Evolution of corticosteroid specificity for human, chicken, alligator and frog glucocorticoid receptors

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Abstract. We investigated the evolution of the response of human, chicken, alligator and frog glucocorticoid receptors (GRs) to dexamethasone, cortisol, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol and aldosterone. We find significant differences among these vertebrates in the transcriptional activation of their full length GRs by these steroids, indicating that there were changes in the specificity of the GR for steroids during the evolution of terrestrial vertebrates. To begin to study the role of interactions between different domains on the GR in steroid sensitivity and specificity for terrestrial GRs, we investigated transcriptional activation of truncated GRs containing their hinge domain and ligand binding domain (LBD) fused to a GAL4 DNA binding domain (GAL4 DBD). Compared to corresponding full length GRs, transcriptional activation of GAL4 DBD-GR hinge/LBD constructs required higher steroid concentrations and displayed altered steroid specificity, indicating that interactions between the hinge/LBD and other domains are important in glucocorticoid activation of these terrestrial GRs.

Short Title: Evolution of steroid specificity for terrestrial GRs

Key Words: Glucocorticoid Receptor, Evolution, Allosteric Regulation, terrestrial vertebrates

1. Introduction

Glucocorticoids (Figure 1) regulate a variety of physiological functions including carbohydrate and protein metabolism, blood pressure, immune function and the body's anti-inflammatory processes via transcriptional activation of the glucocorticoid receptor (GR) [1-5]. The GR and other steroid receptors belong to the nuclear receptor family, a large family of transcription factors, which includes receptors for thyroid hormone, retinoids and other small lipophilic molecules [6-10]. The GR and other steroid receptors have a characteristic modular structure consisting 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) [9, 11-14] (Figure 2). The E domain alone is competent to bind steroids [11, 12, 15-18].

The NTD contains an activation function 1 [AF1] domain, which is a strong transcriptional activator of the GR [19-21]. Interestingly, AF1 is intrinsically disordered, unlike the DBD and LBD [21-23]. Allosteric interactions between AF1 and other domains on the GR and coactivators lead to a conformational rearrangement of AF1 that is important in transcriptional activation of the GR [23-26]. Recent crystal structures of the DBD-Hinge-LBD domains of other nuclear receptors [13, 22] reveal that there is allosteric signaling between the DBD and LBD domains. Moreover, for rat GR, there is evidence that allosteric interactions between DBD and other domains regulates gene transcription [27, 28].

Among terrestrial vertebrates, rodent [29] and human [20, 30-33] GRs have been the main focus of studies on glucocorticoid action, with dexamethasone [DEX] and cortisol [F] as the best studied steroids. Reports of transcriptional activation by corticosteroids of the GR for other terrestrial vertebrates: amphibians, reptiles and birds, are limited [34, 35]. Oka et al. [34] reported half-maximal response (EC50) values for transcriptional activation of full length alligator GR by F, corticosterone [B], 11-deoxycorticosterone [DOC] and aldosterone [Aldo]. Proszkowiec-Weglarz and Porter [35] found that B and Aldo were transcriptional activators of full length chicken GR. Unexpectedly, the EC50 for transcriptional activation of chicken GR by Aldo and B was 0.8 nM and 1.8 nM, respectively, with the level of transcription due to B being about 30% higher than to Aldo. Transcriptional activation by Aldo of alligator GR and chicken GR is surprising because it contrasts with Aldo's low affinity [36] and transcriptional activation of human GR [30]. The EC50 of other corticosteroids for chicken GR was not determined.

