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A Novel Activating Chicken IgY FcR Is Related to Leukocyte Receptor Complex (LRC) Genes but Is Located on a Chromosomal Region Distinct from the LRC and FcR Gene Clusters

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FcRs have multifaceted roles in the immune system. Chicken FcRs were demonstrated on macrophages decades ago; however, only recently the chicken Ig-like receptor AB1, encoded in the leukocyte receptor complex, was molecularly identified as a high-affinity FcR. The present study was initiated to identify additional receptors with the capability to bind chicken immunoglobulins. Based on database searches, we cloned a novel chicken FcR, designated gallus gallus FcR (ggFcR), which was shown to bind selectively chicken IgY. The receptor consists of four extracellular C2-set Ig domains, followed by a transmembrane region containing arginine as a positively charged amino acid and a short cytoplasmic tail. ggFcR associates with the common γ-chain, indicative for an activating receptor, and real-time RT-PCR revealed high expression on PBMC, thrombocytes, and macrophages. The genomic organization is similar to most Ig-like receptor genes, where each Ig domain is encoded by a separate exon. Additionally, the ggFcR signal peptide is encoded by two exons, the second of which is 36 bp, a hallmark for genes encoded in the leukocyte receptor complex. Surprisingly, ggFcR is not encoded in the leukocyte receptor complex, but it is located as a single isolated gene at the extremity of chicken chromosome 20. The Journal of Immunology, 2009, 182: 1533–1540.

Numerous immunoregulatory cell surface receptors have been discovered in recent years. They form multigene families located on distinct chromosomes that comprise both inhibitory and activating family members (1). The inhibitory receptors display a long cytoplasmic tail with ITIM motifs, whereas activating receptors have only a short cytoplasmic tail, but feature a basic amino acid in the transmembrane region, which interacts with an ITAM-containing adaptor molecule, such as the common γ-chain, DAP12, or CD3ζ. The extracellular part of the receptor displays either a C-type lectin structure or a different number of Ig domains. Immunoregulatory families with Ig-like domains are broadly distributed on various immune cells both of the innate and adaptive immune systems. In most cases, the binding partners of the respective immunoregulatory Ig-like receptors are still unknown or were discovered only recently. One exception is the family of classical FcRs. They have long been shown to bind the constant region of different immunoglobulins (IgG, IgE, IgA) and are involved in various immune reactions, such as phagocytosis, Ab-dependent cellular cytotoxicity, and immediate hypersensitivity (2). FcRs differ by their ligand preferences, binding affinity, and signal properties: FcγRI, the high-affinity receptor for IgG; FcεRI, the high-affinity receptor for IgE; and FcγRIIIa, a low-affinity receptor for IgG, are activating receptors that signal via the common γ-chain, whereas FcγRII and its isoform are low-affinity receptors for IgG, which have either ITAMs (FcγRIIa and FcγRIIc) or ITIMs (e.g., FcγRIIb) in their cytoplasmic regions (3). In man, most FcRs are located on chromosome 1 (2). One exception to this is the human FcγRI, which is encoded in the leukocyte receptor complex (LRC)5 on chromosome 19 (4). Another family of immunoregulatory Ig-like receptors, which is closely related to the FcRs, is also located on human chromosome 1. The so-called FcR-like (FCRL) genes are also expressed on a variety of different leukocytes, but, in contrast to the classical FcRs, they have not been shown to bind Ig (5). The functional importance of these proteins is still an unresolved issue.

Recently, an FCRL homolog that is highly related to mammalian FcR and FCRL genes was also identified in the chicken (6, 7). To date, this gene is the only FcR/FCRL gene present on chicken chromosome 25, which represents the syntenic region to the mammalian FcR locus. This receptor has not been shown to bind IgY and is therefore proposed to be an FCRL homolog.

Another large immunoregulatory Ig-like receptor family is formed by the chicken Ig-like receptors (CHIR). These genes are located in the LRC on chicken chromosome 31, and thus they are likely homologous to mammalian leukocyte Ig-like receptors (LILR), killer cell Ig-like receptors (KIR), or other LRC-encoded receptors.

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genes (8, 9). However, in contrast to their mammalian counterparts, CHIR display >60 functional receptor genes with extensive haplotype and allelic variation, including also potentially bifunctional receptors. Recently, we showed that one member, CHIR-AB1, functions as a high-affinity IgY FcR (10). CHIR-AB1 combines features of several mammalian FcRs, such as expression on macrophages, NK cells, and B cells, as well as its potential to signal as inhibitory or activating receptors, based on the presence of a charged transmembrane residue and a cytoplasmic ITIM.

This study was initiated to identify additional FcR-related genes in the chicken based on the analysis of various chicken databases. As shown below, we identified a novel FcR gene in the chicken, designated gallus gallus FcR (ggFcR) (accession no. FM200428), which selectively binds chicken IgY. We demonstrate that it is a potentially activating receptor, which interacts with the common γ-chain and is mainly expressed on chicken PBMC. Surprisingly, ggFcR is highly related to mammalian and chicken LRC-encoded genes, but it is located on chromosome 20, a location different from the chicken FcR and LRC regions.

