Chicken Ig-Like Receptor B2, a Member of a Multigene Family, Is Mainly Expressed on B Lymphocytes, Recruits Both Src Homology 2 Domain Containing Protein Tyrosine Phosphatase (SHP)-1 and SHP-2, and Inhibits Proliferation

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Chicken Ig-Like Receptor B2, a Member of a Multigene Family, Is Mainly Expressed on B Lymphocytes, Recruits Both Src Homology 2 Domain Containing Protein Tyrosine Phosphatase (SHP)-1 and SHP-2, and Inhibits Proliferation1

Birgit C. Viertlboeck,* Richard P. M. A. Crooijmans,† Martien A. M. Groenen,‡ and Thomas W. F. Göbel*‡*

Ig-like inhibitory receptors have been the focus of intensive research particularly in mouse and human. We report the cloning and characterization of three novel inhibitory chicken Ig-like receptors (CHIR) that display a two Ig-domain extracellular structure, a transmembrane region lacking charged residues and a cytoplasmic domain containing two ITIM. The localization of all receptors to a small genomic region and the hybridization pattern indicated that they belong to a multigene family. The genomic structure of the extracellular domain with two exons encoding the signal peptide and single exons for each Ig domain resembled that of all human leukocyte Ig-like receptors and killer cell Ig-like receptors, whereas the exons encoding the C terminus displayed a structure closely resembling killer cell Ig-like receptor genes. A mAb generated against one receptor designated CHIR-B2 reacted with all B cells and a small T cell subset, but not with monocytes, thrombocytes, or various leukocyte-derived cell lines. The mAb immunoprecipitated a 46-kDa protein from bursal cells and transfected cells. The Src homology 2 domain containing protein tyrosine phosphatase (SHP)-2 bound to CHIR-B2 even in unstimulated cells, whereas pervanadate treatment induced the tyrosine phosphorylation and recruitment of several CHIR-B2-associated proteins including SHP-1 and increased levels of SHP-2. Moreover, mAb cross-linking of CHIR-B2 reduced the proliferation of a stable transfected cell line. Together, we have identified a multigene family containing multiple CHIR including one receptor designated CHIR-B2 that is mainly expressed on B lymphocytes and inhibits cellular proliferation by recruitment of SHP-1 and SHP-2. The Journal of Immunology, 2004, 173: 7385–7393.

Leukocytes are regulated by opposing signaling pathways that either activate or inhibit cellular responses. At least two biochemically distinct families mediate cellular inhibition, the Ig-like receptors and the C-type lectins (1–3). Most inhibitory receptors in mammals are encoded by gene clusters with multiple members. The leukocyte receptor complex (LRC) is located on human chromosome 19q13.4, it spans ~1 Mb, and contains over 40 genes (4). These genes can be grouped into four families of Ig-like receptors including the leukocyte Ig-like receptors (LILR; HUGO nomenclature) and Ig-like transcripts (ILT), the killer cell Ig-like receptors (KIR) and glycoprotein VI, NKp46 and FcR, and finally the leukocyte-associated Ig-like receptors (5). Other chromosomal regions encode additional inhibitory receptors such as signal regulatory proteins, CD200R, and triggering receptors expressed by myeloid cells among others (6). A syntenic region of the LRC is located on murine chromosome 7 and contains genes such as the paired Ig-like receptors (PIR) that are closely related to the LILR and ILT, whereas murine homologues to the human KIR genes have not been identified in this complex (7).

KIR and LILR/ILT genes are both encoded in the LRC, share features such as variable numbers of Ig-like extracellular domains, are multigene families with either inhibitory or activating members, and some bind to MHC; however, they also differ in several aspects. The KIR expression is restricted to NK cells and T cell subsets, whereas LILR/ILT genes depending on the individual receptors are confined to the myelomonocytic and lymphocytic lineage (3, 8). A significant feature of KIR genes are the haplotype differences in gene number and the high allelic polymorphism that create a highly diversified KIR repertoire, whereas LILR display lower allelic and haplotype diversity. These features as well as the identification of putative LILR homologues in nonmammalian vertebrates have suggested that KIR genes represent a relatively young and LILR genes an ancient gene family (9, 10).

