

Evolution of Hormone Signaling in Elasmobranchs by Exploitation of Promiscuous Receptors

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Specific interactions among proteins, nucleic acids, and metabolites drive virtually all cellular functions and underlie phenotypic complexity and diversity. Despite the fundamental importance of interactions, the mechanisms and dynamics by which they evolve are poorly understood. Here we describe novel interactions between a lineage-specific hormone and its receptors in elasmobranchs, a subclass of cartilaginous fishes, and infer how these associations evolved using phylogenetic and protein structural analyses. The hormone 1α -hydroxycorticosterone (1α -B) is a physiologically important steroid synthesized only in elasmobranchs. We show that 1α -B modulates gene expression in vitro by activating two paralogous intracellular transcription factors, the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR), in the little skate *Leucoraja erinacea*; MR serves as a high-sensitivity and GR as a low-sensitivity receptor. Using functional analysis of extant and resurrected ancestral proteins, we show that receptor sensitivity to 1α -B evolved millions of years before the hormone itself evolved. The 1α -B differs from more ancient corticosteroids only by the addition of a hydroxyl group; the three-dimensional structure of the ancestral receptor shows that the ligand pocket contained ample unoccupied space to accommodate this moiety. Our findings indicate that the interactions between 1α -B and elasmobranch GR and MR proteins evolved by molecular exploitation: a novel hormone recruited into new functional partnerships two ancient receptors that had previously interacted with other ligands. The ancestral receptor's promiscuous capacity to fortuitously bind compounds that are slight structural variants of its original ligands set the stage for the evolution of this new interaction.

Introduction

Virtually, all cellular functions are driven by specific interactions among biomolecules, such as enzymes and their substrates, transcription factors and their DNA-binding sites, and receptors and their ligands. Despite extensive “top-down” work on the global structure of molecular interaction networks (Barabasi and Oltvai 2004; Cork and Purugganan 2004; Teichmann and Babu 2004; Wilkins 2005), only limited knowledge is available concerning the specific mechanisms and dynamics by which the molecular interactions that constitute these networks evolve (Zhu et al. 2005; Bridgham et al. 2006; Prud'homme et al. 2006; Hittinger and Carroll 2007; McGregor et al. 2007; Ortlund et al. 2007).

Corticosteroid hormones and their receptors provide an elegant model of molecular interactions, and the existence of a lineage-specific corticosteroid in the taxon *Elasmobranchii* offers the opportunity to investigate how new hormone–receptor interactions evolve. Corticosteroids are produced in the adrenal/interrenal gland through an enzyme-mediated biosynthetic pathway and secreted into the blood. The classic actions of corticosteroids are mediated by intracellular corticosteroid receptors (CRs), members of the steroid hormone receptor family that also includes receptors for androgens, progestins, and estrogens (Thornton 2001). These proteins are ligand-activated transcription factors: upon binding to their hormone partners with high specificity and affinity, the receptor changes conformation, dimerizes, binds to specific DNA response elements, and attracts coregulator proteins, resulting in increased expression of nearby target genes (Beato et al. 1995). Like other steroid receptors, CRs have a modular domain structure, consisting of functionally autonomous

conserved domains for DNA-binding and ligand-activated transcription as well as a nonconserved hinge and an N-terminal domain with additional ligand-independent transcriptional regulatory functions (Kumar and Thompson 2005). Most vertebrates possess two paralogous CRs, a glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), which arose from a single ancestral CR in a large-scale gene duplication some 470–440 MYA (Thornton 2001; Bridgham et al. 2006). In bony vertebrates, GR is activated by cortisol and regulates metabolism, immunity, and the stress response; MR controls electrolyte homeostasis and blood pressure and is activated primarily by aldosterone in tetrapods and 11-deoxycorticosterone (DOC) in teleosts (Bentley 1998; Sturm et al. 2005). In addition to their classic receptor-mediated effects, some steroids also trigger rapid “nonclassical” effects through other mechanisms (Thomas et al. 2004, 2005; Prossnitz et al. 2007; Harvey et al. 2008).

A better understanding of steroid hormone–receptor interactions in basal vertebrates would help illuminate how GR, MR, and the physiological processes they regulate have evolved (Baker et al. 2007; Bury and Sturm 2007). Some 40 years ago, it was discovered that the plasma of elasmobranchs—sharks, skates, and rays—contains very high levels of the corticosteroid 1α -hydroxycorticosterone (1α -B), which is not known to be produced in any other taxon (Idler and Truscott 1966). 1α -B is synthesized in the interrenal gland in copious amounts relative to other endogenous hormones (Idler et al. 1967; Truscott and Idler 1972). It differs from other corticosteroids by the addition of a hydroxyl group at the C_1 position of the steroid backbone. The enzyme activity that drives this unusual 1α -hydroxylation reaction is present and functional in in vitro preparations of elasmobranch steroidogenic tissues (Truscott and Idler 1968), but the underlying gene has not been identified. The molecular basis for 1α -B action in elasmobranchs, particularly its potential interactions with MR and GR, remains uncharacterized. It has been speculated that 1α -B functions as a dual ligand in both glucocorticoid and mineralocorticoid signaling pathways (Gelsleichter and