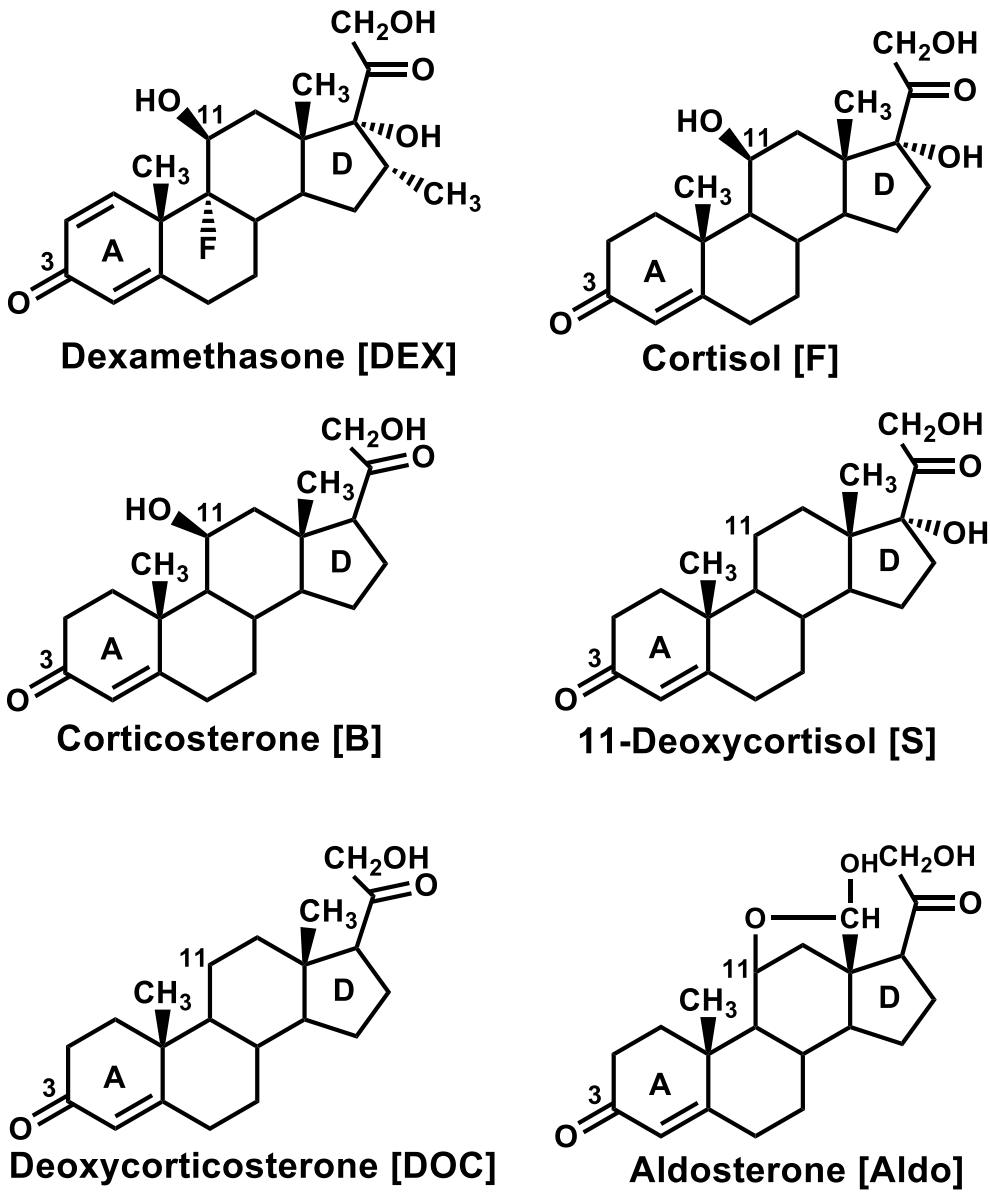


Figure 1. Structures of various corticosteroids.

Cortisol and corticosterone are physiological glucocorticoids in terrestrial vertebrates and ray-finned fish [12, 52, 54]. Aldosterone, 11-deoxycorticosterone and 11-deoxycortisol are physiological mineralocorticoids [12, 38, 45, 48] with high affinity for human GR [30, 36]. 11-deoxycortisol is both a mineralocorticoid and a glucocorticoid in lamprey [55].

