

N-terminal Domain Regulates Steroid Activation of Elephant Shark Glucocorticoid and Mineralocorticoid Receptors

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Abstract. Orthologs of human glucocorticoid receptor (GR) and human mineralocorticoid receptor (MR) first appear in cartilaginous fishes. Subsequently, the MR and GR diverged to respond to different steroids: the MR to aldosterone and the GR to cortisol and corticosterone. We report that cortisol, corticosterone and aldosterone activate full-length elephant shark GR, and progesterone, which activates elephant shark MR, does not activate elephant shark GR. However, progesterone inhibits steroid binding to elephant shark GR, but not to human GR. Together, this indicates partial functional divergence of elephant shark GR from the MR. Deletion of the N-terminal domain (NTD) from elephant shark GR (truncated GR) reduced the response to corticosteroids, while truncated and full-length elephant shark MR had similar responses to corticosteroids. Swapping of NTDs of elephant shark GR and MR yielded an elephant shark MR chimera with full-length GR-like increased activation by corticosteroids and progesterone compared to full-length elephant shark MR. Elephant shark MR NTD fused to GR DBD+LBD had similar activation as full-length MR, indicating that the MR NTD lacked GR-like NTD activity. We propose that NTD activation of human GR evolved early in GR divergence from the MR.

Keywords: glucocorticoid receptor; allosteric; elephant shark; nuclear receptors; evolution

Introduction

Glucocorticoids, such as cortisol and corticosterone, have diverse physiological activities in humans and other terrestrial vertebrates, including regulating glucose metabolism, cognition, immunity and the response to stress (1–5). The physiological actions of glucocorticoids are mediated by the glucocorticoid receptor (GR), which belongs to the nuclear receptor family, a diverse group of transcription factors that also contains receptors for mineralocorticoids (MR), progestins (PR) androgens (AR) and estrogens (ER) (6–8).

The GR is closely related to the MR (9); phylogenetic analysis indicates that the GR and MR evolved from a corticoid receptor (CR) that evolved in a jawless vertebrate in an ancestor of modern lamprey and hagfish (7,10–13). Distinct orthologs of human GR and human MR first appear in cartilaginous fishes, the oldest group of extant jawed vertebrates (gnathostomes) that diverged from bony vertebrates about 450 million years ago (14). Since the emergence of the GR and MR in cartilaginous fishes, these steroid receptors have diverged to respond to different corticosteroids. For example, human MR is activated by aldosterone, the physiological mineralocorticoid in humans, at a concentration at least ten-fold lower than by cortisol (15–18). However, aldosterone has little activity for human GR, which is activated by cortisol and corticosterone (Figure 1) (6,12,16,19,20).

The multi-domain structure of the GR is important in regulating steroid activation of the GR. Like other steroid receptors, the GR consists of an N-terminal domain (NTD) (domains A and B), a central DNA-binding domain (DBD) (domain C), a hinge domain (D) and a C-terminal ligand-binding domain (LBD) (domain E) (5,8,19,21,22) (Figure 2). The GR NTD is noteworthy for containing an activation function domain (AF1) that interacts with the DBD to provide a human GR NTD-DBD protein with constitutive activity (23–26). AF1 also strongly stimulates transcriptional activation by glucocorticoids of the GR in humans (19,24,25,27), other terrestrial vertebrates (21,27–31) and ray-finned fish (21,32). Interaction of the NTD with coactivators as well as post-transcriptional modification of the NTD also regulates transcriptional activation of the GR (5,22,30,33,34). It is not known when strong activation by the NTD of vertebrate GRs evolved.

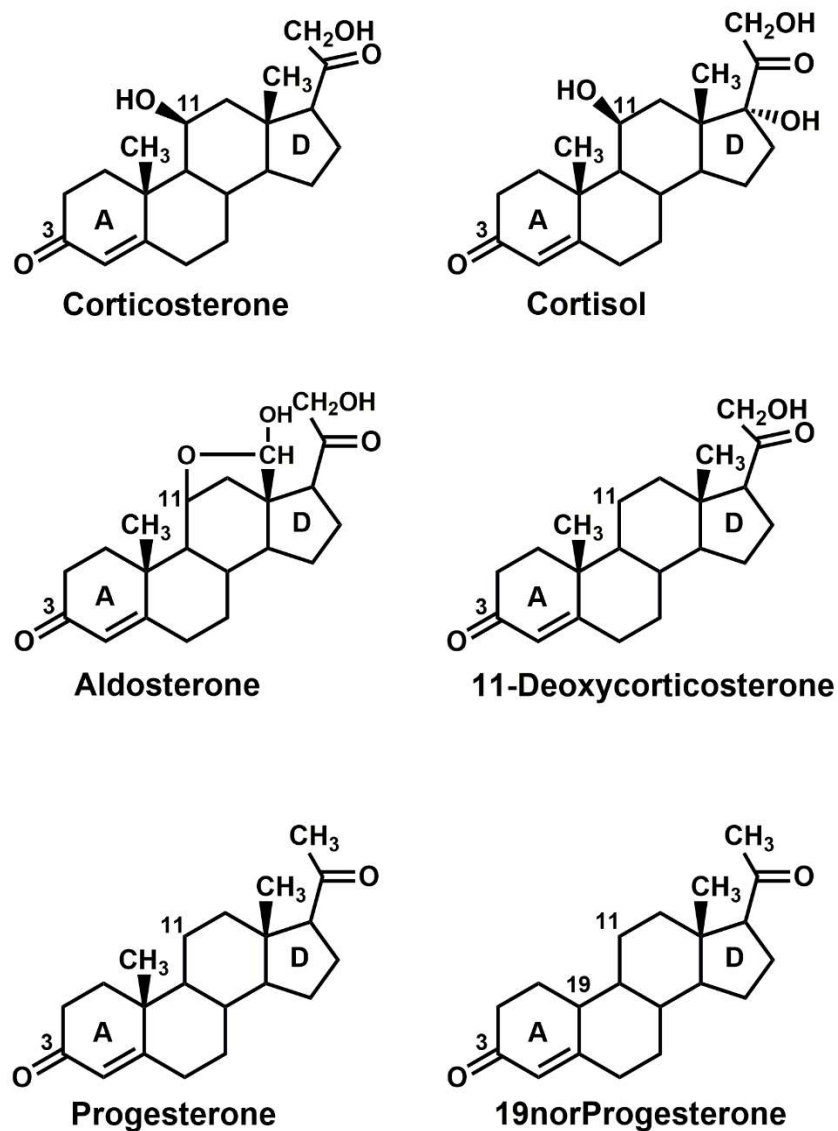


Fig. 1. Structures of Corticosteroids and Progesterone. Cortisol and corticosterone are physiological glucocorticoids in terrestrial vertebrates and ray-finned fish (7,10,35). Aldosterone, 11-deoxycorticosterone are physiological mineralocorticoids (10,12,36,37). Progesterone is female reproductive steroid that also is important in male physiology (38,39).

