# A functional polymeric immunoglobulin receptor in chicken (*Gallus gallus*) indicates ancient role of secretory IgA in mucosal immunity

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Animals are continuously threatened by pathogens entering the body through natural openings. Here we show that in chicken (*Gallus gallus*), secretory IgA (sIgA) protects the epithelia lining these natural cavities. A gene encoding a chicken polymeric Ig receptor (*GG-pIgR*), a key component of sIgA, was identified, and shown to be expressed in the liver, intestine and bursa of Fabricius. All motifs involved in pIgR function are present, with a highly conserved Ig-binding motif in the first Ig-like domain. Physical association of GG-pIgR with pIgA in bile and intestine demonstrates that this protein is a functional receptor. Thus, as shown for mammals, this receptor interacts with J-chain-containing polymeric IgA (pIgA) at the basolateral epithelial cell surface resulting in transcytosis and subsequent cleavage of the pIgR, releasing sIgA in the mucosal lumen. Interestingly, the extracellular portion of GG-pIgR protein comprises only four Ig-like domains, in contrast with the five domain structure found in mammalian pIgR genes. The second Ig-like domain of mammalian pIgR does not have an orthologous domain in the chicken gene. The presence of pIgR in chicken suggests that this gene has evolved before the divergence of birds and reptiles, indicating that secretory Igs may have a prominent role in first line defence in various non-mammalian species.

Key words: antibody, Ig, mucosa, secretory immunoglobulin (sIg), transcytosis.

## INTRODUCTION

Most pathogens invade the body of their host through mucosal surfaces, especially those lining the gastrointestinal, respiratory and genito-urinary tracts. Despite their phylogenetic distance, both mammals and bird species use polymeric IgA (pIgA), produced by plasma cells in the lamina propria underlying the mucosal epithelia [1], in the primary immunological defence against such infections. For mammals it has been shown that mucosal epithelial cells synthesize the polymeric Ig receptor (pIgR). This is an integral membrane glycoprotein binding pIgA at the basolateral epithelial cell surface. The binding of pIgA to pIgR stimulates transcytosis via a signal transduction pathway that is dependent on a protein tyrosine kinase [2]. Receptor Ig complexes are endocytosed and subsequently transcytosed to the apical surface where secretory IgA (sIgA) is released into the mucosal lumen by proteolytic cleavage of the receptor ectodomain [3,4]. This cleaved fragment is called secretory component (SC). The structure of mammalian pIgRs is highly conserved, comprising an N-terminal extracellular region with five Ig-like domains (ILDs), an acidic transmembrane region and a C-terminal cytoplasmic region. Initially pIgR ILD 1 binds pIgA non-covalently [5,6]. Subsequently, covalent bonds are formed between conserved cysteine residues in pIgR ILD 5 and IgA C$\alpha$2 domains [7]. The J-chain, regulating the polymerization of IgA, is also important in the formation of sIgA, as its C-terminus forms a docking site on pIgA for pIgR [8].

In contrast with the detailed information available on the structure and function of mammalian pIgRs, little is known about equivalent structures in birds. In chicken (*Gallus gallus*) particularly, several reports have shown the presence of antigenic determinants associated with high molecular mass IgA complexes [9,10]. Studies on the transport mechanisms of chicken Igs across epithelial layers also suggested the presence of a pIgR-related carrier [11,12]. However, genes encoding the avian pIgR/SC counterparts remain to be identified.

Increased insight into avian mucosal immunity is important, as it can aid in preventing colonisation and, eventually, infection of mucosal epithelia. This is relevant both for animal welfare as well as for human health. Bacteria such as *Salmonellae* and *Campylobacter* and viruses such as the influenza virus can cause zoonosis and thus form a serious threat for humans. Understanding mucosal immunity can aid in improving vaccination of poultry and, in developing and evaluating other means, for instance based on probiotics or prebiotics such as oligosaccharides [13], to enhance immune responses. Consequently, the chances for zoonosis can be reduced.

Here, we report the cloning and characterization of the chicken *GG-pIgR* gene, the first non-mammalian pIgR orthologue. Its association with pIgA, yielding high molecular mass complexes, shows that *GG-pIgR* is the functional orthologue of mammalian pIgRs. As a distinctive feature, this pIgR was found to comprise only four Ig-like domains in its extracellular region, in contrast with all mammalian pIgR proteins consisting of five ILDs. The

Abbreviations used: BAC, bacterial artificial chromosome; CDS, complete coding sequence; EST, expressed sequence tag; GG-pIgR, chicken (*Gallus gallus*) pIgR; ILD, Ig-like domain; Ni-NTA, $Ni^{2+}$-nitrilotriacetate; pIgA, polymeric IgA; pIgR, pIg receptor; RACE, rapid amplification of cDNA ends; SC, secretory component; sIg, secretory Ig

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The nucleotide sequence data reported for *G. Gallus* pIgR cDNA and *G. Gallus* pIgR genomic sequence have been deposited in the GenBank® database under the accession numbers AF303371 and AY233381 respectively.

phylogenetic relationship between the ILDs of GG-pIgR and mammalian pIgR proteins was determined, revealing that the GG-pIgR ILDs are the most distantly related and do not group with any mammalian pIgR-ILD.