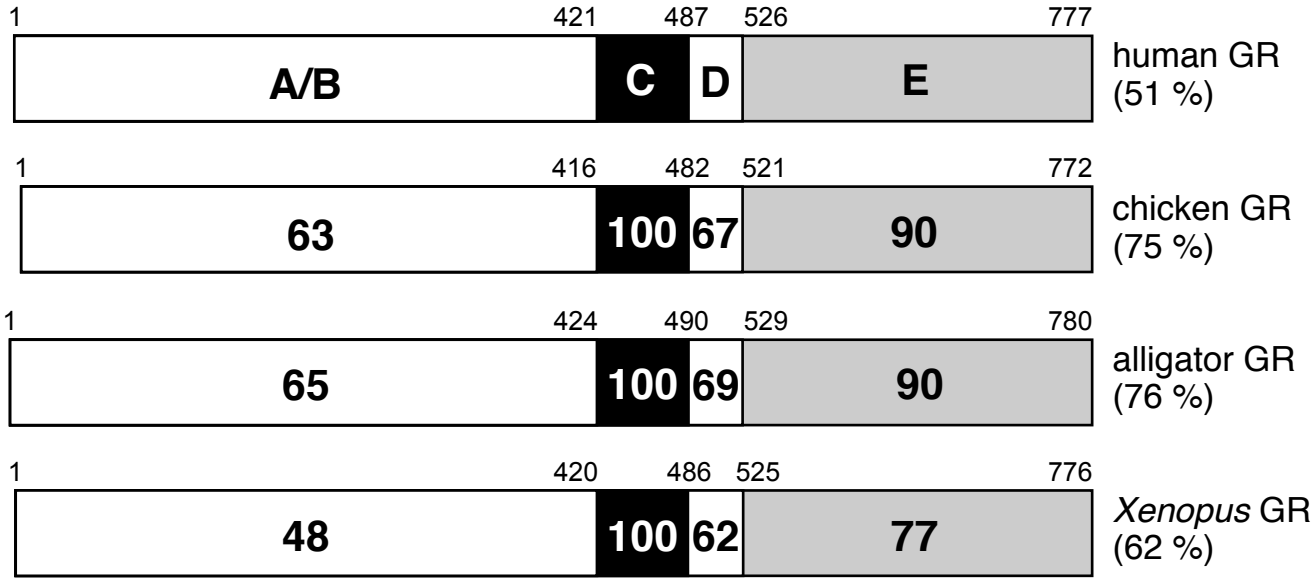


Figure 2. Comparison of domains in some terrestrial vertebrate GRs.
GRs from human, chicken, alligator and X. laevis are compared. The functional A/B domain to E domains are schematically represented with the numbers of amino acid residues and the percentage of amino acid identity is depicted. GenBank accession numbers: human GR (NM_000176), chicken GR (NM_001037826), alligator GR (AB701407), X. laevis GR (NM_001088062).

These unexpected responses of alligator and chicken GRs to Aldo and our interest in the evolution of specificity for corticosteroids in the GR in vertebrates [12, 34, 37-39] motivated us to investigate the response to a panel corticosteroids of the GR from chicken and the amphibian [*Xenopus laevis*] for comparison to human and alligator GR with the goal of clarifying the evolution of corticosteroid specificity in terrestrial vertebrates. In addition, we were interested in investigating the role of allosteric interactions between the domains A-C and domains D and E [13, 21-23, 39-42] in the response of GRs to steroids. The influence of this allosteric interaction on steroid responses has not been studied previously in non-mammalian terrestrial vertebrates. For these studies we constructed a plasmid containing the GAL4 DBD fused to the D domain and E domain of the GR (GR-LBD).

Interestingly, we found significant differences in the EC50s of these full length GRs to corticosteroids indicating that during the evolution of these terrestrial vertebrates there were changes in their response to various corticosteroids. Moreover, in the presence of corticosteroids, truncated GRs containing a GR LBD fused to a GAL4 DBD had a higher EC50 value (weaker activation) than their corresponding full length GRs, indicating altered steroid specificity among these terrestrial vertebrate GRs and that the evolution of the response of terrestrial vertebrate GRs to different steroids was complex, and involved allosteric signaling between the domains D-E and other GR domains.

2. Materials and Methods

2.1 Chemical reagents

DEX, F, corticosterone (B), aldosterone (Aldo), DOC and 11-deoxycortisol (S) 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%.

2.2 Construction of plasmid vectors

The full-coding regions and D/E domains of the GR from *X. laevis*, alligator, chicken and human 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) for the full-coding region or pBIND vector (Promega) for D-E domains [34].

2.3 Transactivation Assay and Statistical Methods

CHO-K1 cells (Chinese hamster ovary cell) were used in the reporter gene assay. Transfection and reporter assays were carried out as described previously [34, 43]. All transfections were performed at least three times, employing triplicate sample points in

each experiment. The values shown are mean ± SEM from three separate experiments, and dose-response data and EC50 were analyzed 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.

3. Results

3.1 Different steroid-response for full length and truncated human, chicken, alligator and *X. laevis* GRs.

3.11 Human GR

In Figures 3A and B, we show corticosteroid-inducible transcriptional activation of full length and truncated (GAL4 DBD-GR LBD) human GRs by DEX, F, B, Aldo, DOC and S. At 10^{-6} M, all corticosteroids induced transcription of full length human GR via the MMTV-reporter gene. In contrast, truncated human GR had a strong response to DEX and F, a much weaker response to B, a small response to Aldo and no response to DOC and S.

3.12 Chicken GR

Transcription of full length chicken GR was activated by all corticosteroids at 10^{-6} M, with a similar strong response to B, Aldo and DOC and a lesser response to DEX, F and S (Figure 3C). Truncated chicken GR was strongly activated by B, F, DEX and Aldo, with a weaker response to DOC and S (Figure 3D).

3.13 Alligator GR

Transcription of full length alligator GR was activated by all corticosteroids at 10^{-6} M, with a similar strong response to B, Aldo and DOC and a lower response to DEX, F and S (Figure 3E). Truncated alligator GR was strongly activated by DEX, F, B and Aldo, with lower response to S and a very weak response to DOC (Figure 3F).

3.14 *X laevis* GR

Transcription of full length *X. laevis* GR was activated by all corticosteroids at 10^{-6} M, with a similar strong response to DEX, F, B, Aldo and DOC and a lower response to DOC and much lower response to S (Figure 3G). Truncated *X. laevis* GR was strongly activated by DEX and B, with a much lower response to F and Aldo and a no response to DOC and S (Figure 3H).