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Musick 1999; Nunez and Trant 1999; Nunez et al. 2006; Manire et al. 2007). 1α -B clearly has a mineralocorticoid-like role in the regulation of salt and osmolyte balance (Hazon and Henderson 1984; Armour et al. 1993), but the evidence for its glucocorticoid-like effects is more speculative. Levels of 1α -B have been shown to increase under stress and reduced osmolarity (Hazon and Henderson 1984; Armour et al. 1993; Manire et al. 2007). Administration of glucocorticoids to elasmobranchs stimulates the regulation of carbohydrate metabolism (Patent 1970), cartilage growth (Gelsleichter and Musick 1999), and immune responses (Walsh et al. 2002). Like the glucocorticoids of other vertebrates, synthesis of 1α -B is regulated by adrenocorticotrophic hormone and angiotensin II from the hypothalamic–pituitary–adrenal axis (Honn and Chavin 1978; Hazon and Henderson 1985; O'Toole et al. 1990; Nunez and Vedeckis 2002). Although cortisol and other recognized glucocorticoids are absent or present at very low concentrations in elasmobranchs, 1α -B circulates at extremely high levels in elasmobranchs (Truscott and Idler 1972; Kime 1977; Armour et al. 1993), just as glucocorticoids do in most other vertebrates.

Here we characterize the functional interactions of 1α -B with the GR and MR of an elasmobranch, the little skate (*Leucoraja erinacea*), and reconstruct how these receptor–hormone interactions evolved. We combine molecular functional assays, ancestral gene resurrection (Thornton 2004), and analysis of protein structure to determine the functions of GR and MR with respect to this hormone and to characterize how the lineage-specific partnership of 1α -B with its receptors evolved.

Methods

Isolation and Reconstruction of CRs

Skate (*L. erinacea*) and hagfish (*Myxine glutinosa*) receptor ligand–binding domains (LBDs) were amplified using degenerate polymerase chain reaction (PCR) and rapid amplification of cDNA ends from liver cDNA (Bridgham et al. 2006). Lamprey (*Petromyzon marinus*) CR cDNA was amplified similarly from a cDNA library (Thornton 2001). Teleost (*Astatotilapia burtoni*) GR2a and MR were provided by R. Fernald, the human MR by R. Evans, and the human GR by B. Darimont.

Reconstruction and synthesis of ancestral receptors were performed as described in (Bridgham et al. 2006; Ortlund et al. 2007). Briefly, ancestral protein sequences were inferred using maximum likelihood (ML) phylogenetic reconstruction and a large data set of steroid and related receptors. Nucleic acid sequences coding for the LBD were synthesized de novo. Ambiguously reconstructed sites were defined as having an alternate amino acid state with a posterior probability greater than 0.20 or as having a different ML state when reconstructed on any tree in the 95% credible set collected by Bayesian Markov chain Monte Carlo analysis. Alternative states were introduced singly into the ML sequence using site-directed mutagenesis (QuikChange II, Stratagene, La Jolla, CA).

Receptor Activation

LBDs (with hinge and carboxy-terminal extension [CTE]) of extant receptors were amplified using high-

fidelity PCR and cloned into pSG5-GAL4DBD (gift of D. Furlow). Ancestral receptor LBDs (with CTE) were cloned into pSG5-GAL4DBD with a human GR hinge region. CHO-K1 cells were grown in 96-well plates in phenol red-free α -MEM plus 10% dextran–charcoal-stripped fetal bovine serum (Hyclone, Logan, UT), then transfected with 1 ng of receptor LBD, 100 ng of pFRluc reporter, and 0.1 ng of normalization vector pHRLtk using Lipofectamine and Plus Reagents (Invitrogen, Carlsbad, CA). After 4-h incubation, cells were treated with fresh medium, allowed to recover overnight, and then treated in triplicate with hormone or vehicle control (ethanol) for 24 h. Reporter expression was measured assayed using Dual-Glo (Promega, Madison, WI) and expressed as the ratio of firefly luciferase to *Renilla* luciferase. Dose–response relationships were analyzed using Prism4 software (GraphPad, La Jolla, CA). Receptors were considered unresponsive if they displayed <2-fold activation at >1 μ M hormone. The 1α -B was synthesized and provided by J. Rimoldi, University of Mississippi.

Quantitative PCR

Expression of MR and GR was measured in various organs of *L. erinacea* provided by J. St. George, Boston University. Total RNA was isolated using RNeasy (Qiagen, Valencia, CA) and cDNA prepared using QuantiTect Reverse Transcription (Qiagen). Skeletal and cardiac muscle samples were digested with proteinase K (Qiagen) to improve yield. Primers were designed to amplify the ligand-dependent activation (AF-2) domain of MR and GR LBDs. Housekeeping gene glyceraldehyde 3-phospho-dehydrogenase (GAPDH GenBank DQ382343) was isolated using degenerate PCR and used as an internal reference for normalization. Quantitative PCR (Q-PCR) was performed on an ABI Prism 7900 HT with $1 \times$ QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.3 μ M each primer, and skate cDNA. Cycling was as follows: 95°/15', (94°/15", 54°/30", 72°/30") \times 39 cycles, followed by melting curve analysis from 65° to 95°. Reactions were run in triplicate and fluorescence detected during extension. Primer efficiencies ($E_{MR} = 1.96$, $E_{GR} = 1.98$, and $E_{Gapdh} = 1.97$) were determined by standard curve analyses (Simon 2003) of serially diluted and linearized skate MR and GR in GAL4-DBD-pSG5 and skate Gapdh gene in pCR2.1 (Invitrogen).

In Silico Structural Analysis

A model of 1α -B was constructed and energy minimized using ChemOffice Ultra (CambridgeSoft, Cambridge, MA) and imported into MacPyMOL (Delano Scientific, Palo Alto, CA) along with the crystal structure of AncCR/DOC (PDB 2Q3Y). 1α -B was aligned to DOC at the C₁₀ carbon with the steroid backbones oriented in the same plane.

