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BRIEF COMMUNICATION

Molecular cloning and expression of the porcine high-affinity immunoglobulin G Fc receptor (FcγRI)

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Abstract Receptors for the Fc region (FcγRs) of immunoglobulin G (IgG) play a crucial role in the immune system and host protection against infection. In this study, we describe the cloning, sequencing, and expression of the high-affinity IgG receptor from pig. By screening a translated Expressed Sequence Tags database with the human FcγRI (CD64) protein sequence, we identified a putative porcine homologue. Subsequent polymerase chain reaction amplification confirmed that the identified full-length cDNA was expressed in porcine cells. Rosetting analysis shows that COS-7 cells transfected with a plasmid containing the cloned cDNA were able to bind chicken erythrocytes sensitized with porcine IgG. Scatchard analysis indicated that monomeric IgG bound to transiently transfected cells with an affinity of approximately 4×10^7 M^{-1}. The porcine FcγRI cDNA is 1,038 nucleotides long and is predicted to encode a 346-amino-acid transmembrane glycoprotein composed of three Ig-like domains, a transmembrane region, and a short cytoplasmic tail. The overall identity of the porcine FcγRI to its human and mouse counterparts at the level of the amino acid sequence was 75% and 57%, respectively. Identification of porcine FcγRI will aid in the understanding of the molecular basis of the porcine immune system and further studies of the receptor function.

Keywords Cloning · Expression · Pig · Receptor

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The GenBank accession number of the nucleotide sequence reported here is DQ026063.

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Receptors for the Fc domain of IgG (FcγRs) are important molecules expressed on hematopoietic cells and a variety of others that provide an essential link between humoral and cellular immunity and the adaptive and innate systems. Cross-linking of FcγRs by immunoglobulin G (IgG)-containing immune complexes triggers a wide variety of effector mechanisms such as phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), regulation of cytokine and antibody production, and antigen processing and presentation. Three distinct but closely related IgG receptors have been intensively characterized in the human and mouse: FcγRI (CD64) is a high-affinity receptor found mainly on myelomonocytic cells, which can bind to monomeric IgG, whereas FcγII (CD32) and FcγRIII (CD16) are of lower affinity, binding primarily aggregated IgG or IgG in immune complexes (Ravetch and Bolland 2001). CD64 is a transmembrane glycoprotein with an extracellular region composed of three Ig-like domains. The first two distal extracellular domains of FcγRI function as broadly specific low-affinity receptors, like FcγRII and FcγRIII, and it has been suggested that the membrane proximal third domain confers the higher affinity to FcγRI (Allen and Seed 1989; Sears et al. 1990).

CD64, a receptor with high affinity for IgG, has been proposed as a potential therapeutic effector target in malignancies. Targeting of tumors to FcγRI with bispecific mAbs can facilitate tumor killing via FcγRI expressing macrophages, and therapeutic humanized bispecific reagents targeting human FcγRI are in clinical trials (Schwaab et al. 2003). Besides a role in antigen clearance, FcγRI can potently enhance MHC class I and II antigen presentation (Rafiq et al. 2002; Wallace et al. 2001). These properties could make FcγRI a powerful candidate target for immunotherapy (Brady 2005).

In contrast with intensive studies on the structure and function of human and mouse FcγRs, there have been few studies on other animals (Kacskovics 2004). FcγRIIIA was the first FcγR cloned and characterized in pig (Halloran et al. 1994a). Recently, an association has been reported between porcine FcγRIII and a molecule that contains significant homology to the cathelin family of antimicrobial proteins on porcine polymorphonuclear (PMN) cells. The presence of a novel FcγRIIIA complex in the porcine system implies that new or unusual responses may be mediated through FcγRs (Sweeney and Kim 2004). Most recently, the cDNA encoding porcine FcγRII has also been cloned by our research group (Qiao et al. unpublished data). In this report, we describe the cloning, sequencing, and characterization of a porcine FcγR, which we believe to represent the porcine homologue of CD64.

