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Extensive Diversification of IgD-, IgY-, and Truncated IgY(ΔFc)-Encoding Genes in the Red-Eared Turtle (Trachemys scripta elegans)

Lingxiao Li,* Tao Wang,* Yi Sun,* Gang Cheng,* Hui Yang,* Zhiguo Wei,† Ping Wang,* Xiaoxiang Hu,* Liming Ren,* Qingyong Meng,* Ran Zhang,* Ying Guo,* Lennart Hammarström,‡ Ning Li,* and Yaofeng Zhao*§

IgY(ΔFc), containing only CH1 and CH2 domains, is expressed in the serum of some birds and reptiles, such as ducks and turtles. The duck IgY(ΔFc) is produced by the same v gene that expresses the intact IgY form (CH1–4) using different transcriptional termination sites. In this study, we show that intact IgY and IgY(ΔFc) are encoded by distinct genes in the red-eared turtle (Trachemys scripta elegans). At least eight IgY and five IgY(ΔFc) transcripts were found in a single turtle. Together with Southern blotting, our data suggest that multiple genes encoding both IgY forms are present in the turtle genome. Both of the IgY forms were detected in the serum using rabbit polyclonal Abs. In addition, we show that multiple copies of the turtle δ gene are present in the genome and that alternative splicing is extensively involved in the generation of both the secretory and membrane-bound forms of the IgD H chain transcripts. Although a single μ gene was identified, the α gene was not identified in this species. The Journal of Immunology, 2012, 189: 000–000.

Immunglobulins are important molecules of the adaptive immune system, and they appeared early in evolution along with the emergence of cartilaginous fish, the most primitive jawed vertebrates (1, 2). Based on a large number of comparative studies of Ig genes in jawed vertebrates, including cartilaginous fish, bony fish, amphibians, reptiles, birds, and mammals, the evolutionary history of Ig genes has been partially tracked and reconstructed. Because both IgM and IgD (W) are present in cartilaginous fish and nearly all other jawed vertebrates (only IgD is missing in certain species) (1, 3–5), they are believed to be the most primordial Ig classes in evolution and thus the ancestors of other classes defined in tetrapods, such as IgY, IgA (X), IgG, and IgE (3).

IgY is a low m.w. serum Ab encoded by the v gene in nonmammalian tetrapods, including birds (6–8), reptiles (9, 10), and amphibians (11–14). The gene is thought to have developed from μ by a duplication event (2, 15) because of their sequence homology and similar gene structures. It is also thought that the v gene gave rise to the IgA-encoding gene (α) through a recombination event with μ and may thus have emerged earlier in evolution than α (16).

IgY plays important roles in the humoral immune response and also mediates immunity to parasitic infection and allergic reactions (15). IgY possesses the functions of IgG and IgE, two Ig classes (encoded by the γ and ε genes, respectively) expressed only by mammals. Because of the functional similarities and relatively high levels of sequence homology between v, γ and ε genes, the v gene is thought to be the evolutionary precursor of the mammalian γ and ε genes and may have diversified through gene duplication events to generate multiple v genes that eventually differentiated into γ and ε. It is highly likely that v evolved to ε because of their similar genetic structures. IgG has three constant domains and a hinge region encoded by a separate exon, which has been thought to be condensed into CH2 of IgY (15). However, our recent work on platypus Ig genes identified a new IgO-encoding gene and proved that the hinge region in IgG was independently formed at the 5’ end of vCH2 and that vCH2 was lost during evolution, probably due to a splice-site mutation (17). Considering that IgG and IgE are present in all mammals, the gene duplication of v that led to development of γ and ε must have taken place earlier than the appearance of mammals. However, there are no reports of the existence of multiple v genes in nonmammalian tetrapods.

In addition to the full-length form, a truncated IgY (5.7S) variant, termed IgY(ΔFc), has also been reported in some species, including ducks and turtles (9, 15, 18, 19). In ducks, IgY(ΔFc) is generated from the alternative use of a transcriptional termination site located between the CH2 and CH3 exons of the v gene, which also produces the full-length IgY (20). A short exon was found to be spliced into CH2, encoding a secretory tail of IgY(ΔFc) (20). A similar tran-
scriptive pattern was also observed in the green anole lizard, in which the secretory tail is not encoded by a separate exon but rather by the sequence immediately downstream of the CH2 exon (21).