Results

High- and Low-Sensitivity Receptors for 1α -B

To determine the intrinsic hormone sensitivity of skate GR and MR, we expressed fusion constructs of receptor

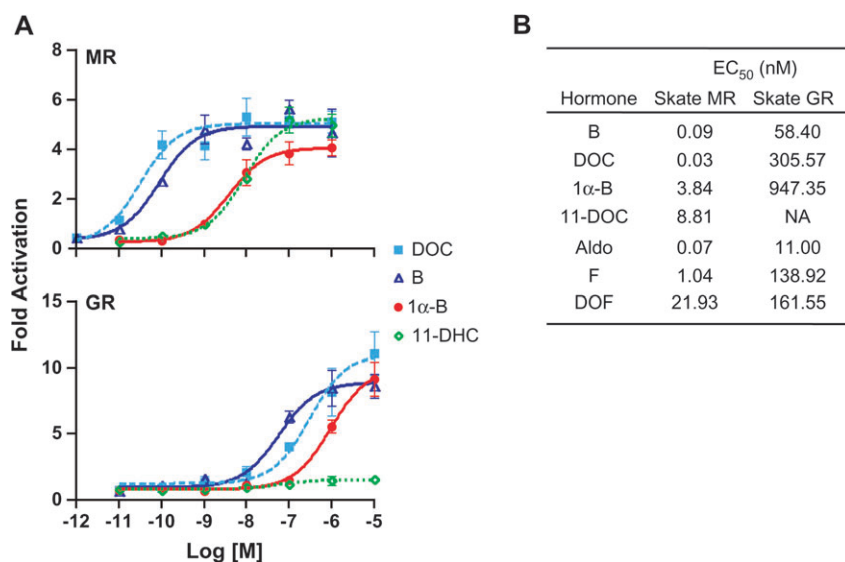


FIG. 1.—Skate MR and GR are high- and low-sensitivity receptors, respectively, for 1 α -B and other corticosteroids. (A) Ligand-dependent transcriptional activity of receptor LBDs was determined in the presence of increasing concentrations of various hormones using a luciferase reporter gene assay. Fold activation is the reporter activity of hormone-treated samples divided by vehicle-only control; points show the mean of three replicates plus standard error of the mean. Four hormones found in elasmobranchs were tested: 11-deoxycorticosterone (DOC), corticosterone (B), 1 α -B, and 11-dehydrocorticosterone (11-DHC). (B) Skate GR and MR hormone sensitivity. The concentration of hormone required for half-maximal reporter activation (EC₅₀) of skate MR and GR is shown in nanomolar (nM). NA, no activation, defined as <2-fold maximal activation or EC₅₀ > 1 μ M of hormone. Aldo, aldosterone; F, cortisol; and DOF, 11-deoxycortisol.

LBDs with the Gal4 DNA-binding domain. We evaluated transcription of a UAS-driven luciferase reporter gene in response to the predominant elasmobranch hormone 1 α -B and several other steroid hormones found at lesser concentrations in elasmobranchs, including 11-deoxycorticosterone (DOC), corticosterone (B), and 11-dehydrocorticosterone (11-DHC) (Truscott and Idler 1972). We also examined several mammalian corticosteroids not present in elasmobranchs, including aldosterone (Aldo), cortisol (F), and 11-deoxycortisol (DOF).

We found that both skate MR and GR LBDs are activated by 1 α -B and other corticosteroid hormones. MR is a high-sensitivity receptor, activating transcription in response to low nanomolar concentrations of all hormones examined, including 1 α -B. GR, in contrast, is activated by the same hormones but requires two to four orders of magnitude higher concentrations to achieve half-maximal activation (fig. 1). Based on these results, endogenous concentrations of 1 α -B and possibly other steroids in elasmobranchs are expected to activate the MR but only low concentrations of 1 α -B are likely to be high enough to activate the GR (see Discussion). The difference in quantitative sensitivity but not hormone specificity between GR and MR in elasmobranchs contrasts with the situation in humans and other bony vertebrates, in which GR and MR have distinct hormone preferences.

MR and GR are Ubiquitously Coexpressed

To determine whether differences in gene expression could be important for generating distinct tissue-specific gluco- or mineralocorticoid responses, we used Q-PCR to measure GR and MR transcripts across skate tissues. We found that both MR and GR are ubiquitously expressed,

with little variation in the relative quantities of the two transcripts. GR and MR transcript levels, normalized to expression of the housekeeping gene *Gapdh*, varied by less than an order of magnitude among tissues, except for in skeletal muscle, where *Gapdh* levels were very high (fig. 2A); a similar pattern was observed when transcripts were not normalized to *Gapdh* (supplementary fig. S1, Supplementary Material online). The ratio of MR to GR expression, which is not affected by differences in *Gapdh* levels, varied by a factor of less than two among tissues (fig. 2B; supplementary fig. S1, Supplementary Material online).

1 α -B Activates CRs from Other Vertebrates

We next sought to determine how the lineage-specific partnership of 1 α -B with CRs was assembled during evolution. We began by determining whether CRs from other species are also sensitive to 1 α -B. MRs from bony vertebrates—both tetrapods (*Homo sapiens*) and teleosts (*A. burtoni*)—activated reporter gene transcription in the presence of submicromolar concentrations of 1 α -B (fig. 3 and table 1), despite the hormone's absence from lineages other than elasmobranchs. The CR of an agnathan—the Atlantic hagfish (*M. glutinosa*), which possesses a single unduplicated gene orthologous to both GR and MR—had similar 1 α -B sensitivity. Only the GRs of bony vertebrates—which are activated only by 17-hydroxylated corticosteroids like cortisol (Bridgham et al. 2006)—and the CR of the sea lamprey (*P. marinus*) were insensitive to 1 α -B.