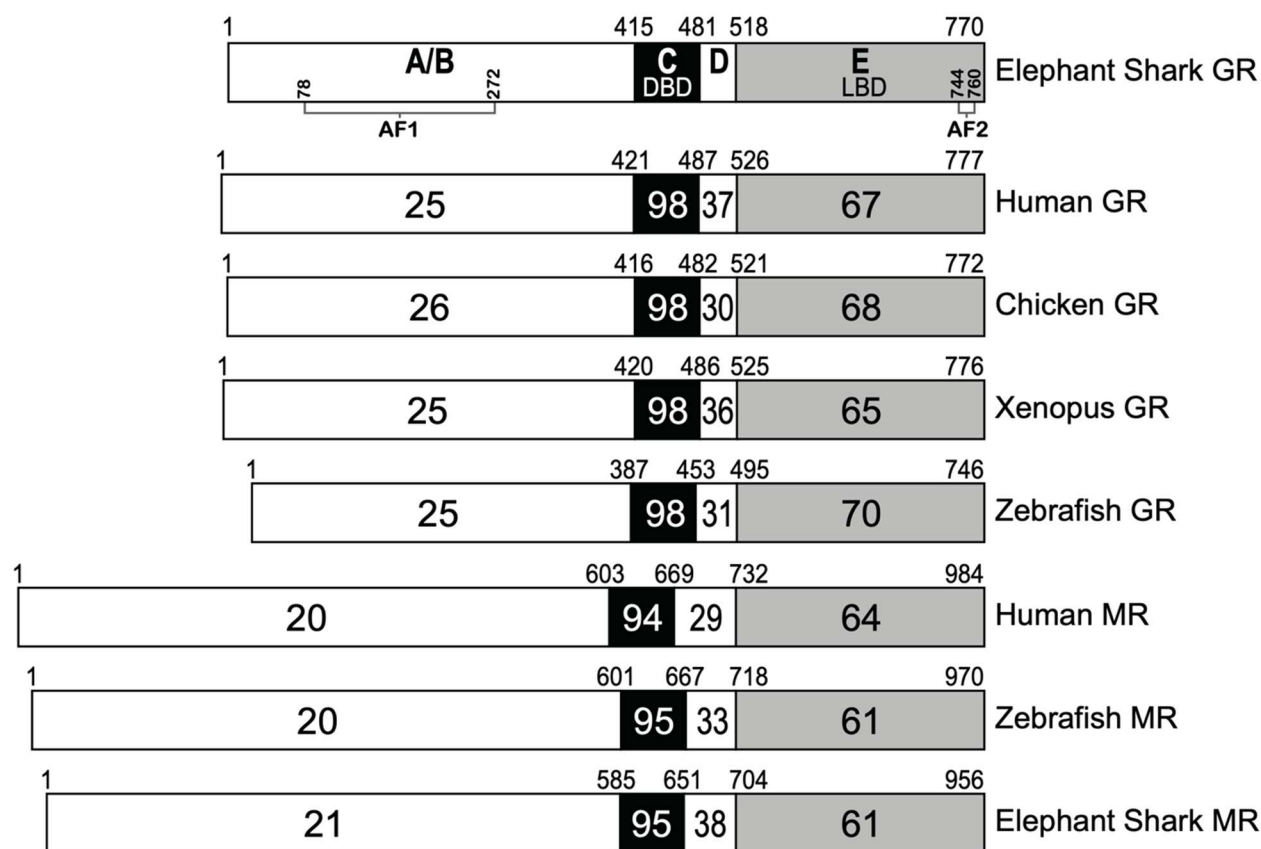


Fig.2. Comparison of domains in elephant shark GR with vertebrate GRs and MRs. GRs from elephant shark, zebrafish, *X. laevis*, chicken and humans and MRs from elephant shark, humans and zebrafish are compared. The functional NTD (A/B), DBD (C), hinge (D) and LBD (E) domains are schematically represented with the numbers of amino acid residues and the percentage of amino acid identity depicted. AF1 (5) and activation function 2 (AF2) (5) domains are shown in human GR.

To investigate corticosteroid signaling via the GR, in the light of the evolution (40), we decided to study activation of full-length and truncated elephant shark (*Callorhinchus milii*) GR by a panel of corticosteroids, which were used in a previous study of elephant shark MR (41). Elephant shark is an excellent system for evolutionary studies because in addition to the key phylogenetic position of elephant shark in vertebrate evolution, as a precursor for ray-finned fish and terrestrial vertebrates, genomic analyses reveal that elephant shark genes are evolving slowly, which makes their genes windows into the past (42). Thus, characterization of steroid activation of elephant shark GR permits analyses relevant to early events in the divergence of the GR and its MR paralog.

Although corticosteroid activation of another cartilaginous fish, skate GR, has been reported (43), this GR consisted of skate LBD fused to a GAL4-DBD. This GR lacked skate NTD and DBD, two domains that are important in AF1 activation of human GR (5,19,23–26). The absence of data on steroid activation of a full-length cartilaginous fish GR prompted our investigation of the role of the NTD and DBD on transcriptional activation of a cartilaginous fish GR to determine if AF1 activity evolved in the NTD in a GR in a basal vertebrate. As the sequences of the NTDs of elephant shark and human GRs are poorly conserved, with 25% sequence identity (Figure 2), it was not evident that there would be strong AF1 activity in the NTD in elephant shark GR. We also investigated the evolution of GR specificity for glucocorticoids through a comparison of steroid activation of full-length elephant shark GR to elephant shark MR and human GR (16,19–21,23,27).

Here we report that full-length elephant shark GR is activated by cortisol and corticosterone, which activate human GR (16,19,20,27). Unexpectedly, aldosterone and 11-deoxycorticosterone, two mineralocorticoids, also activated elephant shark GR. These two mineralocorticoids have little activity for human GR (15,16,27), which is selective for cortisol and corticosterone (16,20,27). Elephant shark GR is not activated by either progesterone or 19norprogesterone, in contrast to elephant shark MR, which also is activated by cortisol and corticosterone (41). However, we find that progesterone and 19norprogesterone inhibit corticosterone activation of elephant GR, while neither progestin inhibits cortisol activation of human GR.

We find that the NTD in elephant shark GR is a strong activator of steroid mediated transcriptional activation. Corticosteroid activation of truncated elephant shark GR, in which the NTD is deleted, was less than 10% of that of full-length GR, in contrast to truncated elephant shark MR, in which deletion of the NTD did not have a major effect on corticosteroid activation (41). To investigate further the specificity of the each NTD for activation of the GR and MR, we studied chimeras of elephant shark GR and MR in which the GR NTD was fused to elephant shark MR DBD-LBD and the MR NTD was fused to GR DBD-LBD. In elephant shark GR NTD fused to MR DBD-LBD activation by cortisol and corticosterone increased by over 10-fold, compared to full length elephant shark MR indicating that this GR NTD contains AF1 activity. Interestingly, the chimera of GR NTD MR DBD-LBD had increased activation by progesterone and 19norprogesterone. In contrast, in MR NTD fused to GR DBD-LBD

corticosteroid activation was reduced by over 90%. These data indicate allosteric regulation by the NTD evolved in elephant shark GR soon after the divergence of the GR from its common ancestor with the MR.

Experimental

Chemical reagents

Aldosterone, cortisol, corticosterone, 11-deoxycorticosterone, progesterone and 19norProgesterone were purchased from Sigma-Aldrich. For the reporter gene assays, all hormones were dissolved in dimethylsulfoxide (DMSO) and the final concentration of DMSO in the culture medium did not exceed 0.1%.

Construction of plasmid vectors

The full-coding regions and DBD-LBD domains of the GR and MR from *C. milii* were amplified by PCR with KOD DNA polymerase (TOYOBO Biochemicals, Osaka, Japan). The PCR products were gel-purified and ligated into pcDNA3.1 vector (Invitrogen) (44).

Transactivation Assay and Statistical Methods

Hek293 were used in the reporter gene assay. Transfection and reporter assays were carried out as described previously (44,45). All transfections were performed at least three times, employing triplicate sample points in each experiment. The values shown are mean \pm SEM from three separate experiments. Data from studies of the effect of different corticosteroid concentrations on transcriptional activation of elephant shark GR and MR were used to calculate EC50s for steroid activation of elephant shark GR and MR using GraphPad Prism. Comparisons between two groups were performed using t-test, and all multi-group comparisons were performed using one-way ANOVA followed by Bonferroni test. $P < 0.05$ was considered statistically significant.

Genbank Accessions for Domain Comparisons

Elephant shark GR (XP_007899521), human GR (BAH02307), chicken GR (ABB05045), *X. laevis* GR (NP_001081531), Zebrafish GR (NP_001018547), elephant shark MR (XP_007902220), human MR (NP_000892), zebrafish MR (NP_001093873).

Results

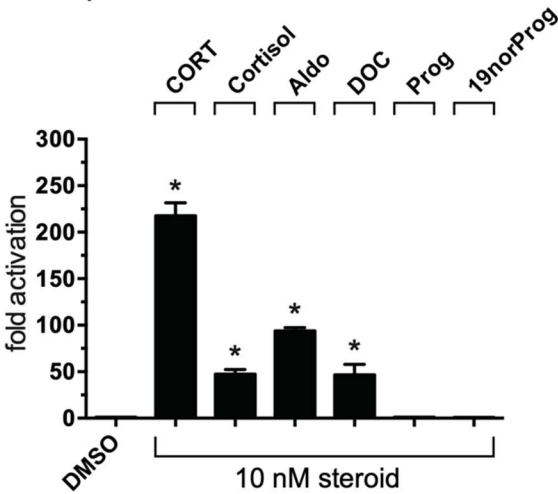
Functional domains on elephant shark GR and other vertebrate GRs.

