

IgY: a key isotype in antibody evolution

Xiaoying Zhang^{1,*}, Rosaleen A. Calvert², Brian J. Sutton² and Katy A. Doré²

¹*Department of Basic Veterinary, College of Veterinary Medicine, Northwest A&F University, Yangling, 712100, China*

²*The Randall Division of Cell & Molecular Biophysics, King's College London, London, SE1 1UL, U.K.*

ABSTRACT

Immunoglobulin Y (IgY) is central to our understanding of immunoglobulin evolution. It has links to antibodies from the ancestral IgM to the mucosal IgX and IgA, as well as to mammalian serum IgG and IgE. IgY is found in amphibians, birds and reptiles, and as their most abundant serum antibody, is orthologous to mammalian IgG. However, IgY has the same domain architecture as IgM and IgE, lacking a hinge region and comprising four heavy-chain constant domains. The relationship between IgY and the mucosal antibodies IgX and IgA is discussed herein, in particular the question of how IgA could have contributed to the emergence of IgY. Although IgY does not contain a hinge region, amphibian IgF and duck-billed platypus IgY/O, which are closely related to IgY, do contain this region, as does mammalian IgG, IgA and IgD. A hinge region must therefore have evolved at least three times independently by convergent evolution. In the absence of three-dimensional structural information for the complete Fc fragment of chicken IgY (IgY-Fc), it remains to be discovered whether IgY displays the same conformational properties as IgM and IgE, which exhibit substantial flexibility in their Fc regions. IgY has three characterised Fc receptors, chicken Ig-like receptor AB1 (CHIR-AB1), the chicken yolk sac IgY receptor (FcRY) and *Gallus gallus* Fc receptor (ggFcR). These receptors bind to IgY at sites that are structurally homologous to mammalian counterparts; IgA/Fc α RI for CHIR-AB1, IgG/FcRn for FcRY and IgE/Fc ϵ RI and IgG/Fc γ R for ggFcR. These resemblances reflect the close evolutionary relationships between IgY and IgA, IgG and IgE. However, the evolutionary distance between birds and mammals allows for the ready generation of IgY antibodies to conserved mammalian proteins for medical and biotechnological applications. Furthermore, the lack of reactivity of IgY with mammalian Fc receptors, and the fact that large quantities of IgY can be made quickly and cheaply in chicken eggs, offers important advantages and considerable potential for IgY in research, diagnostics and therapeutics.

Key words: antibody evolution, IgY, IgM, IgG, IgE, IgA, Fc receptors, hinge region.

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* Address for correspondence (Tel: +86 29 8709 1239; E-mail: zhang.xy@nwsuaf.edu.cn)

I. INTRODUCTION

Immunoglobulin Y (IgY) was first named when Leslie & Clem (1969) suggested that the main serum antibody of chickens, which had thus far been referred to as IgG, differed in several ways from mammalian IgG and displayed similarities to mammalian IgM. This idea was not accepted immediately, with much of the literature still referring to IgY as chicken IgG, due to its functional similarities. However, with the discovery of IgY in non-mammalian vertebrates such as amphibians (Zhao *et al.*, 2006) and reptiles (Wei *et al.*, 2009), and later in the duck-billed platypus (*Ornithorhynchus anatinus*) (Gambón-Deza, Sánchez-Espinel & Magadán-Mompó, 2009), determination of the crystal structure of the sub-fragment of chicken IgY-Fc consisting of the C ν 3 and C ν 4 domains (Fc ν 3-4) that shows similarities to both human IgE and IgG (Taylor *et al.*, 2009b), and phylogenetic evidence that IgY is the evolutionary ancestor of both mammalian IgG and IgE, it is now accepted that IgY is a distinct class of immunoglobulin with a key place in the evolution of immunoglobulins.

This review focuses on structural aspects of IgY, in particular its constant domains and location of its receptor binding sites; these will be compared with those of mammalian IgM, IgG, IgA and IgE and their Fc receptor binding sites, which have been well characterised. This permits consideration of the place of IgY in antibody evolution. However, despite the importance of IgY, there is no crystal structure for the whole Fc region (including the C ν 2 domains), which in terms of the number of domains and lack of a flexible hinge region resembles IgM and IgE; key questions about whether IgY can bend in the Fc region (like IgM and IgE) thus remain unanswered at present. Mechanisms for the generation of diversity in antigen-binding repertoire will only be discussed briefly, although this aspect of IgY structure and function is clearly important for its potential therapeutic, diagnostic and biotechnological applications. The evolutionary distance between IgY and human antibodies and their receptors not only permits the generation of specificity towards conserved mammalian proteins, but also ensures that IgY antibodies do not exhibit reactivity with mammalian Fc receptors for diagnostic applications.

II. HOW IgY FITS INTO THE EVOLUTION OF IMMUNOGLOBULINS IN JAWED VERTEBRATES

In order to understand the evolution of immunoglobulins it can be helpful to group them according to their functional role(s). IgM antibodies can be produced in the absence of antigen challenge, and although of low affinity, nevertheless may be effective due to their multivalency (Fig. 1). They are the first antibodies produced by B cells in an immune response, but also play a role in mucosal immunity and as a serum antibody following affinity maturation. IgZ

(also known as IgT), found in ray-finned fish, IgX in amphibians and IgA in reptiles, birds and mammals, first evolved to provide mucosal immunity, although IgA is also an important serum antibody. IgX is an orthologue of IgA, but the evolutionary relationship between IgZ and IgA is unclear (Mashoof *et al.*, 2013). IgY (and perhaps also IgF), found in amphibians, IgY in birds and reptiles (and early mammals), and IgE and IgG in mammals, evolved as and are still principally serum antibodies produced in response to antigen challenge, although IgE is mainly sequestered on tissue mast cells by its high-affinity receptor Fc ϵ RI. Figure 1 illustrates the progression in overall antibody domain architecture throughout jawed vertebrate evolution, from the most primitive and oligomeric, to the most recent and monomeric molecules. IgD is not included in Fig. 1 because it has a highly variable number of domains and its evolutionary relationship to IgY is not clear (Estevez *et al.*, 2016).

(1) IgM and IgA gave rise to IgY

IgY is thought to have diverged from an ancestral IgM, and although the exact route is not understood, it is a widely held belief that an IgM gene duplication event led to the formation of IgY (Warr, Magor & Higgins, 1995). This was first proposed when IgM was thought to be the most ancient immunoglobulin, from which all others were descended. As our understanding of antibody evolution developed it became clear that this process is more complicated than first thought. We propose that IgA evolved first from a gene duplication event of IgM, and then IgY evolved subsequently from both IgM and an ancestor of IgA. IgA shares features with IgM such as oligomerisation, a C-terminal tailpiece and J-chain (Fig. 1), and its link with IgY is discussed below.