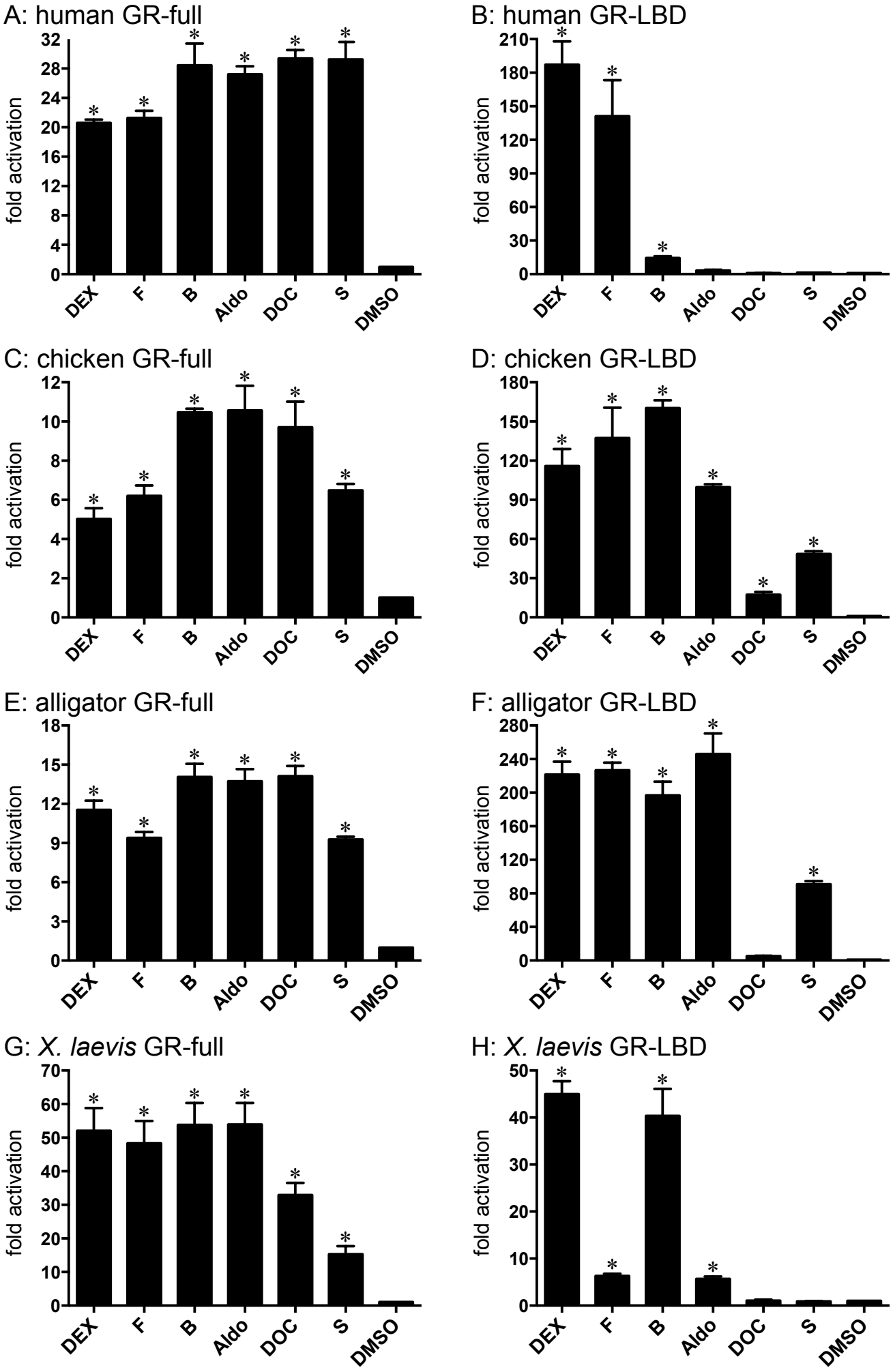


Figure 3

Figure 3. Ligand-specificities of human, chicken, alligator and *X. laevis* full length GRs and LBD GRs.

Full-length human GR (A), chicken GR (C), alligator GR (E), and *X. laevis* GR (G) were expressed in CHO-K1 cells with an MMTV-luciferase reporter. Plasmids for corresponding truncated GRs (human (B), chicken (D), alligator (F) and *X. laevis* (H) containing the D domain and LBD (E domain) fused to a GAL4-DBD were expressed in CHO-K1 cells with a luciferase reporter containing GAL4 binding site. Cells were treated with 10^{-6} M DEX, F, B, Aldo, DOC, S or vehicle alone (DMSO). Results are expressed as means ± SEM, n=3. Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.