In Figure 2, we compare the functional domains of elephant shark GR to corresponding domains in selected vertebrate GRs (human, chicken, *Xenopus*, zebrafish) and MRs (human, zebrafish and elephant shark). Elephant shark GR and human GR have 98% and 67% identity in DBD and LBD, respectively. Elephant shark GR and MR have 95% and 61% identity in DBD and LBD, respectively. This strong conservation of the DBD and LBD contrasts with the low sequence identity between the NTD in elephant shark GR and the NTD in other GRs and MRs. The NTD of elephant shark GR has only 21% sequence identity with elephant shark MR.

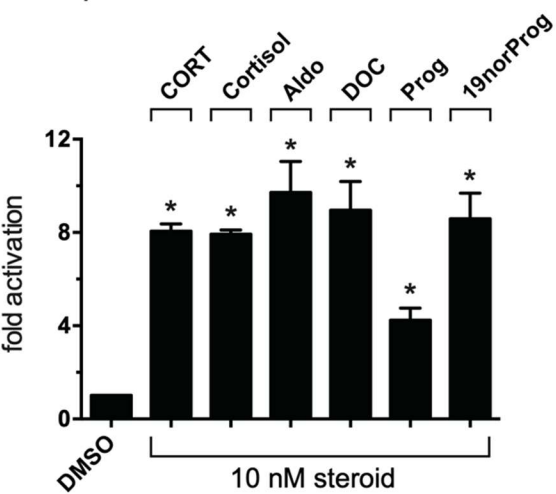
Effect of corticosteroids and progesterone on transcriptional activation of full-length elephant shark GR and MR.

We screened a panel of steroids (cortisol, corticosterone, 11-deoxycorticosterone, aldosterone, progesterone, 19norProgesterone) at 10^{-8} M for activation of full-length elephant shark GR (Figure 3A). For comparison, activation of full-length elephant shark MR by these steroids is shown in Figure 3B. Cortisol, corticosterone, 11-deoxycorticosterone, and aldosterone activated full-length elephant shark GR (Figure 3A) and MR (Figure 3B). However, fold-activation of full-length elephant shark GR by corticosteroids at 10^{-8} M was over 10-fold higher than for full-length elephant shark MR. For example, 10^{-8} M corticosterone activated full-length GR by 225-fold and full-length MR by 8-fold. Interestingly, neither progesterone nor 19norProgesterone activated elephant shark GR, while these steroids activated elephant shark MR, as previously reported (41).

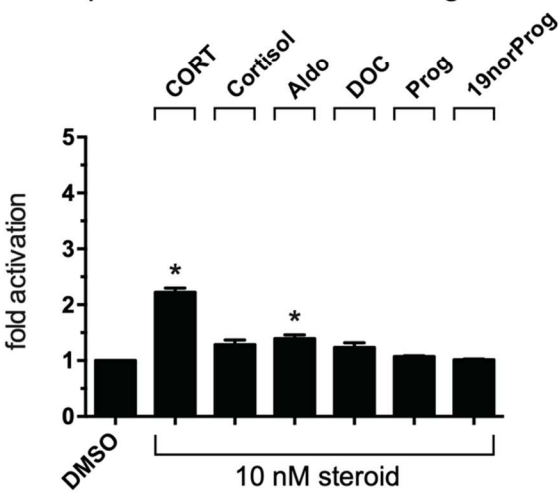
A: elephant shark GR-full



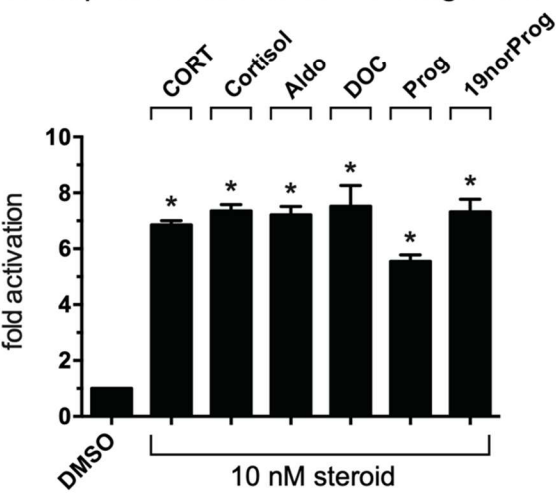
B: elephant shark MR-full



C: elephant shark GR-DBD-hinge-LBD



D: elephant shark MR-DBD-hinge-LBD



E: GAL4-DBD + elephant shark GR-hinge-LBD

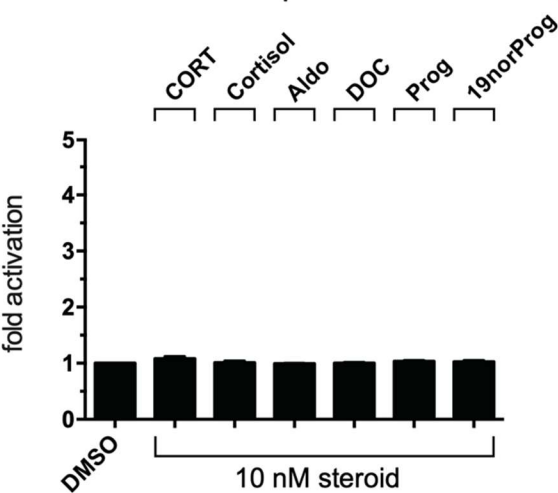


Fig. 3. Ligand specificity of full-length and truncated elephant shark GR and MR.

Plasmids for full-length elephant shark GR or MR or truncated elephant GR or MR, containing the DBD (C domain), D domain and LBD (E domain), were expressed in HEK293 cells with an MMTV-luciferase reporter. Transfected cells were treated with either 10 nM cortisol, corticosterone (CORT), aldosterone (Aldo), 11-deoxycorticosterone (DOC), progesterone (Prog), 19norProgesterone (19norProg) or vehicle alone (DMSO). Results are expressed as means \pm SEM, n=3. Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1. A. Full-length elephant shark GR. B. Full-length elephant shark MR. C. Truncated elephant shark GR. D. Truncated elephant shark MR. E. GAL4-DBD+elephant shark GR-hinge-LBD.

Effect of corticosteroids and progesterone on transcriptional activation of truncated elephant shark GR and MR.

We investigated the role of the NTD in the response to corticosteroids and progestins at 10^{-8} M, of truncated elephant shark GR and MR, in which the NTD was deleted (Figure 3C, D). Figure 3C shows that activation of truncated elephant shark GR by 10^{-8} M corticosterone decreased by over 90%, compared to full-length elephant shark GR (Figure 3A), and there was no significant activation of truncated GR by 10^{-8} M of either cortisol or 11-deoxycorticosterone, both of which activated full-length GR. This indicates that NTD in elephant shark GR contains an AF1 domain. Progesterone did not activate truncated elephant shark GR. In contrast, at 10^{-8} M, corticosteroids and progesterone had similar levels of activation of truncated elephant shark MR (Figure 3D) and full-length MR (Figure 3B), indicating that there is a major difference between NTD activation of transcription of elephant shark GR and MR.

To investigate the effect of the elephant shark DBD on activation of elephant shark GR and MR, we replaced the DBD with GAL4 DBD, which has been used for analysis of transcription of the GR and MR (27,28,43,46). As shown in Figure 3E, there was only a low level of activation by 10 nM corticosterone and aldosterone of GAL4 DBD GR hinge-LBD, and no activation by 11-deoxycorticosterone and cortisol. In contrast, GAL4 DBD MR LBD is activated by aldosterone, 11-deoxycorticosterone and other corticosteroids (41).

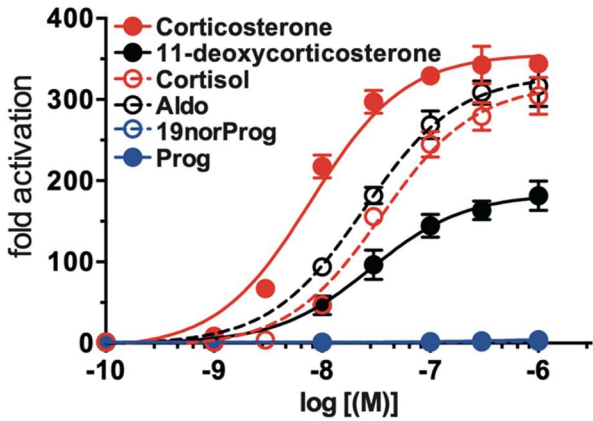
Concentration-dependent activation by corticosteroids and progesterone of full-length and truncated elephant shark GR and MR.