Because IgS play important roles in the adaptive immune system of all jawed vertebrates (1, 2), they have been extensively studied in various groups of animals, including reptiles (16, 21–23). However, little is known about Ig-encoding genes in turtles, except for the description of cDNA sequences (24, 25) and serological studies (9, 10, 19). It has previously been shown that IgY(ΔFc) is expressed in these species, but it is not known whether the expression of the two isofoms of Ig Y is mediated through the same mechanism as in the duck. In this paper, we present a study of the IgH genes of the red-eared turtle (Trachemys scripta elegans) and show that IgY(ΔFc) is expressed from distinct genes and that both IgY- and IgY(ΔFc)-encoding genes are extensively diversified.

Materials and Methods

Animals, isolation of RNA, and reverse transcription

Turtles were purchased from a local Beijing pet market. Total RNA was isolated from pulverized organs using the TRizol Kit (Tiangen Biotech, Beijing, China). Reverse transcription was conducted using Moloney Murine Leukemia Virus Reverse Transcriptase following the manufacturer’s instructions (Invitrogen). An oligo(dT) adapter primer NotI-d(T)18 was used (5′-AAC TGG AAG AAT TCG GCG CAG GAA TAA-3′). The resultant cDNA mixture was used in PCR assays.

Cloning of the turtle μ gene

A pair of primers, JH-forward (5′-GGG GAC GAG AAG GAA CAA TGG TGA CCG TTT CCA-3′) and μ-reverse (5′-ACT TGA TGA AGA GGT CAC GAC GGG-3′), was designed based on conserved sequences among species to amplify a segment of the turtle μ gene. The obtained 1.0-kb PCR product was cloned into the pMD19-T vector (TaKaRa, Dalian, China) and sequenced.

Amplification of the turtle VH sequences

For 5′ RACE, we designed three IgM primers based on the amplified μ gene segment: R1 (5′-TGG AGG CAG GGA CTG ATA G-3′), R2 (5′-CCG AGC AAC CAA GGC TAG CGA TGA-3′), and R5 (5′-AGG GTA GAG TGG GAA AAG AGA AG-3′). cDNA was synthesized from turtle spleen and amplified using a 5′ RACE kit according to the manufacturer’s instructions (Invitrogen).

Construction and screening of the mini-Ig–specific cDNA libraries

A JH-specific primer, JH-primer (5′-TGG GGT GTC AGG CAT CGT ACC GCC TTC ATC GTC ACC GTC ACT-3′), was designed based on the most frequently used JH segment to amplify the C region of turtle Ig H chains using the 3′ RACE System (Invitrogen). Total RNA extracted from the spleen and small intestine was used in the PCR reaction. PCR products were cloned into the pMD19-T vector (TaKaRa) to generate cDNA mini-libraries containing the turtle Ig H chains. The libraries were screened via PCR using IgM-specific primers, IgM-fv50 (5′-GAC CGA CAC GAG CCA AGA TAC TA-3′), and IgM-rev538 (5′-GTC ATT ATC CCA ATC TTC CTC CG-3′). A number of positive and all of the negative clones containing the correct insert size were sequenced.

Genome walking and amplifications of the δ gene

To identify the δ gene, genome walking was initiated using two primers, mTM-1494 (5′-AAA CTT TGC TGC TCT ACT CTG TCA CCT GCG-3′) and mTM-1538 (5′-TGC TCT CGG AAT GGT CTC AGA CTT CG-3′), derived from the 3′ end of the μTM2 region. Walking was performed using the Genome Walker Universal Kit (Clontech) following the manufacturer’s instructions. For IgD cDNA, we designed IgD-specific primers based on the obtained genomic sequences to perform 3′ RACE PCR. IgD-s17 (5′-TTC CCT GCT GAA CCA CTG ATG-3′) and mTM-1538 (5′-TGG AAG AAT TCG GCG CC-3′), derived based on the adaptor sequence of the primer NotI-d(T)18, was used as the sense primer to amplify both secretory and transmembrane IgD. RT-P1 (5′-AAC TGG AAG AAT TCG GCG CC-3′), designed based on the adaptor sequence of the primer NotI-d(T)18, was used as the antisense primer to amplify the transmembrane-encoding IgD H chain.

