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Identification of avian corticosteroid-binding globulin (SerpinA6) reveals the molecular basis of evolutionary adaptations in SerpinA6 structure and function as a steroid-binding protein

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ABSTRACT

Corticosteroid-binding globulin (CBG) was isolated from chicken serum and identified by mass-spectrometry and genomic analysis. This revealed that the organization and synteny of avian and mammalian *SerpinA6* genes are conserved. Recombinant zebra finch CBG steroid-binding properties reflect those of the natural protein in plasma, and confirm its identity. Zebra finch and rat CBG crystal structures in complex with cortisol resemble each other, but their primary structures share only ~40% identity and their steroid-binding site topographies differ in several unexpected ways. Remarkably, a tryptophan that anchors ligands in mammalian CBG steroid-binding sites is replaced by an asparagine. Phylogenetic comparisons show that reptilian CBG orthologs share this unexpected property. Glycosylation of this asparagine in zebra finch CBG does not influence its steroid-binding affinity, but we present evidence that it may participate in protein folding and steroid-binding site formation. Substitutions of amino acids within zebra finch CBG that are conserved only in birds reveal how they contribute to their distinct steroid-binding properties, including their high (nM) affinities for glucocorticoids, progesterone, and androgens. As in mammals, a protease secreted by *Pseudomonas aeruginosa* cleaves CBG in zebra finch plasma within its reactive center loop and disrupts steroid binding: suggesting an evolutionarily conserved property of CBGs. Measurements of CBG mRNA in zebra finch tissues indicate that liver is the main site of plasma CBG production, and anti-zebra finch CBG antibodies cross-react with CBGs in other birds, extending opportunities to study how CBG regulates the actions of glucocorticoids and sex steroids in these species.

Corticosteroid-binding globulin (CBG) is present in the blood of mammals, reptiles, amphibians and birds (1). In these vertebrate subphyla, CBG transports glucocorticoids (cortisol and corticosterone) and regulates their access to tissues and cells (1, 2). To date, only mammalian CBGs have been characterized at the molecular level (3-7). The primary structure of human CBG defines it as a serine proteinase inhibitor (Serpin) family member (3), and its gene (*SERPINA6*) is located in a cluster of related clade A *SERPIN* genes (8, 9) with synteny across other mammalian genomes (10). The crystal structures of rat (11) and human (12, 13) CBGs have been solved in complex with steroid ligands. These and other biochemical studies (14, 15) have revealed how specific residues in mammalian CBGs participate in steroid binding, including a conserved tryptophan that positions and holds steroids in their binding pocket (15). Importantly, this "signature tryptophan" residue distinguishes CBGs from other clade A Serpins in mammals, and its absence in related *SerpinA* sequences of other terrestrial vertebrate species has hampered the identification of their *SerpinA6* genes.

In mammals, plasma CBG has 5-6 sites for N-glycosylation depending on the species (3-5, 7, 9). One of these sites is strictly conserved and appears essential for high affinity steroid-binding

activity (15, 16). In addition, like other Serpins, CBG has an unstructured reactive center loop (RCL) that is targeted by specific proteases (15). Proteolysis of the RCL in many Serpins is an essential step in their inhibition of specific proteases. Proteolysis of the RCL of human CBG by neutrophil elastase (17) or chymotrypsin (18), or exogenous bacterial proteases, such as *LasB* (19), causes a conformational rearrangement in its tertiary structure, as observed in other Serpins (12). However, instead of inhibiting these proteases, proteolysis of the CBG RCL irreversibly disrupts its high affinity steroid-binding activity (17), and serves to promote the delivery of CBG-bound ligands to locations where these proteases are present (2, 17).

In birds, the affinity of CBG for progesterone is as high as for corticosterone if not higher (20). Avian CBGs also bind the androgens, testosterone and 5α-dihydrotestosterone (DHT), with nM affinities (20, 21), and these steroid-binding properties distinguish them from mammalian CBGs. The high affinity of avian CBGs for androgens is considered important because birds lack a sex-hormone binding globulin (SHBG), and CBG is thought to substitute for SHBG in transporting androgens and regulating their actions in these species (21). Plasma corticosterone and CBG levels in birds are sexually dimorphic (22) and undergo marked seasonal changes in parallel with each other (23-25). Given the role of CBG in determining the biological activities of multiple classes of steroids in birds, which are widely used as models to study endocrine, neural and behavioral responses to various stressors (26-28), we set out to identify and characterize the *SerpinA6* genes and their products in avian species. In doing so, we also expected to gain insight into how *SerpinA6* has evolved to accommodate the specialized functions of CBG in different vertebrate classes.

EXPERIMENTAL PROCEDURES

Animals and tissues—Blood and tissue samples from chickens (*Gallus gallus*), song sparrows (*Melospiza melodia*) and zebra finch (*Taeniopygia guttata*) were obtained under protocols approved by The University of British Columbia Animal Care Committee in compliance with regulations established by the Canadian Council on Animal Care. Chicken serum for CBG purification was obtained commercially (Sigma Aldrich). Otherwise, trunk blood samples were taken after decapitation and prepared as heparinized plasma for storage at -80°C until analysis. Tissues samples were snap frozen on dry ice and also stored at -80°C until analysis.

Isolation and identification of chicken CBG—Chicken serum (10 ml) was diluted 1:1 in phosphate buffered saline (PBS) for affinity chromatography using 3-oxo-17β-hydroxy-5α-androstane-17α-(6-hexyn-l-ol) linked to diamino-ethyloxirane-Sepharose CL4B (29). Following extensive washing, 1ml fractions were eluted from the steroid-affinity column with 200 μM cortisol in PBS, and fractions containing cortisol-binding activity were pooled for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were visualized in Coomassie Blue stained SDS-PAGE gels. Three major protein bands, with apparent MWs of ~65, ~55 and ~24 kDa, were excised for mass spectrometry analysis (Fig. 1). Each band was in-gel digested, as described (30) and analyzed by liquid chromatography-tandem mass spectrometry on an Agilent Q-TOF 6550, as described (31). Proteins were identified from tandem mass spectra using Mascot (v2.3, Matrix Science) via Proteome Discoverer (v1.2, Thermo Fisher) to search a database of all SWISS-PROT protein sequences under Metazoa taxonomy.

Recombinant avian CBG production and purification—The ~55 kDa chicken protein isolated using steroid-affinity chromatography corresponded to that encoded by the chicken *SerpinA4* gene (ENSGALG00000010969), and complete cDNA coding sequences for the chicken and zebra finch orthologs were obtained using gene and species specific oligonucleotides (Table S1), together with RNA templates extracted from chicken and zebra finch livers with an RNAeasy miniprep kit (Qiagen). These cDNAs were cloned into pcDNA3 (Life Technologies) for expression in CHO cells, as described for production of recombinant human CBG (32).

The cloned chicken and zebra finch cDNA sequences were compared to those reported at ensemble.org or GenBank. The chicken cDNA sequence (KU180444) is identical to BX935008.1 (GenBank), while the zebra finch cDNA sequence (KU180443) contained 4 non-synonymous single nucleotide variations when compared to ENSTGUT00000013171.1. As indicated below, these cDNAs encode the chicken and zebra finch CBG precursors, respectively.

The cDNAs encoding chicken and zebra finch CBGs without their predicted signal

polypeptide (see Fig. S1) were cloned into the pPIC9 expression vector (Invitrogen) after PCR-amplification with specific oligonucleotides (Table S1) for expression in yeast (*P. pastoris* GS115 cells). Chicken and zebra finch CBG cDNA-pPIC9 constructs were all linearized with *Stu*I prior to transformation of GS115 cells by electroporation. After selection on minimal medium, yeast colonies were grown to an OD600 of 10 in BMGY medium, which was then replaced with methanol-containing BMMY medium to induce protein expression at 30°C for 24h. Yeast cells were removed by centrifugation and supernatants were harvested. The steroid-binding properties of CBG secreted directly in the culture medium were initially determined without further purification. In studies of zebra finch CBG mutants expressed in yeast, a C-terminal His-tag, which has no effect on steroid-binding activity, was added to aid in purification and concentration by Ni-NTA chromatography (Life Technologies) prior to analysis.

For expression in *E. coli* BL21 cells, a cDNA encoding the predicted mature polypeptide sequence of zebra finch CBG was inserted in frame into the HTMpET vector (33). In brief, transformed *E.coli* were grown in LB with 50 µg/ml Kanamycin until an OD600 of 0.5, when 200 µM IPTG was added and cultures were grown overnight at 30°C. After centrifugation, cell pellets were re-suspended in lysis buffer (5 mM imidazole, 50 mM NaH_2PO_4, 500 mM NaCl, pH 8) and left on ice for 30 min prior to sonication. Cell debris was then removed by centrifugation, and the lysate was clarified using a 0.45 µm filter and applied to a Ni-NTA affinity column (Life Technologies). After elution with 500 mM imidazole, the His-MBP-zebra finch CBG fusion protein was treated with Tev protease to release the CBG, as confirmed by SDS-PAGE. The released CBG was purified by affinity column chromatography using 11β-hydroxy-androst-4-en-3-oxo-17β-carboxylic acid-diaminoethyloxirane-Sepharose CL4B (34), and used for rabbit antiserum production by ProSci Inc (Poway, CA).

Mutagenesis—A QuickChange II kit (Agilent Technologies) was used for mutagenesis of cDNAs with oligonucleotide primer pairs (Table S1), and Sanger sequencing confirmed that only the targeted mutations occurred.

