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The Chicken Leukocyte Receptor Complex Encodes a Family of Different Affinity FcY Receptors

Iron C. Viertlboeck,* Sonja Schweinsberg,* Ramona Schmitt,* Friedrich W. Herberg,† and Thomas W. Göbel‡*

Chicken Ig-like receptors (CHIR) form a large family in the leukocyte receptor complex on microchromosome 31 with inhibitory, activating, and bifunctional receptors. Recently, we characterized CHIR-AB1 as a high-affinity, primordial FcY receptor. Given that the CHIR family represents a multigenic family, it is plausible that more than a single receptor binds to IgY. Therefore, after comparing CHIR-AB1-like sequences in databases, we cloned CHIR-AB1 homologues from two individual chickens representing the lines M11 and R11 with primers binding to highly conserved regions. In both lines this approach yielded 18 different CHIR-AB amino acid versions, with one sequence out of each line that was identical with the previously characterized B19 CHIR-AB1 Ig domain and two additional R11-M11 identical sequence pairs. All M11-derived CHIR-AB homologues were then expressed as soluble human Ig fusion proteins. Following standardization of the fusion protein concentration with an ELISA, the IgY, IgM, and IgA binding activities were determined by ELISA. Six fusion proteins recognized IgY, whereas none bound to IgM and IgA. The affinities of selected fusion proteins were determined using surface plasmon resonance yielding an equilibrium binding constant between 25 nM for high binders and 260 nM for low binders. Sequence comparisons and subsequent mutational analysis of selected residues identified five amino acids that are potentially involved in IgY binding. These results imply that multiple FcY receptors of variable affinity are encoded by the CHIR locus and that different chicken lines may express both unique as well as highly conserved FcY receptors. The Journal of Immunology, 2009, 182: 6985–6992.

The Fc receptors, FcRs, have the ability to link the sophisticated recognition of acquired immunity to innate effectors. Almost all Ig isotypes are recognized by specific FcRs (1). These FcRs are not only characterized by their specificity and cellular distribution, but also by their affinity to monomeric Ig and/or immunocomplexes, their signal transduction capabilities, and their chromosomal location. IgG is bound by different receptors that are either high, intermediate, or low affinity receptors and that further show differences in their capability to bind certain Ig subclasses. The FcεRI and the C-type lectin CD23 represent high- and low-affinity IgE FcRs, respectively, whereas IgA is bound by the FcεRII (2, 3). Functionally all FcRs, with the exception of FcγRIIB, lead to cellular activation upon ligand binding due to their association with the common Fcy chain that transduces signals via its intracellular ITAM. The ligation of FcγRIIB, the only FcR expressed on B cells, leads however to cellular inhibition through the cytoplasmic ITIM. Most of the FcRs form a gene family on human chromosome 1. In addition to the classical FcRs, this chromosomal region also includes an Fcα/μ receptor, the poly-Ig receptor, and a group of FcR-like molecules (FCRL) with unknown specificity (4, 5). In contrast, the FcεRI (CD89) is located on human chromosome 19 and thus belongs to the leukocyte receptor complex (LRC) that also encodes multiple immunomodulatory gene families such as the killer cell Ig-like receptors (KIRs) and the leukocyte Ig-like receptors (6).

There is a big discrepancy between the detailed knowledge of nonmammalian Ig isotypes and the scarce information on their respective FcRs. In the channel catfish a FcR homologue that binds IgM has been identified (7). In the chicken, there is information regarding the poly Ig receptor that interacts with J chain-containing polymeric IgA (8). In addition, a chicken yolk sac IgY receptor that transports maternal egg yolk-derived IgY to the yolk sac has been characterized as an orthologue of the mammalian phospholipase A2 receptor that belongs to the mannose receptor family (9, 10). Interestingly, the chicken FcR locus on chromosome 25 may only contain a single FcR/L receptor that seems not to bind IgY (11, 12). We have recently identified a molecule designated CHIR-AB1 as a high affinity IgY FcR (13). CHIR-AB1 combines 4 Abbreviations used in this paper: FCRL, FcR-like molecule; BAC, bacterial artificial chromosome; CHIR, chicken Ig-like receptor; KIR, killer cell Ig-like receptor; LRC, leukocyte receptor complex.