**Materials and Methods**

**Database searches and in silico sequence analysis**

Various chicken expressed sequence tag (EST) databases were used to identify FcR genes by key word search. In particular we used chicken EST databases provided by the Biotechnology and Biological Sciences Research Council (www.chick.uminist.ac.uk/), by the Delaware Biotechnology Institute (www.chick.udel.edu/), and by the Bursal Transcript Database (pheasant.gsf.de/DEPARTMENT/DT40/dt40Transcript.html). Additional ESTs were identified by employing the basic local alignment search tool (BLAST) program limited to “gallus gallus” EST databases with a total of 600,000 ESTs deposited and used to obtain a full-length open reading frame of ggFcR. All resulting ESTs were further analyzed using the Lasergene software package (GATC Biotech), which was manually refined and used for primer design of ggFcR. Deduced amino acid sequence was analyzed for structural elements such as signal peptide, Ig domains, transmembrane regions, and secondary structure elements using SMART (smart.embl-heidelberg.de/ (11), 12), InterProScan (www.ebi.ac.uk/InterProScan/) (13), SignalP 3.0 (www.cbs.dtu.dk/services/SignalP/) (14), THMM 2.0 (www.cbs.dtu.dk/services/TMHMM-2.0/), and Jpred3 (www.compbio.dundee.ac.uk/~www-jpred/) (15). Corresponding genomic DNA was identified using the cloned ggFcR in a BLAST-like alignment tool search of the chicken genome (genome.ucsc.edu/cgi-bin/hgGateway). For phylogenetic analysis, a neighbor-joining tree was constructed using MEGA4 (16). The HomoloGene database (www.ncbi.nlm.nih.gov/sites/entrez?db=homologene) was used for further analysis of the chromosomal region.

**Radiation hybrid (RH) mapping**

The ChickRH6 panel (17) was used to map the ggFcR and the lethal (3) lethalthal3malignant brain-tumor-like protein (L3MBTL) gene. Primer sequences and fragment sizes are given in Table I. Chicken and hamster genomic DNAs were used as positive controls whereas TE buffer was used as a negative control. Twenty-five nanograms of each panel DNA was amplified in an 8-μl PCR reaction containing 1 × PCR Master Mix (ABgene) and 4 μM of each of the two primers. PCR reaction was performed in duplicates. PCR conditions were 3 min at 95°C followed by 35 cycles of 30 s at 95°C, 45 s at 55°C, and 60 s at 72°C, followed by a final elongation step at 72°C. Markers were mapped using the CarthaGene program (www.inra.fr/bia/T/CarthaGene/).

**Animals and cell preparation**

Chickens (line M11, MHC haplotype B2/B2, a kind gift from S. Weigend, Mariensee, Germany) were hatched at the institute and used for experiments at the age of 6–10 wk.

PBMC were prepared by slow-speed centrifugation as described (18), and PBMC were prepared by density centrifugation on Ficoll-Paque (GE Healthcare). PBMC were further used for macrophage preparation by adhering them for at least 48 h on petri dishes (19). Chicken thrombocytes were prepared by short-term cultures of PBMC on petri dishes for 4 h (20). For spleen, liver, and brain RNA preparation, 100 mg of each tissue was taken and directly frozen in liquid nitrogen.

**Cloning procedures**

Total RNA from PBMC was prepared using the Absolutely RNA RT-PCR miniprep kit (Stratagene), and cDNA synthesis was performed with the Thermoscript RT-PCR system (Invitrogen). Herculase enhanced DNA polymerase (Stratagene) was used for PCR at 2 min of denaturation at 95°C, 35 cycles of 10 s at 95°C, 30 s at 55°C, 2 min at 72°C, and a final extension time of 10 min at 72°C. For cloning of ggFcR, oligonucleotides 922s and 923as (Table II and Fig. 1A) were used. The resulting PCR product was TA cloned into a pCRII TOPO vector (Invitrogen), colonies were screened by PCR and restriction analysis, and plasmids from positive colonies were isolated using the Nucleospin plasmid kit (Macherey-Nagel) and sequenced (GATC). The full-length ggFcR-FLAG construct was generated using oligonucleotides 1038s and 1229as with EcoRI site (Table II) as described previously (21). The ggFcR-FLAG-muCD3ζ construct was generated using oligonucleotides 1038s and 931as (Table II) on ggFcR cDNA as described earlier (10), resulting in a N-terminally FLAG-tagged extracellular region of ggFcR fused to the transmembrane region of chicken CD8α and the cytoplasmic domain of murine CD3ζ.

