Aldosterone and Dexamethasone Activate African Lungfish Mineralocorticoid Receptor: Increased Activation After Removal of the Amino-Terminal Domain

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ABSTRACT. Aldosterone, the main physiological mineralocorticoid in humans and other terrestrial vertebrates, first appears in lungfish, which are lobe-finned fish that are forerunners of terrestrial vertebrates. Aldosterone activation of the MR regulates internal homeostasis of water, sodium and potassium, which was critical in the conquest of land by vertebrates. We studied transcriptional activation of the slender African lungfish MR by aldosterone, other corticosteroids and progesterone and find that aldosterone, 11-deoxycorticosterone, 11-deoxycortisol and progesterone have half-maximal responses (EC50s) below 1 nM and are potential physiological mineralocorticoids. In contrast, EC50s for corticosterone and cortisol were 23 nM and 66 nM, respectively. Unexpectedly, truncated lungfish MR, consisting of the DNA-binding, hinge and steroid-binding domains, had a stronger response to corticosteroids and progesterone than full-length lungfish MR, indicating that the N-terminal domain represses steroid activation of lungfish MR, unlike human MR in which the N-terminal domain contains an activation function. BLAST searches of GenBank did not retrieve a GR ortholog, leading us to test dexamethasone and triamcinolone for activation of lungfish MR. At 10 nM, both synthetic glucocorticoids are about 4-fold stronger than 10 nM aldosterone in activating full-length lungfish MR, leading us to propose that lungfish MR also functions as a GR.

Keywords: Lungfish, Lobe-finned Fish, Terrestrial Vertebrates, Aldosterone evolution; mineralocorticoid receptor evolution; evolution

INTRODUCTION

The mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) belong to the nuclear receptor family, a diverse group of transcription factors that arose in multicellular animals [1–3]. The MR and GR have key roles in the physiology of humans and other terrestrial vertebrates and fish [4–11]. The MR and GR evolved from an ancestral corticoid receptor (CR) in a jawless fish (cyclostome), which has descendants in modern lampreys and hagfish [12–14]. A distinct MR and GR first appear in cartilaginous fishes (Chondrichthyes) [1,13,15–17], which diverged from bony vertebrates about 450 million years ago [18,19].

Aldosterone is the main physiological mineralocorticoid in humans and other terrestrial vertebrates [5,6,9,20–23]. Aldosterone activation of the MR in the kidney regulates salt and

water homeostasis by promoting sodium and water reabsorption and potassium secretion, a mechanism that conserves salt and water. Thus, it is puzzling that aldosterone is a potent transcriptional activator of lamprey and hagfish CRs [15], skate MR [16] and elephant shark MR [13,24] because aldosterone is not synthesized by lampreys [15], cartilaginous fishes or ray finned fishes [25]. Aldosterone first appears in lungfish [26–28], which are lobe-finned fish that are forerunners of terrestrial vertebrates [29–31]. The key phylogenetic position of lungfish in the transition of vertebrates from water to land [27,29,30,32] and the role of the MR in maintaining internal electrolyte homeostasis [5,8,33,34] motivated us to investigate the response of the slender African lungfish MR to aldosterone, cortisol and other corticosteroids (Figure 1), as well as activation by progestins, which also activate elephant shark MR [24], ray-finned fish MR [35–39] and chicken MR [24,40].

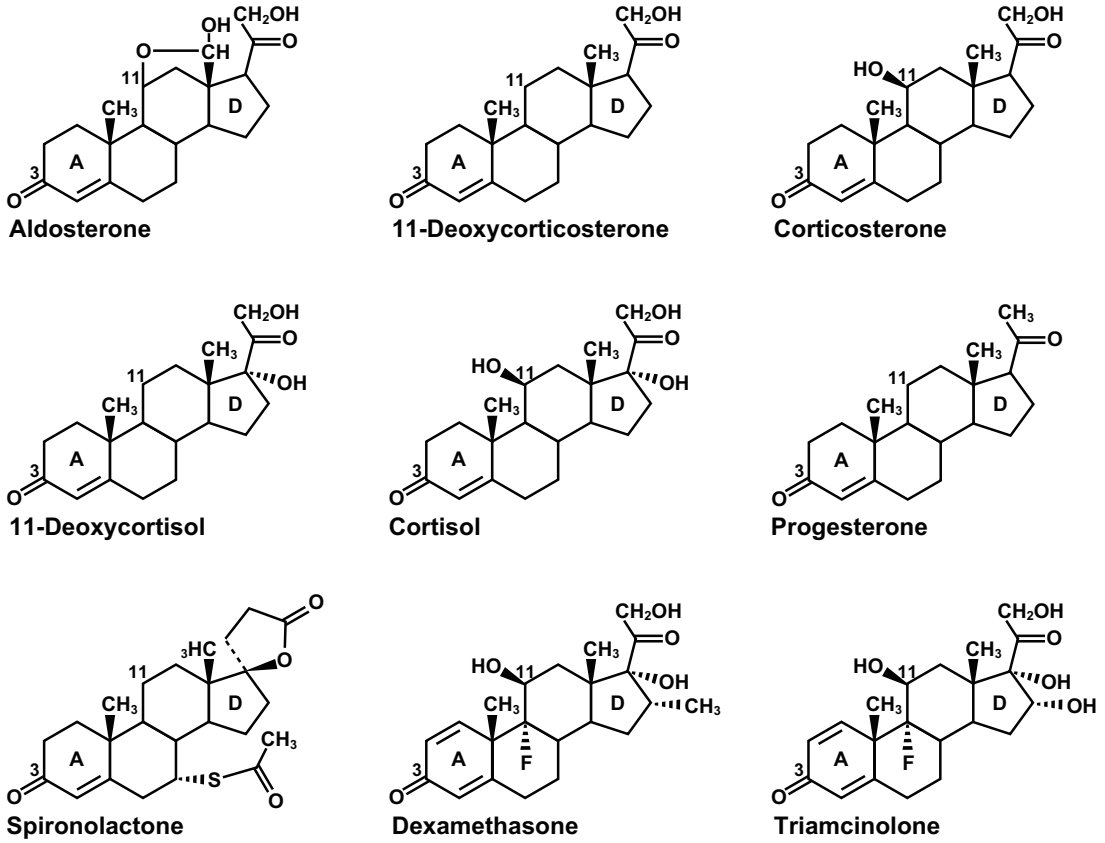

Figure 1. Structures of Corticosteroids, Dexamethasone, Triamcinolone, Progesterone and Spironolactone. Aldosterone and11-deoxycorticosterone are mineralocorticoids [41]. 11-deoxycortisol is a mineralocorticoid in lamprey [42,43]. Cortisol and corticosterone are glucocorticoids in terrestrial vertebrates and ray-finned fish [41,44]. Dexamethasone and