To gain a quantitative measure of NTD activation of elephant shark GR, we determined the concentration dependence of transcriptional activation by corticosteroids and progesterone of full-length and truncated elephant shark GR (Figure 4A, B) and for comparison of elephant shark MR (Figure 4C, D). This data was used to calculate a half maximal response (EC50) for steroid activation of elephant shark GR and MR (Tables 1 and 2). Corticosterone and cortisol, two physiological glucocorticoids in mammals, had EC50s of 7.9 nM and 35 nM, respectively, for full-length elephant shark GR (Table 1). For comparison, the EC50s of corticosterone and cortisol are 0.61 nM and 1.6 nM, respectively, for full-length elephant shark MR (Table 2) (41). However, aldosterone and 11-deoxycorticosterone, two physiological mineralocorticoids in mammals, had EC50s of 24 nM and 28 nM, respectively, for elephant shark GR. Activation by these steroids of elephant shark GR indicates that it retains some properties of its MR paralog. For comparison, the EC50s of aldosterone and 11-deoxycorticosterone are 0.14 nM and 0.1 nM, respectively, for full-length elephant shark MR (Table 2) (41).

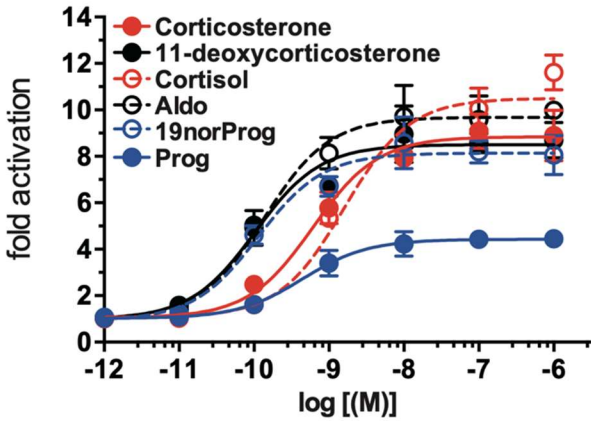
The EC50 of corticosterone for truncated elephant shark GR was 9.4 nM. The EC50s of cortisol and aldosterone for truncated elephant shark GR were 66 nM and 65 nM respectively. In contrast, the EC50s of corticosterone and aldosterone for GAL4-DBD+GR-hinge-LBD were 25 nM and 71 nM, respectively, and cortisol and 11-deoxycorticosterone did not activate GAL4-DBD+GR-hinge-LBD. The higher EC50 for corticosterone and the loss of activation by cortisol and 11-deoxycorticosterone, when elephant shark DBD is replaced with GAL4-DBD indicates that the DBD is important in transcriptional activation of elephant shark GR.

All four corticosteroids, progesterone and 19norProgesterone had EC50s for truncated MR (DBD+LBD) that were either similar to or lower than that of full-length MR (Table 2). EC50s for Gal4-DBD+MR-hinge-LBD were either similar to or lower than that of full-length MR.

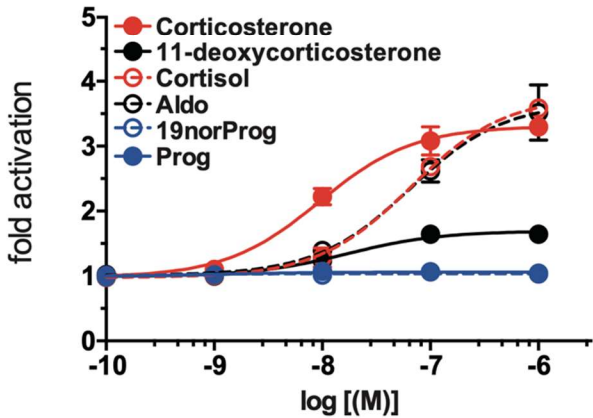
A: elephant shark GR-full



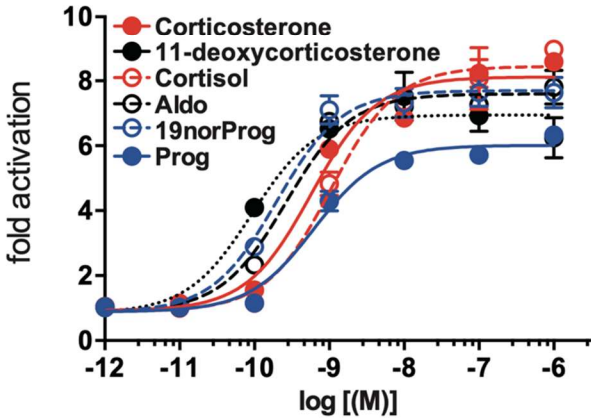
B: elephant shark MR-full



C: elephant shark GR-DBD-hinge-LBD



D: elephant shark MR-DBD-hinge-LBD



E: GAL4-DBD + elephant shark GR-hinge-LBD

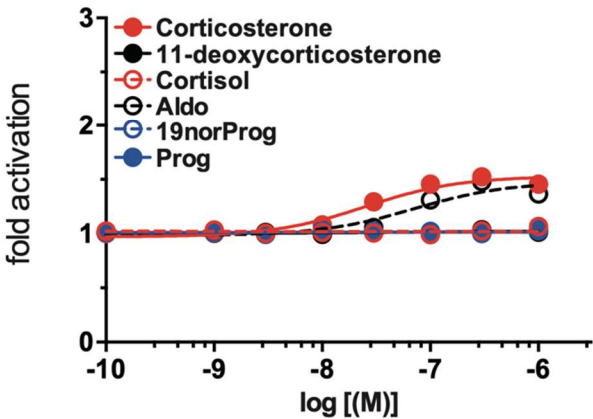


Fig. 4. Concentration-dependent transcriptional activation by corticosteroids and progesterone of full length and truncated elephant shark GR and MR. Plasmids for full-length elephant shark GR or MR or truncated elephant GR or MR, were expressed in HEK293 cells with an MMTV-luciferase reporter. Cells were treated with increasing concentrations of either cortisol, corticosterone, aldosterone, 11-deoxycorticosterone, progesterone, 19norProgesterone or vehicle alone (DMSO). Results are expressed as means \pm SEM, n=3. Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1. A. Full-length elephant shark GR. B. Full-length elephant shark MR. C. Truncated elephant shark GR (Domains CDE). D. Truncated elephant shark MR (Domains CDE). E. GAL4-DBD+elephant shark GR-hinge-LBD.

The dependence of the level of cortisol and 11-deoxycorticosterone activation on the NTD in elephant shark GR (Figures 4A, C) is similar to that of human GR, in which deletion of the NTD reduces cortisol and 11-deoxycorticosterone fold-activation by over 90% (19,27). The diminished activation of GAL4-DBD-elephant shark GR-hinge-LBD (Figure 4E) indicates that the DBD also is important in fold-activation of elephant shark GR. These results indicate that allosteric signaling between the NTD and DBD-LBD in elephant shark GR is critical for its response to corticosteroids. This contrasts to elephant shark MR, in which truncated MR and full-length elephant shark MR have similar levels of activation by corticosteroids and progesterone (Figures 4B, D). Together this indicates that an activation function in the GR NTD and higher EC50s for some corticosteroids that also activated elephant shark MR evolved early in the divergence of the GR from its MR kin.