Phylogenetic analyses— Putative chicken *SerpinA6* orthologs were identified within the genomes of other sub-mammalian species at ensemble.org using the ENSGALG00000010969 sequence, or through BLAST searches against the predicted amino acid sequences of chicken and zebra finch CBG. Multiple sequence alignments were made using Clustal Omega (35) and default parameters with the BOX-SHADE program (www.ch.embnet.org/software/BOX_form.html).

Steroid-binding assays, western blotting and proteolysis—A radiolabeled-steroid saturation assay (36) was used to detect and measure CBG in plasma, culture media or chromatographic fractions obtained during protein purification. In brief, samples were first diluted and incubated in a dextran-coated charcoal (DCC) solution in PBS to remove steroids, if necessary. Samples were then incubated (1 h at room temp) with ~10 nM [^{3}H]-cortisol or [^{3}H]-corticosterone (American Radiolabeled Chemicals), in the absence or presence 200 fold excess of the corresponding unlabeled steroids (to control for non-specific binding), and then placed in ice water for 30 min. Ice cold DCC solution was then added for 10 min at 4˚C, followed by centrifugation to separate and measure CBG-bound [^{3}H]-steroid. This method was also used to determine affinity constants by Scatchard analysis, as well as the steroid-binding specificities of CBGs in competitive ligand-binding assays (36). Affinity constants from Scatchard plots and IC50 determinations were obtained using Gnuplot software.

Western blotting was used to detect CBG in plasma and culture media samples after SDS-PAGE. Rabbit anti-zebra finch CBG antiserum was used at appropriate dilutions, or after affinity purification using yeast-expressed zebra finch CBG immobilized on a hiTrap NH-Activated HP column, as recommended (GE Healthcare). Immunoreactive CBG on western blots was detected using an enhanced chemiluminescence reagent (GE Healthcare) and an ImageQuant LAS4000 (GE Healthcare).

For cleavage of plasma CBG, 199 µl of diluted (1:40) zebra finch plasma was incubated with 1 µl of *P. aeruginosa* medium. After incubation at 37˚C for the indicated times, proteolysis was stopped by addition of EDTA (final concentration 10 mM). *E. coli*-expressed CBG (10 µg) was incubated with *P. aeruginosa* medium under the same conditions. Reaction products were examined by SDS-PAGE. Native and proteolytic fragments of *E. coli*-expressed zebra finch CBG were diluted in 5% acetonitrile and 0.1% formic acid and injected onto a Waters

Xevo GS-2 QTOF mass spectrometer via a NanoAquity UPLC system. Deconvolution of MS spectra with Waters' MaxEnt algorithm revealed a single peak (MW 42,890 Da) in the native sample, while cleaved CBG presented two peaks of 38,595 and 4,308 Da. MS/MS analysis of the 4,308 Da CBG fragment confirmed it was the C-terminal peptide: ISFPPTIEFSHPFLMLIFDRDTNSTLFI GKIVNTITS.

Crystal structure determinations—Because the 23 N-terminal residues of mature zebra finch CBG were predicted as disordered, an N-terminally truncated CBG, i.e. from Glu24, was also expressed in *E. coli* and purified, as described above, for crystallography. Attempts to crystalize this protein were unsuccessful, and the amino acid sequence was optimized for crystallization. For this, we introduced a C28A substitution and several other amino acid substitutions (i.e. E127A, K128A, K130A), as suggested by the SERp server (http://services.mbi.ucla.edu/SER/), into the zebra finch CBG sequence. This approach to aid crystallization relies on the concept of surface entropy reduction, i.e., the replacement of clusters of flexible, solvent-exposed residues with residues that have lower conformational entropy (37). These amino acid substitutions did not affect the cortisol-binding activity of zebra finch CBG.

The zebra finch CBG produced in this way formed needle-shaped crystals overnight at room temperature. Crystals for X-ray diffraction were obtained from hanging drops by equilibrating 1 µl of CBG solution (10 mM Tris, 25 mM NaCl, 5 mM EDTA, 600 µM cortisol, pH 7.5 with CBG at 5mg/ml) and 1 µl of reservoir solution (200 mM NaCl, 1.75 M $(NH_4)_2SO_4$, pH 6.5) against 1 ml of reservoir solution. Before flash freezing, crystals were soaked in a cryoprotective solution (28.5 % glucose, 200 mM NaCl, 1.75 M $(NH_4)_2SO_4$, pH 6.5, 10 µM cortisol).

Diffraction experiments were performed at the Canadian Light Source (Saskatoon) beamline 08ID-1, and datasets were processed using XDS (38). Human SERPINA1 (PDB code 1HP7) was used as a search model where all side chains truncated to alanine residues and loops were deleted. Molecular replacement was performed using Phaser (39) with an LLG value of correct solution of 3580 and LLG value of second unrelated peak of 190. Successive rounds of manual model building in COOT (40) were used to complete the model with refinements using Phenix (41) and REFMAC5(42). No residues were found in disallowed regions of the Ramachandran plot. Structure figures were prepared using PYMOL (DeLano Scientific) with secondary structures assigned through DSSP plugin, and coordinates were deposited in the PDB database (PDB code 5HGC).

Measurement of CBG mRNA in zebra finch tissues—Total RNA from adult zebra finch tissues was obtained with an RNeasy miniprep kit (Qiagen) and used for qPCR measurements of CBG mRNA. In brief, 0.5 µg of RNA, 1 µl of dNTP mix, 2 µl of random primer mix, single strand buffer and 1 µl of Reverse Transcripase II was incubated at 42˚C for 1 h in 20 µl. The resulting cDNA templates (0.5 µl) were added to a Fast SYBR Green Master Mix (Applied Biosystems) and amplified using zebra finch-specific CBG and GAPDH primers (43) both at 500 µM using a Real-Time PCR System (Applied Biosystems) under the following conditions: 95˚C denaturation for 10 min, followed by 40 cycles of 95˚C denaturation (15 sec), 60˚C annealing (40 sec) and 72˚C extension (30 sec). Melting curve analysis was performed to confirm presence of a single amplification product, and PCR products were analyzed by 2% agarose gel electrophoresis to confirm the expected PCR product sizes. The relative abundance of CBG mRNA was calculated using the delta-delta cycle threshold method.

RESULTS

Isolation and identification of steroid-binding proteins in chicken serum—In pilot experiments, a DHT-conjugated affinity matrix (29) proved more effective in isolating CBG from chicken serum, as compared to the corticosterone-based affinity matrix usually employed to isolate mammalian CBGs (34). Our DHT-affinity column removed ~70% of the high-affinity cortisol-binding activity from chicken serum, and some of the chromatography fractions eluting from the column in the presence of either DHT or cortisol exhibited the steroid-binding properties of CBG. When these fractions were subjected to SDS-PAGE, the three major protein bands observed were identified as albumin, SerpinA4, and ApoA1 by mass spectrometry (Fig. 1).

It was no surprise that albumin (~65 kDa) was enriched using DHT-affinity chromatography because it binds all steroid classes with low affinity. The enrichment of ApoA1 (~24 kDa) was unexpected, but human ApoA1 interacts with

steroids (44, 45), and chicken ApoA1 probably bound the DHT-affinity matrix with low affinity. The identification of the ~55 kDa protein band as SerpinA4 was also surprising because *SerpinA6* is the CBG-coding gene in mammals. However, the robust cortisol-binding activity in chromatography fractions containing the ~55 kDa protein suggested that it is CBG (SerpinA6) rather than the serpin peptidase inhibitor, kallistatin (SerpinA4).

Avian SerpinA6 genes—To date, only the mammalian *SerpinA6* genes encoding CBG have been confirmed as being correctly annotated in genome databases. In the chicken genome, the protein we suspected was chicken CBG is encoded by ENSGALG00000010969 currently annotated as *SerpinA4* (ensemble.org). In mammals, *SerpinA4* encodes kallistatin, which is not known to bind steroids. Like *SERPINA6*, *SERPINA4* is located in close proximity to *SERPINA1* on human chromosome 14 but is telomeric to *SERPINA1* while *SERPINA6* is centromeric to *SERPINA1*. This chromosomal arrangement of *SERPINA* genes in humans is conserved across mammalian species, and our findings indicate that synteny within this cluster of *SerpinA* genes extends to avian and reptilian species (Fig. 2A). These observations imply that *SerpinA4* in the current chicken genome database is annotated incorrectly, and that the zebra finch gene, ENSTGUG00000012647, as well as other annotated genes in several other avian species (Fig. S1) are the orthologs of the chicken gene encoding CBG (Fig. 2A).

When compared to the corresponding sequence at ensemble.org, the zebra finch cDNA we cloned contains four non-synonymous single nucleotide variations resulting in the following amino acid differences in the mature zebra finch CBG sequence: A64T, N198S, S319N, V363I. This was not unexpected because of genetic variations between zebra finch colonies (46). In addition, none of these amino acids are in regions that might influence steroid-binding activity, and CBG in plasma from the same colony of birds from which the mRNA was obtained for cDNA synthesis had normal steroid-binding properties.

Based on sequence comparisons and the positioning of *SerpinA6* orthologs in the genomes of multiple avian species, we constructed a phylogram which indicates that the chicken gene encoding CBG (*SerpinA6*) clusters most closely with its related genes in other species of fowl (turkey and duck), while the zebra finch gene is more closely related to the predicted orthologs in other perching birds (Fig. 2B). Extending this type of analysis also allows *SerpinA6* orthologs to be identified in other non-mammalian vertebrates, including reptiles (Fig. 2A and 2C).

Importantly, the avian CBG sequences we have identified display limited (<45%) sequence identity with mammalian CBGs, and alignment of mammalian and avian CBG primary structures indicated that only 5 of the 10-12 residues known to interact with steroids within mammalian CBG steroid-binding sites are conserved in avian CBGs (Fig. 3).

