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ScienceDirect
Developmental and Comparative Immunology 31 (2007) 61–71
Developmental & Comparative Immunology
www.elsevier.com/locate/devcompimm

Identification and characterization of functional CD154 (CD40 ligand) in the Pekin duck

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Received 18 November 2005; received in revised form 19 April 2006; accepted 1 May 2006
Available online 6 June 2006

Abstract

Binding of CD154, a member of the TNF ligand superfamily, to its receptor CD40 is essential for the development and regulation of adaptive immune responses in mammals. The duck CD154 (DuCD154) encoding gene was isolated from activated splenocytes using RT-PCR. Sequence analysis of the cloned DuCD154 gene revealed an open reading frame of 819 base pairs encoding a 272 amino acid protein. The extracellular domain of DuCD154 was identified and expressed for characterization and generation of antibodies. DuCD154 mRNA was predominantly expressed in spleen, thymus and duodenum. DuCD154 protein generated in cell culture was secreted and formed dimers. DuCD154 markedly enhanced proliferative responses in duck splenocytes when used alone or in conjunction with LPS or PHA. These observations suggest that DuCD154 has functional equivalence with mammalian CD154 and that the central role of CD154 as an immunoregulatory protein had already evolved before the divergence of birds and mammals.

Keywords: Cd154; Cd40 ligand; Pekin duck; Splenocyte; Proliferation; Immunoregulation

1. Introduction

The tumor necrosis factor (TNF) superfamily member CD154 (CD40L), is a type II integral membrane glycoprotein, that has an important role in the development and regulation of immune responses in mammals [1,2]. CD154 is transiently expressed on activated T and B cells, but can also be found on other cell types, such as monocytes/macrophages, natural killer cells, activated platelets, endothelial cells and smooth muscle cells [3–6]. Furthermore, a soluble form of CD154 that is biologically active can be generated through proteolytic cleavage of the extracellular domain [7–9]. The cognate receptor CD40 is a transmembrane glycoprotein that shares sequence homology with the TNF-α receptor family [10]. CD40 is

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doi:10.1016/j.dci.2006.05.001

constitutively expressed on resting B cells, dendritic cells (DC), macrophages, and a variety of immune and non-immune cells [11,12]. Both cell-associated and soluble CD154 forms are able to engage the CD40 receptor.

Engagement of CD40 leads to B cell clonal expansion, germinal center formation, isotype switching, affinity maturation and the generation of long-lived plasma cells [13]. Inactivating mutations in CD154 in humans causes the X-linked hyper IgM syndrome, a severe type of immunodeficiency [14–16]. In this syndrome B cell interactions with T cells are compromised, resulting in failure of class-switching from IgM to other Ig classes and the absence of germinal centre formation. The associated defective cellular immune responses in this syndrome highlight the important role of CD154 for the initiation of effective B and T cell responses. CD154 is rapidly up-regulated on activated $CD4^+$ cells [17]. Engagement of CD40 on DCs by CD154 induces DC migration to lymph nodes, enhancement of DC survival, DC maturation and T cell priming [18–20]. Consequently, modulation of CD154–CD40 signaling pathways has been exploited in immunotherapeutic strategies to enhance adaptive immune responses or to induce tolerance [21,22].

In this study we describe the cloning, sequence analysis, expression and tissue distribution of the Pekin duck CD154 orthologue (DuCD154) and provide evidence for conserved biological activity of its extracellular domain. The cloning and characterization of this important immunoregulatory protein provides the basis for further investigation on its role in adaptive immunity and inflammation in the duck, a non-mammalian vertebrate with significant relevance for human diseases.

2. Materials and methods

2.1. Animals and cell lines

Pekin ducks (*Anas platyrhyncos*) used in this study were from a flock maintained at the University of Alberta. BALB/c mice used to generate antibodies to DuCD154 were from an in-house breeding program at University of Alberta. Both species were housed, maintained and handled according to the guidelines of the Health Sciences Laboratory Animal Services, University of Alberta.

The chicken hepatoma cell line LMH was cultured as previously described [23]. The mouse myeloma cell line SP2/0 was grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum and sodium bicarbonate at 37 °C with 5% CO_2 in air.

Duck splenocytes cultured for mRNA expression experiments were isolated by forcing fragments of spleen through a fine wire mesh followed by separation from erythrocytes by Ficoll-Hypaque centrifugation (Amersham Pharmacia, Uppsala, Sweden). Following washes with phosphate-buffered saline, splenocytes were resuspended and plated at $1–5 \times 10^6$ cells/mL in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin, streptomycin and sodium bicarbonate at 37 °C with 5% CO_2 in air. Lipopolysaccharide (LPS, *Escherichia coli* strain O127:B8; Sigma-Aldrich, Oakville, Ontario), concanavalin A (ConA; type IV-S), phytohemagglutinin M (PHA-M; Sigma L-8902), ionomycin (Sigma, I-0634) and phorbol myristic acid (PMA; Sigma, P-8139) were added to final concentrations of 10 μg/mL, 10 μg/mL, 1 μg/mL and 10 ng/mL, respectively.

2.2. Cloning and sequencing of DuCD154

Total RNA was isolated from a 24-hour splenocyte culture grown in the presence of LPS and ConA using Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbaad, CA). First-strand cDNA synthesis was performed using Superscript and oligo dT followed by polymerase chain reaction using Taq polymerase according to the manufacturer's protocols (Invitrogen). Primers FF, FR, IF1 and IR1 were selected based on the chicken CD154 sequence (Genbank AJ243435) while glyceraldehyde-6-phosphate dehydrogenase (GAPDH) primers were taken from the work of Trejbalova et al. (Table 1) [24]. Following agarose electrophoresis (1%), an amplicon was isolated using a gel extraction kit (Qiagen Inc., Mississauga, Ontario) and cloned into the plasmid pCR4-TOPO according to the manufacturer's protocol (Invitrogen). Clones bearing inserts were sequenced and BLAST comparison identified a clone, 13-5, as having an ORF showing homology with the full-length chicken CD154 sequence.

In order to determine the nucleotide sequences at the extreme 3′ and 5′ of the duck CD154 ORF (i.e. to which primers FF and FR bound), both 5′ and 3′ RACE were performed. Total RNA from duck splenocytes treated with PHA-M (6 μg/ml) for 5 hours was used as source material for both 5′ and 3′