(2) The relationship between IgY and IgA

Gambón-Deza, Sánchez-Espinel & Valdueza-Beneitez (2007) reported that the heavy chain C H 1 and C H 2 domains of the leopard gecko (*Eublepharis macularius*) IgA show closer sequence similarity to IgY, while the C H 3 and C H 4 domains show closer sequence similarity to IgM. This IgY-like C H 1/C H 2 and IgM-like C H 3/C H 4 pattern is also seen for the western clawed frog (*Xenopus tropicalis*) IgX (Gambón-Deza *et al.*, 2007; Mashoof *et al.*, 2013). At that time no IgX was known in the axolotl (*Ambystoma mexicanum*) and this gave rise to the suggestion that IgX/IgA formed from a recombination of IgM and IgY. However in 2008, IgX was found in the axolotl (Schaerlinger & Fripiat, 2008), raising the intriguing possibility that IgM led to IgX/IgA, which subsequently led to IgY. Phylogenetic analysis of the four heavy chain domains of IgA, IgY and IgM show that IgM is closer to IgA than to IgY (Fig. 2A), which confirms that mucosal antibodies pre-date IgY. Another mucosal antibody, IgZ, is unique to bony fish. IgZ forms tetramers (as does bony fish IgM; see Fig. 1), is found on mucosal surfaces in the gills and is clearly an ancient mucosal antibody, phylogenetically distant from IgX and IgA (Fig. 3) (Pettinello & Dooley, 2014). In addition

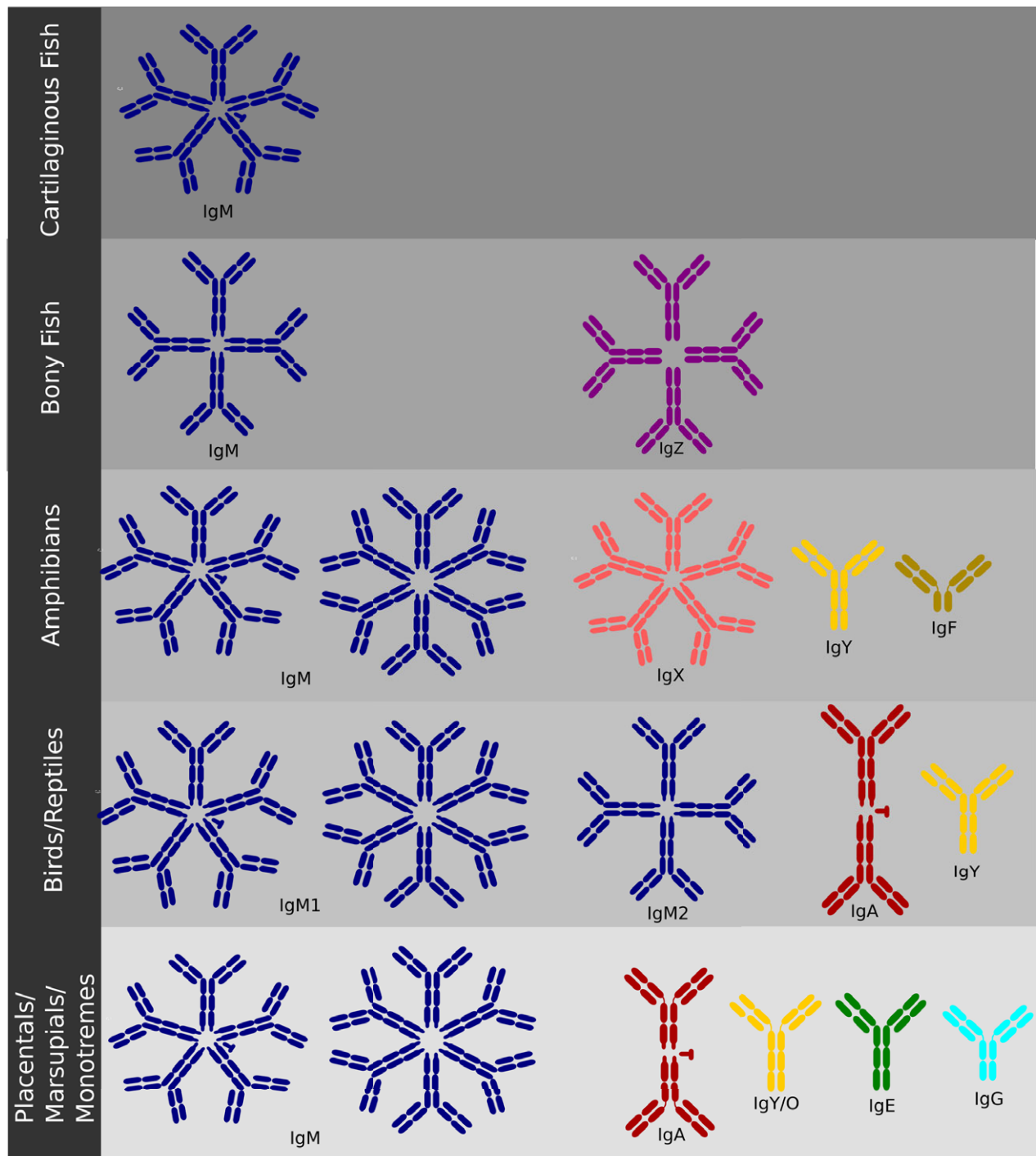


Fig. 1. Antibody domain architecture throughout jawed vertebrate evolution. The domain architecture and polymeric state are indicated and colour-coded consistent with later figures. J-chains are indicated by T-shaped symbols, hinge regions by thin connecting lines, and tailpieces by short stubs at the C-termini. IgD, IgH and IgW are not included.

to the common features of the tailpiece and J-chain (not seen in IgY), IgA and IgM also share two receptors, $Fc\alpha\mu R$ (Sakamoto *et al.*, 2001) and the polymeric immunoglobulin receptor (pIgR) (Akula, Mohammadamin & Hellman, 2014). Thus it seems more likely that two domains of an ancestor of IgA contributed to IgY (Fig. 3).

(3) IgY gave rise to IgE and IgG

IgY is thought to be the precursor of IgG and IgE (Warr *et al.*, 1995), with additional evidence supporting this hypothesis arising from comparison of membrane and cytoplasmic exons (Mussmann *et al.*, 1996) and from analysis of the

