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Green Turtles (*Chelonia mydas*) Have Novel Asymmetrical Antibodies

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Igs in vertebrates comprise equally sized H and L chains, with exceptions such as H chain–only Abs in camels or natural Ag receptors in sharks. In Reptilia, Igs are known as IgYs. Using immunoassays with isotype-specific mAbs, in this study we show that green turtles (*Chelonia mydas*) have a 5.7S 120-kDa IgY comprising two equally sized H/L chains with truncated Fc and a 7S 200-kDa IgY comprised of two differently sized H chains bound to L chains and apparently often noncovalently associated with an antigenically related 90-kDa moiety. Both the 200- and 90-kDa 7S molecules are made in response to specific Ag, although the 90-kDa molecule appears more prominent after chronic Ag stimulation. Despite no molecular evidence of a hinge, electron microscopy reveals marked flexibility of Fab arms of 7S and 5.7S IgY. Both IgY can be captured with protein G or melon gel, but less so with protein A. Thus, turtle IgY share some characteristics with mammalian IgG. However, the asymmetrical structure of some turtle Ig and the discovery of an Ig class indicative of chronic antigenic stimulation represent striking advances in our understanding of immunology. ***The Journal of Immunology*****, 2015, 195: 000–000.**

The study of immunity in nonconventional animals can bring novel and relevant insights to human immunology. For instance, IFN, a treatment routinely applied to autoimmune disorders and certain cancers, was discovered in chickens (1). Central to the study of immunology are Igs that are fundamental to adaptive immune response in all jawed vertebrates (Gnathosomata) (2). In mammals, Ig monomers consist of two L and two H chains, each with C and V regions joined by disulfide bonds (3). For many years, the classical paradigm of dual H and L chain structure of Igs was thought to apply to all vertebrates, but recent findings, such as natural Ag receptors in sharks (4) and H chain–only Igs in camelids (5), suggest that there may be greater plasticity in Ig structure than originally thought.

There is a wealth of literature on Igs of mammals, birds, amphibians, and fish; however, anapsids (turtles) and cold-blooded diapsids (lizards, snakes, and crocodilians) are notably underrepresented in the literature (6). The few studies that do exist on turtle Igs are focused on molecular aspects (7) that shed little light on structure and function. This is unfortunate, because reptiles provide a critical evolutionary bridge between fish and amphibians on the one hand and warm-blooded birds and mammals on the other hand (8); this could, in turn, yield valuable contrasts on immunity of endotherms and ectotherms. Moreover, turtles are a particularly enigmatic group because their evolutionary status is conflicted, with morphology placing them closer to lizards and molecular data placing them closer to birds and crocodiles (9).

Green turtles (*Chelonia mydas*) are one of seven species of sea turtles that have thrived for millennia in the ocean, a hypersaline medium laden with microbes. Their immune defenses allow them to routinely survive severe insults such as traumatic flipper amputations that would kill most mammals. Green turtles are listed as threatened or endangered and are affected by a debilitating and globally distributed tumor disease, fibropapillomatosis, associated with a herpesvirus. This disease leads to immunosuppression and death in severely affected animals (10). Our rudimentary knowledge of the green turtle immune system and lack of laboratory reagents impedes our ability to understand the pathogenesis of fibropapillomatosis or other diseases in this group and could hamper efforts to recover the species, particularly in situations where disease has important demographic effects.

Green turtles have three known types of Ig, assumed to consist of bilaterally symmetrical H and L chains, including a 17S IgM, presumed to be a pentamer, and two monomeric Ig including a 7S IgY, and a 5.7S IgY with truncated Fc (11). Unlike mammals, reptile and avian IgY are thought not to have a hinge (12). Our study challenges established paradigms by showing that green turtles have two types of IgY, one of which is structurally asymmetric. The 7S IgY functions similarly to human IgG and is likely noncovalently associated with a 90-kDa moiety that is produced only during chronic antigenic stimulation. Despite no molecular evidence of a hinge region, turtle IgY display remarkable flexibility of Fab arms. The ability of turtle IgY to bind to protein G contrasts with IgY of birds that do not, thereby suggesting that turtle IgY may have more mammalian than avian characteristics. Our findings of Igs structurally unlike those seen before, as well as the idea that some Igs could be markers of chronic antigenic stimulation, considerably expands the structural and functional repertoire of these molecules in vertebrates. Our findings might also provide tools to enhance understanding of disease pathogenesis in green turtles and possibly anapsids.

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The online version of this article contains supplemental material.

Abbreviations used in this article: EWL, egg white lysozyme; FCA, Freund's complete adjuvant; MB, 2% nonfat milk in borate buffer; RATP, rabbit anti-turtle polyclonal Ab; WB, Western blot.

Materials and Methods

mAbs and ELISAs

Crude IgY was precipitated three times from green turtle plasma using 33% ammonium sulfate (SAS-cut IgY) (13), resuspended in PBS, and mice were immunized with 20–50 μg Ag i.p. with Freund's complete adjuvant (FCA), including two boosters in Freund's incomplete adjuvant 3 wk apart. At 6 wk, the mouse antisera were assayed for reactivity to turtle IgY by indirect ELISA. Briefly, round-bottom 96-well plates (Costar, Corning, NY) were coated to dryness with 1 μg whole-turtle plasma per well and blocked 1 h with 2% nonfat milk in borate buffer (MB). Plates were then incubated 1 h with mouse serum diluted 1:100 and 1:500 each in duplicate wells in MB, washed in borate buffer three times, and incubated 1 h with HRP-conjugated goat anti-mouse IgG (H+L chains) diluted 1:1000 in MB (Alpha Diagnostics). Color development was done by incubating 1 h with 5-aminosalicylic acid and reading absorbance at 405 nm after subtracting blanks consisting of Ag and secondary Ab.

To generate mAbs, spleen cells from immunized mice were fused to P3x63Ag8.653 mouse myeloma cells in the presence of polyethylene glycol to produce mAbs according to established techniques (14). Hybridomas were then selected with hypoxanthine, aminopterin, and thymidine–supplemented medium and allowed to grow on macrophage plates in preparation for ELISA.

Hybridoma supernatants were screened by ELISA for anti-IgY activity (13) using three strategies: 1) ability to detect IgY in whole plasma from sea turtles; 2) ability to detect the specific immune response in archived serum from a captive green turtle that had been immunized with BSA (11); and 3) ability to detect specific immune response of wild green turtles to soluble worm Ag from spirorchiid trematodes that naturally infect the circulatory system of wild green turtles (15). Thus, the first strategy ensured that hybridoma supernatants could detect turtle Igs, and the latter two strategies ensured detection of turtle immune response to known Ags.

To detect turtle Igs in turtle plasma, we used indirect ELISA essentially as described for mouse serum. To assess reactivity of neat hybridoma supernatants to turtle IgY (strategy 1), plates were coated to dryness (1 μg/well) with turtle plasma, blocked 1 h with MB, and then reacted directly with supernatants as described for mouse serum. To detect Ag-specific immune response in sea turtles (strategies 2 and 3), plates were coated to dryness with 1 μg/well of BSA (strategy 2) or soluble worm Ag (strategy 3), blocked 1 h with MB, incubated 1 h with a 1:100 dilution of hyperimmune anti-BSA turtle plasma (11) or green turtle plasma from animals with documented trematode infections (15), and hybridoma supernatants were assayed by ELISA as for mouse serum.

Clones positive for all three ELISA strategies were selected for Ab purification based on their ability to detect H chains of appropriate molecular masses (60 kDa for 7S and 40 kDa for 5.7S) by Western blot (WB) (see below), and mAbs were purified with protein G columns (Pierce) and quantified using a bicinchoninic acid colorimetric assay with bovine γ globulin standard curves (Pierce). From this, single clones showing representative activity against 5.7S or 7S IgYs were expanded and mAbs were purified for subsequent assays. To ensure replicability within green turtles, anti-7S and anti-5.7S mAbs were assayed by WB against sera from multiple green turtles from the Atlantic and Pacific Oceans. To ensure species specificity of mAbs, we assayed serum from a single Pacific leatherback turtle (*Dermochelys coriacea*), Pacific hawksbill turtle (*Eretmochelys imbricata*), and Atlantic loggerhead turtle (*Caretta caretta*).

Characterizing host response with mAbs

To detect immune response in turtles, we used archived serum from four green turtles immunized with egg white lysozyme (EWL) using two different adjuvants (FCA and ISA 70) that showed a detectable immune response when assayed with rabbit anti-turtle polyclonal Ab (RATP) (13). Serum from these turtles was assayed by indirect ELISA as described (13), except that mAbs against 7S and 5.7S turtle IgYs were used at 1 μg/ml instead of RATP.

Gel electrophoresis and WBs

The biochemical organization of turtle 7S and 5.7S IgY and the specificity of the mAbs toward the various turtle Ig subunits or polypeptides were assayed by gel electrophoresis and WB. For reducing SDS-PAGE, 0.5- to 1-μg protein samples were diluted in Laemmli buffer with 2-ME, heated at 100°C, and resolved in SDS-glycine buffer on a 10% polyacrylamide gel overlaid by a 4% stacking gel (16). Nonreducing SDS-PAGE followed identical protocols except that 2-ME was omitted from Laemmli buffer, and proteins were resolved on a 7% or a 4–15% gradient gel. Two-dimensional gels were run to further resolve protein structures as follows: proteins were electrophoresed in a single preparative well on 7% nonreducing SDS-PAGE, the lane was sliced out of the gel, and proteins in the slice were reduced by soaking with Laemmli buffer with 2-ME. The slice was then horizontally overlaid on a 10% gel, protein bands were resolved in a second dimension with reducing SDS-PAGE, and proteins were visualized with silver stain (Bio-Rad). For determination of molecular masses on reducing SDS-PAGE, purified IgY was electrophoresed along with low and high molecular mass markers (Bio-Rad) on 10% gels. For molecular mass determinations on nonreduced SDS-PAGE, purified IgY was electrophoresed along with commercially available pure chicken IgY (Equitech-Bio), protein G affinity-purified mouse IgG, and broad (15- to 250-kDa) range markers (Invitrogen) resolved on a 4–15% gradient. The appearance of a single band for mouse IgG and chicken IgY served as a control for the nonreducing SDS-PAGE.