Confirmation of avian CBG identity—To demonstrate conclusively that the chicken gene, ENSGALG00000010969, and its zebra finch ortholog, ENSTGUG00000012647, both encode CBG, we expressed their respective full-length cDNAs in Chinese hamster ovary (CHO) cells. Scatchard analysis of the recombinant proteins secreted by these cells was performed alongside plasma samples from both bird species, and this confirmed that their affinities for corticosterone are all in the low nM range (Table 1A). We also expressed the predicted mature polypeptide sequences encoded by these two bird genes in yeast, and the affinities of the yeast-expressed proteins are indistinguishable from CBG in the plasma these birds (Table 1A). As a further demonstration that the avian genes we had identified encode CBG, we compared the steroid-binding specificity of the zebra finch protein expressed in yeast with that of CBG in zebra finch plasma, and found them to be virtually identical (Table 1B).

These steroid binding specificity studies (Table 1B) also indicated that zebra finch CBG has a greater affinity for cortisol and progesterone than for corticosterone, the major circulating glucocorticoid in this species. In addition, zebra finch CBG has a remarkably high affinity for the synthetic glucocorticoid, dexamethasone, and moderate affinity for both testosterone and DHT, but does not bind dehydroepiandrosterone, estradiol, or aldosterone. This steroid-binding specificity profile reflects those reported for CBGs in avian species (1, 21, 27, 47), and proves that the chicken ENSGALG00000010969 and zebra finch ENSTGUG00000012647 genes encode CBG and should be annotated as *SerpinA6*.

Influence of glycosylation on avian CBG steroid-binding activity—Avian CBGs have fewer

consensus sites for N-glycosylation than do mammalian CBGs, and all three of them appear to be highly conserved (Fig. S1). Remarkably, the asparagine residue of one of these sites (Asn 385) is positioned in place of the tryptophan residue that plays a critical role within the CBG steroid-binding sites of all mammals (15). We therefore examined the impact of glycosylation on the steroid-binding properties of zebra finch CBGs expressed in yeast and CHO cells.

Each of the three N-glycosylation sites was first disrupted by substitution of the asparagine residues (Asn88, Asn250 and Asn385) with aspartic acid (Table 1C). When expressed in yeast, the N88D and N250D mutants bound steroids with the expected affinity, but the steroid-binding activity of the N385D mutant was undetectable. Although the N385D mutant also exhibited a decreased (< 5% of un-mutated CBG) cortisol-binding capacity (Fig. 4A) when expressed in CHO cells, its affinity (Kd) for cortisol appears to be normal (Table 1C). This suggests that only a small portion of the N385D mutant folds correctly and is capable of binding steroid. To examine this further, we disrupted the N-glycosylation consensus sequence at this site (i.e., Asn-Ser-Thr) by creating a T387P mutant. This also prevents N-linked glycosylation at Asn385, and the T387P mutant bound steroid normally (Table 1C). In addition, a N88D/N250D double mutant that only N-glycosylates at Asn385 binds steroids normally. Deglycosylation of this N88D/N250D mutant with PNGase F results in a reduction in molecular size consistent with the loss of an N-linked oligosaccharide at Asn385 (Fig. 4A and 4B). However, by contrast to the almost complete loss of steroid-binding activity in the N385D mutant, its cortisol-binding activity was unaffected by deglycosylation in this way (Fig. 4A and 4B), despite the fact that PNGase F treatment also converts N385 into an aspartic acid (48). Thus, it appears that while glycosylation at N385 does not affect steroid binding in zebra finch CBG, this asparagine residue may contribute in some way to the formation of high affinity steroid-binding site.

Proteolysis of zebra finch CBG and effect on its steroid-binding activity—Pseudomonas aeruginosa infections are associated with high mortality in birds (49), and the metalloprotease protease, *LasB,* secreted by *P. aeruginosa* cleaves the RCL of human CBG and disrupts its cortisol-binding activity (19). To mimic a biologically relevant situation, we therefore incubated diluted zebra finch plasma in the absence or presence of *P. aeruginosa* culture medium (Fig. 5A). Analysis of the reaction products indicated that ~50% of the zebra finch CBG underwent undergoes proteolysis within 2h, with the appearance of a cleavage product that is ~4kDa smaller than the intact CBG, together with a corresponding reduction in its corticosteroid-binding capacity. This reduction in the apparent molecular size of CBG is consistent with a single cleavage within the RCL sequence, as observed for human CBG treated in this way (19). This proteolysis appeared to continue over 6h with further reductions in corticosteroid-binding capacity by as much as 90% (Fig 4A). Although some additional proteolysis was evident when the reaction was extended for 17h, no intact zebra finch CBG or detectable corticosteroid-binding activity was present at this time point (Fig. 5A). Importantly, when the reaction was performed for 17h in the presence of EDTA, which is known to inhibit the bacterial metalloprotease *LasB* (19), the CBG was completely protected from proteolysis or loss of steroid-binding activity (Fig. 5A).

When the N-terminally truncated zebra finch CBG produced in *E.coli* was incubated for 2h with *P. aeruginosa* medium, a similar ~4kDa reduction in its molecular size was accompanied by a complete loss of its corticosteroid-binding activity (Fig. 5B). In addition, when the resulting proteolytic fragments were analyzed by mass spectrometry, the proteolytic cleavage site was identified between residues P362 and I363 within the C-terminal region of the RCL (Fig. 5C).

Crystal structure of zebra finch CBG in complex with cortisol—Because avian CBG sequences display limited sequence identity with mammalian CBGs, and some of the residues that interact with steroids in mammalian CBGs are not conserved in avian CBG sequences (Fig. 3), it was important to obtain a bird CBG crystal structure in complex with a steroid ligand. To do this, zebra finch CBG was co-crystalized with its preferred glucocorticoid ligand, cortisol (Table 1B). The crystal structure was solved to 2.4Å resolution (Table 2). In this structure, the main chain is organized in a typical Serpin fold with the RCL being fully exposed in the "stressed" or "active" Serpin conformation (Fig. 6A). Only 7 out of the 375 residues in the crystal structure failed to produce a clear electron density; five of these residues are within the unstructured RCL, and this

was expected because the RCL is also disordered in the rat CBG crystal structure (11). The two other residues that could not be modeled are at the N-terminus and C-terminus of the protein. Moreover, an electron density for cortisol was clearly evident (Fig. 6B).

The cortisol in the zebra finch CBG crystal structure is positioned at the interface of helix A, helix H and beta sheet B, as in the rat CBG structure (Fig. 6A). However, while the overall organization of the binding site is conserved in both species there are some important differences. Cortisol in the zebra finch CBG steroid-binding site is slightly tilted when compared to the position of cortisol in the rat CBG structure (Fig. 6A). This can be explained by a different set of interactions with the steroid A ring. In rat CBG, the A ring of cortisol forms hydrophobic interactions with Ala13 and Val17, while in zebra finch CBG it is held in place by the hydrophobic side chains of Val32 and Ala36. Furthermore, the most remarkable difference is the Asn385 in zebra finch CBG (and other avian CBGs) that replaces the Trp362 in rat CBG. In zebra finch CBG, Asn385 is clearly a site for N-linked glycosylation, and our crystal structure data indicate that it is capable of forming a hydrogen bond with the hydroxyl group of cortisol at C17 (Fig. 6B). By contrast, in rat CBG Trp362 forms strong stacking interactions with the surface of the steroid, thus serving to anchor it within its relatively shallow binding site (Fig. 6C).

The zebra finch CBG crystal structure also shows how other key residues interact with oxygen atoms of cortisol via hydrogen bonds, i.e., Ser281 (with the hydroxyl group at C11) and Arg246 (with the hydroxyl group at C21 and the carbonyl group at C20), as well as the positions of several residues that form hydrophobic contacts with the carbon atoms of cortisol (Fig. 7A and B). It is also apparent that cortisol fits tightly into its binding pocket as evidenced by the absence of water molecules bridging amino acid residues and functional groups of the steroid.

Roles of specific amino acid residues in the zebra finch CBG steroid-binding site—Based on our new crystal structure data, we mutated several amino acids in the zebra finch CBG sequence that are likely involved in cortisol binding. Mutation of Ser281 into alanine did not affect cortisol binding (Table 3A). However, although this serine (Ser281) appears to form a hydrogen bond with the cortisol hydroxyl group at C11, when it is substituted with alanine the C11 hydroxyl group of cortisol is likely coordinated by water, as observed in rat CBG, in which the residue corresponding is Gly259 (Fig. 3). In rat CBG, the side chain carboxyl group of Asp256 hydrogen bonds with the C11 hydroxyl of cortisol, and its substitution with alanine causes a loss of steroid-binding activity (11). The corresponding residue in zebra finch CBG is Gln278 (Fig. 6) but it does not hydrogen bond with the hydroxyl group at C11 of cortisol. Instead it creates a surface for hydrophobic interactions with cortisol and its substitution with alanine does not affect cortisol-binding activity (Table 3A). Moreover, the fact zebra finch CBG binds both cortisol and progesterone (which does not have a hydroxyl group at C11) with essentially equal affinity, further suggests that interactions with an hydroxyl group at C11 have little influence on the affinity of avian CBGs for the glucocorticoids.

The affinities of zebra finch CBG for progesterone and cortisol are almost identical, and this characteristic is shared by other avian CBGs (1, 21). In human CBG crystal structures, the hydroxyl group at C21 and the carbonyl group at C20 in cortisol both hydrogen bond with Gln232 (12), while progesterone hydrogen bonds with Gln232 through its carbonyl group at C20 only (13). In zebra finch CBG, the corresponding residue is Arg246, and substitution of this residue with Gln caused a significant decrease in affinity for progesterone while the affinities for cortisol and testosterone were unaffected (Table 3B). Thus, while Arg246 in zebra finch CBG may make similar contact with both cortisol and progesterone, substitution of this highly conserved residue in avian CBGs (Fig. S1) with glutamine explains why the affinities of avian CBGs for progesterone are much higher than for mammalian CBGs.