We screened a translated Expressed Sequence Tags (EST) database (which is composed of sequences from species other than humans or mice) at the National Center for Biotechnological Information (NCBI) using the human CD64 protein sequence and identified several overlapping EST sequences that showed high homology to the CD64. Using this information, a pair of polymerase chain reaction (PCR) primers (S11: 5′-AAC ATG TGG CTC TTA ATA ATT CTG CTC C-3′ and S12: 5′-ATT TGC TTT ATT TAA GAA TGA CAT GCC A-3′) was used to amplify the predicted cDNA. Blood samples were collected from a 6-month-old crossbred pig from a commercial farm, and the peripheral blood leucocytes (PBLs) were isolated from 10 ml of the heparinized blood samples by density centrifugation on Lymphoprep (Nycomed, Norway). The mRNA was extracted from approximately 10^7 of the porcine PBLs using Amersham Pharmacia QuickPrep Micro mRNA Purification Kit (Pharmacia) according to the manufacturer's instructions, and cDNA was subsequently synthesized using an olig-dT primer. PCR amplification with the above primers using porcine cDNA as template gave a single clear band (of ≈1,200 bp) when the reaction product was analyzed by agarose-gel electrophoresis (data not shown). The PCR product obtained was cloned into the pGEM-T easy vector (Promega) prior to sequencing. The amplified cDNA was also subcloned into the mammalian expression vector pCDNA3 (Invitogen) for transfection studies.

The porcine FcγRI cDNA so obtained was found to contain a 1,038-bp open reading frame encoding a 346-amino-acid protein. The predicted amino acid sequence of the protein translated from this cDNA sequence has the typical features of a transmembrane glycoprotein (Fig. 1). The first 15 amino acids were predicted to be an N-terminal leader sequence. The mature receptor would thus begin at Gln(Q)16 and be composed of a 273-amino-acid extracellular region followed by a hydrophobic region of 23 amino acids (representing a putative transmembrane domain) and a 34-amino-acid cytoplasmic tail devoid of known signaling motifs. The extracellular region was found to include six potential N-glycosylation sites (Asn 23, Asn 34, Asn 63, Asn 144, Asn 194, and Asn 225) and six conserved cysteines that form the characteristic disulphide bonds of the immunoglobulin domain structures, spanning 59, 61, and 65 residues for the domains 1, 2, and 3, respectively. The overall identity of the porcine FcγRI with its human, cattle, and mouse counterparts at the amino acid sequence level was 75%, 69%, and 57%, respectively; however, a comparison of the three extracellular domains alone between the four species shows a higher identity of 77%, 74%, and 70%, respectively. This is in agreement with the generally highly conserved nature of the individual FcR binding domains.

In contrast to the high similarity of the extracellular domain of FcγRI, the length of the cytoplasmic region differs dramatically according to species. Mouse FcγRI has the longest cytoplasmic region (84-amino-acid residues), whereas the human receptor has a longer cytoplasmic region (63-amino-acid residues) than that of pig and cattle (34 and 37 amino acids). Interestingly, the untranslated nucleotides following the stop signal represent translated codons for similar amino acid residues found at the 3′ end of the human receptor (Fig. 1, residues in rectangle in the porcine sequence). Finding the identical sequence in all the four clones from two different pigs we have analyzed excludes the possibility of a Taq error due to the reverse transcriptase PCR (RT-PCR) process and suggests that this critical nucleotide underwent a mutation during evolution.

