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Short communication

An interaction between a C-type lectin receptor and leukocyte cell-derived chemotaxin 2 of ayu, *Plecoglossus altivelis*

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ABSTRACT

Yeast two-hybrid (Y2H) screens were used to test for interactions between leukocyte cell-derived chemotaxin 2 (LECT2) and a liver cDNA expression library of ayu, *Plecoglossus altivelis*. Of the 9 independent interacting clones identified, 2 were identical and closely related to C-type lectin receptor (CLR) genes of fish, while the other 7 were partial sequences from transferrin genes. Ayu CLR (aCLR) showed similarity to immune-relevant mammalian receptors in terms of amino acid sequence and overall organization within the C-type lectin-like domain (CTLD). The aCLR transcript was detected with the highest levels in the head kidney and peripheral blood leukocytes (PBLs), and more weakly in the heart, liver and gill. The interaction between aCLR and ayu LECT2 (aLECT2) was confirmed by *in vitro* co-immunoprecipitation of the two proteins. This interaction may be responsible for the “neutrophil-chemotactic” characteristic of LECT2. Y2H assays using different parts of the two proteins showed that the CTLD part of aCLR was involved in the interaction with mature aLECT2, and the contact structure of CTLD was essential for the interaction. The identification of this CLR/LECT2 interaction sheds light on the mechanism of serum LECT2 changes resulting in cellular immune responses.

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Leukocyte cell-derived chemotaxin 2 (LECT2) was first isolated from the culture fluid of the human (*Homo sapiens*) T cell line SKW-3 and was shown to have neutrophil chemotactic activity [1]. Proteins homologous to LECT2 have since been isolated in many other vertebrates. Current evidence suggests that LECT2 may be a multifunctional protein, involved in cell growth, differentiation, damage/repair processes and in the autoimmune response [2–5]. In recent studies, fish LECT2 transcripts were significantly increased after bacterial infection, indicating that LECT2 might participate in immune regulation of fish [6,7].

Ayu (*Plecoglossus altivelis*) is an important cultured freshwater fish in Japan and China. However diseases occur frequently and limit production efficiencies and food quality of this economic fish. Recently, ayu bacterial diseases have been posing a serious threat to cultured ayu in Mainland China [8]. Resistance to infectious diseases would improve ayu production efficiency, but the cellular and molecular processes involved in disease resistance are poorly understood in this species and we have therefore begun to study the ayu genes related to immunology. In this paper, we report studies using a yeast two hybrid (Y2H) system showing that LECT2

interacts with a C-type lectin receptor (CLR) of ayu. The possible roles of this interaction in the immune responses are discussed.

About 30 healthy ayu, weighing 20–25 g each, were obtained from a commercial farm in Huangtian Reservoir, Ningbo City, China. These fish were kept in freshwater tanks at 20–22 °C in a recirculating system with filtered water, fed with pelleted dry food once a day, and acclimatized to laboratory conditions for at least one week before experiments. All fish used in this study were apparently healthy without any pathological signs. The construction of the bait plasmid pGBKT7-aLECT2 and ayu liver cDNA library for Y2H was done as previously described [9].

Using expressed aLECT2 as bait in Y2H screens, 9 independent interacting clones were identified after two separate screenings of an ayu cDNA expression library (2.5×10^6 independent clones). Each clone could induce the two reporter genes, allowing growth on quadruple dropout plates and expression of α -galactosidase activity. Sequencing showed that 2 of 9 were partial sequences from a C-type lectin receptor gene, while the other 7 clones were partial sequences from a transferrin (Tf) gene [9]. Subsequently, the complete cDNA sequence of ayu C-type lectin receptor gene (EMBL accession number: FN396582) was determined. It was 1265 nucleotides in length excluding the poly(A) tail. The ORF was predicted to produce a protein of 256 amino acids with MW 29.30 kDa.