Given the importance of the tryptophan residue in the steroid-binding sites of rat and human CBGs (15), we also produced a zebra finch CBG N385W mutant. When expressed in yeast (Table 3A) or CHO cells (not shown), it was secreted at similar levels to the un-mutated CBG, as assessed by western blotting (not shown), but its steroid-binding affinity was very low (Kd >30 nM) in a Scatchard analysis (Table 3A).

Zebra finch liver is the major site of SerpinA6 expression and anti-zebra finch CBG antibodies detect plasma CBG in other avian species—Quantitative RT-PCR was used to determine the relative abundance of CBG mRNA

in adult male and female zebra finch tissues (Fig. 8A). Single RT-PCR products of the expected sizes were detected in all tissues except skeletal muscle (not shown), but measurements of CBG mRNA in relation to GAPDH mRNA levels in these tissues, indicated that the relative abundance of CBG mRNA in liver is >300 fold higher than in other tissues (Fig. 8A). The amounts of CBG mRNA in kidney, ovary and testis are uniformly low, and undetectable in skeletal muscle. Moreover, while spleen and lung showed greater variability in CBG mRNA levels, they were also very low (Fig. 8A).

A rabbit anti-zebra finch CBG antiserum recognizes CBG in zebra finch plasma with high specificity on western blots (Fig. 5A), and also recognizes plasma CBG in other avian species (Fig. 8B). For this experiment, plasma from the different species was diluted so equal amounts of CBG, as determined in corticosterone-binding capacity assays, were used for western blotting. This showed that both anti-zebra finch antiserum and affinity-purified antibodies recognized CBG in song sparrow plasma slightly less well than in zebra finch plasma, while chicken CBG was recognized weakly only by concentrated affinity-purified antibodies. This is consistent with a higher amino acid sequence identity between zebra finch and sparrow CBGs (95%) than between zebra finch and chicken CBGs (78%).

DISCUSSION

We have found that a chicken gene (ENSTGUG00000012647), currently annotated as *SerpinA4*, encodes the mammalian CBG (SerpinA6) ortholog, and sequence alignments allowed us to identify ENSTGUG00000012647 in a syntenic region of the zebra finch genome as the gene that encodes CBG. Moreover, we can also now predict with confidence the identities of *SerpinA6* genes within the genomes of other avian species, as well as several reptiles. The proximity and synteny of *SerpinA6* and *SerpinA1* in the genomes of these species is intriguing, because the proteins they encode are considered the most closely related amongst SerpinA family members (50). It is also of interest that fish lack CBG while it is present in the blood of amphibians (1). This is consistent with the concept that *SerpinA6* genes originated as a result of *SerpinA* gene duplications within a syntenic region of early terrestrial vertebrate genomes.

As anticipated, the structural organization of avian and mammalian *SerpinA6* genes is identical, with four similarly sized exons encoding the CBG precursor polypeptide. However, chicken *SerpinA6* (ENSTGUG00000012647) appears to comprise a larger 5′ non-coding exon than in mammalian *SerpinA6* genes (ensembl.org). As in mammals (8, 51, 52), this 5′ exon in birds is likely flanked by a conserved promoter sequence that controls the tissue specific and physiologic expression of their *SerpinA6* genes, and the identification of avian *SerpinA6* genes provides opportunities to study their regulation at the transcriptional levels.

The protein encoded by zebra finch *SerpinA6* possesses all the steroid-binding properties previously reported for avian CBGs (1, 20) but its identity was surprising for several reasons. In particular, it did not share several key features of its mammalian orthologs, and one of the three conserved N-glycosylation sites in avian CBGs is located within the steroid-binding site. The latter was most remarkable because the asparagine that is N-glycosylated in this position replaces a tryptophan in mammalian CBGs that interacts directly with steroid ligands (53). Positioning of this tryptophan in mammalian CBGs (11, 13) relies on a cation-pi interaction with an amino-terminal arginine that is also absent in the avian CBG sequences (Fig. 3). By contrast, the asparagine within the avian CBG steroid-binding sites is incapable of forming similar interactions with other residues or the steroid ligand. However, although an N-linked oligosaccharide at N385 does not influence the steroid-binding activity of avian CBG, our results suggest this asparagine may participate in the formation of the steroid-binding site during synthesis in eukaryotic cells.

Our crystal structure and mutagenesis studies explain why avian CBGs have greater affinities for progesterone than mammalian CBGs, and an asparagine instead of a tryptophan residue in the steroid-binding site may allow avian CBGs to bind dexamethasone with high affinity. In addition to these functionally relevant differences in the structures of mammalian and avian CBGs, only ~50% of residues that interact with steroids in mammalian CBGs are conserved in avian CBG sequences. The mechanisms that control the binding and release of steroids may therefore differ between avian and mammalian CBGs.

When the zebra finch and rat CBG crystal

structures in complex with cortisol were aligned, the structural similarity was evident. We used the crystal structure of rat CBG for this comparison because it also has cortisol in the binding site, and it is the only available native CBG structure in which the RCL is not cleaved and inserted in the beta sheet A (11). This revealed differences in their main chain conformations, especially within the C-terminal part of helix D, which is unstructured in zebra finch CBG most likely because of a proline at position 111. In a human CBG crystal structure, in which the RCL was cleaved, and the protein had undergone the typical "stressed to relaxed" Serpin structural conformational rearrangement, and the C-terminal part of helix D was unwound (14). This suggested that the unwinding of helix D causes allosteric rearrangements in the steroid-binding site conformation with a resulting loss of steroid-binding affinity (14), but this has not been formally demonstrated. However, the fact that this region of helix D in the zebra finch CBG structure is unwound, while the RCL appears to be intact, suggests that the conformation of helix D may not influence steroid binding in bird CBGs.

Divergence of CBG structures, while maintaining key functional characteristics, likely reflects how the protein evolved to control steroid transport and bioavailability in vertebrate subphyla in response to environmental pressures or differences in their physiology. During vertebrate evolution, aldosterone first appeared in amphibians (54), and CBG may have appeared at about the same time to protect tissues rich in mineralocorticoid receptors from unnecessary exposures to glucocorticoids. Identification of an amphibian *SerpinA6* gene would therefore help understand the phylogenetic divergence of CBG structures.

The degree of evolutionary change with respect to the primary structures and steroid-binding properties of CBGs within vertebrate subphyla is remarkable compared for instance to the steroid hormone receptors, including the ligand-binding domain of glucocorticoid receptor, which tends to be more highly conserved across the vertebrates (55, 56). It might be argued that evolutionary changes in CBG structure and function compensated for the lack of SHBG in avian species, but reptiles have genes encoding both plasma CBG and SHBG and yet their CBG appears to be more closely related to avian CBGs than mammalian CBGs (Fig. 2C). In addition to sequence identity, the similarity between avian and reptile CBGs extends to the conservation of residues within their steroid-binding sites, the locations of N-glycosylation consensus sequences, and their RCL sequences (Fig S1).

In addition to CBG, other members of the SERPIN clade A family bind hormones; e.g., thyroxine-binding globulin (SERPINA7), and the protein C inhibitor (SERPINA5) that has been reported to have a binding site for retinoic acid (57). If avian orthologs of these other hormone binding SERPINs exist, it would be of interest to determine how their structures and functions may have evolved across vertebrate species, as we have shown for CBG.

In addition to binding anti-inflammatory steroids or steroids involved in stress responses, our data indicate that the irreversible loss of high affinity steroid-binding activity, which occurs when the RCL of CBG is targeted and cleaved by specific classes of proteases, is conserved across vertebrate species. In particular, it is remarkable that the zebra finch CBG RCL appears to be targeted by the virulence factor, *LasB*, secreted by *P. aeruginosa*. It has been proposed that environmental pressures, such as exposures to different sub-sets of pathogens, may explain the apparent accelerated evolutionary change in SerpinA RCL sequences (58). Thus, our data suggest that opportunistic pathogens like *P. aeruginosa* may have been a driving force behind how *Serpina6* genes evolved in terrestrial vertebrates, and that RCL cleavage of CBG by exogenous proteases released by pathogens has been central to the role of CBG in controlling the actions of inflammatory steroids during infectious diseases throughout evolution.

As expected, a survey of adult zebra finch tissues indicated that the liver is the main site of CBG production. However, as in mammals, several other zebra finch tissues also contained low levels of CBG mRNA, the significance of which remains to be determined. In this regard, some extra-hepatic tissues (exocrine pancreas and the convoluted tubules of the kidney) contain remarkable high levels of CBG mRNA at early developmental stages in rodents, and it has been proposed that this may influence the local actions of glucocorticoids on tissue morphogenesis and development (59, 60). It will therefore be of interest to examine the tissue specific expression of *SerpinA6* throughout development in avian species. The identification of avian *SerpinA6*

genes and the production of antibodies against zebra finch CBG, which cross-react with CBGs in other avian species, therefore also provide opportunities to examine the physiological impact of CBG in modulating the actions of multiple classes of steroids in birds.

Birds, and songbirds like the zebra finch in particular, are commonly used as models across diverse fields in the biological sciences (61, 62). Many studies of birds have focused on neural and behavioral responses to environmental and social stressors and reproductive pressures (23-27). Steroid hormones mediate many of the biological responses studied using these models (63), and CBG serves as a primary gatekeeper of the actions of both the major stress (corticosterone) and reproductive (progesterone and testosterone) steroid hormones in birds, by transporting them and regulating their access to target tissues (20-22).