The Ig-like receptors encoded by the LRC are functionally divided into inhibitory and activating receptors, due to differences in the transmembrane and cytoplasmic domains. Inhibitory receptors contain cytoplasmic ITIM with the prototypic sequence motif I/L/V/SxYxxL/V (x denotes any amino acid) (2). Following receptor ligation the tyrosine residues in the ITIM are phosphorylated and provide a binding site to recruit Src homology 2 domain containing protein tyrosine phosphatases (SHP), such as SHP-1 and SHP-2. These phosphatases readily dephosphorylate intracellular substrates and thus stop further cellular activation (11). Whereas...
SHP-2 can associate with both the unphosphorylated and phosphorylated ITIM of the receptors, SHP-1 is recruited only to the phosphorylated ITIM following receptor ligation (12).

In contrast to the inhibitory receptors, the activating receptors lack the ITIM due to a short cytoplasmic domain, and they have a positively charged transmembrane residue that mediates the association with adapter molecules such as DNAX-activating protein of 10 and 12 kDa, CD3ζ, and FceRIγ. The cytoplasmic ITAM of these adapter molecules are phosphorylated upon receptor ligation and activate intracellular signaling cascades (13).

Most of the LRC-encoded genes have only been studied in rodents and humans, with the exception of the recently described bovine KIR (14). To date only few nonmammalian inhibitory Ig-like receptors have been characterized. In the chicken two receptors, designated chicken Ig-like receptor (CHIR)-A and CHIR-B with two Ig-like extracellular domains, have been isolated (15). Whereas CHIR-B displays typical features of an inhibitory receptor with two cytoplasmic ITIMs, CHIR-A has only a short cytoplasmic domain and a charged transmembrane residue. A large family of novel immune-type receptor genes has been characterized in bony fish that are characterized by two Ig ectodomains, one being a typical V-like domain and the membrane proximal Ig domain characterized as a V-like C2 domain that contains J-like elements. Both inhibitory ITIM containing and potential activating members have been characterized (16, 17). All of these studies have been limited by the lack of specific reagents to analyze the biochemical properties and tissue distribution of these nonmammalian receptors in more detail.

We identify three novel CHIRs that display typical features of inhibitory receptors, belong to one multigene family, and analyze their genomic organization. The generation of a novel mAb against one of these receptors designated CHIR-B2 enabled the detailed characterization of CHIR-B2 as an inhibitory receptor that is mainly expressed on B cells. Upon stimulation, this receptor associates with both SHP-1 and SHP-2 tyrosine phosphatases and inhibits the proliferation of a B cell line, indicative of its function as inhibitory receptor on chicken B lymphocytes.

Materials and Methods

Animals and immunizations

Chicken lines H.B19 (B/B), and commercial LSL birds (Lohmann, Cuxhaven) were hatched at the Institute for Animal Physiology, University of Munich (Munich, Germany) and the animals were used for experiments at the age of 3–10 wk. BALB/c mice were raised at the Institute.

Cloning procedures

Two different chicken EST (Expressed Sequence Tag) database repositories (18, 19) were screened by keyword search using “Ig-like”. Candidate sequences were further analyzed using the DNASTAR Lasergene software package (GATC, Konstanz, Germany), including sequence assembly programs. Assembled sequences were used to translate putative coding sequences and to synthesize oligonucleotides for PCR analysis. For cloning, genomic DNA or total RNA was prepared using H.B19 chicken erythrocytes, splenocytes, or bursal cells because one EST database originated from H.B19 bursa (18). Commercial kits were used for genomic DNA kit (Qiagen, Hilden, Germany) and for RNA preparation (TRizol; Invitrogen Life Technologies, Karlsruhe, Germany) and first strand cDNA synthesis (ThermoScript RT-PCR System; Invitrogen Life Technologies). The oligonucleotides used for PCR were all used at 60°C annealing temperature and are summarized in Table I. Following TA cloning using the eukaryotic TA cloning kit (Invitrogen Life Technologies), colonies were screened by PCR and restriction analysis and positive clones were sequenced (GATC).

