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18 **Abstract**

19

20 Many teleost fish possess two glucocorticoid receptors (GR). In the rainbow trout rtGR1 and
21 rtGR2 differ in their affinities to dexamethasone and EC50 values for glucocorticoids in
22 transactivation assays, with rtGR2 being more sensitive. The objective of this study was to
23 identify the molecular traits underlying the sensitivity difference. Domain-swap mutants
24 between rtGR1 and rtGR2 showed that sensitivity was mainly determined by the hormone
25 binding domain (E-domain). Chimeras exchanging three E-domain subregions indicated that
26 all subregions influenced sensitivity, with the most C-terminal region that included AF2
27 having the greatest (12.6-fold) effects on cortisol transactivation EC50. The C-terminal
28 extremity (CTE) in rtGR1 departs from a consensus preserved in other GRs. Introducing the
29 consensus CTE into rtGR1 provoked a 4.2-fold decrease in transactivation EC50,
30 suggesting CTE is one of several determinants of rtGR1's hyposensitivity. GRs with similar
31 unusual CTEs exist in other salmonids, suggesting hyposensitive GR have evolved in this
32 highly successful teleost lineage.

33

34

35 **Keywords:** Steroid receptors, Teleost Fish, Cortisol, Stress response, Activation Function 2,
36 ligand binding domain

37

38

39 1. Introduction

40

41 Glucocorticoids are steroid hormones produced by the adrenal cortex, or the adrenal
42 analogue of teleost fish, the interrenal cells of the head kidney (Bern, 1976). Glucocorticoids
43 have multiple and complex functions, including roles in the regulation of glucose metabolism,
44 growth, development, central nervous function and the immune response (Schoneveld et al.,
45 2004 ; Charmandari et al., 2005). The majority of glucocorticoid effects occur on the
46 transcriptional level and are mediated through the glucocorticoid receptor (GR) (Bamberger
47 et al., 1996; Schoneveld et al 2004). In the absence of hormonal ligands, mammalian GR
48 resides in the cytosol as part of a hetero-oligomeric complex that includes a dimer of heat
49 shock protein (hsp) 90, one co-chaperone p23, and one tetratricopeptide repeat protein
50 (Pratt and Toft, 1997; Heitzer et al., 2007). However, in the teleost rainbow trout unliganded
51 GRs show a subcellular localisation that is both cytosolic and nuclear (Becker et al., 2008).
52 Binding of glucocorticoids facilitates GR dissociation from this complex and provokes the
53 nuclear translocation of the receptor (Schoneveld et al., 2004; Heitzer et al., 2007). In the
54 nucleus, GR can affect transcription of target genes in various ways, including binding to
55 regulatory DNA sequences, and engaging in protein-protein interactions with other
56 transcription factors (Bamberger et al., 1996; Schoneveld et al 2004). Most commonly,
57 homodimers of GR bind to imperfectly palindromic 15-nucleotide sequences called
58 glucocorticoid response elements, which are found in the regulatory regions of glucocorticoid
59 target genes (Bamberger et al., 1996).

60

61 Functional and structural domains have been mapped to specific regions of GR in human
62 and rat. The N-terminal A/B domain of GR contains the receptor's main transactivation
63 function named $\tau 1$ (also called enh2 or AF1) (Giguere et al., 1986; Godowski e tal 1988).
64 The central C domain consists of two zinc fingers involved in GR homodimerisation and DNA
65 binding (Hard et al., 1990), while the C-terminal E-domain functions in ligand binding and
66 contains a second, hormone-dependent activation function called AF2 (Danieleian et al.,

67 1992; Kucera et al., 2002). At the C-terminal end of the E-domain, a further region with roles
68 in transactivation has been identified and was termed $\tau 2$ (Giguere et al 1986; Milhon et al.,
69 1997). The crystal structure of the ligand-binding domain of GR has been elucidated when
70 bound to the synthetic glucocorticoid dexamethasone and a coactivator motif (Bledsoe et al
71 2002), leading to the identification of amino acids involved in ligand binding.

72

73 In contrast to tetrapods, which possess one GR gene only, many teleost fish have two GRs
74 encoded by distinct genes (Ducouret et al., 1995; Bury et al., 2003; Greenwood et al., 2003;
75 Stolte et al., 2008a). Phylogenetic analyses have suggested a common origin of the
76 duplication of GR in a number of teleosts (Bury et al., 2003; Stolte et al 2008a), which may
77 have occurred in the context of a whole-genome duplication that is believed to have taken
78 place in the actinopterygian (ray-finned fish) lineage about 300 Mya (Jaillon et al., 2004), i.e.
79 before the emergence of teleosts (248-213 Mya) but after the divergence of sarcopterygians
80 (lobed finned fish including tetrapods) 420 Mya. The retention of duplicated GR genes in
81 different teleosts, including rainbow trout, Burton's mouthbrooder, pufferfish, green puffer,
82 carp, medaka and stickleback, suggests that duplicated teleosts GRs are not redundant, but
83 have acquired distinct functions by undergoing neo- or subfunctionalisation. Indeed, where
84 data are available, duplicated teleost GRs have shown significant differences in hormone
85 sensitivity and/or tissue expression (Bury et al., 2003; Greenwood et al., 2003; Becker et al
86 2008; Stolte et al., 2008a and b). In rainbow trout, sensitivity differences in receptor
87 transactivation were particularly pronounced, with cortisol having an EC₅₀ of 46 nM in rtGR1
88 and of 0.72 nM in rtGR2 in transactivation assays in the hamster cell line CHO (Bury et al.,
89 2003).

90

91 The objective of this study was to identify the molecular determinants of the sensitivity
92 difference between rtGR1 and rtGR2. In addition, previous studies assessing teleost GR
93 transactivational activities have all been performed in mammalian cell lines and because of
94 the possibility that the receptor co-activator profiles differ between mammalian and fish cells

95 the physiological significance of these results have been questioned. Consequently, a
96 second objective was to assess the transactivational activities of rtGR1 and rtGR2 in a fish
97 cell line. Understanding why the two trout GRs differ in hormone sensitivity, despite their
98 high sequence identity in the central and C-terminal regions of the protein (Bury et al 2003),
99 could help predict the behaviour of duplicated GRs in other teleost species. Moreover, the
100 identification of molecular traits of GR determining hormone sensitivity could further improve
101 the understanding of rare medical conditions based on GR mutations affecting hormone
102 sensitivity, such as generalised glucocorticoid resistance and hypersensitivity (Charmanadari
103 et al 2005 and 2008). In order to unravel the molecular basis of the differential sensitivity of
104 the two trout GR receptors, we created a number of recombinant GRs, either by exchanging
105 homologous regions between rtGR1 and rtGR2, or by introducing single or multiple amino
106 acid mutations into the context of rtGR1 or rtGR2. The resulting mutants were tested in a
107 transactivation assay and dexamethasone binding affinities determined. The results revealed
108 that the E-domain of the receptors was the main determinant of sensitivity, and more
109 specifically, the unusual C-terminal extremity of rtGR1, which departs from a consensus
110 among other known GRs, is important for receptor hyposensitivity.

