The Crystal Structure of an Avian IgY-Fc Fragment Reveals Conservation with both Mammalian IgG and IgE[†,‡]

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Received October 10, 2008

ABSTRACT: Avian IgY is closely related to an ancestor of both mammalian IgG and IgE and thus provides insights into the evolution of antibody structure and function. A recombinant fragment of IgY-Fc consisting of a dimer of the Cυ3 and Cυ4 domains, Fcυ3−4, was expressed and crystallized and its X-ray structure determined to 1.75 Å resolution. Fcυ3−4 is the only nonmammalian Fc fragment structure determined to date and provides the first structural evidence for an ancient origin of antibody architecture. The Fcυ3−4 structure reveals features common to both IgE-Fc and IgG-Fc, and the implications for IgY binding to its receptor are discussed.

The major serum antibody of birds and reptiles, IgY, is the closest extant homologue of the evolutionary ancestor of both IgG and IgE isotypes in mammals, which diverged following a gene duplication fewer than 310 mya[1] (*1*). IgY therefore offers insights into the evolution of the structural and functional differences between mammalian IgG and IgE. We have previously shown that chicken IgY binds to chicken monocytes with IgG-like kinetics (*2*), despite a putative IgE-like structure predicted from the chicken υ heavy chain cDNA sequence (*3*). In mammals, allergen-specific IgE binding to effector cells primes the immune system for an immediate hypersensitivity reaction (*4*). The uniquely slow dissociation of IgE from its receptor, FcεRI, is due in part to the presence of a domain pair (Cε2) that in IgG is condensed to form the flexible hinge region (*5*). In IgY, this additional domain (Cυ2) is also present, but here it does not play the same role (*2*). As a step toward improved understanding of the structural basis for the functional differences among IgY, IgE, and IgG, the X-ray structure of an IgY-Fc fragment was determined. While several crystal structures of mammalian IgG-Fc and IgE-Fc fragments have been reported, this paper presents the first high-resolution structure of the Fc region of a nonmammalian antibody.

MATERIALS AND METHODS

Cloning and Expression. cDNA encoding chicken Fcυ2−4 and Fcυ3−4 was cloned and expressed as described previously (*2*). Briefly, cDNA was amplified from a chicken splenocyte cDNA library, cloned into a mammalian expression vector (pRY), and stably transfected into NS0 mouse myeloma cells, which secreted the IgY-Fc fragments into their culture medium. The fragment described as Fcυ3−4 contains a C340S mutation for reasons described in ref *2*. Site-directed mutagenesis of the Fcυ2−4 cDNA sequence to remove N-linked glycosylation sites (N308Q and N407Q) was performed using a Quikchange kit (Stratagene) and the mutant Fcυ2−4 expressed as described above.

Purification. IgY-Fc fragments were purified from NS0 supernatants by immunoaffinity chromatography using anti-IgY-agarose (Immunology Consultants Laboratory) and size exclusion chromatography using Superdex 200 (GE Life Sciences), with buffer exchange into 20 mM Tris-HCl, 120 mM sodium chloride, and 0.05% sodium azide.

Characterization. Protein concentrations were measured using an ND-1000 spectrophotometer (Thermo Scientific) using molar extinction coefficients described in ref *2*. Proteins were analyzed on denaturing SDS−12% acrylamide gels (SDS−PAGE) with the Laemmli system (*6*). Gels were stained for protein using Coomassie Brilliant Blue R250 (Electran) or for carbohydrates using a periodic acid-Schiff glycoprotein stain kit (Pierce).

Deglycosylation. Fcυ2−4 and human IgG-Fc (Bethyl Laboratories, P80−104) were incubated under nondenaturing conditions [50 mM sodium phosphate (pH 7.5)] with 50 units of PNGase F (New England Biolabs) per microgram of protein for 72 h at 37 °C.

Flow Cytometry (FACS). Chicken MQ-NCSU cells grown in the absence of chicken serum were incubated sequentially with 10 nM IgY-Fc fragments or buffer alone, mouse monoclonal anti-IgY (Sigma), and finally anti-mouse immunoglobulins conjugated to FITC, each for 1 h at 4 °C. Likewise, human U937 cells were incubated with IgG-Fc fragments or buffer alone, followed by anti-IgG-Fc conjugated with FITC, for 1 h at 4 °C. All cells were washed twice in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (by centrifugation at 1000*g* for 5 min)

[†] This work is supported by the Biotechnology and Biological Sciences Research Council (U.K.). A.I.T. was formerly supported by a Medical Research Council (U.K.) studentship.

