STEROID RECEPTORS

Evolution of human, chicken, alligator, frog, and zebrafish mineralocorticoid receptors: Allosteric influence on steroid specificity

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Although multiple steroid ligands of the glucocorticoid, mineralocorticoid, and progestin families bind to and regulate the activity of mineralocorticoid receptors (MRs), the responses to these ligands differ across species. To understand how the different domains of MRs contribute to the ligand-induced activation or inhibition of MR activity, we studied the response to eight steroids (aldosterone, 11-deoxycorticosterone, 11-deoxycortisol, cortisol, corticosterone, progesterone, 19-norprogesterone, and spironolactone) of human, chicken, alligator, frog, and zebrafish full-length MRs and truncated MRs, which lacked the N-terminal domain (NTD) and DNA binding domain (DBD). Compared to full-length MRs, some truncated MRs were not activated by the steroids, and others required higher steroid concentrations for activation. Progesterone, 19-norprogesterone, and spironolactone did not activate full-length or truncated human, alligator, or frog MRs. However, at 10 nM, these steroids activated full-length chicken and zebrafish MRs, whereas at 100 nM, these steroids had little activity for truncated chicken MRs, but they retained activity for truncated zebrafish MRs. This suggests that regulation of the activation of the chicken MR by progestin resides in the NTD-DBD and that of the zebrafish MR resides in the hinge-LBD. Zebrafish and chicken MRs contain a serine corresponding to Ser^{810} in human MR, which is required for the antagonist activity of progesterone for human MR, suggesting a previously uncharacterized mechanism of regulation of progestin activation of chicken and zebrafish MRs. These findings suggest that progesterone may be a physiological activator of chicken and zebrafish MRs.

INTRODUCTION

The mineralocorticoid receptor (MR) belongs to the nuclear receptor family, a diverse group of transcription factors that also include receptors for androgens, estrogens, glucocorticoids, and progestins, as well as other small lipophilic ligands, such as thyroid hormone and retinoids (*1*–*4*). Aldosterone (ALDO) is the physiological activator of the human MR in epithelial tissues, such as the kidney distal collecting tubules and the colon (*5*–*9*). The human MR has strong binding affinities for several corticosteroids (ALDO, cortisol, corticosterone, and 11-deoxycorticosterone) and for progesterone (Prog) (*10*–*12*). These steroids also have similar affinities for rat MR (*13*–*15*) and guinea pig MR (*14*, *15*). Corticosteroids are transcriptional activators of human MR (*10*, *12*, *16*–*18*). In contrast, Prog is an antagonist of human MR (*12*, *17*–*19*). The ability of ALDO to activate human, rat, and mouse MRs is complicated by the substantially higher concentration of cortisol in human serum and of corticosterone in rat and mouse serum. For example, the concentration of cortisol in human serum is from 500- to 1000-fold greater than that of ALDO; under stress, the concentration of cortisol increases further. Consequently, human MR would be expected to be occupied by cortisol, to the exclusion of ALDO (*5*, *8*, *20*–*22*). However, the MR in epithelial cells is selectively occupied by ALDO over cortisol and corticosterone because the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) selectively inactivates cortisol and corticosterone (*5*, *22*–*25*). Neither ALDO nor 11-deoxycorticosterone is a substrate for 11β-HSD2, enabling both steroids to activate the MR in epithelial tissues. The MR is also found in the brain, heart, aorta, lung, adipose tissue, breast, and ovary (*6*, *7*, *9*, *26*), some of which lack 11β-HSD2. In tissues lacking 11β-HSD2, cortisol and corticosterone would be expected to occupy the MR. Another mechanism that enables the selective occupation of the MR by ALDO is the presence of corticosteroid-binding globulin (CBG), which is a serum protein that preferentially sequesters cortisol, corticosterone, and 11-deoxycorticosterone (*13*, *27*). CBG has not been isolated from fish, leaving the role of serum proteins in the regulation of corticosteroid action uncharacterized.

Despite the similar binding affinities of ALDO, cortisol, corticosterone, and 11-deoxycorticosterone for the human MR, there is substantial variation in the half-maximal response (EC_{50}) among these steroids for transcriptional activation of the MR. For example, the binding affinity of ALDO and cortisol for human MR is 0.2 and 0.4 nM, respectively (*28*); however, ALDO has a substantially lower EC_{50} (higher activity) than that of cortisol for human MR (*12*, *16*–*18*, *28*–*30*). Also, fish MRs have a stronger response to ALDO than to cortisol, corticosterone, or 11-deoxycortisol (*18*, *30*–*34*). The basis for this difference among corticosteroids in the transcriptional activation of these vertebrate MRs is not well understood. Prog, 19-norprogesterone (19norProg), and spironolactone (Spiron) are antagonists of human MR (*12*, *17*, *18*), but these steroids are agonists of several fish MRs (*18*, *30*, *34*). Data regarding the regulation of frog, alligator, and chicken MRs by progestins are absent. Thus, the timing of the evolution of antagonist activity of progestins and Spiron for the MR is not known.

An important structural property that influences the transcriptional activation of the MR and other steroid receptors is their modular domain structure, which consists of an N-terminal domain (NTD; also referred to as the A/B domain), a central DNA binding domain (DBD; also referred to as the C domain), a hinge domain (referred to as the D domain), and a C-terminal, ligand-binding domain (LBD; also referred to as the E domain) (*18*, *28*, *35*–*37*).

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Although independently the LBD binds steroids (*20*, *35*, *38*–*41*), allosteric interactions between the LBD and NTD are important in the transcriptional activation of the human and zebrafish MR (*19*, *30*, *37*, *42*), as well as for the glucocorticoid receptor (GR) and other steroid receptors (*28*, *43*–*52*). The effects of cortisol and 11-deoxycorticosterone on transcription by the human MR (*19*, *37*) and zebrafish MR (*30*) differ due to interactions between the LBD and NTD of the receptor. In human MR, 11-deoxycorticosterone and cortisol weakly promote the NTD-LBD interaction and stimulate small increases in gene transcription (*19*). In contrast, in zebrafish MR, cortisol and 11-deoxycorticosterone substantially induce the NTD-LBD interaction and stimulate a large increase in transcription. The molecular basis for these differences between human and zebrafish MR is not known, neither is the effect, if any, of interdomain interactions on corticosteroid- or progestin-mediated transcription in frog, alligator, and chicken MRs.