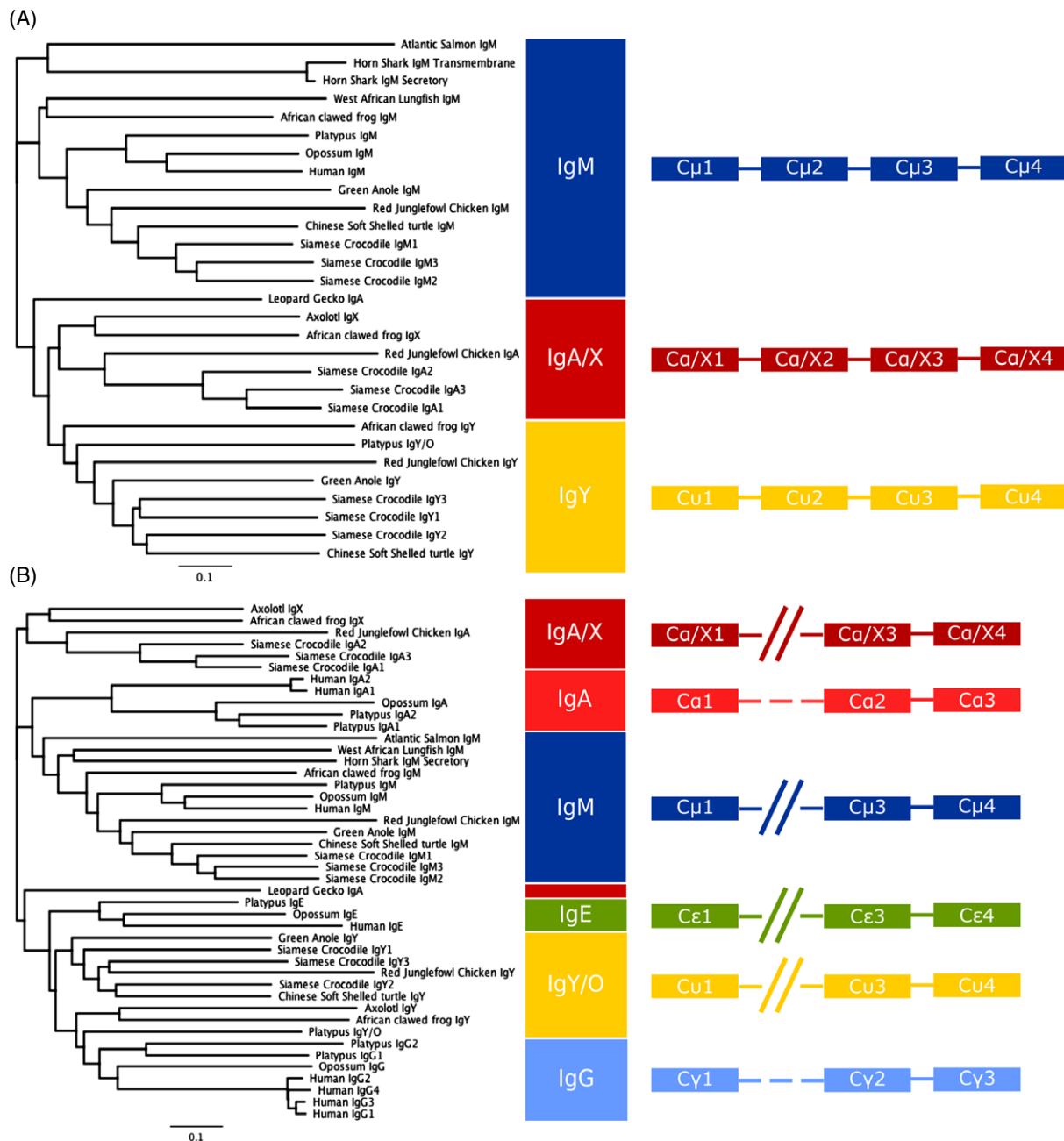


Fig. 2. Phylogenetic trees showing the relationship between IgY and the other isotypes. Phylogenetic relationships for heavy chain domains calculated using either: (A) all four domains, or (B) three domains only. Scale bar indicates substitutions per site. GenBank codes: African clawed frog (*Xenopus laevis*) IgM: AAA49774.1, IgX: s03186, IgY: AAH97629.1; Atlantic salmon (*Salmo salar*) IgM: ADD59895.1; axolotl (*Ambystoma mexicanum*) IgX: CA082107.1, IgY: S31436; Chinese soft-shelled turtle (*Pelodisus sinensis*) IgY: ACU45374.1, IgM: ACU45376.1; green anole (*Anolis carolinensis*) IgM: ABV66128.1, IgY: ABV66132.1; horn shark (*Heterodontus francisci*) IgM transmembrane: P23088.1, secretory: P23087.1; human (*Homo sapiens*) IgA1: P01876.2, IgA2: P01877.3, IgE: AAB59424.1, IgG1: AAG00909.1, IgG2: AAG00910.2, IgG3: AAG00911.1, IgG4: CAC20457.1, IgM: AAS01769.1; leopard gecko (*Eublepharis macularius*) IgA: ABG72683.1; opossum (*Monodelphis domestica*) IgA: AAD21190.1, IgE: AAC79674.1, IgG: AAC78139.1, IgE: AAB59395.1, IgM: CAB37838.1; Platypus (*Ornithorhynchus anatinus*) IgA1: AAL17700.1, IgA2: AAL17701.1, IgE: AAL17702.1, IgG1: AAL17703.1, IgG2: AAL17704.1, IgM: AA037747.1, IgY/O: ACD31541.1; red junglefowl chicken (*Gallus gallus domesticus*) IgA: A46507, IgM: P01875.2, IgY: AHX37590.1; Siamese crocodile (*Crocodylus siamensis*) IgA1: AFZ39174.1, IgA2: AFZ39175.1, IgA3: AFZ39176.1, IgM1: AFZ39177.1, IgM2: AFZ39178.1, IgM3: AFZ39179.1, IgY1: AFZ39180.1, IgY2: AFZ39181.1, IgY3: AFZ39182.1; West African lungfish (*Protopterus annectens*) IgM: AGT56951.1. Drawn with Geneious 2 (with default gap penalties) using cost matrix Blosum62, Jukes-Cantor genetic distance model with neighbour-joining tree-build method (unrooted tree).

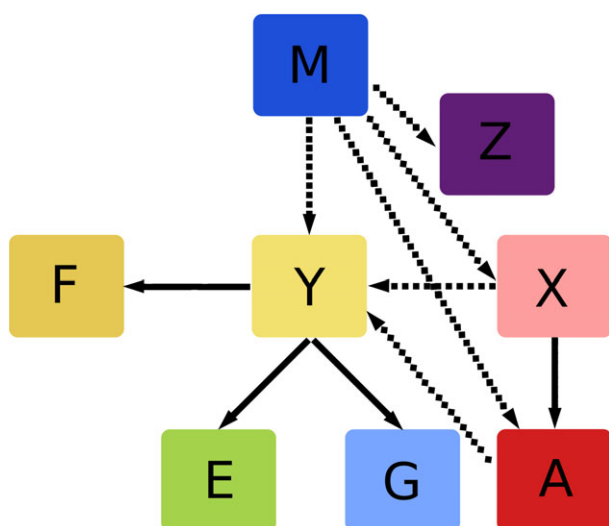


Fig. 3. The central position of IgY in immunoglobulin evolution. Solid arrows indicate an orthologous relationship between isotypes. Broken arrows connect isotypes that have a putative orthologous relationship, not yet verified. The broken arrow that relates IgA to IgY refers to an ancestral form of IgA. Colour coding for antibody isotypes follows that of Fig. 1.

duck-billed platypus genome (Gambón-Deza *et al.*, 2007). There is an immunoglobulin in the heavy chain locus of the platypus in the expected location for IgY, with four constant domains and a hinge (Gambón-Deza *et al.*, 2007), and this has been suggested to represent a distinct class of immunoglobulin known as IgO (Zhao *et al.*, 2009). Zhao *et al.* (2009) argue that the presence of the hinge region, together with sequence similarity of the domains to IgG, imply that this immunoglobulin is not a true IgY. In any case, this antibody offers a unique glimpse into one of the intermediates that probably pre-dated the emergence of IgG and IgE from ancestral IgY. Finally, there is evidence that the hinge-containing IgF found in *Xenopus tropicalis* arose from a gene duplication event from IgY. The hinges in IgG and IgF appear to have arisen separately by convergent evolution (Zhao *et al.*, 2006).