[‡] Atomic coordinates have been deposited in the Protein Data Bank as entry 2W59.

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[1] Abbreviations: Cα/ε/γ/υ, IgA/E/G/Y heavy chain constant domain; Ig, immunoglobulin; mya, million years ago; PDB, Protein Data Bank; rmsd, root-mean-square deviation.

10.1021/bi8019993 CCC: $40.75
Published on Web 12/30/2008

Table 1: Data Collection and Refinement Statistics

space group	$P2_12_12_1$	R_{merge} (%)	10.4 (67.9)[a]
cell dimensions (Å)	$a = 69.48$, $b = 80.10$, $c = 99.14$	R_{pim}[b] (%)	4.3 (17.2)[a]
wavelength (Å)	0.9698	$I/\sigma(I)$	12.9 (2.8)[a]
resolution (Å)	69.50−1.75 (1.84−1.75)[a]	R_{work}/R_{free}[c] (%)	16.9/19.6
no. of observed reflections	384558	rmsd	
no. of unique reflections	56233	bond lengths (Å)	0.006
PBD entry	2W59	bond angles (deg)	1.077
completeness (%)	99.6 (99.6)[a]		

[a] Values in parentheses refer to the highest-resolution shell. [b] Precision-indicating merging R factor (*35*). [c] Calculated from 5% of the unique reflections (*36*).

before and after every antibody incubation step. Cells were fixed in PBS containing 4% paraformaldehyde and analyzed using a FACSCalibur instrument (BD Biosciences).

Crystallization. Fcυ3−4 was crystallized using 96-well MRC sitting drop vapor diffusion plates (Wilden) at 18 °C. Drops (100 nL) of Fcυ3−4 at 3 mg/mL were dispensed using a Mosquito liquid handling robot (Molecular Dimensions) and mixed with equal volumes of an Easyxtal crystallization solution (Qiagen). Crystals were obtained after 3 days using 40 mM potassium phosphate, 16% (w/v) PEG 8000, and 20% (v/v) glycerol. No additional cryoprotectant was used because the mother liquor provided sufficient protection.

X-ray Diffraction Data Collection. X-ray diffraction data were collected from a single crystal cryocooled to 100 K in a nitrogen gas stream at Diamond Light Source beamline I04, using an ADSC Quantum-315 detector. The wavelength was 0.9698 Å, and 360 images of 1° oscillation were collected at a distance of 241 mm. Due to radiation damage at the end of the data collection, only 180 images were used for the data set.

X-ray Data Processing and Refinement. The data were processed with MOSFLM (*7*), SCALA (*8*), and TRUNCATE, from the CCP4 suite of programs (*9*). Molecular replacement was performed using Phaser (*10*), with the IgE-Fc structure (PDB entry 1O0V) as a search model. The structure was refined using Phenix (*11*), and the model was built with Coot (*12*). Validation checks were performed using Procheck (*13*) and MolProbity (*14*). Structural comparisons and alignments were made using SSM (*15*). Figures were generated using PyMol (*16*).

RESULTS

In a manner comparable to that for mammalian IgG, a covalently linked homodimer comprising the two C-terminal heavy chain domain pairs, Fcυ3−4, can be prepared by digesting chicken egg yolk IgY with papain (*17*) and crystallized (*18*). Despite attempts to optimize growth conditions, crystals prepared in this way diffracted poorly, possibly due to heterogeneity resulting from incomplete digestion at the N-terminus (*19*). A recombinant form of the chicken Fcυ3−4 fragment was designed and overexpressed as described previously (*2*), purified, and used to grow crystals that diffracted to 1.75 Å at the Diamond Light Source. The structure was determined by molecular replacement and refined at this resolution ($R_{work} = 16.9\%$; $R_{free} = 19.6\%$). Data collection and refinement statistics are listed in Table 1. The structure was built from residue 349 to 566