To begin to fill in these gaps, we investigated the activation of full-length MRs from human, chicken, alligator, frog (*Xenopus laevis*), and zebrafish. We also evaluated truncated versions, consisting of the GAL4-DBD fused to the D domain and E domain of the MR (MR-LBD), by a panel of corticosteroids [ALDO, cortisol, corticosterone, 11-deoxycorticosterone, 11deoxycortisol, and progestins (Prog and 19norProg)] and the mineralocorticoid antagonist Spiron. Our results indicate that interactions between the NTD-DBD and hinge-LBD domains in vertebrate MRs are important in steroid specificity, with regulation of progestin activation of chicken MR residing in the NTD-DBD and of zebrafish MR in the hinge-LBD. Our data suggest that Prog is a physiological activator of chicken MR. Geller *et al.* (*17*) found that at 1 nM, Prog, 19norProg, and Spiron are transcriptional activators of an S810L mutant human MR. However, both chicken and zebrafish MRs contain a serine corresponding to Ser^{810} in wild-type human MR, indicating that there are alternative mechanisms for the activation of chicken and zebrafish MRs by progestins.

RESULTS

Comparison of vertebrate MR domains

We selected eight ligands to test for activity toward MRs from five vertebrates (Fig. 1, A and B). ALDO is the physiological activating ligand for terrestrial vertebrate MRs. Cortisol, corticosterone, and 11-deoxycorticosterone are agonistic ligands for terrestrial vertebrate MRs. 11-Deoxycorticosterone and cortisol have been proposed to be mineralocorticoid agonists in teleosts (*18*, *53*–*56*) because ALDO is not found in fish (*57*). Prog may be a mineralocorticoid agonist in fish (*11*, *18*, *34*). 11-Deoxycortisol is a ligand for corticosteroid receptor (CR) in lamprey (*58*, *59*). Prog has a high affinity (0.01 nM) for human MR (*12*, *17*, *18*), but it functions as an antagonist. Spiron is an MR antagonist in humans. However, Prog, 19norProg, and Spiron are agonists for gar, sturgeon, zebrafish, and trout MRs (Fig. 1B) (*18*, *30*, *34*).

We selected the human, chicken, alligator, and frog because they are at key nodes in the evolution of terrestrial vertebrates. Zebrafish was selected because it is an important model system for fish (*60*). By comparing the amino acid sequences of the different domains from MRs of each species with that of the human MR, we found that these phylogenetically diverse MRs have strong conservation of the C domain (97 to 100%) and E domain (77 to 93%), with substantially less conservation in the A/B domain (37 to 78%) and D domain (41 to 79%) (Fig. 1C). The 100% identity among the DBDs from human, chicken, and alligator MRs eliminates the possibility of sequence differences in their DBDs contributing to any differences in ligand-dependent regulation of these MRs.

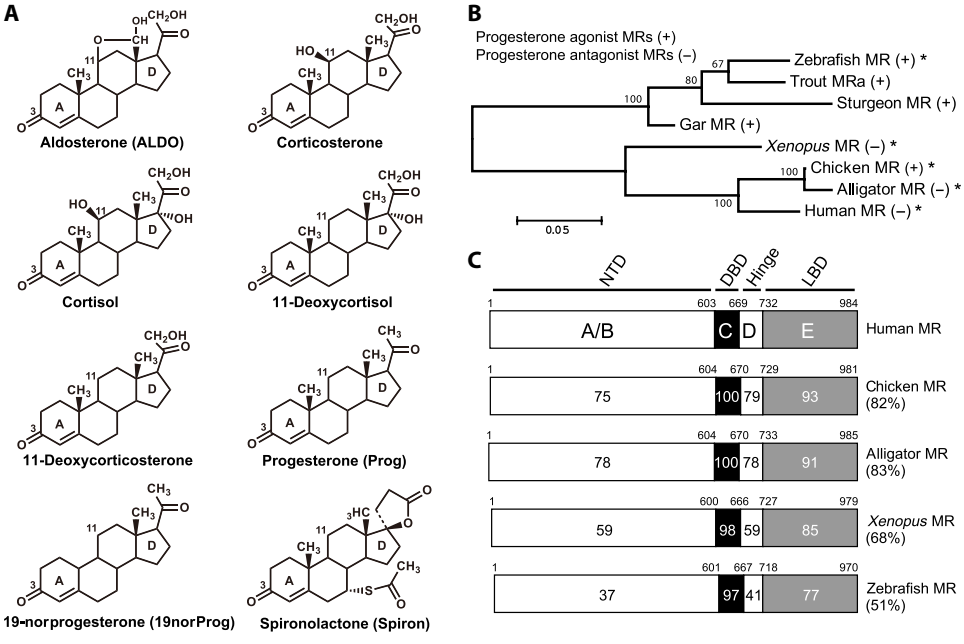

Fig. 1. Steroids and MRs. (**A**) The structures of the steroid ligands. These steroids are called 3-ketosteroids because they contain a ketone at C3. With the exception of Spiron, all of the other steroids are natural ligands. (**B**) Phylogeny of vertebrates investigated for MR activation by Prog. The phylogeny was constructed with the neighbor-joining method (*71*) after sequences were aligned by ClustalW (*72*). Values for 1000 bootstrap runs are shown as percentages at each node. MRs activated by Prog are indicated with (+), whereas those inhibited by Prog are indicated with (–). Those tested in this study are indicated by an asterisk. GenBank accession numbers are as follows: human MR (NP_000892), chicken MR (ACO37437), alligator MR (NP_001274242), *Xenopus* MR (NP_001084074), zebrafish MR (NP_001093873), trout MRa (NP_001117955), sturgeon MR (BAV17690), and gar MR (BAV17691). (**C**) Comparison of the domains in vertebrate MRs. Domains A/B (NTD), C (DBD), D (hinge), and E (LBD) on MRs from the indicated species are compared. Shown are the number of amino acids in each domain and the percentage of identical amino acids compared to the human MR. GenBank accession numbers are as follows: human MR (NP_000892), chicken MR (ACO37437), alligator MR (NP_001274242), *Xenopus* MR (NP_001084074), and zebrafish MR (NP_001093873).

Transcriptional activation of full-length and truncated MRs by corticosteroids

We compared the transcriptional activation of an MR-responsive reporter in human embryonic kidney–293 (HEK293) cells cotransfected with the reporter plasmid and with plasmid encoding either the full-length or truncated MR from each species in response to ALDO or corticosteroids at 1 and 10 nM (Fig. 2, A to E). The responses were compared on the basis of the relative activity of the reporter in the

presence of dimethylsulfoxide (DMSO). ALDO, cortisol, corticosterone, and 11-deoxycorticosterone were similar in their activation of full-length human, chicken, alligator, and zebrafish MRs (Fig. 2, A to C and E). ALDO, cortisol, and corticosterone were strong activators of full-length *Xenopus* MR, whereas 11-deoxycorticosterone was a weaker activator (Fig. 2D). 11-Deoxycortisol stimulated a response similar to that of ALDO for full-length chicken and zebrafish MRs (Fig. 2, B and E), but stimulated a weaker response for full-length human, alligator, and *Xenopus* MRs (Fig. 2, A, C, and D).

