Mapping of AF1 transactivation domains in duplicated rainbow trout glucocorticoid receptors

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Abstract

The glucocorticoid receptor (GR) is a ligand-dependent transcription factor mediating the genomic effects of glucocorticoids. Two activation functions (AFs) are present in the GR. While the N-terminal AF1 is ligand independent, the C-terminal AF2 overlaps with the ligand-binding domain and is ligand dependent. In this study, we have mapped AF1 in duplicated rainbow trout GRs, called rtGR1 and rtGR2, showing a limited homology (24-5%) in the N-terminal domain. Abolition of this domain from rtGR1 or rtGR2 resulted in a marked decrease (> 97%) in maximal hormone-dependent transactivation, but did not affect dexamethasone-binding activity or expression levels. This suggested that, similar to the situation in the human GR (hGR), AF1 is the main AF in the trout GRs. Sequence alignments with hGR suggested a localisation of AF1 to residues 70–230 of rtGR1 and 1–119 of rtGR2. These assignments were generally confirmed in the transactivation experiments with rtGR1- and rtGR2-derived mutants showing partial deletions of their N-terminal domains. In dexamethasone-treated cells (10⁻⁷ M, 2 h), the subcellular distribution of rtGR1 and rtGR2 mutants lacking the entire N-terminal domain, as well that of an rtGR1 mutant lacking the most N-terminal 234 amino acids, was similar to that of the corresponding wild-type GRs, suggesting that the disruption of transactivation activity was not caused by impairment of nuclear access of the mutants. Bioinformatic analyses predicted the presence of potential helical segments in the core of AF1 of rtGR1 and rtGR2, and further revealed that AF1 in rtGR1, rtGR2, and hGR shares a motif composed of hydrophobic and acidic amino acids.

Journal of Molecular Endocrinology (2010) 45, 391–404

Introduction

Glucocorticoids have regulatory roles in many physiological processes, including carbohydrate metabolism, bone turnover, development, cell cycle, immune function, stress response, central nervous functions, growth and reproduction (Charmandari et al. 2005, Heitzer et al. 2007). The majority of effects of glucocorticoids occur at the genomic level and are mediated through the glucocorticoid receptor (GR; Hollenberg et al. 1985, Bledsoe et al. 2002). The GR belongs to the superfamily of nuclear receptors (NRs) that further comprise receptors for other steroid hormones, peroxisome proliferators, vitamin D and thyroid hormones, as well orphan receptors, for which no ligand has been identified (Wurtz et al. 1996, Escriva et al. 1997).

In the absence of hormone, the mammalian GR is located in the cytoplasm, where it is part of a large heteromeric complex involving heat shock protein (hsp)-90, other hsps, hsp-70/hsp-90 organising protein, and different immunophilins (Pratt & Toft 1997, Heitzer et al. 2007). Ligand binding provokes the dissociation of GR from this complex and facilitates the nuclear translocation of the receptor–hormone complex (Pratt & Toft 1997, Heitzer et al. 2007). In the nucleus, the GR can affect the transcription of target genes by forming homodimers and binding to imperfectly palindromic consensus DNA sequences called glucocorticoid response elements (GREs), which are located in the regulatory regions of genes regulated by glucocorticoids. Alternatively, the liganded GR can engage in protein–protein interactions with other transcription factors, thus modulating the expression of genes lacking GREs (Bamberger et al. 1996, Schoneveld et al. 2004).

The GR shares with other NRs a general architecture consisting of domains to which specific functions have been attributed. The N-terminal A/B domain of the GR contains a main transactivation function named activation function 1 (AF1, also called enh2 or τ1; Giguere et al. 1986, Godowski et al. 1988, Hollenberg & Evans 1988). The central C-domain, consisting of two zinc fingers, is involved in DNA binding and receptor dimerisation (Hard et al. 1990). The C-terminal E-domain comprises the site of ligand binding and contains a second, strictly ligand-dependent AF called AF2 (Danielian et al. 1992, Kucera et al. 2002). A further region with roles in transactivation has been mapped to...
the N-terminal extremity of the E-domain and was termed t2 (Giguere et al. 1986, Hollenberg & Evans 1988).