111 2. Materials and Methods

112

113 2.1 Plasmid constructs

114 Expression constructs containing cDNAs of rtGR1 (originally called rtGR) and rtGR2 were
115 available from previous studies (Ducouret et al., 1995; Bury et al., 2003). Using appropriate
116 enzymes, rtGR1 and rtGR2 cDNAs were subcloned into pcDNA3 (Invitrogen) to yield
117 constructs pcrtGR1 and pcrtGR2. Domain swap mutants between rtGR1 and rtGR2 were
118 produced using a two-step PCR protocol based on overlap extension (Sambrook and
119 Russell, 2001) and subcloned into pcDNA3. GRs with single and double amino acid
120 mutations were generated using mutagenic primers and a kit (QuikChange II, Stratagene).
121 Domains were defined as follows: A/B, rtGR1: amino acids 1–386, rtGR2: 1–306; C, rtGR1:
122 387–463, rtGR2: 307–379; D, rtGR1: 464–502, rtGR2: 380–419; E, rtGR1: 503–758, rtGR2:
123 420–669.

124

125 2.2 Cell culture, transactivation and binding assays.

126 COS-7 cells derived from African green monkey kidney and lacking endogenous expression
127 of functional GR were maintained and transfected using the calcium phosphate method as
128 described previously (Becker et al., 2008), or using a modification of this method for 96-well
129 plates (see below). During and after transfection, cells were maintained in Dulbecco's
130 modified Eagle medium (DMEM) / nutrient mix F-12 Ham (Sigma, Poole, UK) containing 2
131 mM glutamine, 3.7 g/l NaHCO₃, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2.5%
132 denatured, dextran/charcoal-treated FCS (DMEM/F12). Per 10 cm² of culture dish surface
133 area, 2.5 µg GR cDNA, 25 µg luciferase reporter pFC31Luc (Gouilleux et al 1991) containing
134 the murine mammary tumour virus (MMTV) promoter, 5 µg control reporter pSVβ (Clontech,
135 Palo Alto, CA), and 21 µg irrelevant DNA (pBluescript SK) were used. In the modified (96-
136 well) method, COS-7 cells were seeded into 6-well plates and transfected. The next
137 morning, cells were trypsinised, resuspended in DMEM/F12, and seeded into the wells of a

138 96-well plate while maintaining the same cell number-to-surface ratio. Cells were allowed to
139 attach for 4 h before starting hormone treatment.

140

141 FHM cells derived from fathead minnow were maintained in DMEM supplemented with 2 mM
142 glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% denatured FCS at 22° C in
143 a humidified atmosphere with 4% CO₂. FHM cells were seeded into 96-well plates (6 x 10⁵
144 cells /well) and transfected with plasmid DNA with the help of lipofectamine 2000
145 (Invitrogen), following the manufacturer's instructions. Per well, 160 ng of pFC31Luc, 30 ng
146 of GR expression plasmid, and 30 ng of control reporter construct pGL4.75 (Promega) were
147 used. After transfection, cells were maintained in the above media in which the FCS was
148 replaced by charcoal/dextran treated, denatured FCS. Hormone treatment started 12 h after
149 transfection and continued for 36 h, after which cells were harvested using a commercial
150 lysis reagent (Promega). Luciferase activities were determined using a modification of a
151 published method (Dyer et al 2000). To measure firefly luciferase activities (FHM and COS-
152 7), 75 µl of cell lysate and 75 µl of reagent one (50 mM TRIS/phosphoric acid pH 8.0, 33 mM
153 dithiothreitol, 4 mM MgSO₄, 2 mM EDTA, 1 mM ATP, 0.2 mM coenzyme A, 0.38 mM beetle
154 luciferin) were added to a black microtitre plate and luciferase activity monitored in a
155 luminometre. In assays with FHM-cells, activity of the control reporter *Renilla* luciferase was
156 determined by adding 75 µl per well of reagent two (0.5 M sodium chloride, 100 mM sodium
157 phosphate (pH 5.1), 4 mM EDTA, 0.33 mg/ml bovine serum albumin, 0.67 mM sodium azide
158 and 1 µM coelentraxine) to the same plate, which was re-read in the luminometre after 10
159 min incubation. β-Galactosidase activities (control reporter in assays with COS-7) were
160 measured as described before (Sturm et al., 2010).

161

162 All experiments were performed using triplicates and repeated at least three times. For each
163 receptor, a solvent control (ethanol) and an optimal glucocorticoid treatment (10⁻⁷ M
164 dexamethasone or 10⁻⁶ M cortisol) were included. In further controls using empty vector
165 instead of receptor plasmid, luciferase activities were negligible regardless of hormone

166 treatment (data not shown). Luciferase activities were divided by β -galactosidase or *Renilla*
167 luciferase activities to correct for differences in transfection efficiency. Luciferase activities of
168 a given receptor/hormone combination were then expressed as percent of maximum
169 activities. In the usual case, activities with the optimal glucocorticoid treatment were the
170 highest in the data set and set to 100%. However, if a lower hormone concentration showed
171 a higher activity, the average of this activity and all activities at higher concentrations was set
172 to 100%. To derive median effective concentrations (EC₅₀), a logistic model was fitted to the
173 transactivation response data for each receptor, versus log concentration [*S*] in, assuming a
174 maximum value of 1 (100%), and a minimum of zero. Assuming no differences between
175 experiments for each receptor, two parameters were to be fitted: a curve-steepness
176 parameter *b* and the EC₅₀ value. The model was:

$$177 \quad B_i = \frac{1}{1 + \exp(-b(\log_{10}[S_i] - \log_{10}EC_{50}))} + \varepsilon_i$$

178
179 The model was fitted for each receptor using a non-linear least-squares curve fitting
180 procedure in the R modelling environment (<http://www.r-project.org/>). The error term ε was
181 assumed to be normally distributed for each data point *i*. 95% confidence intervals for EC₅₀
182 were obtained using the R *confint* function, and are asymmetric due to the transformation of
183 concentration data.

184
185 [³H]-dexamethasone [6,7-³H(N), Perkin-Elmer) binding assays followed the protocols were
186 carried out using a whole cell assay with COS-7 cells transiently expressing the receptors
187 under investigation, as described in detail in Sturm et al., (2010).

188

189

190 **3. Results**

191

192 Median effective concentrations (EC₅₀s) of glucocorticoids in transient transfection assays
193 monitoring the hormonal stimulation of transactivation activity on the MMTV promoter are
194 lower for rtGR2 than for rtGR1 in two mammalian cell lines lacking endogenous functional
195 corticosteroid receptors (CHO derived from chinese hamster ovary, and COS-7 derived from
196 monkey kidney (Bury et al., 2003)). Glucocorticoid EC₅₀ values of the two receptors further
197 differ in transactivation assays with other promoters and in a transrepression assay (Bury
198 and Sturm et al 2007). However, a caveat regarding these finding is that they were obtained
199 with mammalian cell lines that might lack co-factors required for the optimal function of
200 teleost hormone receptors. Therefore, we investigated the hormone dependency of
201 transactivation activity of rtGR1 and rtGR2 in transient transfection assays using the teleost
202 cell line FHM, derived from fathead minnow. In FHM cells, cortisol EC₅₀ values were
203 significantly higher in rtGR1 (16.9 nM [95% confidence limits: 12.1-23.5]) than in rtGR2 (3.6
204 nM [2.4-5.4]) (Fig. 1A). Similar results were obtained with dexamethasone (EC₅₀ values:
205 rtGR1 3.1 nM [2.2-4.2]; rtGR2 0.89 nM [0.57-1.33]) (Data not shown).