Further analyses of the deduced protein sequences were performed with the InterProScan (http://www.ebi.ac.uk/InterProScan) and the Psort (http://psort.nibb.ac.jp/form2.html) computer programs. For the construction of an N-terminal FLAG-tagged CHIR-B2, the chicken MHC class I signal peptide was PCR amplified using an oligonucleotide encoding the eight amino acid FLAG epitope and cloned into the BamHI, EcoRI sites of the pcDNA6/V5-His A vector (Invitrogen Life Technologies). The CHIR-B2 gene lacking the endogenous signal sequence was PCR amplified with the oligonucleotide pair 488 and 489 (Table I) and inserted into the EcoRI site of the vector resulting in addition of the FLAG epitope to the N terminus after cleavage of the MHC class I-derived signal peptide (designated CHIR-B2-FLAG). The genomic CHIR-B2 sequence was obtained by PCR on H.B19 derived genomic DNA with primers 474 and 475 (Table I). Two dimensional screening of the Wageningen chicken Bacterial Artificial Chromosome (BAC) library was performed as described using the oligonucleotides 474 and 475 (20). For Southern blot analysis genomic DNA was digested with EcoRI, PstI, and SfiI (MBI Fermentas, St. Leon-Rot, Germany), separated on 1% agarose gel and blotted onto a Hybond N membrane (Amersham Pharmacia Biotech, Freiburg, Germany). CHIR-B2 cDNA was 32P-labeled with the High Prime DNA labeling kit (Roche, Penzberg, Germany) and hybridized to the membrane using the QuickHyb hybridization solution (Stratagene, Amsterdam, The Netherlands) at 60°C for 2 h.

Cell lines, transfections, and generation of mAb

Human embryonic kidney (HEK) 293 T cells (21) and the reticuloendotheliosis virus transformed chicken B cell line 2D8 (22) were maintained in RPMI 1640 medium with Glutamax (Invitrogen Life Technologies) supplemented with 10% FCS, 1% penicillin/streptomycin in a CO2 incubator at 37°C and 40°C, respectively. HEK 293 T cells were transfected using the Metafectene reagent (Biontex, Munich, Germany) according to the manufacturer’s protocol. For transient transfection, cells were harvested at 48 h posttransfection and analyzed by flow cytometry for high frequency surface expression before using for immunization. 2D8 cells were transfected using the Dosper reagent (Roche) according to the manufacturer’s protocol. For stable transfection, cells were harvested at 24 h posttransfection and plated at 1 × 105 cells/well in a 96-well flat-bottom plate including 10 μg/ml blasticidin S hydrochloride (Sigma-Aldrich, Taulkichen, Germany). Following 2 wk of selection, single colonies were screened by flow cytometry for expression of the FLAG epitope. A stable cell line with high expression designated 2D8-CHIR-B2-FLAG was further used for the experiments. For mAb generation, mice were immunized three times in 3-wk intervals with 1 × 107 HEK 293 T cells transiently transfected with the CHIR-B2-FLAG construct. The mAbs were produced according to standard methods and the hybridoma supernatants were screened by flow cytometry on 2D8-CHIR-B2-FLAG cells as well as untransfected 2D8 cells.

Table I. Oligonucleotides used for cloning

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<th>No.</th>
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<th>Orientationb</th>
<th>Specificity</th>
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<td>pcDNA6/V5-His A-FLAG construct</td>
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<tr>
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<td>AS</td>
<td></td>
</tr>
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<td>474</td>
<td>GGCCGGCCGGCGGACAC</td>
<td>S</td>
<td>CHIR-B-Signal Peptide</td>
</tr>
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<td>AS</td>
<td>CHIR-B2</td>
</tr>
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<td>S</td>
<td>CHIR-B2-FLAG construct</td>
</tr>
<tr>
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<td>AS</td>
<td>CHIR-B3</td>
</tr>
<tr>
<td>498</td>
<td>CCCCCGCTAGGGCACCCTT</td>
<td>AS</td>
<td>CHIR-B4</td>
</tr>
<tr>
<td>574</td>
<td>GCACACCGGACACACTGGCACC</td>
<td>AS</td>
<td></td>
</tr>
</tbody>
</table>

a Restriction sites are underlined; for No. 307, the FLAG epitope is indicated by bold face italics.

b Orientation indicated as S, sense; AS, antisense.
Cell preparations, staining, and proliferation assay

Single cell suspensions of splenocytes, thymocytes, bursal cells, and caecal tonsils were generating by passing the organs through a stainless steel mesh. Mononuclear cells were prepared from the cell suspensions by density centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech). PBL were obtained by speed centrifugation as previously described (23). The mAb used were specific for CD3 (CT3) (24), L chain (both Southern Biotechnology Associates, Birmingham, AL), CHIR-B2 described in this report (3H7, mouse IgG2b), or the FLAG epitope (M2, Sigma-Aldrich). For single staining cells were incubated with the mAb followed by an anti-mouse Ig-FITC conjugate, whereas for double staining, cells were first incubated with a mixture of primary mAb, followed by a mixture of anti-mouse IgG1-PE and anti-mouse IgG2b-FLAG (Con A, Southern Biotechnology Associates). Cells were analyzed with a FacsScan (Becton Dickinson, Heidelberg, Germany) using the CellQuest software.