Database searching and alignment using BLAST indicates that ayu C-type lectin receptor was most similar to the previously

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reported Atlantic salmon (*Salmo salar*) C-type lectin receptor A (sCLR) [10], and it was therefore tentatively named as ayu C-type lectin receptor (aCLR). Mammalian dendritic cell (DC) – specific intercellular adhesion molecule 3 – grabbing non-integrin receptor (DC-SIGN) had the most closely related complete C-type lectin domain (CTLD) compared to that of fish CLR by a BLASTP non-redundant database search. aCLR was predicted to be a monomeric transmembrane receptor like sCLR, as there was no recognizable coiled-coil domain in its extracellular region (Fig. 1). aCLR had a longer N-terminal cytoplasmic tail with a potential tyrosine phosphorylation site (YTSL) that resembles an ITIM-like motif (YxxL) [11]. The CTLD was present at its C-terminus and consisted of 139 residues (amino acid position 118–256). aCLR had a number of conserved residues characteristic of the C-type lectins [12]. Each had six cysteine residues at conserved positions within the CTLD required for formation of the three intramolecular disulfide bonds (C₁₁₈–C₁₂₉, C₁₄₆–C₂₄₈, C₂₂₅–C₂₄₀) that were essential for CTLD fold stability (Fig. 1). It also had four residues essential in coordinating a Ca²⁺ ion at site 1 and five of the six residues at site 2 (Fig. 1). The motif that controls carbohydrate specificity was Glu-Pro-Asn (EPN), which primarily binds mannose and related carbohydrates [10]. aCLR contained such a motif at position 211–213 (Fig. 1).

From the amino acid sequence analysis, aCLR has a relatively higher similarity to that of the sCLR (71.4%) than to that of other fish CLR (41.6–69.5%). It had 21.7–25.3% identity to those immune-related, lectin-like receptors (Illrs) from zebrafish (*Danio rerio*), while less than 15% identity to mammalian DC-SIGN. Phylogenetic analysis showed that most fish CLR formed a large cluster, while zebrafish Illrs formed another separate small cluster. Mammalian DC-SIGN grouped tightly and formed a cluster distantly related to two fish clusters (Fig. 2).

Ayu tissues were dissected from healthy fish, and peripheral blood leukocytes (PBLs) were purified as previously described [10]. The

total RNAs from tissues (5 µg) and cells (2 µg) were purified using RNAiso reagents (TaKaRa, Kyoto, Japan). A 961 bp fragment of the ayu CLR mRNA was then amplified by reverse transcription polymerase chain reaction (RT-PCR) using the RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Kyoto, Japan) with primers pCLR(+): 5'-TGATTGGATACTA GACCAAG-3' and pCLR(-): 5'-CAATCTGCCCTCAATTGAC-3'. As an internal PCR control, primers pActin2(+): 5'-TCGTGCGTGACATC AAGGAG-3' and pActin2(-): 5'-CGCACTTCATGATGCTGTG-3' were used to amplify a 231 bp fragment of the housekeeping β-actin gene. RT-PCR conditions were: denaturing for 2 min at 94 °C, then 30 cycles of denaturing for 30 s at 94 °C, annealing for 30 s at the optimized temperature (57.0 °C for CLR, 60.5 °C for β-actin) and extension for 60 s at 72 °C; the cycles were followed by a final extension step of 10 min at 72 °C. The products were separated on a 1.5% agarose gel and stained with ethidium bromide. The aCLR transcripts were detected with the highest levels in the head kidney and PBLs, and weakly in the heart, liver and gill (Fig. 3). The mRNA expression pattern of aCLR was similar to that of sCLR [10]. To examine the mRNA expression changes following *Listonella anguillarum* infection, the liver and head kidney from artificially infected fish were collected at 6 h post-injection (hpi). Transcript abundances of aLECT2 and aCLR were quantified using quantitative real-time PCR (qPCR), as described previously [13]. Data indicated that bacterial infection caused a significant up-regulation in the mRNA expression level of aLECT2 in liver (up to 67.3-fold) and aCLR in head kidney (up to 12.5-fold) at acute phase (6 hpi), normalized against β-actin.

A plasmid pGADT7-aCLR with the complete aCLR ORF in a pGADT7 vector was constructed subsequently and retransformed back to yeast either alone or in combination with pGBKT7-aLECT2, Lamin C, or the binding domain vector alone. The reporter genes were only induced in combination with pGBKT7-aLECT2. The interaction between aLECT2 and aCLR was confirmed by *in vitro* co-immunoprecipitation (Co-IP) of the two proteins expressed from