79 triamcinolone are synthetic glucocorticoids. Progesterone is female reproductive steroid that
80 also is important in male physiology [45,46]. Spironolactone is a mineralocorticoid antagonist in
81 humans [47,48].
82
83 Our investigation also uncovered an unexpected role of the N-terminal domain (NTD) of
84 lungfish MR in inhibiting transcriptional activation by steroids. Like other steroid receptors,
85 lungfish MR is a multi-domain protein, consisting of an NTD (domains A and B), a central
86 DNA-binding domain (DBD) (domain C), a hinge domain (D) and a C-terminal ligand-binding
87 domain (LBD) (domain E) [49–51] (Figure 2). The NTD in the human MR contains an
88 activation function domain (AF1), which is split into two segments [49–51]. (Figure 2). As
89 described below, we find that in contrast to human MR [24,49,51,52], the NTD in full-length
90 lungfish MR reduces steroid-mediated activation of lungfish MR, compared to truncated lungfish
91 MR-CDE in cells transfected with a 3X-Tyrosine Amino Transferase (TAT3) promoter [53].
92 We also find that lungfish MR is activated by dexamethasone. At 10 nM, dexamethasone
93 activates full-length lungfish MR and truncated lungfish MR with a signal that is 4-fold and 6-
94 fold stronger, respectively, than that of 10 nM aldosterone. This strong response to
95 dexamethasone and the absence of a lungfish GR sequence after a BLAST search of GenBank
96 leads us to propose that lungfish MR also functions as a GR.
97

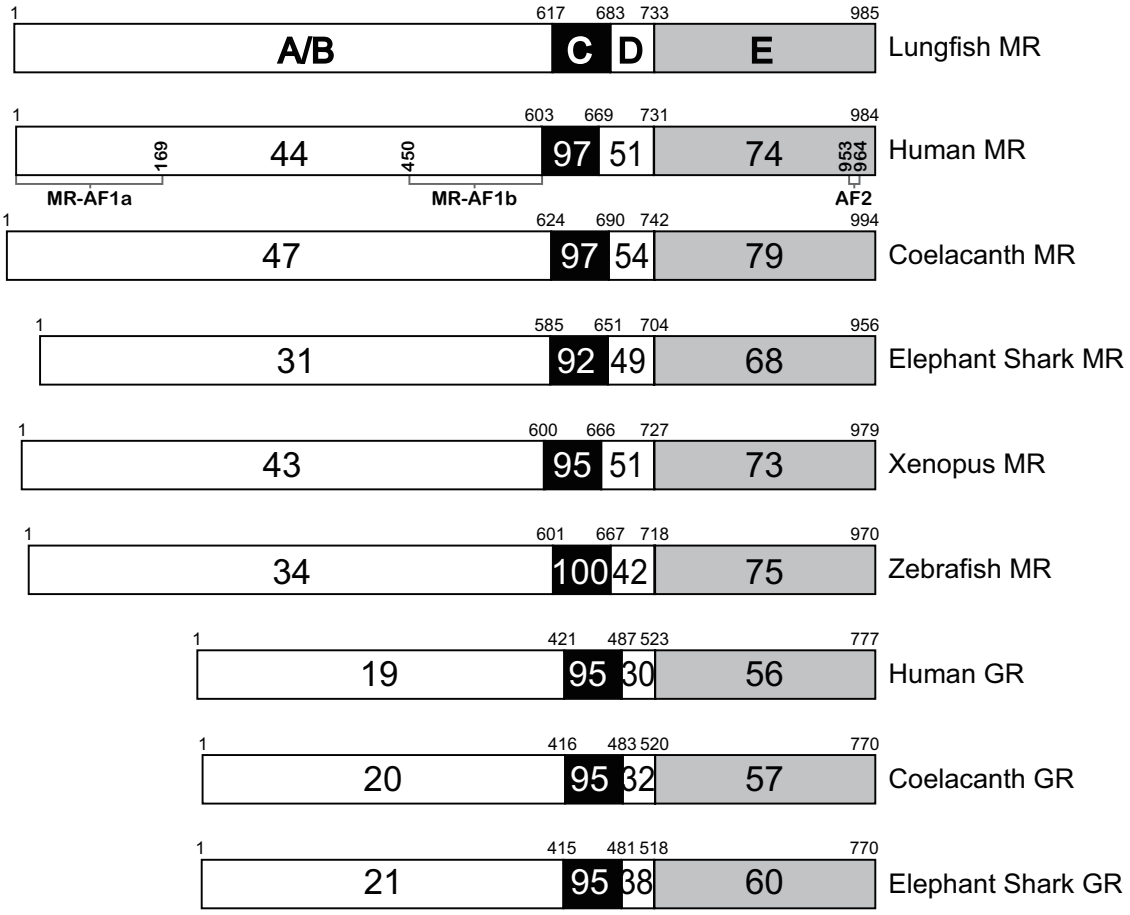

Figure 2. Comparison of the functional domains of lungfish MR to corresponding domains in selected vertebrate MRs (human, coelacanth, elephant shark, *Xenopus*, zebrafish) and GRs (human, coelacanth, elephant shark). Lungfish MR and human MR have 97% and 74% identity in DBD and LBD, respectively. Lungfish MR and elephant shark MR have 92% and 68% identity in DBD and LBD, respectively. This strong conservation of the DBD and LBD contrasts with the low sequence identity of 44% and 47% between their NTDs. There are similar % identities between corresponding domains in lungfish MR and other MRs.

RESULTS

Transcriptional activation of full-length and truncated lungfish MR by corticosteroids, progestins and dexamethasone.

