Immunogenetics (2009) 61:597–601
DOI 10.1007/s00251-009-0382-y

BRIEF COMMUNICATION

Characterization and ligand specificity of sheep IgG2 receptor

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Received: 6 March 2009 / Accepted: 15 June 2009 / Published online: 10 July 2009
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Abstract Neutrophils and macrophages in cattle express a novel class of immunoglobulin Fc receptor, specific for bovine IgG2, termed boFcγ2R. In cows, the ability of neutrophils to kill immunoglobulin-opsonized microorganisms appears to depend largely on this subclass. Although related to other mammalian FcγRs, boFcγ2R belongs to a novel gene family that includes the human killer Ig-like receptor and FcαRI (CD89) proteins. In this study, we describe the presence and characterization of this novel class of FcγR in sheep. The comparative analysis of this novel FcγR has allowed us to begin an exploration of some immunological characteristic of ruminants.

Keywords Cloning · Expression · Sheep · IgG · Fc Receptor

The GenBank accession number of the nucleotide sequence reported here is EF541479 and FJ198054.

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Introduction

Receptors for the Fc domain of IgG (FcγRs) are important molecules expressed on hematopoietic cells that play an important role in immune regulation by providing a link between the humoral immune response and cellular effector functions (Ravetch and Bolland 2001). Four different classes of FcγRs, known as FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and FcγRIV, have been intensively characterized in mice. Whereas FcγRI displays high affinity for the antibody-constant region and restricted isotype specificity, FcγRII and FcγRIII have low affinity for the Fc region of IgG but a broader isotype binding pattern, and FcγRIV is a recently identified receptor with intermediate affinity and restricted subclass specificity (Nimmerjahn and Ravetch 2006).

From a functional point of view, FcγRs can be divided into two major types, the activation receptors and the inhibitory receptor. The activation FcγRs are characterized by the presence of an immunoreceptor tyrosine-based activation motif (ITAM) either intrinsic to the receptor, as in the case of human FcγRIIA (a receptor not found in the mouse), or more commonly, as part of an associated subunit, the γ or ζ chain, as in FcγRI, FcγRIII, and FcγRIV (Nimmerjahn et al. 2005). In human and mouse, there is a single gene for FcγRIIB, an inhibitory FcγR, characterized by containing the inhibitory or ITIM sequence in its cytoplasmic domain (Ravetch and Bolland 2001). The paired expression of activating and inhibitory molecules on the same cell is the key for the generation of a balanced immune response (Nimmerjahn and Ravetch 2008). Additionally, it has recently been appreciated that the IgG Fc receptors show significant differences in their affinity for individual antibody isotypes, rendering certain

isotypes more strictly regulated than others (Nimmerjahn and Ravetch 2005).

Although there is an obvious overall similarity to their human and mouse counterparts, some Fc receptors in domestic animals are unusual. In cattle, neutrophils and macrophages express a novel class of immunoglobulin Fc receptor, specific for bovine IgG2, termed boFcγ2R (Zhang et al. 1994; Zhang et al. 1995). In cows, the ability of neutrophils to kill immunoglobulin-opsonized microorganisms appears to depend largely on this subclass. Although related to other mammalian FcγRs, boFcγ2R belongs to a novel gene family that includes FcαRI (CD89), killer Ig-like receptor (KIR), and leukocyte Ig-like receptor (Kacskovics 2004; Zhang et al. 1995). As a unique class of FcγR, boFcγ2R binds Fc in a distinct manner from the other FcγRs so far characterized. The functional characterization of boFcγ2R showed that it binds bovine IgG2 via the membrane-distal EC1 domain, whereas the others bind IgG via the membrane-proximal EC2 domain (Morton et al. 1999). Considering the active role of boFcγ2R in cellular effector function in cattle and its unique genetic characteristics, it is of interest to investigate whether this unusual FcγR presents in different animal species. Here, we describe the complementary DNA (cDNA) cloning, genomic structure analysis, and characterization of this novel FcγR in other ruminant species: sheep. The comparative analysis of this novel FcγR has allowed us to begin an exploration of some immunological characteristic of ruminants.