Analysis of the truncated MRs, lacking the A/B domain and containing a GAL4-DBD instead of the MR DBD, revealed that 11-deoxycortisol had little transcriptional activity at 10 nM for truncated human and *Xenopus* MRs (Fig. 2, A and D), in contrast to the activity of 11-deoxycortisol for their full-length MRs. 11-Deoxycortisol had similar low activity for truncated and full-length alligator MRs (Fig. 2C). 11-Deoxycorticosterone had decreased activity, compared to ALDO, for truncated human and alligator MRs and exhibited substantially reduced activity for truncated *Xenopus* MR. However, 11-deoxycorticosterone had similar activity, compared to ALDO, for truncated chicken and zebrafish MRs. At 10 nM, cortisol lost about 25% of its activity, compared to ALDO, for truncated human, chicken, and alligator MRs, and almost all activity for truncated *Xenopus* MR (Fig. 2D). The response to corticosteroids by truncated zebrafish MR was different from the response of truncated terrestrial vertebrate MRs. ALDO, cortisol, corticosterone, and 11-deoxycorticosterone produced similar relative increases in activity for truncated and full-length zebrafish MRs (Fig. 2). At 10 nM, 11-deoxycortisol stimulated ~75% of the activity for truncated zebrafish MR compared to the relative activity that 11-deoxycortisol induced for the full-length zebrafish MR. These data indicate that responsiveness to 11-deoxycorticosterone depended on the presence of the DBD or NTD for all of the MRs analyzed. Furthermore, the results suggested that the chicken MR is functionally more similar to the fish MR than it is to the human, alligator, and *Xenopus* MRs. Compared with the other MRs, the zebrafish

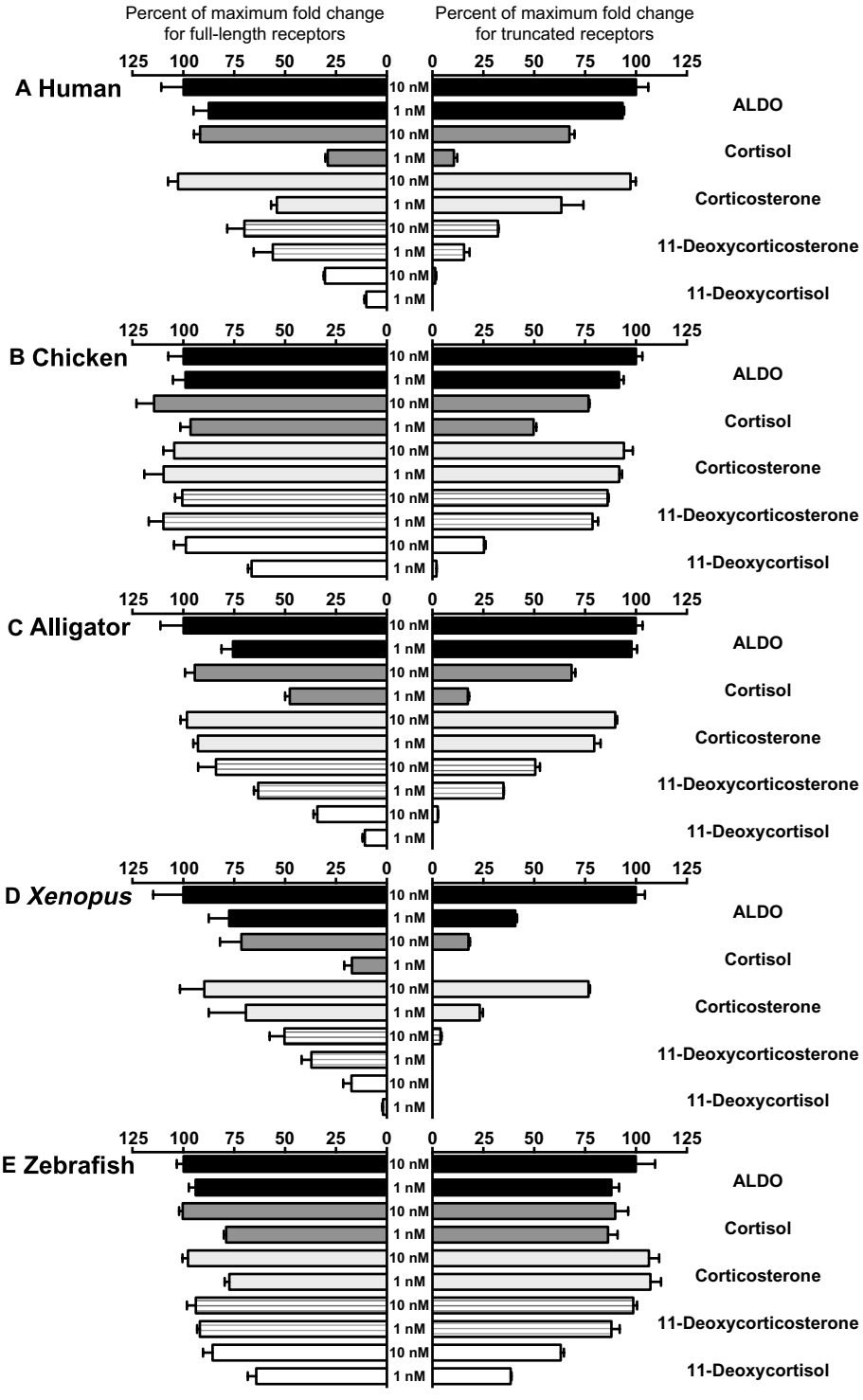

Fig. 2. Corticosteroid activation of human, chicken, alligator, *Xenopus*, and zebrafish full-length MRs and LBD MRs. (**A** to **E**) Full-length (left) and truncated (right) human (A), chicken (B), alligator (C), *Xenopus* (D), and zebrafish (E) MRs. Full-length MR constructs were coexpressed in HEK293 cells with an MMTV-Luc reporter. Plasmids corresponding to the truncated MRs containing the D and E domains fused to a GAL4-DBD were coexpressed in HEK293 cells with a luciferase reporter containing a GAL4-binding site. The cells were treated with the indicated concentrations of ligand or vehicle alone (DMSO) for 44 hours before reporter gene activity was assessed. Data are means ± SEM of three independent experiments and are expressed as the fold activation compared to the activity of cells expressing the control vector and treated with vehicle (DMSO) alone, which was set as 1.