For WB, proteins and color molecular mass markers were transferred onto nitrocellulose membranes, blocked for 1 h in MB, washed in 0.05% Tween 20/borate buffer 3× for 10 min, incubated with primary Abs diluted in MB, washed, reacted 1 h with appropriate HRP-labeled secondary Ab diluted in MB, washed, and color developed using diaminobenzidine (Thermo Fisher Scientific). Primary Abs included either hybridoma supernatants, mAb diluted in MB at 1 μg/ml, or RATP (13) diluted 1:1000 in MB. Secondary Abs included HRP-conjugated goat anti-rabbit or anti-mouse polyclonal IgG (Alpha Diagnostics) diluted 1:1000 in MB. Color development was with diaminobenzidine (Pierce).

Biochemical characterization of IgY

7S and 5.7S Abs were immunoprecipitated from turtle serum using Pierce direct immunoprecipitation kits (Thermo Fisher Scientific) essentially according to the manufacturer's instructions. Briefly, 20 μg anti-7S or 5.7S mAb was covalently coupled to 20 μl Sepharose for 90 min with sodium cyanoborohydrate, washed, incubated overnight at 4°C with 500 μl turtle plasma (1 mg/ml), and eluted. Negative control immunoprecipitations consisted of replicating assays with Sepharose-coupled mouse mAbs of the same isototype (κ L chain) raised to a mock Ag (*Campylobacter* sp.).

To better understand the specific nature of green turtle IgY–Ag interactions, we used archived serum from green turtles that were experimentally inoculated with BSA (11), EWL (13), or naturally infected with spirorchiid trematodes, which are helminth parasites that live in turtle blood vessels and lay eggs that localize in multiple tissues and cause chronic inflammation (15). Sepharose beads were coupled to 20 μg BSA, EWL, soluble adult worm or trematode egg Ag, and reacted with green turtle anti-BSA (11), anti-EWL (13), or anti-spirorchiid hyperimmune serum (15). We also tested the utility of protein A, protein G, or melon gel to purify green turtle IgY using appropriate columns or reagents according to the manufacturers' instructions. Melon gel is a proprietary resin (Thermo Fisher Scientific) for purification of Ig that was used successfully to purify Ig from freshwater turtles (17). All eluates from immunoprecipitations, protein G, protein A, or melon gel were screened by WB using RATP and mAbs to 5.7S and 7S IgY.

Transmission electron microscopy and image analysis

Samples were prepared on continuous carbon films supported on nitrocellulose-coated 400-mesh copper grids (Ted Pella). A 3-μl drop of immunoprecipitated 7S or 5.7S Ab solution was applied to a freshly plasma-cleaned grid for 1 min and blotted to a thin film using filter paper. The sample was washed four times by floating the grid on a droplet of H_2O for 1 min followed by staining on a droplet of 2 or 3% (w/v) uranyl formate for 1 min. The grid was blotted after each incubation and air dried. Transmission electron microscopy was performed using an FEI Tecnai T12 electron microscope operating at 120 kV equipped with an FEI Eagle 4k × 4k CCD camera. Images were collected at nominal magnifications of ×67,000 (0.16 nm/pixel) and ×52,000 (0.21 nm/pixel) using the automated image acquisition software package Leginon (18). Images were acquired at a nominal underfocus of −1.0 to −2.5 μm (×67,000) and −1.5 to −3 μm (×52,000) and electron doses of ~25–35 electrons/$Å^2$.

Image processing was performed using the Appion software package (19). Contrast transfer functions of the images were corrected using Ace2 (20). Individual particles in the ×67,000 images were selected using automated picking protocols, followed by several rounds of reference-free alignment and classification based on the XMIPP (21) processing package to sort them into self-similar groups.

Chromatography

For gel filtration, 5 mg SAS-cut IgY was resolved on a 60 × 1.5-cm Sephacryl 300 column (GE Healthcare, Little Chalfont, U.K.) using an ÄKTA fast protein liquid chromatography system (GE Healthcare).

Peptide identification and endpoint RT-PCR

5.7S and 7S IgY purified by ion exchange chromatography were resolved on reducing SDS-PAGE with 10% precast gels (Bio-Rad), stained with Coomassie blue, H chain bands were excised, and peptides were characterized with liquid chromatography and tandem mass spectrometry after trypsin digestion (Bioproximity). Peptides were blasted (BlastP) in UniProt to identify hypothetical green turtle Ig proteins.

Determining presence of hinge region

To determine whether a hinge was present in IgY, we used peptide sequences obtained by mass spectrometry to query the *C. mydas* whole-genome shotgun contigs (22) with tblastn to identify potential exons (23) and design primers using Primer-BLAST (24). RNA was extracted from the spleen of a single green turtle with TRIzol (Qiagen, Valencia, CA) and used to amplify IgY genes with a one-step RT-PCR kit (Life Technologies) according to manufacturer's instructions using the following three primer pairs: forward 1, 5′-CACATGGCTGAAGGATGGGG-3′, reverse 1, 5′-GGTATTGGCGGTATCGGACA-3′; forward 2, 5′-CACATGGCTGAAGGATGGGG-3′, reverse 2, 5′-GGTATTGGCGGTATCGGACA-3′; forward 3, 5′-GGACCGAAACGAGCCAAGAT-3′, reverse 3, 5′-ACCTTAGGTTCTCCGAGGCT-3′. Briefly, 100 ng RNA was amplified to cDNA for 30 min at 65°C followed by activation of DNA polymerase (95°C for 1 min) with 35 cycles of denaturing (95°C for 30 s), annealing (56°C for 30 s), and extension (72°C for 45 s) followed by 10 min final extension at 72°C. Stringency was increased as appropriate by elevating annealing temperatures. Positive controls for PCR included green turtle GAPDH (Genbank accession no. KT698946; http://www.ncbi.nlm.nih.gov/genbank/). Amplicons were resolved on 1.5% agarose gels with Tris-borate-EDTA buffer and stained with ethidium bromide. Negative controls included water only, whereas positive control included amplification of green turtle GAPDH. All products were sequenced (Sanger) at the University of Hawaii core sequencing facility. In cases where sequences suggested that primers were amplifying multiple products, RT-PCR products were cloned with a TOPO-TA cloning kit (Life Technologies), and plasmids were purified and sequenced. Translated amino acid sequences of cDNA were aligned with human IgG1 H chain constant (GenBank accession no. BAN63131.1) and examined for presence of the proline-rich region characteristic of the Ig hinge (25).

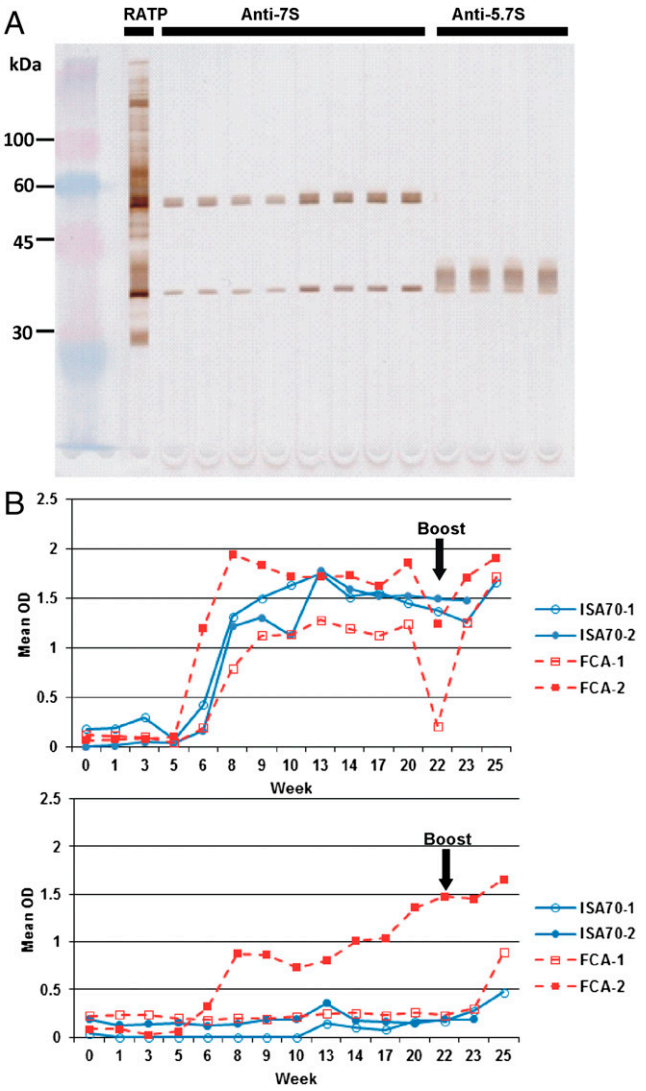

FIGURE 1. (**A**) Reducing SDS-PAGE of green turtle plasma resolved on a 10% gel, blotted onto nitrocellulose, and probed with RATP with color molecular masses (kDa) on left. Note eight clones of anti-7S IgY mAbs recognizing a 60-kDa band and four clones of anti-5.7S IgY mAbs recognizing a 40-kDa band. Note that the anti-7S mAbs also recognize an unexpected 40-kDa band that differs in size and shape from that detected by anti-5.7S mAbs. The RATP sees the 60-, 40-, and a diffuse 30-kDa band along with other nonspecific bands. This is representative of 12 experiments. (**B**) Mean OD of duplicate samples analyzed by ELISA for anti-EWL Ab levels by week using anti-7S and anti-5.7S mAbs for two green turtles each immunized once (day 0) with adjuvants ISA 70 (blue lines) or FCA (red dashed lines); arrow indicates boost. The ELISA was done twice.