The proliferation of 2D8-CHIR-B2-FLAG cells was measured using a standard [3H]thymidine incorporation assay. The 96-well plates were coated with 50 μl of 10 μg/mI mAb (3H7 or isotype-matched control) overnight at 4°C. Following washing 1 x 10^6 cells were incubated in the coated wells for 18 h and in the presence of 1 μCi [3H]thymidine for 6 h. Following harvesting of the cells, [3H]thymidine incorporation was measured with a Top Count NXT Microplate Scintillation and Luminescence Counter (Packard, Dreieich, Germany).

Biotinylation and pervanadate treatment of cells

The biotinylation of primary cells was performed according to standard procedures (25). Briefly, 5 x 10^5 cells were harvested and washed twice in PBS containing 100 mM NaCl, 0.1 mM EDTA, and 0.1% BSA. Three washes were performed by incubation for one min at 4°C with 0.5 mg/ml sulfo-NHS-biotin (Pierce, Rockford, IL) in 6 ml PBS followed by two washes in RPMI 1640 and control staining with streptavidin-PE to check the biotinylation efficiency. For pervanadate treatment, 5 x 10^5 cells in 5 ml of prewarmed medium were stimulated for 5 or 15 min with 0.1 mM Na_2VO_3 containing 0.05% H2O_2 at 37°C. Untreated and treated cells were immediately pelleted by a 4°C centrifugation, followed by a wash in cold PBS containing 0.4 mM EDTA and 0.1 mg/ml of protein G-coupled streptavidin (Upstate Biotechnology, Lake Placid, NY) or 1 mg/ml mouse IgG-1-PE and anti-mouse IgG2b-FLAG (Con A, Southern Biotechnology Associates). The biotinylated cells were washed once with lysis buffer before addition of the mAb followed by goat anti-rabbit Ig-POD (1:5,000; Amersham Pharmacia Biotech). Immunoblotted proteins were visualized using chemiluminescent detection (Super Signal; Pierce).

Results

CHIR-B proteins represent typical two Ig-domain receptors with cytoplasmic ITIMs

Several EST databases were searched by keyword using “Ig-like” to identify partial sequences encoding novel Ig-like receptors. Following this initial search all sequences were aligned to identify overlapping contiguous sequence fragments. The preliminary analysis of the predicted amino acid sequence allowed the selection of those aligned sequences with extracellular Ig domain that contained cytoplasmic ITIMs. Because the sequences analyzed displayed virtually identical signal peptide sequences, a single sense PCR primer was combined with several sequence specific antisense primers (Table I) and various cDNAs derived from different tissues and cell lines were screened for positive signals. The amplified products were subcloned and sequenced and full-length coding regions were obtained by 3’ RACE-PCR.

Using this strategy, three novel sequences were identified and aligned with the previously published CHIR-B sequence (Fig. 1). To extend the previously established nomenclature, the designation CHIR was used and the individual members were numbered according to their identification so that the previously published CHIR-B was renamed CHIR-B1 (15) and the clones identified in this report were designated CHIR-B2 to CHIR-B4 (under agreement with Dr. M. D. Cooper, University of Alabama, Birmingham, AL). All of the CHIR-B clones shared two extracellular Ig domains, which were further classified as C2-type Ig domains according to their cysteine spacing and conserved motifs. CHIR-B1 and CHIR-B4 shared the highest homology of ~70% amino acid identity, whereas the homology of other CHIRs ranged from 54 to 61%. CHIR-B2 had an extra 10 amino acid addition in the second Ig domain, which was also observed on the corresponding genomic sequence (Fig. 1; see Discussion). The 17 (CHIR-B1 to CHIR-B4) amino acid transmembrane region did not contain a charged residue and was followed by a 70 (CHIR-B1 and CHIR-B4), 71 (CHIR-B2), or 68 (CHIR-B3) amino acid long cytoplasmic region with two ITIMs. The predicted molecular mass of the various CHIR-B genes was ~33.5 kDa, and the extracellular domain had two to three potential N-glycosylation sites. A conclusive assignment of the CHIR to mammalian homologues was impossible due to the phylogenetic distance of birds and mammals. The closest homology of ~30 to 35% amino acid identity was found between the CHIR-B sequences and proteins such as human glycoprotein VI, osteoclast-associated receptor-M1, rat IgSF member 1 and horse LILRA. In conclusion, CHIR-B represents typical Ig-like receptors with cytoplasmic ITIMs.

