

Evidence for an early appearance of modern post-switch immunoglobulin isotypes in mammalian evolution (II); cloning of IgE, IgG1 and IgG2 from a monotreme, the duck-billed platypus, *Ornithorhynchus anatinus*

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To trace the emergence of the modern post-switch immunoglobulin (Ig) isotypes in vertebrate evolution we have studied Ig expression in mammals distantly related to eutherians. We here present an analysis of the Ig expression in an egg-laying mammal, a monotreme, the duck-billed platypus (*Ornithorhynchus anatinus*). Fragments of platypus IgG and IgE cDNA were obtained by a PCR-based screening using degenerate primers. The fragments obtained were used as probes to isolate full-length cDNA clones of three platypus post-switch isotypes, IgG1, IgG2, and IgE. Comparative amino acid sequence analysis against IgY, IgE and IgG from various animal species revealed that platypus IgE and IgG form branches that are clearly separated from those of their eutherian (placental) counterparts. However, the platypus IgE and IgG still conform to the general structure displayed by the respective Ig isotypes of eutherian and marsupial mammals. According to our findings, all of the major evolutionary changes in the expression array and basic Ig structure that have occurred since the evolutionary separation of mammals from the early reptile lineages, occurred prior to the separation of monotremes from marsupial and placental mammals. Hence, our results indicate that the modern post-switch isotypes appeared very early in the mammalian lineage, possibly already 310–330 million years ago.

Key words: Monotreme / IgG / IgE / IgY / Evolution

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1 Introduction

The combinatorial immune system defined by bona fide immunoglobulins and T cell receptors seems to have emerged at the appearance of the jawed vertebrates [1–3]. Due to the absence of its components in invertebrates as well as agnathans the combinatorial immune system has been proposed to have appeared in a relatively short period of evolutionary time, possibly in a single step catalyzed by horizontal transfer of recombination genes from prokaryotes into the vertebrate genome. This event has been described as a “Big Bang” in molecular evolution [2, 3]. Since this “Big Bang”, the complexity of the combinatorial immune system has increased gradually throughout vertebrate evolution. Bony and car-

tilaginous fishes express primarily IgM, whereas eutherian (placental) mammals express up to five Ig classes (IgM, IgD, IgG, IgE and IgA). Fish B cells apparently do not undergo isotype switching [4]. However, depending on the species, an IgD-like Ig, IgNAR, IgNARC, IgW or IgR (IgX) may also be expressed, either through differential splicing (the IgD-like Ig) or their presence on loci separate from that of IgM, reviewed in [4].

Amphibians, reptiles and birds all express IgY, the first confirmed post-switch isotype to appear in evolution [5, 6]. Hence, the mechanism of isotype switching seems to have emerged with the tetrapods, or possibly in the putative ancestors of tetrapods, the lungfish or the coelacanth (*Latimeria*) [6]. Early studies in the lungfish have identified a second highly abundant serum Ig in addition to IgM that shows similarities with the truncated form of IgY found in ducks [7–9]. However, further studies in the lungfish and the coelacanth are required to establish at which evolutionary stage the first post-switch isotype did

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appear. In addition, such analyses may help to resolve the origin of tetrapods [10].

IgY shares effector functions characteristic of both mammalian IgG and IgE. Hence, an IgY-like ancestor probably duplicated sometime early in mammalian evolution and formed two separate Ig classes, IgG and IgE [5, 6].

Like IgE and IgM, IgY of birds and amphibians has four constant domains, whereas IgG in eutherian mammals and marsupials has only three. Sequence comparisons among different domains of IgY and IgG suggest that the second domain of the ancestral IgY was deleted in what evolved into the IgG of mammals [5]. A short hinge exon found in mammalian IgG seems to be the only remaining part of the original exon encoding the second constant domain in the ancestral IgY [5].

Frogs (*Xenopus laevis*) but not axolotls, express one additional post-switch isotype called IgX (different from IgX of fish), that probably arose later than IgY [6]. *Xenopus* IgX appears to have similar distribution and effector functions as IgA in mammals and birds, however, it is probably not the direct homologue of mammalian IgA [11].

Most earlier studies of immunoglobulin expression in mammals have focused on eutherian mammals, where all investigated species express IgM, IgG, IgE and IgA. Some eutherian mammals, such as humans and mice, express IgD as well. Apart from the eutherians, there are two other extant mammalian lineages, the marsupials (metatheria) and the monotremes (prototheria).

Marsupials are represented by more than 270 living species, whereas the monotremes, a group of egg-laying mammals, are represented by only three, the duck-billed platypus and two echidna species. The monotremes have been regarded as “reptile-like” or “primitive” mammals simply because they lay eggs. However, they do possess several of the major mammalian features including a well-developed fur coat, a single bone in the lower jaw, three bones in the middle ear and mammary glands. Histological studies show that the spleen, thymus and gut-associated lymphoid tissues in the platypus are well developed and comparable in histological structure to those in therian mammals (eutherians and marsupials) [12]. However, in sites where lymph nodes would be expected in therian mammals, monotremes were found to have lymphoid nodules that resemble the jugular bodies of the amphibians, which indicates that monotremes have a somewhat more primitive immune system [12].