Table 1. EC50 values for steroid activation of full-length and truncated elephant shark GR

	Cortisol	Corticosterone	Aldo	DOC	Prog	19norProg
	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)
Elephant shark GR-full	35 nM	7.9 nM	24nM	28 nM	—	—
95% confidence intervals	27-46 nM	6.1-10.0 nM	19.0-31 nM	19-42 nM	—	—
% Relative Induction	88 %	100 %	92 %	53 %	—	—
Elephant shark GR-DBD-hinge-LBD	66 nM	9.4 nM	65 nM	17 nM	—	—
95% confidence intervals	55-80 nM	7.5-12 nM	44-95 nM	8.6-34 nM	—	—
% Relative Induction	112 %	100 %	109 %	30 %	—	—
Elephant shark Gal4-DBD-GR-hinge-LBD	—	25 nM	71 nM	—	—	—
95% confidence intervals	—	14-45 nM	31-165 nM	—	—	—
% Relative Induction	—	100 %	81 %	—	—	—
Skate GR [Gal4-DBD-GR-hinge-LBD]#	139 nM	58 nM	11 nM	306 nM	N.D.	N.D.
Elephant shark Chimera MR-NTD+GR-DBD-hinge-LBD	93 nM	13 nM	43 nM	60 nM	—	—
95% confidence intervals	58-150 nM	8.0-21 nM	28-67 nM	28-128 nM	—	—
% Relative Induction	108 %	100 %	88 %	20 %	—	—

Values obtained from Mol Biol Evol. 12, 2643-2652 (2008)

Percent relative induction for GR is compared to maximal response to corticosterone.

— = no activation. N.D. = not determined

Aldo = aldosterone, DOC =11-deoxycorticosterone, Prog = progesterone, 19norProg = 19norProgesterone

Table 2. EC50 values for steroid activation of full-length and truncated elephant shark MR

	Cortisol	Corticosterone	Aldo	DOC	Prog	19norProg
	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)
Elephant shark MR-full	1.6 nM	0.61 nM	0.14 nM	0.1 nM	0.45 nM	0.11 nM
95% confidence intervals	0.92-2.8 nM	0.34-1.1 nM	0.069-0.26 nM	0.54-0.2 nM	0.21-0.94 nM	0.06-0.22 nM
% Relative Induction	118 %	88 %	100 %	86 %	38 %	79 %
Elephant shark MR-DBD-hinge-LBD	1.1nM	0.58 nM	0.26 nM	0.1 nM	0.63 nM	0.2 nM
95% confidence intervals	0.67-1.7 nM	0.39-0.86 nM	0.15-0.45 nM	0.05-0.16 nM	0.44-0.9 nM	0.13-0.31 nM
% Relative Induction	117 %	111 %	100 %	77 %	78 %	98 %
Elephant shark Gal4-DBD-MR-hinge-LBD*	0.19 nM	0.1 nM	0.04 nM	0.02 nM	0.48 nM	0.02 nM
95% confidence intervals	0.08-0.2 nM	0.08-0.1 nM	0.02-0.05 nM	0.01-0.03 nM	0.39-0.57 nM	0.01-0.02 nM
% Relative Induction	79 %	90 %	100 %	81 %	40 %	98 %
Skate MR [Gal4-DBD-MR-hinge-LBD]#	1.04 nM	0.09 nM	0.07 nM	0.03 nM	N.D.	N.D.
Elephant shark Chimera GR-NTD+MR-DBD-hinge-LBD	0.3 nM	0.1 nM	0.05 nM	0.03 nM	0.2 nM	0.04 nM
95% confidence intervals	0.2-0.44 nM	0.09-0.16 nM	0.04-0.07 nM	0.02-0.04 nM	0.17-0.27 nM	0.03-0.06 nM
% Relative Induction	100 %	101 %	100 %	97 %	81 %	95 %

*Values obtained from Sci Signal. 12, eaar2668 (2019)

Values obtained from Mol Biol Evol. 12, 2643-2652 (2008)

Percent relative induction for MR is compared to maximal response to aldosterone.

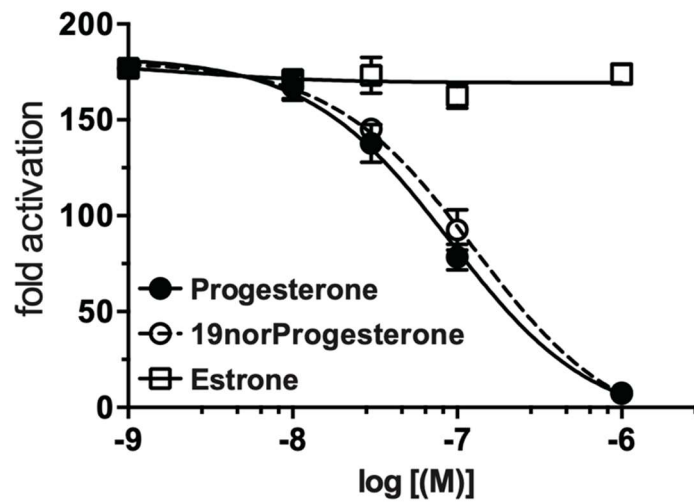
N.D. = not determined.

Aldo = aldosterone, DOC =11-deoxycorticosterone, Prog = progesterone, 19norProg = 19norProgesterone

Progesterone binds to, but does not activate, full-length elephant shark GR.

Neither progesterone nor 19norProgesterone activated transcription by elephant shark GR (Figure 3A), although these steroids are transcriptional activators of elephant shark MR (Figure 3B) (41). The lack of progesterone activation of elephant shark GR could be due to decreased affinity of progesterone for elephant shark GR or to loss of activation of elephant shark GR after binding progesterone. We find that progesterone and 19norProgesterone inhibit activation of elephant shark GR by 10 nM corticosterone (Figure 5A) indicating that elephant shark GR recognizes progesterone. A parallel study showed that neither progesterone nor 19norProgesterone inhibited activation of human GR by 10 nM cortisol (Figure 5B). This indicates that the loss of activation by progesterone preceded the loss of progesterone binding to the GR, which occurred later in vertebrate evolution.

A: Elephant Shark GR (10 nM corticosterone)



B: Human GR (10 nM cortisol)

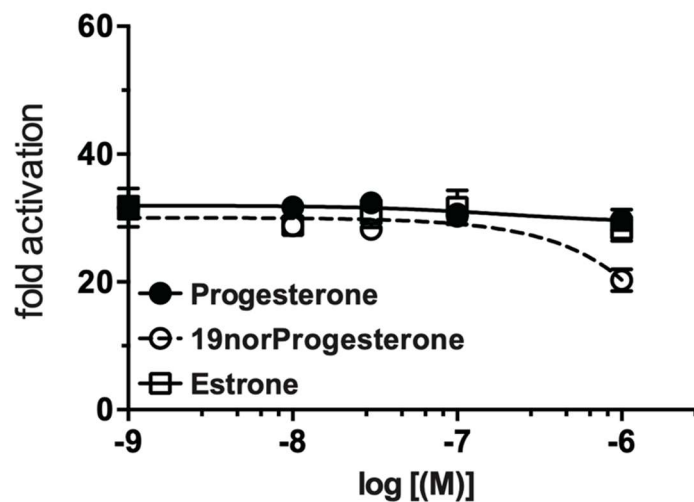


Fig. 5. Progesterone inhibits corticosterone activation of full-length elephant shark GR.

Plasmids encoding full length elephant GR or human GR were expressed in HEK293 cells. Cells with elephant shark GR and human GR were treated with 10 nM corticosterone and cortisol respectively and increasing concentrations of either progesterone, 19norProgesterone or estrone. Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.

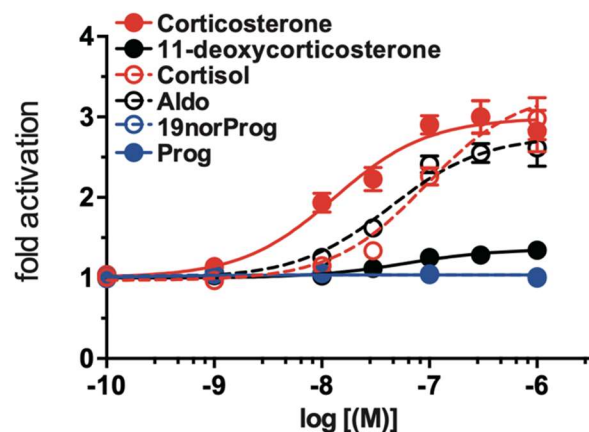
A. Elephant shark GR. B. Human GR.

Corticosteroid activation of chimeras in which the NTD is swapped between elephant shark GR and MR.