We screened a panel of steroids (Figure 1) at 10 nM for transcriptional activation of full-length and truncated lungfish MR containing the CDE domains (MR-CDE) using two promoters: 2X-Mouse Mammary Tumor Virus (MMTV) [54,55] and TAT3 [53], which along with plasmids for both lungfish MRs were transfected into HEK293 cells.

114 As shown in Figure 3A, there was about 2 to 3-fold activation by 10 nM aldosterone,
115 other corticosteroids or progesterone of full-length lungfish MR using the MMTV-luc reporter
116 and less steroid activation of lungfish MR-CDE (Figure 3B).
117 Interestingly, compared to activation of full-length lungfish MR with the MMTV
118 promoter (Figure 3A), transcriptional activation of full-length lungfish MR with a TAT3
119 promoter and 10 nM aldosterone, other physiological corticosteroids or dexamethasone increased
120 by about 1.5 to 2-fold (Figure 3C). Unexpectedly, lungfish MR-CDE with the TAT3 promoter
121 had an additional 2-fold increase in activation by all corticosteroids (Figure 3D). Progesterone
122 activated lungfish MR in accord with the prediction of Fuller et al. [37,39,56]. Together, these
123 experiments show that removal of the NTD increases corticosteroid and progesterone activation
124 of lungfish MR in the presence of the TAT3 promoter.
125 Our results with dexamethasone, which activates human MR [52,57–59], were
126 unexpected. To our surprise, compared to aldosterone, dexamethasone was about 3-fold and 6-
127 fold more active, respectively, in activating full-length lungfish MR (Figure3C) and truncated
128 lungfish MR (Figure 3D) with the TAT3 promoter. Moreover, both cortisol and corticosterone
129 have stronger fold-activation than does aldosterone of lungfish MR using the TAT3 promoter.
130 Under these conditions, lungfish MR appears to have a GR-like response to steroids.
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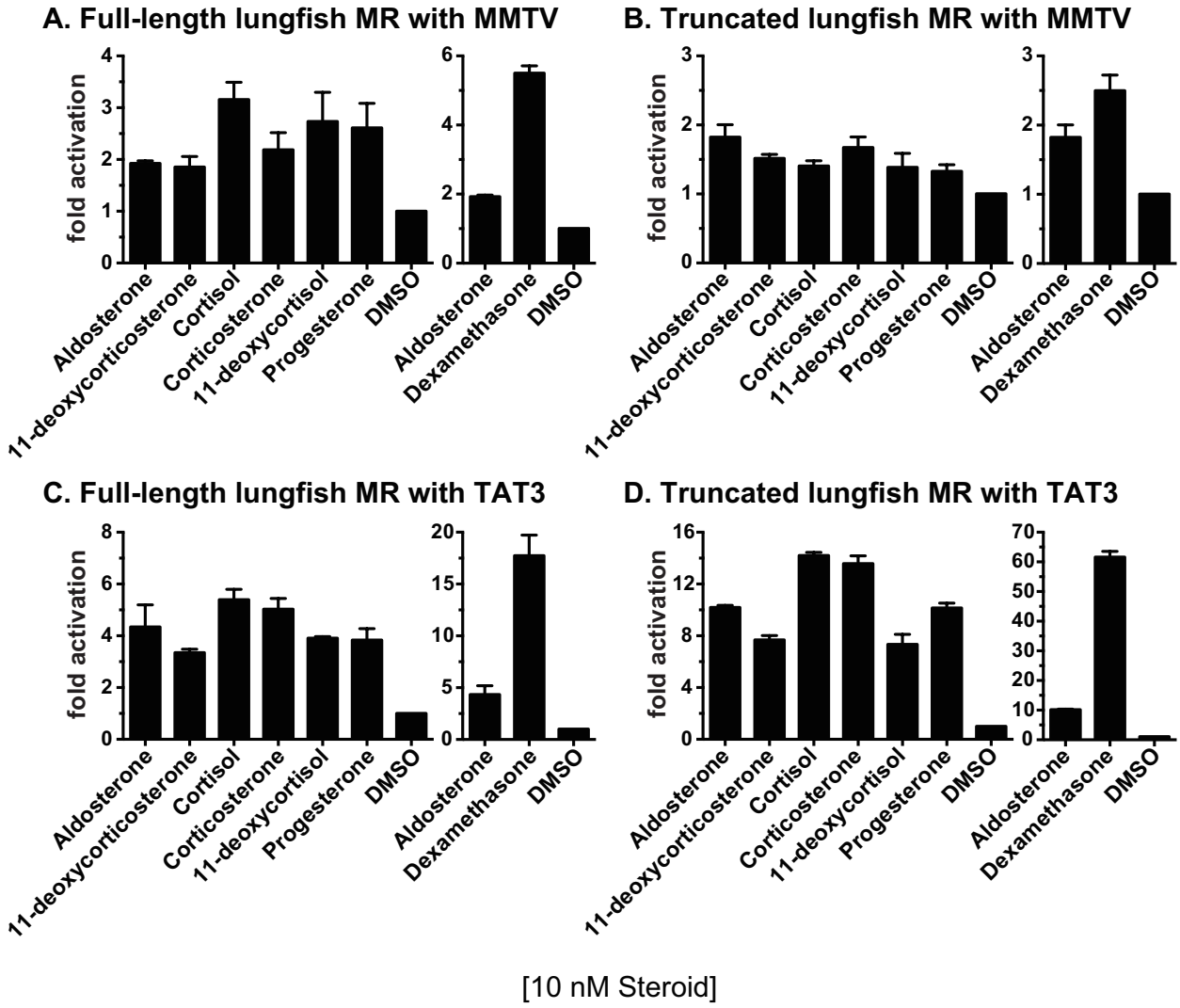

Figure 3. Ligand specificity of full-length and truncated lungfish MR.
Plasmids for full-length lungfish MR or truncated lungfish MR (MR-CDE) were expressed in HEK293 cells with an MMTV-luciferase reporter or a TAT3-luciferase reporter. Transfected cells were treated with either 10 nM aldosterone, cortisol, 11-deoxycortisol, corticosterone, 11-deoxycorticosterone, progesterone, dexamethasone 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 alone as 1. A. Full-length lungfish MR with MMTV-luciferase. B. Truncated lungfish MR (MR-CDE) with MMTV-luciferase. C. Full-length lungfish MR with TAT3-luciferase. D. Truncated lungfish MR (MR-CDE) with TAT3-luciferase.