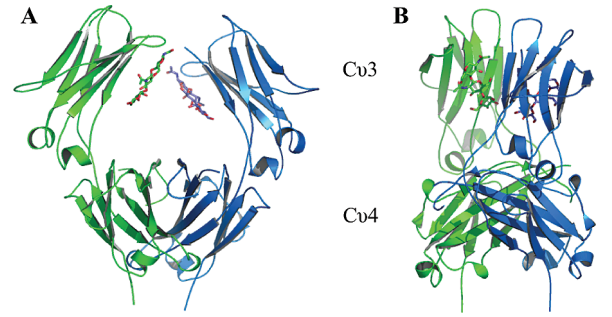

FIGURE 1: Ribbon diagrams showing two orthogonal views of the crystal structure of chicken Fcυ3−4 (A and B). Chain A is colored blue and chain B green, and carbohydrate residues are shown in all-atom representation, colored by chain and atom type.

in both chains, and thus, 11 residues at each N-terminus and two at each C-terminus are disordered. The only break in the electron density occurs between residues 518−520 (chain A) and 517−520 (chain B) inclusive. This lies within an extended loop region in Cυ4, which has no counterpart in human IgG or IgE and represents an overall insertion of nine residues.

The structure of the Fcυ3−4 fragment reveals remarkable conservation with previously determined human IgG- and IgE-Fc fragments (*20*−*22*). Despite the relatively low level of amino acid identity between Fcυ3−4 and the homologous human fragments, IgG-Fc (28−30%, depending on the subclass that is compared) and Fcε3−4 (31%), the immunoglobulin fold within each domain and the overall dimeric "horseshoe" quaternary structure (Figure 1) are highly similar in all three isotypes. The noncovalent interaction observed between the C-terminal domain pair in IgG and IgE is also present between the Cυ4 pair in the Fcυ3−4 structure, resulting from an extensive buried interface (1380 Å^2) of predominantly hydrophobic residues. These residues are particularly well conserved between Cυ4 and Cε4 (five hydrophobic amino acids in the Cυ4−Cυ4 interface are present in the Cε4−Cε4 interface), although two (V508 and L509 in Cυ4) that are not found in Cε4 are conserved between Cυ4 and Cγ3 (V397 and L398). The gross architecture of the Fc region of these antibodies has therefore likely been inherited from a common ancestor, which implies that it dates back at least 300 million years.

Although the Cυ4 domains display local 2-fold symmetry in their pairing, the Cυ3 domains do not (the asymmetric unit of the crystal contains both chains of the Fcυ3−4 dimer). The asymmetry in the Fcυ3−4 structure may clearly be seen in Figure 1, where the only difference between the chains is the orientation of Cυ3 relative to Cυ4. Intriguingly, superpositions of the individual chains of Fcυ3−4 with Fc fragments of IgG and IgE (Figure 2) show that while one chain resembles IgE, the other resembles IgG. Chain A of Fcυ3−4 (right-hand side, Figure 1A) has a quaternary structure that most closely resembles that of IgE-Fc (rmsd = 1.39 Å for chain B in PDB entry 1O0V, calculated on Cα atoms only), whereas the closest structural match to chain B of Fcυ3−4 (left-hand side, Figure 1A) is an IgG-Fc (rmsd = 1.45 Å for PDB entry 1FRT); using the same IgG-Fc and IgE-Fc

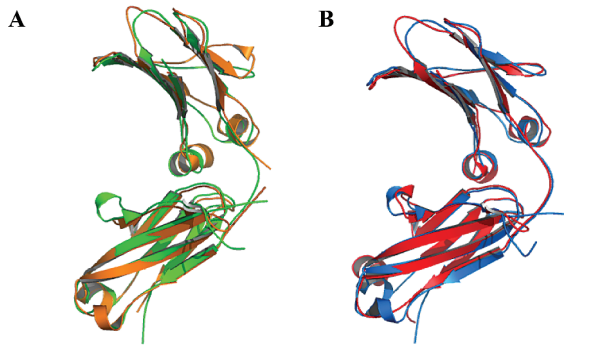

FIGURE 2: Individual chains of Fcυ3−4 superposed on their closest structural matches. (A) Fcυ3−4 chain B (green) on IgG-Fc (1FRT) chain A (orange) and (B) Fcυ3−4 chain A (blue) on IgE-Fc (1O0V) chain B (red).

structures for comparison, Fcυ3−4 chain A matches less well with IgG-Fc (rmsd = 1.94 Å) and chain B less well with IgE-Fc (rmsd = 2.31 Å).