Results

mAbs

We assayed hybridoma supernatants on WB against whole-turtle serum to identify whether mAbs were reacting to 7S or 5.7S IgY. We found eight clones that recognized a 60-kDa band compatible with the H chain of 7S IgY and four clones that recognized a 40-kDa band compatible with the H chain of 5.7S IgY (11). An additional 40-kDa band was also recognized by all mAbs against 7S IgY (Fig. 1A).

Characterizing host response

To confirm that these clones were able to detect an immune response, we purified mAbs from one clone each recognizing 7S or 5.7S IgY and assayed these against archived sera from Hawaiian green turtles immunized with EWL (13). Turtles showed a robust immune response as measured by indirect ELISA with anti-7S mAbs similar to what was observed when assayed with RATP (13); in contrast, anti-5.7S activity only appeared later in the immune response, and only in one turtle immunized with FCA (Fig. 1B).

Dual bands for 7S are common in green turtles

To determine the generality of the unexpected 40-kDa band reacting with anti-7S mAb, we resolved the serum of seven green turtles from Hawaii and two from Florida with reducing SDS-PAGE and assayed them by WB. All but one turtle had varying intensity of the 40-kDa band, with this band being particularly faint

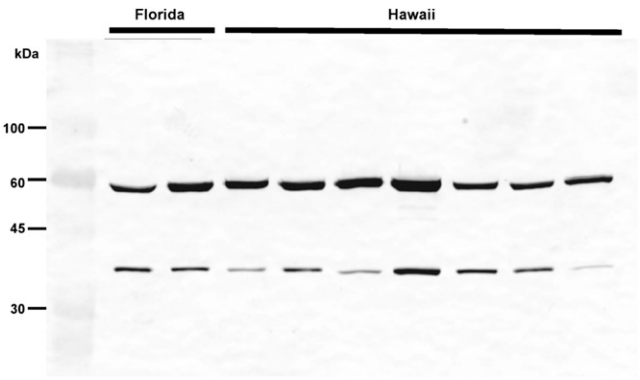

FIGURE 2. WB of plasma (500 μg/well) with color molecular mass standard (*left*) from Florida (*lanes 2* and *3*) and Hawaii (*lanes 4–10*) green turtles resolved on 10% reducing SDS-PAGE, blotted, and probed with anti-7S mAb. Note 40-kDa band of varying intensity with one animal (*last lane*) having a scarcely detectable band. Experiment replicated three times.

in one animal (Fig. 2). To determine species specificity of anti-7S and anti-5.7S mAbs, we probed serum from leatherback (*D. coriacea*), hawksbill (*E. imbricata*), and loggerhead (*C. caretta*) turtles, none of which reacted with either mAb on WB.

7S and 5.7S IgY are antigenically distinct

Having confirmed that anti-5.7S and anti-7S mAbs were capable of detecting an Ag-specific immune response in green turtles, and that an additional unexplained 40-kDa band was consistently detected by anti-7S mAb with multiple clones and in multiple turtles, we further investigated the nature of this 40-kDa band. On immunoprecipitation, anti-5.7S mAb captured proteins from turtle serum that, on reducing SDS-PAGE, resolved into a dense 40-kDa band and diffuse 30-kDa band compatible in appearance on acrylamide gels with H and L chains, respectively (26), whereas a 40-kDa (H chain) band in addition to the expected 60-kDa H chain was noted in eluates from proteins immunoprecipitated with anti-7S mAb (Fig. 3A).

When probed against 7S IgY captured by immunoprecipitation, anti-7S mAb and RATP recognized bands at 60 and 40 kDa (H chains) and RATP recognized an additional band at 30 kDa (L chain), whereas anti-5.7S mAb did not react. When probed against 5.7S IgY captured by immunoprecipitation, anti-5.7S mAb and RATP reacted with a 40-kDa band (H chain) and RATP recognized an additional band at 30 kDa (L chain) and a weak band at 60 kDa (probable aggregate), whereas anti-7S mAb did not react. Anti-7S mAb, anti-5.7S mAb, and RATP all reacted similarly with anti-BSA Abs from archived hyperimmune turtle sera that were immunoprecipitated by BSA coupled to Sepharose (Fig. 3B).

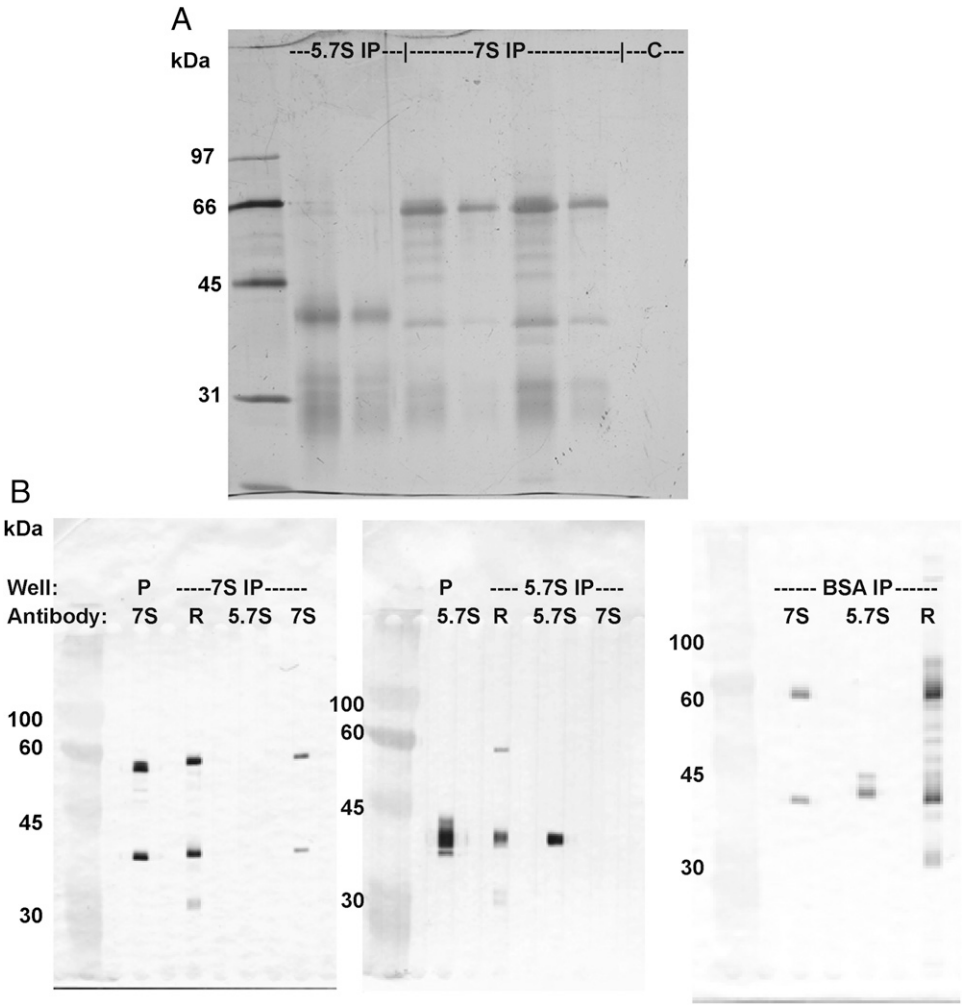

FIGURE 3. Eluates from immunoprecipitations (IP) resolved on reducing SDS-PAGE and stained with silver (**A**) or transferred to blot and probed (**B**). (A) Bio-Rad low molecular mass standards (*lane 1*) and eluates from Sepharose beads coupled with anti-5.7S mAb (*lanes 2* and *3*), anti-7S mAb (*lanes 4–7*), and anti-*Campylobacter* (C) mAb (*lane 8*) as control, all incubated with turtle plasma. Note narrow band at 40 kDa for 5.7S and 7S IgY (H chain), additional narrow 60-kDa band for 7S IgY (H chain) with lighter bands below probable degradation products, diffuse bands at 30 kDa (L chains), and absence of product in control (*lane 8*). This is representative of 12 experiments. (B) Blots with molecular mass standards (*lane 1*). *Left blot*, Turtle plasma (*lane 2*) and 7S IP (*lanes 3–5*) probed with anti-7S mAb (*lanes 2 and 5*), RATP (*lane 3*), and anti-5.7s mAb (*lane 4*); note bands at 40 and 60 kDa detected by anti-7S mAb and RATP (*lanes 2*, *3*, and *5*), 30-kDa band (L chain) detected by RATP (*lane 3*), and lack of reactivity of anti-5.7S mAb to 7S IgY (*lane 4*). *Middle blot*, Turtle plasma (P) (*lane 2*) and 5.7S IP (*lanes 3–5*) probed with anti-5.7S mAb (*lanes 2* and *4*), RATP (*lane 3*), and anti-7s mAb (*lane 5*); note bands at 40 kDa detected by anti-5.7S mAb and RATP (*lanes 2–4*), additional 30-kDa band (L chain) and 60-kDa band (probable aggregate) detected by RATP (*lane 2*), and lack of reactivity of anti-7S mAb to 5.7S IgY (*lane 4*). *Right blot*, Anti-BSA IgY captured from hyperimmune anti-BSA turtle serum incubated with BSA coupled to Sepharose and eluates probed with anti-7S (*lane 2*), anti-5.7S (*lane 3*), and RATP (*lane 4*). Note bands at 60 and 40 kDa seen by anti-7S mAb (*lane 2*) and RATP (*lane 4*), 40 kDa seen by anti-5.7S mAb (*lane 3*) and RATP (*lane 4*), and 300kDa band (L chain) seen by RATP (*lane 4*). Additional bands on *lane 4* are probable protein degradation (turtle serum dates from 1960s). The experiment was repeated three times.