Phylogenetic analysis of the first (C_H1) and last two (C_H3 & C_H4) heavy chain domain sequences shown in Fig. 2B places IgY in relation to IgM, IgX, IgA, IgE and IgG. The IgM sequences from lobe-finned fish to human, unsurprisingly group together. IgA found in the leopard gecko, groups with IgM. Mammalian IgAs group together, and the amphibian IgX groups with crocodilian and chicken IgA, consistent with IgA originating from IgX. IgX/A are closer to IgM than to IgY. IgF found in the western clawed frog groups with IgY, as reported previously (Zhao *et al.*, 2006), based upon alignment of the two C_H domains of IgF with the C_H1 and C_H4 domains of IgY (not shown). Unsurprisingly, the Siamese crocodile (*Crocodylus siamensis*) subclasses IgY1, IgY2 and IgY3 group with the chicken, lizard and turtle IgY, showing a close evolutionary relationship. Platypus IgY/IgO shows closer links with platypus IgG1 and IgG2 and opossum (*Monodelphis domestica*) and human IgG, than with IgE of these

species, or bird, reptilian and amphibian IgY. At some point during the evolution of the mammalian lineage, IgY underwent a gene duplication event and diversified into IgE and IgG. Thus this phylogenetic tree supports the proposal that IgM gave rise to the mucosal antibody IgX and then to IgA, which gave rise to IgY and the serum antibodies IgG and IgE. These relationships are depicted in Fig. 3, which highlights the central role of IgY.

III. THE IgY HEAVY CHAIN

IgY has the typical antibody architecture of two identical light and two identical heavy chains (Fig. 1), with four heavy chain constant domains (C_v1–4), as found in IgM (C_μ1–4) and IgE (C_ε1–4) (Figs 1 and 4). The three-dimensional structure of part of the IgY heavy chain, namely the dimer of the C_v3 and C_v4 domains (Fc_v3–4), is known (Taylor *et al.*, 2009b), but not that of the complete IgY-Fc region, which also includes the C_v2 domains. This crystal structure of Fc_v3–4 (Figs 5 and 6) shows an intriguing similarity to both human IgG-Fc and the Fc_ε3–4 region of human IgE-Fc, adopting a quaternary structure that combines elements of IgG and IgE: the relative orientation of the two constant domains in one chain is more IgG-like, while the other chain is more IgE-like (Taylor *et al.*, 2009b). There is also a truncated form of IgY, IgYΔFc, which does not contain the C_v3 or C_v4 domains at all; this is found in anseriform birds, which are specially adapted for aquatic life (ducks and geese) (Magor *et al.*, 1992), and certain turtles and reptiles (Wei *et al.*, 2009; Li *et al.*, 2012) (Fig. 4). This truncated form is generated by alternative splicing of the heavy chain constant region from a single gene, with the exception of some turtle species that appear to have a separate gene (Li *et al.*, 2012). The function of this immunoglobulin, which lacks the receptor-binding sites (see Section V), is thought to enable pathogen neutralisation without inducing inflammation (Magor, 2011; Li *et al.*, 2012); this may be of significance for human health since it may result in asymptomatic migratory birds transmitting avian influenza virus.

Figure 4 highlights the similarities and differences between the domain structures of IgM, IgZ, IgX, IgA, IgF, IgY, IgG and IgE. IgD and IgW are not included due to the wide variation in the number of their domains, which can be as many as 19 for IgD; they would be situated alongside IgM, and one or other is found in most jawed vertebrates with the exception of certain species of birds (Han *et al.*, 2016) and marsupial mammals (Wang, Olp & Miller, 2009). IgM shows conservation throughout evolution in sequence and domain structure, and there is remarkably little difference between IgM in cartilaginous fish and mammals. IgM forms pentamers in the presence of a J-chain (Fig. 1) in all species apart from ray-finned bony fish, where tetramers are formed instead (Acton *et al.*, 1971) and there is no J-chain (Kaattari, Evans & Klemer, 1998). The lobe-finned bony fish lungfish (*Protopterus dolloi*) and the African and Indonesian coelacanths (*Latimeria chalumnae*, *Latimeria menadoensis*) do

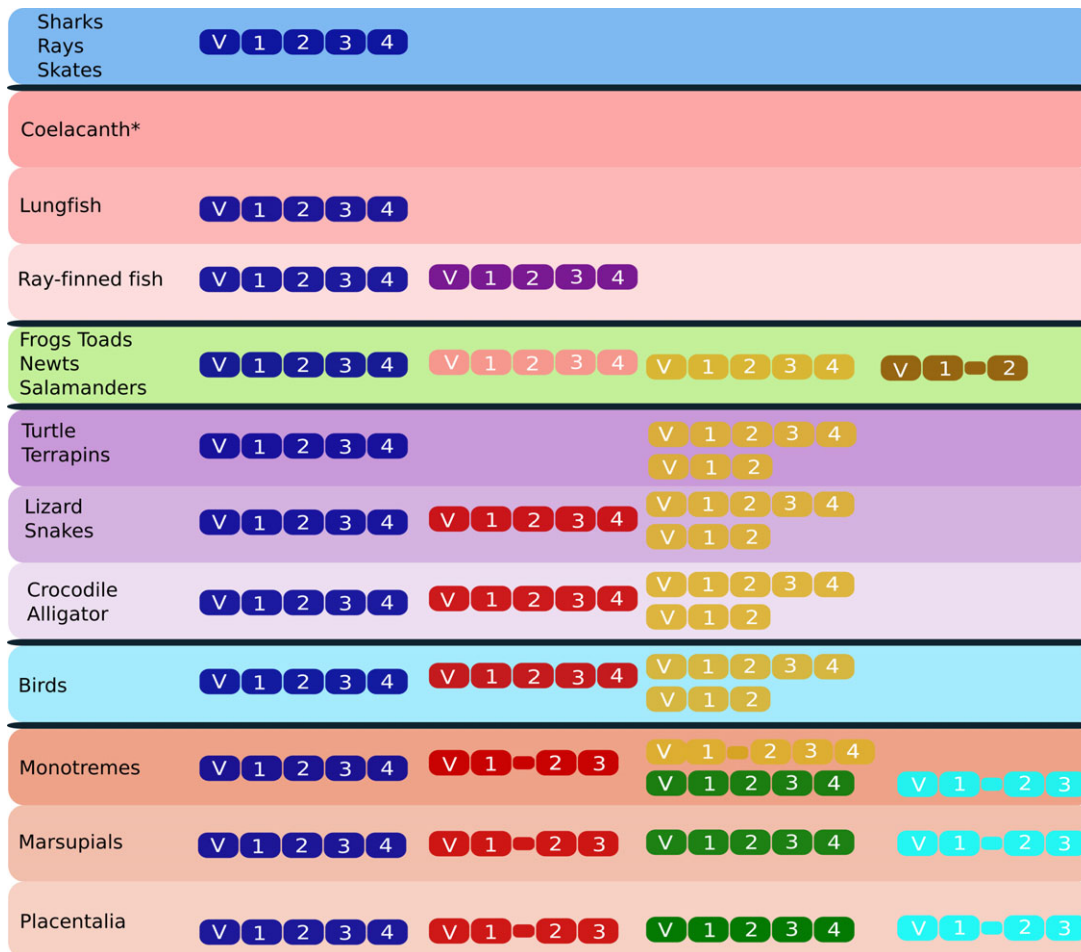


Fig. 4. Antibody heavy chains through evolution. Colour code follows Fig. 1: IgM, dark blue; IgZ, purple; IgX, pink; IgA, red; IgY, yellow; IgF, olive green; IgE, green; IgG, light blue. IgD and IgW are not included. *Coelacanth is reported to have only IgW antibodies. Note that some lizards do not have IgA.

express the J-chain (Tacchi, Larragoite & Salinas, 2013), but in lungfish the IgM polymerisation state is unknown, and the coelacanth has no IgM (Amemiya *et al.*, 2013). In the absence of a J-chain in other species, IgM forms a hexameric structure. This remarkable conservation of IgM is consistent with its critically important protective role (Boes *et al.*, 1998).