148 **Spironolactone and eplerenone are transcriptional activators of lungfish MR.**

149 Because spironolactone, an antagonist of human MR, activates elephant shark MR [24],
150 zebrafish MR [37,40,60] and trout MR [38], we investigated spironolactone for activation of full-
151 length lungfish MR and truncated lungfish MR-CDE. We also studied activation by eplerenone,
152 another antagonist of human MR [61]. As shown in Figure 4, both spironolactone and
153 eplerenone activated lungfish MR with a TAT3 promoter, and there was a further increase in
154 fold-activation by both steroids of lungfish MR-CDE.

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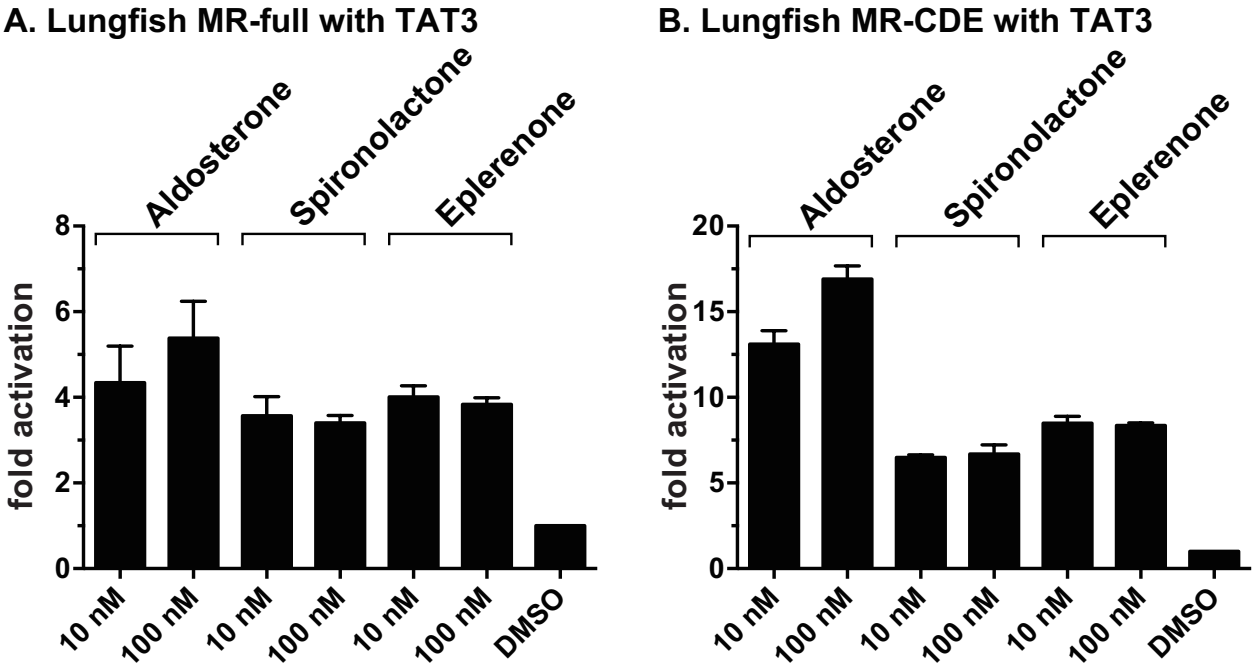

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157 **Figure 4. Spironolactone and eplerenone activation of full-length and truncated lungfish**
158 **MR.** Plasmids for full-length lungfish MR or truncated lungfish MR (MR-CDE) were expressed
159 in HEK293 cells with a TAT3-luciferase reporter. Transfected cells were treated with either 10
160 nM or 100 nM aldosterone, spironolactone or eplerenone or vehicle alone (DMSO). Results are
161 expressed as means ± SEM, n=3. Y-axis indicates fold-activation compared to the activity of
162 control vector with vehicle alone as 1. A. Full-length lungfish MR with TAT3. B. Truncated
163 lungfish MR (MR-CDE) with TAT3.