Southern and Northern blotting

The probes for Southern and Northern blotting were prepared using PCR with primers designed from the above-amplified μ, δ, and ν cDNAs and labeled with digoxigenin using the DIG-High Prime DNA Labeling and Detection Starter Kit (Roche). Hybridization and detection were performed following the manufacturer’s instructions. All Southern and Northern blots were washed at high-stringency conditions (2 × 30 min in 0.1% SSC containing 0.1% SDS at 68 °C).

For Southern blotting, 10 μg liver genomic DNA was digested separately with PstI, PvuII, HindIII, and Dral (NEB), subjected to electrophoresis on a 0.8% agarose gel, and transferred to a positively charged nylon membrane (Roche). For Northern blotting, total RNA was extracted from various tissues, including the heart, liver, spleen, lung, kidney, large intestine, small intestine, testis, and pancreas. The total RNA samples were used for further purification of mRNA with PolyATtract mRNA Isolation System (Promega). One microgram mRNA from each tissue was separated on an agarose/formaldehyde gel and transferred to a nylon membrane.

Western blotting for IgY and IgY(ΔFc) detection in the serum

Polyclonal Abs specific to IgY CH4 and IgY(ΔFc) CH2 were prepared by CoWin Biotech (Beijing, China). Briefly, two peptides (MKNHEGCNETNYT and EQRDGNTQPTKDR) that were respectively derived from conserved sequences in CH4 of IgY and CH2 of IgY(ΔFc) were synthesized and used to immunize rabbits. To confirm the specificity of polyclonal Abs, both IgY and IgY(ΔFc) cDNAs were cloned into pcDNA3.1 and fused with a flag-encoding sequence. The obtained constructs were transfected into 293T cells, and the cell lysates were subject to Western blotting. For Western blotting, serum samples were obtained from an adult turtle and prepared by centrifugation. Serum proteins were separated by electrophoresis on 10% SDS-PAGE gels under reducing conditions and transferred to polyvinyl difluoride membranes (Thermo Scientific). Membranes were blocked with 5% nonfat milk and probed with our polyclonal Abs. The HRP-conjugated secondary Ab used was a goat polyclonal secondary Ab to rabbit IgG-H&L (Abcam).

Sequence and phylogenetic analyses

DNA and protein sequence editing, alignments, and comparisons were performed using DNASTAR’s Lasergene software suite (26) and Boxshade software (http://www.ch.embnet.org/software/BOX_form.html). The identity of turtle VH was identified using IMGT/V-QUEST from the International ImMunoGeneTics database (IMGT; http://www.imgt.org/IMGT_vquest/share/textes/). The BLAST program on the National Center for Biotechnology Information Web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify homologous sequences. Phylogenetic trees were constructed using MrBayes 3.1.2 (27) and viewed in TreeView (28).

Results

Analysis of the expressed H chain V region sequences

To analyze the expressed VH genes in the turtle, we first amplified a genomic fragment of the μ gene, which was subsequently used to design primers for 5′ RACE PCR with spleen RNA isolated from one turtle as the template. The resulting PCR products of ∼500 bp were cloned and sequenced. A total of 190 VH cDNA clones were identified using IMGT/V-QUEST on the IMGT Web site. Among these VH sequences, 115 were unique sequences. The phylogenetic analysis revealed the existence of seven VH families (Fig. 1A), according to the 75% identity criterion (29). The alignment of the amino acid sequences of representatives of each family is shown in Supplemental Fig. 1. To further ascertain how these sequences were related to the VH genes found in other species, a phylogenetic tree of VH genes was constructed using the framework regions, as defined by the IMGT numbering system (30) (Fig. 1B). Unlike human and mouse, in which the VH families have been classified into three major groups or clans (31, 32), the seven turtle VH families were clustered into the five following groups: family 3 (G34) grouped into clan I, family 4 (L3E) and family 6 (P51) grouped into clan II, and family 1 (Q4) and family 7 (L59) grouped into clan III. However, families 2 and 5 were classified into two additional distinct groups with the VH genes of cartilaginous and bony fish.
The 115 unique VH clones accounted for a total of 61 unique CDR3s, which resulted from independent VDJ recombination events. The lengths of the CDR3s (according to the IMGT numbering system) among these clones ranged from 7 to 21 aa (Supplemental Fig. 2), with an average length of 11.4 ± 2.5 aa. Although it was difficult to determine the number of DH segments involved based only on the expressed CDR3s, an analysis of the FR4 regions suggested that there were at least 15 distinct JH genes (Supplemental Fig. 3A, 3B).