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features of several mammalian FcRs such as expression on B cells, monocytes, and NK cells and its potential to signal as an inhibitor or an activating receptor based on the presence of a charged transmembrane residue and a cytoplasmic ITIM.

CHIR-AB1 is a member of a large receptor family designated chicken Ig-like receptors (CHIRs) that are located in the chicken LRC (14, 15) on microchromosome 31. All CHIRs display either one or two C2-type Ig domains. They can be further grouped into activating receptors that possess a basic transmembrane residue and a short cytoplasmic region (CHIR-A), inhibitory receptors with long cytoplasmic tails containing ITIM (CHIR-B), and CHIR-AB, which displays both cytoplasmic ITIM and charged transmembrane residues. The most striking feature of the CHIR family is the large number of genes and the enormous diversity compared with the mammalian LRC (15, 16). The diversity most likely arises from polymorphism for each gene combined with variability in the number of genes between CHIR haplotypes.

Because the chicken LRC contains a large number of highly diverse receptors, it was important to find out whether CHIR-AB1 is an unique FcR gene that is conserved in all chickens and is not affected by haplotypic or allelic variations as in the case of mammalian CD89 or whether there are multiple CHIR-AB1 genes that may bind IgY with different affinities. Our results, presented in this article, consolidate both alternatives in that a single CHIR-AB1 Ig domain sequence was found to be conserved in all chicken lines analyzed, and additional CHIR-AB homologues that bound IgY with different affinities were found to be selectively expressed in different chicken lines.

Materials and Methods

Animals

The chicken lines M11 (B2) and R11 (B15) are homozygous for the MHC allele indicated. M11 and R11 chicken eggs, a gift from S. Weigend (Institute for Animal Science, Marienense, Germany), were hatched at the institute and the animals were used for experiments at the age of 3 to 10 wk.

Database searches

Seven CHIR-specific bacterial artificial chromosome (BAC) clones sequenced previously (16) were used for analysis of CHIR-AB1-like molecules in the chicken genome. In detail, WAG-4C11 (GenBank accession no. BX663523), WAG-93H17 (GenBank accession no. BX663526), WAG-112A23 (GenBank accession no. BX663527), WAG-19H9 (GenBank accession no. BX663529), WAG-52G8 (GenBank accession no. BX663530), WAG-58B13 (GenBank accession no. BX663534), and WAG-88M21 (GenBank accession no. BX897732) were monitored after each batch using SMART (Simple Modular Architecture Research Tool; smart.embl-heidelberg.de/) (17), and the predicted CHIRs were assigned to genes and pseudogenes as illustrated by Laun et al. (16). In total, the coding regions of 10 CHIR-AB1-like genes were extracted from the BAC clone sequences and used for designing primers for conserved regions specific for these genes.

Cell preparations, cloning procedures, and generation of point mutations

PBMC were prepared by density centrifugation on Ficoll-Paque (GE Healthcare). Total RNA was prepared using an Absolutely RNA RT-PCR Miniprep kit (Stratagene) and cDNA synthesis was performed with the ThermoScript RT-PCR system (Invitrogen). For cloning of different CHIR-AB versions, Herculase enhanced DNA polymerase (Stratagene) was used for PCR with 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 (5'-ATGACGACCACATGGCCGTGGCCCTC-3') and antisense primer (5'-GCACGGGATGGCAGCTGGCAT-3'). PCR products were cloned into a pcDNA3.1/V5-His TOPO vector (Invitrogen), colonies were screened by PCR, and plasmids of positive colonies were isolated using the NucleoSpin plasmid kit (Macherey-Nagel) and sequenced (GATC Biotech). Deduced amino acid sequences were further analyzed using Lasergene software (GATC Biotech), SignalP 3.0 (www.cbs.dtu.dk/services/SignalP) (19), THiMM 2.0 (www.cbs.dtu.dk/services/THiMMM-2.0/). jpred3 (www.compbio.dundee.ac.uk/Software/jpred/jpred.html) (20), and MEGA4 (21).

For the soluble CHIR-AB constructs, the full-length cDNA of the different M11 CHIR-AB versions were amplified with primers including the 5' end of the signal sequence (sense, 5'-ATGAATTCAATGGCCGTGGCCCTC-3') and the 3' end of the extracytoplasmic Ig domain including an EcoRI site (antisense, 5'-ATGAATTCAATGGCCGTGGCCCTC-3'). Resulting PCR products were digested with EcoRI and ligated in a pcDNA3.1 vector fusing the extracytoplasmic region of CHIR-AB to the C1α and C1γ domains of human IgG1 as described previously (13). CHIR-AB1 mutants were created using the soluble CHIR-AB constructs as templates and internal mismatch primers and their complements (Tab. 1). To remove a template, PCR products were digested with DpnI before the transformation of bacteria. Plasmids were prepared from selected colonies and the mutations were confirmed by sequencing.