Genomic complexity and BAC location of CHIR-B genes

Inhibitory Ig-like receptors have been grouped into various families that are scattered throughout the genome (6). To test whether the different CHIR-B identified belong to one multigene family, a chicken BAC library was screened with primers specific for CHIR-B. Several CHIR-B2 positive clones were isolated and successively tested by PCR for the presence of the other CHIR-B genes (Fig. 2A). Whereas several clones were positive for two to three CHIR-B genes (clones bW004C11 and bW052G08), a single BAC clone (bW093H17) of ~125 kb was identified that was positive for all four CHIR-B genes (Fig. 2A). The size difference particularly observed for CHIR-B3 may originate by variation of the intron length. In addition, diversity of the CHIR family was also evident following genomic blotting, where multiple hybridizing fragments were observed (Fig. 2B). This result indicates that CHIR-B1 to CHIR-B4 genes are members of a single multigene family.

To characterize the genomic organization of CHIR-B2, the locus was amplifed by PCR on genomic, H.B19 derived DNA because the cDNA was also obtained from H.B19 chickens to facilitate comparison between the corresponding cDNA and genomic DNA. The 2050-bp nucleotide sequence was 99.7% identical with the corresponding cDNA, indicative of an allelic form. CHIR-B2 was
encoded by seven exons. The exon IV specific for the second Ig-like domain contained 30 additional bp encoding the 10 additional amino acids found in CHIR-B2 (Fig. 3). To compare this result to the previously published CHIR-B1, we isolated a genomic clone by PCR that was 92% identical with the CHIR-B1 cDNA sequence, but lacked the 30 bp addition in the second Ig-like domain (Genbank accession number AJ833636), thus indicating that both forms are present in the genome. Generally the intron sequences were very short, as frequently observed for chicken genes. The exon intron boundary sequences (Table II) were identical with those found in KIR and LILR/ILT genes, with exons I to V in phase 1 and exon VI in phase 0. Like in all LILR/ILT and KIR genes, the signal peptide was encoded by two exons and the Ig domains were each encoded by separate exons (Fig. 3). The membrane-proximal extracellular region, the transmembrane domain and part of the cytoplasmic regions were encoded by exon V that was followed by a short exon VI and exon VII that encoded the ITIM (Fig. 3).

CHIR-B2 is mainly expressed on B lymphocytes
Following this initial isolation of the various CHIR, one prototypic inhibitory receptor (CHIR-B2) was selected for further characterization. A chicken B cell line (2D8) was stably transfected with a CHIR-B2 construct that contained an N-terminal FLAG-epitope tag for detection. This cell line expressed high levels of CHIR-B2 on the cell surface as compared with the untransfected cells (Fig. 4). For the generation of CHIR-B2-specific mAb HEK 293 T cells were transiently transfected with the CHIR-B2-FLAG construct for 48 h and used for three successive immunizations. The resulting hybridoma supernatants were then differentially screened by cytometry on 2D8 wild-type cells and 2D8-CHIR-B2-FLAG cells, respectively, and the mAb 3H7 was selected due to its reactivity with the transfected cells but not the wild-type cells (Fig. 4). Because the various CHIR-B that were identified show considerable homology, the 3H7 mAb was tested for its cross-reactivity by transiently transfecting HEK 293 T cells with the various CHIR-B followed by cell surface staining with the 3H7 mAb. Because 3H7 did not react with the other CHIR-B genes transfected into HEK 293 T cells, it is specific for CHIR-B2 (data not shown). 3H7 was also unreactive with a panel of chicken cell lines, including B cell lines (RP9, CU68, DT40), T cell lines (UG9, 855-23), macrophage cell lines (HD11, BM2), and an erythroblast cell line (HD3, data not shown).