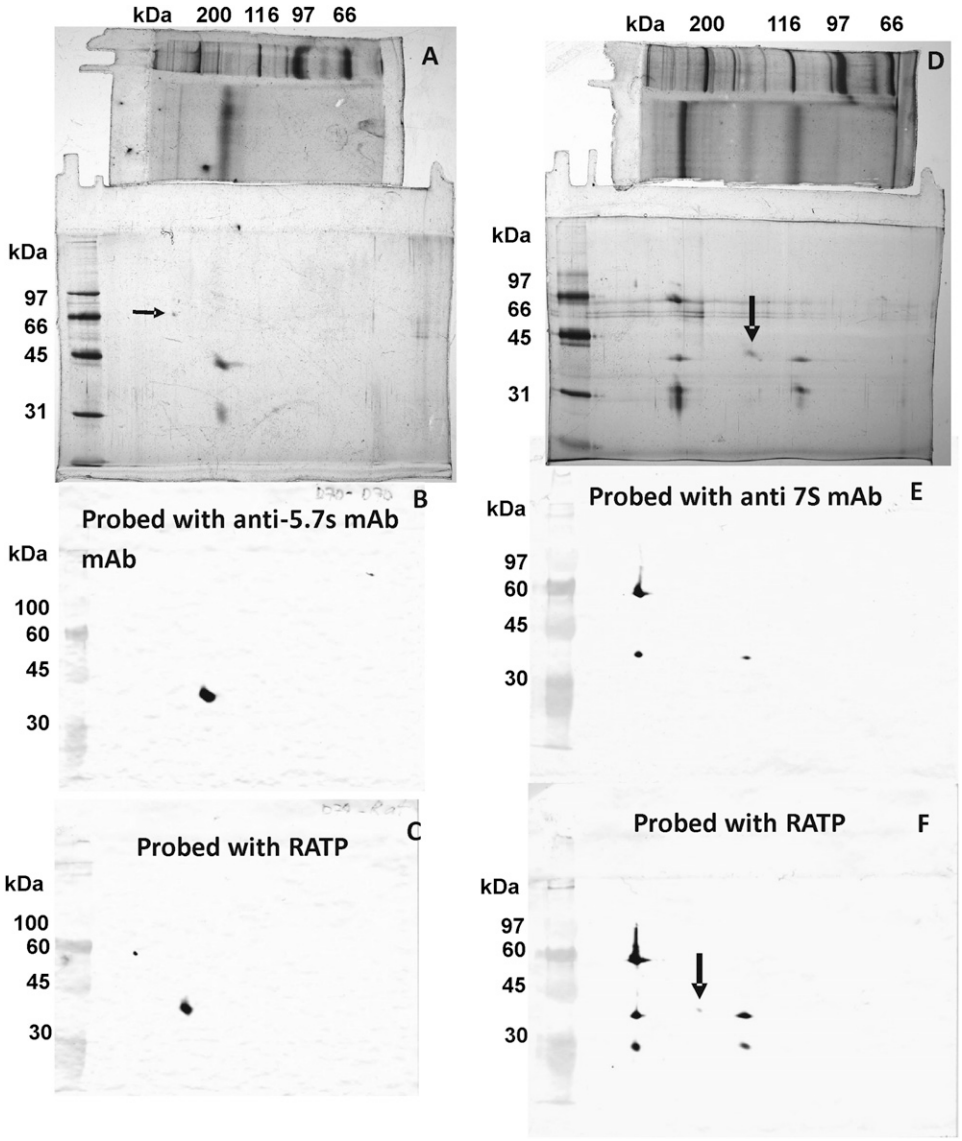

FIGURE 4. Silver stain of 5.7S IgY (**A**–**C**) or 7S IgY (**D**–**F**) immunoprecipitated from turtle serum, resolved in X dimension with nonreducing SDS-PAGE, and then Y dimension with reducing SDS-PAGE that was then transferred to blot and probed with 5.7S mAb (B), anti-7S mAb (E), or RATP (C and F). (A) 5.7S IgY. Note the light band at 200 kDa and stronger 120-kDa band under nonreducing conditions (*top gel horizontal* with Bio-Rad high molecular mass standards); under reducing conditions, the 200-kDa band (possible aggregate) resolves to a small 60-kDa band (arrow), and a 120-kDa band resolves to 40- and 30-kDa bands (Bio-Rad low molecular mass standards on left). (B) Note that 60 kDa stains negative with anti-5.7S but 40 kDa stains positive. (C) Both 60 and 40 kDa stain with RATP; we suspect there was insufficient material for RATP to detect the 30-kDa band. (D) 7S IgY. Note dense bands at 200 and 90 kDa and a weak 120-kDa band under nonreducing conditions (*top gel horizontal* with Bio-Rad high molecular mass standards); under reducing conditions, 200 kDa band resolves to 60 kDa and 40 and 30 kDa, weak 120 kDa resolves to weak 60 kDa (arrow), and strong 90 kDa resolves to 40 and 30 kDa. (E) Anti-7S mAb shows 60- and 40-kDa bands. (F) RATP shows all bands, including weak 60 kDa (arrow). Color molecular mass standards are to the left of blots. The experiment was replicated twice.

Discovery of a 90-kDa IgY antigenically similar to 7S IgY

mAbs against 7S and 5.7S recognized antigenically distinct molecules; however, the multiple bands pulled down by immunoprecipitation of turtle serum with anti-7S mAb suggested presence of aggregates or nonconventional structure for this molecule. To clarify this, we resolved proteins immunoprecipitated by anti-7S or anti-5.7S mAbs in one dimension (X) under nonreducing SDS-PAGE and ran the bands from those gels in a second dimension (Y) using reducing SDS-PAGE.

On nonreducing SDS-PAGE, proteins captured by anti-5.7S mAb resolved into a faint 200-kDa band and strong 120-kDa band. On reducing SDS-PAGE, the 120-kDa band resolved to a 40-kDa (H chain) and 30-kDa diffuse (L chain) band (Fig. 4A), with the 40-kDa band being recognized by both anti-5.7S mAb (Fig. 4B) and RATP (Fig. 4C), whereas the weak 200-kDa band resolved to a weak 60-kDa band recognized by RATP only (Fig. 4C).

On nonreducing SDS PAGE, proteins captured by anti-7S mAb resolved to strong 200- and 90-kDa bands and a weak 120-kDa band (probable aggregate) (Fig. 4D). On reducing SDS-PAGE, the 200-kDa band resolved to a 60-kDa band, and a 40-kDa band was recognized by anti-7S mAb (Fig. 4E) and RATP, whereas the 30-kDa band was detected by RATP only (Fig. 4F). The weak 120-kDa band resolved to a weak 60-kDa band recognized only by RATP. The strong 90-kDa band resolved to a 40-kDa band recognized by both RATP and anti-7S mAb and an additional 30-kDa diffuse band (L chain) recognized by RATP only (Fig. 4F).

To determine whether all turtles had a 90-kDa moiety, we repeated the exercise illustrated in Fig. 4 using plasma from a green turtle that had a very weak 40-kDa band when probed with anti-7S mAb on WB (Fig. 2, *lane 10*). On nonreducing SDS-PAGE, this animal had a single strong band at 200 kDa that on reducing conditions resolved to 60-, 40-, and 30-kDa bands. Both the 60- and 40-kDa bands were recognized by anti-7S mAb and RATP. No 90-kDa band was present, indicating that not all turtles have a 90-kDa moiety (Supplemental Fig. 1).

Two-dimensional SDS-PAGE suggested that 5.7S IgY was a 120- to 140-kDa monomer consisting of two H chains (40 kDa each) and two L chains (30 kDa each). In contrast, anti-7S mAb recognized two antigenically related molecules, a 200-kDa moiety (7S IgY) that resolved into two disulfide-bonded unequally sized H chains (60 and 40 kDa) and L chain (30 kDa) and a separate 90-kDa molecule that resolved into a 40-kDa H chain and a 30-kDa L chain.

Specificity or affinity of 90-kDa moiety varies with antigenic stimulus

To further clarify the respective role of the 200- and 90-kDa molecules in turtle immune response, we immunoprecipitated