Ancient Receptor Sensitivity to 1 α -B

The widespread sensitivity of vertebrate CRs to 1 α -B suggests an ancient origin of 1 α -B responsiveness. To test

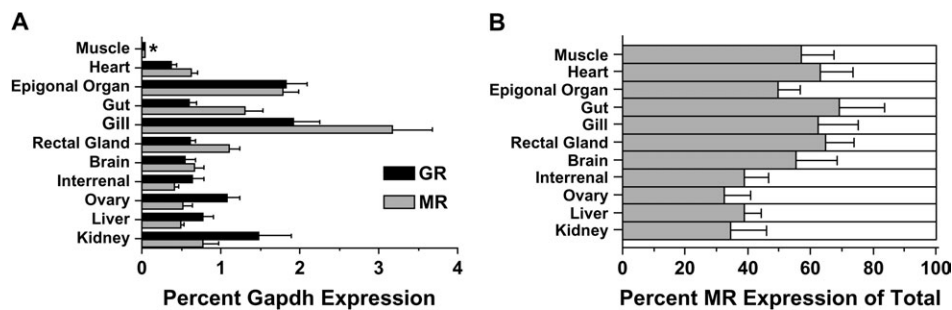


FIG. 2.—MR and GR are widely coexpressed in skate. Abundance of MR, GR, and Gapdh mRNAs were determined in various tissues of adult skate using quantitative PCR. (A) The mean expression level and standard error of the mean (bars) were calculated for MR and GR from triplicate reactions and normalized to Gapdh. Asterisk denotes a tissue-specific increase in Gapdh expression that results in a decrease in normalized MR and GR expression. (B) Relative MR and GR expression in each tissue. Gray bars (with standard error of the mean) show MR expression as percent of total (MR plus GR) expression.

the hypothesis that receptor sensitivity to 1α -B predates the elasmobranch-specific emergence of the hormone, we resurrected and functionally characterized three ancient CRs as they existed in ancestral vertebrates. Specifically, we used a large database of extant receptor sequences and used phylogenetic techniques to infer the ML amino acid sequences of three ancient receptors: the ancestral corticoid receptor (AncCR, the unduplicated ancestral gene from which extant MRs and GRs descend by gene duplication), GR in the last common ancestor of all jawed vertebrates (AncGR1), and GR from the last common ancestor of all bony vertebrates (AncGR2), after their split from cartilaginous fishes (fig. 4). We then synthesized DNAs coding for the LBDs of these reconstructed proteins, expressed them, and characterized their functions using a reporter transcription assay in cell culture (Bridgham et al. 2006; Ortlund et al. 2007).

As predicted, we found that the most ancient receptors—AncCR and AncGR1—are extremely sensitive to 1α -B, activating transcription with EC_{50} s of ~ 20 nM. AncGR2, in contrast, was unresponsive, as expected based on the lack of sensitivity to 1α -B in its descendants, the GRs of tetrapods and teleosts (fig. 4 and table 1). To determine whether the 1α -B sensitivity of AncCR might be an artifact of uncertainty in the inference of the ancestral sequence, we identified sites that were ambiguously reconstructed (defined as having an alternative amino acid state with posterior probability >0.20). In all cases but five, the alternate state is found in other 1α -B-activated receptors and is therefore not sufficient to abolish sensitivity to that hormone. Introducing each of these five alternate states into the AncCR by site-directed mutagenesis had no effect on ligand activation (table 2). Among sites that make contact with the ligand in the AncCR crystal structure (Ortlund et al. 2007), only one was ambiguously reconstructed; introducing this alternate state into AncCR had no effect on sensitivity to 1α -B (table 2). We conclude that AncCR's response to 1α -B is not an artifact of uncertainty in the ancestral reconstruction.

To determine whether AncCR's sensitivity to 1α -B may be due to error in the phylogeny on which the ancestral reconstruction is based, we inferred the ML sequence of AncCR on each of the 467 trees in the 95% credible set from a large Bayesian analysis. At only one sequence site did the ancestral reconstructions differ among trees. We introduced the alternate state at this site (A7V) into AncCR by mutagenesis and found it had no effect on sensitivity to 1α -B (table 2).

We conclude that AncCR and AncGR1 were sensitive to 1α -B, and this ancient sensitivity was retained in most of the lineages descending from those ancestors, including the MR and GR of elasmobranchs. After the divergence of bony from cartilaginous fishes, the GRs of bony vertebrates subsequently lost 1α -B sensitivity, during the same period in which the receptor became corticoid specific (Bridgham et al. 2006; Ortlund et al. 2007). This result indicates that CRs were capable of being activated by 1α -B many millions of years before synthesis of the hormone itself evolved in the elasmobranch lineage.