The mucosal antibodies are classified into three distinct isotypes IgZ, IgX and IgA (Figs 1 and 4). The phylogenetically distant IgZ is unique to the bony fish and forms tetramers. The orthologous IgX and IgA are pentamers and dimers, respectively. Mammalian IgA differs however, despite high sequence similarity with bird and reptilian IgA, in the 'loss' of the Cα2 domain from the four constant domains of antecedent IgA antibodies, and 'gain' of a hinge (Figs 1 and 4). The serum antibodies illustrate the most probable transition from IgY to IgG and IgE. Amphibian IgF, closely related to IgY (Figs 3 and 4) but containing only two heavy chain constant domains with a hinge between them, is an example of the independent, convergent evolution of a hinge region (see Section IV).

IV. THE IgY HINGE REGION

The hinge region is a structure commonly encoded by a discrete heavy-chain exon that lies between the Fab and Fc regions of antibodies including mammalian IgD, IgG and IgA (Figs 1 and 4). The hinge contains inter-heavy-chain disulphide bridges and regions that are conformationally restricted and rich in proline residues, and permits flexibility between the two antigen-binding Fab arms and the Fc. The four subclasses of human IgG are distinguished by hinge regions of different lengths, resulting in a range of conformational flexibility. The earliest evolutionary example of a hinge described to date is in an obscure immunoglobulin found in teleosts, IgH. This novel immunoglobulin has two constant regions with a hinge region between, and two exons encoding a membrane-spanning region; it is expressed in both secretory and membrane forms. It is unusual because it is located upstream of the μ gene in the heavy chain locus (Savan *et al.*, 2005; Rosaria Coscia & Oreste, 2009), but because it has no direct relationship to IgY, IgH has not been included in Figs 1 and 4. The next example

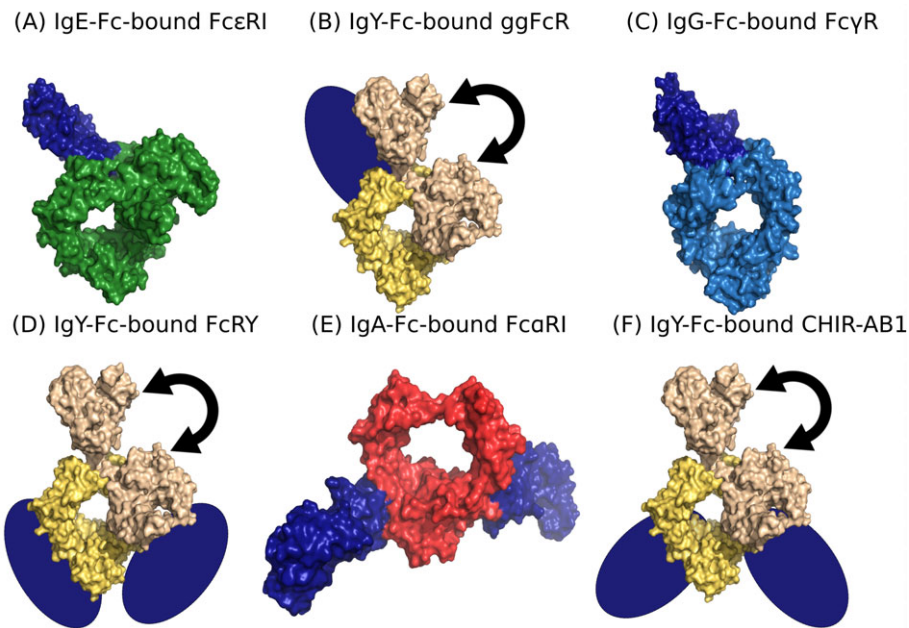


Fig. 5. Comparison of putative IgY-receptor interactions with IgE-, IgG- and IgA-receptor complexes. (A, C, E) Structures of the complexes between IgE-Fc (green), IgG-Fc (light blue) and IgA-Fc (red) and their receptors FcεRI, FcγR and FcαRI (dark blue). (B, D, F) Presumed interactions between IgY-Fc and the receptors ggFcR (Schreiner *et al.*, 2012), FcRY (He & Bjorkman, 2011) and CHIR-AB1 (Taylor *et al.*, 2010) (dark blue ellipses). IgY-Fc is represented here by the known structure of Fcγ3-4 (yellow) and the Cv2 domains (tan) modelled in two orientations corresponding to the 'bent' and 'extended' conformations of IgE-Fc; the double-headed arrows indicate that either conformation, an intermediate or a dynamic ensemble of conformations may be adopted by IgY-Fc.

of a hinge to appear during evolution is in amphibian IgF. Although IgF (Figs 1 and 4) has a similar domain structure to IgH, the sequences are unrelated, with IgF being more IgY-like and IgH being more IgD-like. IgM, IgY, IgE and IgA in turtles, crocodiles, birds and reptiles have no hinge region, but hinges appear extensively in mammals, including in mammalian IgA, IgD and IgG. Although absent from all non-mammalian IgY molecules, there is a hinge in platypus IgY/IgO. This suggests that the hinge region has appeared independently at least three times, in an example of convergent evolution.

The hinge region is highly diverse in its amino acid sequence between classes (and even between subclasses in mammalian IgG) and does not bear any sequence relationship with any other heavy chain region (Adlersberg, 1976; Huck, Lefranc & Lefranc, 1989; Kawamura, Omoto & Ueda, 1990). There are different theories concerning how the hinge developed, and more than one mechanism may be involved. One theory proposes that the hinge is an exon that evolved in parallel with the immunoglobulin genes (Adlersberg, 1976; Sakano *et al.*, 1979), and some evidence from non-human primates suggests that it is related to the variable region (Kawamura *et al.*, 1990). Alternatively it may have evolved from gene duplication of a small constant region (Adlersberg, 1976), or from contraction or extension of a constant domain, most likely C_H2 (Tucker, Slightom & Blattner, 1981; Takahashi *et al.*, 1982; Mansikka, 1992).