## EXPERIMENTAL

### Biological samples

For cloning of the GG-pIgR cDNA, 3-week-old SPF chickens from Cobb broiler breeder parent stock (bred with Hybro parent stock) were used. Northern blots were carried out with tissues from a 10-week-old hen.

### Cloning strategy

The bursa of Fabricius and parts of the intestinal tract (duodenum and jejunum) were collected and immediately frozen in liquid nitrogen. Total RNA extraction was performed using TRIzol®LS Reagent (Life Technologies, Breda, The Netherlands) according to the manufacturer's recommendations, and cDNA was synthesized. By aligning all published pIgR sequences, a conserved region was identified and used to design two degenerate oligonucleotide primers (sense primer 5′-AGCTACGARAAR-TAYTGGTG-3′, corresponding to the amino acid sequence Ser-Tyr-Glu-Lys-Tyr-Trp-Cys (SYEKYWC; human pIgR residues 475–481) and antisense primer 5′-GTAGGCCATRTCXGCYT-CYTCYTT-3′, corresponding to the amino acid sequence Lys-Glu-Glu-Ala-Glu-Met-Ala-Tyr (KEEAEMAY; human pIgR residue 126–133). A PCR reaction was carried out under standard conditions with bursa of Fabricius and jejunum cDNA as templates. The resulting PCR product was isolated, cloned and sequenced. The 5′ RACE (rapid amplification of cDNA ends) system (Life Technologies) was then used to obtain the putative full length chicken cDNA. The obtained fragment was subsequently sequenced and its homology with mammalian pIgR genes determined.

### Cloning *GG-pIgR*

To obtain the full-length genomic sequence of the coding region of the putative *GG-pIgR*, a bacterial artificial chromosome (BAC) library of the chicken genome (kindly provided by Dr R. Crooijmans; http://www.zod.wau.nl/vf/research/chicken/body_bac_library.html) was screened with *GG-pIgR*-specific primers (sense 5′-GGATCCGACGTGCAGATCCAGCTCCTT-CGT-3′ and antisense 5′-TCACCATCATCGACTTCCCAGAGC-AGG-3′) as described previously [14]. A BAC clone was found (AU bw 061 B21) which showed a band of the expected size. The genomic sequence containing the open reading frame of the putative *GG-pIgR* was revealed by using oligonucleotides, based on the cDNA sequence, generating overlapping PCR fragments. Products obtained by PCR were cloned and sequenced and used to construct a contig corresponding to the putative pIgR gene.

### Sequence and structural analyses

DNA sequences were analysed with Vector NTI Suite, version 8 (InforMax). Homology searches were performed using BLAST at the National Center for Biotechnological Information (NCBI). Prediction of coding sequences in the genomic sequence was done using the GENESCAN algorithm, available at http://genes.mit.edu/GENSCAN.html. The structures of the sequences were characterized using SMART (simple modular architecture research tool; http://smart.embl-heidelberg.de). This algorithm allowed identification of putative signal peptides, location of Ig-like domains and transmembrane regions. To compare protein sequences of pIgR Ig-like domains of different species [*Homo sapiens* pIgR (human), NM_002644; *Mus musculus* pIgR (mouse), NM_011082; *Rattus norvegicus* pIgR (rat), P15083; *Oryctolagus cuniculus* pIgR (rabbit), X00412; *Bos taurus* pIgR (cow), LO4797; *Sus scrofa* pIgR (pig), Q9N2H7; *Didelphis* pIgR (opossum), AX282930; *Macropus eugenii* pIgR (wallaby), AF317205; *Trichosurus vulpecula* pIgR (possum), AF091137] multiple alignments were carried out using the BLASTp algorithm at the NCBI server [15]. Standard parameters were applied. The percentage of identity between two sequences was calculated according to Piskurich et al. [16]. The phylogenetic trees were established with neighbour joining analyses of amino acid sequences [17] performed in PAUP* 4.0B10. To obtain the optimal tree, bootstrap analyses were conducted with 1000 replicates.

In addition to the Ig-like domains of the pIgR sequences, two *Xenopus* ESTs (expressed sequence tags) with significant homologies to pIgR Ig-like domains (BU904426 and BG233093 with E-values of $8 \times 10^{-13}$ and $1 \times 10^{-17}$ respectively) were included.

### Northern-blot analysis

RNA was isolated from heart, jejunum, duodenum, caeca, caecal tonsils, bursa of Fabricius, spleen and thymus of a ten-week-old hen. RNA concentrations were equalized by absorbance and verified by ethidium bromide staining of the gels. RNA was blotted onto nylon membranes and hybridized with radio-labelled probes according to standard procedures.

### Production GG-pIgR antiserum ($\alpha$-SC)

A fragment of 354 bp encoding the fourth Ig-like domain of GG-pIgR was cloned in the pBAD/TOPO-vector of the thiofusion expression system (Invitrogen). The resulting plasmid, termed pBADthioSCN4, was introduced into TOP10 *E. coli* cells, and expression of the fusion protein was induced by arabinose. The protein was purified using a Ni-NTA ($Ni^{2+}$-nitrilotriacetate) column under denaturing conditions with 8 M urea according to the manufacturer (Qiagen). After purification the thioredoxin moiety was cleaved using EnterokinaseMAX (Invitrogen), and the remaining pIgR fragment was purified using Ni-NTA mini spin columns (Qiagen). With the purified pIgR fragment a rabbit was immunized according to standard procedures using Freund's Incomplete Adjuvant. Serum was collected two weeks after the last immunization.