Cell lines, transfection, and purification

Human embryonic kidney 293 or 293T cells (22) were maintained in RPMI 1640 (Biochrom) supplemented with 10% low IgG FCS, 1% penicillin/streptomycin in a CO2 incubator at 37°C. 293 and 293T cells were transfected with different soluble CHIR-AB constructs using the Metafectene reagent (Biontex) according to the manufacturer’s protocol. In transient transfections of 293T cells the supernatant was used after 48 h; in stable transfections of 293 cells, these were selected with 800 μg/ml G418 (Appliken) after 24 h and the resulting clones were screened for the expression of a soluble CHIR-AB construct using ELISA. Highly producing clones were expanded and the different soluble CHIR-AB constructs were affinity purified on separate protein G-coupled Sepharose columns by using standard procedures.

ELISA

A sandwich ELISA described previously (13) was used either for expression control of the different soluble CHIR-AB constructs or to test their interaction with chicken Igs. Either the anti-human IgG UNLB (Southern Biotechnology Associates) or the chicken IgY or Fc fragment (Jackson ImmunoResearch Laboratories) was used at 5 μg/ml as a capture reagent and detected with goat anti-human IgG HRP (1/4000) (Southern Biotechnology Associates).

Surface plasmon resonance analysis

Surface plasmon resonance (23) measurements were performed on a Biacore 2000 instrument (GE Healthcare). Purified soluble CHIR-AB constructs were covalently coupled to a total of 750–800 resonance units on a carboxymethylated sensor chip surface (CM5; research grade) using standard N-hydroxysuccinimide (NHS)/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling via primary amines (24). For this purpose the sensor surfaces were activated with 50 μM NHS and 200 μM EDC for 10 min and the CHIR-AB constructs (5 μg/ml) in 10 mM sodium acetate (pH 4.0) were injected for 10 min. The surfaces were deactivated by injecting 1 M ethanolamine–HCl (pH 8.5) for 5 min. The reference cell was activated accordingly and deactivated subsequently in the absence of protein (blank surface).

Kinetic measurements were performed in buffer containing 20 mM HEPES (pH 7.0), 150 mM NaCl, and 0.005% surfactant P20 at 20°C. Increasing concentrations of IgY were injected at a flow rate of 30 μl/min and association and dissociation phase were monitored for 5 min each. The sensor surfaces were regenerated after each binding cycle by three short injections of 10 mM glycine (pH 2.5). After subtracting the reference cell signal, the resulting binding data were fitted to a Langmuir 1:1 binding model. Data evaluation was performed using BIAevaluation software version 4.0.1 (Biacore). The fits yielded apparent association (k on) and dissociation (k off) rate constants from which the equilibrium binding constant can be calculated according to the equation $K_D = k_{off}/k_{on}$.

Results

Database searches reveal additional CHIR-AB1 like receptor genes

CHIRs form a multigene family encoded in the LRC on chromosome 31 (15), a region that has not been assembled in the current
version of the chicken genome project (www.ensembl.org/Gallus_gallus/index.html; assembly of May 2006). The sequence of seven BAC clones covering 550 kb of chromosome 31, however, was recently deposited in nonannotated form in the GenBank database. To identify additional chicken FcRs, these BAC clone sequences were analyzed for receptors similar to the previously described

FIGURE 1. Comparison of 18 M11 CHIR-AB1-like sequences. Amino acid alignment was performed with ClustalW. The primer sequences used for cloning are highlighted by the dashed boxes. Structural features such as Ig β-strands, signal peptide, transmembrane region, and conserved cysteine residues are marked by horizontal arrows, bars, and asterisks. Predicted N-linked glycosylation sites are depicted in boldface (N). The positively charged transmembrane arginine is displayed as a gray-shaded box; the potential YxxM signaling motif is boxed.