V. THREE-DIMENSIONAL STRUCTURE OF IgY-Fc AND BINDING TO Fc RECEPTORS

The crystal structure of the Fcγ3-4 sub-fragment of IgY-Fc is known (Taylor *et al.*, 2009b), but not that of the entire Fc region including the Cv2 domains. However, IgE-Fc, for which several crystal structures including Fcε3-4 sub-fragments have been determined (Sutton & Davies, 2015), may offer a model for the complete three-dimensional structure of IgY-Fc. The crystal structure of IgE-Fc revealed a conformation bent acutely between the Cε2 and Cε3 domains (illustrated in its receptor complex in Fig. 5A) (Wan *et al.*, 2002), but recent biophysical and crystallographic analysis of a complex between IgE-Fc and an anti-IgE antibody Fab have shown that the IgE-Fc can also adopt an extended conformation (Drinkwater *et al.*, 2014). For IgM, although no IgM-Fc crystal structure is known (only individual Cμ2, Cμ3 and Cμ4 domain structures; Müller *et al.*, 2013), electron microscopy (EM) analysis of pentamers indicates that the antibody can flex between extended and bent conformations, although it is unclear whether this occurs between Cμ1 and Cμ2 (Czajkowsky & Shao, 2009), or Cμ2 and Cμ3 (Perkins *et al.*, 1991). The dislocation of IgM pentamers from a planar, extended conformation to a bent structure upon antigen binding is functionally important since it reveals complement binding sites, initiating complement activation. An EM study of green turtle (*Chelonia mydas*) IgY molecules (Work *et al.*, 2015) appears to show

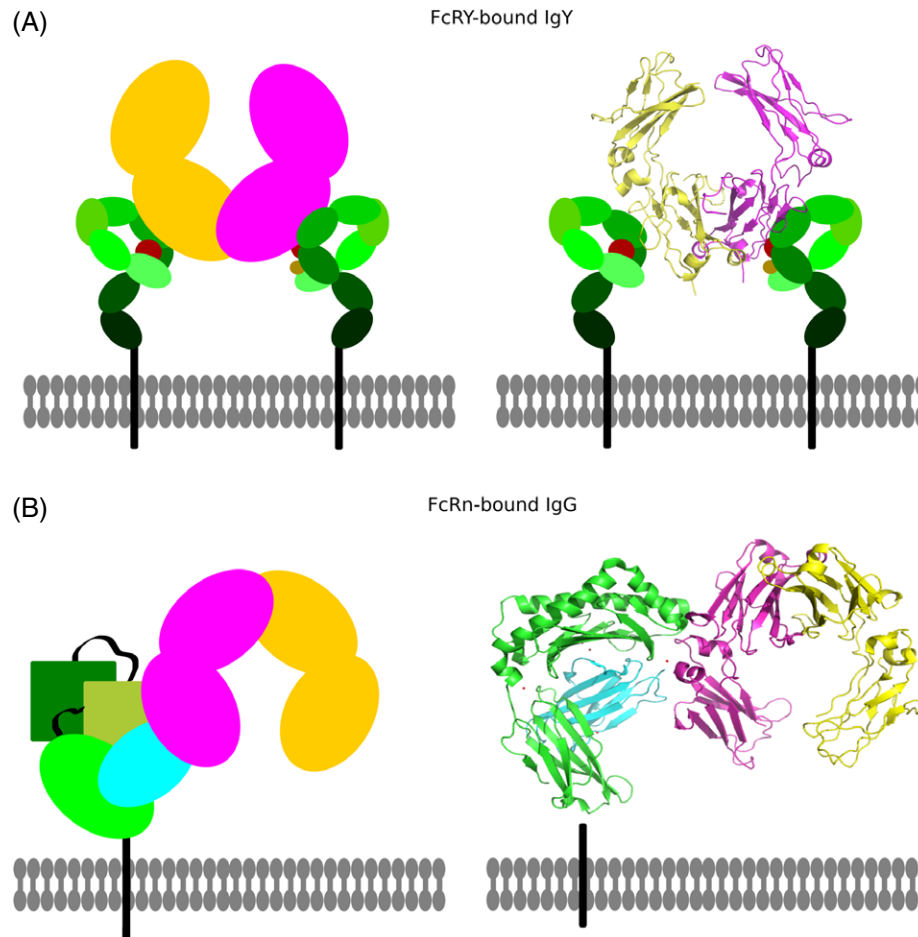


Fig. 6. Comparison of the binding of IgY to FcRY and the binding of IgG to FcRn. Schematic images on the left correspond to the structures on the right. (A) Fc γ 3-4 (yellow and purple) interacting with the tandem array of domains in FcRY (from N-terminal cysteine-rich domain in red, fibronectin type II domain in tan, and tandem array of eight C-type lectin-like domains in light to dark green; from cryo-EM analysis; He & Bjorkman, 2011). (B) IgG-Fc (yellow and purple) bound to FcRn (green domains with β 2-microglobulin in light blue; Martin *et al.*, 2001).

compact, asymmetrical Fc regions, that could be interpreted as bent, akin to the crystal structure of free IgE-Fc. It is possible however, that IgY-Fc can also flex between a bent and an extended conformation, like IgE and IgM. This is depicted in Fig. 5B, D, F, assuming that this occurs, as in IgE-Fc, between C γ 2 and C γ 3.

Both IgY-Fc and IgE-Fc are N-glycosylated in their C H 3 domains (at Asn407 and Asn394, respectively) with high-mannose-type oligosaccharide (Suzuki & Lee, 2004), in contrast to the complex type attached to the homologous site in the C γ 2 domain of IgG-Fc (Asn297). This is another similarity between IgE-Fc and IgY-Fc that may be relevant to conformational flexibility and receptor binding activity. An additional N-linked glycosylation site (Asn308) is present in the C γ 2 domain, which carries a complex type carbohydrate (Suzuki & Lee, 2004).

No high-resolution structures are available for any of the complexes formed between chicken IgY-Fc and its receptors, but some of these interactions have been mapped by mutagenesis or modelled from cryo-electron

microscopy (cryoEM) images. Their proposed locations may be compared to those that are now well determined through crystal structures of mammalian receptor complexes with IgE-Fc, IgG-Fc and IgA-Fc, some of which are depicted in Figs 5 and 6. The homologous Fc ϵ RI and Fc γ R (I, II and III) bind at structurally homologous sites spanning both C ϵ 3 domains in IgE or the C γ 2 domains in IgG, with 1:1 stoichiometry (Fig. 5A, C); Fc α RI (CD89) however binds between the C α 2 and C α 3 domains of IgA (Fig. 5E), at a similar location to that of Fc ϵ R2 (CD23) between the C ϵ 3 and C ϵ 4 domains of IgE (not shown), both with a 2:1 stoichiometry. Fc ϵ R2 (lectin-like superfamily) is not structurally homologous to Fc α RI (Ig superfamily), nor to FcRn [major histocompatibility complex (MHC)-like superfamily] which binds to IgG between the C γ 2 and C γ 3 domains with 2:1 stoichiometry (Fig. 6): thus three structurally distinct receptors (Fc α RI, Fc ϵ R2 and FcRn) bind to their class-specific Fc regions between structurally homologous domains (C α 2/C α 3, C ϵ 3/C ϵ 4 and C γ 2/C γ 3) in IgA, IgE and IgG, respectively. The interactions of IgY

can now be described in relation to the locations of the known receptor binding sites in these other antibody heavy chain classes.