### Western-blot analysis

For detection of the secretory component, serum, bile and faecal extract samples (prepared as described in Dann et al. [18]) were mixed 1:1 with sample buffer (50 mM Tris/HCl, pH 6.8, 2 % (w/v) SDS, 10 % (w/v) glycerol, 0.01 % bromophenol blue) with or without addition of 40 mM DTT. SDS/PAGE was carried out using 7.5 % or 10 % polyacrylamide gels. After electrophoresis the proteins were transferred to PVDF membranes (Millipore) by semi-dry blotting. The membranes were blocked with PBS containing 0.1 % Tween-20 and 5 % low-fat milk powder. SIgA or SC was visualized by incubating the blots with either a 1:1000 dilution of the polyclonal rabbit anti-GG-pIgR serum ($\alpha$-SC) followed by an incubation with a 1:5000 dilution of anti-rabbit

Ig alkaline phosphatase conjugate or with a 1:2000 dilution of goat anti-chicken IgA-alkaline phosphatase conjugate ($\alpha$-IgA). The reaction was visualized using 5-bromo-4-chloro-3-indolyl phosphate as substrate.

### Co-immunoprecipitation

SIgA was co-precipitated using CNBr-activated sepharose 4B beads (Amersham Biosciences). Goat anti-chicken IgA (200 $\mu$g) was coupled to 200 $\mu$l of swollen Sepharose beads according to instructions of the manufacturer. The affinity beads were incubated overnight at 22 °C with 500 $\mu$l of chicken bile in 5 ml of PBS (pH 7.2). Then the beads were washed five times with PBS and subsequently the bound proteins were eluted in 50 $\mu$l of elution buffer (0.2 M $Na_2CO_3$, pH 11.0). Eluted fractions were characterized by Western analysis as described above. Uncoated Sepharose 4B beads were used as a control.

## RESULTS

### The GG-pIgR has four ILDs

In mammals, the pIgR is expressed in mucosal epithelia. For cloning the avian counterpart we therefore synthesized cDNA using mRNA isolated from the bursa of Fabricius, duodenum and jejunum tissue. Using degenerate oligonucleotides, based on the most conserved region of mammalian pIgR sequences, a PCR fragment was amplified showing significant similarity with pIgR sequences. Using a RACE approach the full-length cDNA clone was obtained, henceforth referred to as GG-pIgR. In BLASTp homology searches this clone revealed high levels of identity with mammalian pIgR sequences. Alignment of amino acid sequences (see Figure 1) showed that the motifs involved in pIgR functions are conserved in chicken. These include the calmodulin binding motif, the signal for rapid endocytosis, the initial non-covalent binding site for IgA, and the cysteine residues involved in the formation of intramolecular disulphide bridges. In contrast, the transmembrane region and the putative cleavage site of SC showed low levels of similarity.

The open reading frame of the putative GG-pIgR was approx. 100 amino acids shorter than all known pIgR proteins. To investigate this discrepancy in length, the domain-structure of the putative GG-pIgR was analysed using the SMART algorithm [19]. It was found to comprise an N-terminal signal peptide for secretion, four ILDs, a transmembrane domain and a cytoplasmic C-terminal region. Since all pIgR proteins described so far contain five ILDs, the shorter size of GG-pIgR was attributed to the lack of one ILD.

To investigate which domain was lacking, the levels of sequence identity between individual chicken and mammalian ILDs were compared (see Table 1). Mammalian domains 1, 3, 4 and 5 matched significantly with chicken ILDs a, b, c and d respectively (for overall sequence alignment of several pIgRs see Figure 1 and, Figure 2 for details about the nomenclature used for chicken ILDs). In contrast, the mammalian domains 2 showed relatively low levels of identity with chicken sequences, suggesting that this particular part is missing in bird species.

### The genomic organization confirms four ILDs in GG-pIgR

Alternative splicing has been reported for bovine and rabbit pIgR [20,21]. The finding of a GG-pIgR cDNA, lacking one ILD, prompted us to consider the occurrence of alternative splicing in the GG-pIgR transcript. To clarify this, a BAC clone containing the *pIgR* gene (AU bw 061 B21) was selected from a chicken BAC library. The sequence of a 10.4 kb genomic DNA fragment containing the complete coding sequence (CDS) of *GG-pIgR* was determined. The GENESCAN algorithm predicted a CDS consistent with the cDNA. No regions showing Ig-like homology could be found in the predicted introns. It was concluded that *GG-pIgR*, unlike the mammalian pIgR genes, comprises only four ILDs. The predicted genomic structure of *GG-pIgR* in comparison with its human counterpart is depicted in Figure 1. Exons 1 and 2 encode the 5′ untranslated region. The initiator AUG codon and the N-terminal part of the leader peptide is also encoded by exon 2. The four ILDs are encoded by exons 3, 4, 5 and 6 respectively. It is worth noticing that in human and mouse *pIgR*, exon 4 encodes two ILDs.

