Molecular determinants of hormone sensitivity in rainbow trout glucocorticoid receptors 1 and 2

Armin Sturm, Louise Colliar, Michael Leaver, Nic R. Bury

► To cite this version:

Armin Sturm, Louise Colliar, Michael Leaver, Nic R. Bury. Molecular determinants of hormone sensitivity in rainbow trout glucocorticoid receptors 1 and 2. *Molecular and Cellular Endocrinology*, 2011, 333 (2), pp.181. 10.1016/j.mce.2010.12.033 . hal-00668059

HAL Id: hal-00668059

<https://hal.science/hal-00668059>

Submitted on 9 Feb 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Molecular determinants of hormone sensitivity in rainbow trout glucocorticoid receptors 1 and 2

Authors: Armin Sturm, Louise Colliar, Michael Leaver, Nic R. Bury

PII: S0303-7207(11)00002-5
DOI: doi:10.1016/j.mce.2010.12.033
Reference: MCE 7738

To appear in: *Molecular and Cellular Endocrinology*

Received date: 26-10-2010
Revised date: 2-12-2010
Accepted date: 28-12-2010

Please cite this article as: Sturm, A., Colliar, L., Leaver, M., Bury, N.R., Molecular determinants of hormone sensitivity in rainbow trout glucocorticoid receptors 1 and 2, *Molecular and Cellular Endocrinology* (2010), doi:10.1016/j.mce.2010.12.033

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Molecular determinants of hormone sensitivity in rainbow trout glucocorticoid
receptors 1 and 2**

Author list: Armin Sturm^{1,2}, Louise Colliar¹, Michael Leaver¹, Nic R. Bury^{2†}

1 University of Stirling, Institute of Aquaculture, Stirling, Scotland, UK

2 King's College London, Nutritional Sciences Division, London, England, UK

† Corresponding author.

Tel. 00 44 20 78 48 40 91.

Fax. 00 44 20 78 48 45 00.

Email: nic.bury@kcl.ac.uk

Abstract

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

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

1. Introduction

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

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

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

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

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

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

2. Materials and Methods

2.1 Plasmid constructs

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

2.2 Cell culture, transactivation and binding assays.

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

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

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

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

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

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

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

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

3. Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

Acknowledgements

This work was supported by Grant S18960 from the UK Biotechnology and Biological Sciences Research Council (to N.R.B.) and by Grant NE/F008287/1 from the UK Nature and Environment Research Council (to L.C.). We thank different colleagues for kindly placing plasmids at our disposal. Dr. B. Ducouret supplied pCMrtGR, Dr. P. Prunet gave pCMrtGR2, and pFC31Luc was a gift from Dr. F. Gouilleux. We are grateful to Professor M.-E. Rafestin-Oblin and Dr. J. Fagart for their fruitful comments on the manuscript. We thank Dr. D. M. Green for advice on biometrical analyses in the modelling environment R.

References

- Bamberger C.M., Schulte, H.M., Chrousos, G.P. 1996. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocr. Rev.* 17:245-61
- Barton, B.A., Iwama, G.K. 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases* 1:3-26
- Becker, H., Sturm, A., Bron, J.E., Schirmer, K., Bury, N.R. 2008. The A/B domain of the teleost glucocorticoid receptors influences partial nuclear localization in the absence of hormone. *Endocrinology* 149:4567-4576
- Bern, H.A. 1967. Hormones and endocrine glands of fishes. *Science* 158:455-462
- Charmandari, E., Tsigos, C., Chrousos, G. 2005. Endocrinology of the stress response. *Annu. Rev. Physiol.* 67:259-284
- Bledsoe, R.K., Montana, V.G., Stanley, T.B., Delves, C.J., Apolito, C.J., McKee, D.D., Consler, T.G., Parks, D.J., Stewart, E.L., Willson, T.M., Lambert, M.H., Moore, J.T., Pearce, K.H., Xu, H.E. 2002. Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. *Cell* 110:93-105
- Bury, N.R., Sturm, A., Le Rouzic, P., Lethimonier, C., Ducouret, B., Guigen, Y., Robinson-Rechavi, M., Laudet, V., Prunet, P. 2003. Evidence for two distinct functional glucocorticoid receptors in teleost fish. *J. Mol. Endocrinol.* 31:141-156
- Bury, N.R., Sturm, A. 2007. Evolution of the corticosteroid receptor signalling pathway in fish. *Gen. Comp. Endocrinol.* 153:47-56