**Cell lines, transfections, and cell stainings**

Human embryonic kidney 293T cells (22) and the mouse thymoma cell line BWZ.36 (23) were maintained in RPMI 1640 (Biochrom) supplemented with 10% FCS and 1% penicillin/streptomycin in a CO2 incubator at 37°C. 293T cells were double transfected with the full-length ggFcR-FLAG construct and either the common γ-chain-V5 construct (FcγRIy) (8) or a mock-V5 control using the Metafectene reagent (Biontex Laboratories). According to the manufacturer’s protocol. After 24 h of transient transfection, cells were used for staining with a mouse anti-FLAG mAb (Sigma-Aldrich) followed by an anti-mouse Ig-FITC conjugate (SouthernBiotech), and analyzed with a FACScan (BD Biosciences) using the CellQuest software. For stably expressing reporter cells, 3 × 106 BWZ.36 cells were electroporated with 25 μg of ggFcR-FLAG-muCD3ζ construct at 200 V with 950 μF capacitance. Cells were selected with 800 μg/ml g418

**Table I. Oligonucleotides used for RH mapping**

| Specificity | No. | Sequence | Fragment Size |
|------------|-----|----------|--------------|
| ggFcR (exon 6) | 1171a | AGCCCTGCTCCCTGAGCCCTTA | 401 bp |
| ggFcR (intron 6) | 1232as | CCCCCAAGGTCTCTGTCCTG | |
| ggL3MBTL (exon 7) | 1230s | GAGTGGCCAGTGGAGAAAT | 496 bp |
| ggL3MBTL (intron 8) | 1231as | TGATGAGCTTGGGGACCTGA | |

a Orientation indicated as s, sense, as, antisense.

**Table II. Oligonucleotides used for cloning and real-time RT-PCR**

| No. | Sequence |
|-----|----------|
| 922s | GAAATGTCCTCGCTCATACTC |
| 923as | TTCCAATCTAGGCTCGCATGG |
| 1038s | ATGAAATTTCACTGCTCGAGT |
| 1229as | ATGAAATTTCACTGCTCGAGT |
| 931as | ATGAAATTTCACTGCTCGAGT |
| 1165s | CTCGACCCACACACCTCCATCC |
| 1166as | CTCGACCCACACACCTCCATCC |
| 870s | CATGTCCTAGTACACAACACCGCCGTA |
| 871as | GGGCTGCTGGGCTGACATTA |

a EcoRI restriction site underlined.

b Orientation indicated as s, sense, as, antisense.
FIGURE 1. Sequence features of ggFcR. A, Coding nucleotide (nt) sequence and deduced amino acid (aa) sequence of ggFcR. The primer sequences used for cloning are indicated by vertical bars. Nucleotide positions are shown at the end of each line, and amino acid positions are labeled in distances of 50 in boldface below the amino acid sequence. The putative methionine initiation residues are marked with /H11002 \underline{56}, /H11002 \underline{24}, /H11002 \underline{23}, and /H11002 \underline{17}. The methionine at /H11002 \underline{24}, which is most likely the start codon, is highlighted in boldface. Arrows above the nucleotide sequence indicate the exact beginning of the respective exons. Arrows below the amino acid sequence mark the C2-set Ig domains. The cysteine residues forming the disulfide bonds are encircled. The transmembrane region is boxed and the positively charged arginine is shaded gray.

B, Genomic organization of the ggFcR gene. The lengths of exons (differently shaded boxes) and introns (horizontal lines) are drawn to scale and indicated with nucleotide numbers. The exons are numbered and described, containing signal peptide (SP), Ig domains (IG), and transmembrane region (TM).
(AppliChem), and single clones were screened for expression by cell staining as described above.

**Reporter gene assay**

Stably expressing BWZ.36-ggFcR cells were used in reporter gene assays as follows: 3 × 10⁶ reporter cells were cultivated for 24 h in 24-well cell culture plates either left uncoated (control) or coated with anti-FLAG mAb (Sigma-Aldrich) or chicken IgA, IgM, and IgY (all 10 μg/ml) or stimulated with 10 ng/ml PMA (Sigma-Aldrich) and 0.5 μg/ml Ca ionophore (Sigma-Aldrich) as an unspecific positive control. Purified chicken IgM and IgA was kindly provided by B. Kaspers (Munich, Germany). Chicken IgY was purchased from Jackson ImmunoResearch Laboratories. β-galactosidase was measured using the high sensitivity β-galactosidase assay kit (Stratagene) after an incubation period of 18 h in a 37°C incubator. The activity of β-galactosidase (U) was calculated by dividing the amount of chlorophenol red formed (nmol) by a specific time length of incubation (minutes). The amount of chlorophenol red (in nmol) equals the concentration of chlorophenol red formed (nmol/ml) × total assay volume (ml). The concentration of chlorophenol red formed (nmol/ml) is calculated by the OD × 55.