3.15 EC50 values for transcriptional activation of full length human, chicken, alligator and *X. laevis* GRs

Next we examined the concentration-dependence of transcriptional activation of full length terrestrial vertebrate GRs by DEX, F, B, Aldo, DOC and S (Figure 4, Table 1). Compared to the other steroids, DEX has the lowest EC50 for all of the full length GRs (Table 1). Interestingly, there are significant differences among the GRs of the EC50s for other corticosteroids, including F and B, which are the major physiological glucocorticoids in terrestrial vertebrates. For example, for full length GRs, B has a lower EC50 than F for *X. laevis* GR, while F has a lower EC50 than B for human, chicken and alligator GR.

Table 1. EC50 values for transcriptional activation by corticosteroids of terrestrial vertebrate GRs

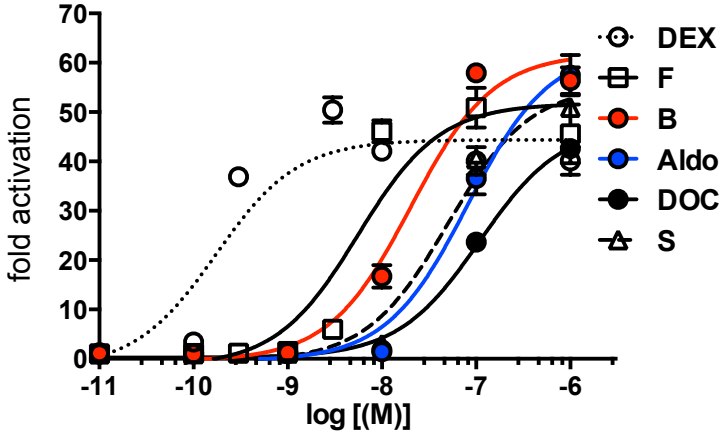
A. Full length GR (A-E domains)

	DEX	F	B	Aldo	DOC	S
Human GR-Full	1.7x10-10	5.6x10-9	2.0x10-8	8.2x10-8	1.1x10-8	5.0x10-8
Chicken GR-Full	2.8x10-11	6.0x10-11	2.3x10-10	2.0x10-9	6.3x10-10	1.7x10-10
Alligator GR-Full	1.4x10-10	2.0x10-10	3.5x10-10	2.7x10-9	2.6x10-9	3.5x10-10
Xenopus GR-Full	7.3x10-9	5.6x10-8	5.1x10-9	4.4x10-8	2.3x10-8	5.3x10-7

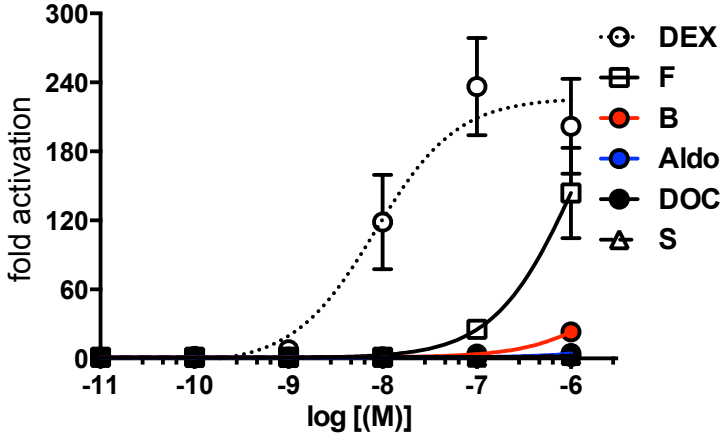
B. Truncated GR (GAL4-DBD+GR-D+E domains)

	DEX	F	B	Aldo	DOC	S
Human GR-LBD	8.3x10-9	1.2x10-6	-	-	-	-
Chicken GR-LBD	2.5x10-10	1.6x10-9	6.5x10-9	7.7x10-8	-	6.6x10-8
Alligator GR-LBD	3.1x10-9	7.7x10-9	4.9x10-8	1.6x10-7	-	1.2x10-7
Xenopus GR-LBD	6.7x10-8	-	4.8x10-8	-	-	-