In respect to corticosteroid signalling, teleost fish show a number of differences from mammals. Cortisol is well established as the main teleost corticosteroid, having gluco- and mineralocorticoid effects (Mommsen et al. 1999). By contrast, aldosterone, the tetrapod mineralocorticoid hormone, is most likely absent in teleosts, which lack the enzymes required for the final steps of aldosterone biosynthesis (Jiang et al. 1998, Nelson 2003). Surprisingly, however, teleosts as well as elasmobranchs possess a homologue of the human mineralocorticoid receptor (MR; Arriza et al. 1987, Colombe et al. 2000, Bridgham et al. 2006), suggesting that MRs have evolved before the emergence of the mineralocorticoid aldosterone, and might have originally functioned as a high-affinity receptor for glucocorticoids (Bridgham et al. 2006, Ortlund et al. 2007). Teleosts further differ from tetrapods in the number of GR genes. While tetrapods including human have one GR gene (Hollenberg et al. 1985), two GRs encoded by distinct genes exist in a number of teleosts, including rainbow trout, Burton’s mouthbrooder and common carp, as well as a number of species with available genome sequences (puffer fish, green puffer, medaka, stickleback; Ducouret et al. 1995, Colombe et al. 2000, Bury et al. 2003, Greenwood et al. 2003, Stolte et al. 2006, 2008a). Evolutionary analyses have suggested a common origin of duplicated teleost GRs (Stolte et al. 2006, 2008a), which may have emerged during a whole genome duplication event believed to have taken place in the early evolutionary history of teleosts (Jaillon et al. 2004). In contrast, the zebrafish genome contains only one GR gene (Schaaf et al. 2003, 2009), which could indicate a secondary loss of one of the GR gene copies. The retention of two GRs in many teleosts suggests a selective advantage of having two GRs, and implies neo- or subfunctionalisation of the duplicated genes. Indeed, where data are available, they suggest differences in tissue distribution and/or hormone sensitivity exist between the duplicated GRs of teleosts (Bury et al. 2003, Greenwood et al. 2003, Stolte et al. 2008a,b).

The two GRs of rainbow trout, called rtGR1 (initially called rtGR) and rtGR2, show a high degree of sequence similarity in the C-domain and E-domain (>89% amino acid identity; Ducouret et al. 1995, Bury et al. 2003). By contrast, the D-domain (58-5% identity) and the A/B domain (24-4% identity) are less conserved between the receptors. Transactivation by rtGR1 and rtGR2 differed markedly (at least about tenfold) in glucocorticoid sensitivity with the mouse mammary virus promoter, or tyrosine kinase promoter constructs containing two or three GREs (Bury et al. 2003, Bury & Sturm 2007). The two trout GRs further differ in their subcellular distribution. While the localisation of the GR in mammals is predominantly cytosolic in the absence of hormone, unliganded green fluorescent protein (GFP)-tagged recombinant rtGR1 and rtGR2 were distributed between the cytoplasm and the nucleus, with rtGR2 showing a higher propensity to a nuclear localisation than rtGR1 (Becker et al. 2008).

The aim of this study was to characterise the AF1 of rtGR1 and rtGR2. We have previously shown that the A/B domains of rat GR mutant C656G and those of rtGR1 or rtGR2 can be substituted for each other without major changes in transactivation properties (Becker et al. 2008). Similar results have been obtained when exchanging the A/B domain between rtGR1 and rtGR2 (A Sturm & NR Bury, unpublished data). Together, these results suggested a functional equivalence of the A/B domains of teleost and mammalian GRs with respect to transactivation. However, sequence homology of the A/B region was moderate between teleost and mammalian GRs, or between duplicated teleost GRs, with 25% amino acid identity between rtGR1 and rtGR2, and 30 or 22% amino acid identity between human GR (hGR) and rtGR1 or rtGR2 respectively. Consequently, the precise localisation and extension of AF1 in the trout GRs remained uncertain. In this study, we mapped AF1 of rtGR1 and rtGR2 by generating mutants derived from these receptors, in which the A/B domain was truncated at its N- or C-terminus. The results are complemented by bioinformatic analyses correlating the findings to the predicted secondary structural features of the A/B domains.