FIGURE 2. Six M11 CHIR-AB receptors bind to IgY. A. For expression control serial dilutions (log2) of soluble M11 CHIR-AB, fusion proteins were tested on anti-human IgG (Anti-huIgG)-coated plates. The dilutions of the supernatants were adjusted to have equal concentrations used for the subsequent IgY ELISA. B. Eighteen soluble CHIR-AB fusion proteins were reacted with plate-bound IgY.
FcY receptor CHIR-AB1. In addition to CHIR-AB1 present in the BAC clones (CHIR1C10), the coding regions of nine further CHIR-AB1 like sequences (CHIR1C1 to CHIR1C9 according to the nomenclature used by Laun et al. in Ref. 16) were extracted. All of them displayed a single extracytoplasmic Ig domain, a trans-membrane region containing arginine as positively charged amino acid, and a long cytoplasmic tail encoding one YxxM motif and one ITIM. The overall amino acid sequence identity between these CHIR-AB-like sequences is 85–97%, indicating a close relationship. We used these 10 sequences and the previously described B19 CHIR-AB1 sequence (GenBank accession no. AJ745094; Ref. 13) to design primers binding to conserved CHIR-AB-specific regions. The sense primer is located at the 5’ end of the signal sequence and the antisense encodes at the 3’ end of the CHIR-AB-specific YxxM motif (Fig. 1).

Identification of 18 CHIR-AB1 like receptors in a single animal PBMC-derived cDNA of one individual M11 chicken was amplified with the primers described above and the PCR product of ~580 bp was subcloned. Sequencing of 46 different clones yielded a total of 18 different CHIR-AB amino acid sequences (Fig. 1).Awaiting a final CHIR nomenclature once the entire locus has been sequenced, the receptors were designated by the chicken line, the receptor type, and an internal number (e.g., M11 CHIR-AB-500, derived from M11 chicken line, CHIR-AB type, number 500). The comparison of the 18 different M11 CHIR-AB sequences revealed an amino acid identity between 83 and 99% (Fig. 1). All sequences displayed a conserved potential N-linked glycosylation site (Fig. 1; N40, boldface) and two further N-linked glycosylation sites (Fig. 1; N21 and N85, bold) that are only present in some of the M11 CHIR-AB sequences.

A minimum of six FcY receptors are encoded in the CHIR family

To determine whether the cloned CHIR-AB1-like receptors bind to Ig, soluble CHIR-Ig-fusion proteins were produced by linking the extracytoplasmic CHIR Ig domain to the human IgG1 C1r2 and C1r3 domains. An anti-human IgG capture ELISA was used to control the expression and to adjust the concentration of the 18 different constructs after expression by transient transfection (Fig. 2A). Similar concentrations of all constructs were successively tested on plate-bound IgY, IgM, and IgA in an ELISA (Fig. 2B).
Of the 18 constructs, six (M11 CHIR-AB-500, M11 CHIR-AB-502, M11 CHIR-AB-579, M11 CHIR-AB-581, M11 CHIR-AB-586, and M11 CHIR-AB-599) showed binding to IgY (Fig. 2), whereas none of the receptors bound to IgA or IgM (data not shown). One of the newly cloned receptors, M11 CHIR-AB-586, which bound IgY, was found to be identical with the previously identified CHIR-AB1 Ig domain cloned from an B19 chicken (13, 15).

We next compared the Ig domain amino acid sequences of the IgY binders and nonbinders (Fig. 3A) to gain insight in potential interaction sites of CHIR-AB and IgY. This analysis indicated that there is one region between residues 80 and 85 where all of the IgY binders display identical amino acids, whereas in nonbinders one or several of these residues are different from this consensus motif. In addition, M11 CHIR-AB-582 and M11 CHIR-AB-600 are both nonbinders that each differ in a single amino acid (A313V31 in M11 CHIR-AB-582 and S763L76 in M11 CHIR-AB-600; Fig. 3A, shaded columns) compared with the IgY-binding M11 CHIR-AB-586.

To further identify residues that are important for IgY binding, we used mutational analyses of single amino acids (Table I). These mutations revealed five amino acids (A31, S76, W80, S82, and S85; Fig. 3A, shaded columns) that are critical for IgY binding. We used the crystal structure of B19 CHIR-AB1 (25) to visualize the potential binding sites to IgY (Fig. 3B). The amino acids potentially involved in binding to IgY are in close proximity to each other and are placed on one side of the Ig domain, presuming that the binding of IgY is located in that region. In conclusion, at least six CHIR versions function as FcY receptors in M11 chickens, all of which share five critical residues for potential IgY interaction.

