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# The Pekin duck programmed death ligand-2: cDNA cloning, genomic structure, molecular characterization and expression analysis

Qingxia Yao[a,c], Karl P. Fischer[b,c], D. Lorne Tyrrell[a,b,c], Klaus S. Gutfreund[a,c,*]

[a] Depts. of Medicine, University of Alberta, Edmonton, AB, Canada
[b] Medical Microbiology & Immunology, University of Alberta, Edmonton, AB, Canada
[c] Li Ka Shing Institute of Virology, University of Alberta, Edmonton, AB, Canada

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ABSTRACT

Programmed death-1 (PD-1), upon engagement by its ligands, programmed death ligand-1 (PD-L1) and programmed death ligand-2 (PD-L2), provides signals that attenuate adaptive immune responses. Here we describe the identification of the Pekin duck PD-L2 (duPD-L2) and its gene structure. The duPD-L2 cDNA encodes a 321 amino acid protein that has an amino acid identity of 76% and 35% with chicken and human PD-L2, respectively. Mapping of the duPD-L2 cDNA with duck genomic sequences revealed an exonic structure similar to that of the human P*dcd1lg2* gene. Homology modelling of the duPD-L2 protein was compatible with the murine PD-L2 ectodomain structure. Residues known to be important for PD-1 receptor binding of murine PD-L2 were mostly conserved in duPD-L2 within sheets A and G and partially conserved within sheets C and F. DuPD-L2 mRNA was constitutively expressed in all tissues examined with highest expression levels in lung, spleen, cloaca, bursa, cecal tonsil, duodenum and very low levels of expression in muscle, kidney and brain. Lipopolysaccharide treatment of adherent duck PBMC upregulated duPD-L2 mRNA expression. Our work shows evolutionary conservation of the PD-L2 ectodomain structure and residues important for PD-1 binding in vertebrates including fish. The information provided will be useful for further investigation of the role of duPD-L2 in the regulation of duck adaptive immunity and exploration of PD-1-targeted immunotherapies in the duck hepatitis B infection model.

## 1. Introduction

The inhibitory receptor programmed death-1 (PD-1, CD279) [1] and its ligands programmed death ligand-1 (PD-L1, B7-H1, CD274) [2] and programmed death ligand-2 (PD-L2, B7-DC, CD273) [3] play a key role in the attenuation of adaptive immune responses and peripheral tolerance. The B7 family members PD-L1 and PD-L2 are currently the only known ligands for PD-1. However, PD-L1 can also engage B7-1 (CD80) [4] and PD-L2 has recently been shown to interact with repulsive guidance molecule B (RGMb), thereby regulating respiratory T cell immunity [5] beyond the currently known co-inhibitory pathways of the B7: CD28 family [6].

The two PD-1 ligands are immunoglobulin superfamily (IgSF) members that contain tandem IgV-and IgC-like IgSF domains and a cytoplasmic tail of unknown function. PD-L2 is encoded by the P*dcd1lg2* gene on human chromosome 9 in close proximity to the P*dcd1lg1* gene that encodes PD-L1 [7]. The human P*dcd1lg2* gene is comprised of seven exons and six introns, whereas the murine P*dcd1lg2* gene is comprised of 6 exons and 5 introns and its exon 5 contains a stop codon that results in a truncated cytoplasmic domain of 5 aa. The crystal structures have been determined for the complexes formed by the ectodomains of murine PD-L2 (mPD-L2) and murine PD-1 (mPD-1) [8], human PD-L1 (hPD-L1) and mPD-1 [9], and those of hPD-1 and hPD-L1 [10].

PD-L1 is constitutively and inducibly expressed in a wide range of hematopoetic and non-hematopoetic cells, whereas PD-L2 expression was initially thought to be more restricted and mainly found on antigen presenting cells, such as activated dendritic cells (DCs) and macrophages, and murine B1 B cells [11]. Additionally, PD-L2 has been shown to be expressed on various other human cells including endothelial cells, epithelial cells, fibroblasts and T cells; and PD-L2 is inducibly expressed on monocytes and macrophages by T-helper

*Abbreviations:* GM-CSF, granulocyte-macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cells; ORF, open reading frame; RACE, rapid amplification of cDNA ends; TLR, Toll-like receptor

* Correspondence to: Liver Unit, Division of Gastroenterology, Department of Medicine, University of Alberta, Zeidler Ledcor Centre, 130 University Campus, Edmonton, Alberta, Canada T6G 2XB.
*E-mail address:* klaus.gutfreund@ualberta.ca (K.S. Gutfreund).