### Phylogenetic relationship between different pIgR domains

To gain insight in the phylogenetic relationships among the different Ig-like domains in polymeric Ig receptors, individual Ig-like domains from GG-pIgR and seven different mammalian pIgRs were used in the generation of similarity matrices. In addition, the derived amino acid sequences of two *Xenopus leavis* ESTs, having significant homology with pIgR ILD 1 and ILD 4, were included in these analyses. The exact boundaries of every ILD were determined using available structural or genomic information. The resulting unrooted phylogenetic trees are presented in Figure 3. The chicken ILDs do form a separate branch in the trees for domains 1, 3, 4 and 5. This also applies to the *Xenopus* ESTs in the trees for domains 1 and 4. Within each branch formed by the mammalian ILDs, marsupial and murine ILDs always form separate subgroups. It is worth noticing that rabbit ILDs do not cluster together with other mammalian species.

### GG-pIgR is expressed in jejunum, bursa of Fabricius, liver and thymus

RNA samples from different chicken organs were isolated and used in Northern-blot analysis with a gene-specific probe to determine which organs express GG-pIgR. As can be seen in Figure 4, it is expressed as a single transcript in jejunum, bursa of Fabricius, liver and thymus, but not in heart, caeca, caecal tonsils and spleen. The highest abundance of transcript was found in thymus and liver.

### pIgA associates with GG-pIgR

Upon transcytosis to the basolateral surface of the epithelium, the extracellular, ligand-binding domains of the pIgR are cleaved off. The resulting soluble form, known as SC, is found predominantly associated with pIgs (pIgA and pIgM), known as sIgs. To investigate the association of chicken SC with pIgA to sIgA, a rabbit antiserum ($\alpha$-SC) was raised against the ILD d of GG-pIgR expressed in *E. coli*. The presence of sIgA in serum, bile and fecal extracts was analysed by SDS/PAGE followed by immunoblotting using $\alpha$-SC and $\alpha$-IgA antibodies. Under non-reducing conditions, high molecular mass products were found in bile and faecal extracts upon blotting with either antisera (Figure 5a). These bands presumably correspond to chicken sIgA, with an estimated size of 400–500 kDa. In bile we also found substantial amounts of free chicken SC, while in faeces mainly proteins corresponding in size to monomeric IgA and the $\alpha$ heavy chain were found. The physical interaction between SC and chicken IgA was confirmed in a co-immunoprecipitation