Materials and methods

Plasmid constructs

Plasmids containing the whole open reading frame of rtGR1 and rtGR2 (GenBank accession no. CA90937 and AAR87479) were available from previous studies (Ducouret et al. 1995, Bury et al. 2003). Using appropriate enzymes, cDNAs of rtGR1 and rtGR2 were subcloned into pcDNA3 (Invitrogen) to yield expression constructs pCRTGR1 and pCRTGR2. N-terminal and internal deletion mutants of rtGR1 and rtGR2 were generated by simple or overlap-extension PCR using appropriate primers (Sambrook & Russell 2001), and subcloned into the pcDNA3. In the naming of N-terminal deletion mutants, numbers denote the last amino acid removed from the receptor before the addition of an initiating methionine to the N-terminus. In the naming of internal deletion mutants, numbers refer to the still included amino acids on both sides of the deletion, e.g. mutant GR1del63_370 combines amino acids 1-63 and 370-758 of the 758-amino acid wild-type rtGR1 sequence. In analogy to the hGRa mutant ΔGR
(Alksnis et al. 1991), the A/B domain deletion mutant GRdelA/B comprised amino acids 381–758 of rtGR1 and GR2delA/B comprised 301–669 of rtGR2, to which two additional N-terminal amino acids were added (the initiating methionine and a valine). Internal deletion mutants in which the A/B domain was successively truncated from its C-terminus paralleled the mutational strategy in a previous study (Hollenberg et al. 1987), introducing internal deletions N-terminal of amino acid 381 of rtGR1 or 301 of rtGR2 (homologous to amino acid 404 of hGRa). Plasmid constructs encoding trout GRs, N-terminally tagged with enhanced GFP (GFP-rtGR1 and GFP-rtGR2), were available from a previous study (Becker et al. 2008). To generate GFP-tagged versions of mutants GR1delA/B and GR2delA/B from the above pcDNA3-based constructs, PCR was used to introduce terminal restriction sites into the cDNAs encoding these receptors, allowing in-frame insertion downstream of the sequence encoding GFP in a commercial vector (pEGFP-C1, Clontech). The plasmid pcI-nGFP-C656G encoding ratGR mutant C656G tagged by another variant of GFP (Htun et al. 1996) was a generous gift from Prof. G. Hager. The cDNA encoding ratGR C656G derived from this construct and 2 µg of irrelevant DNA (pBluescript SK) were used. Forty-eight hours after the transfection, the cells were harvested and whole-cell extracts were prepared using a lysis buffer (20 mM Tris–HCl (pH 7.5), 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100) supplemented with a commercial protease inhibitor cocktail (Thermo Scientific Pierce, Cramlington, UK). After centrifugation of the cell extracts (5 min at 10 000 g and 4 °C), proteins (30–50 µg) from the supernatant were resolved on 10% SDS–PAGE and transferred to nitrocellulose. Membranes were blocked with 5% non-fat dry milk (TBS–T–DM), rinsed in TBS and incubated for 1 h at room temperature with the appropriate dilution of primary antibody in TBS–T–DM. After four washing steps in TBS, membranes were incubated for 1 h at room temperature with the appropriate dilution of the matching HRP-labelled secondary antibody in TBS–T–DM. Following the washing, membranes were treated with chemiluminescent reagent (Thermo Scientific Pierce) and subjected to autoradiography.

Antibodies

A polyclonal antibody was raised in sheep against an oligopeptide derived from rtGR1 (271GVIKQENDRRSFC283) conjugated to BSA. Immunoglobulins were obtained from the antiserum and purified by affinity chromatography. A monoclonal antibody cross reacting with GFP was commercially obtained (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). Peroxidase-conjugated secondary anti-goat/sheep and antimouse antibodies were obtained from Sigma–Aldrich, while biotinylated secondary anti-sheep antibody was purchased from AbD Serotech (Kidlington, UK).