206

207 The binding of ³H-dexamethasone to rtGR1 and rtGR2 was studied using a whole cell assay
208 with COS-7 transiently transfected with rtGR1 or rtGR2 (Sturm et al., 2010). Apparent
209 affinities were expressed as the dissociation constant K_d (rtGR1: 30.4 nM, rtGR2 8.6 nM)
210 (Table 1).

211

212 In a transactivation assay in COS-7 cells established previously (Bury et al., 2003; Sturm et
213 al., 2005), rtGR1 and rtGR2 showed a more marked sensitivity difference with cortisol (28.8-
214 fold) (Fig. 1C) and dexamethasone (12.6-fold, Fig. 2). This difference is far greater than that
215 observed in FHM cells (4.7-fold for cortisol and 3.5-fold for dexamethasone). Moreover,
216 much higher induction rates of transactivation activity were found in COS-7 (>100-fold) than
217 in FHM (~15-fold) (Fig. 1B), with maximally induced transactivation activity in COS-7 being

218 higher for rtGR1 than for rtGR2 (Fig. 1D), despite both receptors being expressed at similar
219 levels, as determined by there being no significant difference in B_{max} in the binding studies
220 (Table 1). In contrast, similar maximum activities were observed with both receptors in FHM
221 cells (Fig. 1B).

222

223 To identify receptor regions affecting glucocorticoid sensitivity, we generated a series of GR
224 chimeras that combined the N-terminus of either rtGR1 or rtGR2 with the C-terminus of the
225 other receptor, with different switching points coinciding with boundaries of main receptor
226 domains (Fig. 2A). The transactivation properties of mutants were assessed using the
227 transient transfection assay with COS-7 cells, which because of the significantly higher
228 induction rates offers a better signal-to-noise ratio than the assay in FHM cells (Fig. 1C, D),
229 and further possessed the advantage of greater difference between the EC50s of the two
230 receptors (Fig. 1C). Transactivation by all mutants was stimulated by cortisol and
231 dexamethasone in a dose-dependent fashion, confirming that the chimeras were functional
232 (Fig. 2B and C). Recombinant receptors in which the N-terminus of rtGR2 was combined
233 with the E-domain of rtGR1 showed cortisol EC50 values similar to that of rtGR1 (Fig. 2B),
234 while the converse mutants, which combined the rtGR1 N-terminus with the E-domain of
235 rtGR2, resembled rtGR2 in sensitivity (Fig. 2B). Identical trends were observed with
236 dexamethasone (Fig. 2B). Maximum transactivation activities of mutants after treatment with
237 optimal cortisol or dexamethasone levels varied between those observed for rtGR1 and
238 rtGR2 with no a discernible correlation to the domain origin of mutants (data not shown).
239 Dexamethasone binding assays revealed that mutant ABCD(GR1)E(GR2) resembled rtGR2
240 in glucocorticoid affinity, while ABCD(GR2)E(GR1) resembled rtGR1, as apparent from
241 similar K_d values (Table 1).

242

243 Together, these transactivation and hormone binding results from domain-swap mutants
244 (Fig. 2, Table 1) suggested a main role of the E-domain in the glucocorticoid sensitivity of
245 receptors. Further experiments sought to identify subregions of the E-domain, affecting the

246 hormone sensitivity of receptors. The E-domain is highly similar between rtGR1 and rtGR2
247 (89% amino acid identity), and between the human GR (hGR) and either trout GR (>70%
248 amino acid identity, Fig. 3). To elucidate which subregions of the E-domain might play a role
249 in determining receptor sensitivity to glucocorticoids, homologous E-domain regions were
250 exchanged between receptors, paralleling a mutational strategy that has been used to
251 unravel determinants of hormone specificity of MR and GR (Rogerson et al., 1999). For the
252 design of mutants, three E-domain subregions were defined, the first consisting of the $\tau 2$
253 region (Milhon et al 1997), the second of the middle portion of the E domain ranging from
254 helices 3 to the middle of helix 10, and the third comprising the AF2 region (Kucera et al.,
255 2002) and the C-terminus of the receptor (Fig. 3). Recombinant GRs were generated, in
256 which above E-domain region 3, or combined regions 2 and 3, were exchanged between
257 rtGR1 and rtGR2 (Fig. 4A). All chimeras showed a dose-dependent stimulation of
258 transactivation by glucocorticoids, with maximal activities between those of rtGR1 and rtGR2
259 (data not shown). Transactivation EC50s for cortisol and dexamethasone (Fig. 4B) were
260 compared between wild type receptors, mutants exchanging parts of the E-domain, and
261 previous mutants exchanging the entire E-domain. Results are arranged in two series of
262 receptors. In series one, consisting of rtGR1, AB-t2-M(GR1)AF2-CT(GR2), AB-t2(GR1) M-
263 AF2(GR2), and ABCD(GR1)E(GR2), the E-domain in rtGR1 is successively replaced with
264 that of rtGR2 (Fig. 4A), which coincided with progressive increases of sensitivity (Fig. 4B). In
265 series two, comprised of rtGR2, AB-t2-M(GR2)AF2-CT(GR1), AB-t2(GR2) M-AF2(GR1), and
266 ABCD(GR2)E(GR1), the gradual substitution of the rtGR2 E-domain with that of rtGR1 lead
267 to incremental losses of sensitivity (Fig. 4A, B).

268

269 The dimension of the effect of exchanging a particular E-domain region depended on the
270 glucocorticoid and the receptor context. In series one, the differences in dexamethasone
271 sensitivity between the individual receptors suggested about similar effects of all subregions
272 (2.2- to 3.0-fold shifts of EC50) (Fig. 4B). By contrast, in the same series exchange of the
273 most C-terminal E-domain region 3 had a far greater effect on cortisol sensitivity (12.6-fold

274 shift of EC50) than that of region 1 (2.7-fold) or region 2 (1.5-fold, not significant) (Fig. 4B). In
275 series two, exchange of the middle region had the greatest effect with both hormones,
276 causing sensitivity shifts of 8.1- to 8.6-fold (Fig. 4A, B). Swapping the most C-terminal region
277 had medium effects (shifts of 1.8- to 4.6-fold), while effects of replacing the τ 2 region were
278 marginal (~1.2-fold, significant only for cortisol) (Fig. 4A, B). Despite these complexities, the
279 results taken together suggested that region 2 and region 3 were more important for
280 sensitivity than region 1. Dexamethasone binding properties were analysed for AB-t2-
281 M(GR1)AF2-CT(GR2) and AB-t2-M(GR2)AF2-CT(GR1) (Table 1). Paralleling the findings on
282 dexamethasone EC50s of transactivation activity, K_d values for these receptors were
283 intermediary between rtGR1 and rtGR2 (Table 1).