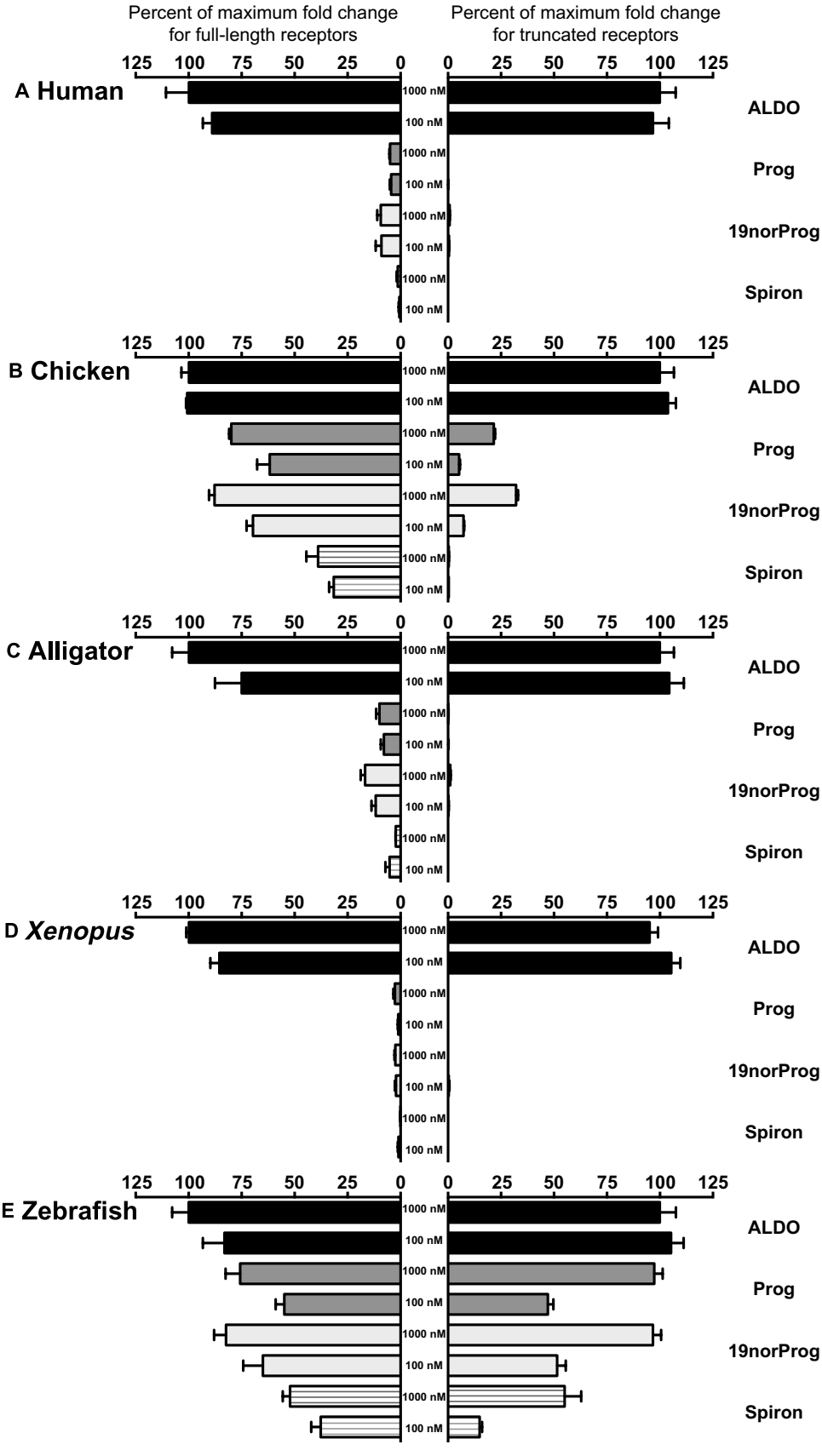

Fig. 3. Prog, 19norProg, or Spiron activation of human, chicken, alligator, *Xenopus*, and zebrafish full-length and truncated MRs. (**A** to **E**) Full-length (left) and truncated (right) human (A), chicken (B), alligator (C), *Xenopus* (D), and zebrafish (E) MRs. HEK293 cells were transfected with the appropriate constructs and reporters as described for Fig.2. The cells were treated with the indicated concentrations of Prog, 19norProg, or Spiron or vehicle alone (DMSO) for 44 hours before gene reporter activity was measured. Data are means ± SEM of three independent experiments and are expressed as the fold activation compared to the activity of cells expressing the control vector and treated with vehicle (DMSO) alone, which was set as 1.

MR exhibited less dependency on the DBD and NTD for regulation by corticosteroids.

Transcriptional activation of full-length and truncated MRs by progestins and Spiron

We tested Prog, 19norProg, and Spiron at concentrations of 10, 100, and 1 μM for transcriptional activation of full-length and truncated terrestrial vertebrate and zebrafish MRs (Fig. 3, A to E). At 1 μM, Prog and 19norProg were weak transcriptional activators of full-length human and alligator MRs, and both steroids displayed no activity for *Xenopus* MR. At 1 μM, Spiron did not activate transcription by the full-length human, *Xenopus*, and alligator MRs. As expected, Prog, 19norP, and Spiron activated transcription by full-length zebrafish MR (Fig. 3E) (*30*). Unexpectedly, Prog, 19norP, and Spiron activated full-length chicken MR (Fig. 3B). Our finding that Spiron activated full-length chicken MR is consistent with an earlier report (*61*).

Prog, 19norProg, and Spiron did not stimulate the activity of truncated human, alligator, or *Xenopus* MRs (Fig. 3, A, C, and D). At 1 μM, Prog and 19norProg stimulated ~30% of the transcriptional activity of that stimulated by 10 nM ALDO for truncated chicken MR, whereas Spiron was inactive (Fig. 3B). Prog and 19norProg stimulated similar relative responses of truncated zebrafish MR, and Spiron stimulated less activity for the truncated zebrafish MR than for the full-length receptor (Fig. 3E). These data indicate that the DBD or NTD of