We searched the translated EST database at the National Center for Biotechnological Information with the bovine Fcγ2R protein sequence and identified several overlapping homologous sequences in the sheep. The 3′-rapid amplification of cDNA ends (RACE) was performed using the three-site adaptor primer and a gene-specific forward primer (5′-ACC ACG GAG GAG CCC GAG ACA A-3′) designed according to the identified EST sequences. Blood samples were collected from a 5-month-old sheep from a commercial farm. Ovine peripheral blood leukocytes were isolated by centrifugation of peripheral blood anticoagulated with EDTA and hypotonic lysis of erythrocytes as suggested (Carlson and Kaneko 1973). Methods for total leukocytes RNA extraction and cDNA synthesis were as described previously (Qiao et al. 2006). The resulting PCR product was isolated, cloned, and sequenced. The smart 5′-RACE system (Clontech) was then used to obtain the putative full-length ovine cDNA. The obtained fragment was subsequently cloned and sequenced. Based on the newly obtained sequence for the full-length cDNA, a pair of PCR primers, SH1 (5′-TCC TCC ATC CCC ACA GCC TGG AC-3′) and SH2 (5′-GGT GCT CAG CTC CGG GCC TT-3′), were designed to amplify the sequence covering the open reading frame (ORF) of ovine Fcγ2R. The amplified cDNA was also subcloned, in the correct orientation, into the mammalian expression vector pCDNA3 (Invitrogen) for transfection studies.

The ovine Fcγ2R cDNA so obtained is 1,527 bp long, including a 126-bp 5′ untranslated region and a 621 bp 3′ untraslated region terminating in a poly-A tract. The cDNA contains a single ORF, which extends 780 bp, encoding a 259-amino-acid protein (GenBank accession number EF541479). The first 23 amino acids were predicted to be an N-terminal secretory signal peptide. The mature protein is composed of a 202-amino-acid extracellular region followed by a hydrophobic region of 19 amino acids containing a single positively charged residue (Arg204) representing a putative transmembrane domain and a short cytoplasmic tail of 15 amino acids devoid of known signaling sequences. The extracellular region was found to include three potential *N*-glycosylation sites (Asn82, Asn117, and Asn126) and four conserved cysteines that form the characteristic disulfide bonds of the immunoglobulin domains. From the conserved immunoglobulin domain structure, it is suggested that Cys26 is linked to Cys75 to form domain 1 (EC1) and that Cys122 is paired with Cys173 to form domain 2 (EC2). The level of identity between ovFcγ2R and boFcγ2R at the amino acid level is 84% for the complete protein, 81% for the extracellular domains alone, and 83% for the extracellular and transmembrane regions together. Site-directed mutational analysis has elucidated that the EC1 F-G loop of both boFcγ2R and boFcαR is clearly involved in ligand binding (Zhang et al. 2006a,b). As the residues within bovine and ovine Fcγ2R F–G loop are fully conserved, it is possible that the sequence in this region of ovFcγ2R is also involved in the interaction with its ligand. In contrast to the high similarity of the binding region of Fcγ2R, the length of the linker region connecting EC1 and EC2 differs dramatically. In boFcγ2R, there is a five-amino-acid insertion (EEPAG) that increases the linker strand from five to ten amino acids (Fig. 1). The significance remains unknown.

The presence of a positively charged residue in the transmembrane domain is a characteristic of a number of related receptors, including CD89 and several KIRs. For many of these receptors, it has been shown that they associate with specialized signaling molecules via these charged residues. Both the FcR γ chain and DAP-12 contain an ITAM (within their cytoplasmic domains), and cross-linking of these proteins via their associated receptors is generally considered to be a potent cellular activation signal (Morton et al. 2004; Zhang et al. 1995). As sheep also possesses an FcR γ chain (McAleese and Miller 2003), it therefore seems probable that it can associate with ovFcγ2R and that they form an activation receptor complex on the surface of immune phagocytes.