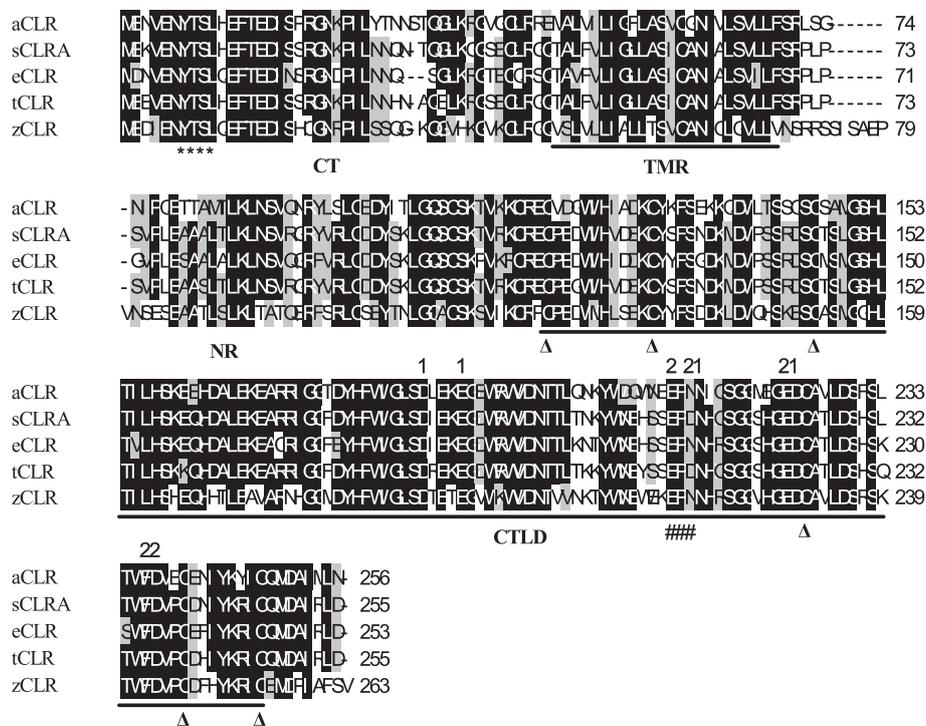


Fig. 1. Predicted amino acid sequence of ayu CLR (aCLR) aligned with the homologous sequences from Atlantic salmon (*Salmo salar*) CLR (sCLR) (NM_001123579), Northern pike (*Esox lucius*) CLR (eCLR) (BT079281), rainbow trout (*Oncorhynchus mykiss*) CLR (tCLR) (AY593994), and zebrafish (*Danio rerio*) CLR (zCLR) (BC134972) using the ClustalW program. Threshold for shading is >70%, similar residues are marked as gray shadow, identical residues as black shadow and alignment gaps as "Δ". CT: cytoplasmic tail; TMR: transmembrane region; NR: neck region; CTLD: C-type lectin domain. Conserved Cys residues are marked as "Δ". A novel YXXL-dependent signaling cascade is marked as "*****". A mannose-binding lectin motif "EPN" is marked as "####". The numbers 1 and 2 above the alignment indicate the residues normally conserved among CTLDs which coordinate Ca²⁺ at sites 1 and 2.