FcγRI is known to exist in the form of multi-subunit complexes containing a unique ligand binding α-chain and the promiscuous immunoreceptor tyrosine-based activation motif (ITAM) bearing γ-chain that is indispensable for phosphotyrosine-based signaling (Ravetch and Bolland 2001), whereas recent reports showed that the cytoplasmic tail of the FcγRI α-chain plays an important role in endocytosis, phagocytosis, and the induction of the secretion of IL-6 (Edberg et al. 1999, 2002). This raises the question of whether the porcine FcγRI still contains the entire important motif necessary for its functioning. Van Vugt et al. (1999) have reported that human FcγRI

cytoplasmic domain harbors an antigen presentation motif in the membrane proximal ~34 amino acids. Beekman et al. (2004) defined a periplakin binding site in the membrane proximal region of the FcγRI cytoplasmic tail which could possibly further modulate the functional activity of the receptor and possibly its ligand binding affinity (Fig. 1, residues underlined with a wave line). Receptor truncation experiments indicated FcγRI c-terminal residues 333–374 to be fully dispensable for the interaction of FcγRI with periplakin. This motif in porcine FcγRI is very similar to the human counterpart, suggesting that the porcine FcγRI would still be fully functional with regard to binding of and functional modulation by periplakin.

Rosetting analysis was performed to confirm the identity of the porcine cDNA as a putative IgG receptor. COS-7 cells cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma) supplemented with 10% fetal bovine serum in 96-well culture plates were transiently transfected with 0.4 μg of porcine FcγRI cDNA constructs per well using the Lipofectamine 2000 kit (Invitrogen) according to the manufacturer'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 IgG (from hyperimmune serum collected from a piglet that had been inoculated with chicken erythrocytes and was purified by ammonium