164

165 **Concentration-dependent activation by corticosteroids and progestins of full-length and**
166 **truncated lungfish MR**.
167 To gain a quantitative measure of corticosteroid and progestin activation of full-length
168 and truncated lungfish MR, we determined the concentration dependence of transcriptional
169 activation by corticosteroids and progestins of full-length lungfish MR and lungfish MR-CDE
170 using TAT3 (Figure 5). This data was used to calculate a half maximal response (EC50) for
171 steroid activation of lungfish MR with a TAT3 promoter (Table 1). For full-length lungfish MR,
172 the four lowest EC50s were for aldosterone (0.04nM), 11-deoxycorticosterone (0.04 nM), 11-
173 deoxycortisol (0.17nM) and progesterone (0.03nM). These low EC50s are consistent with a
174 physiological role for one or more of these steroids as ligand for lungfish MR. In contrast,
175 corticosterone and cortisol, two physiological corticosteroids in terrestrial vertebrates, had EC50s
176 of 23.1nM and 66.1nM, respectively. Two synthetic glucocorticoids, dexamethasone and
177 triamcinolone, had EC50s of 4.7nM and 1.3nM, respectively.
178 For truncated lungfish MR, there were similar low EC50s for aldosterone (0.24nM), 11-
179 deoxycorticosterone (0.013nM), 11-deoxycortisol (0.27nM) and progesterone (0.04nM). EC50s
180 for corticosterone and cortisol were 85.5nM and 86.7nM, respectively. EC50s for
181 dexamethasone and triamcinolone were 7.7nM and 2.4nM, respectively.
182 Overall, these results reveal that the EC50s of aldosterone, 11-deoxycorticosterone, 11-
183 deoxycortisol and progesterone for full-length lungfish MR and lungfish MR-CDE are similar
184 and that one or more of these steroids could be a physiological mineralocorticoid in lungfish.
185 Although EC50s for full-length lungfish MR of triamcinolone and dexamethasone were at least
186 10-fold higher than that of aldosterone, deoxycorticosterone, 11-deoxycortisol and progesterone,
187 compared to these steroids, dexamethasone and triamcinolone have a several fold higher
188 activation of full-length and truncated lungfish MR (Figure 5). Consistent with data in Figure 3,
189 deletion of the NTD to form truncated lungfish MR-CDE increased fold-activation by
190 aldosterone, the other corticosteroids, progesterone, dexamethasone and triamcinolone.
191 However, deletion of the NTD did not have a large effect on their EC50s.
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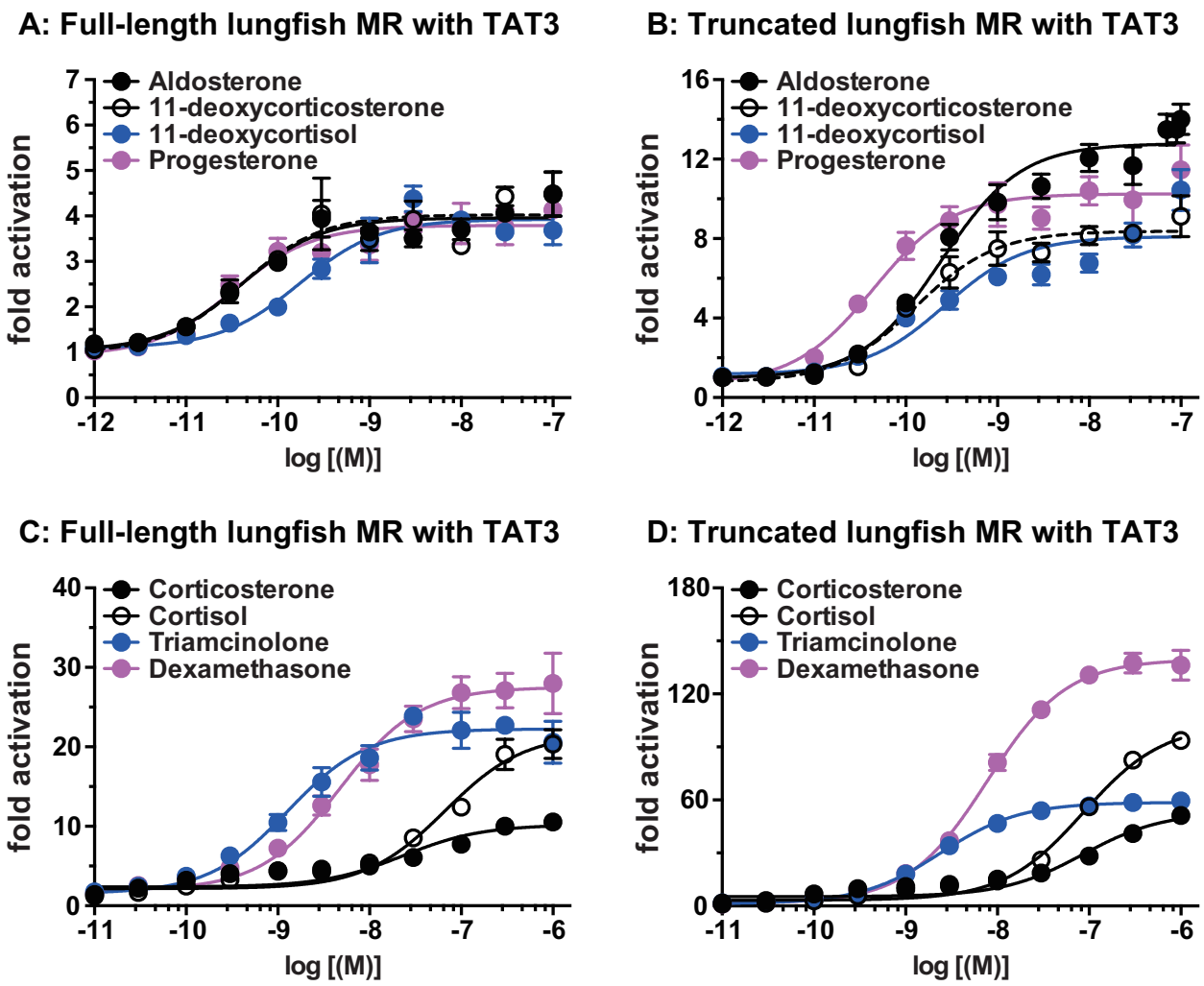

Fig. 5. Concentration-dependent transcriptional activation by corticosteroids, progesterone, dexamethasone and triamcinolone of full length and truncated lungfish MR. Plasmids for full-length lungfish MR or truncated lungfish MR, were expressed in HEK293 cells with a TAT3-luciferase promoter. Cells were treated with increasing concentrations of either aldosterone, cortisol, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol, progesterone, dexamethasone and triamcinolone 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. A. Aldosterone, 11-deoxycorticosterone, 11-deoxycortisol and progesterone with full-length lungfish MR with TAT3-luc. B. Aldosterone, 11-deoxycorticosterone, 11-deoxycortisol and progesterone with truncated lungfish MR (Domains CDE) with TAT3-luc. C. Cortisol, corticosterone, dexamethasone and triamcinolone with full-length lungfish MR with TAT3-luc. D. Cortisol, corticosterone, dexamethasone and triamcinolone with truncated lungfish MR (Domains CDE) with TAT3-luc.

212 **Table 1. EC50 values for steroid activation of full-length and truncated lungfish MR with the TAT3**
213 **promoter.**

	Aldosterone	11-deoxycorticosterone	11-deoxycortisol	Progesterone
	EC50	EC50	EC50	EC50
MR-full length	0.04 nM	0.04 nM	0.17 nM	0.03 nM
95% confidence interval	0.02-0.07 nM	0.02-0.09 nM	0.1-0.3 nM	0.02-0.06 nM
MR-CDE	0.24 nM	0.13 nM	0.27 nM	0.044 nM
95% confidence interval	0.17-0.35 nM	0.08-0.2 nM	0.14-0.53 nM	0.026-0.076 nM

214

	Corticosterone	Cortisol	Triamcinolone	Dexamethasone
	EC50	EC50	EC50	EC50
MR-full length	23.1 nM	66.1 nM	1.3 nM	4.7 nM
95% confidence interval	11.3-47.1 nM	44.5-98.4 nM	0.9-1.9 nM	3.3-6.9 nM
MR-CDE	85.5 nM	86.7 nM	2.4 nM	7.7 nM
95% confidence interval	60.0-121.8 nM	74.3-101.2 nM	2.1-2.8 nM	6.6-9.1 nM

215
216
217 **Transcriptional activation of full-length and truncated human MR and full-length and**
218 **truncated elephant shark MR by corticosteroids and progestins.**

219 To gain an evolutionary perspective on activation of lungfish MR by steroids, we
220 screened a panel of steroids, at 10 nM, for transcriptional activation of full-length human and
221 elephant shark MRs and truncated human and elephant shark MR-CDEs using two reporters:
222 MMTV-luc and TAT3-luc.