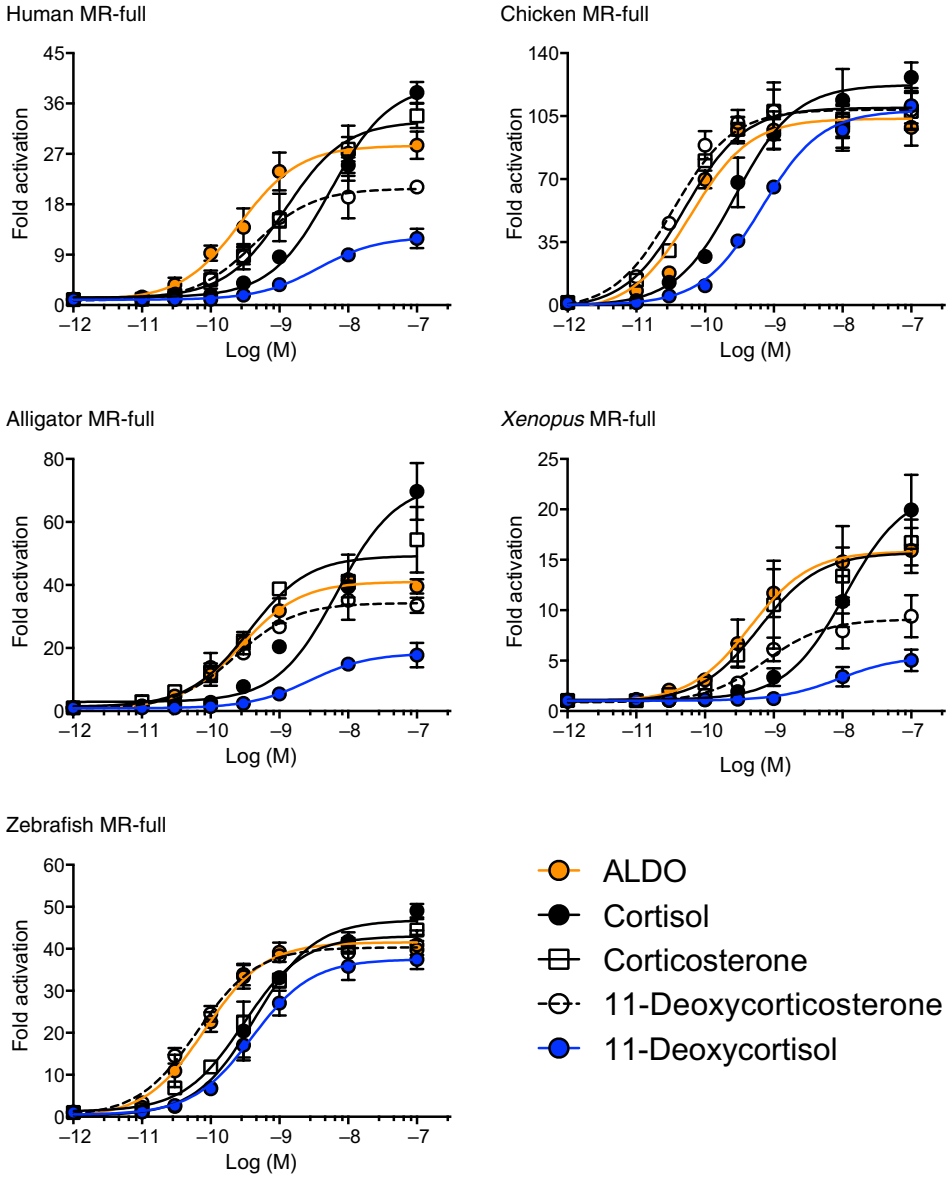

Fig. 4. Concentration-dependent transcriptional activation by corticosteroids of full-length human, chicken, alligator, *Xenopus*, and zebrafish MRs. HEK293 cells were cotransfected with plasmids encoding the indicated full-length MRs together with the reporter plasmid. The cells were then treated with increasing concentrations of the indicated steroids or with vehicle (DMSO) alone before gene reporter activity was measured. Data are means ± SEM of three independent experiments and are expressed as fold activation compared to the activity of cells expressing the control vector and treated with vehicle (DMSO) alone, which was set as 1.

Table 1. EC_{50} values for the 3-ketosteroid transcriptional activation of full-length vertebrate MRs. The percentage relative induction is compared to the maximal response to ALDO.

MR	ALDO	Corticosterone	Cortisol	11-Deoxycorticosterone	11-Deoxycortisol
	EC_{50} (M)	EC_{50} (M)	EC_{50} (M)	EC_{50} (M)	EC_{50} (M)
Human	2.7×10^{-10}	1.2×10^{-9}	5.5×10^{-9}	4.2×10^{-10}	3.6×10^{-9}
	100%	119%	133%	74%	42%
Chicken	6.2×10^{-11}	5.1×10^{-11}	2.8×10^{-10}	3.4×10^{-11}	6.7×10^{-10}
	100%	109%	128%	110%	112%
Alligator	2.8×10^{-10}	3.6×10^{-10}	6.9×10^{-9}	2.3×10^{-10}	2.7×10^{-9}
	100%	138%	176%	85%	45%
Xenopus	4.6×10^{-10}	6.2×10^{-10}	1.1×10^{-8}	7.6×10^{-10}	9.1×10^{-9}
	100%	105%	126%	59%	31%
Zebrafish	8.2×10^{-11}	3.0×10^{-10}	4.4×10^{-10}	6.3×10^{-11}	4.0×10^{-10}
	100%	112%	123%	103%	94%

human, alligator, and *Xenopus* MRs are required for regulation of activity by these three steroids. In contrast, chicken and zebrafish MRs retained responsiveness to progestins and Spiron even in the absence of these two domains, indicating a distinct mode of regulation of these MRs by Prog, 19norProg, and Spiron.

EC_{50} values for corticosteroid activation of full-length and truncated MRs

To gain a quantitative measure of the corticosteroid activation of vertebrate MRs, we determined the concentration-dependent activation of full-length vertebrate MRs by the corticosteroids and compared these to the response to ALDO (Fig. 4 and Table 1). Although chicken and zebrafish are phylogenetically distant vertebrates (Fig. 1B), full-length chicken and zebrafish MRs had EC_{50} values <1 nM for ALDO and the corticosteroids. Human, alligator, and *Xenopus* MRs exhibited the greatest responses to ALDO, corticosterone, and cortisol, whereas they had weaker responses to 11-deoxycorticosterone and 11-deoxycortisol. Thus, for the human MR, the maximal response induced by 11-deoxycorticosterone was only 74% of that induced by ALDO (Table 1). For 11-deoxycortisol, the maximal response was only 42% of the maximal response induced by ALDO. In contrast, the maximal response induced by the corticosteroids for full-length chicken or zebrafish MR was similar with even 11-deoxycortisol producing a maximal response (Fig. 4 and Table 1).

To investigate the role of the NTD and DBD on ligand affinity and efficacy, we determined the concentration-dependent transcriptional activation of truncated terrestrial vertebrate MRs by ALDO, cortisol, corticosterone, 11-deoxycorticosterone, and 11-deoxycortisol. Consistent with our screening studies (Fig. 3), transcriptional activation by 11-deoxycortisol, 11-deoxycorticosterone, and cortisol was reduced for some terrestrial vertebrate MRs that lacked the NTD and DBD (Fig. 5 and Table 2). For example, whereas 11-deoxycortisol had an EC_{50} value of 3.6 nM with a 42% maximal response for full-length human MR, this steroid stimulated only 8% of maximal response that was so low an EC_{50} could not be calculated. A similar pattern occurred for truncated alligator and *Xenopus* MRs. In contrast, truncated chicken and truncated zebrafish MRs retained responsiveness to corticosteroids (Table 2).