**Real-time RT-PCR**

Total RNA was extracted from 100 mg of tissue or 1 × 10⁷ cells by using TRIzol (Invitrogen). The RNA quality was determined with the 2100 Bioanalyzer (Agilent Technologies). RNA with an integrity number 2100(Bioanalyzer) obtaining the cycle thresholds (Ct) for each tissue. The relative amounts of gene-of-interest mRNA were calculated by means of the ΔΔCt method as described previously (10).

**Results**

**Cloning of ggFcR, a putative FcR, which displays genomic organization similar to LRC-encoded genes**

Initially, various chicken EST databases were searched by using the term “Fc Receptor”, and this yielded seven EST clones of the Biotechnology and Biological Sciences Research Council EST database. Analyzing those resulted in two different contigs containing four and three EST clones, respectively. Four EST clones (BU221313, BU32940, BU332675, BU459232) were identified to represent the previously described ggFcR/L gene (6), whereas the other three clones (BU239980, BU422666, BU424877) represented a yet undefined protein. To obtain a full-length protein, additional EST clones were identified by BLAST search (AJ394599, BU372165, BU450696, BU455112, BX273743, BX273744, CF250847, CV891153, CK607089), and a manually refined consensus sequence of all EST clones was used to design primers for ggFcR. PCR with oligonucleotides 922s and 923as amplified a 1525-bp transcript with an open reading frame of 1428 bp. The deduced protein sequence consists of a signal peptide, four C2-set Ig domains, and a transmembrane region with arginine as a positively charged amino acid followed by a short cytoplasmic tail (Fig. 1A). Note that there are four possible ATG initiation codons encoding methionine residues at positions −56, −24, −23, and −17 (Fig. 1A). After applying different signal peptide prediction programs, methionine −24 seems to be most likely the initiation site of ggFcR.

The corresponding genomic DNA was identified on chicken chromosome 20, bp 22,577−25,509. The coding sequence of the genomic DNA differed only in two nucleotides from the cloned ggFcR, one of them resulting in a nonsynonymous change (C82 → R82) in the g strand of the Ig1 domain. The exon/intron organization was obtained by comparison of the cloned ggFcR with the

**Phylogenetic comparison of ggFcR with chicken and human genes encoded in the LRC and FcR region, respectively.** Individual Ig domains were aligned to the ClustalW algorithm. The neighbor-joining tree with 1000 bootstrap replicates and pairwise gap deletions was built using MEGA4. An unweighted pair group method with arithmetic mean (UPGMA) and a minimum evolution tree was also constructed, but they were essentially the same as the neighbor-joining tree in the major branching patterns and are not presented here. Accession numbers of genes included in the tree are FM200428 (ggFcR), NM_002000 (huFcAR), NM_000566 (huFcGRIA), AM412311 (ggFcR/L), and AJ745094 (ggCHIR-AB1).
FIGURE 3. Comparative analysis of chicken and human chromosome 20. Predicted gene order and orientation (arrows) as presented in the HomoloGene database. Corresponding gene blocks in chicken and human are shown in boxes and connected with lines. The ggFcR gene is located at the very beginning of chicken chromosome 20 and is the only gene that is not connected by a line to a corresponding human gene. The size of the respective gaps, which were not analyzed in detail, is labeled. The entire gene designation is according to HomoloGene (in order of appearance on chicken chromosome 20): L3MBTL, lethal(3)malignant brain tumor-like protein; SFRS6, splicing factor, arginine/serine-rich 6; EPB41L1, erythrocyte membrane protein band 4.1-like 1; C20orf4, chromosome 20 open reading frame 4; DLGAP4, discs, large homolog-associated protein 4; MYL9, myosin, L chain 9, regulatory; GGT7, myosin, L chain 9, regulatory; NCOA6, nuclear receptor coactivator 6; PIK3C3, phosphatidylinositol-3-kinase, catalytic, subunit gamma; PIGU, phosphatidylinositol glycan anchor biosynthesis, class U; SAMHD1, SAM domain and HD domain 1; RBL1, retinoblastoma-like 1 (p107); SAMHD1, SAM domain and HD domain 1; RBL1, retinoblastoma-like 1 (p107); KIAA1219, hypothetical protein KIAA1219; KIAA1219, hypothetical protein KIAA1219; NDRG3, N-myc downstream-regulated gene 3; RBM12, RNA-binding motif protein 39; C20orf52, chromosome 20 open reading frame 52; NFS1, nitrogen fixation 1 homolog (Saccharomyces cerevisiae); RBM39, RNA-binding motif protein 39; C20orf52, chromosome 20 open reading frame 52; NFS1, nitrogen fixation 1 homolog (Saccharomyces cerevisiae); RBM12, RNA-binding motif protein 12; TOX2, TOX high mobility group box family member 2; MYBL2, MYB binding leucine zipper containing 2; IFT52, intraflagellar transport 52 homolog (Chlamydomonas); SGK2, serine/threonine-protein kinase 2; KCNK15, potassium channel, subfamily K, member 15; WISP2, WNT1 induced signaling pathway protein 2; ADA, adenosine deaminase; SERINC3, serine incorporator 3; HNF4A, hepatocyte nuclear factor 4, alpha; GDAP1L1, ganglioside-induced differentiation-associated protein 1-like 1; JPH2, junctophilin 2.