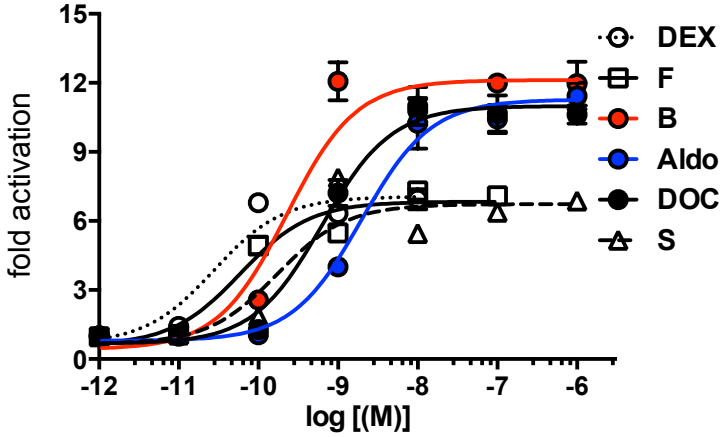
A: human GR-full



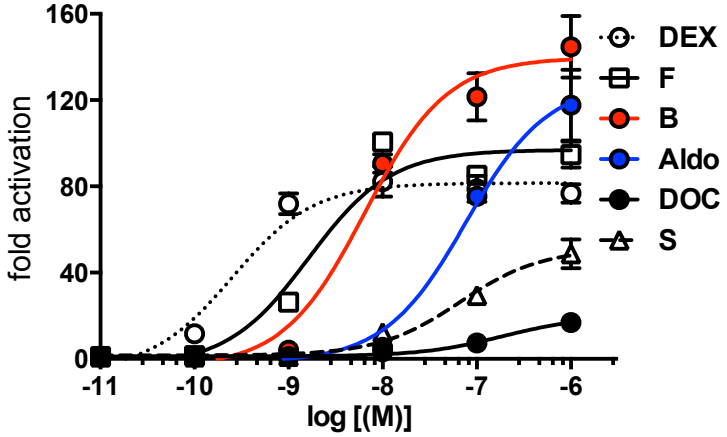
B: human GR-LBD



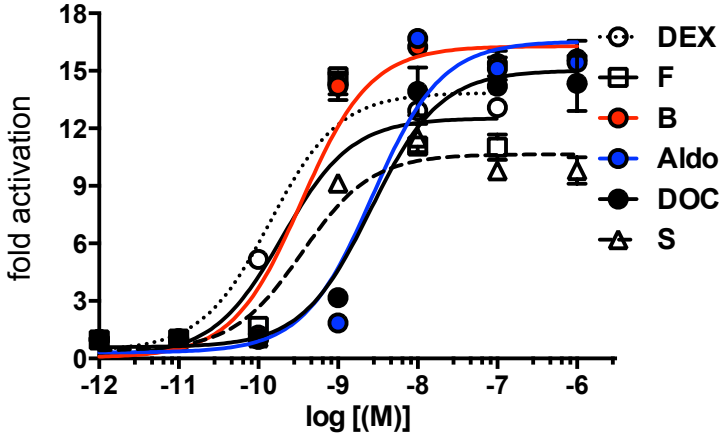
C: chicken GR-full



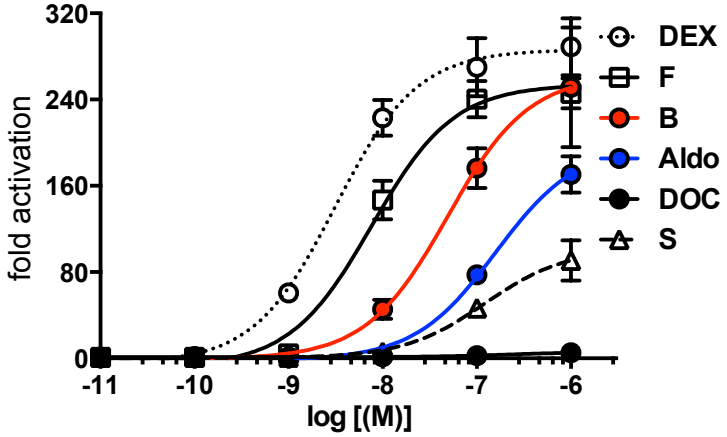
D: chicken GR-LBD



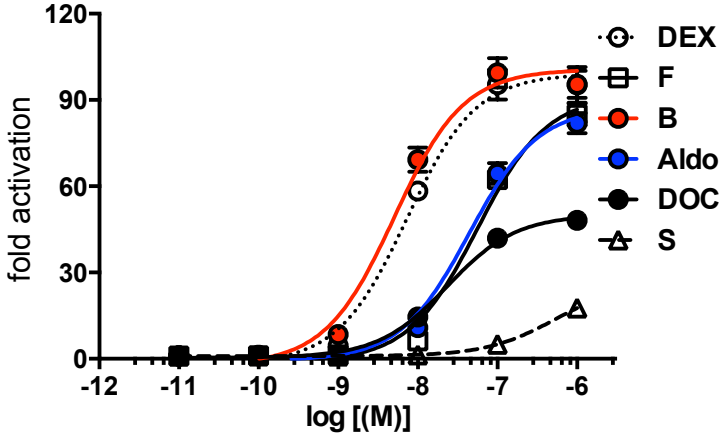
E: alligator GR-full



F: alligator GR-LBD



G: *X. laevis* GR-full



H: *X. laevis* GR-LBD

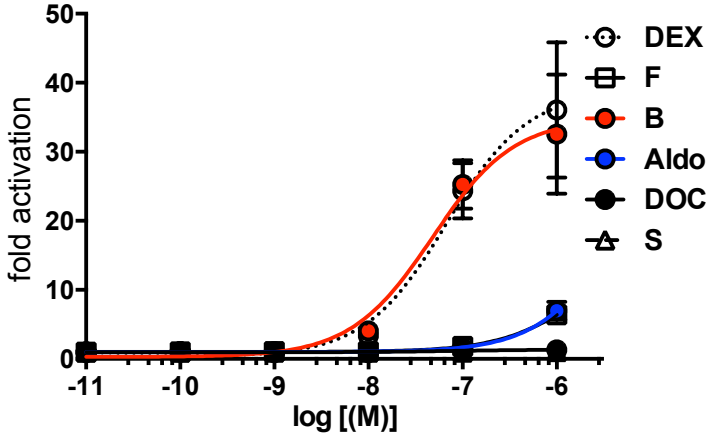


Figure 4

Figure 4. Concentration-dependent transcriptional activation by corticosteroids of full length and truncated human, chicken, alligator and *X. laevis* GRs.
Plasmids encoding full length GRs (A: human GR, C: chicken GR, E: alligator GR, G: *Xenopus* GR) or plasmids encoding the GAL4-DBD fused to the D domain and LBD of GRs (B: human GR, D: chicken GR, F: alligator GR, H: *Xenopus* GR) were expressed in CHO-K1 cells. Cells were treated with increasing concentrations of F, B, Aldo, DOC, S or vehicle alone (DMSO). Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.

Aldo, which is a mineralocorticoid, has an EC50 of 2.7 nM and 44 nM respectively, for alligator GR and *X. laevis* GR and an EC50 of 2 nM and 82 nM, respectively, for chicken and human GR. DOC, which also is a mineralocorticoid, has an EC50 of 2.6 nM and 23 nM, respectively, for alligator GR and *X. laevis* GR, and an EC50 of 0.63 nM and 110 nM, respectively, for chicken GR and human GR. Interestingly, S has an EC50 of 0.17 nM and 0.35 nM, respectively, for chicken and alligator GR, and a much higher EC50 for human GR [50 nM] and *X. laevis* GR [530 nM].

3.16 EC50 values for transcriptional activation of truncated (GAL4 DBD-GR LBD) terrestrial vertebrate GRs

The concentration-dependence of transcriptional activation of truncated terrestrial vertebrate GRs by DEX, F, B, Aldo, DOC and S is shown in Figure 4 and Table 1. Transcriptional activation by several steroids was dramatically different among the terrestrial vertebrate GRs that lacked the A-C domains. For example, truncated human GR has a strong response to DEX (EC50 = 8.3 nM) and a very