Immunoblot

COS-7 growing in six-well plates were transfected by lipofectamine 2000 following the manufacturer’s instructions. For one well of a six-well plate containing COS-7 at 95% confluency, 2 µg of GR expression construct and 2 µg of irrelevant DNA (pBluescript SK) were used. Forty-eight hours after the transfection, the cells were harvested and whole-cell extracts were prepared using a lysis buffer (20 mM Tris–HCl (pH 7.5), 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100) supplemented with a commercial protease inhibitor cocktail (Thermo Scientific Pierce, Cramlington, UK). After centrifugation of the cell extracts (5 min at 10 000 g and 4 °C), proteins (30–50 µg) from the supernatant were resolved on 10% SDS–PAGE and transferred to nitrocellulose. Membranes were blocked in Tris-buffered saline (TBS; 10 mM Tris–HCl (pH 7.4), 2 mM EDTA, 150 mM NaCl) containing 0.05% Tween-20 and 5% non-fat dry milk (TBS–T–DM), rinsed in TBS and incubated for 1 h at room temperature with the appropriate dilution of primary antibody in TBS–T–DM. After four washing steps in TBS, membranes were incubated for 1 h at room temperature with the appropriate dilution of the matching HRP-labelled secondary antibody in TBS–T–DM. Following the washing, membranes were treated with chemiluminescent reagent (Thermo Scientific Pierce) and subjected to autoradiography.

Cellular localisation and nuclear transfer studies

Two approaches were used to elucidate whether GR mutants with partial or full deletions of the A/B domain show altered subcellular localisation in the presence or absence of glucocorticoids. COS-7 cells transiently expressing the receptor of study were either subjected to immunocytochemistry (ICC) to reveal subcellular
receptor localisation or GFP-tagged receptors were created and studied by confocal fluorescence microscopy. COS-7 cells were seeded into 24-well plate containing circular glass coverslips at a density of 2×10^4 cells (ICC experiments) or 8×10^5 cells per well (experiments with GFP-tagged receptors). The following day, the cells were transfected with polyfect reagent (Qiagen) according to the manufacturer’s instructions. The total DNA transfected consisted of 25% of receptor DNA and 75% of irrelevant stuffer DNA (pBluescript SK). After 48 h of incubation at 37°C in fully supplemented DMEM (ICC) or 36 h of incubation in fully supplemented DMEM and 12 h of incubation in DMEM lacking serum (experiments with GFP-tagged GRs), cells were washed with PBS and treated for 2 h with 10^-7 M dexamethsone or 0.1% ethanol (solvent control) in DMEM without supplements. After washing with PBS, cells were fixed with 3% paraformaldehyde for 30 min at 4°C, followed by incubation with PBS containing 0.2 M glycine for 10 min at 20°C.

**Immunocytochemistry**

Following fixation, specimens were treated with 0.02% Triton X-100 for 20 min at room temperature, washed in PBS and blocked with 0.2% normal rabbit serum. Incubation with primary antibody (anti-rTGR1, 1:2000) was overnight at 4°C. The next day, cells were washed three times with PBS (5 min each) before being incubated with biotinylated secondary antibody (1:250) for 30 min. After PBS washes, samples were incubated with HRP–streptavidin (Vector Laboratories UK, Peterborough, UK; 1:250) for 30 min. Following washing with PBS, a commercial kit (VIP substrate, Vector Laboratories, UK, Peterborough, UK; 1:250) for 30 min. Following fixation and mounting, cells were examined under a compound light microscope with white light or a mercury source with a Hoechst 33342 stain and excitation using a 405 nm diode laser with emission recorded at 411–453 nm. GFP fluorescence (500–560 nm) was recorded after argon laser excitation at 488 nm. Per coverslip, 25 image-stacks comprising 25 z-sections were captured on three random positions using the sequential scanning mode to prevent crosstalk and maximum projections derived. Two replicate coverslips were examined per treatment in each experiment. After double-blinded encryption of digital micrographs, the intracellular localisation of signal was rated according to five scoring categories (see above).

**Confocal microscopy**

Following fixation and mounting, cells were examined using a TCS SP2 confocal microscope (Leica, Heidelberg, Germany). Nuclear position was established using a Hoechst 33342 stain and excitation using a 405 nm diode laser with emission recorded at 411–453 nm. GFP fluorescence (500–560 nm) was recorded after argon laser excitation at 488 nm. Per coverslip, 25 image-stacks comprising 25 z-sections were captured on three random positions using the sequential scanning mode to prevent crosstalk and maximum projections derived. Two replicate coverslips were examined per treatment in each experiment. After double-blinded encryption of digital micrographs, the intracellular localisation of signal was rated according to five scoring categories (see above).