FIGURE 4. GgFcR associates with the common γ-chain. 293T cells were transfected with ggFcR-FLAG and either cotransfected with a V5-tagged mock control (left panel) or with a V5-tagged common γ-chain (right panel). Twenty-four hours after transfection, the surface expression of ggFcR was analyzed by staining with an anti-FLAG mAb (filled area) and compared with a control staining with an irrelevant mAb (gray line). The frequency of positive cells and the mean fluorescence intensity calculated for all cells of the anti-FLAG staining are indicated.

The positively charged arginine in the beginning of the transmembrane region of ggFcR (Fig. 1A) suggested that this is a potentially activating receptor, which interacts with the ITAM-containing common γ-chain (FceR1γ). This is similar to the situation in CHIR-A2 (8) and CHIR-AB1 (10), which both contain arginine at the beginning of the transmembrane region and were shown to interact with the common γ-chain. To test common γ-chain association, we used a FLAG-tagged full-length construct of ggFcR for cotransfection of 293T cells with either a common γ-chain-V5 construct or a mock-V5 control. Staining of the transiently transfected cells with anti-V5 mAb showed equal expression of the V5 construct (data not shown), whereas anti-FLAG staining revealed that ggFcR-FLAG surface expression is only reconstituted if co-transfected with the common γ-chain (Fig. 4, right panel) but not with mock-V5 control (Fig. 4, left panel). Since the Ig binding capabilities are an important classification parameter, which distinguishes FcR from FCRL genes, we established a reporter gene assay for ggFcR to address this question. We produced BWZ.36 reporter cells, which are stably expressing a chromosome 19 and chicken chromosome 31, respectively. The highly related ggFcR gene, however, is located at the very beginning of chicken chromosome 20 (bp 22,577–25,509), which has by now not been characterized to contain any Ig-like receptor family members. The syntenic region to chicken chromosome 20 is human chromosome 20 (26), so we decided to examine these two chromosomal regions for the presence of Ig-like receptor genes by using the HomoloGene database. The result is shown in Fig. 3. Apart from the ggFcR gene, all genes on the first 1 Mb of chicken chromosome 20 (Fig. 3, FCR to RBM12, boxes 1–8, left panel) are also present on human chromosome 20, but with a different order and orientation (Fig. 3, line-connected boxes 1–6, right panel). Since the ggFcR gene is located adjacent to the 3’ end of the L3MBTL gene, we were particularly interested in the human genes next to the 3’ end of this gene. This 5-Mb region on human chromosome 20 (Fig. 3, SGK2 to KCNK15, boxes 7–9, right panel) does not contain any Ig-like receptor genes, but it displays genes present on chicken chromosome 20 (2.4 Mb–5.5 Mb) (Fig. 3, boxes 9–11, left panel). To confirm the unusual chromosomal location of an LRC-related FcR gene, we also performed RH mapping using genomic amplicons of the ggFcR gene and the adjacent ggL3MBTL gene, which mapped both on chicken chromosome 20. ggFcR associates with the common γ-chain and binds chicken IgY.
ggFcR-FLAG-muCD3ζ construct. Plate-bound chicken IgY, IgA, and IgM were tested as potential ligands for ggFcR reporter cells, and a specific β-galactosidase induction was observed only for plate-bound IgY (Fig. 5). The calculated activity of β-galactosidase (U) for IgY was 0.01839, which is comparable to the activity of the two positive controls, anti-FLAG (U = 0.02018) and PMA/Ca-Ionophore (U = 0.02166). In comparison, chicken IgY induced a much higher β-galactosidase expression in CHIR-AB1 reporter cells (10), with U = 0.56595, which is more than 30-fold higher than IgY-induced β-galactosidase expression in ggFcR reporter cells.

We also used a soluble ggFcR construct in a sandwich ELISA to determine the interaction of ggFcR with the Fc fragment of IgY, as shown for CHIR-AB1, but we could not detect any binding in this assay (data not shown). This is most likely due to the fact that the interaction between ggFcR and IgY is much weaker compared with IgY-CHIR-AB1 and cannot be detected by ELISA.

Expression analysis of ggFcR by real-time RT-PCR

Several immune-relevant tissues and cells were tested for expression of ggFcR by real-time RT-PCR. Oligonucleotides 1165s and 1166as are intron-spanning primers, which amplify 190 bp of the Ig3 and Ig4 domain of ggFcR. The normalized results showed the highest level of ggFcR expression in PBMC (Fig. 6A). Furthermore, in spleen and macrophage preparations we also observed ggFcR expression, whereas in brain and PBL the expression was negligible (Fig. 6A). Chicken PBL were prepared by slow speed centrifugation and comprise a relatively pure fraction of chicken lymphocytes. PBMC, however, were prepared by density centrifugation with Ficoll-Paque and contain, besides lymphocytes, also monocytes and thrombocytes. To further evaluate the high level of ggFcR expression in PBMC, which is not monocyte/macrophage derived (Fig. 6A), we prepared chicken thrombocytes and compared the ggFcR expression of PBMC, PBL, and thrombocytes. The normalized results were calibrated on PBL, and it appears that chicken thrombocytes are the major source of ggFcR-expressing cells in PBMC (Fig. 6B).