To investigate further the origins of NTD activation of transcription by the GR, we studied corticosteroid activation of chimeras of elephant shark GR and MR, in which the GR NTD was fused to MR DBD-hinge-LBD and the MR NTD was fused to GR DBD-hinge-LBD (Figure 6). We find that despite low sequence similarity between the NTDs in elephant shark GR and MR, their NTDs had dominant effects on transcription of the DBD-hinge-LBD in each chimera (Figure 6, Tables 1 and 2). Thus, in the GR NTD-MR DBD-hinge-LBD chimera (GR NTD fused to MR DBD-hinge-LBD), cortisol and corticosterone increased activation by over 30-fold, compared to full length elephant shark MR (Figures 3B, 4B and 6B). Moreover, the GR NTD-MR DBD-hinge-LBD chimera also had a higher level of activation in the presence of progesterone and 19norProgesterone. In addition, the EC₅₀s for corticosteroids and progestins were lower in the GR NTD-MR DBD-hinge-LBD chimera indicating that the NTD affects the affinity of steroids for the chimera, as well as the level of transcriptional activation (Table 2).

In contrast, the MR NTD reduced activation by corticosterone and other corticosteroids of the MR NTD-GR DBD-hinge-LBD chimera by over 90% compared to full-length GR (Figures 3A, 4A and 6A, Table 1). These data indicate that increased activation by the NTD in elephant shark GR evolved soon after it diverged from the MR.

A: elephant shark MR-A/B fused to elephant shark GR-hinge-LBD



B: elephant shark GR-A/B fused to elephant shark MR-hinge-LBD

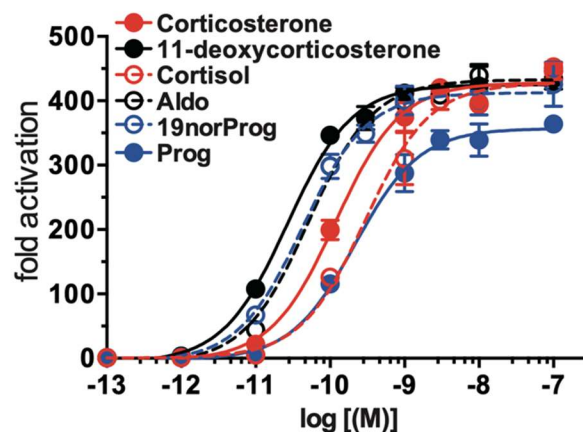


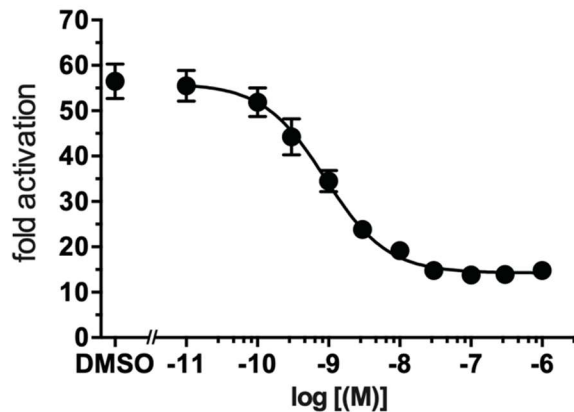
Fig. 6. Concentration-dependent transcriptional activation by corticosteroids of chimeras of GR and MR. Plasmids for chimeric GR or MR were expressed in HEK293 cells with an MMTV-luciferase reporter. Cells were treated with increasing concentrations of either cortisol, corticosterone, aldosterone, 11-deoxycorticosterone, progesterone, 19norProgesterone or vehicle alone (DMSO). Results are expressed as means \pm SEM, n=3. Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1. A. Chimera of MR-A/B fused to GR-DBD-hinge-LBD. B. Chimera of GR-A/B fused to MR-DBD-hinge-LBD.

RU486 is a glucocorticoid antagonist of full-length elephant shark GR.

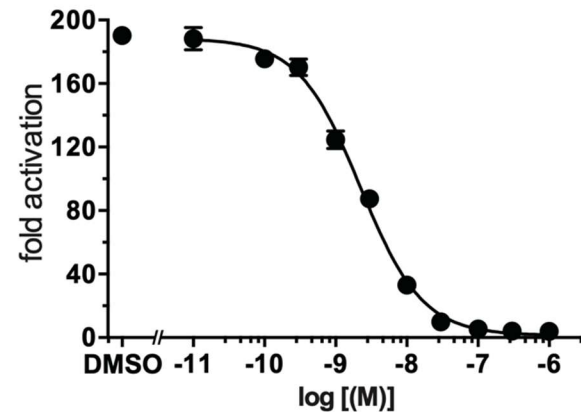
To gain another measure of the similarities and differences between elephant shark GR and human GR, we investigated the effect of RU486 on transcriptional activation of elephant shark GR. RU486, originally developed as a progesterone antagonist (47,48) also is used as an antagonist for glucocorticoid activation of human GR (47), although at high concentrations (>100nM), RU486 is a weak GR agonist. We found that RU486 inhibited activation by 2 nM dexamethasone of elephant shark and human GR (Figure 7A, B). At 0.9 nM, RU486 inhibited dexamethasone activation of human GR by 50% (Figure 7A). At 2.2 nM, RU486 inhibited dexamethasone activation of elephant shark GR by 50% (Figure 7B).

Concentration dependent activation by RU486 of elephant shark GR and human GR, shown in Figure 7C and D, was used to calculate EC50 values for RU486. The EC50 of RU486 for elephant shark GR is 8.7 nM and for human GR is 0.55 nM. Maximal transcriptional activation by RU486 of elephant shark GR (Figure 7D) is considerably lower than for activation of human GR (Figure 7C). Nevertheless, although RU486 is not as active towards elephant shark GR as towards human GR, the responses of elephant shark GR to RU486 mimic those of human GR.

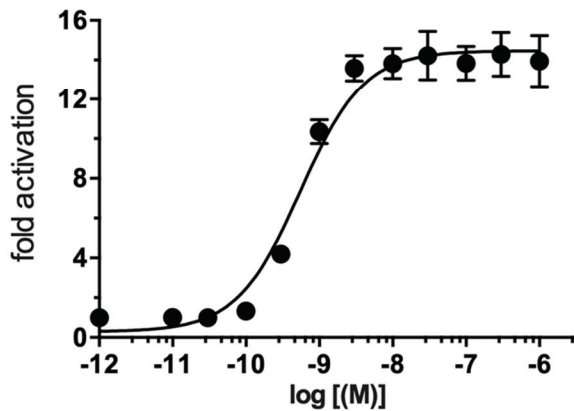
A: human GR (RU486 + 2 nM DEX)



B: elephant shark GR (RU486 + 2 nM DEX)



C: human GR (RU486 transactivation)



D: elephant shark GR (RU486 transactivation)

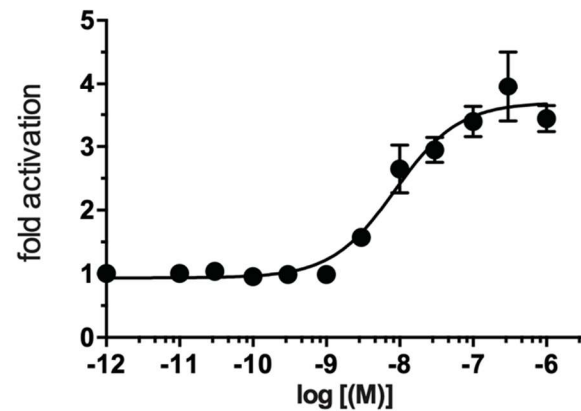


Fig. 7. RU486 inhibition of dexamethasone activation of elephant shark GR and human GR. Plasmids encoding full length elephant GR or human GR were expressed in HEK293 cells. Cells with elephant shark GR or human GR were treated with 2 nM dexamethasone + increasing concentrations of RU486 (panels A, B) or increasing concentrations of RU486 (panels C, D). Results are expressed as means \pm SEM, $n=3$. Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.