223 **Comparison of human MR and lungfish MR.**

224 Overall, compared to lungfish MR, fold activation of human MR was significantly higher
225 for aldosterone and other corticosteroids. For example, compared to 2-fold activation by
226 aldosterone of full-length lungfish MR with the MMTV promoter (Figure 3A), activation of full-
227 length human MR by aldosterone was about 70-fold with the MMTV promoter (Figure 6A).
228 Although fold-activation by steroids for truncated human MR (Figure 6B) decreased compared
229 to full-length human MR (Figure 6A), activation by aldosterone and other corticosteroids of
230 truncated human MR with the MMTV promoter (Figure 6B) was about 7-fold higher than for
231 truncated lungfish MR (Figure 3B).

232 Unlike for lungfish MR, deletion of the NTD in human MR resulted in a loss of
233 activation by aldosterone and other corticosteroids for human MR-CDE with both promoters
234 (Figure 6A-D), consistent with the presence of two activation function domains in the NTD
235 (Figure 2) [49–52]. The relative loss of activation of human MR was greater with the MMTV
236 promoter than with the TAT3 promoter. For example, at 10 nM aldosterone, activation of full-
237 length human MR with the MMTV reporter was 70-fold (Figure 6A), which decreased to 14-fold
238 for human MR-CDE (Figure 6B). In contrast, at 10 nM aldosterone, fold-activation of human
239 MR-CDE with the TAT3 promoter was about 75% of activity for full-length human MR (Figure
240 6C, D). However, 11-deoxycorticosterone and 11-deoxycortisol lost substantial activity for
241 human MR-CDE with the MMTV and TAT3 promoters (Figure 6D).
242 There also was higher fold-activation by aldosterone of full-length and truncated human
243 MR with the TAT3 promoter (Figure 6C, D) compared to full-length and truncated lungfish MR
244 (Figure 3C, D). Aldosterone activation of full-length human MR with the TAT3 promoter
245 (Figure 6C) was about 45-fold higher than that for full-length lungfish MR with the TAT3
246 promoter (Figure 3C). Aldosterone activation of human MR-CDE with the TAT3 promoter
247 (Figure 6D) was about 15-fold higher than that for lungfish MR-CDE (Figure 3D).
248 The relative activation by aldosterone and dexamethasone of human MR and lungfish
249 MR was reversed. Aldosterone was more active than dexamethasone in stimulating transcription
250 by full-length human MR and human MR-CDE with the TAT3 promoter (Figure 6C, D). In
251 contrast, for lungfish MR dexamethasone was more active than aldosterone for full-length
252 lungfish MR and lungfish MR-CDE with the TAT3 promoter (Figure 3C, D).
253
254

255 **Comparison of elephant shark MR and lungfish MR.**

256 Activation by corticosteroids and progesterone of elephant shark MR with the MMTV
257 promoter has some similarities with their activation of lungfish MR. Like lungfish MR,
258 corticosteroids have a similar activation of about 10-fold for full-length and truncated elephant
259 shark MR, with little difference in potency among the corticosteroids. However, unlike lungfish
260 MR, aldosterone is stronger than dexamethasone in activating full-length and truncated elephant
261 shark MR with the MMTV promoter (Figure 6E, F).

262 At a 10 nM steroid concentration, aldosterone and other corticosteroids activated full-
263 length elephant shark MR with the TAT3 promoter by 9 to 12-fold (Figure 6G), which was
264 similar to activation with the MMTV promoter (Figure 6E). Activation of full-length elephant
265 shark MR by progesterone was about 5-fold with the TAT3 and MMTV promoters (Figure 6E,
266 G). Aldosterone was about 2-fold more active than dexamethasone.

267 However, deletion of the NTD from elephant shark MR resulted in a significant increase
268 is activation by steroids in the presence of the TAT3 promoter (Figure 6H). Thus, truncated
269 elephant shark MR with the TAT3 promoter was activated from 300 to 350-fold by aldosterone
270 and other corticosteroids and about 200-fold by progesterone and dexamethasone (Figure 6H),
271 indicating that like lungfish MR, the NTD in elephant shark inhibits activation by
272 corticosteroids. However, unlike lungfish MR, compared to aldosterone, dexamethasone was
273 less active for full-length and truncated elephant shark MR with the MMTV promoter and
274 truncated elephant shark MR with the TAT3 promoter.