**Repertoire analysis detects conserved and unique CHIR-AB molecules**

Due to the polymorphic nature of the CHIR gene family locus (15), we were interested in whether the subgroup of CHIR involved in IgY binding is fixed in the genome or if there is variation in different chicken lines. As noted above, one of the M11-CHIR-AB sequences was identical with the original B19 CHIR-AB1 Ig domain, thus indicating that at least one FcY receptor may be conserved. To extend these analyses, the cloning strategy used for M11 CHIR-AB was repeated using a single R11 chicken. Sequencing of 27 colonies resulted in 18 different amino acid sequences with an overall identity between 74 and 99%. Sixteen R11 CHIR-AB versions showed high similarity to CHIR-AB1, displaying a single extracytoplasmic Ig domain, arginine as positively charged transmembrane residue, and the YXXM cytoplasmic motif, whereas two receptors (R11 CHIR-AB-850 and R11 CHIR-AB-921) encoding a transmembrane lysine were found to be more related to the previously described CHIR-AB2 (94.4–98.5% identity) (15), which does not bind IgY (13). For further comparison, a phylogenetic tree was constructed using the single Ig domain sequences (Fig. 4). Three pairs of identical Ig domain sequences were found in M11 and R11 chickens, two of them belonging to

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**Table I. Effect of CHIR-AB mutations on IgY binding**

<table>
<thead>
<tr>
<th>Position</th>
<th>Template</th>
<th>IgY Binding of Template</th>
<th>Mutation</th>
<th>IgY Binding of Mutation</th>
<th>Primer (5′—3′)*b</th>
</tr>
</thead>
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<tr>
<td>31</td>
<td>CHIR-AB-582</td>
<td>—</td>
<td>V to A</td>
<td>+ + +</td>
<td>TGCCCGCAGTGGTCGCTGGTCCAGC</td>
</tr>
<tr>
<td>76</td>
<td>CHIR-AB-600</td>
<td>—</td>
<td>L to S</td>
<td>+ + +</td>
<td>GGTACACAGTGGTCAAGGCCACTCTGG</td>
</tr>
<tr>
<td>77</td>
<td>CHIR-AB-586</td>
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<td>+ + +</td>
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<td>CHIR-AB-586</td>
<td>+ + +</td>
<td>W to R</td>
<td>—</td>
<td>TCAGAGCCACCTGCAAGCATCAAAAGAG</td>
</tr>
<tr>
<td>82</td>
<td>CHIR-AB-586</td>
<td>+ + +</td>
<td>S to T</td>
<td>—</td>
<td>CATCTGGGACAACAAATGCAAGT</td>
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<tr>
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<td>+ + +</td>
<td>NQ to KK</td>
<td>+ + +</td>
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<tr>
<td>85</td>
<td>CHIR-AB-586</td>
<td>+ + +</td>
<td>S to C</td>
<td>—</td>
<td>CATCTGGGACAACAAATGCAAGT</td>
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<tr>
<td>82/85</td>
<td>CHIR-AB-586</td>
<td>+ + +</td>
<td>SNQ to TNQC</td>
<td>—</td>
<td>CATCTGGGACAACAAATGCAAGT</td>
</tr>
</tbody>
</table>

* A dash (—) denotes no binding activity; + + + denotes strong binding activity.

* Forward primers are shown and mutations are in boldface.

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**FIGURE 4.** Phylogenetic comparison of the CHIR-AB Ig domains. All M11, R11, and B19 CHIR-AB1 like sequences were compared using a neighbor-joining tree constructed using MEGA4 with 1000 bootstrap replicates and pairwise gap deletions. IgY binding capabilities of M11 CHIR-AB are indicated as IgY+ and IgY−. Identical sequences are indicated by dotted boxes.
IgY binders (Figs. 4 and 5; IgY/H11001). Among these, R11 CHIR-AB-915 and M11 CHIR-AB-586 are also identical with the previously characterized FcY receptor B19 CHIR-AB1 Ig domain (13), thus demonstrating that this receptor has been conserved in at least three different chicken lines. The third pair consisting of R11 CHIR-AB-907 and M11 CHIR-AB-487 resembled a nonbinder (Figs. 4 and 5; IgY/H11002).