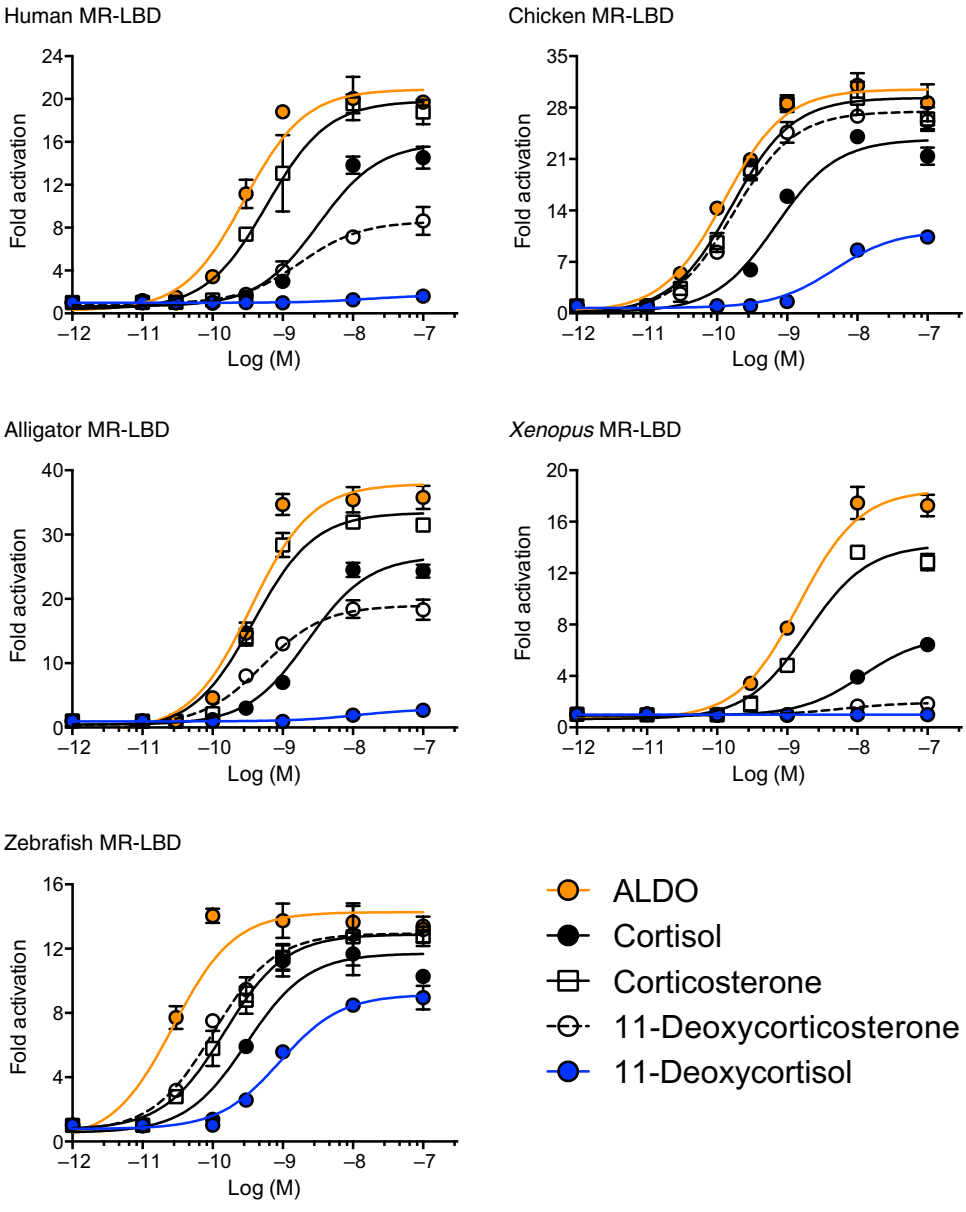

Fig. 5. Concentration-dependent transcriptional activation of truncated human, chicken, alligator, *Xenopus*, and zebrafish MRs. HEK293 cells were cotransfected with plasmids encoding the GAL4-DBD fused to the D and E domains of the indicated MRs together with the reporter plasmid. The cells were then treated with increasing concentrations of the indicated steroids or with vehicle (DMSO) alone before gene reporter activity was measured. Data are means ± SEM of three independent experiments and are expressed as fold activation compared to the activity of cells expressing the control vector and treated with vehicle (DMSO) alone, which was set as 1.

Effects of progestins, corticosteroids, and Spiron on the ALDO-mediated activation of terrestrial vertebrate MRs

The absence of the NTD and DBD differentially impaired the induced response of the MRs for the specific steroids Prog, 19norProg, Spiron, 11-deoxycortisol, and 11-deoxycorticosterone. Thus, we investigated whether these steroids bound to the truncated MRs that were not activated in the reporter assay. We inferred binding on the basis of the ability of these steroids to alter the transcriptional activation by ALDO of truncated human, alligator, and *Xenopus* MRs. We used

Table 2. EC_{50} values for the 3-ketosteroid transcriptional activation of GAL4-DBD-MR-LBD of vertebrate MRs. The percentage relative induction is compared to the maximal response to ALDO.

MR	ALDO	Corticosterone	Cortisol	11-Deoxycorticosterone	11-Deoxycortisol
	EC_{50} (M)	EC_{50} (M)	EC_{50} (M)	EC_{50} (M)	EC_{50} (M)
Human	2.8×10^{-10}	5.9×10^{-10}	3.2×10^{-9}	1.8×10^{-9}	—
	100%	95%	74%	44%	8%
Chicken	1.3×10^{-10}	1.6×10^{-10}	6.9×10^{-10}	1.7×10^{-10}	4.7×10^{-9}
	100%	92%	75%	92%	36%
Alligator	3.5×10^{-10}	3.8×10^{-10}	2.3×10^{-9}	5.2×10^{-10}	—
	100%	88%	68%	51%	8%
Xenopus	1.5×10^{-9}	1.9×10^{-9}	1.2×10^{-8}	—	—
	100%	74%	37%	10%	6%
Zebrafish	2.7×10^{-11}	1.5×10^{-10}	3.1×10^{-10}	1.0×10^{-10}	9.1×10^{-10}
	100%	96%	77%	99%	67%