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**Table 1**
PCR primers and cycle conditions.

| Name(position) | Sequence(5′→ 3′) |
|---|---|
| FF (1–24) | ATGTTCCAAATCCTGACACTGCTG |
| FFB(1–20) | CGggatccATGTTCCAAATCCTGACACT |
| F1 (735–758) | ATCCCTGATCTTATTTATCATCGC |
| F2 (774–795) | AACAGTCCTTCTCTCTGCCTTA |
| FR (966–946) | TCACAGACTCACACTCCCACT |
| FRE(966–946) | CGgaattcTCTCAGACTCACACTCCCACT |
| R1 (385–367) | CACCCTGGTAGTCAATAAG |
| R2 (252–235) | AGATGGAGGGAGTGCTTT |
| R3 (150–133) | CAACGAGCCATTCACAGG |

Denaturation – 94 °C, 3 min; amplification 1–35 cycles – 94 °C, 30 s; 58 °C, 30 s; 72 °C, 60 s; final extension – 72 °C, 5 min.

**Table 2**
Real-time PCR primers, probes and cycle conditions.

| Name(position) | Sequence(5′→ 3′) |
|---|---|
| qF-PDL2(14–35) | TGACACTGCTGTTGCTGGAAAT |
| qR-PDL2(99–77) | CACAGCAACATACAGCTGCTGAG |
| qP-PDL2(38–72) | /6-FAM/ AGCTCTGGGTGGTTTCAGGTTTATTTACAGTTGAA/ TAMRA-sp/ |
| qF2-GAPDH (284–305) | TCCACCGGTGTCTTCACCA |
| qR2-GAPDH (308–333) | GAGATGATGACACGCTTAGCACC |
| qP2-GAPDH (359–337) | /6-FAM/TGGAGAAGGCTGGTGCTCACCTGAAG/TAMRA-sp/ |

50 °C 2 min; 95 °C 10 min; 95 °C 15 s and 60 °C 1 min for 45 cycles. Primers and probes are based on duck PD-L2 (GenBank ID, KU674826), and duck GAPDH sequence deposited by BGI (ENSAPLT00000014139, Pre Ensembl release 63 - September 2011).

cytokines, common γ-chain cytokines, GM-CSF and TLR ligands, and upon T-cell activation [12].

Inhibitory signaling through PD-1 is recognized for its important role in T cell exhaustion in persistent viral infections and tumor immunity [13]. Recently, therapeutic targeting of the PD-1: PD-L1 pathway has led to breakthroughs in the treatment of cancer [14]. To date, three humanized monoclonal antibodies (Mabs), nivolumab and pembrolizumab targeting PD-1 and atezolizumab targeting PD-L1, have obtained FDA approval. Nivolumab has been explored as a treatment for chronic hepatitis C [15]. Albeit in this small pilot study antiviral effects were highly variable, further development of therapeutic targeting of co-stimulatory pathways is of interest for chronic viral infections for which the induction of a functional cure or a sustained viral clearance remain an unattained treatment goal. Blockade of PD-1 has been shown to reverse the exhausted T cell phenotype in patients with chronic hepatitis B in *in vitro* studies [16]; and to enhance viral clearance in woodchucks chronically infected with the woodchuck hepatitis B virus [17].

Ducks infected with duck hepatitis B virus (DHBV) serve as a valuable natural disease model for hepatitis B infection amenable for the exploration of novel therapies for chronic hepatitis B [18,19]. In spite of the recent sequencing of the duck genome [20], studies on viral immunopathogenesis remain hampered by a limited repertoire of characterized immune genes and reagents available to study duck immune responses. Here we describe the cloning, sequence analyses and 3D modelling of the Pekin duck PD-L2 (duPD-L2). Furthermore, we analyze duPD-L2 mRNA expression in tissue and in lipopolysaccharide (LPS)-treated adherent and non-adherent peripheral blood mononuclear cells (PBMC). The identification of duPD-L2 will facilitate further work on the role of PD-1 signaling in the regulation of duck adaptive immunity relevant to studies on animal and human health.