The identification and characterization of avian CBGs therefore helps explain their distinct steroid-binding properties when compared to the CBG in mammalian species, and provides the tools to study how CBG controls plasma concentrations and activities of steroid hormones in a range of biological studies in birds. Furthermore, recent advances in avian genomic biodiversity promise to provide unprecedented insight into vertebrate evolution (64). In this regard, differences in the topography of the mammalian and avian CBG steroid-binding sites are shared at least by reptilian CBGs, suggesting that evolutionary adaptations in CBG functions occurred to accommodate physiological and endocrine changes during the evolution of mammals, and our work provides a foundation for studies to further explore this.

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Author contributions: G.V. designed the study, performed the majority of the experiments and prepared the paper; S.D performed the crystallographic data collection and solved the structure together with G.V., F.V.P. supervised the X-ray crystallographic part, K.M.M. and J.C.R. performed the mass spectrometry and interpreted that data, M.D.T. collected tissues samples, provided reagents and assisted with preparation of the paper, K.K.S. provided reagents and assisted with preparation of the paper, L.J.F. designed and supervised the mass spectrometry, G.L.H. supervised the project, guided the experimental design and the preparation of the paper.

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FIGURE LEGENDS

FIGURE 1. Isolation and identification of chicken CBG. Coomassie blue stained SDS-PAGE gel of proteins isolated by steroid-affinity chromatography from chicken serum. Lane 1, Molecular size markers (kDa values on the left); Lane 2, 3 µl affinity chromatography eluate; Lane 3, 12 µl affinity chromatography eluate. Protein bands excised for mass spectrometric analysis and their identifications are indicated on the right.

FIGURE 2. Phylogenetic comparisons of *SerpinA6* genes and CBG sequences. **A.** Chromosomal locations *SerpinA* genes illustrating the syntentic conservation of *SerpinA10*, *SerpinA6* (encoding CBG) and *SerpinA1* in humans, chickens, zebra finch and the anole lizard. In all species, *SerpinA* loci are flanked by *PPP4R4* (protein phosphatase 4, regulatory subunit 4) and *GSC* (goosecoid homeobox) genes. Orthologs of *PPP4R4, GSC, SerpinA10*, *SerpinA6* and *SerpinA1* are colored identically. **B.** A neighbor-joining phylogram of CBG in avian species. Phylogenetic analyses were performed using ClustalW2 Phylogeny (65) with default settings, and the phylogram was created with the PHY.FI online tool (66). **C.** Amino acid sequence identity of CBG precursor polypeptides from different species. Pairwise sequence alignments were produced using Clustal Omega (35). Accession numbers for the sequences used in this figure: Chicken (KU180444), Turkey (XP_003206753.1), Mallard (EOA99912.1), American crow (XP_008627951.1), Zebra finch (KU180443), White throated sparrow (XP_005483238.1), Medium ground finch (XP_005417914.1), Rock dove (XP_005506178.1), Saker falcon (XP_005438961.1), Chinese softshell turtle (XP_006133744.1), Anole lizard (XP_008105229.1), Rat (NP_001009663.1), Human (P08185.1).

FIGURE 3. Multiple sequence alignment of zebra finch, chicken, human and rat CBGs. N-terminal residues (predicted or experimentally determined) are shown in red and are numbered as residue 1. Residues in the secretion signal polypeptide are numbered negatively with respect to residue 1. Amino acids whose side chains interact with cortisol are highlighted in green, and consensus sequences for N-linked glycosylation are shown in blue letters. Arginine, which forms cation-pi interaction with Trp362 in rat CBG and Trp371 in human CBG, is highlighted in cyan. Black boxes highlight conserved amino. The 3 conserved amino acids that interact with cortisol are shown in bold.

FIGURE 4. Glycosylation and cortisol-binding capacity of zebra finch CBG and its glycosylation-deficient mutants **A**. Western blotting of media from CHO cells with a rabbit anti-zebra finch antiserum. Deglycosylation of the N88D/N250D mutant, which has only one N-glycosylation site at Asn385, with PNGase F resulted in ~2-3 kDa reduction in apparent molecular size with only minor loss of cortisol-binding capacity. The molecular size of the deglycosylated N88D/N250D mutant is ~45 kDa consistent with a complete loss of N-linked oligosaccharides. **B.** The N88D/N250D mutant expressed in yeast examined by SDS-PAGE and Coomassie blue staining before and after deglycosylation with PNGase F. Note that it also retained almost full cortisol-binding capacity after deglycosylation.

FIGURE 5. Proteolysis within the reactive center loop (RCL) disrupts the corticosterone-binding activity of zebra finch CBG. **A**. Diluted (1:40) zebra finch plasma (199 µl) was incubated with 1 µl *P. aeruginosa* culture medium. Proteolysis was stopped by addition of EDTA at predetermined times (0 - 17 h). In one

17 h reaction (*), EDTA was added prior to incubation with *P. aeruginosa* culture medium. Reaction products were analyzed by western blotting and a corticosterone-binding capacity assay. The reduction in apparent molecular size of ~4 kDa after incubations of 2-17 h, is consistent with cleavage occurring in the RCL. At 17 h, additional proteolysis is evident, but proteolysis and loss of corticosterone-binding capacity was almost completely blocked by pre-treatment with EDTA even after 17 h. **B.** Incubation of un-glycosylated N-terminally truncated zebra finch CBG (i.e. lacking the first 23 amino-terminal residues) expressed in *E. coli* with *P. aeruginosa* medium for 2h resulted in a molecular size reduction of ~4 kDa, as shown by SDS-PAGE and Coomassie staining, together with a complete loss of corticosterone-binding activity. **C.** Location of the proteolytic cleavage site within the RCL as determined by mass spectrometric analysis of proteolytic fragments obtained after incubation of *E. coli*-expressed zebra finch CBG with *P. aeruginosa* medium, as in B.

FIGURE 6. Comparisons of zebra finch (red; PDB ID 5HGC) and rat (blue; PDB ID 2V95) CBG crystal structures (*A*) and their steroid-binding sites (*B, C* and *D*). **A**. Overall structural alignments of zebra finch and rat CBG with cortisol in the binding sites. Reactive center loop regions without clear electron density are shown with a dotted line. Structural alignment was performed in Pymol using all residues. **B.** Portion of the final 2Fo-Fc electron density map contoured at 1.0 σ shows electron density corresponding to cortisol. **C**. Cortisol in the zebra finch CBG steroid-binding site showing a hydrogen bond between N385 and the hydroxyl group at C17 of cortisol. **D**. Cortisol the rat CBG steroid-binding site showing how W362 forms both a hydrogen bond and a stacking interaction with the ligand.

FIGURE 7. Detailed structure of the zebra finch CBG steroid-binding site (*A*), and a schematic representation of how cortisol interacts with specific amino acid residues (*B*). **A**. Cortisol is in purple with amino acids participating in cortisol binding in orange. **B**. A schematic diagram drawn with LigPlot (EMBL-EBI, Cambridge, UK) shows the carbon and oxygen atoms of cortisol (purple) as black and red circles, respectively. Amino acids participating in hydrophobic interactions with cortisol are shown as orange open half circles. Amino acids forming hydrogen bonds (green dotted lines) with cortisol are depicted as schematic structures, where black, red and blue circles represent carbon, oxygen and nitrogen atoms of the amino acids, respectively.

FIGURE 8. A. Levels of CBG mRNA in adult zebra finch tissues (*A*) and immuno-detection of CBG in plasma from different avian species (*B*). **A**. CBG mRNA levels are expressed relative to GAPDH mRNA levels. Error bars represent the SEM and numbers of tissues from different animals are indicated in parenthesis. **B**. Plasma samples were diluted for SDS-PAGE (0.3-0.4 µl) to ensure similar amounts of CBG were analyzed based on their corticosterone-binding capacity measurements. Western blotting was performed using anti-zebra finch CBG antiserum (1:5,000 dilution) or immuno-affinity purified anti-zebra finch CBG antibodies (1:250 dilution).

TABLE 1. Steroid-binding properties of avian CBGs.

A. Dissociation constants (Kd) of CBG for [^{3}H]-corticosterone in diluted (1:100) chicken and zebra finch plasma versus the corresponding CBGs expressed in yeast (*P. pastoris*) or Chinese hamster ovary (CHO) cells.

Species	Plasma Kd (nM)	Yeast-expressed Kd (nM)	CHO-expressed Kd (nM)
Chicken	1.9 ± 0.2	1.5 ± 0.2	0.9 ± 0.1
Zebra Finch	2.6 ± 0.2	3.0 ± 0.3	3.2 ± 0.5

B. Competition of different steroids for zebra finch CBG in diluted (1:100) plasma versus recombinant zebra finch CBG expressed in yeast (*P. pastoris*). The mean ± asymptotic standard error IC_{50} values (nM) are presented for unlabeled steroids in competitive steroid binding assays using [^{3}H]-corticosterone as the labeled ligand.