For the remaining 15 R11 CHIR-AB sequences no identical counterpart could be detected in the M11 sequences, and it thus remains speculative if they can bind to IgY (Fig. 5; rows labeled "IgY?"). These comparative analyses reveal that different chicken lines may have both unique and highly conserved FcY receptors.

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**FIGURE 5.** Sequence alignment of the Ig domains of 18 R11 CHIR-AB homologues. Features are highlighted as in Fig. 3A. The consensus sequence displays the amino acid sequence that is present in more than nine of the sequences. The sequences are subgrouped in blocks designated as IgY+, IgY?, and IgY− to indicate the prediction of IgY reactivity. Bold labeling of R11 CHIR-AB highlights sequences identical with those of M11 CHIR-AB.

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**FIGURE 6.** Biosensor affinity measurement of M11 CHIR-AB fusion proteins. A, Anti-human IgG (left panel) and chicken IgY (right panel) were used as capture agent and reacted with decreasing amounts of selected purified CHIR-AB fusion proteins. B, A kinetic analysis of the interaction of IgY with the different immobilized M11 CHIR-AB fusion proteins was performed by injecting varying concentrations of analyte (as indicated). Two high-affinity (M11 CHIR-AB-586 and CHIR-AB-599; left panels) and one low-affinity receptor (M11 CHIR-AB-581; upper right panel) could be identified. M11 CHIR-AB-583 was demonstrated to be a nonbinder (lower right panel). Apparent Kd values were calculated to 25, 27, and 260 nM for M11 CHIR-AB-586, CHIR-AB-599, and CHIR-AB-581, respectively. Obtained binding curves are shown as solid black lines and the resulting fits as dotted gray lines.
Low and high affinity FcY receptors are expressed in single animals

Based on the CHIR fusion protein binding to IgY in the ELISA, three different groups of high, intermediate, and nonbinders could be differentiated (Fig. 2B). Because these results were obtained using supernatants of transfected cells, in a further step four receptors representing these groups were selected, stably expressed, purified, and assayed in the human IgG capture ELISA (Fig. 6A). The four fusion proteins were then tested for their binding to IgY. When the binding was assayed using plate-bound IgY, the descending order of binding was M11 CHIR-AB-599 and M11 CHIR-AB-586, followed by M11 CHIR-AB-581 (Fig. 6A). To obtain more accurate affinity predictions, surface plasmon resonance analysis was performed, injecting IgY over the immobilized CHIR-AB fusion proteins. For the IgY interaction with M11 CHIR-AB-586, CHIR-599, and CHIR-581, apparent \( K_D \) values could be obtained by a kinetic binding analysis (Fig. 6B). After fitting the resulting data to a 1:1 binding model, the \( K_D \) values for IgY binding were calculated to 25 nM for M11 CHIR-AB-586, 27 nM for M11 CHIR-AB-599, and 260 nM for M11 CHIR-AB-581, respectively (Fig. 6B), thus confirming the ELISA results. IgY displayed no interaction with M11 CHIR-AB-583 (Fig. 6B).

In conclusion, these results support the differentiation of three different groups of those CHIR versions acting as FcRs in high, intermediate, and nonbinders as they show significant differences in affinity to their ligands.

Discussion

The CHIR locus has been characterized as the chicken counterpart to the mammalian LRC (15). Both loci harbor a large set of activating and inhibitory Ig-like receptors that are expressed by a wide variety of leukocytes (15, 26). Some of the LRC receptor families such as the KIR and the CHIR display a high degree of heterogeneity due to allelic polymorphism and haplotypic variation in gene content. In man, rat, and cattle, the FcεRI (CD89) is located adjacent to the KIR family and shares more homology to the LILR (leukocyte Ig-like receptor) and KIR genes than to the other FcRs located on chromosome 1. In addition, the bovine FcγR2 is encoded within the LRC. Therefore, it was not unexpected that one of the CHIR versions (CHIR-AB1) was found to act as a high-affinity FcR for chicken IgY. In contrast to the mammalian LRC with 30 Ig-like receptors in man, the chicken LRC has been massively expanded with hundreds of CHIR genes (16). The present study was initiated to clarify whether we had serendipitously found a single monomorphic FcR within the chicken LRC or if this genomic region encodes multiple FcRs.