284

285 The effects of region 2 differed between the two series of mutants shown in Fig. 4.
286 Therefore, the effects of this part of the E-domain, which shows 11 amino acid differences
287 between rtGR1 and rtGR2 (Fig. 3), were analysed in two further sets of mutants. The first set
288 of recombinant GRs was derived from rtGR1 by introducing rtGR2-specific residues as
289 single amino acid mutations or double mutations of adjacent amino acids (Table 2). In the
290 final mutant of the first set, called GR1(M_GR2), the entire region 2 of rtGR1 was replaced
291 by that of rtGR2. A second set of recombinant receptors consisted of the converse mutants
292 in a rtGR2-background (Table 2). Maximum activities of the rtGR1-derived mutants were
293 similar to those of rtGR1, while maximum activities of rtGR2-derived mutants resembled
294 those in rtGR2 (data not shown). Whereas the introduction of the rtGR2 sequence had
295 paradoxical desensitising effects in GR1_Y635H and GR1_T667S, EC50 values of all other
296 rtGR-derived mutations were not significantly different from that of rtGR1 (Table 2). Among
297 the rtGR2-derived mutants, GR2_I451L/R461Q, GR2_D509N/N511G and GR2(M_GR1)
298 were slightly less sensitive than rtGR2 (1.6- to 2.5- fold) (Table 2).

299

300 Within E-domain region 3, the AF2 domain differs in two adjacent amino acids between
301 rtGR1 (Gln⁷⁰⁹Ile⁷¹⁰) and rtGR2 (Asp⁶²⁶Phe⁶²⁷) (Fig. 3). The second of these residues

302 corresponds to hGR Tyr⁷³⁵ (Fig. 3), which is involved in ligand binding (Ray et al., 1999;
303 Bledsoe et al., 2002). To explore potential effects of this two-amino acid-difference on
304 receptor sensitivity, the mutants GR1_Q709D/I710F and GR2_D626Q/F627I were made.
305 According to EC50 values obtained in our transactivation assay, GR1_Q709D/I710F was
306 more sensitive than rtGR1 to cortisol, but less sensitive to dexamethasone (Fig. 5B).
307 Similarly, GR2_D626Q/F627I was less sensitive to cortisol than rtGR2, but more sensitive to
308 dexamethasone (Fig. 5B). Maximal transactivation activities of rtGR1 and
309 GR1_Q709D/I710F were in a similar range, as were those of rtGR2 and GR2_D626Q/F627I
310 (data not shown). Apparent dexamethasone binding affinities, expressed as K_d , of receptors
311 GR1_Q709D/I710F and GR2_D626Q/F627I only showed slight, statistically insignificant
312 changes compared to the affinities of the corresponding wild-type receptor (Table 1).
313

314 A second striking difference between rtGR1 and rtGR2 in region 3 regards the C-terminus,
315 where the sequence of rtGR1 is highly unusual. Here, rtGR2 conforms to a consensus
316 amongst other known GRs, which have Gln-Lys or Gln-Arg as the terminal amino acids
317 (Milhon et al 1997; Kucera et al 2002). By contrast, rtGR1 shows the amino acids Ala-Leu at
318 the corresponding positions, and further possesses six additional C-terminal amino acids
319 lacking in other GRs (Fig 3). Mutants were derived from rtGR1 introducing changes to the C-
320 terminus (Fig. 5A). In GR1_A751Q/L752K, change of residues 751 and 752 to the
321 consensus had moderately sensitising effects on transactivation EC50s with cortisol (2.1-
322 fold) and dexamethasone (2.5-fold) (Fig. 5B). The further ablation of the additional C-
323 terminal residues in GR1-CTcons resulted in additional sensitivity shifts of transactivation
324 EC50s of about 1.5-fold (Fig. 5B), making GR1-CTcons 3.1- and 4.2-fold more sensitive than
325 rtGR1 to cortisol and dexamethasone, respectively (Fig. 5B). GR1_A751Q/L752K and GR1-
326 CTcons were similar to rtGR1 regarding maximal transactivation activities (data not shown).
327 Combining rtGR1 mutations Q709D, I710F, A751Q and L752K resulted in a receptor being
328 4.9-fold more sensitive to cortisol in transactivation assays than rtGR1, but only 1.4-fold
329 more sensitive to dexamethasone (Fig. 5B). Mutants GR1_A751Q/L752K and GR1-CTcons

330 displayed 2.6-fold and 2.0-fold lower dexamethasone K_d values, respectively, than the wild
331 receptor rtGR1 (Table 1).

332

333 4. Discussion

334

335 Despite their high sequence similarity in the central and C-terminal regions, rtGR1 and
336 rtGR2 markedly differ in their EC50 values for glucocorticoids in transactivation and
337 transrepression assays in mammalian cell lines (Ducouret et al 1995; Bury et al 2003). The
338 present study confirmed this finding for the first time in a teleost cell line (Fig. 1A), excluding
339 the possibility that the difference in transactivation assays sensitivity reported earlier had
340 been caused by the heterologous expression. In the current study the two GRs also showed
341 a significantly different (3.5-fold) apparent dissociation constant for dexamethasone; a result
342 that contrasts to our original observation of a smaller difference (1.6-fold) in dexamethasone
343 affinity between the receptors (Bury et al., 2003). The reasons for the discrepancy are
344 unknown, but the current study uses a whole cell assay for assessment of dexamethasone
345 binding characteristics, whereas our previous study used a cell free expression system.
346 However, distinct dexamethasone binding affinities would help explain the downstream
347 differences in transactivation and transrepression assays EC50 values seen in previous
348 studies (Bury et al., 2003; Bury and Sturm, 2007). In accordance with this hypothesis,
349 domain-swap mutants between rtGR1 and rtGR2 show that the main determinant of
350 glucocorticoid transactivation EC50 sensitivity resides in the hormone binding (E) domain of
351 the receptors, and there is a clear correlation ($R^2=0.86$) between the transactivation EC50
352 value and the K_d value of dexamethasone among all wildtype and mutant GRs tested (Fig.
353 6). However, experiments in which three subregions of the E-domain were defined and
354 exchanged between rtGR1 and rtGR2 demonstrated that all subregions contributed to the
355 overall sensitivity shift. Effects of the most N-terminal E-domain subregion were strongly
356 dependent on the receptor context, while those of the central and C-terminal subregions
357 showed effects in all exchange mutants. At the C-terminal extremity, rtGR1 deviates from a

358 consensus sequence present in all other known GRs except for a few GRs in species closely
359 related to rainbow trout (Fig. 7). Here we show for the first time a role of this unique
360 sequence as a determinant of the relatively cortisol hyposensitivity of rtGR1.