Unlabeled Competitor	Zebra finch (plasma)	Zebra finch CBG (yeast-expressed)
Corticosterone	4.2 ± 0.3	4.0 ± 0.3
Cortisol	1.8 ± 0.2	1.7 ± 0.2
Progesterone	1.6 ± 0.1	2.0 ± 0.3
Dexamethasone	3.7 ± 0.8	2.4 ± 0.6
5α-dihydrotestosterone (DHT)	11.3 ± 2.7	16.4 ± 2.0
Testosterone	18.5 ± 3.0	20.8 ± 4.1
Estradiol	>200	>200
Dehydroepiandrosterone	>200	>200
Aldosterone	>200	>200

C. Influence of N-glycosylation on zebra finch CBG steroid-binding activity. Yeast-expressed zebra finch CBG and CBG mutants with specific N-glycosylation sites disrupted were subjected to Scatchard analyses with [^{3}H]-cortisol to determine affinity constants (Kd). Because the CBG N385D mutant expressed in yeast had no detectable cortisol-binding activity, the zebra finch CBG and CBG mutants were also produced in CHO cells. The amount of the CBG N385D mutant produced by CHO cells with cortisol-binding activity was low, but its affinity for cortisol was normal, as it was for the other mutants. Mean ± asymptotic standard error dissociation constant (Kd) values are listed.

Expression system	CBG Kd (nM)	CBG N88D Kd (nM)	CBG N250D Kd (nM)	CBG N385D Kd (nM)	CBG T387P Kd (nM)	CBG N88D/N250D Kd (nM)
Yeast	2.2 ± 0.2	2.5 ± 0.3	2.5 ± 0.3	undetectable	2.5 ± 0.2	2.0 ± 0.3
CHO cells	2.8 ± 0.2	3.4 ± 0.1	3.3 ± 0.4	2.4 ± 0.2	3.6 ± 0.2	1.7 ± 0.1

TABLE 2. Data collection and refinement statistics.

Data collection	
Space group	$P6_3$
Cell dimensions	
a, *b*, *c* (Å)	116.2, 116.2, 72.7
α, β, γ (°)	90, 90, 120
Resolution (Å)	58.0 -2.43
R_{sym} or R_{merge}	19.9 (138.1) [a]
$I / \sigma I$	11.1 (1.9)
Completeness (%)	100 (100)
Redundancy	11.3 (11.1)
Wilson B factor	32.6
Refinement	
Resolution (Å)	50.3 -2.43
No. reflections	21,312
R_{work} / R_{free}	16.2/21.7
No. atoms	
Protein	2996
Water	186
Ligand	26
B-factors	
Protein	39.29
Water	39.02
Ligand	34.11
R.m.s. deviations	
Bond lengths (Å)	0.017
Bond angles (°)	1.968
Ramachandran plot (% residues)	
Most favored regions	97.3
Allowed regions	2.7
Outlier regions	0

[a] Values in parentheses are for highest-resolution shell.

TABLE 3. Steroid binding properties of zebra finch CBG and its mutants expressed in *P. pastoris*.
A. Mean ± asymptotic standard error dissociation constant (Kd) values for [^{3}H]-cortisol binding to zebra finch CBG and various zebra finch CBG mutants. **B**. IC50 values for different steroids measured for zebra finch CBG and the CBG R246Q mutant. Competition studies with unlabeled competitors were performed with [^{3}H]-cortisol as the labeled ligand.

A

Protein	Kd (nM)
CBG	2.2±0.2
CBG Q278A	2.5±0.2
CBG S281A	2.2±0.2
CBG R246Q	2.8±0.3
CBG N385W	>30

B

Unlabeled Competitor	IC50 (nM)	
	CBG	CBG R246Q
Cortisol	5.5±0.7	6.3±0.7
Progesterone	7.1±0.4	27.0±2.8
Testosterone	43.7±3.0	44.5±4.4

Figure 1

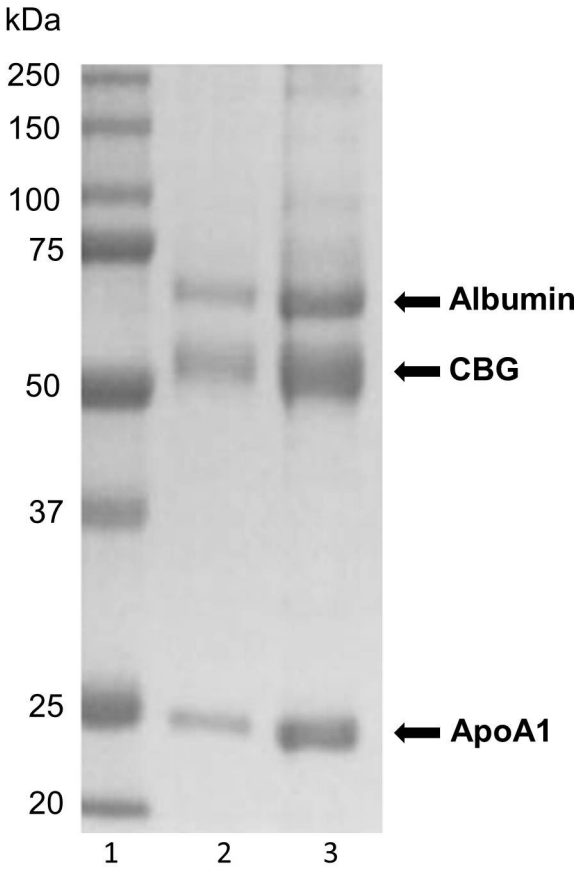
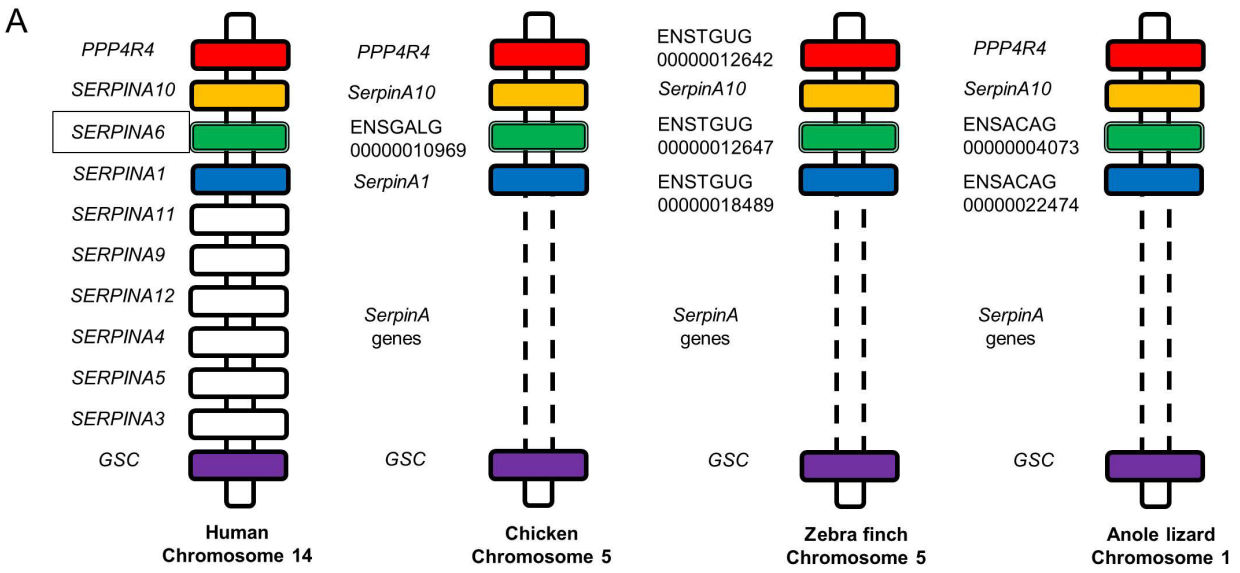
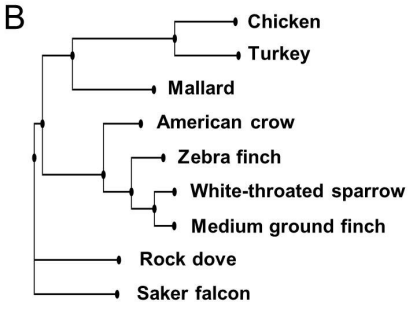


Figure 2





C

	Zebra finch	Chicken	Turtle	Anole lizard	Rat	Human
Zebra finch	100	78	66	51	40	42
Chicken		100	62	51	41	43
Turtle			100	54	42	47
Anole lizard				100	37	45
Rat					100	60
Human						100