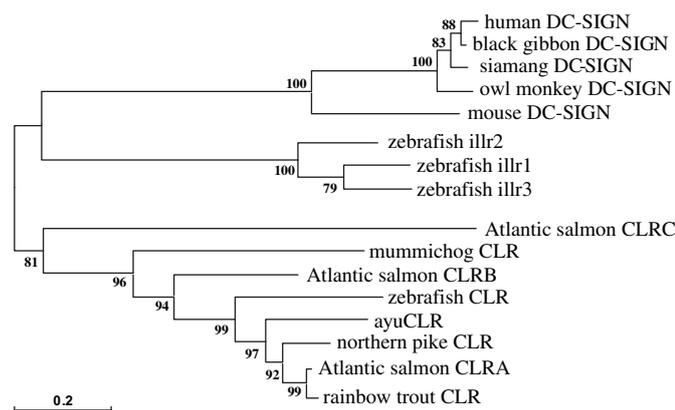


Fig. 2. Phylogenetic (neighbor-joining) analysis of C-type lectin receptor amino acid sequences using the MEGA4.0 program. The values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping (1000 replicates; shown only when >60%). The scale bar shows the number of substitutions per base. Accession numbers of sequences used are mouse (*Mus musculus*) DC-SIGN, AF373408; siamang (*Hylobates syndactylus*) DC-SIGN, AY078884; owl monkey (*Aotus trivirgatus*) DC-SIGN, EU041930; human (*Homo sapiens*) DC-SIGN, AF209479; black gibbon (*Hylobates concolor*) DC-SIGN, AY078891; zebrafish (*Danio rerio*) CLR, BC134972; zebrafish illr1, NM_001040050; zebrafish illr2, NM_001128371; zebrafish illr3, AL928975; mummichog (*Fundulus heteroclitus*) CLR, AY735176; ayu (*Plecoglossus altivelis*) CLR, FN396582; rainbow trout (*Oncorhynchus mykiss*) CLR, AY593994; Atlantic salmon (*Salmo salar*) CLRA, NM_001123579; Atlantic salmon CLRB, NM_001123580; Atlantic salmon CLRC, NM_001123581; Northern pike (*Esox lucius*) CLR, BT079281.

the TNT T7 Coupled Reticulocyte Lysate System [9]. Proteins of the expected sizes of 18.6 kDa (c-Myc-aLECT2) and 30.9 kDa (HA-aCLR) were obtained, but were not detected in the control (Fig. 4). aLECT2 and aCLR were prokaryotically expressed, and polyclonal antisera against them were then raised in mice (Balb/C), respectively. Western blots using these two polyclonal antisera were performed on Co-IP product, and confirmed that the bands noted were indeed the proteins of interest (Fig. 5).

The aLECT2 gene was divided into two parts (Table 1) and each was inserted into a pGBKT7 vector. The aCLR gene was divided into several parts (Table 1) according to the deduced structure (Fig. 1) and each was inserted into a pGADT7 vector. Y2H assays using different parts of the two proteins (Table 1) showed that the CTLD part of aCLR was involved in the interaction with mature aLECT2, and the contact structure of CTLD was essential for the aCLR/aLECT2 interaction (Table 1).

In accordance with the previous reports, ayu LECT2 and CLR transcripts were significantly increased after bacterial infection, indicating that they two might participate in immune regulation [6,7,10]. Although there is little information on fish LECT2 and CLRs, studies on well-characterized mammalian LECT2 and CLRs should provide functional clues. Besides its neutrophil chemotactic activity [1], some investigations further indicate that mammalian LECT2 should play a role in immune regulation. In LECT2-deficient mice, the proportion of NKT cells in the liver increased and hepatic injury was exacerbated in severe concanavalin A-induced hepatitis [5].

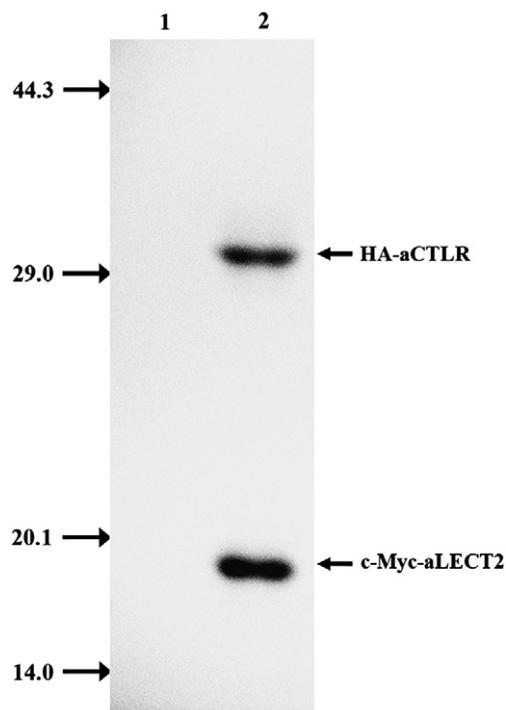


Fig. 4. Colorimetric (BCIP/NBT) analysis of aLECT2 co-immunoprecipitates with aCLR. 1, c-Myc-aLECT2 immunoprecipitated with anti-HA; 2, co-immunoprecipitates of the biotinylated HA-aCLR and c-Myc-aLECT2. The sizes of the HA and c-Myc epitope tags were 1.5 and 1.6 kDa respectively. The positions of protein size markers (kDa) are shown on the left.