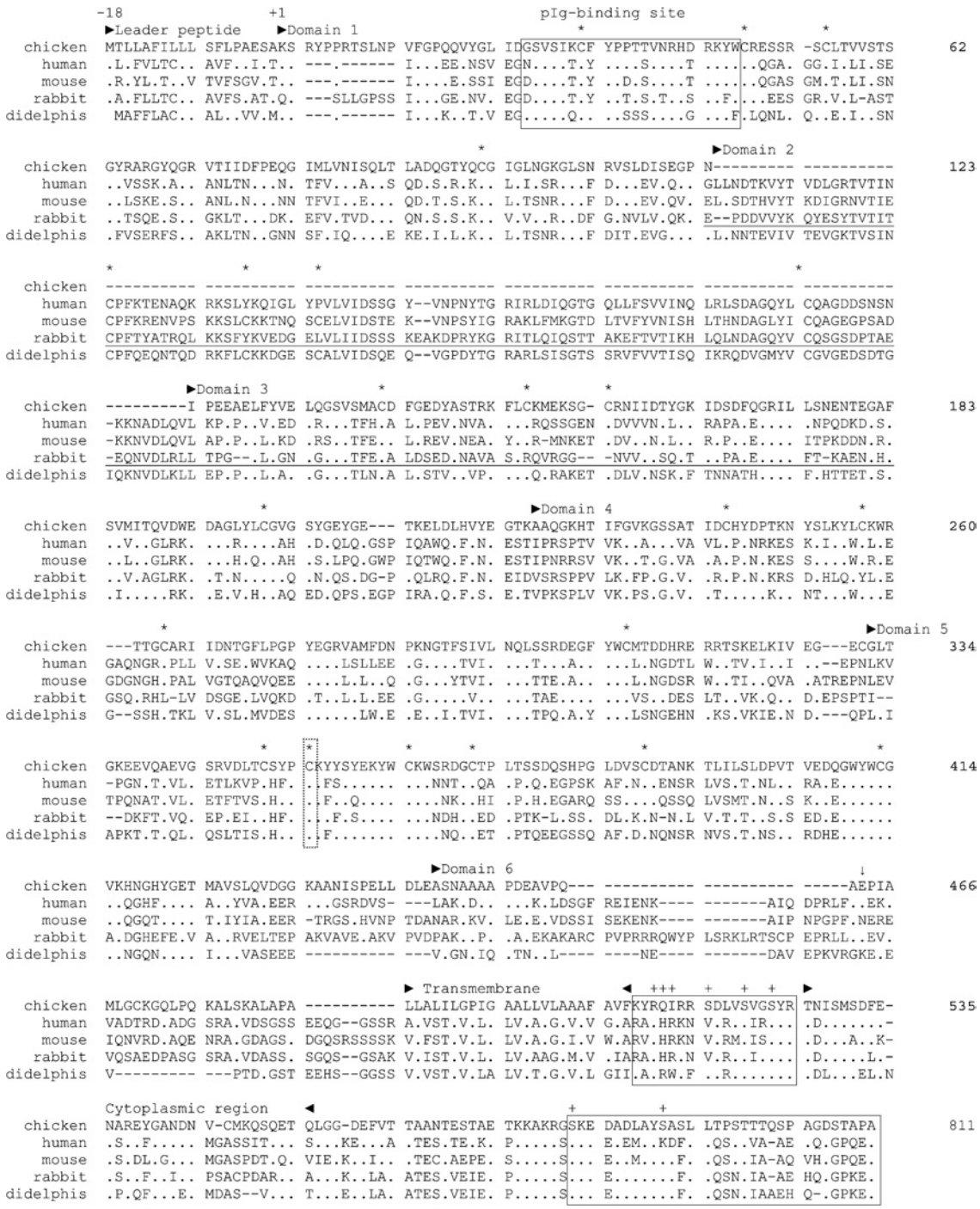


**Figure 1 Interspecies alignment of pIgR amino acid sequences**

The sequences of human, mouse and rabbit pIgRs were aligned as described in Piskurich et al. [16], including sequences of GG-pIgR and *Didelphis* pIgR. Numbering is based on the GG-pIgR sequence beginning with the N-terminal Lys of the mature protein. Dots indicate identity with the GG-pIgR sequence, and dashes indicate gaps in the sequence homology. The noncovalent Ig-binding site in domain I is boxed, the boxed residues in the cytoplasmic region denote sequences shown to be required for transepithelial trafficking of rabbit pIgR. Other residues found to be critical for pIgR trafficking are indicated with plus signs. Asterisks mark the perfectly conserved cysteine residues [7]; the cysteine residue in the dotted box within domain 5 is responsible for covalent IgA binding. The underlined part of the rabbit sequence indicates the two domains that are spliced out in the short form of pIgR.

experiment. Bile samples were subjected to immunoprecipitation with $\alpha$-IgA coupled to Sepharose beads followed by immunoblotting. As shown in Figure 5(b), SC co-immunoprecipitated with chicken IgA, confirming that the secretory component assembles with pIgA into sIgA complexes in chicken.

## DISCUSSION

Cloning of the avian *pIgR* orthologue, *GG-pIgR*, revealed a protein with four ILDs that assembles with chicken IgA to form a sIg complex. The motifs involved in pIgR functions are conserved.

**Table 1 Interdomain comparison of chicken, human, rabbit and opossum immunoglobulin-like domains**

The amino acid sequences of individual pIgR ILDs were compared by pairwise alignment. Percentage identity was calculated using the formula: 100× [(number of identical amino acid residues)/(total length of alignment-gaps)]. n.s., no significant alignment possible.

| ILD | Species | Chicken ILD a | Chicken ILD b | Chicken ILD c | Chicken ILD d |
|---|---|---|---|---|---|
| 1 | Human | 51.8 | 34.4 | 36.6 | 35.4 |
| | Mouse | 50.5 | 33.8 | 34.5 | 34.4 |
| | Rabbit | 49.1 | 34.2 | 34.1 | 34.0 |
| | Opossum | 47.3 | 34.0 | 32.1 | 25.0 |
| 2 | Human | 34.1 | 33.7 | 33.3 | n.s. |
| | Mouse | 29.0 | 29.8 | 30.4 | n.s. |
| | Rabbit | n.s. | 32.2 | n.s. | n.s. |
| | Opossum | 33.3 | 36.9 | 23.1 | 25.5 |
| 3 | Human | 36.7 | 44.7 | 29.6 | n.s. |
| | Mouse | 31.5 | 48.9 | 32.4 | n.s. |
| | Rabbit | 28.7 | 40.9 | 33.3 | n.s. |
| | Opossum | 26.7 | 46.2 | 31.3 | 27.6 |
| 4 | Human | 36.3 | 30.1 | 44.7 | 34.7 |
| | Mouse | 32.7 | 32.4 | 43.3 | 35.8 |
| | Rabbit | 38.8 | 30.0 | 42.6 | 34.1 |
| | Opossum | 39.2 | 33.3 | 46.4 | 34.8 |
| 5 | Human | 22.8 | 25.3 | 35.2 | 48.3 |
| | Mouse | 25.0 | 28.6 | 44.9 | 48.4 |
| | Rabbit | 28.6 | 31.7 | 35.8 | 46.1 |
| | Opossum | 23.4 | 27.7 | 45.2 | 45.8 |