Discussion

FcRs represent a typical immunoregulatory Ig-like receptor family, which signal via activating and inhibitory receptors to set thresholds for cell activation, generating a well-balanced immune response. These FcR genes are encoded on a specific region on human chromosome 1 intermingled with the highly related FCRL genes. The pivotal difference between FcR and FCRL receptors is their capability to bind the Ig constant region. Recently, two groups showed that the region on chicken chromosome 25 syntenic to the human FcR locus contains only a single FCRL gene present, which does not bind chicken immunoglobulins and therefore is considered to be an FCRL homolog (6, 7). The present report describes the first cloning and characterization of a novel FcR in chicken, designated ggFcR. Comparison of the newly cloned ggFcR with the recently identified ggFCR/L showed only 21% overall identity on amino acid level. A crucial step to distinguish between an FcR or FCRL homolog is the identification of the ligand-binding properties of ggFcR. This was done using a reporter gene assay on the basis of BWZ.36 cells, which we already applied on the IgY FcR CHIR-AB1 (10). The results clearly show that ggFcR selectively binds chicken IgY, inducing a specific β-galactosidase expression in the reporter cells.

The capability of binding Ig constant regions, however, is not restricted to genes encoded in the FcR region on human chromosome 1, but is also present in genes encoded in the LRC. The FcRI gene (CD89) in primates, horses, cows, and rats and the bovine FcγR2 are two examples in mammals (27, 28). Additionally, we have previously shown that a CHIR family member, designated CHIR-AB1, which is also encoded in the LRC, binds IgY with high affinity. Here, we show that ggFcR is an additional IgY FcR present in chicken, which is highly related to genes encoded in the LRC and not to genes in the FcR region. This relationship is validated by various findings. The first refers to the exon/intron organization of ggFcR (Fig. 1B). One aspect of the genomic organization, which distinguishes the FcR family on human chromosome 1 from the LRC-encoded genes on human chromosome 19, is a short mini-exon that encodes the second half of a split signal peptide. For all FcR and FCRL family members except of FCRLA, this mini-exon is 21 bp in size (5). This size also applies to ggFcR/L on the FcR syntenic region on chicken chromosome 25 (6, 7). On the other hand, all genes encoded in the LRC, including the human FcαR, display also a mini-exon for the split signal peptide, but in contrast to the FcR/FCRL genes, this is 36 bp in size (www.ncbi.nlm.nih.gov/books/bookres.fcgi?mono_003/ch1d1.pdf and Refs. 24, 25). This 36bp mini-exon is also present in all genes encoded in the chicken LRC on chromosome 31 (8). Consequently, the presence of a
36-bp mini-exon in the ggFcR gene clearly indicates a relationship to LRC-encoded genes (Fig. 1B). Additionally, this relationship was also shown by a phylogenetic analysis of the Ig domains of various FcR- and LRC-encoded genes. The analyses demonstrated that the Ig domains of ggFcR are highly related to LRC, but not FcR-encoded genes (Fig. 2). Although all Ig domains of ggFcR clearly cluster with the LRC-encoded genes, the amino acid sequence identities between ggFcR and various CHIR genes range only between 20% and 40% (data not shown), indicating that ggFcR is distinct from the CHIR gene family, whose members are usually related 65–99%. The third fact, which indicates that ggFcR is mostly related to LRC genes but is distinct from the CHIR family, is the organization of the transmembrane region displaying the amino acids NIVR (Fig. 1A), which interact with the common γ-chain. As described by Guselnikov et al., the presence of an NxxR motif in the transmembrane region of activating family members is specific for most LRC-encoded genes, but it is missing in FcR genes (29). This NxxR motif appears to be an ancestral element of a primordial FcR/LRC family, which has been lost in CHIR, classical FcR and KIR, but is present in Xenopus FcR-like (XFL)- and LRC-encoded genes, like huIL1R, huFcαR, huGPVL1, and muP2R and in the newly cloned ggFcR.