The evolutionary separation of the marsupials from the placental mammals is generally dated back to approxi-

mately 130 to 175 million years ago (Belov et al., “echidna IgM”, submitted for publication) [13, 14], whereas the major radiation of the placental mammals probably occurred 60 to 120 million years ago [14]. The evolutionary relationship between marsupials and monotremes has not yet been fully resolved. The Marsupio-nata hypothesis supported mainly by mitochondrial data and DNA-hybridization studies suggests that marsupials and monotremes are sister lineages [15–18]. The opposing Theria hypothesis proposes that the separation of the monotremes from the marsupial and eutherian mammals occurred much earlier in evolution. The Theria hypothesis initially emerged from morphological data and has more recently gained additional support from paleontological data [19, 20] and a number of nuclear gene sequences (Belov et al., “echidna IgM” and “echidna IgA”, both submitted for publication) [13, 21–24]. The nucleotide substitution rate in mitochondrial DNA is generally more rapid and the tendency for repeated mutations in the same site is higher than what is observed in nuclear DNA [25]. Consequently, time estimates based on mitochondrial data may be less accurate over large evolutionary distances. In fact, Springer and colleagues [25] showed that mitochondrial genes have less resolving power than certain nuclear exons in resolving deep-level mammalian clades. Studies based on IgM (Belov et al., “echidna IgM”, submitted for publication) and protamine sequences estimate the time of divergence of the monotremes from the other mammalian lineages to around 150 to 170 million years ago [13]. This estimate fits quite well with dating based on fossil records and anatomical features [19, 20, 26].

In a previous study of marsupial immune functions, we found that the American short-tailed gray opossum expresses four Ig isotypes, IgM, IgG, IgE and IgA, which all conform to the basic structure of the corresponding isotypes in placental mammals [27]. The findings in the opossum revealed that all of the major changes in the Ig isotype pattern that emerged after the separation of the mammals from the early reptile lineages had occurred prior to the separation of marsupials from placental mammals. Hence, the duplication of IgY and the deletion of the second constant domain in the ancestral IgG and IgA probably took place more than 130 million years ago.

To further trace the evolution of modern post-switch isotypes and the appearance of the full range of functionally separated Ig isotypes found in therian mammals, referred to as “a second Big Bang” by Marchalonis and colleagues [3], we initiated a study of Ig isotypes in monotremes. As a first step, we describe here the cloning of IgE, IgG1 and IgG2 from the duck-billed platypus, *Ornithorhynchus anatinus*.

2 Results

2.1 Cloning and nucleotide sequence analysis of the ϵ - and γ -chains of platypus IgE, IgG1 and IgG2

A PCR-based cloning strategy was adopted to isolate cDNA clones encoding partial platypus IgE and IgG heavy chains. Nucleotide sequence comparisons of the ϵ - and γ -chain sequences from a panel of different mammals had been conducted earlier [27]. Based on these comparisons, two regions with particularly high sequence conservation within the respective isotypes were selected as target regions for synthesis of degenerate oligonucleotide primers. The degenerate primers were used in PCR amplifications using double-stranded cDNA from Australian platypus splenocytes as template. This resulted in the isolation of one partial cDNA clone each for platypus IgE and IgG. The clone generated with the ϵ -chain primers was approximately 470 base pairs (bp), encoding a region stretching from the CH3 to CH4 central regions of the ϵ -chain. The partial IgG clone was approximately 540 bp stretching from the N-terminal part of the CH2 domain to the C-terminal part of the CH3 domain.

The partial platypus IgE and IgG cDNA clones were used as probes to screen a platypus spleen cDNA library. Screening of about 130,000 plaques (16,250 plaques/filter) resulted in an average of 16 and 115 positive signals/filter using the ϵ - and γ -chain probes, respectively. Four positive clones, two from each screening, were selected for further analysis. The complete nucleotide sequences of the ϵ - and γ -chains were determined from one full-length clone of the respective isotypes (GeneBank AY055780 and AY055781).

Upon further screening of the library using a full-length IgG clone as probe, two clones for a novel γ -chain isotype were isolated and sequenced, together with nine additional clones of the previously isolated γ -chain isotype. The initially isolated γ -chain and the novel γ -chain isotype were designated IgG1 and IgG2, respectively. The complete nucleotide sequence of one full-length γ 2-chain was determined (GeneBank AY055782).

The amino acid sequence of the variable domains of the full-length ϵ , γ 1 and γ 2 clones are shown in Fig. 1 and the amino acid sequence of the constant domains of IgE and the two IgG isotypes are shown in Fig. 2 and 3, respectively.

Following the isolation of full-length clones for IgG1, IgG2 and IgE, the platypus cDNA library was screened

with a variable region (V-region) probe. Based on previous screenings using IgG1, IgG2 and IgE constant-region-specific probes, IgG and IgE negative V-region positive clones were isolated. To avoid abolition of closely related isotypes the filters were washed at high stringency following all constant region hybridizations. Thirteen of the V-region-positive, γ - and ϵ -chain-negative clones were subcloned and sequenced using a primer directed against the region spanning the J segment. All of these thirteen clones were clearly identified as α -chains of two different IgA-like isotypes: three α 1-chains (IgA1) and ten α 2-chains (IgA2). Hence, no additional IgG or IgE isotypes were identified and we failed to identify any IgM or IgD like isotypes. IgM is present in all mammals investigated so far, including another monotreme, the Echidna (Belov et al., submitted for publication), and therefore likely to be present also in the platypus. However, a similar screening strategy was used to isolate the various Ig isotypes expressed by the opossum (*Monodelphis domestica*) [27, 28] and in this screening 9 out of 12 IgG- and IgE-negative clones were identified as IgM, whereas the remaining 3 turned out to be IgA (unpublished observations). Therefore, our failure to identify IgM in platypus is an intriguing subject for ongoing investigation.

The complete primary structure of platypus IgA1 and IgA2 will be presented elsewhere (manuscript in preparation).

2.2 Sequence comparisons and structural analysis of the ϵ - and γ -chains of the platypus IgE and IgG

The complete amino acid sequences of the constant regions of platypus IgE, IgG1 and IgG2 were compared with amino acid sequences of the corresponding isotypes of other mammals (Fig. 2 and 3). The overall structures of the platypus IgE and IgG conform to the structures of the previously characterized eutherian and marsupial IgE and IgG.