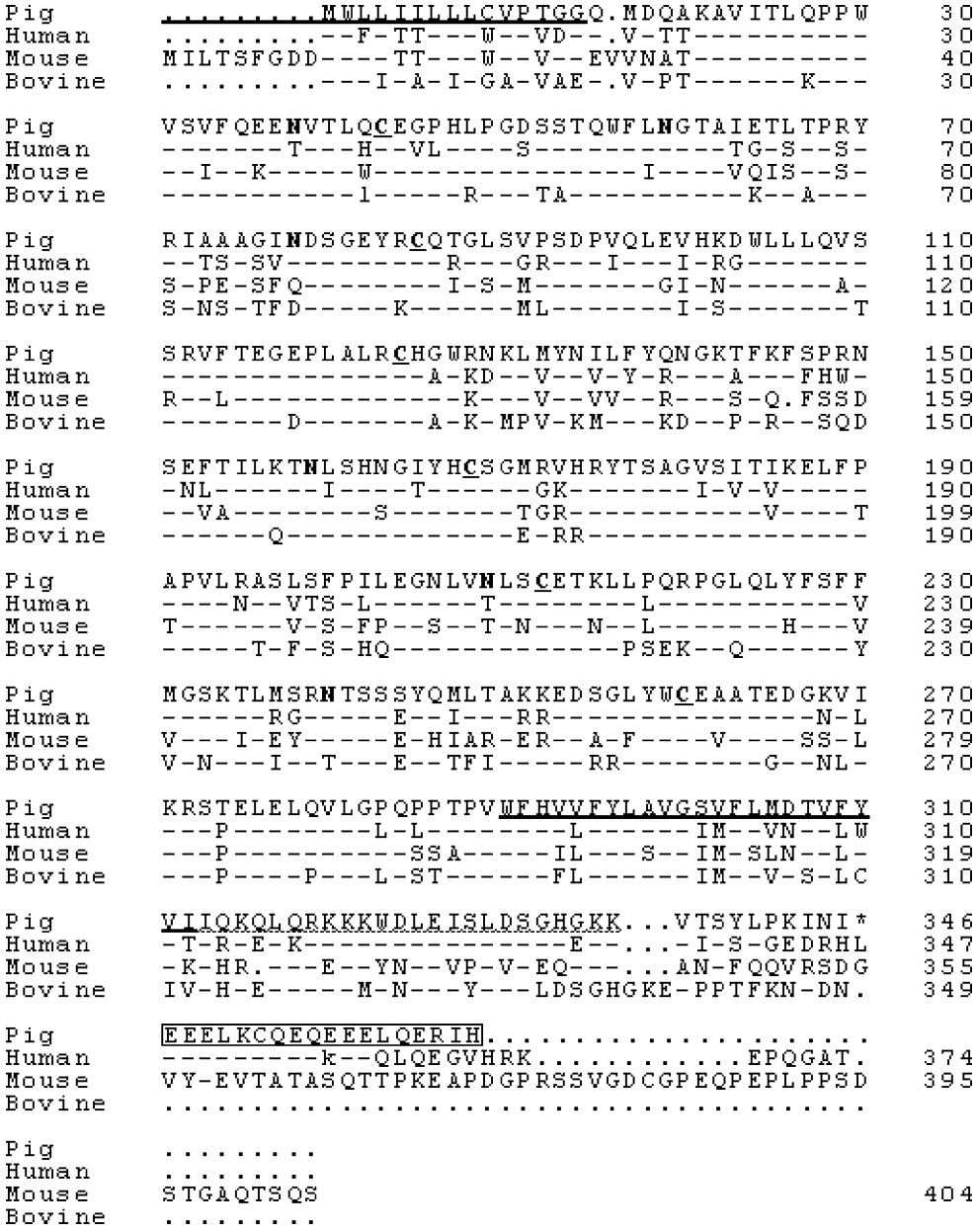

Fig. 1 Comparison of amino acid sequence of FcγRI from pig, human, mouse, and cattle. Amino acid sequence determined from the porcine FcγRI cDNA (accession no. DQ026063) is shown on the *top line*. Amino acid sequence for human FcγRI (accession no. NM_000566), mouse FcγRI (accession no. M14216), and bovine FcγRI (accession No. AF162866) are shown *below*. Cysteine residues involved in Ig-like domain disulphide bonds are *underlined twice*. Potential N-linked glycosylation sites are in *bold*. The putative signal sequence and transmembrane region are *underlined*. The cytoplasmic motif for binding periplakin is *underlined with a wave line*. The *asterisk* represents the stop signal in the porcine sequence. Residues in an *open box* following the stop signal show the untranslated but conserved carboxyl end of the porcine CYT domain. *Dashes* indicate identity of amino acid to pig FcγRI. *Dots* indicate the gaps inserted to maintain alignment