361

362 To map determinants of receptor hormone sensitivity to specific regions of the E-domains,
363 we generated recombinant GRs that exchanged homologous parts of the E-domain between
364 rtGR1 and rtGR2 (Fig. 4). The effects of exchanging region 1 were highly dependent on
365 receptor context, suggesting that they involve other parts of the receptor, and do not directly
366 reflect differences in the strength of the τ 2-region between rtGR1 and rtGR2. In line with this
367 interpretation, amino acids involved in hormone binding and transactivation in the mouse GR
368 (Milhon et al., 1997) are either conserved in both trout GRs, or show conservative
369 substitutions (Fig. 3). The τ 2-region has been identified as the main determinant of
370 glucocorticoid hyposensitivity in the guinea pig GR, and it has been noted that this receptor
371 is more similar to rtGR1 than to hGR in this region (Keightley et al., 1998). However, the τ 2-
372 domain of the later isolated rtGR2 also is highly similar to that of the guinea pig GR,
373 suggesting that the similarity of the τ 2-domains of rtGR1 and the guinea pig GR is not
374 directly related to the low hormone sensitivity of the receptors.

375

376 Exchanging E-domain region 2 also had variable effects, depending mainly on the receptor
377 context. There was a marked difference in glucocorticoid EC50 values of transactivation
378 between recombinant receptors containing the rtGR1 region 2 compared to those with rtGR2
379 region 2 for those chimeras in which the N-terminal part of the receptor was from rtGR2 and
380 the C-terminal part was from rtGR1. In contrast, there was little if any effect of the origin of
381 region 2 on glucocorticoid EC50 values in the converse mutants having an N-terminus
382 originating from rtGR1 and a C-terminus derived from rtGR2. The effects of region 2 of the-E
383 domain were further explored in mutants, in which single or multiple amino acids of region 2
384 of the respective other receptor were introduced into a rtGR1 or rtGR2 background. In this
385 region, rtGR1 and rtGR2 differ in 11 amino acids, none of which is involved in ligand binding

386 (Bledsoe et al., 2002). Interestingly, exchanging the entire region 2 of rtGR1 for that of rtGR2
387 had no effect on dexamethasone EC50s in transactivation assays, while the converse
388 mutant in which region 2 of rtGR2 was substituted for that of rtGR1 showed a significantly
389 increased EC50 (Table 2). This suggests that region 2 is necessary but not sufficient in
390 conferring to a receptor the capability to respond to dexamethasone at lower concentrations.

391
392 Among chimeras differing in parts of the E-domain, exchanging the third, most C-terminal,
393 region had the most consistent effects in that differences in glucocorticoid EC50s in
394 transactivation assays and K_d values in binding studies. In this region, one sequence
395 difference between the two trout receptors (rtGR1 Ile⁷¹⁰, rtGR2 Phe⁶²⁷) corresponds to a
396 residue involved in ligand binding in hGR (Tyr⁷³⁵, Fig. 2, (Ray et al., 1999; Bledsoe et al.,
397 2002). Indeed, mutations that converted this and the preceding amino acid in one of the
398 trout GRs to the sequence found in the other trout GR, GR1_Q709D/I710F and
399 GR2_D626Q/F627I, provoked moderate sensitivity shifts of transactivation EC50s in the
400 expected direction with cortisol (~2.0-fold, Fig. 5). Surprisingly, however, and difficult to
401 explain, EC50s of dexamethasone were shifted in the opposite direction.

402
403 Another striking difference in region 3 between rtGR1 and rtGR2 regards the C-terminal
404 extremity of receptors. While in all other known GRs, Gln-Lys or Gln-Arg are conserved at
405 the two most C-terminal amino acids, rtGR1 shows the amino acids Ala-Leu at the
406 corresponding positions, and further has six additional amino acids C-terminal to this site.
407 Introducing the consensus C-terminus into rtGR1 had a consistent, if moderate, effect on
408 glucocorticoid EC50 values in transactivation assays (3.1- to 4.2-fold) and on apparent
409 dexamethasone binding affinities (2.0- to 2.6-fold). In the crystal structure of hGR, a C-
410 terminal β -strand following the AF2 helix formed a conserved β -sheet with a β -strand
411 between helices 8 and 9, suggesting its importance in receptor activation by stabilising the
412 “active” conformation of the AF2-helix (Bledsoe et al., 2002). The divergent C-terminus of
413 rtGR1 is located directly C-terminally of the C-terminal β -strand (Fig. 3). Our observation that

414 the non-consensus C-terminus of rtGR1 contributes to its hyposensitivity suggests the
415 possibility that it might affect the stability of the “active” conformation of the AF2-helix.
416 Previous studies have identified mutations located in the C-terminal part of GR that
417 negatively affect hormone sensitivity, such as mouse GR Y770N (corresponding to pos. 764
418 of hGR) from a glucocorticoid resistant cell line (Zhang et al., 1996), and hGR L773P from a
419 patient showing signs of generalised glucocorticoid resistance (Charmanadari et al., 2005).

420

421 To identify other GRs deviating from the C-terminal sequence consensus, tblastn searches
422 were carried out on the website of the National Centre for Biotechnology Information, using
423 E-domain region 3 of rtGR1 (Fig. 3) as the query. This yielded two GR sequences departing
424 from the C-terminal consensus, one in brown trout (*Salmo trutta*) and one in Atlantic salmon
425 (*Salmo salar*), two salmonid species closely related to rainbow trout (Fig. 7A). The brown
426 trout GR C-terminus is identical to that of rtGR1. The Atlantic salmon sequence resembles
427 rtGR1, but shows an insertion of one His after the conserved Leu-Leu-Phe and a far longer
428 C-terminal extension than rtGR1 (33 amino acids compared to 6 in rtGR1) (Fig. 7A). In
429 addition to the above salmonid GR sequences, the tblastn search hit a number of teleost
430 nucleotide GR sequences, in which reading the translation frame past the stop codon
431 yielded a predicted amino acid sequence with striking similarities to the C-terminal extension
432 found in rtGR1 (Fig. 7B). This suggests that the peculiar rtGR1 sequence might have
433 evolved by a mutation of the consensus stop codon.

434

435 A previous study provided an evolutionary analysis of available teleost GR sequences
436 including those from genome assemblies for zebrafish, puffer, green puffer, Japanese
437 medaka and three-spined stickleback (Stolte et al., 2006). Teleost GRs formed two well
438 supported clades, suggesting that duplications of GR in teleosts trace back to one ancient
439 gene duplication. The retention of duplicated GR genes in many species suggests these are
440 not redundant, but have undergone neo- or subfunctionalisation. In contrast, the zebrafish
441 genome contains one GR gene only (Schaaf et al., 2008 and 2009), which could indicate a

442 secondary loss of one of the GR gene copies. Duplicated GRs have been cloned and
443 functionally characterised in rainbow trout and two further teleost species, Burton's
444 mouthbrooder and common carp (Bury et al., 2003; Greenwood et al., 2003). In contrast to
445 trout, the maximal variability in cortisol sensitivity among GR isoforms was relatively
446 moderate in Burton's mouthbrooder (2.6-fold) and carp (7.3-fold) (Greenwood et al., 2005;
447 Stolte et al., 2008). This shows that the pronounced sensitivity difference found between
448 rtGR1 and rtGR2 is a specific trait of GRs in this teleost. The identification of a C-terminal
449 sequence significantly contributing to the hyposensitivity of rtGR1, which departs from a
450 consensus among GRs except for similar sequences in two other salmonids, suggest that
451 following the duplication of GRs in an ancestor of teleosts, one of the two GRs might have
452 mutated into a hyposensitive GR in the salmonid lineage.