Three receptors for chicken IgY have been characterised: CHIR-AB1, FcRY and ggFcR (Härtle *et al.*, 2013); a further receptor for uptake of IgY from the maternal circulation into egg yolks has been inferred (Murai *et al.*, 2013; Takimoto *et al.*, 2013). The Fc receptor pIgR, which transports the other two chicken antibody isotypes, IgM and IgA, but not IgY, is evolutionarily conserved, in contrast to the IgY receptors. Sequences of homologues of mammalian Fc and FcRL receptors have been identified in chicken and amphibian genomes (Akula *et al.*, 2014), one of which has been characterized and does not bind IgY (Taylor *et al.*, 2007); many of these receptors likely mediate other functions unrelated to binding antibodies. What can the IgY receptor proteins additionally tell us about the mechanisms of antibody evolution?

(1) CHIR-AB1

The presence of human Fc α RI (CD89) and bovine Fc γ RII receptor genes in the human leukocyte receptor cluster (LRC) encouraged Viertlboeck *et al.* (2007) in their study of possible ligands for chicken Ig-like receptors (CHIR) in the chicken LRC on chromosome 31 to include IgY, IgA and IgM in their assay. CHIR-AB1 was the first chicken IgY immune receptor discovered. It is expressed on monocytes and other chicken innate immune cells and has activatory activity and the potential for inhibitory activity. Further IgY binding proteins were identified in the same very large cluster (>100 CHIR genes), but no other ligands have been identified to date. The kinetics of the single extracellular Ig domain of CHIR-AB1 binding to IgY was measured by surface plasmon resonance analysis and was found to be similar to IgY binding to monocytes (Taylor *et al.*, 2008). Soluble CHIR-AB1 could inhibit most of the IgY binding to the chicken monocyte cell line MQ-NCSU, suggesting that CHIR-AB1 is the predominant monocyte IgY receptor (Taylor *et al.*, 2009a). Interestingly, the stoichiometry was 2:1 (Taylor *et al.*, 2009a), the same as for the binding of Fc α RI to IgA (Herr, Ballister & Bjorkman, 2003a; Herr *et al.*, 2003b).

The crystal structures of Fc ν 3-4 (Taylor *et al.*, 2009b) and CHIR-AB1 (Arnon *et al.*, 2008) were superimposed onto the structure of the IgA-Fc/Fc α RI complex (Herr *et al.*, 2003a) (Fig. 5E), and residues were selected for mutation to test whether the interactions might be structurally homologous: the results supported this hypothesis (Pürzel *et al.*, 2009; Taylor, Sutton & Calvert, 2010). The proposed mode of binding is indicated in Fig. 5F.

(2) FcRY

A second Fc receptor for IgY was identified in chicken, FcRY, functionally similar to the mammalian MHC-like protein, FcRn, responsible for transport of maternal IgG to the foetus (West, Herr & Bjorkman, 2004; Tian & Zhang, 2012). FcRY was purified from yolk sacs and the sequence of the

N-terminal peptide used to search an expressed sequence tag (EST) database, revealing a protein of the mannose receptor family, a structural homologue of the phospholipase A₂ receptor (PLA₂R). It consists of 10 extracellular domains: an N-terminal cysteine-rich domain, a fibronectin type II domain and eight C-type lectin-like domains (Fig. 6). As for FcRn and IgG, FcRY binds IgY at pH 6 and releases it at pH 8, but the domains that constitute these functionally equivalent proteins are from entirely different gene families – an example of convergent evolution. The histidine residue responsible for the pH sensitivity of IgG binding to FcRn is absent in FcRY; a pH-dependent structural change has been observed using small-angle X-ray scattering (SAXS) analysis (He & Bjorkman, 2011), evidently taking place by a different mechanism. CryoEM analysis of the complex between two molecules of soluble FcRY and IgY at pH 6 indicated interaction with the C ν 4 domains in each heavy chain (He & Bjorkman, 2011), but some interaction with C ν 3 could not be ruled out at this low resolution. The similarity between the FcRY/IgY and FcRn/IgG interactions is depicted in Fig. 6, and the location of the site in IgY-Fc is compared with that of the other receptors in Fig. 5.

FcRY transports IgY from the egg yolk to the embryo, but there must be a receptor that first transports IgY from the maternal ovarian follicle into the yolk, and this has not yet been isolated. However, several amino-acid residues have been identified on IgY that affect yolk uptake of IgY (Murai *et al.*, 2013; Takimoto *et al.*, 2013). Recent gene targeting in chickens using clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) (Oishi *et al.*, 2016) should facilitate the production of chickens with modified IgY-Fc sequences and consequently higher IgY yields.

(3) ggFcR

A further receptor, ggFcR, was unexpectedly located to chromosome 20 (Viertlboeck *et al.*, 2009), a location unrelated to the LRC or FcR loci. The receptor consists of four extracellular Ig-like domains, and its binding site in IgY-Fc, mapped by mutagenesis, was found to involve residues of C ν 3 that suggest a structurally homologous location to the binding sites of Fc ϵ RI in C ϵ 3 (Fig. 5A) and Fc γ R in C γ 2 (Fig. 5C) (Schreiner, Viertlboeck & Göbel, 2012). This putative location is shown in Fig. 5B. Although ggFcR bound moderately well to Fc ν 3-4, residues in C ν 2 were also found to contribute to stronger binding to the whole IgY-Fc, and thus the ggFcR binding mode is slightly different. This similarity to IgE/Fc ϵ RI and IgG/Fc γ R is depicted in Fig. 5A–C, although of course the involvement of the C ν 2 domains will depend critically upon whether IgY-Fc is bent like IgE-Fc, as it is in the Fc ϵ RI complex (Holdom *et al.*, 2011) (Fig. 5A), or in a more extended conformation (Fig. 5B).

CHIR-AB1 (Viertlboeck *et al.*, 2007) and ggFcR (Viertlboeck *et al.*, 2009) require the FcR γ -chain to be expressed on the membrane, and then monomeric and aggregated IgY, respectively, can activate these receptors. Both have an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic sequence, but an inhibitory signal has not

been demonstrated. Interestingly, CHIR-AB1 and ggFcR are found in both myeloid and haematopoietic lineages, whereas FcR γ is only present in myeloid lineages, suggesting that CHIR-AB1 function may be cell-type dependent.

In the evolution of the relationship between IgY and its receptors there is thus evidence of both convergence and divergence, even working independently with respect to both structure and function. The complexity of the situation is illustrated by the three characterised IgY/receptor pairs, which present three different scenarios. IgY is of course related through divergence of both structure and function to the other antibody isotypes (IgA, IgG and IgE) for which receptor complexes are structurally characterised. However, the FcRY receptor and mammalian FcRn present a clear case of convergence: polypeptides from entirely different lineages (and thus unrelated structures) that converge in function and with respect to the region of the respective antibodies to which they bind (Figs 5D and 6).

The genes for CHIR-AB1 and Fc α RI are both found in the LRC and presumably evolved by divergence when the LRC expanded. These two structurally homologous receptors bind to IgY and IgA, respectively, at structurally homologous sites (Fig. 5E, F), but it is not known whether they evolved together as an antibody/receptor pair, or whether antibody and receptor evolved separately, ‘discovering’ their binding sites independently.