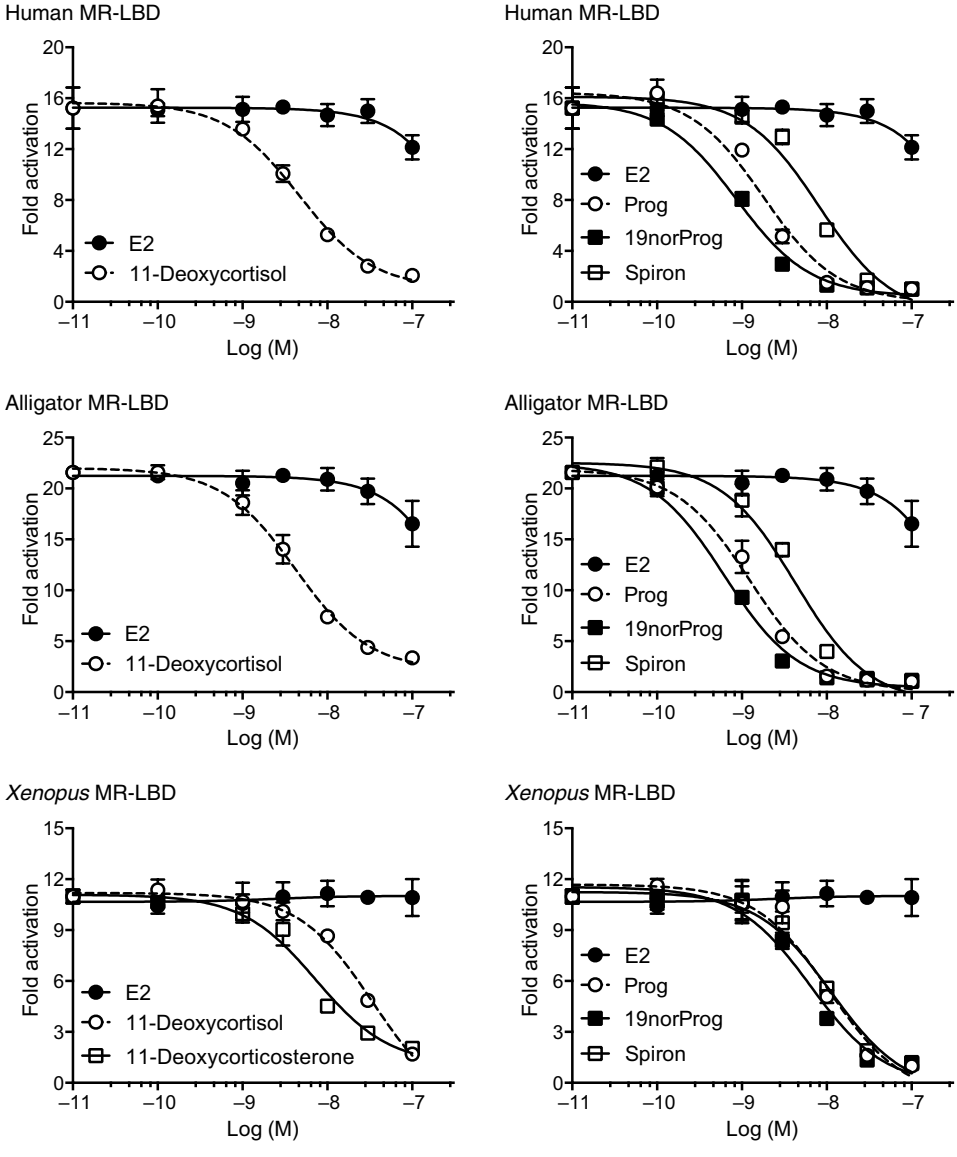

Fig. 6. Competition by Prog, 19norProg, Spiron, or 11-deoxycortisol for transcriptional activation by ALDO of truncated human, alligator, and *Xenopus* MRs. HEK293 cells were cotransfected with plasmids encoding the GAL4-DBD fused to the D and E domains of the indicated MRs together with reporter plasmid. Cells expressing human or alligator MR were treated with 0.5 nM ALDO and increasing concentrations of the competing ligands Prog, 19norProg, Spiron, or 11-deoxycortisol, as indicated. Cells expressing *Xenopus* MR were treated with 5 nM ALDO and with increasing concentrations of the competing ligands Prog, 19norProg, Spiron, 11-deoxycortisol, or 11-deoxycorticosterone. E2 was used a control in all experiments. Data are means ± SEM of three independent experiments and are expressed as fold activation compared to the activity of cells expressing the control vector and treated with vehicle (DMSO) alone, which was set as 1.

0.1 to 100 nM E2 as a positive control because E2 does not bind to the MR. Each of the steroids tested inhibited ALDO-induced activation of the truncated MRs, indicating that these steroids bound to the truncated MRs (Fig. 6).

DISCUSSION

Although the human MR was cloned in 1987 (*10*), data on the transcriptional activation of vertebrate MRs by corticosteroids and progestins are modest. For the most part, the focus has been on the activation of full-length human MR by ALDO and cortisol, and in some studies by corticosterone, 11-deoxycorticosterone, and 11-deoxycortisol (*11*, *16*, *18*, *19*, *37*, *38*, *62*). Transcriptional activation by ALDO and corticosterone of full-length chicken MR (*61*) and by ALDO, corticosterone, cortisol, and 11-deoxycorticosterone of full-length alligator MR (*63*) also have been studied. Data for Prog, 19norProg, and Spiron in terrestrial vertebrates are limited to human MR, for which Prog and 19norProg have low activity, whereas Spiron is an MR antagonist (*12*, *17*, *18*, *39*). In contrast, Prog, 19norProg, and Spiron activate the MR in zebrafish, trout, gar, and sturgeon (*18*, *30*, *34*). Data on the influence of the NTD on transcriptional activation by corticosteroids and progestins are limited to human, rat, and zebrafish MRs (*19*, *30*, *37*, *64*), with no data on chicken, alligator, and *Xenopus* MRs. Here, we fill in some gaps in our knowledge of the transcriptional activation by corticosteroids and progestins of full-length and truncated chicken, alligator, and *Xenopus* MRs and of truncated zebrafish MR. The DBDs in human, chicken, and alligator MRs are identical, and the DBDs in other vertebrate MRs are strongly conserved (Fig. 1C), suggesting that differences in transcriptional activation by corticosteroids and progestins of full-length and truncated MRs are mainly due to interactions between the NTD and hinge-LBD.

The higher EC_{50} values for corticosteroid activation of truncated terrestrial vertebrate MRs compared to those of full-length MRs (Tables 1 and 2) suggest that the NTD and LBD are important in the transcriptional activation of the MR. The loss of activity for truncated MRs varies with the steroid and the vertebrate. ALDO and corticosterone have the smallest change in EC_{50} value for full-length and truncated terrestrial vertebrate MRs (Tables 1 and 2). Unexpectedly, 11-deoxycortisol has less than 10% of the activity of ALDO for truncated human, alligator, and *Xenopus* MRs, and 36 and 67%, respectively, for chicken and zebrafish MRs, which are the two MRs that are activated by Prog. 11-Deoxycorticosterone lost activity for truncated *Xenopus* MR (10% of ALDO) and had diminished activity for truncated human MR (44% of ALDO). In contrast to terrestrial vertebrate MRs, truncated zebrafish MR retained activity for 11-deoxycorticosterone, 11-deoxycortisol, and the other corticosteroids.

Our results are consistent with those of Pippal *et al.* (*19*) and Rogerson and Fuller (*37*), who found that truncated (GAL4-DBD-MR-LBD) human and zebrafish MRs had lower responses to ALDO than had truncated MRs incubated with the NTD domain. Rogerson and Fuller (*37*) also reported that ALDO could not activate transcription by the truncated human Gly^{962}→Ala mutant MR, but ALDO could activate the NTD + truncated Gly962Ala MR, demonstrating the importance of the human MR NTD in transcriptional activation. 11-Deoxycorticosterone and cortisol promoted an increase in transcriptional activation for the NTD + GAL4–DBD–zebrafish MR–LBD, but only had a weak effect on the NTD + GAL4–DBD–human LBD (*30*). Our results extend this role of the NTD to the transcriptional activation of chicken, alligator, and *Xenopus* MRs by ALDO, cortisol, corticosterone, 11-deoxycorticosterone, and 11-deoxycortisol and transcriptional activation of human MR by corticosterone and 11-deoxycortisol (Tables 1 and 2, and Figs. 4 and 6).

