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Complexity of expressed CHIR genes

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ABSTRACT

The chicken leukocyte receptor complex (LRC) encodes an unprecedented number of chicken Ig-like receptor (CHIR) genes compared to the mammalian LRC. Although there are at least 100 CHIR genes in the LRC, only little information is available about the number and variability of expressed CHIR. Recently, we showed that CHIR with one Ig domain encode a variety of different affinity IgY receptors, which are highly variable in different chicken strains. The current report focused on expressed CHIR with two Ig domains. Oligonucleotides specific for conserved regions at the 5′ end of Ig1 and 3′ end of Ig2 were used on PBMC mRNA obtained from two individual chickens with different MHC haplotypes (M11, R11). Sequencing of 142 colonies of M11 and 117 of R11 yielded 98 and 70 different CHIR2D amino acid sequences, respectively. Comparing a total of 219 CHIR sequences, including also a genomic dataset from an LSL chicken, revealed a single amino acid sequence identical between all three chicken strains, and four sequence pairs either shared between M11 and R11 or between M11 and LSL. Calculating Wu-Kabat variability revealed three amino acid positions, which were highly variable and the analysis of synonymous/non-synonymous ratio indicated positive selection. This analysis of expressed CHIR genes in different chickens demonstrates an unusual polymorphism of expressed receptors, where only few are conserved between chickens.

1. Introduction

The human leukocyte receptor complex (LRC) is located on chromosome 19q13.4 and contains two multigene families: the killer cell Ig-like receptors (KIR) and the leukocyte Ig-like receptors (LILR). Apart from several mammalian species, LRC orthologous regions have also been characterized in other vertebrates such as teleost fish and amphibians, where in all cases highly diverse multigene families have been identified [1–4]. The corresponding chicken LRC, which was located to chromosome 31 by FISH analysis in LSL chicken, is vastly expanded and encodes the chicken Ig-like receptors (CHIR) [5,6]. In the current version of the chicken genome, originated from Red Jungle Fowl, chromosome 31 is still unassembled. Structurally, there are four major types of CHIR genes [5,7]: CHIR-A, displaying two extracellular C2-type Ig-domains, a basic transmembrane residue (arginine) and a short cytoplasmic tail indicative for an activating receptor, when associated with an ITAM bearing co-receptor (e.g. CHIR-A2). CHIR-B, also displaying two C2-type Ig-domains, but followed by an uncharged transmembrane region and a long cytoplasmic tail with ITIMs or ITSMs representing an inhibitory receptor (e.g. CHIR-B2). CHIR-AB, containing only a single C2-type Ig-domain, a basic residue in the transmembrane region and a long cytoplasmic tail with one ITIM and one YXXM motif (e.g. CHIR-AB1) and finally, CHIR-AB with two C2-type domains, a basic residue in the transmembrane region and ITIMs or ITSMs in the long cytoplasmic tail (e.g. CHIR-AB3). The latter two are supposed to have bifunctional potential, since they display features of both inhibitory and activating receptors. The multigene family character of CHIR was demonstrated by Southern blot analysis with DNA samples from single animals of four different chicken lines homozygous for different MHC haplotypes [5]. Hybridization with the Ig1 domain of CHIR-A2 showed multiple bands under highly stringent conditions. Surprisingly, besides common bands, found in all four chicken lines, there were multiple line-specific bands, indicative of the polymorphic nature of the CHIR gene locus. It is to note that restriction fragment length polymorphism is not automatically reflecting the genes themselves, but a common way to show polymorphism, especially when detailed information of the genomic locus is missing. Although the chicken genome project, which represents red jungle fowl, was finished in 2004, up to now CHIR genes are only represented on chromosome unassigned. To gain more insight in the chromosomal organization Laun et al. [6] screened the Wageningen Chicken BAC library, which was originated from a single LSL chicken [8]. They obtained 18 CHIR positive BAC clones. Sequencing of seven clones yielded an unfinished

Abbreviations: PBMC, peripheral blood mononuclear cells; LRC, leukocyte receptor complex; CHIR, chicken Ig-like receptor; KIR, killer cell Ig-like receptor; LILR, leukocyte Ig-like receptor.

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fragmented insight into the chicken LRC covering 550 kb in four groups. In total 103 CHIR genes were identified: 25 CHIR-A, 22 CHIR-B, 10 CHIR-AB with one Ig domain, 5 CHIR-AB with two Ig domains and 41 pseudogenes. Similar to the situation known from mammalian LILR genes, CHIRs are expressed on various different chicken cells as shown by RT-PCR [5]. Moreover mAb 3H7, specific for CHIR-B2, showed protein expression mainly on B lymphocytes [9], whereas mAb 8D12, specific for CHIR-AB1, was used to detect CHIR-AB1 on B lymphocytes, monocytes, macrophages and NK cells in the gut [10].

One of our recent studies focused on the identification of CHIR ligands. By employing BWZ.36 reporter assays we showed that CHIR-AB1, representing a CHIR with one extracellular Ig domain, is a high affinity primordial FcY receptor [10]. Interestingly, the interaction of CHIR-AB1 and IgY is similar to the human IgA:FcαRI interaction [11]. Furthermore we could show that there are additional CHIR-AB1-like receptors also involved in FcY binding [12]. Functional data about CHIRs with two extracellular Ig domains is only present for CHIR-B2, which recruits tyrosine phosphatases SHP-1 and -2 and inhibits proliferation of a B cell line [9]. The ligand and the physiological function of CHIR-B2 still remains elusive.