The immunofluorescence analysis of various chicken tissues with the 3H7 mAb revealed a percentage of positive cells that matched the anticipated frequency of B cells in these tissues. Therefore double staining using 3H7 together with either B cell-specific (IgL) or T cell-specific (CD3) mAb was performed on cells isolated from bursa, spleen, PBL, and caecal tonsils (Fig. 4). In all of these organs, virtually all Ig positive cells coexpressed the 3H7 Ag. In addition, a small subset of CD3 H11001 cells coexpressed CHIR-B2 (Fig. 4). Further analyses revealed that these 3H7 H11001/CD3 H11001 cells were present in low frequencies in the CD4, CD8, TCR-1, TCR-2, and TCR-3 T cell subpopulations, and they were only detected in the spleen and not in PBL (data not shown). The 3H7 staining intensity was rather low on the B cells thus indicating a low Ag density on the cell surface. These results identify CHIR-B2 as a receptor expressed on all B cells and a small T cell subset.

CHIR-B2 is a 46-kDa glycosylated protein
The biochemical analyses were performed using the 2D8-CHIR-B2-FLAG cells as well as primary cells. 2D8-CHIR-B2-FLAG lysates were immunoprecipitated on anti-FLAG beads, blotted and detected with an anti-FLAG-POD conjugate. Compared with the wild type 2D8 control, a single major protein was detected that had an apparent molecular mass of 46 kDa under both nonreducing and reducing conditions (Fig. 5, left panel). Under nonreducing conditions, in addition to the predominant 46 kDa form, a higher molecular mass protein of 85 kDa was also observed, which may have resulted from an aggregation of CHIR-B2 protein.
resemble a dimeric form. Treatment with PNGaseF to remove N-linked carbohydrates reduced the molecular weight to 35 kDa, which is close to the calculated molecular weight of 34 kDa based on the amino acid sequence. Identical results were obtained by immunoprecipitation of biotinylated 2D8-CHIRB2-FLAG cell lysates with an anti-FLAG mAb followed by streptavidin-HRP detection (data not shown).

These data obtained from a cell line transfected with an epitope-tagged CHIR-B2 were compared with primary cells. For this purpose, bursal cells or thymocytes were biotinylated, lysed, and immunoprecipitated with the 3H7 mAb. Following visualization with a streptavidin-POD conjugate a 46-kDa protein was clearly detectable under reducing conditions in bursal lysates, but not in thymocyte-derived lysates (Fig. 5, right panel).

Together these data characterized the CHIR-B2 protein as a glycosylated protein of 46 kDa with the potential to form homodimers.

**CHIR-B2 phosphorylation leads to the inhibition of cellular responses by SHP-1 and SHP-2 recruitment**

Some inhibitory receptors such as PIR-B and KIR are known to recruit SHP-1 and SHP-2 upon tyrosine phosphorylation. To test whether similar signal transduction pathways are used in the case of CHIR-B2, the transfected cells were treated with pervanadate for different time intervals (Fig. 6). The anti-FLAG detection served as an internal control for loading equal amounts of protein following each incubation period. In untreated cells, only weak tyrosine phosphorylation was detected that increased after 5 min and particularly 15 min of pervanadate treatment. To identify some of the tyrosine phosphorylated proteins that associated with CHIR-B2, SHP-1- and SHP-2-specific Abs were used. CHIR-B2 did not bind SHP-1 in untreated cells but following 5 and 15 min of pervanadate treatment.

**Table II. Genomic organization of CHIR-B2 with exon/intron phases**

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<th>Exon</th>
<th>5’ Intron</th>
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<td>lyTrp</td>
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<tr>
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*Length of exons and introns (in base pairs) is given in parentheses, exon-intron boundary sequences are underlined.
pervanadate treatment, it associated to CHIR-B2. In contrast, basal levels of SHP-2 were associated to CHIR-B2 even in untreated cells and a 5 min pervanadate stimulation lead to maximum levels of associated SHP-2. Interestingly, CHIR-B2 was not phosphorylated in freshly isolated bursal cells as analyzed after 3H7 immunoprecipitation and Western blot analysis (data not shown).

In a further experiment, the functional consequences of CHIR-B2 cross-linking in 2D8-CHIR-B2-FLAG cells were analyzed by incubating the cells in the presence of plate-bound 3H7 or control mAb. 3H7 cross-linking for 24 h lead to a marked reduction of cellular proliferation as compared with an isotype-matched control or medium alone (Fig. 7). The comparison of treated vs untreated cells indicated that 3H7 cross-linking did not affect the viability and the rate of apoptosis as detected by annexin V and PI labeling (data not shown). These results indicate that CHIR-B2 recruits both SHP-1 and SHP-2 and inhibits cellular proliferation.