Surprisingly, the ggFcR gene is not encoded in the chicken LRC on chromosome 31, but it is located as a single gene on a unique position on chromosome 20. As depicted in Fig. 3, chicken chromosome 20 represents the syntenic region of human chromosome 20, displaying similar genes in a different order. Detailed analysis of the chromosomal region presented in Ensembl even shows that the ggFcR gene is located on the same contig as the neighboring L3MBTL gene (data not shown). To completely exclude a misassembly of the chicken genome in that area, we also proofed by RH mapping that both the L3MBTL gene and the ggFcR gene are located on chicken chromosome 20. Interestingly, a survey of various genomes from mammals, fish, and amphibians via Ensembl revealed no Ig-like receptor at the 3' end of the respective L3MBTL gene (data not shown). In all genomes analyzed, the SGK2 gene was located at this position, which is also present at this location in the human genome (Fig. 3).

The ggFcR gene was already annotated within the Ensembl and HomoloGene databases as a novel gene located on chromosome 20. Due to prediction programs based on closest hits of this gene to mammalian databases, it was predicted to be similar to Ig superfamily member 1 (gene ID 419114) and to venom myotoxin inhibitor DM64 (XM_417301.2). By this report we demonstrated that this gene is predicted wrongly and is a true Ig FcR. Interestingly, the closest hit of a blastn search to a nonpredicted chicken gene was CHIR-AB3 (AJ879909), clearly indicating again a close relationship to LRC-encoded genes. The origin of the ggFcR gene on chromosome 20 remains elusive, but the relationship to LRC-encoded genes and the presence of the NxxR motif in the transmembrane region indicate that it is a dispersed gene from an ancient LRC locus.

Further characterization of the expression pattern of the activating ggFcR by real-time RT-PCR showed typical expression on chicken PBMC, macrophages, and spleen cells. Interestingly, ggFcR is also expressed on thrombocytes, which represent nucleated blood cells phylogenetically closely related to platelets. In various, partly contradictory reports, chicken thrombocytes have long been suspected to have phagocytic activities (20, 30, 31) and there are also reports from human platelets with phagocytic capabilities. Recently, it was shown that platelets do engulf bacteria, but do not kill them, because a true phagosome is not generated (32). Interestingly, human platelets also express the activating FcγRIIA. The function of this receptor on platelets is still not resolved completely. Most of the work was done in transgenic mouse models, since mice lack the genetic equivalent of human FcγRIIA. These studies revealed that FcγRIIA plays a significant role in the immune clearance of platelets in vivo (33) and that Abs that activate platelets in an FcγRIIA-dependent manner lead to thrombosis, shock, and death (34).

In conclusion, ggFcR represents a potentially activating IgY FcR, which is highly related to LRC encoded genes but is located on chicken chromosome 20. It is expressed on chicken PBMC, macrophages, spleen cells, and thrombocytes, but its actual function on these cells still needs to be resolved.

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Disclosures

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References

1. Colonna, M. 2003. TREMs in the immune system and beyond. Nat. Rev. Immuno- nol. 3: 445–453.
2. Daeron, M. 1997. Fc receptor biology. Annu. Rev. Immunol. 15: 203–234.
3. Nimmerjahn, F., and J. V. Ravetch. 2008. Fcy receptors as regulators of immune responses. Nat. Rev. Immunol. 8: 34–47.
4. Monteiro, R. C., and J. G. Van De Winkel. 2003. IgA Fc receptors. Annu. Rev. Immunol. 21: 177–204.
5. Davis, R. S. 2007. Fc receptor-like molecules. Annu. Rev. Immunol. 25: 525–560.
6. Taylor, A. L., H. J. Gould, B. J. Sutton, and R. A. Calvert. 2007. The first avian Ig-like Fc receptor family member combines features of mammalian FcR and FCRL. Immunogenetics 59: 323–328.
7. Fayngerts, S. A., M. Nancikewie, and A. V. Taranin. 2007. Species-specific evolution of the FcR family in endothelial vertebrates. Immunogenetics 59: 493–506.
8. Viertelboeck, B. C., F. A. Habermann, R. Schmitt, M. A. Groenew, L. De Pasquier, and T. W. Gobel. 2005. The chicken leukocyte receptor complex: a highly diverse multigene family encoding at least six structurally distinct receptor types. J. Immunol. 175: 385–393.
9. Laun, K., P. Coggill, S. Palmer, S. Sims, Z. Ning, J. Ragoussis, E. Volpi, N. Wilson, S. Beck, A. Ziegler, and A. Volz. 2006. The leukocyte receptor complex in chicken is characterized by massive expansion and diversification of immunoglobulin-like loci. PLoS Genet. 2: e73.
10. Viertelboeck, B. C., S. Schweinsberg, M. A. Hanczurak, R. Schmitt, L. De Pasquier, F. W. Herberg, and T. W. Gobel. 2007. The chicken leukocyte receptor complex encodes a primordial, activating, high-affinity IgY Fc receptor. Proc. Natl. Acad. Sci. USA 104: 11718–11723.
11. Lefrancois, L., and B. C. Pienaar. 2003. C. C. P Ponting, and P. Bork. 2004. SMART 4.0: toward genomic data integration. Nucleic Acids Res. 32: D142–D144.
12. Schultz, J., F. Milpitz, P. Bork, and C. P. Ponting. 1998. SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl. Acad. Sci. USA 95: 5857–5861.
13. Zdobnov, E. M., and R. Apweiler. 2001. InterProScan: an integration platform for the signature-recognition methods in InterPro. Bioinformatics 17: 847–848.
14. Bentsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak. 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340: 783–795.
15. Cuff, J. A., M. E. Clamp, A. S. Siddiqui, M. Finlay, and G. J. Barton. 1998. JPPred: a consensus secondary structure prediction server. Bioinformatics 14: 892–893.
16. Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596–1599.
17. Morisson, M., A. Lemiere, S. Bosco, M. Galan, F. Plisson-Petit, P. Pinton, C. Delcroix, K. Feve, F. Pitel, V. Fillon, et al. 2002. ChickRHB: a chicken whole-genome radiation hybrid panel. Genet. Sel. Evol. 34: 521–533.
18. Gobel, T. W., C. H. Chen, and M. D. Cooper. 1996. Expression of an avian CD6 candidate is restricted to ab T cells, splenic CD8+ γδ T cells and embryonic natural killer cells. Eur. J. Immunol. 26: 1743–1747.
19. Peck, R., K. K. Murthy, and O. Vainio. 1982. Expression of B-L (Ia-like) anti- gens on macrophages from chicken lymphoid organs. J. Immunol. 129: 4–5.
20. Grecchi, R., A. M. Saliba, and M. Mariano. 1980. Morphological changes, surface receptors and phagocytic potential of fowl mono-nuclear phagocytes and thrombocytes in vivo and in vitro. J. Pathol. 130: 23–31.
21. Viertelboeck, B. C., M. A. Hanczurak, C. C. Schmitt, R. Schmitt, and T. W. Gobel. 2008. Characterization of the chicken CD200 receptor family. Mol. Immunol. 45: 2097–2105.
22. DuBridge, R. B., P. Tang, H. C. Hsia, P. M. Leong, J. H. Miller, and M. P. Calos. 1987. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol. Cell. Biol. 7: 379–387.
23. Iizuka, K., O. V. Naidenko, B. F. Plougastel, D. H. Fremont, and W. M. Yokoyama. 2003. Genetically linked C-type lectin-related ligands for the NKRPI family of natural killer cell receptors. *Nat. Immunol.* 4: 801–807.