Structural Analysis of 1α -B Docked in the AncCR

To determine why ancient receptors were activated by 1α -B, we examined the previously solved crystal structure of AncCR (Ortlund et al. 2007). We hypothesized that AncCR was structurally "preadapted" to bind 1α -B because of that hormone's similarity to DOC, an ancient hormone that is the putative ancestral ligand for AncCR (Ortlund et al. 2007). We generated a structural model of 1α -B docked into the LBD of the AncCR with DOC. The 1α -B differs from DOC only by the presence of hydroxyl groups at the C_1 and C_{11} positions. The structure shows that AncCR's ligand pocket contains ample room to accommodate the 11-hydroxyl of 1α -B; this conclusion is supported by the receptor's previously identified ability to bind cortisol and aldosterone, which also carry the 11-hydroxyl, without conformational adjustment (Ortlund et al. 2007). The ligand pocket also contains unoccupied space in the alpha plane above the C_1 carbon (fig. 5A, 5C); in the model of AncCR with 1α -B, this space is occupied by and adequate to accommodate the 1α -hydroxyl (fig. 5B, 5D). A slight hydrophobic clash of this hydroxyl with AncCR's Leu32 and Phe92 is the likely cause of the receptor's slightly reduced sensitivity to 1α -B compared with other ligands that lack the 1α -hydroxyl. That the receptor retains nanomolar sensitivity to 1α -B, however, indicates that minor adjustments of the receptor backbone or side chain rotamers are sufficient to relieve the clash.

Discussion

Our analysis indicates that the lineage-specific partnership of 1α -B with MR and GR in elasmobranchs evolved

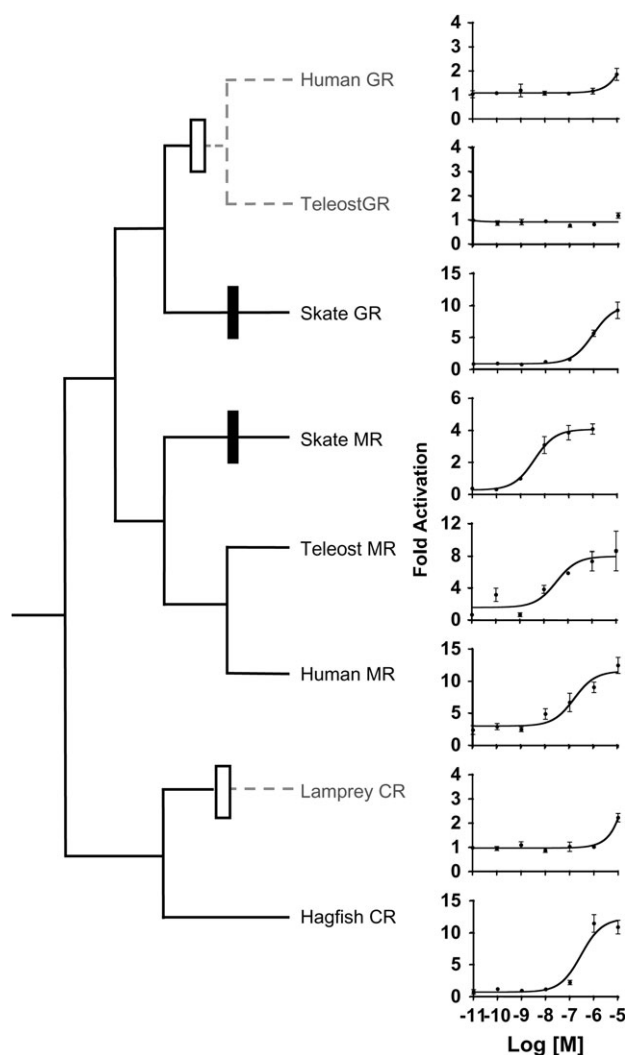


FIG. 3.—GR and MR sensitivity to 1α -B is taxonomically widespread. Sensitivity of CRs from a variety of vertebrates to 1α -B was assessed using a luciferase reporter assay. Black and gray branches show receptors sensitive and insensitive to 1α -B, respectively. Open boxes mark evolutionary loss of ancestral activation by 1α -B in the most parsimonious scenario. Black boxes denote the evolution of 1α -B synthesis in elasmobranchs.

due to molecular exploitation—recruitment of older molecules, which previously had different functions, into new functional relationships (Thornton 2001; Bridgham et al. 2006). Both the widespread taxonomic distribution of 1α -B sensitivity and experimental analysis of resurrected ancestral receptors indicate that the ancestral gene from which both GR and MR evolved was already sensitive to 1α -B, millions of years before synthesis of the hormone itself evolved (fig. 6). Sensitivity to 1α -B has been retained to the present in numerous descendants of AncCR, including those in numerous species that lack 1α -B, such as mammals, in which administration of exogenous 1α -B elicits a strong mineralocorticoid response (Idler et al. 1967). Our results indicate that the partnership of 1α -B with its receptors in elasmobranchs evolved when a newly synthesized hormone-recruited preexisting receptors, which previously had different ligands, into new functional

Table 1
Sensitivity of Extant and Ancestral Receptors to 1α -B

Receptor	EC ₅₀ (nM)	GenBank ID	Species
Skate MR	3.81	ABD46745	<i>Leucoraja erinacea</i>
AncCR	20.6	ABD46748	Ancestral reconstruction
AncGR1	25.1	ABU96169	Ancestral reconstruction
Teleost MR	32.5	AAM27890	<i>Astatotilapia burtoni</i>
Human MR	153	NP_000892	<i>Homo sapiens</i>
Hagfish CR	288	ADB46742	<i>Myxine glutinosa</i>
Skate GR	947	ABD46744	<i>L. erinacea</i>
Lamprey CR	No activation	AAK20929	<i>Petromyzon marinus</i>
AncGR2	No activation	ABU96170	Ancestral reconstruction
Teleost GR2	No activation	AAM27888	<i>A. burtoni</i>
Human GR	No activation	NP_000167	<i>H. sapiens</i>