499

500 Charmandari, E., Raji, A., Kino, T., Ichijo, T., Tiulpakov, A., Zachman, K., Chrousos, G.P.
501 2005. A novel point mutation in the ligand-binding domain (LBD) of the human glucocorticoid
502 receptor (hGR) causing generalized glucocorticoid resistance: The importance of the C
503 terminus of hGR LBD in conferring transactivational activity. *Journal of Clinical*
504 *Endocrinology and Metabolism* 90:3696-3705

505

506 Charmandari, E., Ichijo, T., Jubiz, W., Baid, S., Zachman, K., Chrousos, G., Kino, T. 2008 A
507 novel point mutation in the amino terminal domain of the human glucocorticoid receptor
508 (hGR) gene enhancing hGR-mediated gene expression. *Journal of Clinical Endocrinology &*
509 *Metabolism* 93:4963-4968

510

511 Danielian, P.S., White, R., Lees, J.A., Parker, M.G. 1992. Identification of a Conserved
512 Region Required for Hormone Dependent Transcriptional Activation by Steroid-Hormone
513 Receptors. *EMBO J.* 11:1025-1033

514

515 Ducouret, B., Tujague, M., Ashraf, J., Mouchel, N., Servel, N., Valotaire, Y., Thompson, E.B.
516 1995. Cloning of a teleost fish glucocorticoid receptor shows that it contains a
517 deoxyribonucleic acid-binding domain different from that of mammals. *Endocrinology*
518 136:3774-3783

519

520 Dyer, B.W., Ferrer, F.A., Klinedinst, D.K., Rodriguez, R. 2000. A noncommercial dual
521 luciferase enzyme assay system for reporter gene analysis. *Anal. Biochem.* 282:158-161

522

523 Giguere, V., Hollenberg, S.M., Rosenfeld, M.G., Evans, R.M. 1986. Functional Domains of
524 the Human Glucocorticoid Receptor. *Cell* 46:645-652

525

- 526 Godowski, P.J., Picard, D., Yamamoto, K.R. 1988. Signal Transduction and Transcriptional
527 Regulation by Glucocorticoid Receptor-Lexa Fusion Proteins. *Science* 241:812-816
528
- 529 Gouilleux, F., Sola, B., Couette, B., Richard-Foy, H. 1991. Cooperation between structural
530 elements in hormono-regulated transcription from the mouse mammary tumor virus
531 promoter. *Nucleic Acids Res.* 19:1563-9
532
- 533 Greenwood, A.K., Butler, P.C., White, R.B., DeMarco, U., Pearce, D., Fernald, R.D. 2003.
534 Multiple corticosteroid receptors in a teleost fish: distinct sequences, expression patterns,
535 and transcriptional activities. *Endocrinology* 144:4226-36
536
- 537 Hard, T., Kellenbach, E., Boelens, R., Maler, B.A., Dahlman, K., Freedman, L.P.,
538 Carlstedt-Engvall, J., Yamamoto, K.R., Gustafsson, J.A., Kaptein, R. 1990. Solution Structure of
539 the Glucocorticoid Receptor DNA-Binding Domain. *Science* 249:157-160
540
- 541 Heitzer, M.D., Wolf, I.M., Sanchez, E.R., Witchel, S.F., DeFranco, D.B. 2007. Glucocorticoid
542 receptor physiology. *Reviews in Endocrine & Metabolic Disorders* 8:321-330
543
- 544 Jaillon, O., Aury, J.M., Brunet, F., Petit, J.L., Stange-Thomann, N., Mauceli, E., Bouneau, L.,
545 Fischer, C., Ozouf-Costaz, C., Bernot, A., Nicaud, S., Jaffe, D., Fisher, S., Lutfalla, G.,
546 Dossat, C., Segurens, B., Dasilva, C., Salanoubat, M., Levy, M., Boudet, N., Castellano, S.,
547 Anthouard, V., Jubin, C., Castelli, V., Katinka, M., Vacherie, B., Biemont, C., Skalli, Z.,
548 Cattolico, L., Poulain, J., De Berardinis, V., Cruaud, C., Duprat, S., Brottier, P., Coutanceau,
549 J.P., Gouzy, J., Parra, G., Lardier, G., Chapple, C., McKernan, K.J., McEwan, P., Bosak, S.,
550 Kellis, M., Volff, J.N., Guigo, R., Zody, M.C., Mesirov, J., Lindblad-Toh, K., Birren, B.,
551 Nusbaum, C., Kahn, D., Robinson-Rechavi, M., Laudet, V., Schachter, V., Quetier, F.,
552 Saurin, W., Scarpelli, C., Wincker, P., Lander, E.S., Weissenbach, J., Roest Crollius, H.