24. Torkar, M., Z. Norgate, M. Colonna, J. Trowsdale, and M. J. Wilson. 1998. Isotypic variation of novel immunoglobulin-like transcript/killer cell inhibitory receptor loci in the leukocyte receptor complex. *Eur. J. Immunol.* 28: 3959–3967.

25. de Wit, T. P., H. C. Morton, P. J. Capel, and J. G. van de Winkel. 1995. Structure of the gene for the human myeloid IgA Fc receptor (CD89). *J. Immunol.* 155: 1203–1209.

26. Bourque, G., E. M. Zdobnov, P. Bork, P. A. Pevzner, and G. Tesler. 2005. Comparative architectures of mammalian and chicken genomes reveal highly variable rates of genomic rearrangements across different lineages. *Genome Res.* 15: 98–110.

27. Wines, B. D., and P. M. Hogarth. 2006. IgA receptors in health and disease. *Tissue Antigens* 68: 103–114.

28. Zhang, G., J. R. Young, C. A. Tregaskes, P. Sopp, and C. J. Howard. 1995. Identification of a novel class of mammalian Fcγ receptor. *J. Immunol.* 155: 1534–1541.

29. Guselnikov, S. V., T. Ramanayake, A. Y. Erilova, L. V. Mechetina, A. M. Najakshin, J. Robert, and A. V. Taranin. 2008. The Xenopus FcR family demonstrates continually high diversification of paired receptors in vertebrate evolution. *BMC Evol. Biol.* 8: 148.

30. Traill, K. N., G. Bock, R. Boyd, and G. Wick. 1983. Chicken thrombocytes: isolation, serological and functional characterization using the fluorescence activated cell sorter. *Dev. Comp. Immunol.* 7: 111–125.

31. DaMatta, R. A., S. H. Seabra, and W. de Souza. 1998. Further studies on the phagocytic capacity of chicken thrombocytes. *J. Submicrosc. Cytol. Pathol.* 30: 271–277.

32. White, J. G. 2006. Why human platelets fail to kill bacteria. *Platelets* 17: 191–200.

33. McKenzie, S. E., S. M. Taylor, P. Malladi, H. Yuhan, D. L. Cassel, P. Chien, E. Schwartz, A. D. Schreiber, S. Surrey, and M. P. Reilly. 1999. The role of the human Fc receptor FcγRIIA in the immune clearance of platelets: a transgenic mouse model. *J. Immunol.* 162: 4311–4318.

34. McKenzie, S. E. 2002. Humanized mouse models of FcR clearance in immune platelet disorders. *Blood Rev.* 16: 3–5.