Discussion

Sequence analysis revealed that the MR and GR are kin (9,13). A distinct GR and MR first appear in cartilaginous fish, which occupy a key position at an ancestral node from which ray-finned fish and terrestrial vertebrates diverged (14,49,50). Genomic analyses reveal that elephant shark genes are evolving slowly (14). Thus, analysis of steroid activation of elephant shark GR can provide insights into the evolution of steroid activation of the ancestral GR early in its divergence from the MR. As a consequence, we studied activation of elephant shark GR by

physiological glucocorticoids (cortisol, corticosterone) and mineralocorticoids (aldosterone, 11-deoxycorticosterone), as well as progesterone, which we compared to similar experiments with elephant shark MR (41). Because the NTD has a strong AF1 for human GR (19,23–26), unlike human MR (19,41), we also studied elephant shark GR, in which the NTD was deleted. After finding that corticosterone activation of truncated elephant shark GR declined by over 90% from the activity of full-length elephant shark GR, we investigated the evolution of the NTD in chimeras of elephant shark GR and MR, in which their NTDs were swapped. Despite low sequence identity between the GR and MR NTDs, AF1 in elephant shark GR NTD could transfer its activity to elephant shark MR, when it was substituted for the MR NTD. This indicates that a strong AF1 appeared early in the evolution of the GR in a basal jawed vertebrate (Figure 6, Tables 1 and 2).

Overall, we find that the response to corticosteroids and RU486 (47,51) by elephant shark GR has some similarities and some differences to human GR (15,19,21,27), as well as to elephant shark MR (41). In comparison to corticosteroid activation of elephant shark MR, corticosteroids have higher EC50s for elephant shark GR, indicating reduced sensitivity to corticosteroids [Tables 1 and 2]. Interestingly, the level of transcriptional activation by corticosteroids of elephant shark GR is over 10-fold higher than for elephant shark MR (Figure 3A, B). In this respect, elephant shark GR and MR are similar to human GR and human MR in which cortisol has a higher EC50, as well as a higher level of transcriptional activation for human GR, compared to human MR (15,16,19,20,52).

Activation of elephant shark GR by aldosterone is a notable difference with human GR. Although 10nM aldosterone has little activity for human GR (Figure 3A) (15,27), aldosterone and cortisol have a similar level of activation of elephant shark GR. Aldosterone, the physiological mineralocorticoid in terrestrial vertebrates, also is a weaker activator of truncated skate GR than of truncated skate MR (43) and of lamprey CR (7), which is ancestral to the GR and MR (7,13). Thus, elephant shark GR retains some responses, albeit diminished, of elephant shark MR and of lamprey CR (7). We conclude that elephant shark GR is transitional from elephant shark MR towards human GR in specificity for glucocorticoids and mineralocorticoids.

A novel property of elephant shark MR is that it is activated by progesterone and 19norProgesterone (41,53). Neither steroid activates elephant shark GR. Unexpectedly,

progesterone and 19norProgesterone inhibit activation of elephant shark GR by corticosterone (Figure 5A), which suggests that progestins may influence activation of elephant shark GR. Neither progesterone nor 19norProgesterone inhibit activation of human GR by cortisol (Figure 5B). The timing of the loss of recognition of progestins by the GR needs further study.

The evolution of a distinct GR and MR in cartilaginous fish has provided an opportunity to investigate early events in the divergence of these two steroid receptors from their common ancestor. Cartilaginous fish, including elephant shark, also contain a PR and AR, which diverged from a common ancestor. The PR is a close relative of the MR. Comparison of steroid activation of elephant shark PR and AR with each other and with the MR and GR could provide valuable insights into their evolution as important transcription factors in vertebrates.

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References

1. Cain DW, Cidlowski JA. Immune regulation by glucocorticoids. *Nat Rev Immunol.* 2017;17(4):233-247. doi:10.1038/nri.2017.1.
2. de Kloet ER. From receptor balance to rational glucocorticoid therapy. *Endocrinology.* 2014;155(8):2754-2769. doi:10.1210/en.2014-1048.
3. Gross KL, Cidlowski JA. Tissue-specific glucocorticoid action: a family affair. *Trends Endocrinol Metab.* 2008;19(9):331-339. doi:10.1016/j.tem.2008.07.009.

4. Hunter RG, Seligsohn M, Rubin TG, et al. Stress and corticosteroids regulate rat hippocampal mitochondrial DNA gene expression via the glucocorticoid receptor. *Proc Natl Acad Sci U S A*. 2016;113(32):9099-9104. doi:10.1073/pnas.1602185113.
5. Weikum ER, Knuesel MT, Ortlund EA, Yamamoto KR. Glucocorticoid receptor control of transcription: precision and plasticity via allostery. *Nat Rev Mol Cell Biol*. 2017;18(3):159-174. doi:10.1038/nrm.2016.152.
6. Baker ME, Nelson DR, Studer RA. Origin of the response to adrenal and sex steroids: Roles of promiscuity and co-evolution of enzymes and steroid receptors. *J Steroid Biochem Mol Biol*. 2015;151:12-24. doi:10.1016/j.jsbmb.2014.10.020.
7. Bridgham JT, Carroll SM, Thornton JW. Evolution of hormone-receptor complexity by molecular exploitation. *Science*. 2006;312(5770):97-101. doi:10.1126/science.1123348.
8. Evans RM. The steroid and thyroid hormone receptor superfamily. *Science*. 1988;240(4854):889-895. doi:10.1126/science.3283939.
9. Arriza JL, Weinberger C, Cerelli G, et al. Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science*. 1987;237(4812):268-275. doi:10.1126/science.3037703.
10. Baker ME, Funder JW, Kattoula SR. Evolution of hormone selectivity in glucocorticoid and mineralocorticoid receptors [published correction appears in *J Steroid Biochem Mol Biol*. 2014 Jan;139:104]. *J Steroid Biochem Mol Biol*. 2013;137:57-70. doi:10.1016/j.jsbmb.2013.07.009.
11. Kassahn KS, Ragan MA, Funder JW. Mineralocorticoid receptors: evolutionary and pathophysiological considerations. *Endocrinology*. 2011;152(5):1883-1890. doi:10.1210/en.2010-1444.
12. Rossier BC, Baker ME, Studer RA. Epithelial sodium transport and its control by aldosterone: the story of our internal environment revisited. *Physiol Rev*. 2015;95(1):297-340. doi:10.1152/physrev.00011.2014.
13. Baker ME, Katsu Y. 30 YEARS OF THE MINERALOCORTICOID RECEPTOR: Evolution of the mineralocorticoid receptor: sequence, structure and function. *J Endocrinol*. 2017;234(1):T1-T16. doi:10.1530/JOE-16-0661.
14. Venkatesh B, Lee AP, Ravi V, et al. Elephant shark genome provides unique insights into gnathostome evolution [published correction appears in *Nature*. 2014 Sep 25;513(7519):574]. *Nature*. 2014;505(7482):174-179. doi:10.1038/nature12826.
15. Arriza JL, Simerly RB, Swanson LW, Evans RM. The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. *Neuron*. 1988;1(9):887-900. doi:10.1016/0896-6273(88)90136-5.

16. Hellal-Levy C, Couette B, Fagart J, Souque A, Gomez-Sanchez C, Rafestin-Oblin M. Specific hydroxylations determine selective corticosteroid recognition by human glucocorticoid and mineralocorticoid receptors. *FEBS Lett.* 1999;464(1-2):9-13. doi:10.1016/s0014-5793(99)01667-1.
17. Katsu Y, Oka K, Baker ME. Evolution of human, chicken, alligator, frog, and zebrafish mineralocorticoid receptors: Allosteric influence on steroid specificity. *Sci Signal.* 2018;11(537):eaao1520. Published 2018 Jul 3. doi:10.1126/scisignal.aao1520.
18. Lombes M, Kenouch S, Souque A, Farman N, Rafestin-Oblin ME. The mineralocorticoid receptor discriminates aldosterone from glucocorticoids independently of the 11 beta-hydroxysteroid dehydrogenase. *Endocrinology.* 1994;135(3):834-840. doi:10.1210/endo.135.3.8070376.
19. Rupprecht R, Arriza JL, Spengler D, et al. Transactivation and synergistic properties of the mineralocorticoid receptor: relationship to the glucocorticoid receptor. *Mol Endocrinol.* 1993;7(4):597-603. doi:10.1210/mend.7.4.8388999.
20. Rupprecht R, Reul JM, van Steensel B, et al. Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. *Eur J Pharmacol.* 1993;247(2):145-154. doi:10.1016/0922-4106(93)90072-h.
21. Oka K, Hoang A, Okada D, Iguchi T, Baker ME, Katsu Y. Allosteric role of the amino-terminal A/B domain on corticosteroid transactivation of gar and human glucocorticoid receptors. *J Steroid Biochem Mol Biol.* 2015;154:112-119. doi:10.1016/j.jsbmb.2015.07.025.
22. Gross KL, Lu NZ, Cidlowski JA. Molecular mechanisms regulating glucocorticoid sensitivity and resistance. *Mol Cell Endocrinol.* 2009;300(1-2):7-16. doi:10.1016/j.mce.2008.10.001.
23. Hollenberg SM, Giguere V, Segui P, Evans RM. Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. *Cell.* 1987;49(1):39-46. doi:10.1016/0092-8674(87)90753-7.
24. Eliezer D. Proteins acting out of (dis)order. *Elife.* 2017;6:e32762. Published 2017 Nov 21. doi:10.7554/eLife.32762.
25. Li J, White JT, Saavedra H, et al. Genetically tunable frustration controls allostery in an intrinsically disordered transcription factor [published correction appears in *Elife.* 2018 Feb 12;7:]. *Elife.* 2017;6:e30688. Published 2017 Oct 12. doi:10.7554/eLife.30688.
26. Dahlman-Wright K, Almlöf T, McEwan IJ, Gustafsson JA, Wright AP. Delineation of a small region within the major transactivation domain of the human glucocorticoid receptor that mediates transactivation of gene expression. *Proc Natl Acad Sci U S A.* 1994;91(5):1619-1623. doi:10.1073/pnas.91.5.1619.