Fig. 1 Alignment of amino acid sequence of ovFcγ2R with those of boFcγ2R and boFcαR. The GenBank accession numbers of nucleotide sequences of these three FcγRs are EF541479, NM_001001138, and NM_001012685. Cysteins residues involved in Ig-like domain disulfide bonds are in *bold*. The putative signal sequence and transmembrane region are *underlined*. *Dashes* indicate identity of amino acid to ovFcγ2R. *Italics* define the F–G loop regions, which have been show to be important for binding to ligands. *Dots* indicate the gaps inserted to maintain alignment

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ovFcγ2R   MAPALPALLCLGLIVGLRTQVQAGTLRKPTIWAEPGSVVT    40
boFcγ2R   ---T---------S-----------FP--I-----S---P    40
boFcαR    ---RDIT-F--V-CL-QKI-A-DENFPI-I-S-T-S--IP    40

ovFcγ2R   RGSPVTIWCQGPPNANSFSLNKEGTLAPWNAQPPVEPWDR    80
boFcγ2R   L--S---L------TK--------DST---IH-SL----K    80
boFcαR    WNGS-K-L---TLESYLYQ-EILEN-TYKQVEKKLGFQEV    80

ovFcγ2R   ASFFIKRVTEQQAGRYHCSHFIGVNWSEPSEPLELLVAG.   119
boFcγ2R   -N---SN-R------------------------D-----E   120
boFcαR    -E-I-NHMDTNT----Q-RYRREHR--A---A---VAT-.   119

ovFcγ2R   ....RLRGSPSLSVQPGPSVAPGENVTLLCQSGNR.TDTF   154
boFcγ2R   EPAG---DR-----R-S------------------.----   159
boFcαR    .....-YDK-F--TDG-HV-M----ISFQ-S-AYISF-R-   154

ovFcγ2R   LLSKEEAAHRPLRLRSQDQDGQYQAEFSLSPVTSAHGGTY   194
boFcγ2R   -----G---------------W------------------   199
boFcαR    S--RPGG-T.....L-RHR-ARL-GD-T-G--NLSFS-V-   189

ovFcγ2R   RCYRSLSTDPYLLSQPSEPLALVVSD.....YTVQNLIRM   229
boFcγ2R   --------N-------------L-A-.....--M------   234
boFcαR    T--GWH-GH--VW-A--NA-E---T-TTSQDH-TE-WV--   229

ovFcγ2R   GLAASVLLLLGILLCQARHDHGGARDKARS             259
boFcγ2R   -------------------------EA---             264
boFcαR    -V-GL---A-LAI-AEN-LGPQLPHQEDQQDLPDLSWSWQ   269

boFcαR    RSQTERTFGLTPKDHQGDSWS                      290
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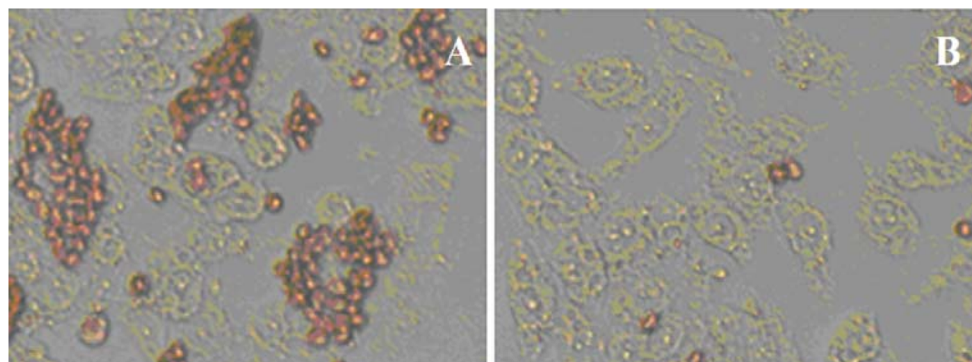

Fig. 2 COS-7 cells transfected with ovFcγ2R bind ovine IgG2-sensitized chicken erythrocytes but not IgG1-sensitized erythrocytes. **a** COS-7 cells transfected with ovFcγ2R incubated with erythrocytes specifically sensitized with ovine IgG2. Extensive binding of erythrocytes to individual cells was evident. **b** COS-7 cells transfected with ovFcγ2R incubated with erythrocytes specifically sensitized with ovine IgG1. No binding of erythrocytes was evident. Control COS-7 cells that had not been transfected did not bind ovine IgG2-sensitized erythrocytes (data not shown)