The gene for ggFcR is the only one discovered on chromosome 20 to date that has the characteristics of an Fc receptor: extracellular Ig-like domains that bind antibody, a transmembrane domain and the ability to transmit an activating signal. It is thought to have translocated from the LRC, thus originating from a different cluster from mammalian Fc γ R and Fc ϵ RI. Any divergent evolutionary relationship is presumably very distant. However, to the extent that ggFcR and mammalian FcR bind at structurally homologous sites on their respective antibodies (Fig. 5A–C) and can transmit an activating signal, both structural and functional convergence is clear (Schreiner *et al.*, 2012), although the receptors have different tissue distributions and their functions may well differ.

A further intriguing aspect of antibody/receptor co-evolution is the possibility that a major shift may have occurred involving ‘migration’ of receptor binding activity, by a member of a different receptor family, from one site (e.g. between C_H3 and C_H4) to another (e.g. between C_H2 and C_H3). If, as we have previously suggested (Taylor *et al.*, 2010), this occurred in an IgY-like ancestor of IgG and IgE, leading to the emergence of novel receptor-mediated activities, IgY might have played an even more significant role in the evolution of antibody structure and function than previously thought.

VI. DIVERSIFICATION OF IgY SPECIFICITY

It has been known for some time that chickens produce little diversification by combinatorial joining of their

variable, diversity and joining (VDJ) gene segments, but instead use pseudogenes to enable gene conversion, in both the heavy chain (Reynaud *et al.*, 1989) and light chain (Reynaud *et al.*, 1987), as do rabbits (Knight & Winstead, 1997; Flajnik, 2002). Chickens can produce IgY antibodies against proteins that are highly conserved between mammals because of their evolutionary distance from mammals (Larsson & Sjöquist, 1990), and also because gene conversion leads to a different bias in amino acid usage, sometimes giving rise to disulphide bonds either within the complementarity-determining regions (CDRs) or bridging the CDRs and framework regions (Finlay & Almagro, 2012). Three crystal structures confirm this observation (Shih *et al.*, 2012; Conroy *et al.*, 2014). Gene conversion is responsible for most of the diversification in CDR1 and 2, and somatic hypermutation and N-region diversity in CDR3 (Leighton *et al.*, 2015). Gene conversion is recapitulated in the chicken cell line DT40 (Buerstedde *et al.*, 1990) and this has lately been repeated with both human V sequences and synthetic pseudogenes. It has been shown on a small scale (Schusser *et al.*, 2013) and subsequently by deep sequencing (Leighton *et al.*, 2015) that almost all of the introduced pseudogenes are involved in gene conversion events. A patent has been issued for a transgenic chicken to exploit this system, which should yield novel human V regions to generate monoclonal antibodies for diagnostics and therapy (Leighton, Harriman & Etches, 2014).

Diversification of chicken antibodies in DT40 cells has been used to investigate the mechanism of gene conversion and somatic hypermutation, enabling identification of the targets of activation-induced cytidine deaminase (AID) and an understanding of the control of its activity, where studies in transgenic mice had failed (Buerstedde *et al.*, 2014). As a result, AID-binding DNA sequences can now be investigated in mice, and thus a key question in mammalian immunology has found some answers by recourse to the chicken.

VII. THERAPEUTIC AND DIAGNOSTIC APPLICATIONS OF IgY

The evolutionary distance of IgY from mammalian proteins enhances its applications in research and diagnostics, and purification of antibody from egg yolk permits high-yield production both conveniently and ethically. Not only can antibodies be raised to conserved mammalian proteins more easily than in mammalian species, but also IgY does not react with mammalian receptors, eliminating background cross-reactivities in immunological assays (Larsson & Sjöquist, 1990; Spillner *et al.*, 2012).

IgY can be used for oral passive immunotherapies (Rahman *et al.*, 2013). The advantage of this type of therapy is that IgY is easily generated in chicken eggs, and is well tolerated since chicken eggs are a natural part of the human diet. As the purified IgY does not contain egg albumin, it can be used in patients with egg allergies, as the allergic reaction is commonly to the chicken albumin. IgY does not react with

the human complement system or Fc receptors, and so the risk of further inflammation is minimal. It is thought to work by binding to the bacteria or viruses and allowing them to be eliminated through the gut, preventing bacterial replication or virus spread. Furthermore, as the IgY is produced in yolk granules, it is protected from pH and thermal denaturation in the digestive system. It has distinct advantages over vaccination in some cases as it is more specific, has a rapid and local onset of reaction, and so can be given to patients with active infection. It is also applicable to infants and immune-compromised adults. IgY has successfully been used to treat cystic fibrosis patients suffering from chronic *Pseudomonas aeruginosa* infections (Kollberg *et al.*, 2003), gastroenteritis in infants (Rahman *et al.*, 2013) and *Helicobacter pylori* infections (Horie *et al.*, 2004; Suzuki *et al.*, 2004; Nomura *et al.*, 2005). It has been used prophylactically in dental caries in children (Nguyen *et al.*, 2011) and periodontitis (Yokoyama *et al.*, 2007).

A number of efforts have been made to generate monoclonal IgY antibodies in the same way that monoclonal IgG has previously been produced. Such molecules could combine the advantages of both monoclonal antibodies and avian IgY (Zhang *et al.*, 2010). Genetically engineered single-chain fragment variable IgY (IgY-scFv) has been successfully generated based on phage display technologies by different research groups. One recent example is the generation of anti-gentamicin IgY-scFv. This scFv showed high binding affinity in immuno-assays and high specificity in recovery assays, indicating that such an antibody reagent could be used for the detection of antibiotic residue or chemical contaminants to ensure food safety (Li *et al.*, 2016).

VIII. CONCLUSIONS

(1) IgY occupies a central position in antibody evolution, implicated in the evolution of present-day mammalian serum IgG and IgE antibodies, yet directly linked both to more primitive mucosal IgX and IgA, and also to the ancestral IgM antibodies.

(2) The precise relationship of IgY to IgA and the mucosal antibodies such as IgX is complex, but in light of recent information, we propose that IgY is partially derived from an IgA ancestor.

(3) Although the heavy chain of IgY generally consists of four constant domains and no hinge, there is a variant with a hinge, and others with truncated Fc regions. Considering its importance and structural versatility, surprisingly little is known about the three-dimensional structure of IgY, apart from the C ν 3-4 region of chicken IgY-Fc. Since the C ν 2 domains are missing from this structure, it is unknown whether IgY-Fc adopts a predominantly bent conformation like IgE-Fc, or indeed can flex between bent and extended structures like IgM. As IgY is a direct link between 'ancient' IgM and 'modern' IgE, this is a fascinating question.

(4) Whether IgY is bent, extended or flexible also has important implications for understanding how it interacts

with its three characterised receptors, CHIR-AB1, FcRY and ggFcR. Intriguingly, IgY binds to CHIR-AB1 in an IgA/Fc α RI-like manner, to FcRY in an IgG/FcRn-like manner, and to ggFcR in an IgE/Fc ϵ RI- and IgG/Fc γ R-like manner. Not enough is known at present about IgM/receptor interactions to extend this comparison.

(5) The evolutionary position of IgY in relation to mammals, and humans in particular, renders it particularly valuable in relation to both its Fab-mediated ability to recognise conserved mammalian proteins, and its Fc-mediated lack of reactivity with mammalian receptors. However, detailed structural information about IgY and its receptor interactions would clearly be valuable regarding both its diagnostic and therapeutic applications, for which there is growing interest and exciting potential.

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