FIGURE 4. CHIR-B2 expression on transfected and primary cells. A, The untransfected or CHIR-B2-FLAG transfected 2D8 B cell lines were stained with either anti-FLAG or 3H7 mAb and analyzed by flow cytometry. B, Immunofluorescence analyses of various chicken tissues as indicated using the CHIR-B2-specific 3H7 mAb in combination with mAb specific for the Ig L chain and CD3, respectively. Numbers indicate the percentage of cells in the respective quadrants.
Discussion

Multiple families harboring Ig-like inhibitory and activating members have been recently described in mammals, whereas only few receptors such as novel immune type receptors in fish (17) and CHIR in chickens (15) were isolated in other vertebrates. To our knowledge the data presented in this study resemble the most conclusive characterization of a nonmammalian inhibitory receptor extending the previous molecular data by the generation of a specific mAb that was used to define the biochemical properties, the signal transduction pathways, functional aspects, and the cellular distribution of a single representative receptor designated CHIR-B2.

This study was initiated to identify novel Ig-like receptors in the chicken with the help of EST databases. The three novel receptors together with the previously characterized CHIR-B1 most likely resemble individual genes of a multigene family. The CHIR-B2 sequence contained an addition of 10 amino acids in the second Ig-like domain. These 10 amino acids were also observed in the corresponding genomic DNA sequence (Fig. 3) although they were absent in the genomic sequences of the CHIR-B1 gene. These extra amino acids represent an extraordinary addition to an Ig-like domain. The amino acid homology of 54–70% between the different individual CHIR-B sequences is indicative of individual genes rather than allelic variants. In contrast, the corresponding CHIR-B2 genomic sequence that shows >99.7% identity and originates from the same chicken line, most likely represents an allelic form. The colocalization of all four CHIR-B sequences to a single BAC clone suggests that they are located on a relatively short chromosomal region and therefore represent members of a multigene family. The high homology is also evidence that they form one family of receptors. Finally, CHIR-B2 hybridized with multiple genomic fragments in Southern blot analysis indicative of genomic diversity.

In addition to inhibitory receptors, mammalian Ig-like receptor families also include corresponding receptors with putative activating function due to the lack of ITIM and the association to adaptor molecules through a positively charged transmembrane residue (5). The functional consequences of these activating/inhibitory receptor pairs are not fully understood. A similar situation is also found in the fish novel immune type receptor family (17) and seems to be likely for the CHIR. The previously characterized CHIR-A sequence that shares 57–75% amino acid identity to the various CHIR-B sequences, lacks a cytoplasmic ITIM, and contains a positively charged transmembrane residue is likely to represent such an activating receptor (15). Taken together, these data indicate that the CHIR family is a typical Ig-receptor family that is composed of multiple Ig-like inhibitory and activating receptor genes.

It is more challenging to find a true mammalian homologue of the CHIR family. This cannot be based primarily on sequence identity or homology due to the phylogenetic distance of mammals and birds of ~330 million years. Therefore other factors such as

FIGURE 5. The CHIR-B2 protein resembles a 46-kDa protein. The CHIR-B2-FLAG transfected 2D8 cell line and untransfected 2D8 cells were lysed and immunoprecipitated using the anti-FLAG mAb. PNGaseF treatment (+) was used for deglycosylation and the proteins were analyzed under nonreducing (NR) or reducing (R) conditions, blotted and reacted with an anti-FLAG-POD conjugate (left). Cells isolated from thymus or bursa were surface biotinylated, lysed, and immunoprecipitated with the 3H7 mAb. Western Blot analysis was successively performed with a streptavidin-POD conjugate (right).

FIGURE 6. CHIR-B2 constitutively associates with SHP-2 and recruits SHP-1 upon phosphorylation. 2D8-CHIR-B2-FLAG cells were treated with pervanadate for the times indicated, lysed, and immunoprecipitated with an anti-FLAG mAb. Following Western blot analysis the proteins were detected using the Abs indicated.