NOTE.—Sensitivity is presented as the EC₅₀—the concentration at which half-maximal reporter gene activation (EC₅₀) is achieved.

partnerships. This evolutionary dynamic is similar to that previously observed for the interaction between aldosterone and the MR of tetrapods (Bridgham et al. 2006). Other steroid hormone–receptor interactions evolved when intermediates in the biosynthesis of ligands for more ancient receptors were recruited into partnerships with newly duplicated receptors (Thornton 2001; Thornton et al. 2003). Several other apparent examples of evolutionary recruitment of ancient molecules into new partnerships have recently been described (Krasowski et al. 2005; Cai et al. 2007; Cardoso et al. 2007), suggesting that the evolution of specific molecular interactions by molecular exploitation may be a dominant theme in the emergence of biological systems.

Molecular exploitation is greatly facilitated by untapped promiscuity in ancient proteins, which allows them to accommodate new partners that are minor variants of their original binding partners. 1α -B is identical to the likely ancestral ligand DOC, except for additional oxygen atoms at the C₁ and C₁₁ positions. Our structural analysis of AncCR indicates that the ancestral ligand pocket had ample room to accommodate these modifications when the hormone evolved later in the elasmobranchs. AncCR's promiscuous response to steroids that had not yet evolved contrasts with its unresponsiveness to the more ancient androgens, estrogens, or progestins, which differ from the corticosteroids at other key locations on the steroid backbone (Bridgham et al. 2006). AncCR therefore appears to have been only as specific as it needed to be: it excluded other endogenously produced hormones that would inappropriately activate the receptor but not other potential ligands whose synthesis had not yet evolved. When steroids that fortuitously fit the pocket appeared later, this promiscuity set the stage for the evolution of novel receptor–ligand partnerships with physiological or developmental functions. Structural promiscuity—a ligand pocket that contains extra space or flexibility, allowing it to accommodate a range of ligands—is a common protein feature and is likely to have facilitated the evolution of new functions in both receptors and enzymes (Brzozowski et al. 1997; James and Tawfik 2001; Bledsoe et al. 2002; Copley 2003; Khersonsky et al. 2006; Pereira de Jesus-Tran et al. 2006; Bloom et al. 2007; Nettles et al. 2007; Suino-Powell et al. 2008).