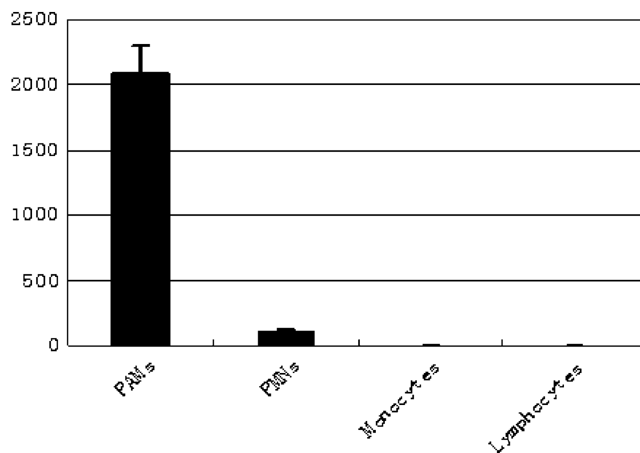

Fig. 3 Transcription pattern of ovFcγ2R mRNA. Each sample was individually assayed in triplicate. All samples were normalized using GAPDH expression as an internal control in each real-time PCR. Relative level of ovFcγ2R mRNA was analyzed by the $2^{-\Delta\Delta Ct}$ method

Rosetting analysis was performed to confirm the identity of the ovine cDNA as a putative IgG receptor and characterize the ligand specificity of this receptor. Ovine IgG1 and IgG2 were purified from hyperimmune serum collected from a sheep that had been inoculated with chicken erythrocytes and used to specifically sensitize erythrocytes. IgG was precipitated with ammonium sulfate $(NH_4)_2SO_4$, and IgG1 and IgG2 were further purified by diethylamino ethanol chromatography followed by fractionation on protein-A Sepharose (Pharmacia) to which ovine IgG2 but not IgG1 bound (Schmerr and Goodwin 1991). COS-7 cells were maintained in Dulbecco's modified Eagle's minimal essential medium (Sigma) as described previously (Zhang et al. 2006a,b) and were transiently transfected with 0.4 μg of ovine Fcγ2R cDNA constructs per well using the Lipofectamine 2000 kit (Inivtrogen), according to the manufacture's protocol. Cells were incubated in medium containing 10% fetal calf serum at 37°C in a humidified CO_2 atmosphere for 24 h and then incubated in medium without serum for 2 h. Chicken erythrocytes were sensitized with ovine IgG1 or IgG2 resuspended in serum-free medium and added to the transfected COS-7 cell monolayer. After 45–60 min incubation at room temperature with occasional gentle agitation, non-adherent erythrocytes were washed off with phosphate-buffered saline. The monolayer cells were fixed with methanol for 10 min, incubated with serum free medium for 2 h, and the cells stained with horseradish peroxidase-conjugated rabbit anti-sheep IgG (Sigma), washed and incubated with 3-amino-9-ethylcarbazole (Sigma). Bovine IgG2 binding was performed as described (Zhang et al. 1995). As is shown in Fig. 2, COS-7 cells transfected with the ovine Fcγ2R cDNA were able to bind ovine IgG2-sensitized chicken erythrocytes but not ovine IgG1-sensitized erythrocytes or nonsensitized chicken erythrocytes (data not shown). Rosetting analysis also confirmed that COS-7 cells transfected with ovFcγ2R was able to bind similar amounts of bovine IgG2-sensitized chicken erythrocytes (data not shown).

In addition, to investigate the genomic organization of ovine Fcγ2R, a genomic clone was amplified from ovine genomic DNA purified from peripheral blood cells using blood genome DNA extraction kit (Takara) with a pair of PCR primers SH1 and SH2. The PCR fragments of about 2.3 kb were isolated and ligated into pMD18-T (Takara) for sequencing. The exon–intron structures were identified with the GenScan program (http://genes.mit.edu/GENSCAN.html). As shown in Fig. 3, the ovFcγ2R gene spans 2.2 kb (from the start methionine residue to the stop codon) and consists of five exons and four introns (GenBank accession number FJ198054). The leader sequence is encoded by exons 1 and 2. The rest of the extracellular domain and cytoplasmic domain are encoded by exons 3, 4, and 5. All the introns in the ovFcγ2R gene obey the “GT-AG” rule. Fine structure mapping of the ovFcγ2R gene revealed the canonical split signal exon of classical FcR genes, two extracellular domains encoded on separate exons, and a single exon encoding the transmembrane and cytoplasmic domains, features conserved in the CD89 gene (Morton et al. 2005).