weak response to F (EC50 = 1.2 µM), and no significant response to B, Aldo, DOC or S. This contrasts to truncated chicken GR, which has nM EC50s for DEX, F and B, and a weaker but significant response to Aldo and S. Only DOC does not activate truncated chicken GR. Truncated alligator GR has nM EC50s for DEX and F, a weaker but significant response to B (EC50 = 49 nM), a weak response to Aldo (EC50 = 0.16 µM) and S (EC50 = 0.12 µM) and no response to DOC.

These results suggest that allosteric signaling between the hinge/LBD and one or more of the A, B and C domains influences the response of terrestrial vertebrate GRs to corticosteroids.

4. Discussion

There are several reports of the response to different corticosteroids of the mammalian GR [20, 29-33, 39, 44]. However, the EC50s for steroid activation of GRs from other terrestrial vertebrates have not been studied in depth. Proszkowiec-Weglarz and Porter [35] investigated the EC50 of B and Aldo for chicken GR. Other steroids were not studied. Oka et al. [34] investigated transcriptional activation of alligator GR by F, B, Aldo, DOC, and S, but not by DEX. Here we report differences in the response of full length GRs from *X. laevis*, alligator, chicken and humans to a panel of corticosteroids, providing evidence for the evolution of selectivity of terrestrial vertebrate GRs for F, B, Aldo, DOC and S. We confirm that Aldo has nM EC50s for both full length chicken and alligator GR [34, 35]. This contrasts with full length human and *X. laevis* GR, for which the EC50 of Aldo is 82 nM and 44 nM, respectively. In addition, we find that DOC, another mineralocorticoid [38, 45, 46], also has a lower EC50 (0.6 nM) for full length chicken GR than for human GR (110 nM) and *X. laevis* GR (23 nM). S also has a substantially lower EC50 for chicken GR (0.17 nM) and alligator GR (0.35 nM) compared to human GR (50 nM) and *X. laevis* GR (953 nM). Together these data indicate that there were significant changes in the response to corticosteroids during the evolution of terrestrial vertebrates.