2004. Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early
vertebrate proto-karyotype. *Nature* 431:946-57
- Keightley, M.C., Curtis, A.J., Chu, S., Fuller, P.J. 1998. Structural determinants of cortisol
resistance in the guinea pig glucocorticoid receptor. *Endocrinology* 139:2479-85
- Kucera, T., Waltner-Law, M., Scott, D.K., Prasad, R., Granner, D.K. 2002. A point mutation
of the AF2 transactivation domain of the glucocorticoid receptor disrupts its interaction with
steroid receptor coactivator 1. *J. Biol. Chem.* 277:26098-26102
- Milhon, J., Lee, S., Kohli, K., Chen, D., Hong, H., Stallcup, M.R. 1997. Identification of amino
acids in the tau 2-region of the mouse glucocorticoid receptor that contribute to hormone
binding and transcriptional activation. *Mol. Endocrinol.* 11:1795-805
- Pratt, W.B., Toft, D.O. 1997. Steroid receptor interactions with heat shock protein and
immunophilin chaperones. *Endocr. Rev.* 18:306-360
- Prunet, P., Sturm, A., Milla, S. 2006. Multiple corticosteroid receptors in fish: from old ideas
to new concepts. *Gen. Comp. Endocrinol.* 147:17-23
- Ray, D.W., Suen, C-S, Brass, A., Soden, J., White, A. 1999. Structure/function of the human
glucocorticoid receptor: Tyrosine 735 is important for transactivation. *Mol. Endocrinol.*
13:1855-1863
- Rogerson, F.M., Dimopoulos, N., Sluka, P., Chu, S., Curtis, A.J., Fuller, P.J. 1999. Structural
determinants of aldosterone binding selectivity in the mineralocorticoid receptor. *J. Biol.*
Chem. 274:36305-11