453

454 Whether a hyposensitive receptor offers specific selective advantages is unclear, and awaits
455 further physiological studies to elucidate the physiological roles of the two receptors in
456 salmonids and other teleosts.

457

458

459

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461

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620 **Figure legends**

621

622 **Figure 1.** Functional properties of rtGR1 and rtGR2. Transactivation profiles (A and C) and
623 maximum induced transcriptional activities at 10^{-6} M cortisol or dexamethasone (B and D) in
624 FHM cells (A and B) derived from fathead minnow and COS-7 cells (C and D). Cells were
625 transiently transfected with GR, a firefly luciferase reporter containing the MMTV promoter,
626 and in FHM cells *Renilla* luciferase internal reporter or in COS-7 cells β -galactosidase
627 internal reporter to correct for transfection efficiency. Cells were treated for 36 h with cortisol.
628 After determination of reporter gene activities, transactivation activities were obtained by
629 normalising luciferase activity to activity of the internal reporter and expressed as percent of
630 maximal activities. AU: arbitrary units. Values represent mean \pm SE of at least 3 separate
631 experiments.

632

633 **Figure 2.** Transactivation properties of domain-swap chimeras between rtGR1 and rtGR2.
634 Transactivation assays were carried out in COS-7 cells as described in the legend of Figure
635 1. (A). Graphical representation of the domain composition of recombinant domain-swap
636 mutants. Percentages indicate amino acid identities between rtGR1 and rtGR2 for the main
637 receptor domains. (B). Median effective concentrations (EC50s) and 95% confidence limits
638 of the stimulation of transactivation activity by cortisol and dexamethasone. Numbers at bars
639 denote the EC50 in nM. EC50s are considered significantly different if confidence limits do
640 not overlap. Values represent mean \pm SE of at least 3 separate experiments.

641

642 **Figure 3.** Alignment of the E-domains of rtGR1, rtGR2 and human GR alpha, prepared with
643 ClustalW. Conserved residues are marked with "*", while "." denotes conservative amino
644 acid changes. The τ 2 region (Nilhon et al., 1997) and the AF2 region (Kucera et al., 2002)
645 are marked by grey shadowing. The crystal structure of hGR (Bledsoe et al., 2002) has
646 identified amino acids involved in ligand binding (bold underlined), receptor homo-
647 dimerisation (embossed/underlined) and coactivator binding (italics/underlined). The position

648 of helices (H) and beta-strands (B) of hGR are according to the Protein Knowledgebase
649 (UniProtKB, <http://www.uniprot.org/>, Accession number P04150). For the generation of
650 mutants described in Figure 4, the following subregions of the E-domain were defined:
651 Region 1 coincides with the $\tau 2$ domain, region 3 is comprised of the AF2 domain and the C-
652 terminal extremity of the receptor, and region 2 is the middle portion of the E-domain
653 enclosed by regions 1 and 3.

654

655 **Figure 4.** Transactivation properties of chimeras exchanging parts of the E-domain between
656 rtGR1 and rtGR2. Transactivation assays were carried out in COS-7 cells as described in
657 the legend of Figure 1. (A). Graphical representation of the domain composition of chimeras
658 where M represents the middle portion of E-domain. (B). Median effective concentrations
659 (EC50s) and 95% confidence limits of the stimulation of transactivation activity of receptors
660 and mutant receptors by cortisol and dexamethasone. Numbers at bars denote the EC50 in
661 nM. EC50s are considered significantly different if confidence limits do not overlap. Values
662 represent mean \pm SE of at least 3 separate experiments.

663

664 **Figure 5.** Transactivation properties of rtGR1- and rtGR2-derived mutants with changes in
665 the AF2 domain or C-terminal extremity. Transactivation assays were carried out in COS-7
666 cells as described in the legend of Figure 1. (A). Sequence information for C-terminal of
667 wildtype rtGR1 and rtGR2 and mutants generated. (B), Median effective concentrations
668 (EC50s) and 95% confidence limits of the stimulation of transactivation activity of receptors
669 and mutant receptors by cortisol and dexamethasone. GR1_709/710, GR2_626/627,
670 GR1_709/710/751/752 refers to specific site directed mutations; see 3. Results for more
671 details. Numbers at bars denote the EC50 in nM. EC50s are considered significantly
672 different if confidence limits do not overlap. Values represent mean \pm SE of at least 3
673 separate experiments.

674

675 **Figure 6.** The relationship between the dexamethasone binding affinity (K_d) and the
676 dexamethasone EC50 of transactivation activity for wild-type and recombinant GRs studied.

677

678 **Figure 7.** Homology of the C-terminal extremity of rtGR peptide to other sequences. (A).

679 Alignment of hGR α and GRs from salmonid fish. Dots represent residues or gaps identical to

680 hGR α (top). btGR, brown trout (*Salmo trutta*) GR (GenBank accession number AY863149) ;

681 asGR, Atlantic salmon GR (*Salmo salar*) (EG879323). (B). The homology of rtGR1 to the

682 conceptual translation of different teleost mRNA GR sequences extends to the region past

683 the stop codon (#). bm, Burton's mouthbrooder (AF263738), zf zebrafish (EF436284), fm

684 fathead minnow (AY533141). Conserved residues are marked with "*", while "." denotes

685 conservative amino acid changes.

Table 1. Dexamethasone binding to wild type and mutant trout GRs, as determined in a whole cell assay with COS-7 cells transiently expressing the receptors.

Receptor	n	K_d (nM)	B_{max} (nmoles/well)
rtGR1 ^a	4	30.4 (23.0 – 37.8) ^b	1.21 ± 0.80 ^c
rtGR2 ^a	4	8.6 (6.6 – 10.6)	1.85 ± 0.79
ABCD(GR1)E(GR2)	3	9.5 (8.2 – 10.8)	2.33 ± 0.90
ABCD(GR2)E(GR1)	3	35.9 (26.7 – 45.1)	1.33 ± 0.75
AB-t2-M(GR1)AF2(GR2)	3	18.4 (13.0 – 23.7)	2.40 ± 0.46
AB-t2-M(GR2)AF2(GR1)	3	13.7 (12.1 – 15.2)	3.59 ± 1.72
GR1_709/710	3	36.1 (27.2 – 45.1)	1.57 ± 1.08
GR2_626/627	2	8.1 (6.7 – 9.2)	2.81 ± 2.09
GR1-CTcons	3	14.9 (12.9 – 17.0)	1.62 ± 1.49
GR1_751/752	3	11.6 (9.1 – 14.0)	1.47 ± 0.94

^a Data from Sturm et. al, 2010

^b Values in brackets are 95% confidence limits.

^c Values are mean and standard deviation. Differences in B_{max} between receptors were not significant.

Table 2. Median effective concentrations (EC50) of dexamethasone-dependent stimulation of transactivation activity of wild-type and mutant GRs.

