an adenovirus, caused new type gosling viral enteritis (NGVE) which is a serious disease of gosling less than 30 days of age (Chen et al., 2008a). Moreover, the immunological function of CD4 during NGVE virus infection was identified. These data will expand our knowledge of the T cell-mediated immune responses in waterfowl during viral infection and the results from this study will contribute to provide information for the development of some immunological toolbox which will enabled investigation of immune cell subsets.

## 2. Materials and methods

### 2.1. Animals and virus strain

The study was conducted with Sichuan White Goose and Gosling (the Chinese goose, *A. cygnoides*). The birds were euthanized and tissues were collected by using fresh, or snap frozen in liquid $N_2$.

The NGVEV-CN strain (a high virulence field isolate) was provided by the Avian Diseases Research Centre of Sichuan Agricultural University. The minimal lethal dose of the virus suspension ($LD_{50}$) was $10^{-6.5\pm1.3}$/0.5 ml. This strain has been previously described (Chen et al., 2008b, 2008c; Cheng et al., 2001).

### 2.2. Cloning of goose CD4 cDNA

Total RNA was isolated from goose thymus by using TRIzol Reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). About 2 μg of RNA was subjected to reverse transcription with the use of Oligo$(dT)_{18}$ as the primer and SuperScript II Reverse Transcriptase (Invitrogen). The partial sequence of goose CD4 was amplified by the degenerate primers (CD4 F and CD4 R; all primer sequences used in this study were listed in Table 1), which were designed based on the conserved regions of its counterparts in Mallard (GenBank Accession no. FJ527912), Muscovy duck (GenBank Accession no. AY738736), Chicken (GenBank Accession no. Y12012), and Zebra finch (GenBank Accession no. XM_002193001). The target PCR fragment was purified using the Universal DNA Purification Kit (Tiangen, Beijing, China) and cloned into pGEM-T Easy Vector (Promega, USA), and the selected clones were sequenced by using the ABI 3730 XL sequencer (Applied Biosystems, Foster City, CA). Subsequently, 3′ and 5′ rapid amplification of cDNA ends (RACE) was performed to obtain the full-length cDNA sequence of goose CD4. Based on the sequence obtained, the Gene Specific Primers (GSPs) including 3′-N1, 3′-N2, 5′-N1, 5′-N2 and 5′-N3 were designed to pull out the full-length of goose CD4 cDNA. For 3′-RACE, the first strand cDNA was synthesized using the Adapter Primer (AP). The 3′-end of goose CD4 was amplified by the method of nested PCR using the primers 3′-N1 and 3′-N2 with AP1 and AP2 respectively. For 5′-RACE, the first-strand cDNA was synthesized by using the primer 5′-N1 and SuperScript II Reverse Transcriptase (Invitrogen), and then a homopolymeric tail was added to the 3′-end of the cDNA using TdT and dCTP (Invitrogen). The 5′-end of goose CD4 was also obtained by two rounds of nested PCR with the use of the primers 5′-N2 and Abridged Anchor Primer (AAP), and 5′-N3 and Abridged Universal Amplification Primer (AUAP). The PCR fragments were sequenced and the full-length cDNA sequence was assembled. Finally, the full-length coding sequence of goose CD4 was amplified by using KOD-Plus-DNA polymerase (TOYOBO Co., LTD, Japan).

**Table 1**
Primers and sequences.

| Method | Primer name | Nucleotide sequence(5′–3′) |
|---|---|---|
| Reverse transcription | Oligo$(dT)_{18}$ | TTTTTTTTTTTTTTTT |
| Partial sequence | CD4 F | TGCTGTCTTCKTGSTTCTGC |
| | CD4 R | CCACGGTGTAATKAARGCTGA |
| 3′ RACE | AP | GCTGTCAACGATACGCTACGTAACGGCATGACAGTG (T)18 |
| | AP1 | GCTGTCAACGATACGCTACGTAACG |
| | AP2 | CGCTACGTAACGGCATGACAGTG |
| | 3′ N1 | AACGCCACAGCAGATGGACAG |
| | 3′ N2 | AGCCAGACATTGTTTGAAATACCT |
| 5′ RACE | AAP | GGCCACGCGTCGACTACGGGIIGGGIIGGGIIGGGIIG |
| | AUAP | GGCCACGCGTCGACTAGTAC |
| | 5′ N1 | GCATACAAGGACAAAC |
| | 5′ N2 | AGCTTCAGTACGTATTTTTGAGGGG |
| | 5′ N3 | CCCGTTGAGAGCTGTATAATAGTAG |
| Tissue distribution | CD4 GSP F | AGCCAGACATTGTTTGAAATACCT |
| | CD4 GSP R | CCACGGTGTAATGAAGGCTGA |
| | Goose β-actin F | AGATGACGCAGATCATGTTTG |
| | Goose β-actin R | GAAGGATGGCTGGAAGAGG |

### 2.3. Sequence analysis of goose CD4 cDNA

Amino acid sequence of goose CD4 cDNA and open reading frame (ORF) were deduced by using DNAStar software and the Sequence Manipulation Suite (http://www.bioinformatics.org/sms/). The similarity was analyzed with the Basic Local Alignment Search Tool (BLAST) program from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Multiple alignment of deduced amino acid sequence was performed by using Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The functional sites or the domains were predicted by CBS Prediction Servers software online (http://genome.cbs.dtu.dk/services/). Phylogenetic tree was constructed by MEGA 5 and based on the Neighbor-Joining method with the bootstrap of 1000 repetitions (Tamura et al., 2011).

### 2.4. Tissue expression of goose CD4 mRNA

13-Day-old gosling and adult goose were killed and the selected tissues were collected including the thymus, spleen, harderian gland, bursa, cecal tonsil, pancreas, heart, kidney, brain, liver, lung, small intestine, cecum, trachea and skin. Briefly, total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) from the samples. The cDNA samples were prepared by reverse transcription as described above. The GSPs were designed (CD4 GSP F and CD4 GSP R, shown in Table 1) for detecting the expression of CD4 in these tissues by a semi-quantitative RT-PCR method. To optimize the cycle number for RT-PCR, PCR amplification profiles were analyzed by using the mixture of cDNAs from all selected tissues as the template. Amplification of β-actin was used as the internal control.