An N-linked glycosylation site in the Cυ3 domain of IgY (position 407) is well-conserved in the homologous domains in IgE (Cε3) and IgG (Cγ2), although the type of attached oligosaccharide differs between isotypes; IgG contains biantennary complex-type sugars at this position, whereas IgE contains high-mannose-type sugars at the equivalent site. Two *N*-acetylglucosamine and one mannose unit were modeled into the electron density extending from N407 in each chain of Fcυ3−4, which confirms the presence of an N-linked oligosaccharide at this site. The lack of further electron density for these sugars may indicate flexibility and/or static disorder. Previous mass spectrometry findings have indicated that IgY-Fc contains high-mannose-type oligosaccharides (*17*). The orientation of the "core" sugars in IgY-Fc relative to the protein is the same as in IgG-Fc and IgE-Fc, covering surface hydrophobic residues. The difference between the types of N-linked sugars in IgY and IgG is noteworthy given the growing body of evidence relating Fc-linked oligosaccharides in IgG to a number of structural, biological, and pharmacological effects (*23*). Studies of IgG and IgE that have removed glycosylation either using endoglycosidases or through site-directed mutagenesis have shown that the removal of sugars from IgG eliminates Fc receptor binding (*24*−*26*), whereas deglycosylated IgE fragments retain Fc receptor binding, albeit with lower affinity (*27*). This difference may be explained by the relative contributions made by the two types of oligosaccharides to the quaternary structure of the Fc region; the IgG-Fc region collapses as sugars are removed, thus sterically restricting the accessibility of the Fc receptor binding site (*28, 29*), whereas it appears that in IgE-Fc the Cε3 domains adopt a more compact conformation even with the carbohydrate present (*21, 22*). Like IgE, chicken IgY-Fc fragments were found to retain the ability to bind to Fc receptors when N-linked oligosaccharides were removed or absent (Figure 3). The fragments were either deglycosylated using PNGase F or expressed without glycosylation following mutagenesis of all putatively glycosylated asparagines to glutamine. Despite these treatments, the fragments bound to MQ-NCSU chicken monocytes, which express Fc receptors for IgY (*30, 2*), as detected by immunofluorescence and flow cytometry (FACS). These findings are consistent with the structural similarity between IgE and IgY.