Receptor	EC50 (nM)
rtGR1	4.4 (3.8 – 5.3)*
rtGR2	0.60 (0.48 – 0.76)
GR1_L534I/Q544R	3.9 (3.1 – 4.8)
GR1_S555A	5.9 (4.6 – 7.7)
GR1_N592D/G594N	4.6 (3.5 – 6.3)
GR1_D606Q/E607D	4.0 (3.1 – 5.2)
GR1_T615A	4.8 (3.8 – 5.9)
GR1_T626S	5.0 (4.3 – 5.8)
GR1_Y635H	18.2 (15.4 – 21.5)
GR1_T667S	6.1 (5.4 – 7.0)
GR1 (M_GR2)	3.7 (2.8 – 4.7)
GR2_I451L/R461Q	0.98 (0.80 – 1.20)
GR2_A472S	0.62 (0.48 – 0.81)
GR2_D509N/N511G	1.0 (0.85 – 1.2)
GR2_Q523D/D524E	0.34 (0.20 – 0.56)
GR2_A532T	0.34 (0.21 – 0.54)
GR2_S543T	0.51 (0.34 – 0.78)
GR2_H552Y	0.55 (0.23 – 1.2)
GR2_S584T	0.57 (0.45 – 0.72)
GR2 (M_GR1)	1.5 (1.3 – 1.6)

* Values were derived from data of at least three independent experiments, and numbers in brackets are 95% confidence limits of the EC50.

Fig. 1

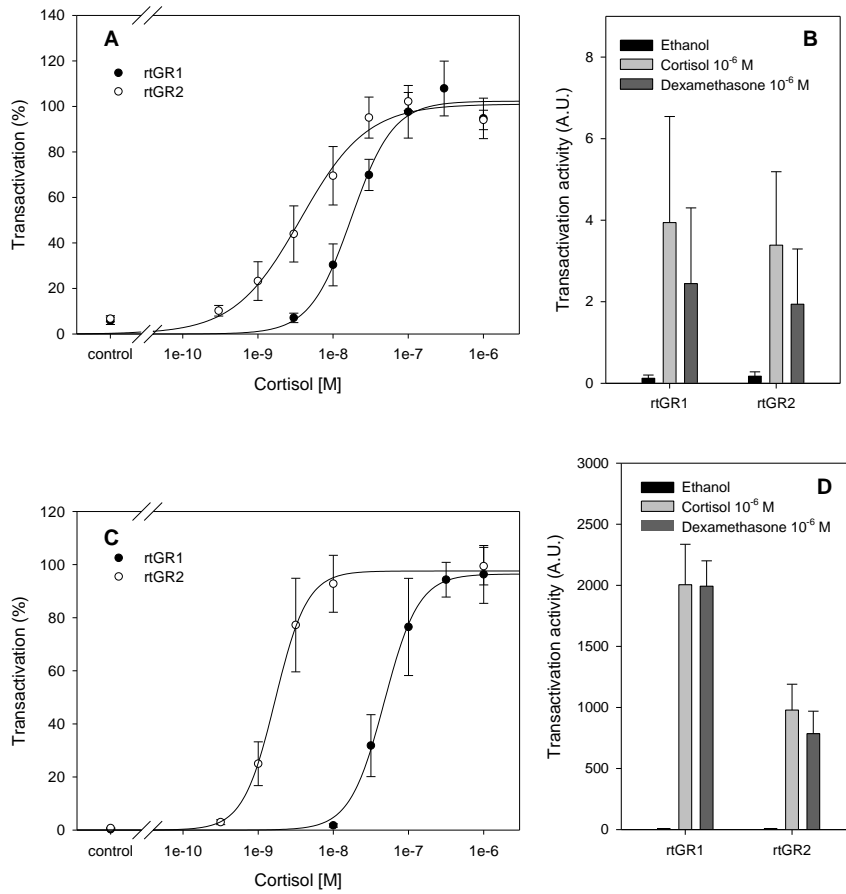
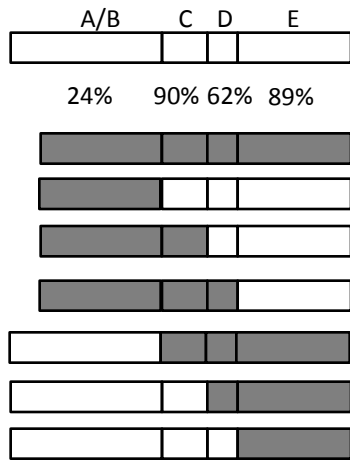
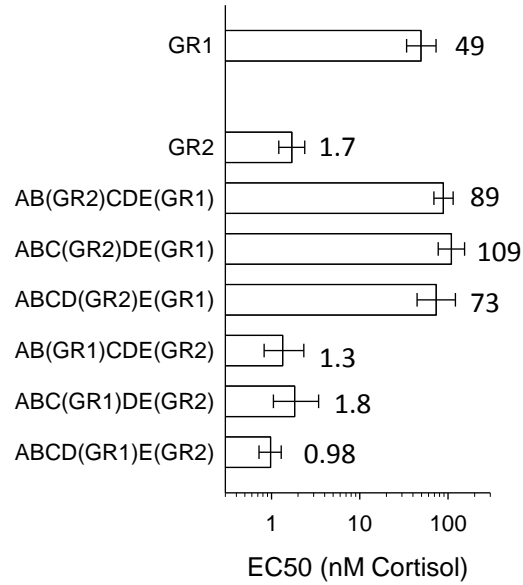


Fig 2

A



B



C

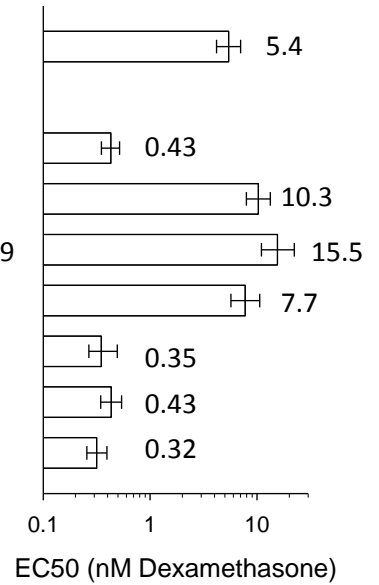


Fig 4

A



B

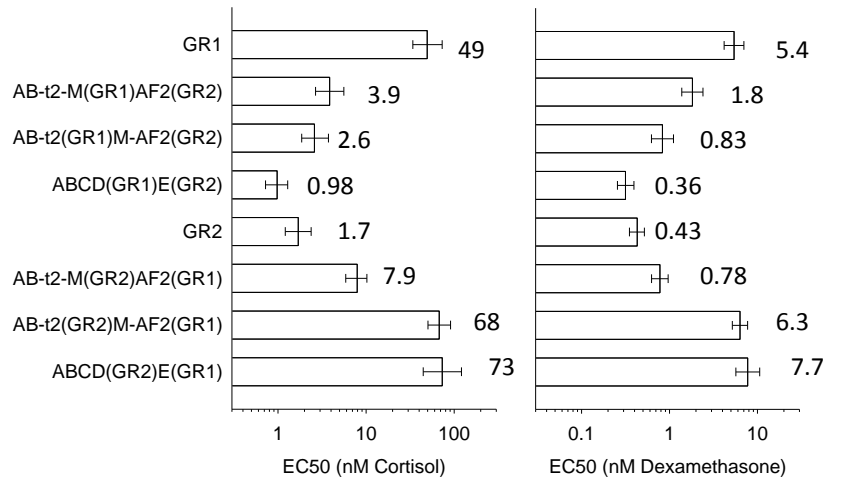


Fig. 5

A

```

GR1      ...KDGSVKPLLFHALNHDTMP 758
GR2      ...KAGSVKPLLFHQK----- 669
GR1-CTcons ...KDGSVKPLLFHQK 752
GR1_A751Q/L752K ...KDGSVKPLLFHQKNHDTMP 758
    
```