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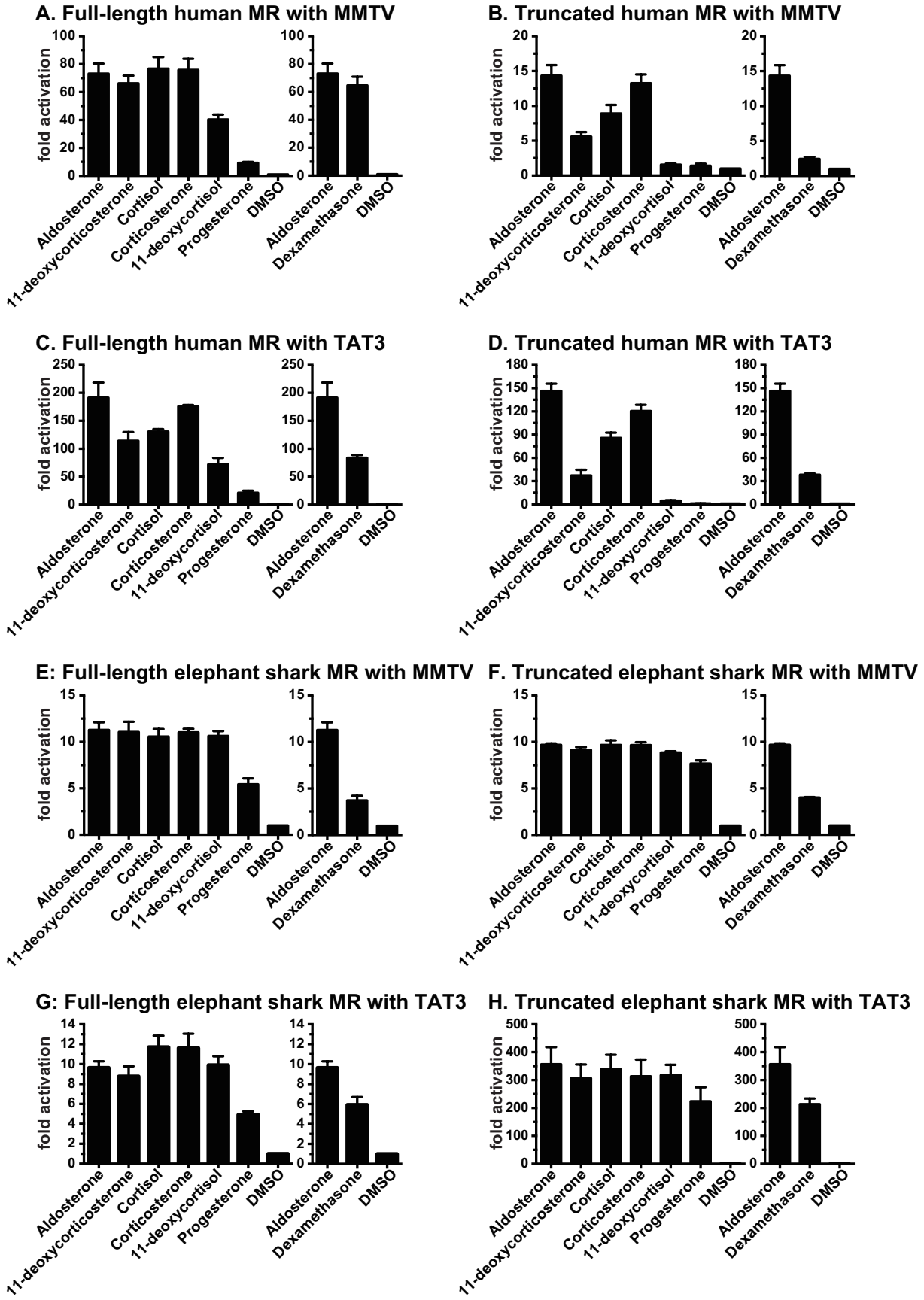
A. Full-length human MR with MMTV
B. Truncated human MR with MMTV
C. Full-length human MR with TAT3
D. Truncated human MR with TAT3
E: Full-length elephant shark MR with MMTV
F. Truncated elephant shark MR with MMTV
G: Full-length elephant shark MR with TAT3
H. Truncated elephant shark MR with TAT3
fold activation
Aldosterone
11-deoxycorticosterone
Cortisol
Corticosterone
11-deoxycortisol
Progesterone
DMSO
Aldosterone
Dexamethasone
DMSO

277

278 **Figure 6. Ligand specificity of full-length and truncated human MR and elephant shark**
279 **MR.** Plasmids for full-length human and elephant shark MR or truncated human and elephant
280 shark MR (MR-CDE) were expressed in HEK293 cells with an MMTV-luciferase reporter or a
281 TAT3-luciferase reporter. Transfected cells were treated with either 10 nM aldosterone, cortisol,
282 11-deoxycortisol, corticosterone, 11-deoxycorticosterone, progesterone, dexamethasone or
283 vehicle alone (DMSO). Results are expressed as means ± SEM, n=3. Y-axis indicates fold-
284 activation compared to the activity of control vector with vehicle alone as 1. A. Full-length
285 human MR with MMTV-luciferase. B. Truncated human MR (MR-CDE) with MMTV-
286 luciferase. C. Full-length elephant shark MR with TAT3-luciferase. D. Truncated elephant
287 shark MR (MR-CDE) with TAT3-luciferase.
288

289 **Does lungfish contain a separate GR gene?**

290 We used sequences of human GR, coelacanth GR and elephant shark GR as probes in a
291 BLAST search of GenBank and did not retrieve a lungfish GR sequence. The absence of a
292 lungfish GR ortholog coupled with the strong response of lungfish MR to dexamethasone and
293 triamcinolone (Figure 5) leads us to propose that lungfish MR also functions as a GR.
294

295 **Discussion**

296 Dobzhansky's aphorism "Nothing in Biology Makes Sense Except in the Light of
297 Evolution" [62] explains the importance of the evolution of aldosterone in lungfish because
298 aldosterone activation of the kidney MR in terrestrial vertebrates regulates sodium, potassium
299 and water transport, which is critical in maintaining internal electrolyte homeostasis in terrestrial
300 vertebrates [30,34,63–65] an activity that was important in the transition from water to land.
301 Here we report that aldosterone, 11-deoxycorticosterone, and progesterone have EC50s below 1
302 nM for lungfish MR (Table 1), which makes these steroids potential physiological ligands for
303 lungfish MR. Another potential physiological steroid is 11-deoxycortisol, which is a steroid for

304 the CR in Atlantic sea lamprey [42,43]. 11-deoxycortisol has EC50 of 0.17 nM for full-length
305 lungfish MR (Table 1).
306 A functional advantage of 11-deoxycorticosterone, 11-deoxycortisol and progesterone as
307 ligands for the MR is that they lack an 11β-hydroxyl group, and thus, like aldosterone, they are
308 inert to 11β-hydroxysteroid dehydrogenase-type 2, unlike cortisol and corticosterone [66–69].
309 Indeed, this inertness to 11β-hydroxysteroid dehydrogenase-type 2 and the low EC50s of these
310 steroids for lungfish MR suggests that more than one corticosteroid and progesterone [39] may
311 be physiological mineralocorticoids.
312 Like ray-finned fish MRs [24,37,38,60] and elephant shark MR [24,39], lungfish MR is
313 activated by spironolactone (Figure 4), and, as reported here, by eplerenone [47,48,61].
314 We also find important differences between the response of lungfish MR and human MR
315 to aldosterone, 11-deoxycorticosterone, 11-deoxycortisol and progesterone, indicating that
316 further selectivity for aldosterone in human MR occurred during the evolution of terrestrial
317 vertebrates [13,15,37,40,52,69,70].
318 An unexpected difference between lungfish MR and human MR is the substantial
319 increase in fold-activation by steroids of lungfish MR after deletion of the NTD, in contrast to
320 human MR in which the NTD contains an activation function domain (Figure 2) [49–52].
321 Deletion of the NTD in elephant shark MR also resulted in a substantial increase in fold-
322 activation by corticosteroids and progesterone using the TAT3 promoter (Figure 6), but not for
323 the MMTV promoter [17]. These data with lungfish MR and elephant shark MR suggest that
324 early in the evolution of the MR there was an allosteric interaction between the LBD and NTD
325 [71,72] that repressed steroid activation of the MR, and that the activation function in the NTD
326 as found in human MR [49–52] evolved later in terrestrial vertebrates, along with changes in