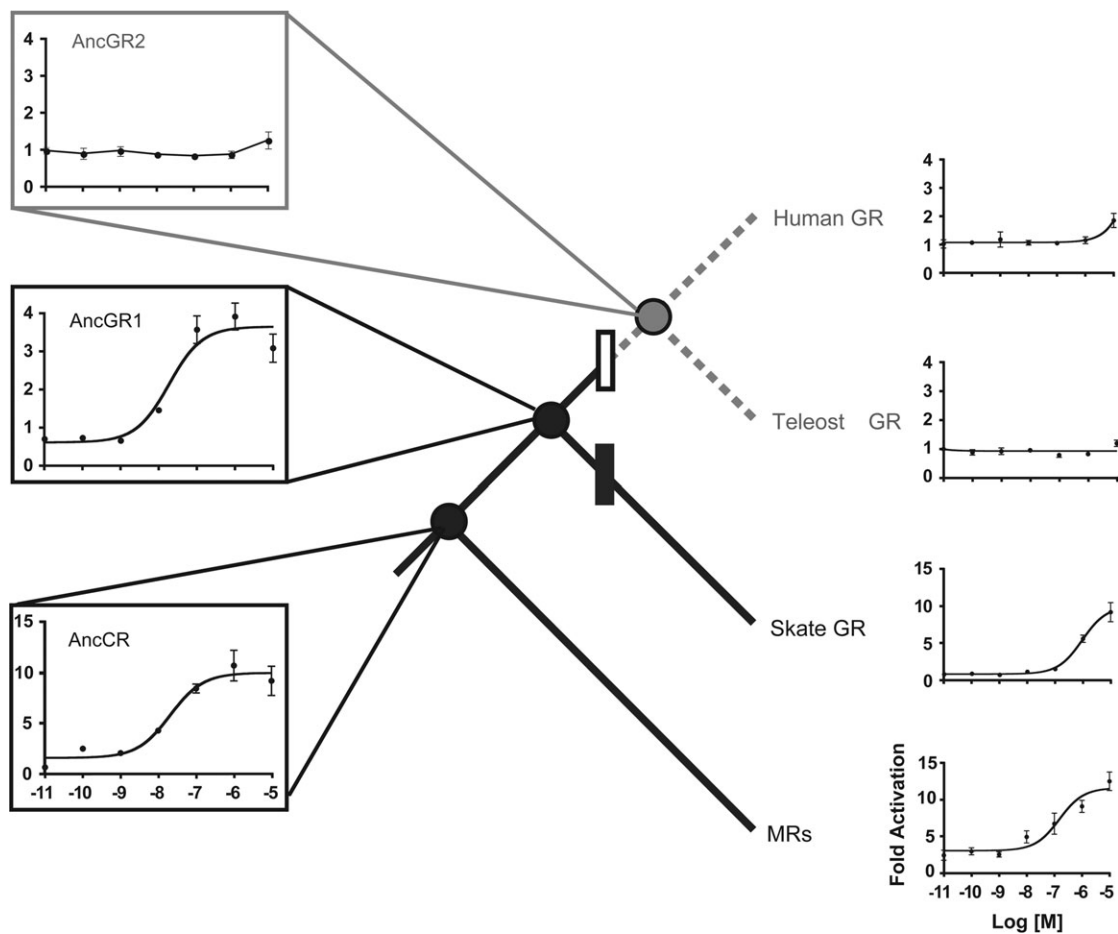


FIG. 4.—CR sensitivity to 1α -B predates the evolution of 1α -B synthesis. Three ancestral receptors from early vertebrates (circled nodes) were resurrected by ancestral sequence reconstruction and gene synthesis. Graphs depict luciferase reporter activity in the presence of increasing 1α -B concentrations, relative to vehicle-only control. Dose–response curves for selected extant receptors are also shown. Black and gray circles denote ancestral receptors sensitive and insensitive, respectively, to 1α -B. White rectangle shows loss of 1α -B activation; black rectangle, origin of 1α -B synthesis. AncCR represents the unduplicated ancestral gene from which GR and MR descend; AncGR1 is GR in the last common ancestor of jawed vertebrates; and AncGR2 is GR in the last common ancestor of bony vertebrates.

Our results shed light on the functional roles of corticosteroids and their receptors in elasmobranchs. The LBDs of skate MR and GR differ little in hormone specificity but radically in sensitivity: MR is activated by much lower corticosteroid concentrations than are required to activate GR. Analysis of serum steroid concentrations in a wide variety of elasmobranch species have indicated that 1α -B levels are very high—up to several hundred nanomolar (Truscott and Idler 1968; Kime 1977)—and that corticosterone, DOC, and 11-DHC are present at orders of magnitude lower concentrations (Idler et al. 1967; Truscott and Idler 1972; Kime 1977; Hazon and Henderson 1984; Armour et al. 1993; Rasmussen and Crow 1993; Snelson et al. 1997). Our results indicate that skate MR has corticosteroid sensitivity similar to that of tetrapod and teleost MRs (Bridgham et al. 2006), whereas the skate GR's sensitivity is orders of magnitude lower. Although *in vitro* reporter assays using fusion proteins are not precise predictors of *in vivo* dose–response relationships, physiological 1α -B levels, at least under some conditions, are likely to be well beyond those necessary to activate skate MR. It is possible that DOC and

corticosterone may also serve as MR ligands. Skate GR, in contrast, is likely to be activated only by elevated concentrations of 1α -B (and possibly corticosterone) such as those that occur under conditions of stress (Manire et al. 2007). Our findings suggest the hypothesis that low corticosteroid levels regulate MR to control osmolarity, whereas stress-induced increases in the same hormones regulate GR to control the stress response. Like the elasmobranch GR, the GR of bony vertebrates is a low-sensitivity receptor that activates the stress response only when cortisol levels are very high (Bentley 1998). Unlike the elasmobranch GRs, however, GRs in bony vertebrates evolved novel specificity as well, making them insensitive to even high doses of mineralocorticoids (Bridgham et al. 2006). Other factors, such as posttranslational modification and the availability of coregulators, may also contribute to differences in GR and MR function.

MR and GR are ubiquitously coexpressed, at roughly equal proportions, in a wide variety of skate tissues. This is largely consistent with patterns of expression found in teleosts (Bury et al. 2003; Greenwood et al. 2003; Sturm et al.

Table 2
AncCR's Sensitivity to 1α -B Is Robust to Statistical and Phylogenetic Uncertainty

Receptor	EC50 (nM) ^a	Source of Uncertainty ^b	Receptors Insensitive to 1α -B with Alternate State ^c
AncCR	20.6	—	—
AncCR A7V	23.3	Phylogenetic	Pma CR
AncCR A36G	3.0	Stochastic—LBP	—
AncCR S20T	10.5	Stochastic—extant	Abu GR; Pma CR
AncCR K38R	12.3	Stochastic—extant	Has GR
AncCR S76A	4.5	Stochastic—extant	Hsa GR; Pma CR
AncCR V137A	9.8	Stochastic—extant	Pma CR
AncCR V224A	8.1	Stochastic—extant	Abu GR; Pma CR

NOTE.—Alternate reconstructions of ancestral states were introduced into the ML reconstruction of AncCR by site-directed mutagenesis and their sensitivity to 1α -B determined with a reporter gene assay.

^a Sensitivity to 1α -B is reported as the concentration required for half-maximal activation of a luciferase reporter gene.

^b Phylogenetic uncertainty refers to ML reconstructions that differ among trees in the 95% credible set. Stochastic uncertainty refers to nonoptimal reconstructions with posterior probability >0.2 on the ML phylogeny. LBP, sites in the ligand-binding pocket. Extant, alternate reconstruction is present in one or more receptors insensitive to 1α -B.

^c Abu, *Astatotilapia burtoni*; Hsa, *Homo sapiens*; and Pma, *Petromyzon marinus*.

2005) and tetrapods (de Castro et al. 1996; Le Menuet et al. 2000). These data provide no evidence for subfunctionalization of expression domains as important in the maintenance of the GR and MR after duplication. We cannot rule out the possibility, however, that GR and MR may be differentially expressed on a finer scale, in specific cell types or groups of cells or at specific stages.