B

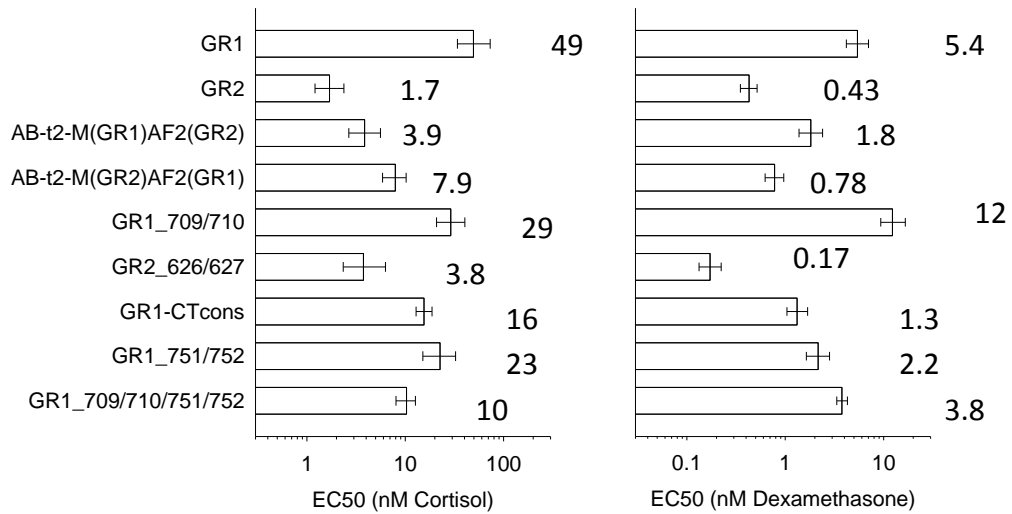


Figure 6

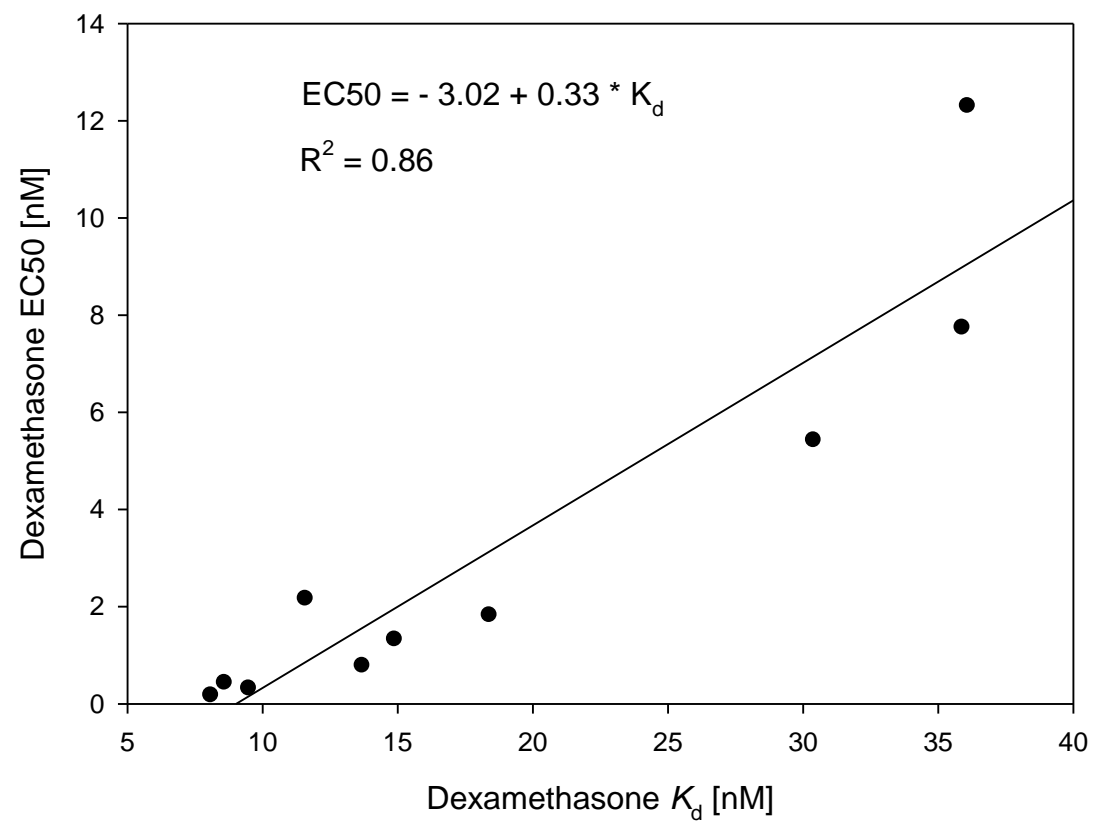


Fig. 7

A

	H11	H12
hGRa	EVVENLLNYCFQTFLDKTSIEFPPEMLAEIITNQIPKYSNGNIKKLLF-HQK	
rtGR1	.M.GG..QI..Y..VN.SL.V.....S..L..FKD.SV.P.....ALNHDTMP	
rtGR2	.M.GG..DF..Y..VN.SL.V.....S..L..FKA.SV.P.....	
btGR	.M.GG..QI..Y..VN.SL.V.....FKD.SV.P.....ALNHDTMP	
asGR	.M.GG..QI..Y..VN.SL.V.....FKD.SV.P...H.ALNHDNALKQPPLSPARPTCFRRRPTRSPTSTD	

B

rtGR1	EMVGGLLQICFYTFVNKSLSVFPEMLAEIISNQLPKFKDGSVKPLLFH--ALN---HDTMP
bmGR1	EMVGGLLSFCFYTFVNKSLSVFPEMLAEIISNQLPKFKAGSVKPLLFHQ#LTPV-TDTMP
zfGR	DLVGGLLNFCFYTFVNKSLSVFPEMLAEIISNQLPKFKDGSVKPLLFHQ#ISSQQDTMP
fmGR	DMVGGLLNFCFFTFVNKSLSVFPEMLAEIISNQLPRFRAGSVKALLFH#ISSQQDTMP
	::*****.:**.:*****:*****.:*: ****.**** :. ****