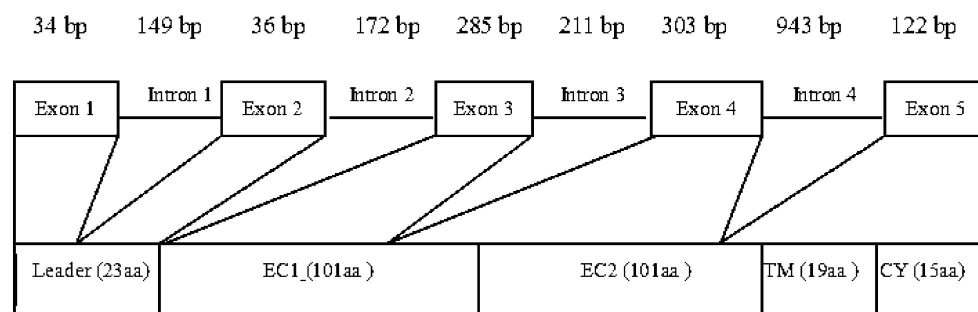

Fig. 4 Genomic organization of the ovFcγ2R gene. *Boxes* represent the exons, and *lines between the boxes* represent introns. The length of each gene segment is described above its corresponding exon or intron. The exons and their corresponding coding regions are depicted. Amino acid (*aa*) lengths of each domain (*EC* extracellular domain, *TM* transmembrane domain, *CY* cytoplasmic domain) are indicated. The genomic sequence data is available from GenBank (FJ198054)

In order to examine the cell distribution of ovine Fcγ2R, real-time PCR was performed on messenger RNA (mRNA) from selected cellular subsets using primers specific for either ovine Fcγ2R (forward primer 5′-CAG CTT CTA TGC AGG CAA CC-3′; reverse primer 5′-TGG AGC AGC AGC CAG TCA-3′) or glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (forward primer 5′-ATG TTT GTG ATG GGC GTG AA-3′; reverse primer 5′-AGC AGT TGG TGG TGC AGG AG-3′). All real-time PCR reactions were performed on a Bio-Rad I cycler real-time PCR instrument (Bio-Rad) using SYBR green system (Takara). The thermal cycling conditions included an initial denaturation step at 95°C for 30 s, 40 cycles at 95°C for 5 s, and 63.5°C for 25 s. Data from three independent experiments were calculated using the comparative Ct method (Livak and Schmittgen 2001). As shown in Fig. 4, the ovFcγ2R mRNA was detected in pulmonary alveolar macrophages (PAMs) and polymorphonuclear neutrophils (PMNs). The PAMs had the higher ovFcγ2R mRNA abundance than PMNs. However, undetectable levels of ovFcγ2R at mRNA expression were observed in the monocytes and lymphocytes. This result is somewhat similar to the previously observation that both ovine macrophages and neutrophils express FcγRs for IgG2 and mediate the process of killing immunoglobulin-opsonized microorganisms (Fleit et al. 1986; Jungi et al. 1992).

In sheep, the heavy chain constant region genes of IgG1 and IgG2 have been sequenced, and the sequence data indicate that IgG1 isotype is typically IgG-like, but the IgG2 isotype is the most divergent of all known IgGs with significant shortening of the lower hinge (Clarkson et al. 1993). Functional studies have shown that IgG2 is more efficient than IgG1 in promoting phagocytosis and antibody-dependent cell-mediated cytotoxicity (Singh et al. 2006). It is likely that the FcγR evolved to accommodate the changes in IgG2 structure that resulted in the failure of IgG2 to bind to FcγRs that bind IgG1, and the protective role of ovine IgG2 in immune response could be explained by its interaction with FcγR. Thus, the further investigation of ovine FcγR system and mapping the expression patterns on various cell types will no doubt help us to understand the immunological role of ovine IgG subclass.

In conclusion, we have isolated an ovine IgG2 receptor in sheep, which we have termed ovFcγ2R. This receptor binds both bovine and sheep IgG2, and we consider it to represent the ovine homologue of boFcγ2R. The presence of this new mammalian receptor in the other ruminant animal may be reflecting some immunological characteristic of ruminants.

Acknowledgments This work was supported by a grant of key program from the National Natural Science Foundation of China (no. 30730068).

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