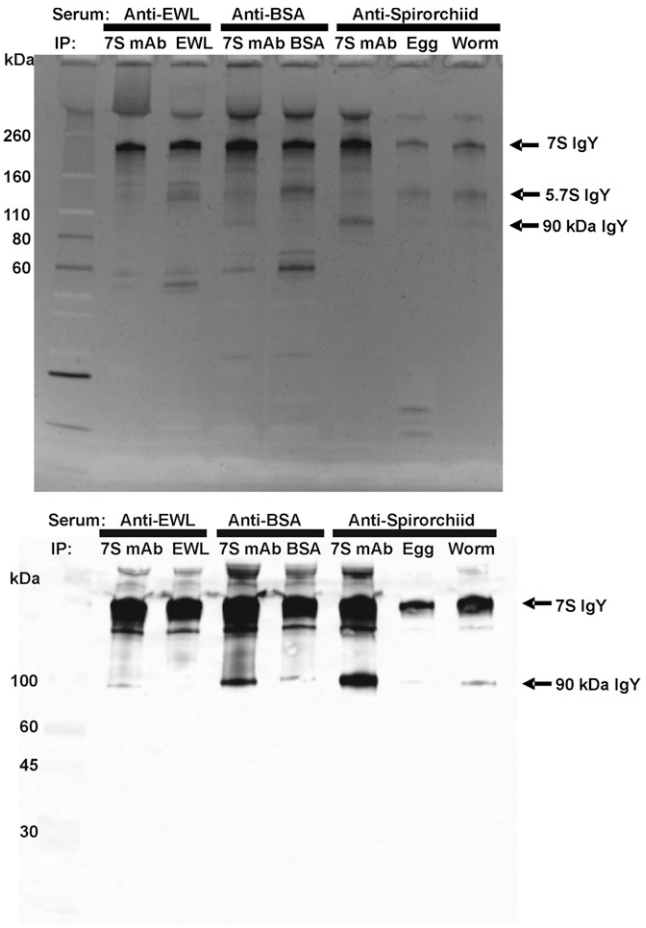

FIGURE 5. Anti-EWL serum (*lanes 2* and *3*), anti-BSA serum (*lanes 4* and *5*), and anti-spirorchiid serum (*lanes 6–8*) were immunoprecipitated (IP) with anti-7S mAb or corresponding Ags, eluates were resolved on a nonreducing 4–15% gradient SDS-PAGE, and gel was stained with silver (*top*) or the blot was probed with anti-7S mAb (*bottom*) with molecular mass standards (*lane 1*). Both 7S and 90-kDa IgY are captured by anti-7S mAb (*lanes 2*, *4*, and *6*), albeit weakly for the anti-EWL plasma (*lane 2*). For Ag-specific immunoprecipitation, 90-kDa IgY is captured by BSA (*lane 5*) and spirorchiid egg and worm (*lanes 7* and *8*) but not by EWL (*lane 2*); 7S IgY is captured by all Ags. Additional bands on gel are likely aggregates. The experiment was replicated twice.

hyperimmune turtle anti-BSA serum, anti-EWL serum, and anti-soluble spirorchiid worm or anti-soluble spirorchiid worm egg serum against their respective Ags or anti-7S mAb coupled to Sepharose beads. Eluates from these immunoprecipitations were then probed on WB using anti-7S mAb. The 200-kDa band was captured by all four Ags (EWL, BSA, soluble spirorchiid worm and egg Ag, and anti-7S mAb). In contrast, the 90-kDa band was only captured by anti-7S mAb, BSA, or soluble spirorchiid worm and egg Ag (Fig. 5), with the weakest 90-kDa presence in anti-EWL plasma.

7S IgY has asymmetrical Fc

Based on electrophoresis and WBs, turtle IgY seemed to have unconventional structures. To clarify this, we immunoprecipitated 7S and 5.7S IgY with respective mAbs and examined them by transmission electron microscopy. Two-dimensional class averages of individual 5.7S IgY molecules showed that the Ab consists of five domains arranged in a Y configuration with a truncated Fc arm and Fab arms that demonstrated a striking degree of flexibility (Fig. 6A–C).

The 7S mAb captured 7S IgY molecules that comprised seven domains, five of which were in a Y configuration with an asymmetrical Fc consisting of domains whose exact conformation could not be ascertained based on two-dimensional class averages (Fig. 6D). Similar to the 5.7S molecule, the Fab portion also manifested considerable flexibility (Fig. 6E). A minor population of molecules precipitated by 7S mAb formed two-dimensional class averages that were smaller, consisting of perhaps two to three asymmetrical domains, compatible with a size of 50–80 kDa (Fig. 6F). It is possible that these smaller molecules represented the 90-kDa IgY.

Turtle IgYs can be purified with protein G and melon gel but not with protein A

Unlike birds and reptiles, mammalian IgGs bind to proteins A and G (27). To test this, we assayed turtle serum on matrices commonly used to purify mammalian IgG, including protein G, protein A, and melon gel. Protein G and melon gel captured 7S and 5.7S IgY, whereas protein A very weakly captured 7S IgY and not 5.7S IgY (Fig. 7).

IgY does not have a hinge

To gain insight into the protein and nucleic acid sequences of turtle IgY, representative bands of H chains resolved on reducing SDS-PAGE were excised and analyzed by mass spectrometry after trypsin digestion. Peptides were then used to identify hypothetical IgY proteins in Swiss-Prot that were then blasted (tblastn) against whole-genome shotgun contigs of green turtles to design primers to amplify mRNAs (Supplemental Table I). Fifty-one peptides from digests of heavy bands blasted to 21 different hypothetical green turtle IgY proteins, of which 17, 2, and 12 were unique to 7S 60-kDa, 7S 40-kDa, and 5.7S 40-kDa H chains, respectively. Additionally, one peptide was common to both H chains of 7S, six were common to the 60-kDa H chain of 7S and the 40-kDa H chain of 5.7S, one was common to the 40-kDa H chains of 7S and 5.7S, and 12 were common to all chains of both IgYs (Supplemental Table I).

Because IgY manifested flexibility of Fab on electron microscopy, we explored whether this molecule might have a hinge. We used the information from the peptides above to query the whole-genome shotgun contig of green turtle and design primers to amplify cDNA coding for turtle IgY. This yielded two sequences of IgY (GenBank accession nos. KT698944 and KT698945; http://www.ncbi.nlm.nih.gov/genbank/) that when aligned with human IgG1 H chain (GenBank accession no. BAN63131.1) overlapped with, but did not have, the proline-rich AA region characteristic of a hinge as defined in humans (28) (Supplemental Fig. 2).

Chromatography shows that we were not detecting IgM

Because some of the peptides we obtained blasted to IgM, we resolved the 7S and 5.7S IgY on gel filtration chromatography where IgM elutes in void volume (29). Crude SAS-cut IgY was resolved on a Sephacryl 300 gel filtration column, and column fractions were probed with anti-7S and anti-5.7S mAbs. Gel filtration chromatography revealed two peaks, a void volume containing large (70-kDa) H chains characteristic of green turtle IgM (29), with 7S IgY eluting slightly before but concomitantly with 5.7S IgY in a second peak. Lack of a lone 40-kDa band only reacting to anti-7S mab indicated that the 90-kDa moiety did not elute separately on gel filtration (Supplemental Fig. 3).

Discussion

Abs of turtles (and other vertebrates) have historically been thought to consist of bilaterally symmetrical H and L chains (11, 30). In the

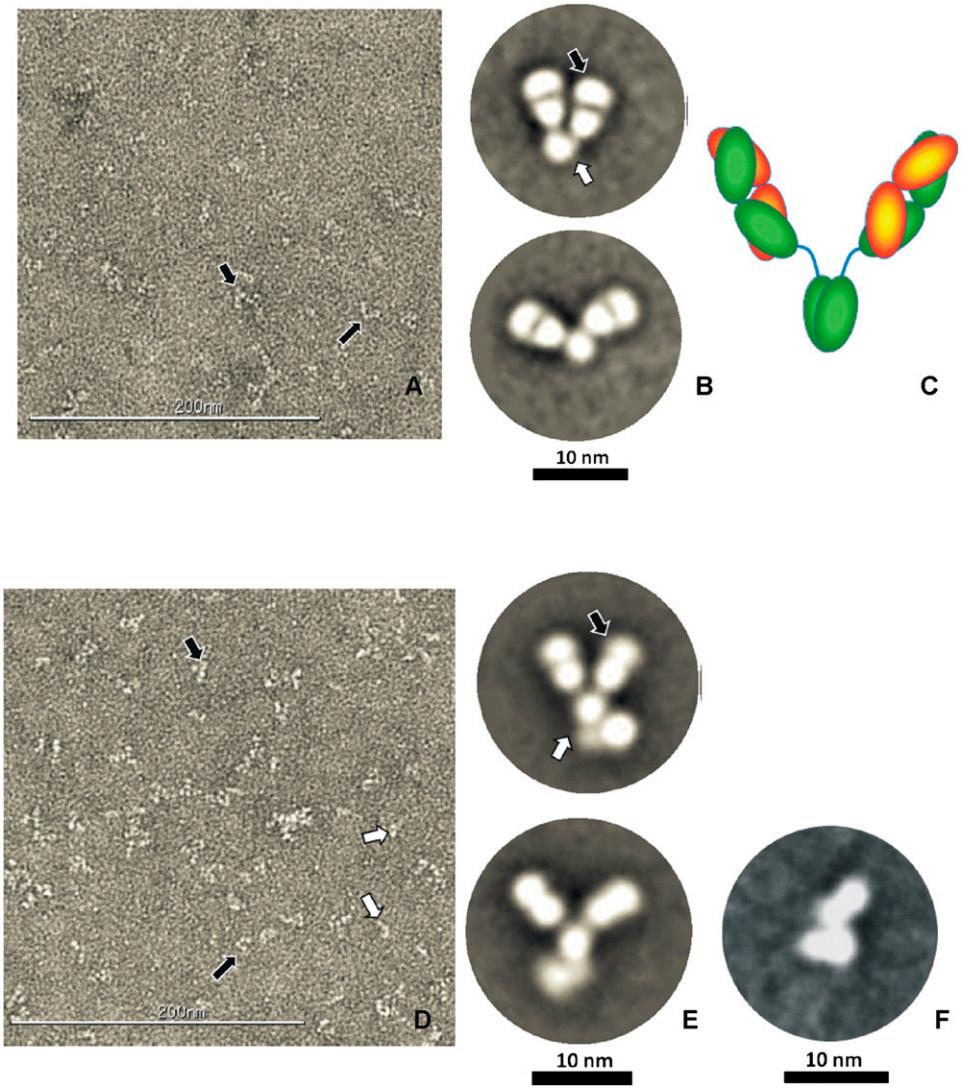

FIGURE 6. Electron micrographs of 5.7S IgY (**A**–**C**) and 7S IgY (**D**–**F**) captured by immunoprecipitation. (A) Negatively stained 5.7S IgY (arrows). (B) Exemplary two-dimensional class averages for 5.7S IgY. Arrow points to putative Fab (black arrow) connected to truncated Fc (white arrow). Note flexibility of Fab arms (contrast *top* and *bottom*). (C) Hypothesized structure of 5.7S IgY with disulfide-linked dual H chains (green) linked by disulfide bonds to L chains (orange). The entire molecule has an estimated molecular mass of 120–140 kDa that resolves under reducing SDS-PAGE to 40-kDa H and 30-kDa L chains. (D) Negatively stained 7S (black arrows) (white arrow points to smaller particle also detected in the preparation). (E) Exemplary two-dimensional class averages for 7S IgY. Arrow points to putative Fab (black arrow) connected to asymmetrical Fc (white arrow). Note flexibility of Fab arms (contrast *top* and *bottom*). Although the Fab arms of 7S appears to have some commonalities with 5.7S IgY, the actual structure of the Fc portion cannot be definitively deduced based on available data. (F) Two-dimensional class averages for 90-kDa IgY. These smaller particles appear to have a molecular mass anywhere from 50–80 kDa based on sizes of domain structure. The experiments were done twice.