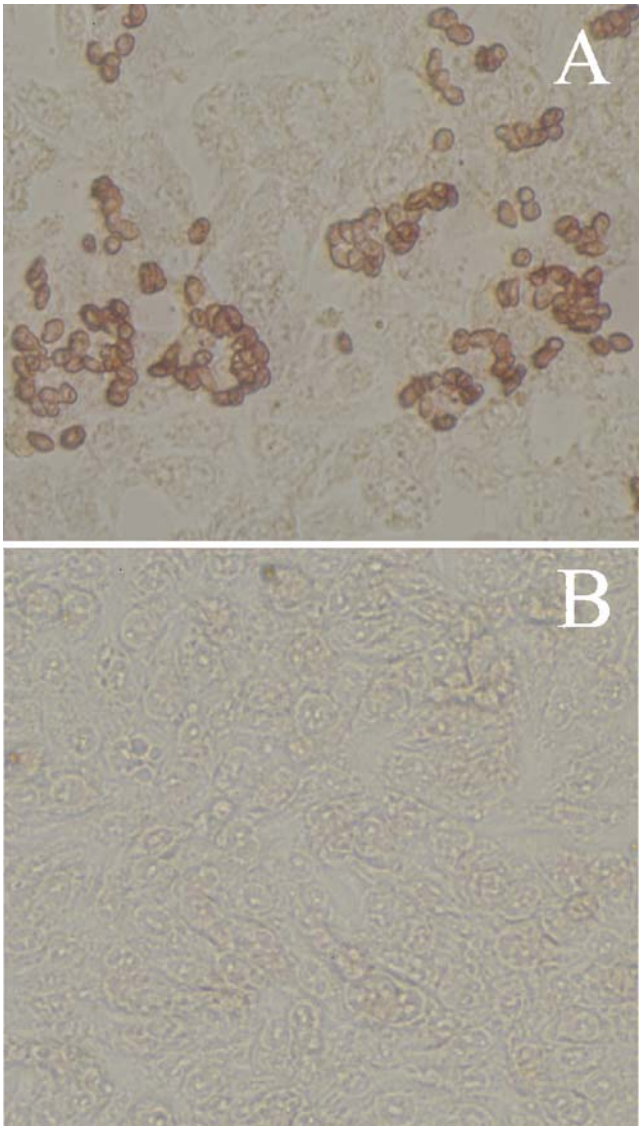

Fig. 2 COS-7 cells transfected with the gene porcine FcγRI bind IgG-sensitized erythrocytes. **a** COS-7 cells transfected with the cloned porcine FcγRI gene and incubated with erythrocytes sensitized with porcine IgG. Extensive binding of erythrocytes to individual cells was evident. **b** Control COS-7 cells transfected with the pcDNA 3 plasmid and incubated with IgG-sensitized erythrocytes. No binding of erythrocytes was evident. Control COS-7 cells that had not been transfected did not bind porcine IgG-sensitized erythrocytes (data not shown)

sulphate precipitation and DEAE chromatography), resuspended in serum-free medium, and added to the transfected COS-7 cell monolayer. After 45–60 min of incubation at room temperature with occasional gentle agitation, nonadherent erythrocytes were washed off with phosphate-buffered saline (PBS). The monolayer was fixed with methanol for 10 min and incubated with serum-free medium for 2 h, and the cells were stained with HRP-conjugated goat swine-specific IgG (Sigma) and incubated with 3-amino-9-ethylcarbazole (AEC; Sigma). As shown in

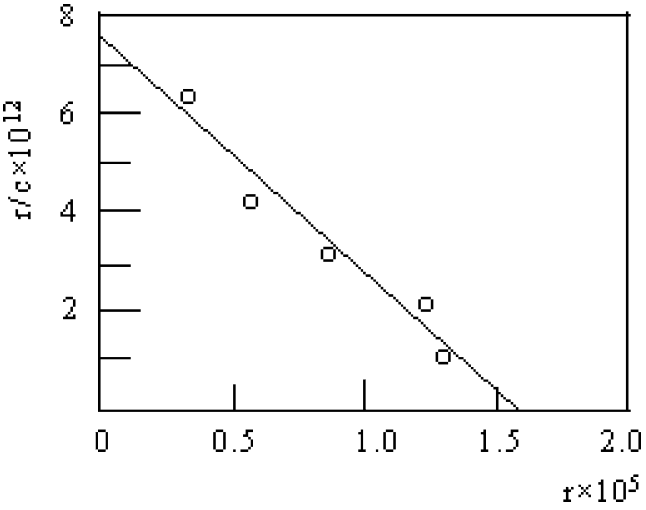

Fig. 3 Scatchard analysis of monomeric IgG bound to the transfected COS-7 cells. *r* is the number of IgG molecules bound per cell and *c* is the concentration of free IgG in the incubation mixture (assuming a molecular weight for IgG of 1.5×10^5 and using Avogadro's number=6×10^{23}). COS-7 cells were transiently transfected with the cloned porcine FcγRI gene and after 48 h were incubated with serial dilutions of monomeric ^{125}I-labeled porcine IgG as described

Fig. 2, COS-7 cells transfected with the pig FcγRI cDNA were able to bind chicken erythrocytes sensitized with porcine IgG. Nonsensitized erythrocytes did not attach to the transfected COS-7 cells, and sensitized red cells did not attach to the untransfected COS-7 cells.