Prog, 19norProg, and Spiron are enigmatic ligands for vertebrate MRs. These steroids are antagonists for human MR (*12*, *17*–*19*, *30*) and, as reported here, for alligator and *Xenopus* MRs (Fig. 4), and are agonists for zebrafish MR (*30*), trout MR (*34*), sturgeon MR, and gar MR (*18*) and, as reported here, for chicken MR, which was unexpected (Fig. 4). Chicken MR also is unusual because coexpression of chicken MR with GR does not affect their transcriptional activity (*61*), which differs from the clear transcriptional effect of coexpressing human MR with GR (*65*–*67*) and trout MR with GR (*66*).

Prog may be a transcriptional activator of chicken MR in tissues that contain 11β-HSD2, which converts corticosterone to the inactive 11-dehydrocorticosterone (*5*, *8*, *22*, *24*). Because of the absence of an 11β-hydroxyl on Prog, it is inert to 11β-HSD2, which would convert about 90% of corticosterone to 11-dehydrocorticosterone (*14*, *68*). The concentrations of Prog and corticosterone in chicken blood are 4.7 and 15.3 nM, respectively (*69*), and an upper limit for ALDO in chicken blood is 0.4 nM (*70*). Thus, the physiological concentration of Prog is sufficient to occupy chicken MR. For terrestrial vertebrate MRs, the only previous example of a Prog-activated MR is the S810L mutant human MR, which was studied by Geller *et al.* (*17*). The S810L MR is activated by 1 nM Prog, 19norProg, and Spiron. However, both chicken and zebrafish MRs, as well as other Prog-activated fish MRs (Fig. 2) (*18*, *30*, *34*), contain a serine corresponding to Ser^{810} in wild-type human MR. This indicates that alternative mechanisms are involved in the activation of chicken, zebrafish, and other fish MRs by progestins. Our experiments with truncated chicken and zebrafish MRs provide clues to the regulation of chicken and zebrafish MR activation by Prog. The lack of activation of truncated chicken MR by Prog, 19norProg, and Spiron suggests that allosteric interactions between the NTD and the hinge-LBD domains of full-length chicken MR are important in the transcriptional activation by these steroids, whereas the activation of truncated zebrafish MR by Prog, 19norProg, and Spiron suggests that the hinge-LBD domains are important in the transcriptional activation of full-length zebrafish MR.

MATERIALS AND METHODS

Chemical reagents

Steroids with their following Chemical Abstracts Service numbers: cortisol, 50-23-7; corticosterone, 50-22-6; ALDO, 52-39-1; 11-deoxycorticosterone, 64-85-7; 11-deoxycortisol, 152-58-9; Prog, 57-83-0; 19norProg, 472-54-8; and Spiron, 57-01-7 were purchased from Sigma-Aldrich. For reporter gene assays, all hormones were dissolved in DMSO, and the final concentration of DMSO in the culture medium did not exceed 0.1% (v/v).

Construction of plasmids

The full-coding regions and D/E domains of the MRs from human, chicken, alligator, frog (*Xenopus*), and zebrafish were amplified by polymerase chain reaction (PCR) with KOD DNA polymerase. The PCR products were gel-purified and ligated into the pcDNA3.1 plasmid (at the Kpn I–Not I site for human MR and the Bam HI–Not I site for chicken, alligator, frog, and zebrafish MRs) for the full-coding region or into the pBIND vector (at the Mlu I–Not I site for human, chicken, frog, and zebrafish MR, and at the Mlu I–Kpn I site for alligator MR) for D/E domains. The E domain begins at human MR

(732), chicken MR (729), alligator MR (733), frog MR (727), and zebrafish MR (718) (Fig. 1C).

Transactivation assay and statistical methods

HEK293 cells were used in the reporter gene assays, and transfection and reporter assays were carried out as described previously (*18*, *51*). HEK293 cells were seeded in 24-well plates at 5×10^4 cells per well in phenol red–free Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% charcoal/dextran-treated fetal bovine serum (Hyclone). After 24 hours, the cells were transfected with 200 ng of MMTV-Luc, 50 ng of pRL-TK (as an internal control to normalize for variation in transfection efficiency; contains the *Renilla reniformis* luciferase gene with the herpes simplex virus thymidine kinase promoter; Promega), and 100 ng of pcDNA3.1-MR using PEI-max transfection reagent (Polysciences Inc). After 4 hours of incubation, various steroid hormones were applied to the medium. After 44 hours, the cells were collected, and the luciferase activity of the cells was measured by a chemiluminescence assay with Dual-Luciferase Reporter Assay System (Promega). For the GAL4 assay, HEK293 cells were transfected with 50 ng of pBIND-MR-LBD and 200 ng of the pG5-luc reporter containing a GAL4-binding site. After 4 hours of incubation, various steroid hormones were applied to the medium. After 44 hours, the cells were collected, and the luciferase activity of the cells was measured by a chemiluminescence assay with Dual-Luciferase Reporter Assay System. Luminescence was measured with a Turner Designs Luminometer TD-20/20 (Promega). Promoter activity was calculated as firefly (*Photinus pyralis*) luciferase activity/sea pansy (*R. reniformis*) luciferase activity. All transfections were performed at least three times, with triplicate sample points in each experiment. The values shown are means ± SEM from three separate experiments, and the dose-response data and EC_{50} values were analyzed with GraphPad Prism. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was performed for statistical analyses. Differences were considered statistically significant if $P < 0.05$.

Phylogenetic analysis

The phylogeny was constructed with the neighbor-joining method (*71*) after sequences were aligned by ClustalW (*72*). Values for 1000 bootstrap runs are shown as percentages at each node.

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Evolution of human, chicken, alligator, frog, and zebrafish mineralocorticoid receptors: Allosteric influence on steroid specificity

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Evolving steroid responses

Mineralocorticoid receptors (MRs) are members of the nuclear receptor family of transcription factors, and they are activated by steroid hormones including mineralocorticoids, such as aldosterone, and glucocorticoids, such as cortisol. Katsu *et al*. studied the effects of eight different steroids on the transcriptional activity of full-length and truncated MRs from five different species. Truncated MRs generally showed weaker activation than full-length receptors, and, among terrestrial vertebrate receptors, only chicken MR was activated by progesterone, a property shared by the zebrafish receptor. Together, these findings suggest that interactions between different domains within the MR were important in determining steroid specificity.