To analyze whether different turtles or different tissues in one individual could show different usage of VH and JH segments, 5′ RACE reactions were performed separately on the RNA samples isolated from the spleen and small intestine of a second turtle. Totally, 94 and 64 clones with unique CDR3 were obtained from the spleen and small intestine, respectively. Similar to the first turtle, the VH genes used in the spleen clones belonged to seven families. However, only the members of families 1–5 were found in the clones from the small intestine. Compared with the first turtle, some common and additional JH segments were identified in both tissues of the new individual, showing different profiles of JH usage (Supplemental Fig. 3A–C).

Analysis of the IgH isotypes in the turtle

In birds and reptiles such as lizards, IgH transcripts of all isotypes can be detected in the spleen plus intestine (21, 33, 34). To analyze the IgH isotypes in the turtle, we designed a sense primer based on the sequence of the most frequently used JH segment, which was used in 5′ RACE using the RNA isolated from the spleen and small intestine. We amplified two major bands of 1.5 and 0.9 kb from both tissues. These bands were cloned to construct IgH-specific mini-cDNA libraries. A subsequent analysis showed that all of the amplified 0.9-kb fragments from both tissues were IgY H chains containing only two CH domains. The analysis of 960 clones in the spleen library containing the 1.5-kb inserts revealed 915 IgM H chain, 29 IgY (with 4 CH domains), and 16 non-Ig sequences. Additionally, 458 clones of the small intestine libraries were analyzed and showed 446 IgM H chain, 3 IgY (with four CH domains), and 9 non-Ig sequences. No IgD or IgA H chains were identified in either library.

The turtle μ gene

The IgH C region of turtle μ encodes 451 aa and shows overall sequence identities of 47.3 and 45.3% to the gecko and lizard μ genes, respectively. The alignment of the turtle μ gene (inferred amino acids) and those of other species showed a conserved
distribution of cysteine and tryptophan residues (Supplemental Fig. 4). The turtle C domains all had the conserved Cys residues for intra- and inter-H chain disulfide bonds and for covalent binding of the L chain. The turtle sequence also had the conserved Cys residues found in CH3 and the secretory tail that covalently polymerizes pentameric IgM in mammals (35). Six putative N-linked glycosylation sites (N-X-S/T) were present in the turtle, and three of them are also highly conserved in mammals. The transcripts of the membrane-bound form of the IgM H chain were also amplified using 3′ RACE (Supplemental Fig. 4).

Although RT-PCR showed that IgM (both membrane-bound and secretory forms) was expressed in all of the tissues examined (data not shown), Northern blotting confirmed that the gene was highly expressed in the spleen and the large intestine and relatively weakly in the small intestine and kidney, but barely detectable in other tissues (Fig. 2).

We also performed Southern blotting using one probe of the CH1–3 sequence and one probe of a single CH1 sequence, which revealed that it is likely that there is only a single copy of μ in the turtle genome (Fig. 3).

Multiple copies of δ genes are present in the genome

Despite its absence in birds, the δ gene, consisting of up to 11 CH exons, has been identified in several reptiles (21, 22). We failed to obtain δ gene transcripts from the constructed cDNA mini-libraries, probably due to a low level of expression. To clone the δ gene, we performed genome walking starting from the 3′ end of the μTM2 region toward downstream regions. A δCH-encoding exon was identified ∼6.2 kb from μTM2. BLAST searches and phylogenetic analysis (Fig. 4) confirmed this exon to be turtle δ.

Through continuous walking experiments, we obtained a δ genomic sequence that spanned ∼11 kb of DNA and consisted of six CH exons and a typical TM1 exon (Fig. 5). Although the walking process was based on overlapping sequences, nucleotide mismatches were often observed in the overlapping regions. This result suggested that there could be more than one δ allele or δ
FIGURE 4. Phylogenetic analysis of the turtle IgH constant genes. The credibility value of each node is shown. The amino acid sequences were used for tree construction. Unless otherwise indicated, all CH domains of Ig classes other than IgD are used. For those species that express IgD (and IgW) longer than four CH domains, only the first four CH domains were used. The accession numbers of the sequences used were as follows: m: nurse shark: M92851; skate: M29679; rainbow trout: X65261; zebrasfish: AF281480; X. laevis: X15114; X. tropicalis: AAH89670; axolotl: A46532; platypus: AY168639; duck: AJ314754; chicken: X01613; human: X14940; mouse: V00818; opossum: AAD24482; lizard: ABV66128; gecko: ABY74509; catfish: X52617; ratfish: AAC12892.1; cod: CAA41680.1; waltl: CAE02865.1; pig: BAI82566.1; cow: AAN60017.1; horse: AAU09792.1; rabbit: (Figure legend continues)
gene in the turtle genome, and it is possible that the genomic δ sequence we obtained was chimeric.