Although parts of the chicken LRC have been assembled and sequenced (16), a complete genomic map is not yet available, most likely because of the difficulties to assemble such a multigene family. Due to this limitation we focused on expressed CHIR in individual chickens originating from different lines, accepting that this approach can only give a minimal estimate of the CHIR-AB gene number. Eighteen different CHIR-AB homologues could be identified in both lines, indicating that these are encoded by at least nine different loci. This number is close to the 10 CHIR-AB loci described in the partial genomic sequence (16). Interestingly, the CHIR-AB sequences form a separate phylogenetic group when compared with all CHIR sequences available, including those with two Ig domains (data not shown).

The Ig binding capacities of the CHIR-AB homologues as measured by ELISA and surface plasmon resonance could be grouped into high, intermediate, and nondetectable binding categories, a situation that closely resembles the various FcRs and FcRL proteins in man (1, 4). The function of FCRL proteins is largely unknown (4), and likewise the function of CHIR-AB homologues that do not bind IgY has to be determined. The preferential and competitive interaction of the various mammalian FcRs with different Ig subclasses enables a highly specific immune response ranging from cellular inhibition to activation. The different CHIR-AB homologues may enable a similar range of functional responses, especially since their intracellular signaling capabilities with ITIM and potential association to the common Fcγ chain leave both options open. This would imply that there is IgY heterogeneity in the chicken. IgY subclasses have not been reported; however, the respective genomic region has not been sequenced and at least several IgY allotypes have been defined (27). Even in the presence of only one IgY, there are alternative explanations for the various CHIR-AB homologues expressed in single animals. Variations in the promoter regions may allow differential expression of individual CHIR-AB on various immune cells and up- or down-regulation upon cell activation, respectively. Moreover, even subtle affinity differences between distinct CHIR-AB receptors may have a big influence on the immune response.

To evaluate the binding data of IgY injected over various CHIR-AB-Ig fusion proteins, we used a 1:1 binding model instead of a stepwise or sequential binding model. However, it has been demonstrated that higher apparent \( K_D \) values may be expected due to avidity effects (25). The recent determination of IgY binding affinity to the chicken cell line MQ-NCSU has revealed apparent \( K_D \) values comparable to the calculations using the 1:1 binding model (12), indicating that avidity effects may be important on the cell membrane, where CHIR may be clustered in lipid rafts. It is of note that this cell line reacts with the CHIR-AB1-specific mAb 8D12 (13) and that the IgY binding can be blocked by preincubation with the mAb (data not shown), suggesting that in fact IgY-CHIR-AB1 interactions were measured.

The evolution of the LRC and FcR gene families has been recently analyzed in different vertebrate species (28, 29). Following the separation of mammals and birds, the FcR function may have migrated from the LRC to the FcR locus present on human chromosome 1 (12). The syntenic region of chromosome 1 in chicken harbors only a single FcεRL gene with no apparent binding capability to IgY (11), and an additional chicken FcεR is located on chromosome 25, a region that contains no FcR gene in man (30). The LRC-encoded FcRs such as CD89, CHIR-AB1, or bovine FcγR2 are more related to the LRC genes than to the FcR genes, and their binding site to IgG or IgA is created by residues of the two C-terminal domains. In contrast to the classical FcεR, they bind their ligand with a 2:1 stoichiometry, as has also been demonstrated for CHIR-AB1 (25). Therefore, it seems possible that the CHIR-AB1-IgY interaction more closely reflects that of mammalian CD89 to IgA. A crystal of the receptor ligand complex will answer this question and should be feasible, because both have been successfully crystallized individually (25, 31).

Multigene families are frequently shaped by intergenic and intragenic recombinations causing alterations in the number of genes and domain shuffling, respectively. These processes seem to have extensively occurred in the chicken LRC leading to multiple CHIR-AB genes, some of which bind to IgY, and similar processes may have shaped the mammalian FcεR region. This extraordinary CHIR diversity most likely reflects a pathogen-driven selection process. CHIRs are expressed on a wide variety of leukocytes, including innate immune cells, and may thus be in part responsible for innate immune recognition. In fact, FcRs are essential in bridging innate and adaptive immune responses. It is thus plausible that
rapid birth and death evolution within the chicken LRC has generated multiple CHIR subgroups that have acquired diverse functions such as Ig binding, innate immune recognition, and immune modulation of innate and adaptive responses.

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Disclosures
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