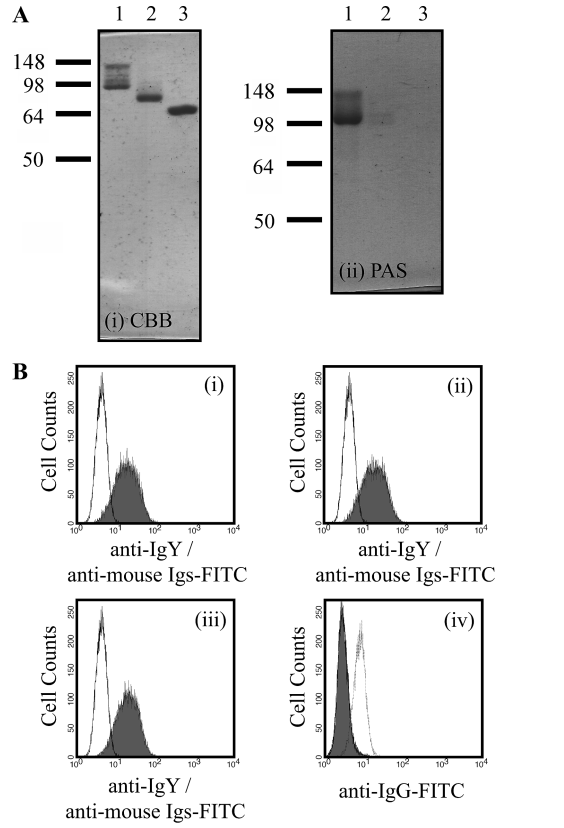

FIGURE 3: (A) SDS−PAGE gels stained for protein [A (i)] or carbohydrate [A (ii)]. Lanes were loaded as follows: untreated Fcυ2−4 (lane 1), enzymatically deglycosylated Fcυ2−4 (lane 2), Fcυ2−4 with putative N-linked glycosylation sites (N308, N407) mutated to glutamine (lane 3). (B) FACS histograms showing binding of untreated [B (i)], deglycosylated [B (ii)], or aglycosylated mutant Fcυ2−4 [B (iii)], to MQ-NCSU chicken monocytes, and human IgG-Fc before or after enzymatic deglycosylation, as verified by SDS−PAGE (not shown), to U937 human monocytes [B (iv)]. Cells treated with IgY fragments were incubated with mouse monoclonal anti-IgY-Fc (filled peaks in parts i–iii), or buffer alone (solid line, unfilled peaks in parts i–iii), followed by anti-mouse Igs-FITC. Cells treated with glycosylated (dotted, unfilled peak in part iv) or deglycosylated (filled peak in part iv) IgG fragments or buffer alone (solid line, unfilled peak which overlaps with the filled peak precisely in part iv) were stained with anti-IgG-FITC.

DISCUSSION

A receptor for IgY on chicken monocytes, CHIR-AB1, has recently been identified (*31*), but the structure of the complex is currently unknown. Both IgG and IgE bind to their receptors in an almost identical manner, involving subsites in the N-terminal regions of both Cγ2 and Cε3 domains, respectively, and thus, a similar mode of binding might be expected for their IgY-like ancestor involving the Cυ3 domains. However, Fcυ3−4 contains an interchain disulfide bond between Cys347 in each Cυ3 domain, whereas Fcε3−4 does not. Although there was no electron density for this residue (the model starts at residue 349), the existence of this bond was confirmed by SDS−PAGE and an Ellman assay (*19*), and the proximity of Pro349 residues (Cα−Cα = 8.6 Å) is consistent with an intact

interchain disulfide bond in the crystal structure. The Cε3 domains of IgE-Fc "open up" upon receptor binding, moving away from each other and allowing access to both subsites (*32, 22*); similar rearrangement of the Cυ3 domains would clearly be constrained by the interdomain disulfide bond, and this may preclude an IgG- or IgE-like mode of receptor interaction for IgY.

The intriguing possibility that IgY binds to its receptor in a manner more analogous to that of IgA, a more distant homologue, is suggested by the discovery that CHIR-AB1 is a member of the same gene family as the Fc receptor for IgA, CD89, rather than the "classical" Fc receptors for IgG and IgE (*31*). The crystal structure of CHIR-AB1 has recently been determined by Arnon et al. (*33*). Although this study suggests rather poor structural homology between CHIR-AB1 and CD89 (rmsd = 2.7 Å), based upon comparison of the uncomplexed forms, superposition of CHIR-AB1 (PDB entry 2VSD) on the bound structure of CD89, taken from the complex with IgA-Fc (PDB entry 1OW0) (*34*), reveals a closer match (rmsd = 1.9 Å). Furthermore, despite a fairly low level of structural similarity between IgA-Fc and Fcυ3−4 (rmsd = 1.99 Å for chain B and 3.11 Å for chain A, on PBD entry 1OW0), many of the contact residues in the Cα2 and Cα3 domains of IgA (e.g., L256, R382, L384, and eight residues in the Cα3 FG loop) are identical and accessible in Fcυ3−4. An IgY−CHIR-AB1 interaction that is structurally homologous to the IgA−CD89 interaction would indicate that the mammalian IgG- and IgE-like mode of interaction is a relatively recent evolutionary innovation (*2*). A crystal structure of the receptor complex will resolve this issue.

In summary, we have determined the structure of the first nonmammalian Fc region of an antibody and discovered that IgY, the closest extant homologue of the evolutionary ancestor of mammalian IgG and IgE, exhibits structural features of both.

ACKNOWLEDGMENT

This work was carried out with the support of the Diamond Light Source. We thank Dr. B. Dhaliwal, Mr. E. Girardi, and the staff of DLS beamline I04 for assistance during data collection.

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BI8019993