327 steroid specificity, such loss of MR activation by progesterone [37,39,70]. The different
328 responses of full-length and truncated lungfish MR, human MR and elephant shark MR with the
329 MMTV and TAT3 promoters indicate that the NTD and the promoter are important regulators of
330 steroid activation of these MRs. Corticosteroid activation of these MRs in the presence of other
331 promoters merits investigation.

332 The stronger response of lungfish MR to dexamethasone compared to aldosterone and the
333 absence a lungfish GR ortholog sequence are puzzling. At a 10 nM concentration, fold-
334 activation by dexamethasone and triamcinolone is substantially higher than that of cortisol,
335 corticosterone, as well as aldosterone, 11-deoxycorticosterone, 11-deoxycortisol and
336 progesterone for lungfish MR (Figure 5). One explanation is that lungfish MR also has a GR
337 function.

338

339 **Materials and Methods**

340 **Chemical reagents**

341 Aldosterone, cortisol, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol and
342 progesterone, spironolactone and eplerenone were purchased from Sigma-Aldrich. For reporter
343 gene assays, all hormones were dissolved in dimethyl-sulfoxide (DMSO); the final DMSO
344 concentration in the culture medium did not exceed 0.1%.

345

346 **Animal**

347 A slender spotted African lungfish, *Protopterus dolloi,* was purchased from a local
348 commercial supplier. Lungfish were anesthetized in freshwater containing 0.02% ethyl 3-
349 aminobenzoate methane-sulfonate from Sigma-Aldrich, and tissue samples were quickly

350 dissected and frozen in liquid nitrogen. Animal handling procedures conformed to the guidelines
351 set forth by the Institutional Animal Care and Use Committee at the University of Tokyo.
352
353 **Molecular cloning of lungfish mineralocorticoid receptor**
354 Two conserved amino acid regions, GCHYGV and LYFAPD of vertebrate MRs were
355 selected and degenerate oligonucleotides were used as primers for PCR. First-strand cDNA was
356 synthesized from 2 μg of total RNA isolated from the liver after amplification, and an additional
357 primer set (CKVFFK and LYFAPD) was used for the second PCR. The amplified DNA
358 fragments were subcloned with TA-cloning plasmid pGEM-T Easy vector, sequenced using a
359 BigDye terminator Cycle Sequencing-kit with T7 and SP6 primers, and analyzed on the 3130
360 Genetic Analyzer (Applied Biosystems). The 5'- and 3'-ends of the mineralocorticoid receptor
361 cDNAs were amplified by rapid amplification of the cDNA end (RACE) using a SMART RACE
362 cDNA Amplification kit. Genbank accessions for this lungfish MR are: Nucleotide ID:
363 LC630795 and Protein ID: BCV19931.
364 **Construction of plasmid vectors**
365 The full-length and truncated MRs were amplified by PCR with KOD DNA polymerase. The
366 PCR products were gel-purified and ligated into pcDNA3.1 vector (Invitrogen). The truncated
367 MR proteins were designed to possess methionine and valine residues at the N-terminus and
368 contain a DNA-binding domain, a hinge-region, and a ligand-binding domain. The truncated
369 MRs were amplified by PCR with KOD DNA polymerase by using the following primers:
370 lungfish MR forward primer (5'-
371 C<u>AAGCTT</u>ACCATGGTGTGTCTGGTGTGTGGTGACGAAG-3' containing *Hind*III site) and
372 lungfish MR reverse primer (5'-CCTACTTCCTGTGAAAGTACAATGAC -3' containing stop

373 codon), human MR forward primer (5'-

374 CGGATCCACCATGGTGTGTTTGGTGTGTGGGGATGAG-3' containing *Bam*HI site) and

375 human MR reverse primer (5'-CTCACTTCCGGTGGAAGTAGAGCGGC -3' containing stop

376 codon). The amplified DNA fragments were subcloned with TA-cloning plasmid pGEM-T Easy

377 vector and sequenced, and then subcloned into pcDNA 3.1 vector by using *Hind*III-*Not*I sites for

378 lungfish MR truncated form or *Bam*HI-*Not*I sites for human MR truncated form. Mouse

379 mammary tumor virus-long terminal repeat (MMTV-LTR) was amplified from pMSG vector by

380 PCR, and inserted into pGL3-basic vector containing the *Photinus pyralis* lucifease gene. 3X-

381 Tyrosine Amino Transferase (TAT3) promoter containing reporter vector named pGL4.23-

382 TAT3-Luc was constructed as described previously [53]. All cloned DNA sequences were

383 verified by sequencing.

384

385 **Transactivation assay and statistical methods**

386 Transfection and reporter assays were carried out in HEK293 cells, as described previously

387 [40,73]. All experiments were performed in triplicate. The values shown are mean ± SEM from

388 three separate experiments, and dose-response data, which were used to calculate the half

389 maximal response (EC50) for each steroid, were analyzed using GraphPad Prism.

390 **DECLARATION OF INTEREST**

391 We have no conflict of interest.

392

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396

397 **AUTHOR CONTRIBUTIONS**

398 Y.K., S.O., and M.E.B. carried out the research and analyzed data. S.H. aided in the collection
399 of animals. X.L. constructed plasmid DNAs used in this study. Y.K. and M.E.B. conceived and
400 designed the experiments. Y.K. and M.E.B. wrote the paper. All authors gave final approval for
401 publication.

402

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