**Dexamethasone-binding assays**

COS-7 cells were grown in 24-well plates and transfected using lipofectamine 2000 as described above. A total of 20 µg receptor cDNA were used per plate. After transfection, cells were incubated in fully supplemented DMEM for 48 h. Cells were then washed twice with PBS, followed by exposure (1 h at 37°C) to different concentrations of [3H]dexamethasone [6,7-3H(N)] (Perkin-Elmer, Boston, MA, USA) (1.56, 3.12, 6.25, 12.5, 25 and 50 nM) in the absence or presence of 500-fold excess unlabelled dexamethasone, dissolved in DMEM lacking PBS (Bland & Hewison 2001). After two washing steps with PBS, tritiated hormone was extracted from the cell monolayer with ethanol and quantified by scintillation counting. Specific binding was derived as the difference between total binding (cells exposed to tritiated hormone alone) and non-specific binding (cells exposed to tritiated hormone with an excess of unlabelled hormone). Confirming the lack of endogenous GR in COS-7 cells, total and non-specific binding were undistinguishable in non-transfected cells and in a similar range to the values of non-specific binding in GR-transfected cells (data not shown). In COS-7 transiently expressing wild-type or mutant trout GR specific binding followed Michaelis–Menton-type saturation kinetics in accordance with the presence of a single binding site. However, the upper asymptote differed significantly between experiments, most likely reflecting differences in transfection efficiency. To account for this, a non-linear least squares model-fitting approach was used to fit a Michaelis–Menton-type model of binding B versus substrate concentration [U_i,j] for measuring jin experiment i. The model allowed for a different asymptote B_{max,i} (estimated number of binding sites) for each experiment i but a single kinetic parameter K_i:

\[ B_{ij} = \frac{B_{max,i} [U_{ij}]}{[U_{ij}] + K_i} + \varepsilon_{ij} \]

The error term \( \varepsilon \) was assumed to be normally distributed. Models were fitted using the R modelling
environment (http://www.r-project.org/). A benefit of this model over a transformation of the data to a linear model is that it does not put undue weight on the data points in a particular range of B.

Bioinformatic analyses

Pairwise protein alignments were carried out using the program EMBOSS::needle (global) with the Blosum62 matrix, as implemented on the website of the European Bioinformatics Institute (http://www.ebi.ac.uk/). Physico-chemical parameters of the A/B domains of rtGR1 and rtGR2 were analysed using the ProtParam tool provided on the Expert Protein Analysis System proteomics server of the Swiss Institute of Bioinformatics (http://expasy.org/). Secondary structure consensus predictions were performed using the Network Protein Sequence Analysis platform (Combet et al. 2000) available on the website of the Pôle Bioinformatique Lyonnais Gerland (http://pbil.ibcp.fr/). Protein regions of naturally disordered structure (Dunker et al. 2002) were predicted using the trained neural network program PONDR (http://www.pondr.com).

Statistical analysis

To compare transactivation activities after optimal (10⁻⁶ M) dexamethasone treatment among receptors, data were log-transformed and subjected to repeated measure ANOVA, followed by post hoc comparisons with Bonferroni’s test. Means were considered to be significantly different when the probability value (P) was < 0.05. To derive median effective concentration (EC₅₀) of hormonal stimulation of transactivation activity, sigmoidal curves of a log-logistic model were fitted to the data in the regression module of SigmaPlot for Windows, version 10.