The amino acid sequence of platypus IgE reveals that the cysteine residues involved in the various disulfide bridges that are conserved in most mammalian IgE have been conserved also in platypus IgE. Furthermore, both of the putative inter-chain disulfide bridges present in the CH2 domain of IgE in all species analyzed so far are conserved also in platypus IgE (Fig. 1 and 2). Five potential N-linked glycosylation sites were identified in the constant region of platypus IgE: two in the C2 domain, one in the C3 domain and two in the C4 domain (Fig. 2). The site in the CH3 domain is conserved among all ϵ -chain sequences isolated so far, suggesting that glycosylation

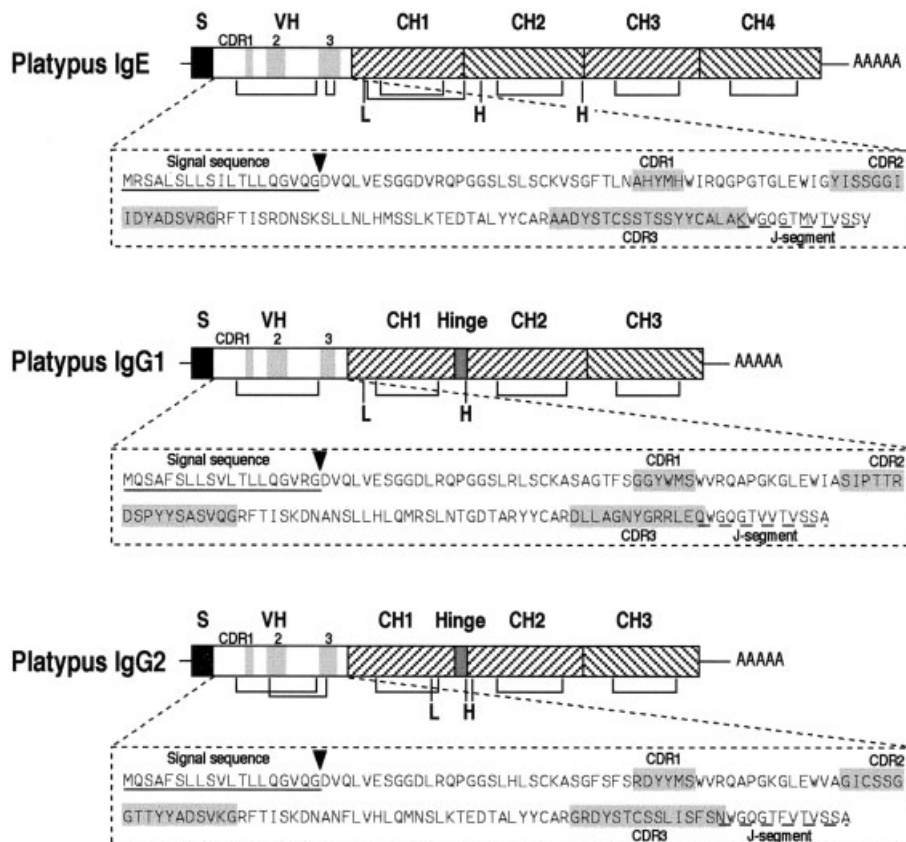


Fig. 1. Schematic maps (in scale) of the platypus ϵ , $\gamma 1$ and $\gamma 2$ heavy-chain clones and the amino acid sequence of the variable domains encoded by the V, D and J gene segments of the respective full-length clones. The signal sequences (S) are represented in black, the variable domain (VH) is represented in white with light gray regions denoting the positions of the complementarity-determining regions (CDR), the constant domains are illustrated by striped boxes and the hinge region (in IgG) is indicated in dark gray. Brackets indicate the positions of putative intra-chain disulfide bridges. Cysteine residues involved in putative inter-chain disulfide bridges connecting the two heavy chains and the heavy chain with the light chain are denoted by H and L, respectively. The amino acid sequences of the variable domains are shown below the schematic maps. A small arrow marks the position of the signal sequence cleavage site and the amino acid residues corresponding to CDR1, 2 and 3 are indicated by light gray boxes. CDR borders were set according to Kabat et al. [29].

at this position is of importance for protein structure and/or solubility (position 276 in Fig. 2).

Like the previously characterized eutherian and marsupial IgG, the heavy chains of the platypus IgG isotypes, IgG1 and IgG2, contain three constant domains (Fig. 1 and 3). However, although the platypus IgG conform to the general IgG structure, some important differences were observed. For instance, in platypus IgG2 the cysteine residue forming the disulfide bridge that connects the heavy (H) chain with the light (L) chain is positioned in the C-terminal region of the CH1 domain (Fig. 1 and 3). In contrast, in most, if not in all, previously characterized mammalian IgG molecules, this cysteine residue occurs in the N-terminal part of the CH1 domain (Fig. 3). One potential inter-chain disulfide bridge-forming cysteine

residue is found in the hinge region of platypus IgG1, whereas two cysteines are present in the same region of IgG2 (Fig. 1 and 3). On the whole, the two IgG isotypes found in platypus display a remarkable divergence in sequence compared to IgG isotypes of other mammalian species.