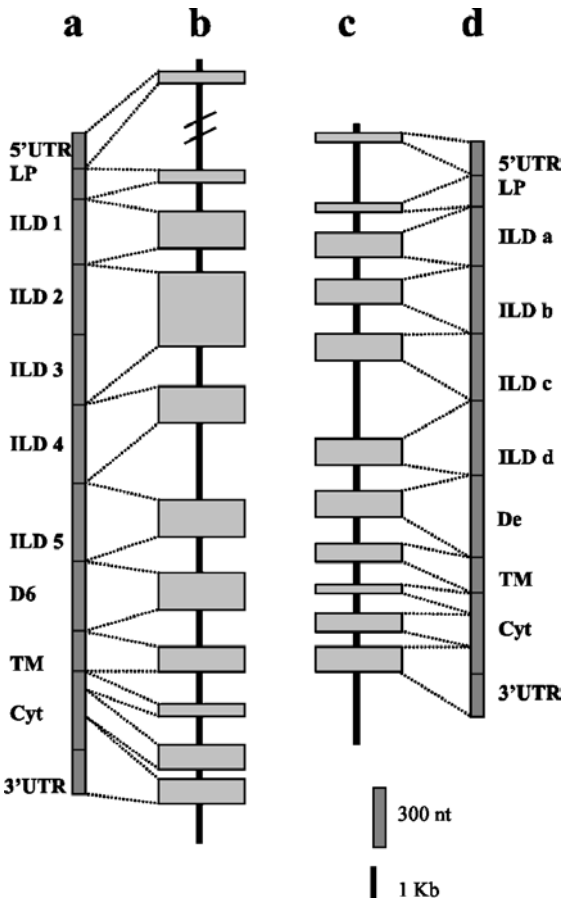


**Figure 2 Schematic representation of human and *GG-pIgR* genes and corresponding mRNAs**

(**a**) Domain structure of human mRNA, (**b**) intron/exon distribution in human *pIgR* gene, (**c**) intron/exon distribution in *GG-pIgR* gene and, (**d**) domain structure of chicken mRNA. Light grey boxes represent exons, dark grey boxes represent domains of the protein. Neither the human nor the chicken 3′-terminus of the exon coding the 3′UTR (untranslated) region of pIgR mRNA has been mapped so far. Human data from Krajci et al. [25]. LP, leader peptide; De/D6, extracellular domains with SC cleavage sites; TM, transmembrane region; Cyt, cytoplasmic domain.

It was concluded that the equivalent of mammalian ILD 2 is absent. This does not compromise the ability of GG-pIgR to bind IgA, which is not surprising, provided that differentially spliced forms of mammalian pIgRs lacking ILD 2 and ILD 3 are known to retain IgA binding capacity [5]. Also, in other studies, it was demonstrated that ILD 2 and ILD 3 do not play a critical role in the assembly with pIgA. Crottet and Corthésy [22] found that the insertion of recombinant epitopes in murine ILD 2 and ILD 3 did not affect non-covalent binding of pIgA. In addition, both full-length human and the short version of rabbit pIgR translocate dimeric IgA with comparable efficiency [23]. We have not been able to show that GG-pIgM associates with chicken SC. In concordance with the situation in mice and rats [24], no or only poor binding is expected because GG-pIgR is expressed in the liver. Binding of IgM by GG-pIgR in this organ and subsequent transport into bile would compromise the protective role of IgM in the systemic circulation. In addition, none of the residues in the motif involved in IgM binding, amino acid residues 61–64 (Figure 1), is conserved in chicken when compared with its mammalian counterparts.

The overall organization of the *GG-pIgR* gene is comparable with that of human and murine pIgR genes. All three genes have their CDS interrupted by 11 introns. The chicken gene spans, excluding the promoter region, 10.4 kb, thus being considerably shorter than the human and mouse pIgR genes (19 kb and 32 kb respectively) [25,26]. This shorter size was to be expected, as the length of introns in avian species is generally shorter than in mammals [27]. In chicken, the four extracellular ILDs are encoded by exons 3, 4, 5 and 6, thus complying with the one domain/one exon rule characteristic of the Ig superfamily [28]. In contrast, the exceptionally large exon 4 in mammalian pIgR genes encodes two ILDs. In this sense, the mammalian exon 4 seems unique. Interestingly, Bruce et al. [29] reported that an 84 nt deletion in the murine 654 nt pIgR exon 4, activated cryptic 5′ and 3′ splice sites within this exon. As a consequence, two smaller exons of 158 and 334 nt were generated, separated by a 78 nt intron. The activated cryptic 3′ splice site was located at the N-terminal end of ILD 2, which is exactly the location of intron 4 in the *GG-pIgR* gene. The occurrence of cryptic splice sites in mouse exon 4 could be interpreted as relics of ancient active splice sites, which may have become inactive as a consequence of duplication events. The finding of a four-domain structure in GG-pIgR reinforces this hypothesis. It seems reasonable to assume that the unusually large exon 4 found in mammals appeared as a result of one or more duplications within a primitive, single-domain exon. The lack of a homologue of ILD 2 in chicken could be explained assuming that ILD 2–3 duplication resulting in the extant four domain structure of GG-pIgR either never took place in the bird lineage or was rapidly reverted. As previously mentioned, in some mammalian species the large exon 4 is entirely spliced out as a consequence of differential splicing, giving rise to a shorter version of pIgR encoding only ILD 1, ILD 4 and ILD 5 [5,21].