Results

In rainbow trout, two GRs called rtGR1 and rtGR2 have been described (Ducouret et al. 1995, Bury et al. 2003), which markedly differ in glucocorticoid sensitivity in transactivation assays (Fig. 1A). The amino acid sequence of rtGR1 and rtGR2 shows little homology (< 25% identity) in the A/B domain, a receptor region known to contain a main transactivation function called AF1 in hGR (Giguere et al. 1986). To test the hypothesis that the A/B domains of rtGR1 and rtGR2 also harbour major transactivation functions, we generated receptor mutants lacking this domain, called GR1delA/B and GR2delA/B. The ability of mutant receptors to stimulate transcriptional activity of a reporter gene was assessed in a co-transfection assay in the monkey kidney cell line COS-7, which lacks endogenous corticosteroid receptors. The deletion mutants retained the ability to induce transcription in a glucocorticoid-dependent manner (Fig. 1B), but transactivation activity at optimal hormone levels (10⁻⁶ M dexamethasone) was reduced to < 3% of the corresponding wild-type receptor activity (Fig. 1A). Moreover, ablation of the A/B domain resulted in a right shift of the dose–response relationship, as evident from increased median EC₅₀ of receptor transactivation in the mutants (GR1delA/B 15.7 ± 1.9 nM, GR2delA/B 3.6 ± 0.7 nM, Fig. 1B).

Figure 1 Transactivation activities of rtGR1, rtGR2 (A) and rtGR1- and rtGR2-derived mutants lacking the A/B domain, called GR1delA/B and GR2delA/B (B). COS-7 cells were transiently transfected with wild-type or recombinant GRs, a luciferase reporter containing the MMTV promoter, and a β-galactosidase internal reporter to correct for transfection efficiency. Cells were treated for 36 h with different concentrations of dexamethasone or vehicle control. Transactivation activities were determined by normalising luciferase to galactosidase activities, and expressing values as percent of the corresponding wild-type receptor activity with 10⁻⁶ M dexamethasone. Each point is the mean and S.E.M. of three separate experiments. Median effective concentrations (EC₅₀ ± S.E.M.) of the dexamethasone stimulation of transactivation were 3.6 ± 0.3 nM for rtGR1, 0.30 ± 0.05 nM for rtGR2, 15.7 ± 1.9 nM for GR1delA/B and 3.6 ± 0.7 nM for GR2delA/B.
when compared to wild-type receptors (rtGR1 3·6 ± 0·3 nM, rtGR2 0·30 ± 0·05 nM, Fig. 1A). The ligand-binding affinities of wild-type or mutant GRs were assessed in whole-cell [3H]dexamethasone-binding assays using COS-7 cells transiently expressing the receptors. Specific binding of tritiated hormone was obtained as the difference between total and non-specific binding (Fig. 2A). Exemplary data for one experiment are shown as a Scatchard plot (Fig. 2B). However, for any given receptor, maximum specific binding significantly varied between experiments, most likely reflecting differences in transfection efficiency. To obtain experiment-wide estimates of the dissociation constant $K_d$, a modified Michaelis–Menten model allowing $B_{\text{max}}$ to vary between experimental repeats was designed (see section Materials and methods), and fitted to the data using non-linear least squares regression (Table 1). The dexamethasone-binding affinities, expressed as the dissociation constant $K_d$, were similar between rtGR1 and GR1delA/B, and between rtGR2 and GR2delA/B (Table 1). Estimates for the number of binding sites, $B_{\text{max}}$, did not differ significantly among receptors (Table 1). Together, results from the dexamethasone-binding assays demonstrated that GR1delA/B and GR2delA/B were expressed at similar levels as rtGR1 and rtGR2, and showed similar hormone-binding affinities to the corresponding wild-type receptors. This is in accordance with the hypothesis that the loss of transactivation activity in GR1delA/B and GR2delA/B resulted from the loss of AF1 functions located in the A/B domains of rtGR1 and rtGR2.

The activity of AF1 is itself hormone independent; however, in the context of the intact hGR AF1 activity is suppressed by the E-domain in the absence of ligand (Hollenberg & Evans 1988). Accordingly, mutants of hGR lacking the E-domain show constitutive transactivation activity (Hollenberg et al. 1987). In this study, mutants of rtGR1 and rtGR2 lacking the E-domain, called GR1delE and GR2delE, showed appreciable transactivation in the absence of hormone (GR1delE, 8·7%; GR2delE, 16·3%; percent of the activity of the corresponding wild-type receptor after optimal hormone treatment of 10−6 M dexamethasone; Fig. 3, left panel). By contrast, in the absence of hormone the activity with wild-type receptors rtGR1 and rtGR2 was <1% of optimally hormone-induced activities (Fig. 1A). Immunoblot analysis using an anti-rtGR1 antibody confirmed that COS-7 cells transiently transfected with GR1delE expressed an immunoreactive protein fraction of approximately the expected apparent molecular mass, at levels somewhat higher than those of rtGR1 in the same expression system (Fig. 3, right panel).