Our studies with truncated GRs (hinge-LBD) indicate that one or more of the A, B and C domains are important in the response of the GR to corticosteroids. We find that all of the truncated GRs (hinge-LBD) have substantially higher EC50s for all corticosteroids. For example, the EC50s of DEX and F for truncated human GR increased to 8.3 nM and 1.2 μM, respectively. Moreover, Aldo, B, DOC and S have an EC50 greater than 1 μM for truncated human GR. Also, F, Aldo, DOC and S have an EC50 greater 1 μM for truncated *X. laevis* GR. DOC has an EC50 greater 1 μM for truncated chicken and alligator GR. Among the corticosteroids that we studied, transcriptional activation of the GR by DEX is least sensitive and by DOC is most sensitive to the loss of the A, B and C domains.

Analysis of the crystal structures of a protein containing the C-D-E domains of peroxisome proliferator-activated receptor gamma (PPARγ)-retinoid X receptor (RXR), hepatocyte nuclear factor 4 (HNF-4α) and RXR-liver X receptor (LXR) reveal interactions between the C and E domains [13, 22, 47]. Although a structure of a GR containing the C-D-E domains has not been solved, several laboratories have reported that that the amino terminal A-B domains influence transcriptional activation of the GR [21, 23, 24, 26]. In addition the C domain on rat GR has allosteric effects on gene transcription [27, 28, 42]. Together this supports a complex mechanism in which

allosteric interactions between A, B, C and E domains in the GR regulate the specificity of the transcriptional response to corticosteroids.

4.1 Evolution

It is interesting that human mineralocorticoid receptor [MR] [48-50] and zebrafish MR [51] have an interaction between the domains A and B and the LBD, which regulates transcriptional activation by Aldo. The A/B domains on human and zebrafish MR can interact with each other's LBD, indicating that this is an ancient property of the MR [47]. The GR and MR are descended from a common ancestor [12, 37, 52, 53], which suggests that the role in transcriptional activation of the interaction between the A/B and LBD domains arose in their common ancestor. Further studies of the role in transcriptional activation of the A, B and C domains on the GR and MR should provide insights into the evolution of steroid specificity in these receptors.

Author Contributions

Yoshinao Katsu and Michael E. Baker conceived and designed the experiments and wrote the paper. Satomi Kohno and Kaori Oka carried out the research.

Declaration of interests: The authors have no conflict of interest to declare.

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Figure Legends

Figure 1. Structures of various corticosteroids.

Cortisol and corticosterone are physiological glucocorticoids in terrestrial vertebrates and ray-finned fish [12, 52, 54]. Aldosterone, 11-deoxycorticosterone and 11-deoxycortisol are physiological mineralocorticoids [12, 38, 45, 48] with high affinity for human GR [30, 36]. 11-deoxycortisol is both a mineralocorticoid and a glucocorticoid in lamprey [55].

Figure 2. Comparison of domains in some terrestrial vertebrate GRs.

GRs from human, chicken, alligator and *X. laevis* are compared. The functional A/B domain to E domains are schematically represented with the numbers of amino acid residues and the percentage of amino acid identity is depicted.

GenBank accession numbers: human GR (NM_000176), chicken GR (NM_001037826), alligator GR (AB701407), *X. laevis* GR (NM_001088062).

Figure 3. Ligand-specificities of human, chicken, alligator and *X. laevis* full length GRs and LBD GRs.

Full-length human GR (A), chicken GR (C), alligator GR (E), and *X. laevis* GR (G) were expressed in CHO-K1 cells with an MMTV-luciferase reporter. Plasmids for corresponding truncated GRs (human (B), chicken (D), alligator (F) and *X. laevis* (H) containing the D domain and LBD (E domain) fused to a GAL4-DBD were expressed in CHO-K1 cells with a luciferase reporter containing GAL4 binding site. Cells were treated with 10^{-6} M DEX, F, B, Aldo, DOC, S or vehicle alone (DMSO). Results are expressed as means ± SEM, n=3. Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.

Figure 4. Concentration-dependent transcriptional activation by corticosteroids of full length and truncated human, chicken, alligator and *X. laevis* GRs.

Plasmids encoding full length GRs (A: human GR, C: chicken GR, E: alligator GR, G: *Xenopus* GR) or plasmids encoding the GAL4-DBD fused to the D domain and LBD of GRs (B: human GR, D: chicken GR, F: alligator GR, H: *Xenopus* GR) were expressed in CHO-K1 cells. Cells were treated with increasing concentrations of F, B, Aldo, DOC, S or vehicle alone (DMSO). Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.