We subsequently performed 3’ RACE to amplify the δ H chain transcripts using primers derived from the δCH1 exon. This amplification resulted in two major bands of 2.2 and 1.0 kb. Sequencing of the 2.2-kb bands (35 clones) revealed multiple, highly similar (sharing >96% sequence identity), but distinct IgΔ H chain transcripts. These sequences all encoded secretory IgH chains with six CH domains in which a secretory tail was encoded by the sequence immediately downstream of δCH6. Among the 35 cloned cDNA sequences, there were 25 distinct clones that shared a sequence identity of <99% (Fig. 6). It was difficult to determine exactly how many distinct δ genes were transcribed based on these cDNA sequences amplified using PCR because the PCR process may create chimeric sequences when more than one highly similar sequence is present in the template. To confirm whether multiple δ genes were present in the turtle genome, we therefore performed Southern blotting using both δCH1-3 and δCH1 cDNA as probes, both of which, as expected, detected multiple bands (Fig. 3). This strongly suggests the presence of multiple copies of δ genes in the turtle genome, although some distinct bands could potentially be explained by allelic polymorphisms. To analyze the 1.0-kb amplified bands, 12 clones were sequenced, revealing three different transcripts. All three transcripts encoded two CH domains (δCH1 and δCH3). Two of these transcripts differed from each other by only 1 nt, but both showed 5-nt differences from the third transcript. Surprisingly, none of the three short transcripts could be matched to any of the 35 cloned 2.2-kb transcripts because they contained several consistent nucleotides distinct from the long transcripts (Fig. 6).

To analyze the transcripts encoding the δ membrane-bound form, RT-PCR was performed using primers derived from δCH1 and δTM1 and spleen cDNA as the template. This amplification generated four bands of 1.0, 1.3, 1.6, and 2.0 kb. All bands were cloned and sequenced and showed the presence of three CH domains, four CH domains, five CH domains, and six CH domains, respectively (Fig. 5). Similar to the secretory IgH chain transcripts, the transcripts of each length did not have a unique sequence and appeared to be transcribed from distinct δ genes.

Northern blotting showed that the turtle δ gene was only expressed in the spleen, and in addition to a major band, some weaker bands were also observed (Fig. 2), confirming the alternative splicing of the δ gene.

IgY- and IgYΔFc-encoding genes in the turtle

Previous studies examining turtle serum Igs have suggested the presence of both intact IgY and truncated IgY (ΔFc) (9, 10, 19). Four CH domain-encoding IgY cDNA transcripts were obtained from the screening of the Ig-specific mini-libraries with 1.5-kb inserts, whereas all 0.9-kb-amplified PCR bands were shown to be IgY(ΔFc) H chain cDNAs. Surprisingly, the sequencing of more clones also revealed extensive diversification of both IgY and IgY (ΔFc) subclasses; at least eight IgY and five distinct IgY(ΔFc) cDNAs could be identified in the same animal (Fig. 7). This result is consistent with Southern blotting results, in which both multiple- and single-CH probes detected multiple bands (Fig. 3).

To our surprise, sequence comparisons of turtle IgY and IgY (ΔFc) revealed that IgY(ΔFc) was not expressed from the IgY-encoding genes because they displayed distinct sequences (Fig. 7). This result is different from the case in ducks, in which both intact IgY and IgY(ΔFc) H chain cDNAs are expressed from the same gene using different transcriptional termination sites (15, 20).

Northern blotting was performed to further examine the expression of the turtle IgY- and IgY(ΔFc)-encoding genes using an IgY CH1-CH3 cDNA probe. Because all of the cloned IgY and IgY(ΔFc) genes shared a highly similar CH1 exon, this probe was expected to detect both the intact and truncated transcripts. The hybridization revealed that intact IgY transcripts were detected in the spleen, kidney, and large intestine, whereas the IgY(ΔFc) transcripts were mainly detected in the large intestine (Fig. 2).