present study, we show that, in addition to confirming morphologically that 5.7S IgY has a truncated Fc (11), green turtles also have a structurally asymmetrical 7S IgY consisting of a 200-kDa moiety with two differently sized H chains and an antigenically related 90-kDa moiety with at least one H and L chain. The molecular masses of the denatured 5.7S IgY conformed to two 40-kDa H chains and two 30-kDa L chains adding up to a molecule of 120–140 kDa under nonreducing conditions. In contrast, there was a molecular mass discrepancy for the 7S IgY. At a minimum, this molecule would consist of single 60-kDa H chain, a single 40-kDa H chain, and one to two 30-kDa L chains for a total of 130–160 kDa, which is less than the 200 kDa seen under nonreducing conditions. Because gel electrophoresis does not allow one to tease out just how many of each component comprise the 7S IgY, one possible explanation is that additional L or H chains exist to bring the molecular mass to 200 kDa. This would in part explain the unconventional structure of this molecule on electron microscopy.

Another potential explanation for the unconventional Fc structure of 7S IgY might be that this region is flexible. IgY is evolutionarily most closely related to IgG and IgE (2). Similar to IgY, IgE does not have a hinge, and recent studies indicate that the Fc portion of this molecule folds back upon itself (31), which may explain the unusual appearance of the Fc in 7S IgY. Other Igs also have flexible Fc. For instance, when binding to Ag, the Fc portion of pentameric human IgM flexes to adopt a staple form (28).

Turtle IgY did not have the proline-rich AA sequence that characterizes the hinge in other vertebrates (32). However, the lack of a hinge region in IgY clearly did not preclude flexibility of Fab arms of both 7S and 5.7S IgY as seen in electron microscopy. The proline-rich region characteristic of the human Ig hinge is thought to confer flexibility to the molecule (32). However, electron microscopy studies of human IgE and IgM, both of which do not have a hinge, revealed that both also have considerable flexibility in the Fab region (33). Indeed, the authors of that study suggested that compared with electron microscopy, other methods used to predict structural movements of Igs such as x-ray crystallography or AA sequences may not be as reliable.

Although unconventional Abs have been seen in other animals such as H chain–only Ig in camels (5), or natural Ag receptors in sharks (34), all of these have been bilaterally symmetrical. Humans have asymmetric Igs with a different glycosylation pattern in one of the Fab arms, making them univalent and unable to precipitate Ags; these Abs play important roles in maternal immunity (35). However, unlike the asymmetric IgY of green turtles, the asymmetry in human Abs is only in glycosylation and not the H chain. Thus, to our knowledge, our findings are the first to document naturally occurring structurally asymmetrical Abs in vertebrates where the asymmetry is manifested in the polypeptide backbone of the molecule. It is entirely possible that other similar asymmetrical H chain Abs exist and have been previously

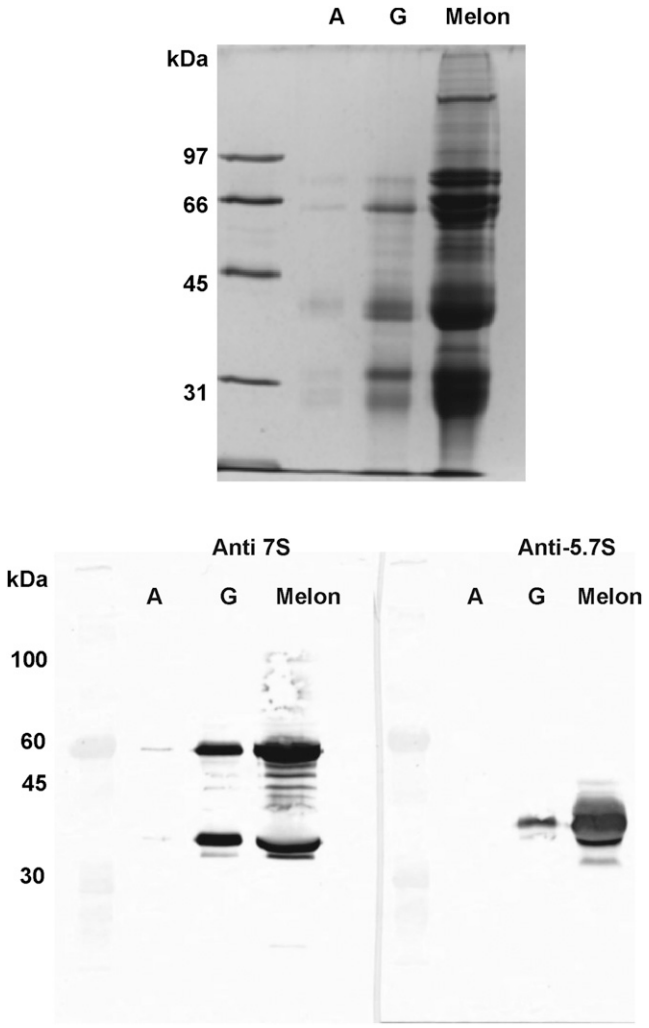

FIGURE 7. Eluates of turtle serum incubated with protein A, G, and melon run on 10% reducing denaturing SDS-PAGE and stained with silver (*top*) or transferred to blots and probed with anti-7S or anti-5.7S mAb with molecular mass standards (*lane 1*). Note that protein G and melon gel capture both 7S and 5.7S IgY, whereas protein A much less so. All matrices were incubated with 500 μg turtle serum proteins and eluates run at 1:2 dilution. Results are representative of three experiments.

misidentified. For instance, Abs similar to what we saw on reducing SDS-PAGE for IgY in sea turtles have been observed in chickens (36), snakes (37), ducks (38), sea turtles (29), and nurse sharks (39), but they have been explained away as contaminants or ignored.

Based on antigenic relationships by radioimmunoassays, reptile Igs were thought to be more closely related to birds (40); however, molecular analyses place reptilian Igs closer to those of amphibians and mammals (41). The reality is that turtle Igs probably contain characteristics of both mammalian and avian Igs. For example, similar to mammals (42), both 7S and 5.7S turtle IgYs appear to bind preferentially to protein G over protein A, which contrast with Igs of birds that are not known to bind to either proteins (27). However, other studies show that both the full and truncated forms of duck IgY will bind to protein A but less so to protein G (38), and snake IgY will bind to protein A (37), whereas snapping turtle 5.7S IgY will not (43). Similar to mammals (but not birds), turtle Ig can be purified using substrates such as melon gel (17). Unlike birds and mammals, green turtle Igs require high salt to bind to Ags in laboratory assays such as ELISA (13, 29).

The exact nature of the 90-kDa moiety antigenically related to 7S IgY remains enigmatic. Nonreducing SDS-PAGE of 7S IgY specifically purified with either cognate Ag or by anti-7S immunoprecipitation suggests that the 7S IgY complex consists of noncovalently associated 200- and 90-kDa moieties. Upon reduction, the 200 kDa resolves to 60- and 40-kDa H chains and a 30-kDa L chain, whereas the 90 kDa resolves to a 40-kDa H and 30-kDa L chain (Fig. 4). Support for the idea that the 200 and 90 kDa are noncovalently associated is seen in the WB profile of gel filtration (Supplemental Fig. 1) where the 90- and 200-kDa moieties appear to elute concomitantly. If the 90-kDa moiety did exist independently in serum, we would have expected to see it late in the elution profile manifested as a single 40-kDa band reacting with anti-7S mAb under reducing SDS-PAGE. Clearly, however, not all green turtles have equal amounts of the 90-kDa moiety.

The findings of the 90-kDa moiety reacting with soluble spirorchiid egg and worm Ag and BSA but not EWL does not necessarily detract from the idea that it is noncovalently associated with the 200-kDa portion of 7S IgY, but rather may be evidence of some form of affinity maturation. The only difference we could see with these Ags was the amount and intensity of antigenic stimulation. Turtles are exposed to spirorchiid Ags chronically over their lifetime (15), and, in the study by Benedict and Pollard (11), turtles were immunized with BSA multiple times, although a specific immunization regimen was not provided. In contrast, turtles immunized with EWL received just two injections (one initially and then a booster). Thus, the 90-kDa moiety could be a marker of chronic Ag exposure in sea turtles or simply have higher affinity to these particular Ags. Interestingly, when green turtle Igs were first characterized using centrifugation studies, four molecules were detected: 17S, 7S and 5.7S corresponding to IgM, 7S and 5.7S IgY, and an additional smaller 3.8S molecule that was never further investigated (11). The 90-kDa molecule in our study may be related to this 3.8S moiety. Teasing this apart will require developing methods to better differentiate affinity of reptilian Ab to various Ags.