The cloning of *GG-pIgR*, the first non-mammalian pIgR, allows studying phylogenetic relationships. It is worth noting in this respect that a phylogenetic relationship between the two Ig

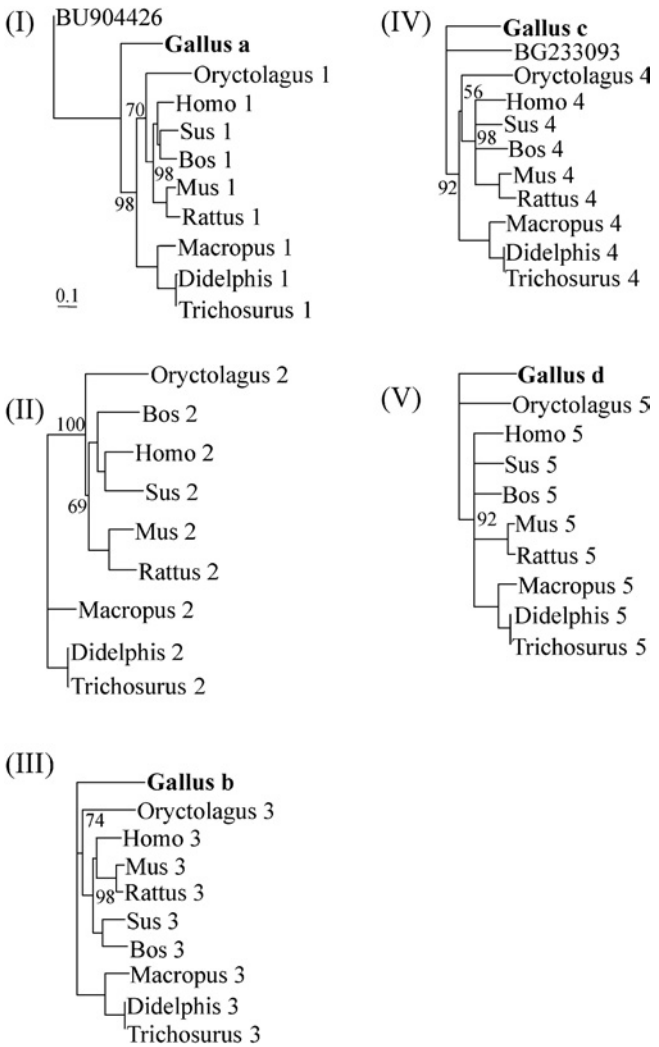


**Figure 3 Domain-based phylogenetic trees comparing ILDs from eight pIgRs and two *Xenopus leavis* translated ESTs**

Analysis was done with neighbour joining distance matrices for the generation of unrooted trees. The trees are numbered I, II, III, IV and V for ILDs 1–5. Numbers at branch nodes represent confidence level of 1000 bootstrap replications; bar indicates the number of amino acid changes per site. Arabic numbers and letters represent domain identity as depicted in Figure 1 for mammalian and GG-pIgRs. Homo, human; Mus, mouse; Rattus, rat; Oryctolagus, rabbit; Bos, cow; Sus, pig; Didelphis, opossum; Macropus, wallaby; Trichosurus, possum.

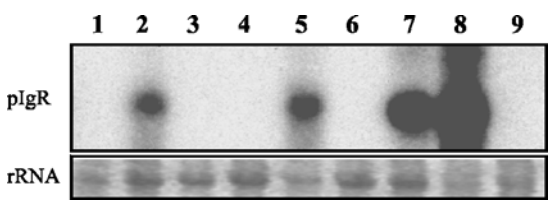


**Figure 4 Expression profiling of *GG-pIgR***

Northern-blot analysis of GG-pIgR mRNA in different organs: bone narrow (lane 1), jejunum (lane 2), caecal tonsils (lane 3), caeca (lane 4), bursa of Fabricius (lane 5), heart (lane 6), liver (lane 7), thymus (lane 8) and spleen (lane 9). Ethidium-bromide staining of ribosomal RNA (rRNA; bottom panel) shows equal loading of the samples.

families binding Fc fragments, pIgR and Fc$\alpha/\mu$ receptors [30] has been suggested. This hypothesis is based both on sequence similarities and on their topological proximity within the human