Table 1 Dexamethasone binding to wild-type and mutant trout glucocorticoid receptors (GRs)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$n$</th>
<th>$K_d$ (nM)</th>
<th>$B_{\text{max}}$ (nmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rtGR1</td>
<td>4</td>
<td>30·4 (23·0–37·8)</td>
<td>1·21 ± 0·80b</td>
</tr>
<tr>
<td>rtGR2</td>
<td>4</td>
<td>8·6 (6·6–10·6)</td>
<td>1·85 ± 0·79b</td>
</tr>
<tr>
<td>GR1delA/B</td>
<td>4</td>
<td>34·4 (26·8–41·9)</td>
<td>1·89 ± 0·78</td>
</tr>
<tr>
<td>GR2delA/B</td>
<td>3</td>
<td>10·1 (8·3–11·8)</td>
<td>3·23 ± 0·74</td>
</tr>
</tbody>
</table>

See the legend of Fig. 2 for experimental details.

aValues in brackets are 95% confidence limits.

bValues are mean and s.d. Differences in $B_{\text{max}}$ between receptors were not significant.
Together, the above results suggested the presence of an AF1 in the A/B regions of rtGR1 and rtGR2. While the A/B domain of the GR is fairly well conserved among mammalian species (e.g. >80% amino acid identity between rat and hGRs), it shows only moderate conservation between hGR and rtGR1 (30-2% amino acid identity), hGR and rtGR2 (22-1% amino acid identity) or rtGR1 and rtGR2 (24.5% amino acid identity). The AF1 domain has been mapped to amino acids 77 to 262 of hGR (Hollenberg et al. 1987), with a core region essential for transactivation spanning from 187 to 227 of hGRa; (Dahlman-Wright et al. 1994). To obtain tentative localisations of the AF1 region and its core, the trout receptors were aligned with hGR (Fig. 4A and B). The hGR AF1 was aligned with amino acids 70 to 230 of rtGR1 and 1–119 of rtGR2, while its core matched amino acids 176–206 of rtGR1 and 47–75 of rtGR2 (Fig. 4A and B).

Using bioinformatic methods, we attempted to identify structural and secondary structural features in the A/B domain of rtGR1 and rtGR2 related to the transactivation function localised to this domain. Generally, steroid receptor A/B domains tend to be rich in proline and serine residues (together >10%; Lavery & McEwan 2005). These amino acids were also frequent in the A/B domains of the trout receptors (21-8% in rtGR1 and 25-2% in rtGR2). Moreover, the AF1 of steroid receptors often show acidic isoelectric points (Lavery & McEwan 2005). On the basis of tentative localisations of AF1 (Fig. 4A and B), this AF also had acidic properties in rtGR1 and rtGR2 (pI of 4.13 and 3.75 respectively). The AF1 function of GR, but also MR, AR and ERa, comprises a mixture of potential α-helices and β-strands (Lavery & McEwan 2005). Moreover, extended regions of naturally disordered structure (40 or more residues) have been found in the AF1 domains of steroid receptors (Lavery & McEwan 2005). Bioinformatic analyses confirmed the presence of the above structural features in the A/B domains of rtGR1 and rtGR2 (Fig. 4C; hGR shown for comparison). Particularly noteworthy as a common trait across the receptors is the presence of predicted helices in the AF1 and its core in hGR, and in regions aligning to AF1 and its core in the trout receptors (Fig. 4C). By contrast, while predicted extended naturally disordered regions were present in all of the receptors, no common pattern was discernible regarding the positions of these regions relative to the total length of the A/B domain or the localisation of AF1 (Fig. 4C).