The significantly exacerbated arthritis and altered expression of inflammatory cytokines were also found in LECT2-deficient mice [4]. These two reports revealed that LECT2 might be an important regulator of leukocyte differentiation and activation. However, as a cytokine, the receptor of LECT2 remains unclear. Mammalian DC-SIGN contains the most closely related complete CTLD to that of fish CLRs, which recognize specific carbohydrate structures of pathogens to internalize pathogens for degradation in lysosomal compartments to enhance antigen processing [14]. The DC-SIGN cytoplasmic tail also carries a YxxL motif, which is known to play a role in the induction of intracellular signals related to DCs maturation [15]. Therefore, we suggest that DC-SIGN or similar CLRs are possible receptors of LECT2. The interaction between CLR and LECT2 of ayu suggests that serum LECT2 may influence the immune system through the activation of CLRs and intracellular signaling pathways, and their roles in immune regulation of fish might be closely related to macrophage activation and polarization.

In conclusion, CLRs may be an important component involved in LECT2 signaling transduction, and CLR/LECT2 interaction may be responsible for the “neutrophil-chemotactic” characteristic of LECT2. Our study at least sheds some light on the mechanism of serum LECT2 changes resulting in cellular immune responses, but much more investigations are needed.

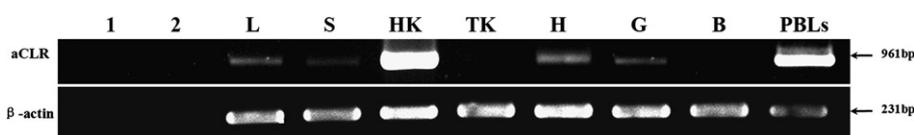


Fig. 3. RT-PCR analysis of aCLR mRNA expression in various tissues (or cells) of ayu fish. 1, ayu genomic DNA as template; 2, without template; L, liver; S, spleen; HK, head kidney; TK, trunk kidney; H, heart; G, gill; B, brain; PBLs, peripheral blood leukocytes. β -actin amplification included as an internal control. The size of the products is shown on the right.

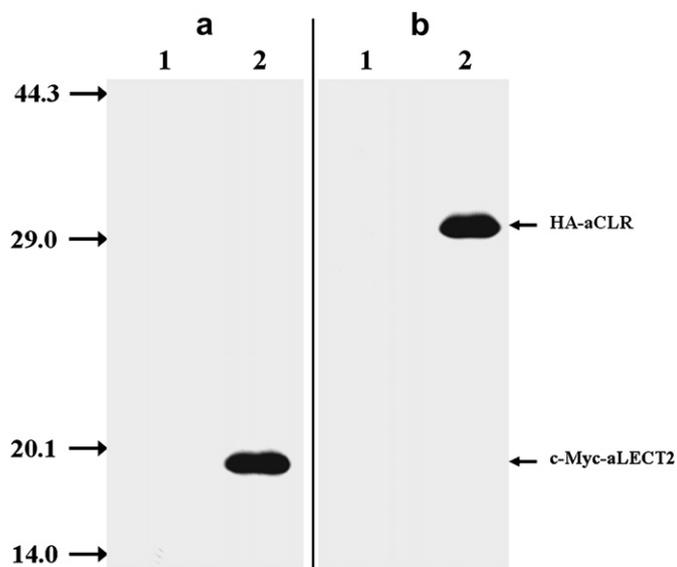


Fig. 5. Western blot analysis using an anti-aLECT2 (a) or anti-aCLR (b) polyclonal antiserum on Co-IP product. 1, c-Myc-aLECT2 immunoprecipitated with anti-HA; 2, co-immunoprecipitates of the biotinylated HA-aCLR and c-Myc-aLECT2. The sizes of the HA and c-Myc epitope tags were 1.5 and 1.6 kDa respectively. The positions of protein size markers (kDa) are shown on the left.

Table 1

Interactions (negative, –; positive, +) determined from Y2H experiments between different regions of the aCLR and aLECT2. Human lamin C is included as a non-specific control. The numbers are the amino acid positions in the protein sequence. CT: cytoplasmic tail; TMR: transmembrane region; NR: neck region; CTLD: C-type lectin domain; aa: amino acid.

	CT	TMR	NR	CTLD		
	aa1–45	aa46–69	aa70–117	aa118–256	aa118–188	aa189–256
aLECT2 aa22–156	–	–	–	+	–	–
aLECT2 aa1–21	–	–	–	–	–	–
lamin C	–	–	–	–	–	–

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