FIGURE 7. Cross-linking of CHIR-B2 causes inhibition of cell proliferation. The CHIR-B2-FLAG 2D8 cells were seeded on microwell plates previously coated with 10 μg/well 3H7 mAb or an isotype-matched control mAb or medium alone. The proliferation was measured by [3H]thymidine incorporation for 6 h before harvesting. The mean ± SD is shown for triplicates. One representative of three independent experiments is shown.
genomic organization, tissue distribution and function may provide additional evidence to clarify family relationships. The CHIR-B2 gene has a genomic structure in which the exons I to IV are very similar to mammalian ILT and KIR genes in terms of exon intron boundary sequences and structures encoded (http://www.ncbi.nlm.nih.gov/book/bookres.fcgi?mono_003/ch1d1.pdf) (26). The genomic organization represented by exons V to VII encoding the C terminus of the protein is different to the situation found in ILIR/ILT genes, but similar to the KIR gene organization. Strikingly, the CHIR-B2 genomic organization is identical with the KIR3DL3 exon/intron structure (27). In contrast to the two Ig-like domains present in the CHIR-B genes, some ILT and KIR have large extracellular regions with more than two Ig-like domains; however, this number seems to be an unconserved feature.

The first draft of the chicken genome project is an exciting new development that provides 6.6 coverage of the 1.1 × 10⁹ bp genome and will certainly contribute to clarify the CHIR identity (http://pre.ensembl.org/Gallus_gallus). A database search of the chicken genome using the CHIR-B2 genomic sequence revealed a large number of homologues, providing additional evidence for the hypothesis that CHIR resemble a multigene family. At this point, however, the different contigs that are homologous to the CHIR-B2 sequence cannot be assigned to a chicken chromosome. Once the chicken genome is completed, the definitive location of CHIR locus to a chromosome will identify the homologues mammalian genes by synteny.

The cellular expression of inhibitory receptors may also be indicative for a certain family assignment. Primate KIR are expressed on NK cells and T cell subsets, however, it seems rather unlikely that lower vertebrates will contain true KIR genes because they are absent in mice (5). The restricted expression of CHIR-B2 on B cells and a small T cell subset is unique compared with the different mammalian inhibitory receptors. Human LILRB1 (ILT-2) and mouse PIR-A and PIR-B receptors are expressed on B lymphocytes, but in addition, on a variety of other cell types (3, 28). The previously published analysis of CHIR-A and CHIR-B has indicated expression of these genes in B cell and T cell lines (15). Our analysis using a monoclonal CHIR-B2 specific mab revealed that virtually all B cell expressed CHIR-B2. In addition, a small 3H⁷⁺ T cell subset was detected in splenocytes, but not in PBL. Taken together, the expression pattern mainly confined to B cells and a small T cell subset suggests that the CHIR-B2 may represent ILT/PIR homologues.

The question remains why an inhibitory receptor is expressed on virtually all B lymphocytes, including developingursal and naive peripheral B cells as well as recently activated effector cells from caecal tonsils. Because the density of CHIR-B2 seems to be rather low as detected by flow cytometry, CHIR-B2 may be up-regulated following activation. This seems unlikely because cells derived from caecal tonsils that contain high numbers of activated cells do not express higher CHIR-B2 levels as compared with bursal cells. Moreover, stimulation of splenocytes with phorbol esters did not lead to a CHIR-B2 increase on the cell surface (data not shown). If the receptor density is not affected, the regulation could also be mediated through modulation of a potential ligand. Therefore future characterizations of CHIR-B2 will also try to identify their ligands, however, given that only few ligands have been identified for mammalian inhibitory receptors, this may be a difficult task. Interestingly, the CHIR-B2 molecules were not phosphorylated on bursal cells indicating that the ligand of CHIR-B2 may not be expressed in the bursa.

Apart from the question relating to the CHIR homology, it will be most important to characterize the signal transduction and functional features of CHIR. The data provided in this study clearly identify the inhibitory capability of CHIR-B2. As has been reported for KIR, CHIR-B2 constitutively associates with basal levels of SHP-2, and SHP-1 binding is induced following activation (11). The inhibition of cellular proliferation by inhibitory receptors as shown for CHIR-B has also been reported in mammals. Such an arrest of B cell proliferation is dependent on the ITIM pathway through the activation of the adaptor protein Dok that leads to the inactivation of MAPKs (2, 29). Although this provides important evidence for a putative CHIR-B2 in vivo function, currently this is only observed as a transient feature of a transfected cell line. Future studies should further clarify the function of CHIR-B2 in B cells, especially the consequences of cocross-linking Ig and CHIR-B2. However, this is a difficult task because primary B cells are short lived in culture and the 2D8 cell line used in this study does not express surface Ig molecules.

In conclusion, this report provides an extensive analysis of a nonmammalian multigene inhibitory receptor family and provides evidence for a predominant expression of CHIR-B2 on B cells that inhibits cellular proliferation by recruitment of SHP-1 and SHP-2.

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References

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