Kinetics of Ab response for Pacific green turtles mirrored those of Atlantic green turtles where a robust 7S IgY response occurs 5–7 wk postimmunization, whereas the 5.7S response arises 8–10 mo postimmunization in Atlantic greens (29) and ~5 mo after inoculation and boosting in Pacific greens. However, direct comparisons between kinetics of immune response in Florida and Hawaiian green turtles is complicated by the fact that both studies used vastly different immunization regimens: Florida turtles were immunized biweekly (29), whereas Hawaiian turtles received a single immunization and one booster 21 wk later, which allowed the detection of an anamnestic response (13). There was a marked drop of 7S Abs at week 22 for Hawaiian turtles. Boosting was not responsible for this, because animals were bled just prior to boosting on the day they were boosted. It is unlikely that the use of mAbs in ELISA was responsible, because identical results to the 7S IgY response were obtained with RATP using the same set of samples (13). Rather, we suspect we happened to boost turtles at a time when Abs were naturally declining following single immunization. Indeed, a similar Ab decline after a peak response has been documented in the snake *Psammophis sibilans* (44) and lizard *Calotes versicolor* (45) receiving a single immunization of rat RBCs.

The role of 5.7S IgY in host response is unclear, and this Ab is found only in ducks (46) and turtles (29, 30). In ducks, truncated IgY is thought to be involved in skin sensitization (12), predominates over IgY late in the immune response, and does not have effector functions such as opsonization, complement fixation, or hemagglutination (47). The presence of 5.7S response only late in the course of immunity against egg white lysozyme suggests that this Ab in green turtles probably functions similarly to that of anseriforms.

Other investigators have shown that 5.7S and 7S IgY in turtles share antigenic epitopes (11, 30). In contrast, our mAbs against 7S do not react with 5.7S IgY and vice versa, indicating that these two molecules are antigenically distinct. We suspect in this case that the difference in findings is due to methodology and possible cross-contamination of 7S with 5.7S. Prior studies purified 7S and 5.7S using DEAE or Sephadex, but these two molecules are notoriously difficult to separate by column chromatography (30). In contrast, we used immunoprecipitation to capture IgY. Presumably, had shared epitopes been present, we should have detected them on molecules captured with one mAb and probed with the other.

Most contemporary studies on phylogeny and structure of reptilian Ig have focused on molecular aspects (6), with no functional bases upon which to interpret molecular findings in part because studies such as ours focusing on function and structure are relatively rare. Future attempts to interpret molecular biology of reptilian Ig in light of form and function could yield a richer and more complete picture of the immune system of reptiles. Such approaches might also provide answers to broader questions such as host response to pathogens such as the tumor-associated herpesvirus in green turtles and the role of host immunity in explaining the presence of or disappearance of disease in animal populations (48).

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Disclosures

The authors have no financial conflicts of interest.

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Supplementary Table 1. Peptides obtained for heavy chain bands from 7S and 5.7S IgY by mass spectrometry and Blastp result of peptides against uniProt with lowest E values.

7S-40 kDa	7S-60 kDa	5.7S-40 kDa	Peptides (multiple peptides separated by "**/**")	Uniprot	Name
		x	ATISVDPSKNEFYLR**/** SLAAADTGRYYCAR**/** SSQWYTNYASSLQSR**/** TTIAQDTSKNQFSLQLR**/** QVQLVESGAGVKKPGDSLR	M7AHU3	Ig heavy chain V-II region ARH-77
x	x	x	LLIYLASTLQSGVPAR	M7ANJ7	Ig kappa chain V-IV region
		x	FSGSGSGTDYTLTINR		
x			EFSLQLSTLTTADTATYYCAR	M7AP69	Ig heavy chain V-II region COR
	x		LTCSVSGYSLTSR		
		x	LLIYSASTRPSGIPDR	M7AQD6	Ig kappa chain V-III region SIE
		x	LLIYSASTRPSGVPDR	M7AV00	Ig kappa chain V-IV region B17
x	x	x	GLLQSDTDLAKKDADGHTFSTR	M7AYG9	Ig epsilon chain C region
x	x		HPGTIQLVCFISGFYPEPLTVQWLVNGER		
	x		SNASVSQDEWLEGKTYTCQVYHPGTGSK		
x	x	x	NHKADESMEYFTTQPIKDGAGDSNFFLYSK**/** DWDAGETFTCKVEHSELPSPLIK**/** ASWNSGDTYTCMVIHEALQMKFTQR**/** GFFPEDISVQWMKNHK**/** RSGPGIYLLRPHNDELSSSEDSVSLTCLVR	M7AZC8	Ig epsilon chain C region**/** Ig gamma-1 chain C region, membrane-bound form
		x	VEHSELPSPLIKSISK		
	x		HNDELSSSEDSISLTCLVR		
x			LTCLVVNLPSDSGLQVVWSR	M7B4Z6	Ig gamma-1 chain C region, membrane-bound form
x	x	x	ADESMEYFTTQPIKDGAGDSNFFLYSKLK		
	x		EKPGSIVPDPLTLTEQFNSSFTASSSMPIFTR		
	x		LKVNKASWNSGDTYTCMVIHEALQMK		
x	x	x	QQDTSDSTYSLSSTLTLTK	M7BF29	Ig kappa chain C region
x		x	ESAKPSVFIFHPSADQLSGGSATVVCLVSGFYPSALNVVWK		
	x		QAPGKGLEWVADIK**/** KLIYLQMTGLKPEDTGR**/** QAPGQGLEWVSAINGATKYYGDSVKGR	M7BHK9	Ig heavy chain V region 3

		x	GLEWVADIKSDGSSQWYSPAVQGR		
	x	x	QVQLLESGGDVKKPGDSLR		
	x		TSGFNIDSYWMSWVR		
	x		NRVTISTENSISTVYLQLR	M7BMN6	Ig heavy chain V-I region HG3
	x	x	SSVSNHYSPIVQGR**/** QVVLTQSGPEVK**/**	M7BS21	Ig heavy chain V region 3
	x		GLQWLVSYWK		
		x	DTSKSEVYLEMRGMEAGDSGIYYCAR**/** QVKLLQTGAAQVKPSQTLR**/**	M7BU10	Ig heavy chain V-II region SESS
	x	x	LTISRDTSKSEVYLEMR		
x	x	x	FSGSGSGTDFTLTISR	M7BUG8	Ig kappa chain V-III region SIE
	x		FSGSGSGTDYTFTISR		
x	x	x	TGYATITANEFKGR	M7BW25	IgW heavy chain V region W26
	x		RPSGIPDRFSGSGSGTDFTFTISR	M7BYT0	Ig kappa chain V-III region HIC (Chelonia mydas)**/** Uncharacterized protein (Pelodiscus sinensis)
	x	x	QIVLTQTPESLAVSPGDRVTINCK		
	x		QVVLTRSGPALK**/** QAPGKGLEWIGEIHPTSTTINYAQPFK**/** GLEWIGEIHPTSTTINYAQPFKGR	M7BZD4	Ig heavy chain V region 3
	x	x	FTISRDNPNNLLYLQMTGLKSEDTAR		
	x		VVHPDLIFPIEK	M7C1F4	Ig mu chain C region
	x		QPVTNSHAPSIYIFPPPSEQLALR	M7CCQ8	Ig mu chain C region (Chelonia mydas)
	x		EVQLVQSGAEMK	M7CKZ0	Ig heavy chain V-I region HG3**/** Ig heavy chain V-I region HG3

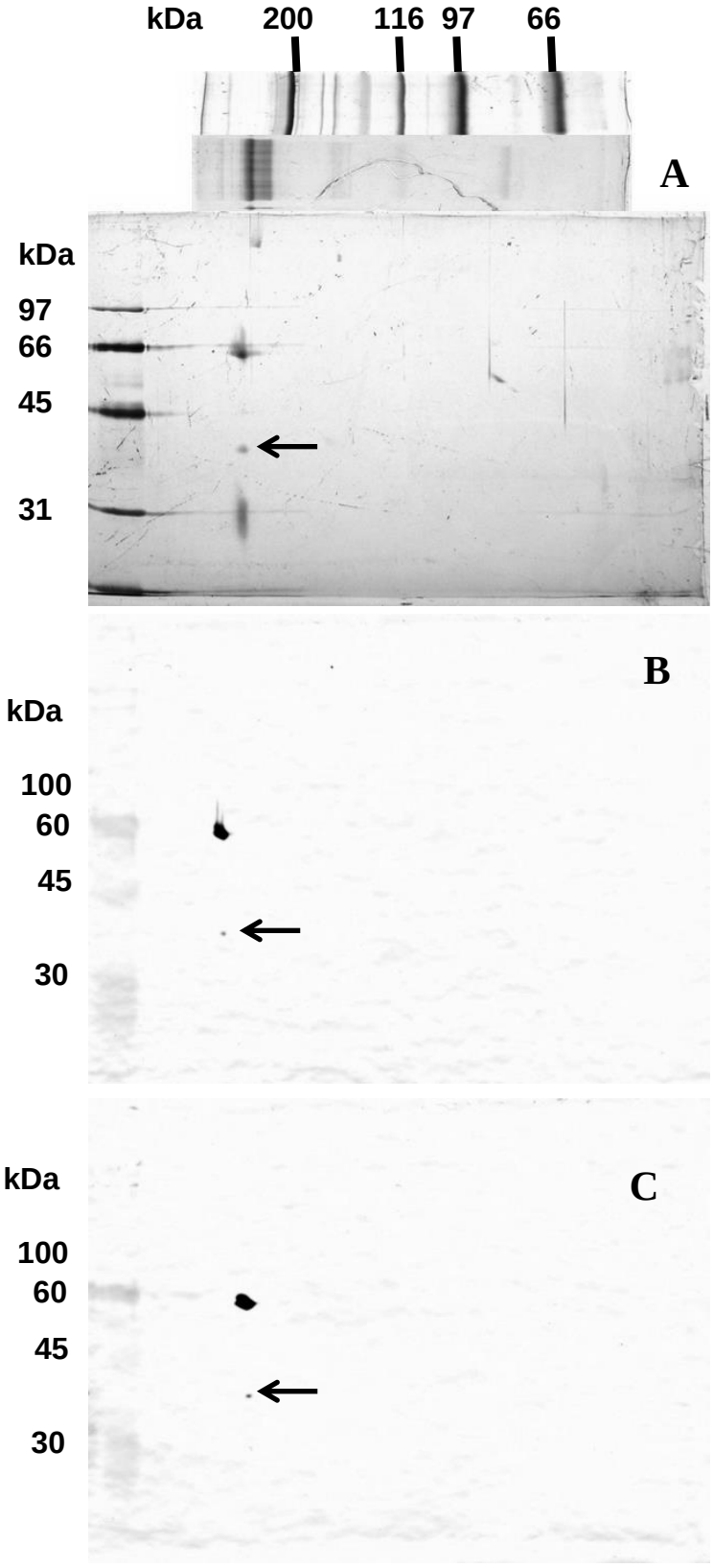

Figure S1, Plasma from a green turtle (Lane 9, Fig 2) with weak 40 kDa band resolved horizontally under non-reducing conditions and then vertically under reducing conditions. Note

presence of strong 200 kDa band that resolves to 60 kDa, 40 kDa, and 30 kDa on silver stain (A). The 60 kDa and faint 40 kDa bands (arrows) are recognized by both anti-7S mAb (B) and RATP (C) whilst 30 kDa band is not recognized by either mAb. Note absence of or very weak secondary bands on silver stain under non-reducing conditions. This experiment was replicated twice.