Figure 3

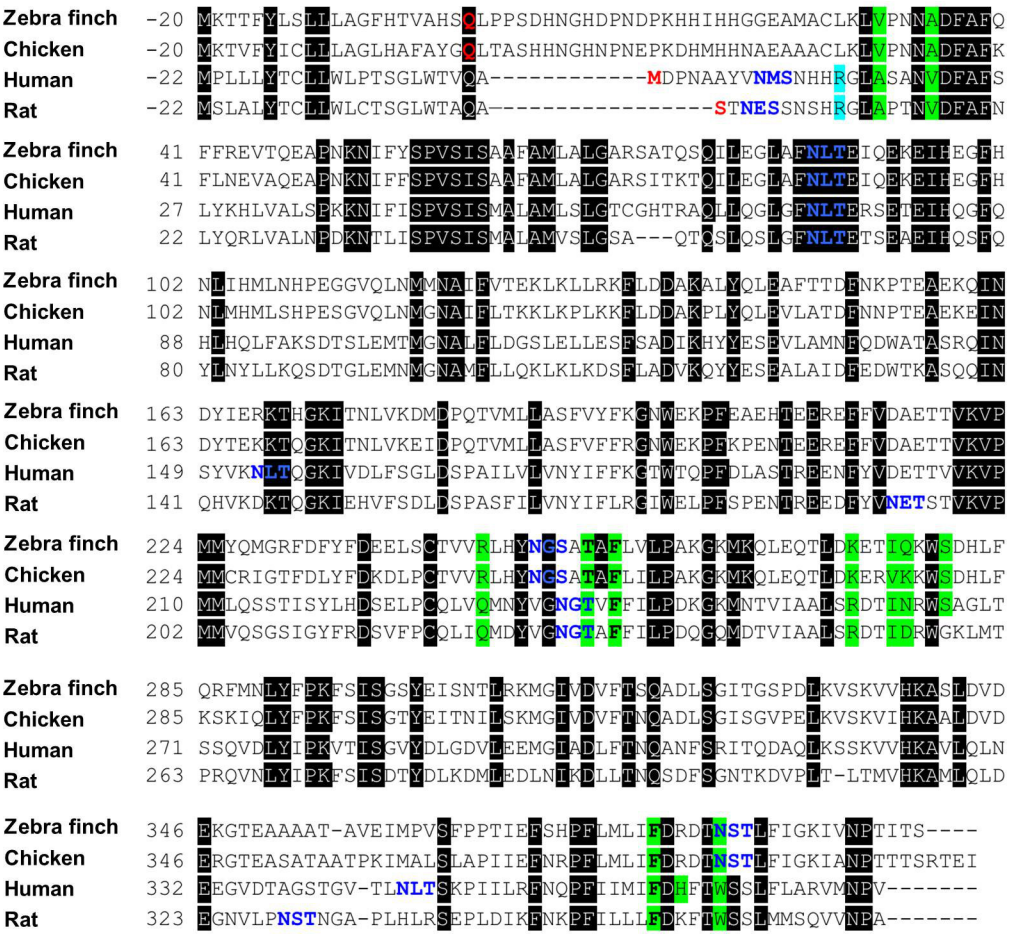


Figure 4

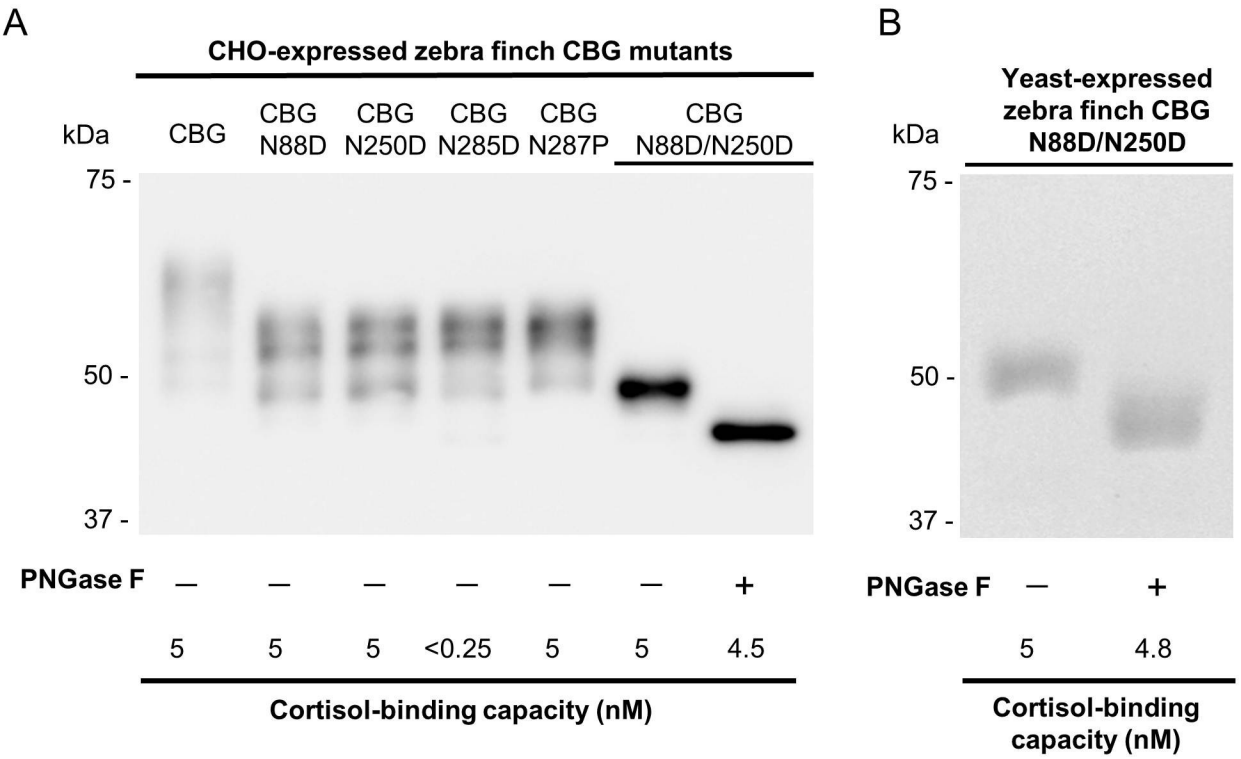


Figure 5

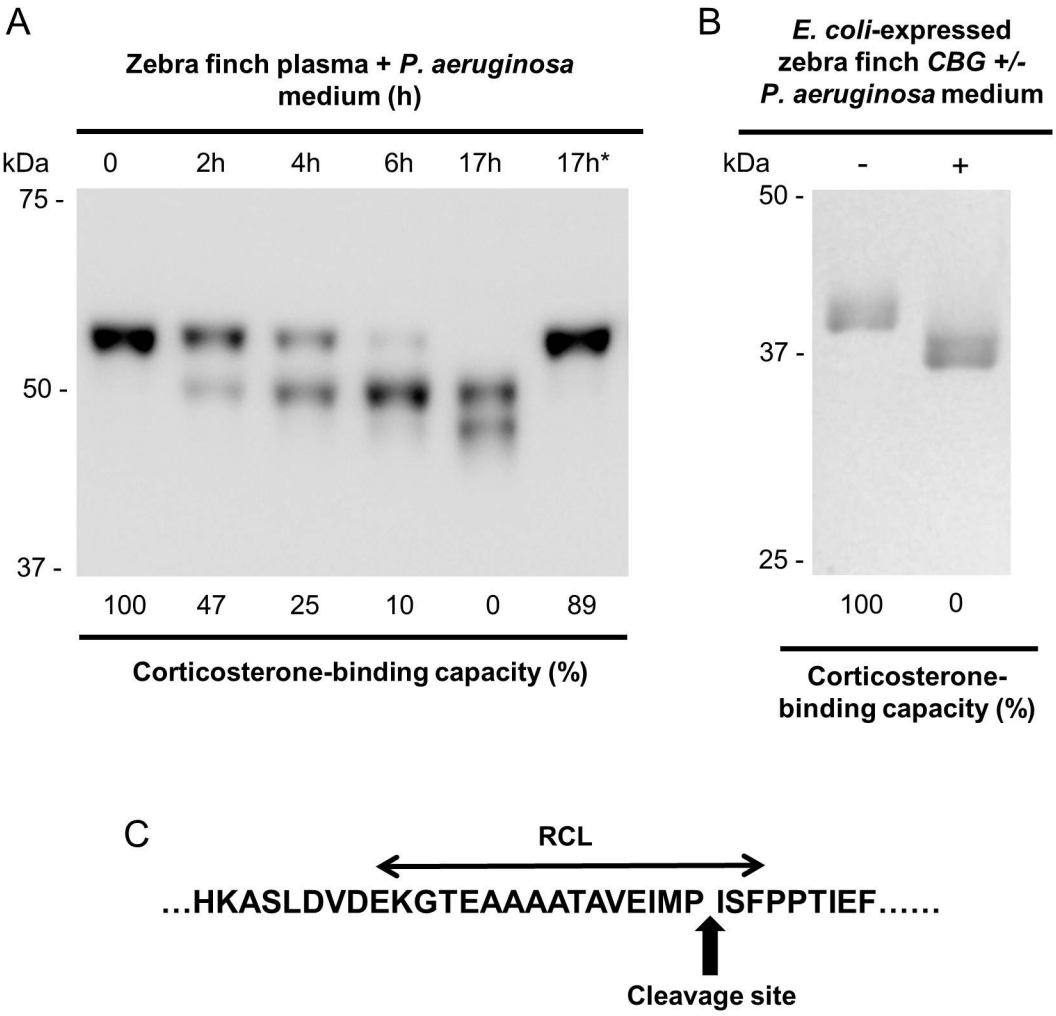


Figure 6

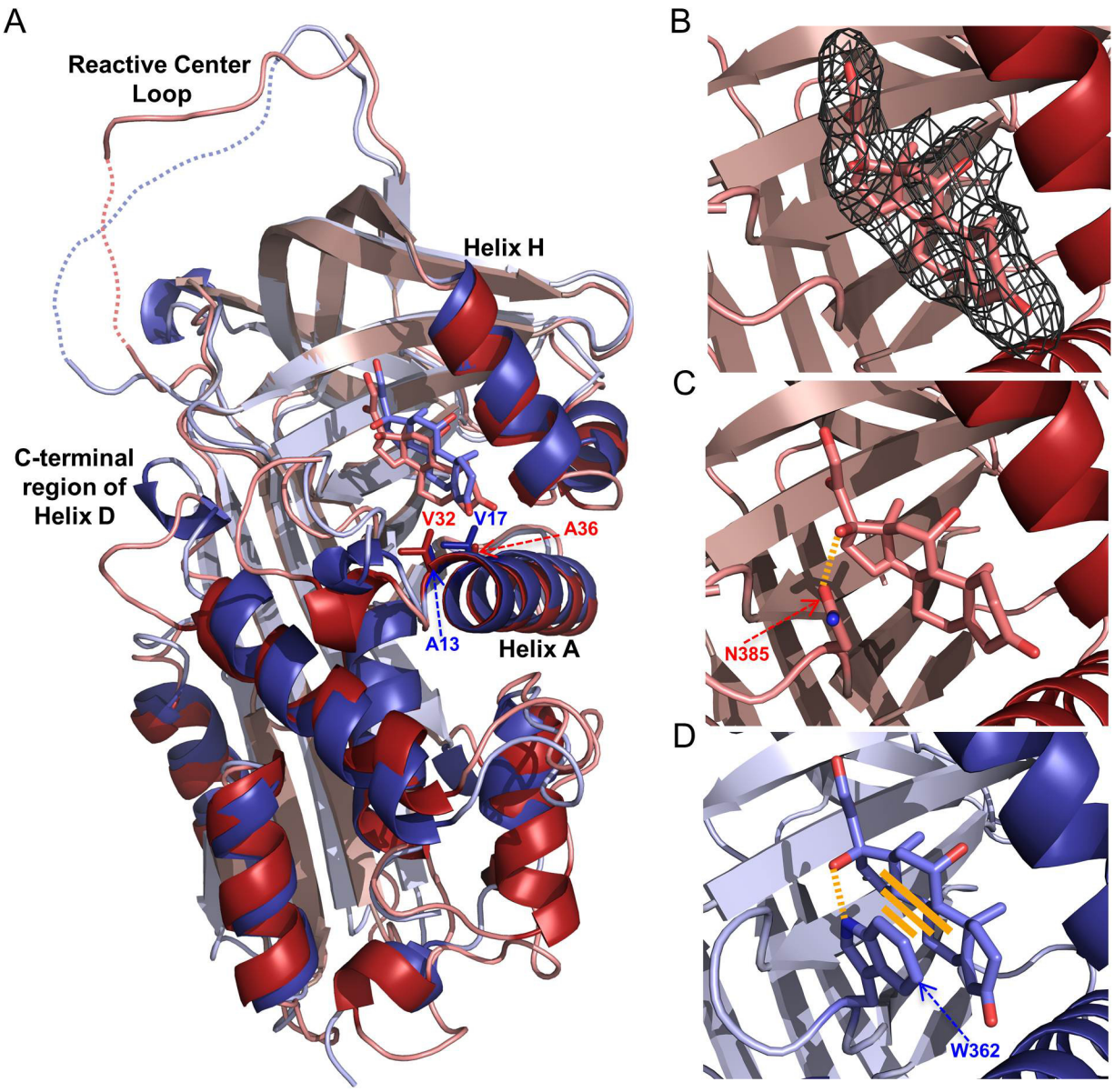


Figure 7

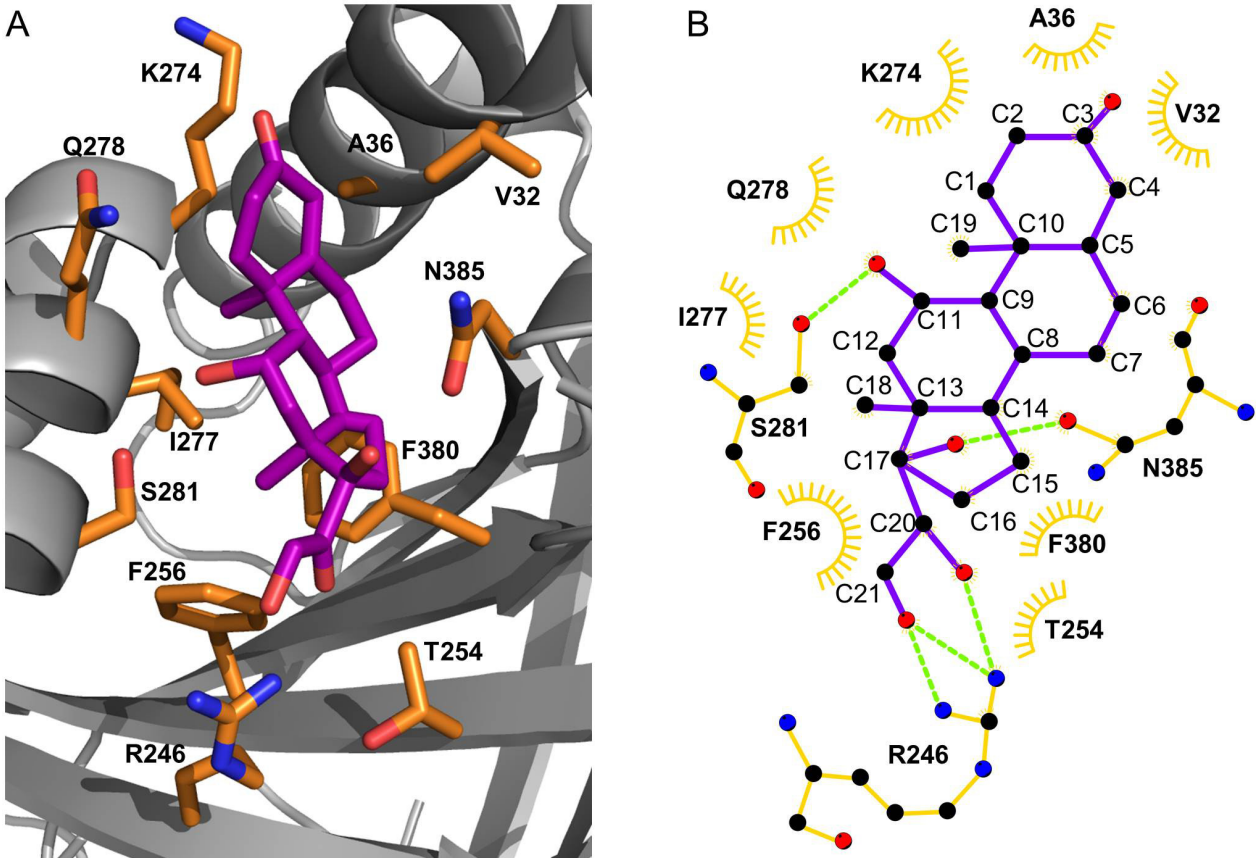