Scatchard analysis was performed using monomeric ^{125}I-labeled porcine IgG prepared with the chloramine-T method (Hunter and Greenwood 1962). Transfected or nontransfected COS-7 cell suspensions (10^6 cells per well) were mixed with varying concentrations of monomeric ^{125}I-labeled porcine IgG incubated at 0°C for 60 min with

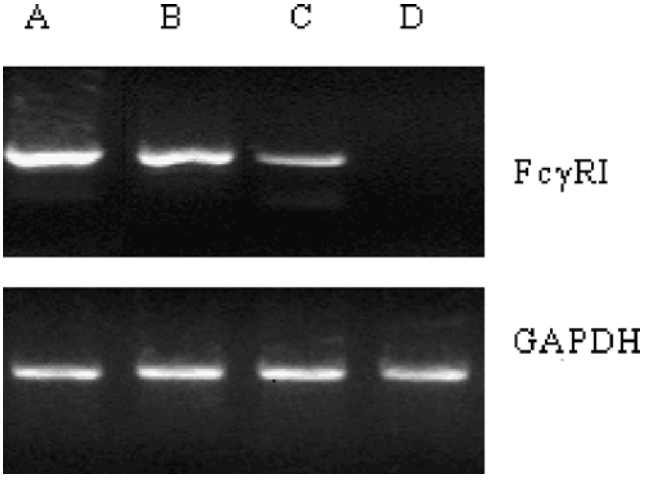

Fig. 4 Expression pattern of porcine FcγRI in different cells: PAM (*lane A*), monocytes (*lane B*), PMN (*lane C*), lymphocytes (*lane D*). Total mRNA was prepared and cDNA was synthesized from various cells. This cDNA was then used as a template in PCR with porcine FcγRI or porcine GAPDH specific primers

occasional agitation. The cells were then washed twice and their radioactivity was measured. Nonspecific binding was determined using identical experimental conditions in the presence of a large excess of unlabeled porcine IgG (Allen and Seed 1989; Sears et al. 1990). The results showed that radiolabeled porcine monomeric IgG bound to the transfected cells with an affinity of approximately 4×10^7 M^{-1} (Fig. 3), indicating that porcine monomeric IgG can bind swine FcγRI with a high affinity.

In order to examine the cell distribution of porcine FcγRI, RT-PCR was performed on mRNA from selective cellular subsets using primers specific for either porcine FcγRI (forward primer 5′-AAC ATG TGG CTC TTA AT AAT TCT GCT CC-3′; reverse primer 5′-AAG GCC AGA GGT TCC CTT CAG TAA AGA C-3′) or glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (forward primer 5′-TTC CAC GGC ACA GTC AA-3′; reverse primer 5′-GCA GGT CAG GTC CAC AA-3′). Porcine monocytes and lymphocytes were isolated from porcine peripheral blood cells as described previously (Halloran et al. 1994b). Porcine PMN cells were prepared by dextran sedimentation following Ficoll-Hypaque separation of whole blood. Pulmonary alveolar macrophages (PAM) were collected by lung lavage using ice-cold PBS (Halloran et al. 1994b). Cells that were less than 90% viable prior to experimentation were not used. As can be seen in Fig. 4, porcine FcγRI is expressed in PAM, monocytes, and PMN. A higher abundance of transcripts was found in PAM and monocytes than in PMN, where only a trace transcript was found. RT-PCR using GAPDH specific primers generated specific bands of similar intensity in all tissues.

In conclusion, we have cloned an IgG Fc receptor from pig that shows high homology to the human, bovine, and mouse high-affinity Fc gamma receptor. Rosetting analysis shows this receptor binds antigen-complexed porcine IgG. Scatchard analysis indicated that monomeric IgG bound to transiently transfected cells with an affinity of approximately 4×10^7 M^{-1}. Therefore, we consider it to represent the porcine homologue of human FcγRI (CD64). Future studies will identify the detail expression pattern of this FcγR, the IgG subclass that it binds, and its function in porcine immune responses.

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