Sequence analysis of the V-regions also revealed some interesting features. In addition to the canonical cysteine residues, the V-regions of both the IgG2 and the IgE clone have two extra cysteine residues located in the complementarity-determining region (CDR)2 and/or CDR3 loop structures (Fig. 1). Considering the high reactivity of thiol groups and the structural flexibility of the loop structures, additional intra-chain disulfide bridges probably occur in these V-regions. Moreover, the addi-

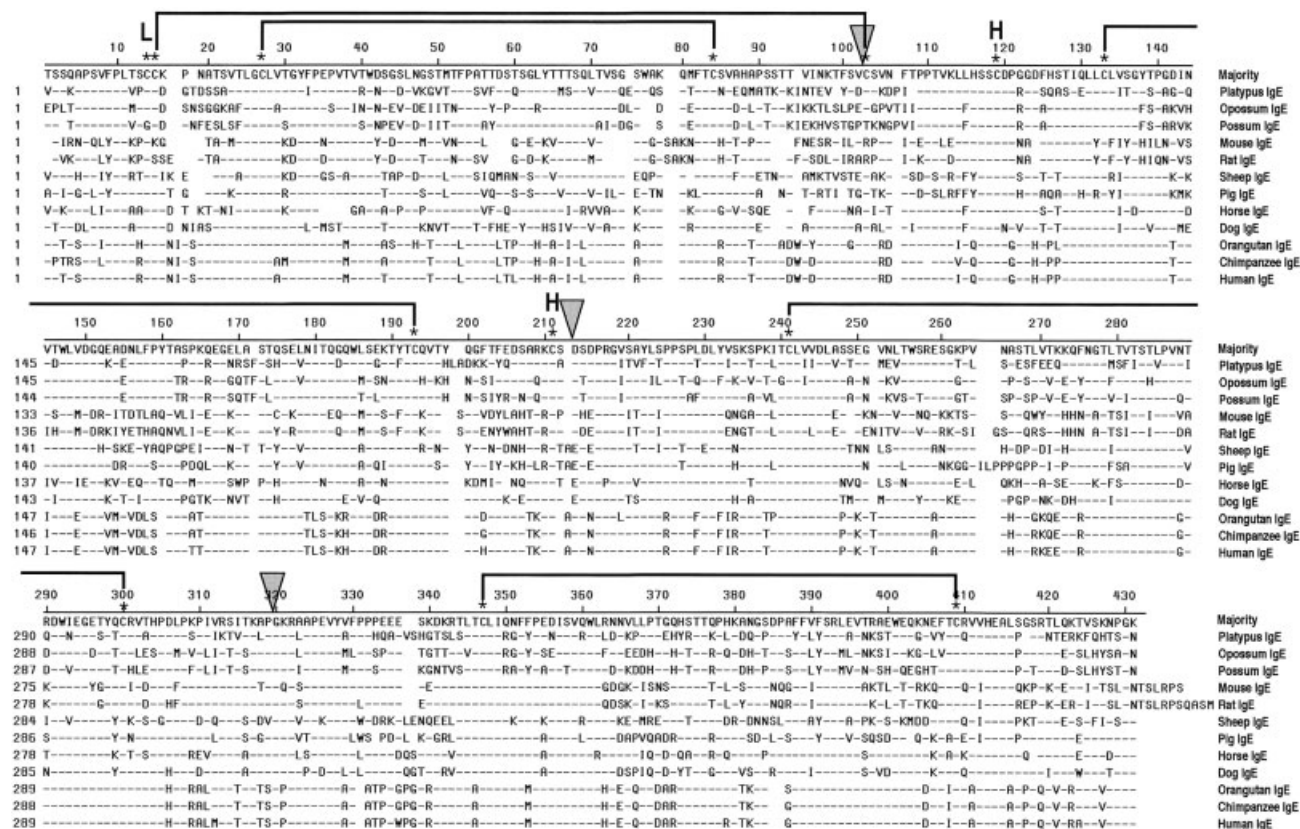


Fig. 2. Amino acid sequence alignment of a selected panel of ϵ -chains. The top sequence is generated by the amino acid residues that are represented in the majority of the sequences. Residues that match with the consensus sequence are indicated by a dash. The positions of the exon/intron boundaries (based on sequences in which gene structure has been determined) are indicated by arrowheads above the sequence. All cysteine residues involved in putative inter-chain and intra-chain disulfide bridge formations are marked by asterisks and the intra-chain disulfide bridges are indicated by brackets. Cysteine residues involved in the formation of inter-chain disulfide bridges connecting the heavy chains and the heavy chain with the light chain are denoted by H and L, respectively.

tional cysteine residue in the CDR2 of platypus IgG2, residue 52 according to [29], appears to face the CDR3 [30]. A similar arrangement of cysteine residues in the heavy chain V-regions of the llama was suggested to be compatible with a disulfide bridge between CDR2 and CDR3 [31]. The presence of intra-CDR3 disulfide bridges has been indicated in the heavy chain V-regions of shark [32], cow [33] and members of Camelidae [31, 34]. Hence, a disulfide bridge may form between the CDR2 and CDR3 of the IgG2 clone and an intra-CDR3 cysteine bridge probably occurs in the IgE clone (Fig. 1). A more detailed analysis of the V-gene repertoire in platypus revealed that additional cysteine residues that form putative disulfide bridges, either between the CDR2 and CDR3 loop structures or within the CDR3, were found in 9 of 25 clones analyzed [35].

A phylogenetic tree was generated by comparing the complete amino acid sequence of the platypus IgE and

IgG constant regions with those of a selection of the presently available mammalian IgE and IgG sequences, along with two IgY sequences from birds (Fig. 4). The platypus IgE and IgG form separate branches, outside of all the eutherian IgE and IgG. Strikingly, the branch for the platypus IgG isotypes occurs outside of both marsupial and eutherian IgG, whereas the platypus IgE sequence indicates that monotremes and marsupials are sister lineages.