- 581 Sambrook, J., Russell, D.W. 2001. Molecular cloning, Third ed. Cold Spring Harbor
582 Laboratory Press, Cold Spring Harbor, NY, USA
583
- 584 Schaaf, M.J.M., Champagne, D., van Laanen, I.H.C., van Wijk, D., Meijer, A.H., Meijer, O.C.,
585 Spaink, H.P., Richardson, M.K. 2008. Discovery of a functional glucocorticoid receptor beta-
586 isoform in zebrafish. *Endocrinology* 149:1591-1599
587
- 588 Schaaf, M.J.M., Chatzopoulou, A., Spaink, H.P. 2009. The zebrafish as a model system for
589 glucocorticoid receptor research. *Comparative Biochemistry and Physiology a-Molecular &*
590 *Integrative Physiology* 153:75-82
591
- 592 Schoneveld, O., Gaemers, I.C., Lamers, W.H. 2004. Mechanisms of glucocorticoid
593 signalling. *Biochimica Et Biophysica Acta-Gene Structure and Expression* 1680:114-128
594
- 595 Stolte, E.H., Verburg van Kemenade, B.M.L., Savelkoul, H.F.J., Flik, G. 2006. Evolution of
596 glucocorticoid receptors with different sensitivity. *J. Endocrinol* 190. 17-28.
597
- 598 Stolte, E.H., de Mazon, A.F., Leon-Koosterziel, K.M., Jesiak, M., Bury, N.R., Sturm, A.,
599 Savelkoul, H.F.J., van Kemenade, B., Flik, G. 2008a. Corticosteroid receptors involved in
600 stress regulation in common carp, *Cyprinus carpio*. *J. Endocrinol.* 198:403-417
601
- 602 Stolte, E.H., Nabuurs, S.B., Bury, N.R., Sturm, A., Flik, G., Savelkoul, H.F.J., Kemenade, B.
603 2008b. Stress and innate immunity in carp: Corticosteroid receptors and pro-inflammatory
604 cytokines. *Molecular Immunology* 46:70-79
605
- 606 Sturm, A., Bury, N., Dengreville, L., Fagart, J., Flouriot, G., Rafestin-Oblin, M.E., Prunet, P.
607 2005. 11-deoxycorticosterone is a potent agonist of the rainbow trout (*Oncorhynchus*
608 *mykiss*) mineralocorticoid receptor. *Endocrinology* 146:47-55

609

610 Sturm, A., Bron, J.E., Green, D.M., Bury, N.R. 2010. Mapping of AF1 transactivation

611 domains in duplicated rainbow trout glucocorticoid receptors. J Mol Endocrinol 45, 1- 14

612

613 Zhang, S.M., Liang, X.Y., Danielsen, M. 1996. Role of the C terminus of the glucocorticoid

614 receptor in hormone binding and agonist antagonist discrimination. Mol. Endocrinol. 10:24-

615 34

616

617

618

619

Figure legends

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

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

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

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

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

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

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

Figure 7. Homology of the C-terminal extremity of rtGR peptide to other sequences. (A). Alignment of hGR α and GRs from salmonid fish. Dots represent residues or gaps identical to hGR α (top). btGR, brown trout (*Salmo trutta*) GR (GenBank accession number AY863149) ; asGR, Atlantic salmon GR (*Salmo salar*) (EG879323). (B). The homology of rtGR1 to the conceptual translation of different teleost mRNA GR sequences extends to the region past the stop codon (#). bm, Burton's mouthbrooder (AF263738), zf zebrafish (EF436284), fm fathead minnow (AY533141). Conserved residues are marked with "*", while "." denotes 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

Figure

Fig 2

Fig. 3

Fig 4

Figure
Fig. 5

A

GR1	...KDGSVKPLL F HALNHD TMP	758
GR2	...KAGSVKPLL F HQK-----	669
GR1-CTcons	...KDGSVKPLL F HQK	752
GR1_A751Q/L752K	...KDGSVKPLL F HQKNHD TMP	758

Figure 6

Fig. 7

A

	H11	H12
hGRa	EVVENLLNYCFQTFDLKTSIEFPPEMLAEIITNQIPKYSNGNIKKLLF-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.ALNHDNALKPQPPLSPARPTCFPRRRPTRSPTSTD	

B

rtGR1	EMVGGLLQICFYTFVNKSLSVFPEMLAEIISNQLPKFKDGSVKPLL FH--ALN---HDTMP
bmGR1	EMVGGLLSFCFYTFVNKSLSVFPEMLAEIISNQLPKFKAGSVKPLL FHQR#LTPV-TDTMP
zfGR	DLVGGLLNFCFYTFVNKSLSVFPEMLAEIISNQLPKFKDGSVKPLL FHQK#ISSQQQDTMP
fmGR	DMVGGLLNFCFFTFVNKSLSVFPEMLAEIISNQLPRFRAGSVKALLFH HK#ISSQQQDTMP
	::*****.:**.:*****:*****:*.: ****.**** :. ****