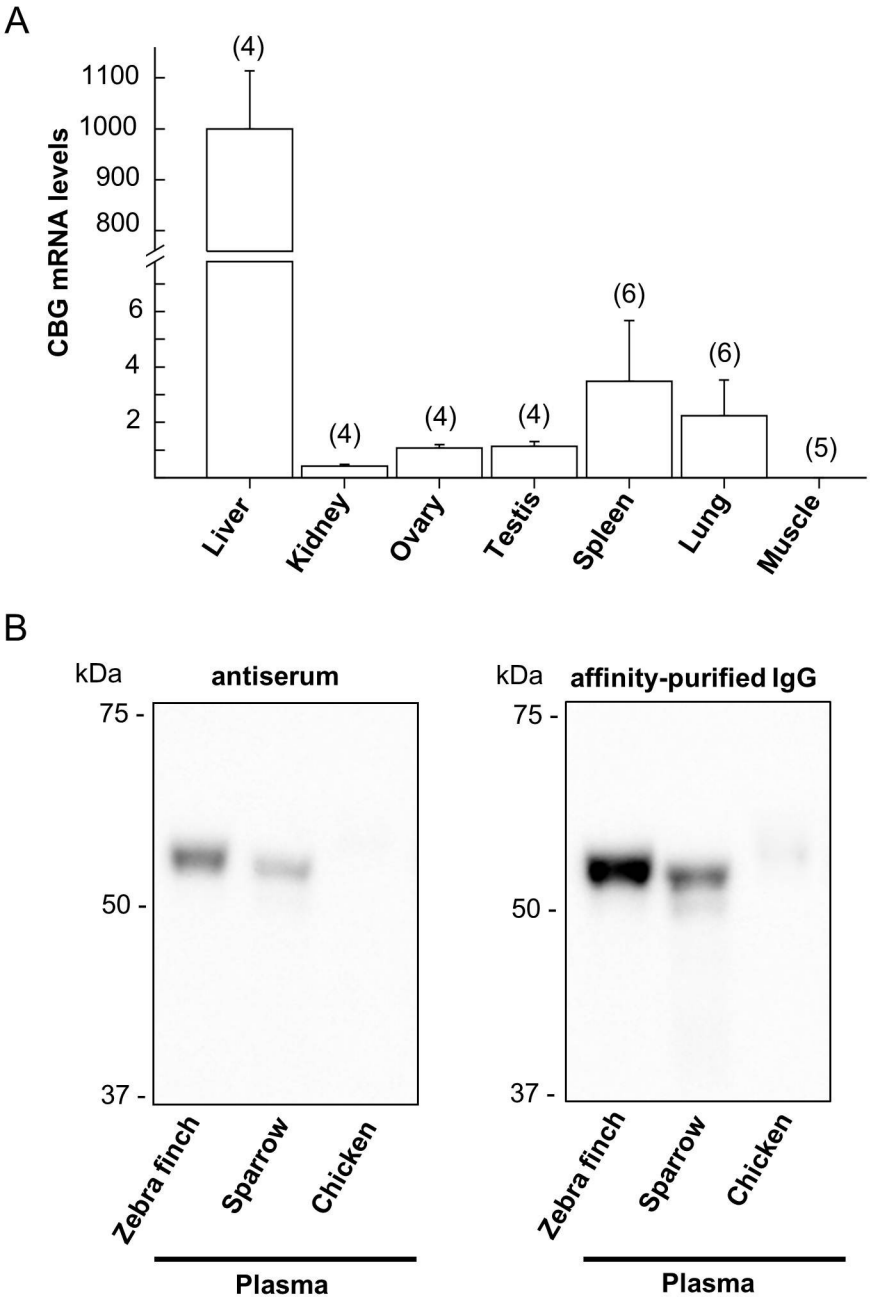


Figure 8



Identification of avian corticosteroid-binding globulin (SerpinA6) reveals the molecular basis of evolutionary adaptations in SerpinA6 structure and function as a steroid-binding protein

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