2.3 Relative expression levels of platypus IgE, IgG1 and IgG2 in the spleen of a free-living Tasmanian platypus and Northern blot analysis

The relative expression levels of the various isotypes in the spleen of a free-living platypus were estimated by screening the non-amplified spleen cDNA library with

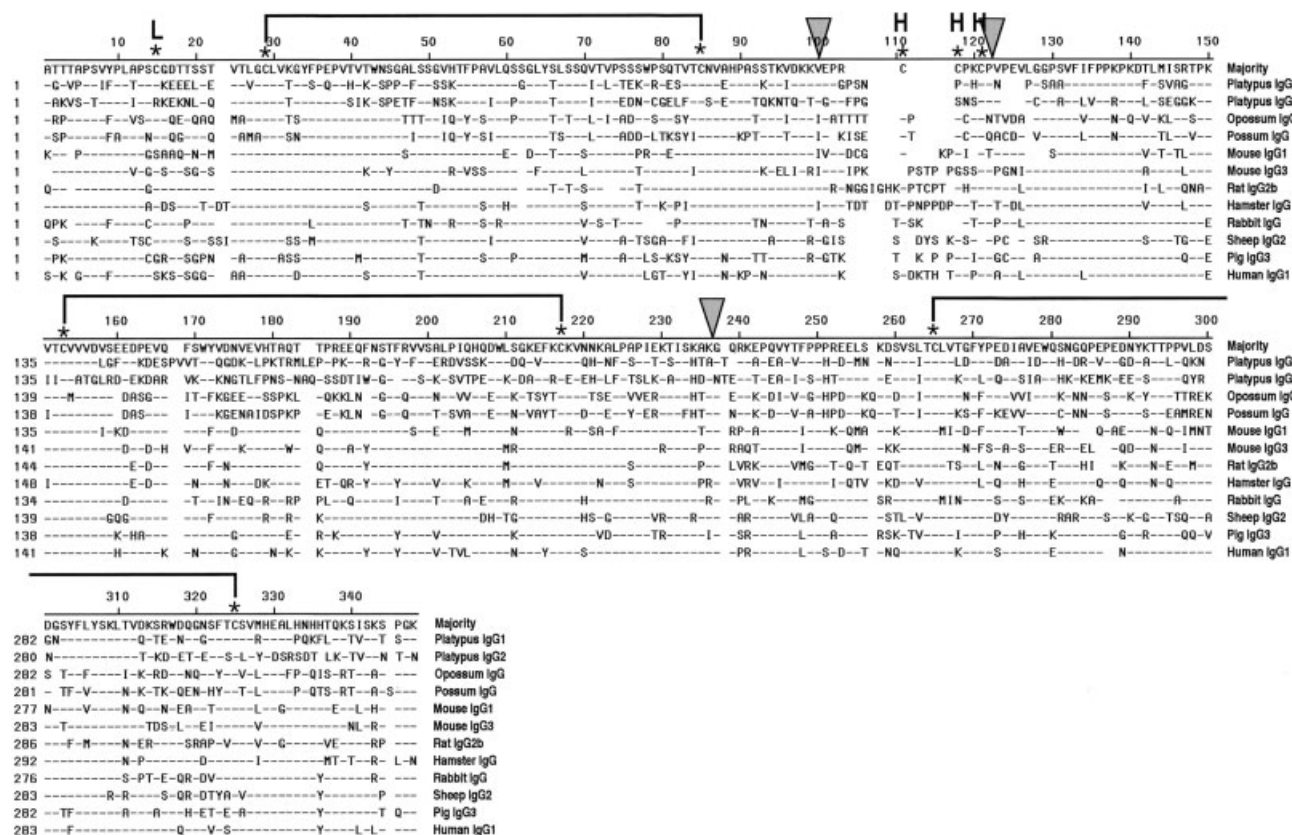


Fig. 3. Amino acid alignment of a selected panel of γ -chain sequences. The top sequence is generated by the amino acid residues that are represented in the majority of the sequences. Residues that match with the consensus sequence are indicated by a dash. The positions of the exon/intron boundaries (based on sequences in which gene structure has been determined) are indicated by arrowheads above the sequence. All cysteine residues involved in putative inter-chain and intra-chain disulfide bridge formations are marked by asterisks and the intra-chain disulfide bridges are indicated by brackets. Cysteine residues involved in the formation of inter-chain disulfide bridges connecting the heavy chains and the heavy chain with the light chain are denoted by H and L, respectively.

isotype specific probes. No cross-hybridization to other isotypes was observed with the isotype-specific probes. The screenings revealed that IgE, IgG1 and IgG2 are expressed at relative levels of 10, 60 and 1. Hence, IgG1 seems to be the predominant plasma IgG isotype in the platypus, whereas IgG2 is much less abundant. IgG1 is expressed at six times the level of IgE and the expression level of IgG2 is only one tenth of that observed for IgE. However, it should be emphasized that these values are based on the relative abundances of mRNA and need not reflect the serum levels of the various Ig isotypes.

None of the 28 analyzed Ig clones contained exons corresponding to the membrane region, indicating that the expression levels of the membrane-bound forms are comparatively low. A Northern blot analysis using isotype-specific probes was performed to estimate the relative abundance of transcripts encoding membrane-

bound versus secreted forms of the platypus IgE, IgG1 and IgG2 isotypes, respectively. However, no transcripts encoding the membrane-bound forms of the three isotypes were detected (data not shown). Based on our results we estimate that in the spleen less than 5% of the total mRNA pool for each of the respective isotypes corresponds to the membrane-bound forms, which appears to be in agreement with the situation in other mammals.

3 Discussion

We have demonstrated the presence of IgE and two separate IgG isotypes, IgG1 and IgG2, in a monotreme *Ornithorhynchus anatinus*, the duck-billed platypus.