In the current report, we applied a cloning strategy to show that a large number of CHIR with two domains (CHIR2D) is expressed in individual chickens. The comparison between MHC defined chickens lines shows only few conserved sequence pairs, with only one sequence identical between three lines. This work emphasizes the excessive amount and variability of CHIR expressed in an individual chicken, but also indicates that there are few conserved CHIR, potentially representing CHIR with conserved ligands and/or functions in all strains.

2. Materials and methods

2.1. Animals

Chicken lines M11 (B2) and R11 (B15) are homozygous for the MHC allele indicated. M11 and R11 chicken eggs are a kind gift from S. Weigend (Mariensee, Germany). Chickens were hatched at the institute and the animals were used for experiments in the age of 6–10 weeks.

2.2. Database searches

CHIR with two extracellular Ig domains (CHIR2D) were identified by Blast Analysis in seven CHIR specific BAC clones sequenced previously [6]. Since these sequences are not annotated in the GenBank database, the respective BAC clones (WAG-4C11, Acc. No. BX663523; WAG-93H17, Acc. No. BX663526; WAG-112A23, Acc. No. BX663527; WAG-19H9, Acc. No. BX663529; WAG-52G8, Acc. No. BX663530; WAG-58B13, Acc. No. BX663534; WAG-88M21, Acc. No. BX897752) were analysed in detail. The sequences were introduced to GENSCAN Analysis (http://genes.mit.edu/GENSCAN.html) [13]. Predicted exons were further investigated for the existence of Ig-like receptor genes using SMART (http://smart.embl-heidelberg.de/) [14] and the predicted CHIR were assigned to genes and pseudogenes as illustrated in Laun et al. [6]. In total, the coding regions of 51 CHIR2D were extracted from the BAC clone sequences. The extracytoplasmic parts were used for designing primers specific for conserved regions at the 5′ end of the Ig1 domain and at the 3′ end of the Ig2 domain.

2.3. Cell preparations and cloning procedures

PBMC (peripheral blood mononuclear cells) were prepared by density centrifugation on Ficoll-Paque (GE Healthcare). Total RNA was prepared using Absolutely RNA RT-PCR Miniprep Kit (Stratagene) and cDNA synthesis was performed with the ThermoScript RT (Invitrogen), with an estimated error rate of 1 error in 15,500 bp. For cloning of different CHIR2D, Herculase® Enhanced DNA Polymerase (Stratagene), a proofreading polymerase with an estimated error rate of only 2×10^{-6} mutations per bp was used for PCR at 2 min of denaturation at 95 °C, 35 cycles of 10 s at 95 °C, 30 s at 60 °C, 2 min at 72 °C and a final extension time of 10 min at 72 °C using sense (GAATTCCTGCCCCGACCCTCCCTG) and antisense primer (GAATTCTGCAGGTGTG GGTGTCAC). Restriction sites as underlined were introduced to facilitate subcloning for subsequent applications. PCR products were cloned into a pcDNA3.1/V5-His TOPO Vector (Invitrogen), transformed in One Shot® TOP10 chemically competent cells and cultivated overnight on 4 Petri dishes containing LB agar and 100 μg/ml ampicillin (Applichem). All colonies were screened by PCR, plasmids of positive colonies were isolated using the NucleoSpin® Plasmid Kit (Macherey-Nagel) and sequenced (GATC, Konstanz). Deduced amino acid sequences were further analysed using the Lasergene Software (GATC, Konstanz) and jpred3 (http://www.compbio.dundee.ac.uk/~www-jpred/) [15] for secondary structure prediction. The different CHIR2D were compared using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html) and a phylogenetic tree was constructed using MEGA4 [16]. The consensus sequence of all CHIR2D was used to create a 3D model using Swiss Model automated mode (http://swissmodel.expasy.org/) [17,18] and visualized using the SwissPdb Viewer (http://www.expasy.org/spdbv/) [19]. For further analysis of highly variable regions of the different CHIR2D sequences Wu-Kabat variability was calculated using the protein variability server (http://imed.med.ucm.es/PVS/) [20].

The variability coefficient is computed using the following formula:

$$Variability = \frac{N * k}{n}$$

where N is the number of sequences in the alignment, k is the number of different amino acids at a given position and n is the frequency of the most common amino acid at that position. For highly variable amino acid positions the number of synonymous and non-synonymous substitutions was determined manually.

3. Results

Full length CHIR sequences currently present in the databases, represent either genomic sequences retrieved from a sequencing project of CHIR specific BACs obtained from a single LSL chicken [6] or CHIR mRNA sequences cloned randomly from different sources [5,9,10,12]. There is also a limited number of CHIR present in the chicken genome project representing an individual red jungle fowl chicken, but since they are all localized on chromosome unassigned and mostly fragmented sequences, they were not included in the initial analysis. Analysing 7 different LSL BAC clones revealed a total of 51 different CHIR2D representing 21 potentially inhibitory CHIR2B, 25 potentially activating CHIR2A and 5 potentially bifunctional CHIR2C. It is to note that the nomenclature for the bifunctional receptors differs between Laun et al. and our group, naming them CHIR-AB (used herein) and not CHIR1C. The nucleotide sequences of the two extracytoplasmic Ig domains of the 51 CHIR2D obtained from LSL BACs were combined with the respective region of CHIR previously cloned by our group (Acc. Nos.: AJ745093, CHIR-A2; AJ639837, CHIR-B2; AJ639838, CHIR-B3; AJ639839, CHIR-B4; AJ879908, CHIR-B5; AJ879910, CHIR-B6; AJ879909, CHIR-AB3) and aligned by ClustalW (data not shown). The 5′ end of the first Ig domain and the 3′ end of the second Ig domain were used to design primers which match to a majority of the CHIR2D sequences. These primers were used in PCR using