We developed rabbit polyclonal Abs specific to turtle IgY and IgY(ΔFc). The specificity of the rabbit polyclonal Abs was first confirmed by Western blotting on the cell lysates of 293T cells, which were transfected by IgY and IgY(ΔFc) cloned in pcDNA3.1 (Fig. 8A). The polyclonal Abs were then used in the Western blotting detection of serum proteins. The results showed that both IgY and IgY(ΔFc) were present in the serum with molecular mass of 62 and 38 kDa, respectively, both of which were in accordance with predicted results (Fig. 8B).

Discussion

In this paper, we performed a detailed study of IgH genes in the red-eared turtle and showed that this species expressed three IgH isotypes, including μ, δ, and ν. Unlike the μ gene, both the δ and ν genes are present as multiple copies. Surprisingly, unlike in ducks, in which the same ν gene produces both IgY and IgY(ΔFc) H chains, multiple distinct genes have been developed to encode truncated IgY(ΔFc) in the turtle. This study thus reveals a more complex set of Ig genes in the turtle than other reptiles and birds. The red-eared turtle has a diploid genome, with a total of 50 chromosomes (36). Neither the genome sequence nor a bacterial artificial chromosome library is available for this species. It is therefore currently not possible to determine the exact copy numbers of the δ and ν genes or how these genes are organized in the genome.

The δ gene is as ancient as the μ gene, and it has now been identified in nearly all jawed vertebrates except birds and a few mammalian species (3, 5, 37–40). An exceptional feature of the δ gene compared with other isotypes appears to be its highly variable structure with regard to the number of CH exons throughout the evolution of jawed vertebrates, which can range from 2 in mice to 16 in zebrafish (41–43). A large number of studies have revealed that the δ gene tends to have more CH-encoding exons in nonmammalian species [including the most primitive living...
mammal, the duck-billed platypus, in which 10 CH-encoding exons have been identified for its δ gene (17), but no more than 3 in placental mammals (44).

The δ gene has previously been analyzed in three reptilian species, including the green anole lizard, the leopard gecko, and the Chinese soft-shelled turtle (21, 22, 25). In the first two species, the δ gene contains 11 CH-encoding exons; however, in the green anole lizard, only the first 4 CH exons are included in the IgD H chain transcripts (21). In the Chinese soft-shelled turtle, six CH-encoding exons were observed in cloned IgD H chain cDNA (25). Additionally, two δ genes were observed in a single IgH gene locus in the leopard gecko, whereas only a single δ gene was found in the green anole lizard (21–23). The current study revealed the presence of multiple copies of the δ gene in the genome of the red-eared turtle, and alternative splicing was extensively involved in gene expression, suggesting that IgD may play a significant role in immunological defense of turtles.

The δ gene has been thought to be functionally redundant and merely act as a genetic backup for μ as δ-knockout mice do not show apparent immunological defects (45). Recently, however, it was shown that IgD regulates a surveillance system at the interface between immunity and inflammation and that this mechanism appears to be conserved in jawed vertebrates (46). In addition to enhancing mucosal immunity, secretory IgD is able to bind basophils and other innate immune cells to stimulate release of immunoregulating, proinflammatory, and antimicrobial mediators (46). Although it is not known how the multiplication of the turtle δ genes occurred, these multiple copies of δ genes are most likely located together within a common IgH locus, as only a single μ gene was observed in the turtle. It may be expected that some of the δ genes would be expressed through class-switch recombination, which would generate IgM-IgD+ B cells in the turtle. As multiple cDNA transcripts encoding secretory IgD were cloned, it is reasonable to speculate that IgD would mediate immunity or inflammation in this species. More importantly, the turtle δ genes show extensive sequence variations, especially at some functional sites (for instance, glycosylation sites), which may indicate functional divergence among these different δ-encoding genes.