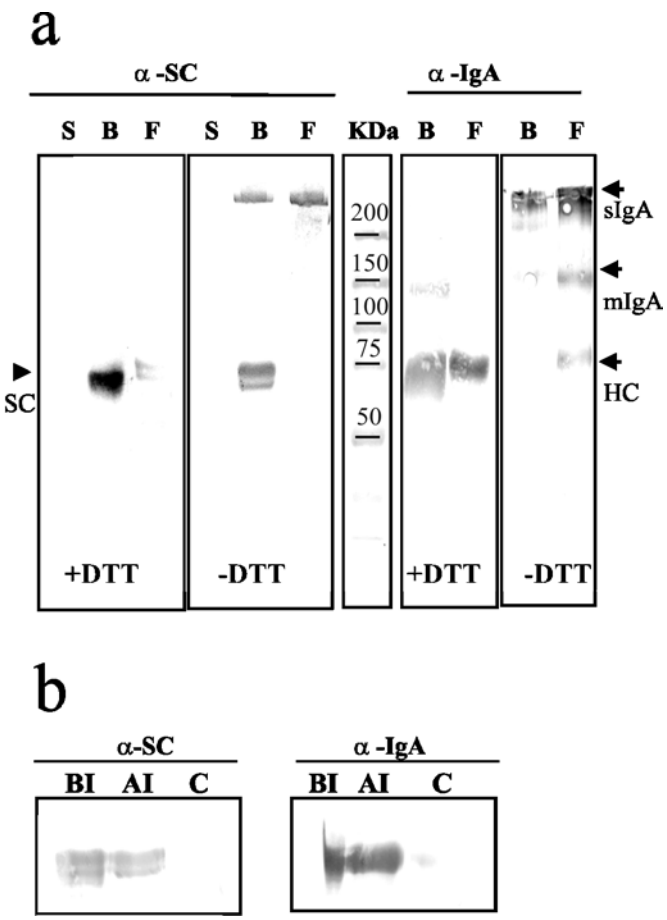


**Figure 5 Association of the chicken secretory component ($_{GA}$SC) and pIgA**

(**a**) Western-blot analysis of serum (S), bile samples (B) and faecal extracts (F) under reducing (+DTT) and non-reducing (−DTT) conditions. mIgA, monomeric IgA; HC, IgA heavy chain. (**b**) Western blot of co-immunoprecipitation of SC and IgA from bile with anti IgA antiserum under reducing conditions (BI, before immunoprecepitation; AI, after immunoprecipitation; C, control. Blots were developed with antisera $\alpha$-SC or $\alpha$-IgA.

genome [30,31]. The unique Ig-like domain of Fc$\alpha/\mu$ receptors is related to the N-terminal ILD of all known pIgR proteins. This conservation, likely maintained by strong selection pressure, is most evident in the CDR1-like loop of the first ILD, where the initial binding site of IgA/M is located. The equivalent motif in GG-pIgR, between residues 25 and 46 (see Figure 1), is highly conserved (77 % identity with human pIgR), suggesting a similar role in pIg binding.

From the domain-based sequence comparison of the pIgRs it became apparent that the GG-pIgR ILDs form a separate branch in the unrooted phylogenetic trees. A similar result was obtained for the two ESTs from *Xenopus leavis* showing significant homology to the mammalian and chicken ILD 1 and ILD 4 of pIgR. This is not surprising considering the phylogenetic distance existing between mammals and, respectively, birds and amphibians. The fact that *Xenopus* ESTs do show homology may be a first indication for the presence of pIgR-like proteins in amphibians. This is relevant in view of the fact that in *Xenopus*, an IgA analogue, IgX [32], has been described, but no transport system has been identified so far. The same applies to a hypothetical protein from the *Fugu rubripes* genome project (Scaffold_687_1) showing both sequence and structural similarities with pIg receptors (results not shown). The GENESCAN algorithm

predicted a 763 amino-acid protein containing a signal peptide, four ILDs, one transmembrane domain and a cytoplasmic domain. The ILDs in the *Fugu* hypothetical protein are highly self-similar and seem to have arisen from recent duplication events. Although no conclusions can be drawn from these findings regarding the presence of pIgR-like molecules in poikilotherm vertebrates, they are at least indicative of the versatility of pIgR-related ILD combinations. Our observations point at an ancient origin of pIgR and suggest that this gene is present in clades that have evolved before the divergence of birds and reptiles [33].

An explanation for a smaller but still functional GG-pIgR may be found in the protective role of SC against IgA degradation. With three constant domains and a hinge region, mammalian IgA differs structurally from chicken IgA having four constant domains and no hinge region [34]. Crottet and Corthésy [35] showed that SC exerts its protective role by delaying cleavage in the hinge/Fc region of the $\alpha$-chain. Since chicken IgA has no hinge region, the absence of an ILD could be related to a less demanding role for the secretory component in IgA protection.

We have shown that upon translocation pIgA associates with GG-pIgR, forming high molecular mass sIgA complexes, which can be detected in bile and faecal samples. Additionally, free chicken SC was found in bile. This has also been described for mammals [36] and a role as microbial scavenger has been suggested. As expected from a receptor involved in transcytosis, GG-pIgR was found to be mainly expressed in epithelia associated with lymphoid organs. Some peculiarities in GG-pIgR expression and accumulation patterns were found when compared with the mammalian counterparts ([1] and references therein). The low level of chicken SC in faeces contrasts with the abundance in bile (Figure 5a), but is in agreement with the mRNA expression patterns of intestine and liver tissues respectively (Figure 4a). This could be indicative of a distinct balance in the roles of GG-pIgR in hepatobiliar transport and gut protection. The expression of GG-pIgR in bursa of Fabricius, the central lymphoid organ for B-cell lymphopoiesis in birds, is difficult to explain considering the known functions of this organ. A role of pIgR in antigen sampling should be considered, as it is known that antigen presentation takes place in bursal epithelia during lymphopoiesis [37]. Similarly, the expression in the thymus is hard to explain and additional research is required to clarify the roles played by GG-pIgR both in thymus and bursa of Fabricius.

In conclusion, GG-pIgR is the functional homologue of mammalian pIgRs, the first non-mammalian pIgR described, and provides additional insight in the functioning of the avian mucosal immune system. Conservation of motifs for the various pIgR functions indicates that assembly to sIgs and transcytosis take place in a similar fashion as in mammals. The indications are that this gene evolved before the divergence of birds and reptiles, leading to the hypothesis that sIgs have a prominent role in the defence of mucosal epithelia in other jawed vertebrates.

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