27. Katsu Y, Kohno S, Oka K, Baker ME. Evolution of corticosteroid specificity for human, chicken, alligator and frog glucocorticoid receptors. *Steroids*. 2016;113:38-45. doi:10.1016/j.steroids.2016.06.005.
28. Cho S, Blackford JA Jr, Simons SS Jr. Role of activation function domain-1, DNA binding, and coactivator GRIP1 in the expression of partial agonist activity of glucocorticoid receptor-antagonist complexes. *Biochemistry*. 2005;44(9):3547-3561. doi:10.1021/bi048777i.
29. Christopoulos A, Changeux JP, Catterall WA, et al. International Union of Basic and Clinical Pharmacology. XC. multisite pharmacology: recommendations for the nomenclature of receptor allosterism and allosteric ligands. *Pharmacol Rev*. 2014;66(4):918-947. doi:10.1124/pr.114.008862.
30. Khan SH, Awasthi S, Guo C, et al. Binding of the N-terminal region of coactivator TIF2 to the intrinsically disordered AF1 domain of the glucocorticoid receptor is accompanied by conformational reorganizations. *J Biol Chem*. 2012;287(53):44546-44560. doi:10.1074/jbc.M112.411330.
31. Miesfeld R, Godowski PJ, Maler BA, Yamamoto KR. Glucocorticoid receptor mutants that define a small region sufficient for enhancer activation. *Science*. 1987;236(4800):423-427. doi:10.1126/science.3563519.
32. Sturm A, Bron JE, Green DM, Bury NR. Mapping of AF1 transactivation domains in duplicated rainbow trout glucocorticoid receptors. *J Mol Endocrinol*. 2010;45(6):391-404. doi:10.1677/JME-09-0152.
33. Kumar R, Thompson EB. Folding of the glucocorticoid receptor N-terminal transactivation function: dynamics and regulation. *Mol Cell Endocrinol*. 2012;348(2):450-456. doi:10.1016/j.mce.2011.03.024.
34. Simons SS Jr, Kumar R. Variable steroid receptor responses: Intrinsically disordered AF1 is the key. *Mol Cell Endocrinol*. 2013;376(1-2):81-84. doi:10.1016/j.mce.2013.06.007.
35. Sturm A, Colliar L, Leaver MJ, Bury NR. Molecular determinants of hormone sensitivity in rainbow trout glucocorticoid receptors 1 and 2. *Mol Cell Endocrinol*. 2011;333(2):181-189. doi:10.1016/j.mce.2010.12.033.
36. Fuller PJ, Yao Y, Yang J, Young MJ. Mechanisms of ligand specificity of the mineralocorticoid receptor. *J Endocrinol*. 2012;213(1):15-24. doi:10.1530/JOE-11-0372.
37. Hawkins UA, Gomez-Sanchez EP, Gomez-Sanchez CM, Gomez-Sanchez CE. The ubiquitous mineralocorticoid receptor: clinical implications. *Curr Hypertens Rep*. 2012;14(6):573-580. doi:10.1007/s11906-012-0297-0.
38. Conneely OM, Mulac-Jericevic B, DeMayo F, Lydon JP, O'Malley BW. Reproductive functions of progesterone receptors. *Recent Prog Horm Res*. 2002;57:339-355. doi:10.1210/rp.57.1.339.

39. Publicover S, Barratt C. Reproductive biology: Progesterone's gateway into sperm [published correction appears in *Nature*. 2011 Mar 31;471(7340):589]. *Nature*. 2011;471(7338):313-314. doi:10.1038/471313a.
40. Dobzhansky.T (1973) Nothing in Biology Makes Sense except in the Light of Evolution. *Am Biol Teach* 35(3):125-129.
41. Katsu Y, Kohno S, Oka K, et al. Transcriptional activation of elephant shark mineralocorticoid receptor by corticosteroids, progesterone, and spironolactone. *Sci Signal*. 2019;12(584):eaar2668. Published 2019 Jun 4. doi:10.1126/scisignal.aar2668.
42. Venkatesh B, Lee AP, Ravi V, et al. Elephant shark genome provides unique insights into gnathostome evolution [published correction appears in *Nature*. 2014 Sep 25;513(7519):574]. *Nature*. 2014;505(7482):174-179. doi:10.1038/nature12826.
43. Carroll SM, Bridgham JT, Thornton JW. Evolution of hormone signaling in elasmobranchs by exploitation of promiscuous receptors. *Mol Biol Evol*. 2008;25(12):2643-2652. doi:10.1093/molbev/msn204.
44. Oka K, Kohno S, Urushitani H, et al. Molecular cloning and characterization of the corticoid receptors from the American alligator. *Mol Cell Endocrinol*. 2013;365(2):153-161. doi:10.1016/j.mce.2012.10.014.
45. Katsu Y, Kubokawa K, Urushitani H, Iguchi T. Estrogen-dependent transactivation of amphioxus steroid hormone receptor via both estrogen and androgen response elements. *Endocrinology*. 2010;151(2):639-648. doi:10.1210/en.2009-0766.
46. Arterbery AS, Fergus DJ, Fogarty EA, et al. Evolution of ligand specificity in vertebrate corticosteroid receptors. *BMC Evol Biol*. 2011;11:14. Published 2011 Jan 14. doi:10.1186/1471-2148-11-14.
47. Baulieu EE. Contragestion and other clinical applications of RU 486, an antiprogesterone at the receptor. *Science*. 1989;245(4924):1351-1357. doi:10.1126/science.2781282.
48. Cadepond F, Ulmann A, Baulieu EE. RU486 (mifepristone): mechanisms of action and clinical uses. *Annu Rev Med*. 1997;48:129-156. doi:10.1146/annurev.med.48.1.129.
49. Inoue JG, Miya M, Lam K, et al. Evolutionary origin and phylogeny of the modern holocephalans (Chondrichthyes: Chimaeriformes): a mitogenomic perspective. *Mol Biol Evol*. 2010;27(11):2576-2586. doi:10.1093/molbev/msq147.
50. Shaughnessy CA, Barany A, McCormick SD. 11-Deoxycortisol controls hydromineral balance in the most basal osmoregulating vertebrate, sea lamprey (*Petromyzon marinus*). *Sci Rep*. 2020;10(1):12148. Published 2020 Jul 22. doi:10.1038/s41598-020-69061-4.
51. Gaillard RC, Riondel A, Muller AF, Herrmann W, Baulieu EE. RU 486: a steroid with antigluccorticosteroid activity that only disinhibits the human pituitary-adrenal system at a

- specific time of day. *Proc Natl Acad Sci U S A*. 1984;81(12):3879-3882. doi:10.1073/pnas.81.12.3879.
52. Kiilerich P, Triqueneaux G, Christensen NM, et al. Interaction between the trout mineralocorticoid and glucocorticoid receptors in vitro. *J Mol Endocrinol*. 2015;55(1):55-68. doi:10.1530/JME-15-0002.
 53. Baker ME, Katsu Y. Progesterone: An enigmatic ligand for the mineralocorticoid receptor. *Biochem Pharmacol*. 2020;177:113976. doi:10.1016/j.bcp.2020.113976.