This study also generated two interesting findings regarding the ν genes in the turtle. First, similar to the δ gene, the ν gene has also been extensively diversified to generate multiple subclass-encoding genes. Second, distinct ν genes have been developed in the turtle for the production of IgY(ΔFc). This situation is clearly different from the case of ducks, in which the same ν gene is used to produce both IgY and IgY(ΔFc) H chains (20). Extensive diversification of the ν gene has significant evolutionary implications when considering the gene to be the evolutionary precursor of mammalian γ and ε, encoding IgG and IgE, two functionally divergent Ig classes (15). The presence of multiple ν genes in the turtle suggests that the functional divergence of IgY may have already been initiated in nonmammalian tetrapods. Indeed, our unpublished data also revealed the presence of multiple IgY subclasses at both the DNA and protein level in other reptiles (such as snakes and crocodiles). Because IgY can mediate humoral responses, it would be interesting to investigate whether some IgY subclasses are specialized in mediating allergic response in reptiles, similar to the ability of IgE in mammals.

IgY(ΔFc) is a truncated form of IgY that lacks the last two CH domains compared with four intact CH domains in IgY. The Fc region is missing in this truncated form, which is believed to have only Ag-binding activity but no effector functions. IgY(ΔFc) is
produced at the protein level in various birds and reptiles, including ducks and turtles (15, 18, 39, 47). The present study clarifies that turtles possess a different mechanism than ducks for the production of the truncated IgY form. Nonetheless, the most interesting question of the immunological benefits of IgY(D\(\text{Fc}\)) remains to be addressed.

**FIGURE 6.** Amino acid sequence alignment of the cloned IgD transcripts. Conserved cysteine and tryptophan residues are shaded. Potential N-linked glycosylation sites are underlined. Dots are used to denote identical amino acids, whereas dashes are used to adjust the sequence alignments. sIgD, Short IgD.
This study also adds the turtle to the list of reptilian species, including lizards and snakes (21, 48), in which an IgA-encoding gene could not be identified. The consistent lack of IgA in reptiles is surprising, because IgA, as a specialized Ab class in mucosal immunity, has been observed in all mammals and birds examined so far (1, 4). Another Ab class, IgX, has also been implicated in mucosal immunity of amphibians (49, 50). Even in the most primitive bony vertebrates, teleost fish, IgT, was found to be functionally specialized in mucosal immunity (51). There are two questions with regard to the absence of IgA in reptiles: 1) why or how the IgA encoding gene was lost? and 2) which Ab class is used at the mucosal sites in the absence of IgA? The answer for the first question may be derived from a comparative analysis of Ig genes in both reptiles and birds. Reptiles are believed to share a direct common ancestor with modern birds, among which all examined species show an inversion of the IgA-encoding gene and an absence of the IgD-encoding gene (34, 39, 52, 53). It seems that the IGHC gene locus in both reptiles and birds has experienced some genetic rearrangements during their evolutionary process. It is not known whether these rearrangements are accidental or deliberate events but somehow lead to loss of δ or α genes or inversion of a gene in these species. Our Northern data may provide some potential answers to the second question. As a strong IgM expression was detected in the large intestine and also in the small intestine, it may be speculated that IgM is able to compensate for the absence of IgA at mucosal sites in turtles. This is a reasonable speculation, as only IgM and IgA share the same polymeric Ig receptor, which allows transport into mucosal secretions (54). In conclusion, this study provides a detailed analysis of IgH genes in the red-eared turtle, and it aids in the understanding of how flexible IgH genes can be in different species and the evolutionary process of IgH genes in tetrapods.

**FIGURE 7.** Amino acid sequence alignment of different IgY and IgY(ΔFc) H chain transcripts. Conserved cysteine and tryptophan residues are shaded. Potential N-linked glycosylation sites are underlined. Dots are used to denote identical amino acids, whereas dashes are used to adjust the sequence alignments.

**FIGURE 8.** Western blotting detection of turtle IgY and IgY(ΔFc) in serum. (A) Specificity confirmation of the developed polyclonal Abs. Fragments of IgY and IgY(ΔFc) were separately cloned into pcDNA3.1 and fused with a flag sequence, which were transfected into 293T cells. The cell lysates were subject to Western blotting. 1, IgY detected by the developed polyclonal Abs; 2, IgY detected by anti-flag Abs; 3, IgY(ΔFc) detected by the developed polyclonal Abs; 4, IgY(ΔFc) detected by anti-flag Abs. (B) Detection of serum IgY and IgY(ΔFc) by the developed polyclonal Abs.
Disclosures

The authors have no financial conflicts of interest.

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