Sequence comparisons of the platypus isotypes and a selection of the presently available mammalian ϵ - and γ -

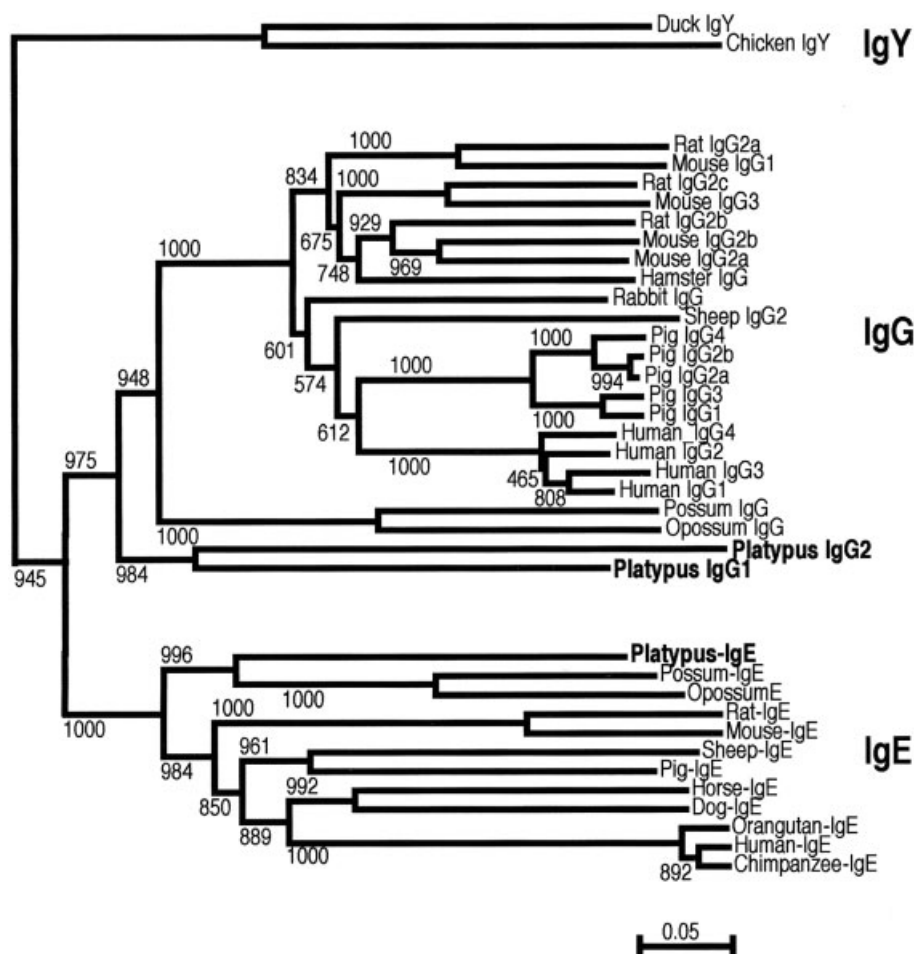


Fig. 4. A comparative analysis of the amino acid sequence of the entire constant region of a selected panel of mammalian IgE and IgG sequences and two IgY sequences from birds presented in the form of a phylogenetic tree. The bootstrap tree is based on 1,000 independent comparisons and the bootstrap values are added at each branch point. The sequence distance is represented by the length of the branches according to the scale at the bottom of the figure, which corresponds to a sequence divergence of 5%.

chain sequences indicate that only minor changes in the overall structures of IgE and IgG have occurred since the evolutionary separation of the monotremes from the other mammalian lineages (Fig. 2 and 3). Apart from several small insertions and deletions (1–4 full codons) and a few changes in the positions and numbers of disulfide bridges and putative glycosylation sites, few changes other than single point mutations have occurred. However, due to the relatively high number of point mutations, the divergence among the various primary nucleotide and amino acid sequences is substantial (Fig. 2 and 3).

The most striking differences among the various ϵ -chain sequences in Fig. 2 occur in rodent IgE, which has lost one of the intra-chain disulfide bridges in the CH1 domain (position 15 and 103) and has substantially lon-

ger C terminus than all other mammalian IgE. These characteristic features probably appeared in a common rodent ancestor after the major radiation of the placental mammals. Interestingly, the recent finding that this intra-chain disulfide bridge is absent also in a marsupial ϵ -chain, that of the Australian brushtail possum, suggests that it has been lost at least twice during mammalian evolution [36].

The mRNA level for IgE was surprisingly high in the platypus spleen. Only a sixfold difference in mRNA levels was observed between the predominant serum antibody in platypus, IgG1, and IgE. This is intriguing, since, in general, the serum concentration of IgE is significantly lower than that suggested by the mRNA level. In human adults, the average serum level of IgG is 9.5–12.5 mg/ml, whereas IgE is present at concentrations that are 30,000

times lower (10–400 ng/ml). The mRNA levels presented here are based on studies in a free-living platypus. Hence, increased expression of IgE as a result of parasitic infections may account for some elevation in the mRNA level. Furthermore, in humans, the turnovers of IgG and IgE differ quite remarkably. Serum IgG has a half-life of about 3 weeks (23 days), whereas the half-life of IgE in the circulation has been estimated at 2–2.5 days [37]. The expression level of IgE is thus much higher than can be expected on the basis of serum levels alone.

Several findings indicate a common phylogenic history of the avian and amphibian ν -chains and the mammalian ϵ - and γ -chains [5, 6, 9]. In analogy with IgG in mammals, IgY is the major serum antibody in birds and amphibians, whereas with regard to structure (the number of exons and inferred positions of disulfide bridges) IgY is more similar to mammalian IgE. Comparison studies of the trans-membrane exons of the ν in duck and the corresponding regions of mammalian γ and ϵ genes revealed several shared characteristic features [9]. In similar studies of *Xenopus* IgY no particular similarities with either the duck ν or the mammalian γ and ϵ trans membrane exons were observed. However, conserved residues in the trans-membrane and cytoplasmic domains that are absent in other isotypes were identified [6]. The authors conclude that *Xenopus* IgY, duck IgY and mammalian IgG and IgE probably all share a common precursor and that this IgY-like ancestor arose by a gene duplication of IgM that occurred before the anurans or even the amphibians appeared [6].