The interaction of CRs, their ligands, and the DNA response elements they regulate represent a relatively simple but physiologically essential molecular network. Gene duplication provides raw material for the genesis and expansion of such networks (Wagner 1994; Teichmann and Babu 2004), but duplication alone is not enough to make a network more elaborate or generate a new one. To increase complexity, functional interactions must diversify, either by the evolution of new interactions or by the partitioning of ancestral functions among new network nodes generated by duplication. We have shown that the corticosteroid hormone/receptor network was elaborated when receptor gene duplication and divergence, together with the extension of a biosynthetic pathway, produced new receptors and hormones that were slight structural and functional modifications of their ancestral forms. The receptors' promiscuity set the stage for these new hormones to be integrated into the existing receptor signaling network. Subsequently, divergence of the two sister receptors—leading to reduced sensitivity in the GR—resulted in markedly different quantitative sensitivities to corticosteroid hormones and the possibility of distinct network responses to varying levels of hormone. These observations indicate that the complex molecular networks that drive physiological process can evolve by mechanisms as simple as molecular exploitation and the partial degradation of function after gene duplication.

Supplementary Material

Supplementary figure S1 is available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

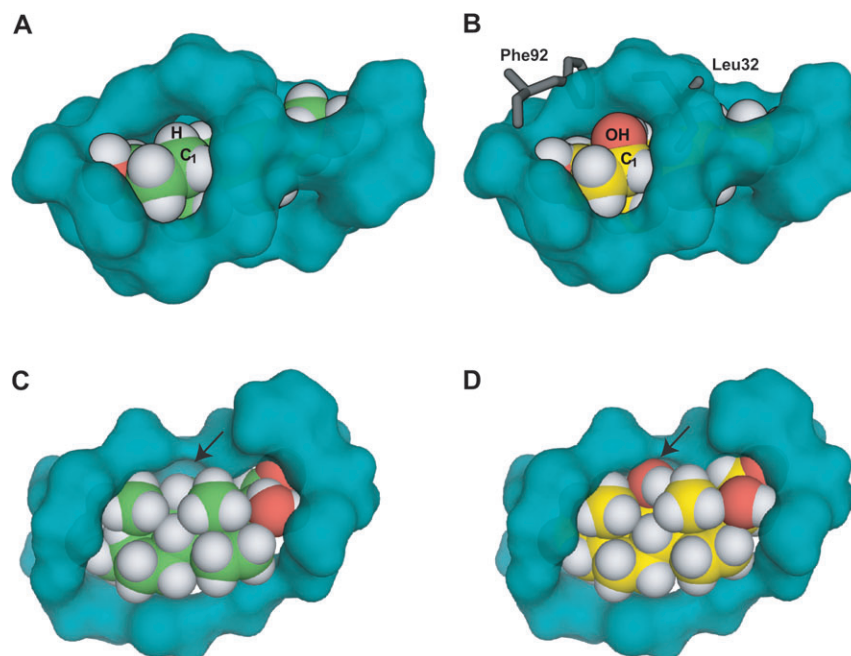


FIG. 5.—The structural basis for receptor promiscuity. The crystal structure of AncCR's ligand pocket in complex with DOC (green, *A* and *C*) and an in silico model of AncCR with 1α -B (yellow, *B* and *D*) are shown from two perspectives (*A*, *B* and *C*, *D*). Electron density of atoms within 4 angstroms of ligand is shown as a teal surface; some atoms from the foreground surface have been excluded to aid viewing. Red, oxygen atoms; white, hydrogen atoms. In (*A*), empty space above the C_1 carbon of DOC is apparent (black arrow), which can accommodate the added hydroxyl of 1α -B (*B*, labeled OH). In (*C*), empty space (arrow) above C_{11} and its hydrogen (labeled H) accommodates the added hydroxyl of 1α -B (*D*).

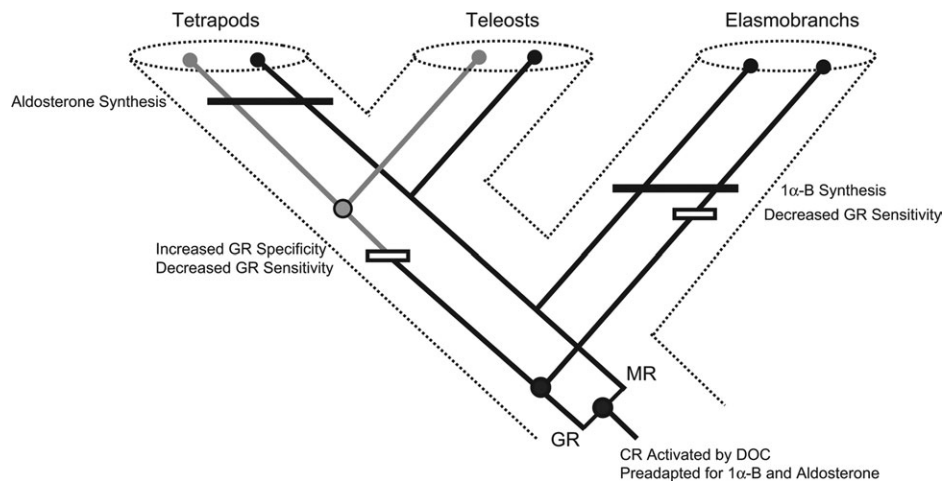


FIG. 6.—Receptor exploitation in the evolution of corticosteroid signaling. The phylogeny traces the evolution of receptor genes and their ligand sensitivity (black and gray lines, sensitive and insensitive to 1α -B, respectively) within the divergence of major vertebrate lineages (funnels). Closed circles at nodes on the phylogeny indicate ancestral receptors that were resurrected and functionally characterized. Open boxes mark functional shifts in the GR lineage. The origin of aldosterone and 1α -B synthesis is shown by black bars in the tetrapod and elasmobranch lineages, respectively.

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