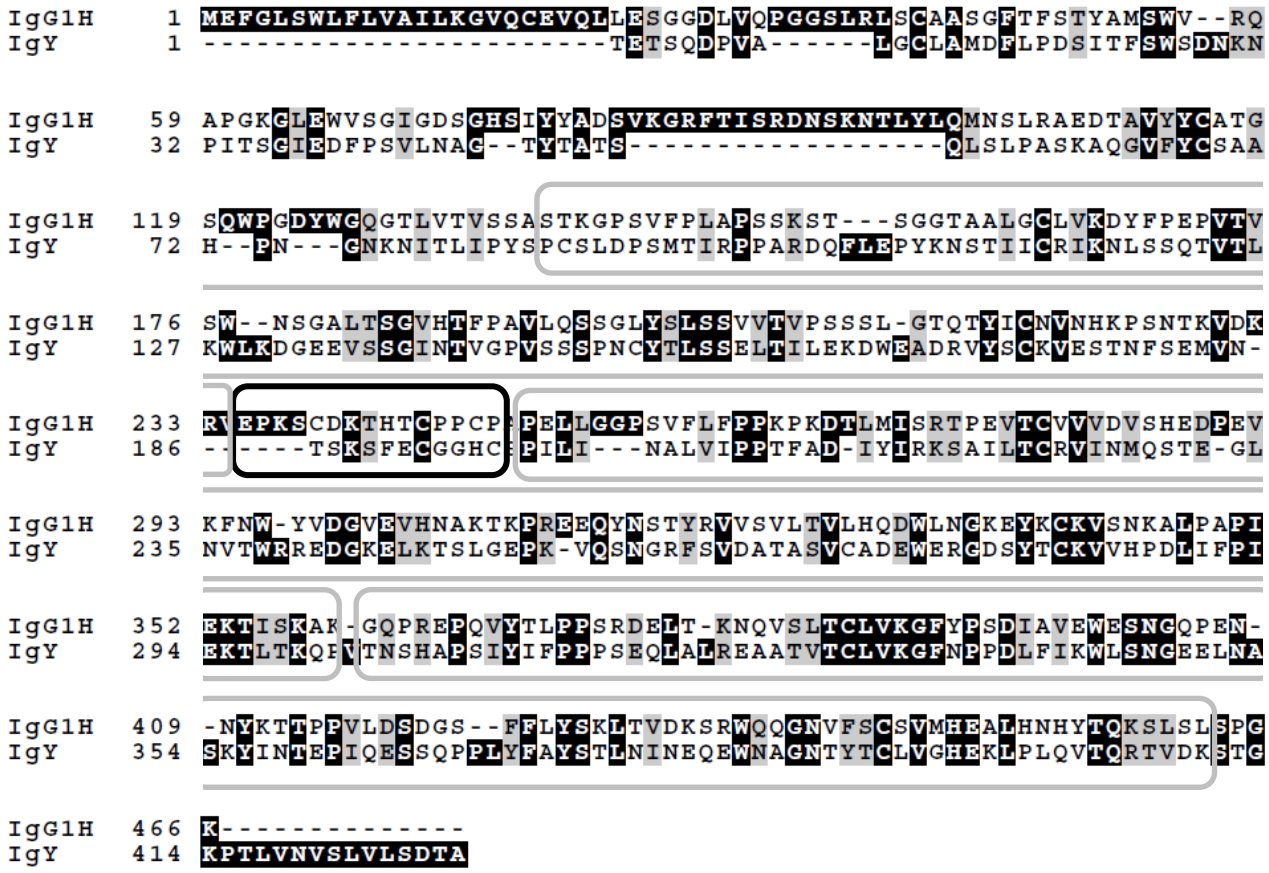

Figure S2. Alignment of turtle IgY to human IgG1 heavy chain with constant regions 1-3 (top to bottom) circled in grey and hinge region (circled in black). Note lack of proline-rich region (CPPCP) characteristic of IgG1 lower hinge in turtle IgY.

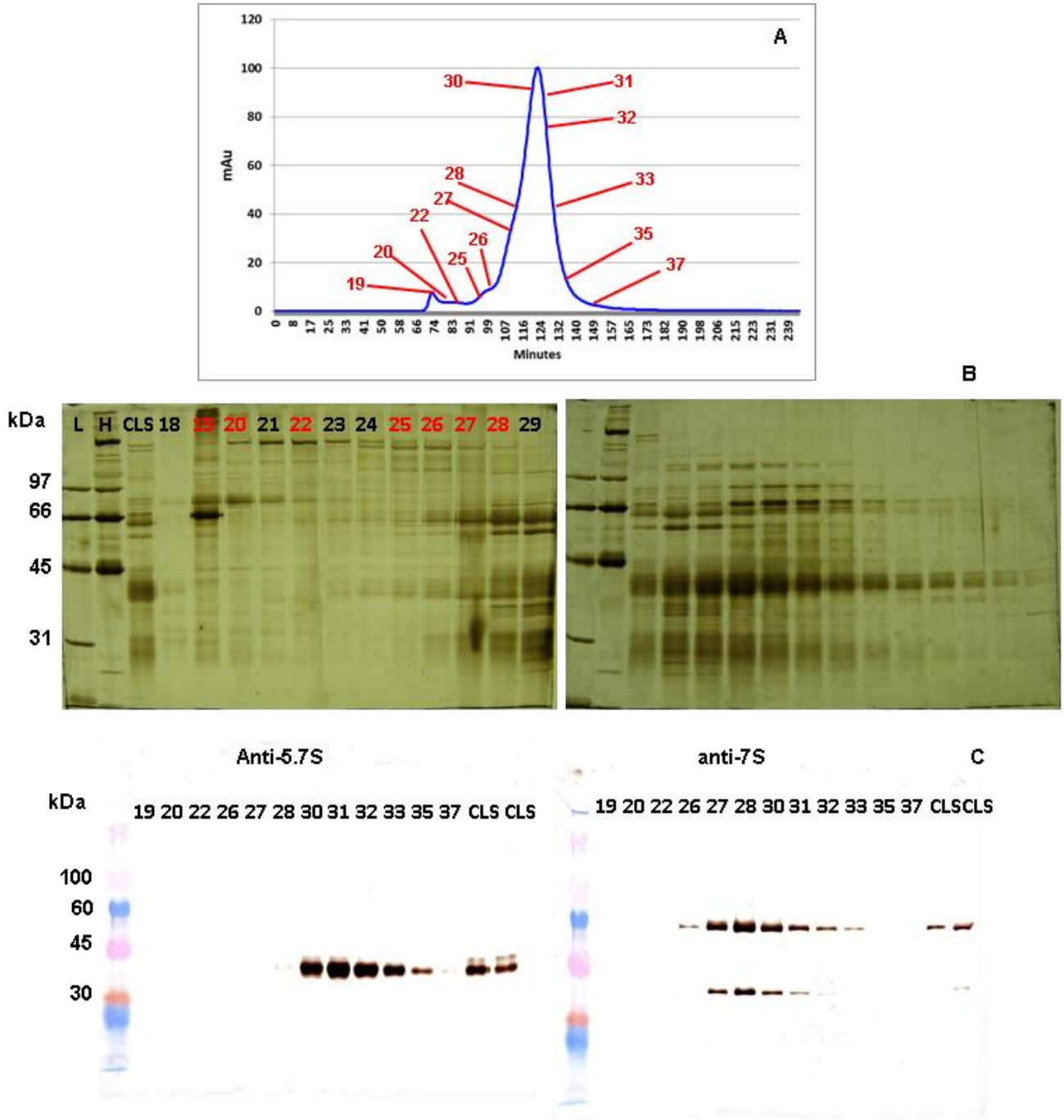

Figure S3. (**A**) Elution profile of 33% SAS cut crude IgY from the same turtle as in Figure 4 on Sephacryl 300 with red numbered fractions selected for reducing-SDS-PAGE and WB. (**B**) Silver stain of 10% gel of fractions (red numbers are those fractions assayed by WB). (**C**) WB

of fractions probed with anti-5.7S and anti-7S mAbs . Note that 7S and 5.7S IgY co-elute after the void volume (peak 1) indicating they are not IgM. Note also that the 90 kDa moiety fails to elute separately as evidenced by the absence of a single 40 kDa band reacting only with anti-7S mAb. CLS indicate column loading samples and molecular weight markers on 2 left lanes. Experiment done twice.