Although IgG is the predominant serum Ab in mammals, the role of regulating mast cell degranulation falls mainly on IgE, an antibody that is present at low concentrations. The separation of the effector functions of an IgY-like ancestor into two separate isotypes, IgG and IgE, may therefore have been of selective advantage. Furthermore, the conservation of the overall structure of IgE is strongly indicative of an evolutionary advantage of maintaining the function of the IgE mast cell system. In some mammalian species where mast cells are triggered by both IgE and IgG, as in the guinea pig, a second immunization with an antigen can result in massive anaphylactic reactions [38]. In contrast, in species where mast cells are triggered exclusively by IgE, mast cell degranulation is normally kept under more stringent control. So far, only one IgE isotype has been found in all mammalian species studied, now including the platypus. Hence, restricting regulation of mast cell degranulation to one isotype and to have only one gene for this isotype may have a selective advantage. The potentially very high turnover of IgE raises questions regarding the role of this isotype in the inflammatory response. Does IgE function as a “doorkeeper”, scanning the antigen repertoire of the

environment and preparing the individual for potentially harmful pathogens [39]? A rapid turnover may then more adequately prepare the individual to deal with its present environment.

Like IgG in other mammals, both of the IgG heavy chains found in platypus ($\gamma 1$ and $\gamma 2$) have only three constant domains (Fig. 1 and 3). Hence, the second domain in the Ig ancestral to platypus IgG1 and IgG2 was apparently deleted prior to the evolutionary separation of monotremes from the other mammalian lineages. Alternatively, two or three independent deletions occurred, one in each of the three major mammalian lineages (or one in the common ancestors of marsupials and placental mammals and one in monotremes). However, the high overall structural similarity of the platypus IgG to marsupial and placental IgG strongly favors the first alternative.

In mouse, rat, pig and human the different IgG isotypes are relatively closely related within a single species. In contrast, the sequence divergence between the two platypus IgG isotypes is remarkably high (Fig. 3). In fact, the sequence divergence between the two IgG isotypes in platypus is actually greater than that between the mouse and human IgG isotypes (Fig. 4), indicating that the two platypus IgG isotypes did appear as separate isotypes before the major radiation of placental mammals 60–120 million years ago. Although quite speculative, the distances in the phylogenetic tree presented in Fig. 4 suggest that the platypus IgG isotypes were formed by a gene duplication shortly after the separation of monotremes from the common mammalian ancestor, possibly as long as 150–170 million years ago. The different IgG isotypes in rodents, pigs and humans may instead have arisen from relatively recent duplications within each separate subgroup of eutherians (Fig. 4).

The high degree of sequence divergence between the two platypus IgG isotypes is also reflected in several structural differences. For instance, the position of the cysteine residue involved in the putative disulfide bridge connecting the heavy chain with the light chain differs between the two isotypes. In IgG1, this cysteine residue is located in the N-terminal region of the CH1 domain, whereas in IgG2 it is located in the C-terminal region of the same domain (Fig. 1B and C). To our knowledge, the latter position occurs only in the platypus IgG2 and is thus unique among mammalian IgG molecules. The N-terminal cysteine residue in the CH1 domain of mouse IgG1 has also been lost, but it is not replaced by a cysteine residue in the C-terminal region (Fig. 3). Hence, the heavy chain of mouse IgG1 seems to rely entirely on non-covalent interactions with the light chain, a feature that has been observed in other Ig isotypes as well. For example, disulfide bridges between the heavy and light

chains are absent in the IgM-like Ig and the two low molecular weight Ig isotypes of the bullfrog (*Rana catesbeiana*) [40].

The two platypus IgG isotypes also differ with respect to the number of potential N-linked glycosylation sites. Platypus IgG2 has four potential N-linked glycosylation sites, whereas IgG1 has only one site located in the C2 domain (Fig. 3). However, the lone N-linked glycosylation site in IgG1 is conserved between the two platypus isotypes and among other Ig isotypes as well, including opossum IgG (Fig 3). In fact, this site corresponds to the site in the ϵ -chain that is conserved among all IgE molecules isolated so far (position 276 in Fig. 2).

In summary, the primary structures of the cloned platypus γ 1-, γ 2- and ϵ -chains suggest that all the major evolutionary changes that gave rise to the Ig isotypes expressed by present day mammals emerged prior to the separation of monotremes from marsupials and placental mammals. Furthermore, the presence of three constant domains in both of the highly divergent IgG isotypes found in platypus supports the conclusion that deletion of the second domain of IgG occurred very early in mammalian evolution. The results from the phylogenetic analysis of platypus IgE and IgG are not conclusive with respect to the evolutionary relationship between monotremes and marsupials, indicating that further comparative studies of a large panel of nuclear genes are required to fully resolve the relationship (Fig. 4). However, the platypus IgG sequences and several other unrelated nuclear gene sequences, echidna IgM (Belov et al., submitted for publication), echidna IgA (Belov et al., submitted for publication), protamine, neutropin, α -lactalbumin, β -globin and mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R) all support the Theria hypothesis [13, 21–24], whereas it is opposed only by platypus IgE and two dental genes, amelogenin and dentin matrix protein 1 [41, 42]. Estimates based on protamine and IgM (Belov et al., submitted for publication) sequences indicate that the monotremes separated from the ancestors of marsupials and placentals about 150–170 million years ago [13], whereas the separation of early mammalian ancestors from early reptiles may have occurred as far back as 310–330 million years ago [14]. Provided that the Theria hypothesis is correct, this leaves us with an evolutionary window for the appearance of modern post-switch isotypes of about 150 million years. Unfortunately, no more living groups of mammals exist today and we cannot obtain information regarding individual molecules from the fossil record. However, further clues may be gained from other molecules that have co-evolved with the various antibody isotypes. For this purpose, we have initiated a study of the high affinity receptor for IgE, Fc ϵ RI, in

mammals distantly related to placentals. At present, however, we are only able to speculate based on available Ig sequences. Hence, the modern post-switch isotypes may have appeared in the early mammalian ancestors essentially in their present form as early as 310–330 million years ago, shortly after the split from the early reptile lineages.