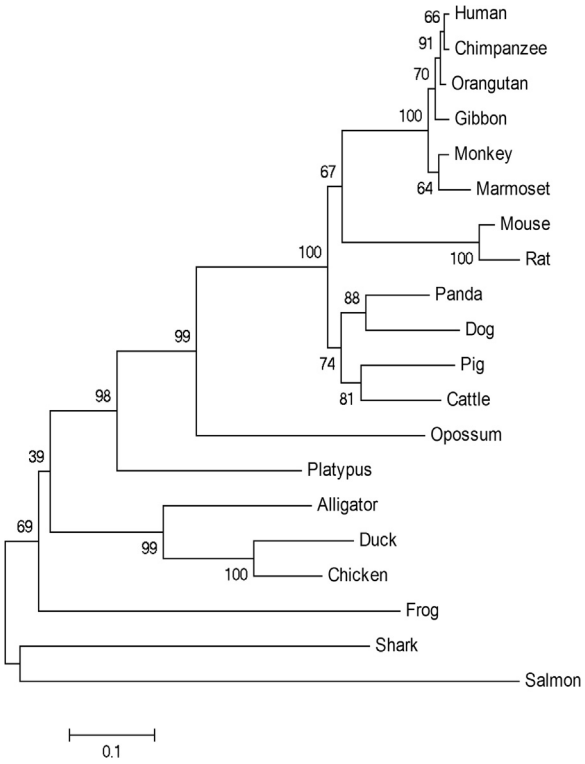


**Fig. 1. Phylogenetic tree of PD-L2 proteins.** Amino acid sequences were aligned using ClustalW and the phylogenetic tree was generated with MEGA 4 using the neighbor-joining method. Branches were validated by bootstrap analysis from 500 repetitions and are represented by numbers at the branch nodes. Bar indicates 0.1 substitutions per site. Protein sequences were extracted from GenBank with the following accession numbers: Human (*Homo sapiens*, NP_079515); chimpanzee (*Pan troglodytes,* XP_001140776); orangutan (*Pongo abelii*, XP_002819857); gibbon, (*Nomascus leucogenys*, XP_003273875); monkey (*Macaca mulatta*, NP_001077068); marmoset (*Callithrix jacchus*, XP_002742957); mouse (*Mus musculus*, NP_067371); rat (*Rattus norvegicus*, NP_001101052); panda (*Ailuropoda melanoleuca*, XP_002923722); dog (*Canis familiaris*, XP_852105); pig (*Sus scrofa*, NP_001020391); cattle (*Bos taurus*, BAO74173); opossum (*Monodelphis domestica*, XP_001371408); platypus (*Ornithorhynchus anatinus*, XP_001506123); chicken (*Gallus gallus*, XP_004949124.1); alligator (*Alligator mississippiensis*, XP_006268964); frog (*Xenopus tropicalis*, XP_012811615); shark (*Callorhinchus milii*, XP_007904718); salmon (*Salmo salar,* XP_013980940). The sequence for duPD-L2 is deposited in GenBank under accession number KU674826.

## 2. Materials and methods

### 2.1. Animals, tissues and cell cultures

Pekin ducks (*Anas platyrhynchos*) were from a flock maintained at the University of Alberta. Animals were housed, maintained and handled according to the guidelines of the Health Sciences Laboratory Animal Services (HSLAS), University of Alberta. Tissues from two-month-old Pekin ducks were resected at necropsy, snap-frozen in liquid nitrogen and stored at −70 °C. PBMC from healthy ducks were isolated by using Ficoll-Hypaque (GE Healthcare, Baie d'Urfe, Quebec, Canada) and centrifugation, suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin, streptomycin and sodium bicarbonate and cultured at 37 °C with 5% $CO_2$ in air. Adherent cells were generated from PBMC cultured in 6-well plates for 6 h, that were then washed three times with PBS and adherent cells were then cultured for 18 h with or without LPS (final concentration 5 μg/ml; Sigma, Oakville, Ontario, Canada) and then harvested in Trizol (Invitrogen, Burlington, Ontario, Canada). Scrapings of frozen tissues were placed directly in Trizol and homogenized with a micropestle before RNA isolation [21]. Total RNA was isolated from tissues and PBMC using Trizol reagent according to the manufacturer's protocol.