4 Materials and methods

4.1 Primer design and PCR amplification

Partial cDNA clones of the platypus ϵ - and γ -chains were isolated by PCR using degenerate primers. The design and sequence of the primers have been described previously [27]. A 10- μ l aliquot of the same double-stranded cDNA that was used to generate the platypus spleen cDNA library was used as template in the PCR amplifications. The PCR amplifications were carried out in 100 μ l under the following conditions: 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.25 mM each of the four nucleotide triphosphates, approximately 1 μ g of each of the two degenerate oligonucleotide primers and 1 unit of Taq DNA polymerase (Promega, Madison, WI). The reactions were initiated with a 2-min incubation at 90°C followed by 45 cycles of 40 s at 94°C, 60 s at 52°C and 90 s at 72°C in an MJ Research minicycler (MJ Research, Inc., MC). The PCR products were purified from 1% low-melting agarose gels and cloned into the EcoRI and BamHI sites of Bluescript SK(+/-) (Stratagene, La Jolla, CA). Purified ϵ - and γ -chain fragments were labeled with ³²P by random priming (Megaprime, Amersham Int., GB) and used as probes to screen the platypus cDNA library.

4.2 Isolation of full-length cDNA for platypus IgE, IgG1 and IgG2 (ϵ - and γ chains)

Total cellular RNA from platypus splenocytes was isolated by the guanidium thiocyanate method as described previously [43] and poly(A)⁺ RNA was purified using oligo dT-coupled magnetic beads according to the manufacturer's protocol (PolyATtract, mRNA Isolation System II, Promega). Double-stranded cDNA was synthesized using the time-saver cDNA synthesis kit (Pharmacia-Biotech, Uppsala, Sweden). The cDNA was ligated into the single EcoRI site of the λ -gt 10 vector and the recombinant vectors were packaged into phage particles using an *in vitro* packaging system (Stratagene). Approximately 130,000 plaques from this unamplified cDNA library were spread as a monolayer of the *E. coli* C600 Hfl strain with a titer of approximately 17,000 plaques/plate on eight plates. The plaques were transferred to Hybond N+ filters (Amersham Int.). Five of the filters were screened with the ³²P-labeled, platypus ϵ -chain fragment and three filters were screened with the partial γ -chain clone. The filters were washed at high stringency (0.1 $\times$ SSC,

0.1% SDS). Autoradiography was performed for 24–48 h on Kodak Exomat AR film (Eastman-Kodak Company, Rochester, NY). Positive plaques were purified and the inserts were excised by EcoRI and/or NotI cleavage and subcloned into Bluescript SK(+/-) (Stratagene) or a pGEM-2 vector with a modified multicloning site. The obtained cDNA clones were sequenced from both ends using T7 and T3 or Sp6 primers. Based on the obtained sequences, new sequence primers were designed and used to complete the entire insert sequences. In order to identify additional isotypes, the library was screened with a full-length γ -chain clone (IgG1: 11). Novel signals (negative for the ϵ - and γ -chain fragment probes) were subcloned and sequenced as above. All nucleotide sequences presented in this study were established by sequencing both strands of the DNA insert.

4.3 Amino acid sequence alignment

The amino acid sequences of the constant regions from a panel of IgE, IgG and IgY sequences were compared using the CLUSTAL W program. This program performs progressive pair-wise comparisons in order of divergence, initiating with the most closely related sequence [44]. The neighbor-joining method was used to construct the evolutionary tree and to estimate the individual branch lengths. A bootstrap tree based on 1,000 independent calculations was constructed using this program (Fig. 4).

The GenBank accession numbers for the various Ig amino acid sequences are listed below.

IgY: duck (X65219) and chicken (X07174).

IgG: platypus 1 (AY055781), 2 (AY055782); rat 2a (M28669), 2b (M28671), 2c (X07189); mouse 1 (J00453), 2a (J00470), 2b (J00461), 3 (J00451); hamster (U17166); rabbit (L29172); sheep 2 (X70983); pig 1 (U03778), 2a (U03779), 2b (U03780), 3 (U03781), 4 (U03782); human 1 (J00228), 2 (J00230), 3 (X03604), 4 (K01316); possum (AF191648) and opossum (AF035195).

IgE: platypus (AF055780), possum (AF157617), opossum (AF035194), rat (K02901), mouse (X01857), sheep (M84356), pig (U96100), horse (U15150), dog (L36872), orangutan (M15399), human (L00022) and chimpanzee (M15398).

4.4 Northern blot analysis

Poly(A)⁺ RNA derived from platypus splenocytes was isolated as described above. Aliquots of approximately 2 μ g of this RNA were loaded in separate lanes on an 1.0% agarose gel containing 0.2 M formaldehyde. The fractionated RNA was blotted onto Hybond-N⁺ filters using 0.05 M NaOH as transfer solution. The RNA was fixed to the filter by UV cross-linking at 150 mJoule and hybridized overnight at 65°C in 6×SSC, 0.5% SDS, 3×Denhardt's solution, 2 mM EDTA

and 100 μ g/ml yeast RNA. Random primed ³²P fragments of 450–750 bp derived from IgG1, IgG2 and IgE clones, respectively, were used as probes. The filters were washed at high stringency (0.1×SSC, 0.1% SDS) prior to autoradiography. As an internal control of the amount of RNA loaded in each lane, one of the filters was rehybridized with a platypus actin fragment (data not shown).

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