To experimentally test the above tentative localisations of AF1 in rtGR1, the A/B domain was truncated N- or C-terminally in a series of mutant receptors (Fig. 5A). Immunoblot analyses with an anti-rtGR1 antibody demonstrated expression of a subset of rtGR1-derived mutants in transiently transfected COS7 cells at levels roughly comparable to those of rtGR1 (GR1Ndel63 and GR1Ndel166), or at somewhat higher levels (GR1Ndel234; Fig. 5C). The remaining mutants lacked the portion of rtGR1 recognised by the anti-rtGR1 antibody, precluding their study in immunoblots. Ablation of the N-terminal 63 amino acids of rtGR1, which leaves the region aligning with AF1 intact, had only marginal effects on transactivation activity (GR1Ndel63, Fig. 5B and D). The N-terminal truncation of rtGR1 by 166 residues, which removes about half of the region aligning with AF1, but preserves the portion matching the AF1 core, resulted in a drop of activity by about 50% (GR1Ndel166, Fig. 5B and D). Further N-terminal deletions in mutants GR1Ndel234 and GR1Ndel314, which further ablated the portion aligning with the AF1 core, were functionally indistinguishable from GR1NdelA/B (Fig. 5B and D). The C-terminal truncation of the A/B domain of rtGR1 by 134 amino acids resulted in a loss of about 80% of wild-type activity, despite leaving the region aligning with AF1 intact (GR1del 234_370, Fig. 5B and D). Larger C-terminal deletions resulted in receptors indistinguishable from GR1delA/B (GR1del166_370; GR1del234_370; Fig. 5B and D). Taken together, the behaviours of mutants were in accordance with a tentative localisation of an AF in the A/B domain of rtGR1 from alignments, insofar as the AF does not appear to extend N-terminally beyond residue 63. At the same time, the data show that regions on the C-terminal side of residue 234 are required for optimum transactivation activity, suggesting that the AF of the A/B domain of rtGR1 extends C-terminally further than predicted from alignments with hGR.

A similar set of A/B domain truncation mutants was derived from rtGR2 (Fig. 6) and tested for transactivation activity following treatment of cells with serial dilutions of dexamethasone. For space
reasons, only maximum transactivation activities are shown (Fig. 6). With rtGR2, truncating about 25% of the region aligning with AF1 N-terminally, but preserving the region aligning with the AF1 core, provoked a loss of activity of about 30% (GR2Ndel29, Fig. 6). Removing 60 amino acids from the N-terminus of rtGR2, which included a part of the region aligning with the AF1 core, caused activity to drop by more than 80% (GR2Ndel60, Fig. 6). N-terminal deletions of 90 or more amino acids resulted in receptors indistinguishable from a GR2delA/B (GR2Ndel90 and GR2Ndel202, Fig. 6). When the rtGR2 A/B domain was truncated by almost one-third at its C-terminal end, the resulting receptor still contained the region aligning with AF1, and retained more than 60% of the wild-type activity (GR2_del202_290, Fig. 6). GR2del90_290, possessing only the N-terminal 90 amino acids of the A/B domain including the region aligning with the core of AF1, retained about 25% of the wild-type rtGR2 (Fig. 6). Taken together, the results suggested that an AF is present in the region defined by residues 1–119 of rtGR2 predicted from alignments with hGR.

While our results on transactivation activities of rtGR1- and rtGR2-derived mutants appeared to confirm the localisation of AF1 derived from bioinformatics, a caveat is that hormone-dependent transactivation requires nuclear translocation. To exclude the possibility that impaired transactivation in mutant receptors reflected a disruption of nuclear access rather than a specific effect on AF, we examined receptor subcellular localisation in the absence of hormone, and after 2 h of treatment with 10⁻⁷ M dexamethasone.

Figure 4 Alignment of the A/B domain of hGR with that of rtGR1 (A) and rtGR2 (B), prepared with the programme needle. ‘|’ Denotes identical and ‘:’ similar residues between sequences. In hGR, the underlined sequence corresponds to the AF1 domain, also called -1 (Hollenberg et al. 1987), and the area shaded in grey shows the core of AF1 (Dahlman-Wright et al. 1994). (C) Consensus secondary structure prediction for the A/B domains of hGR, rtGR1 and rtGR2. The large black and medium-sized grey bars represent α-helices and β-strands respectively (Combet et al. 2000). Dotted lines show the position of predicted extended naturally disordered regions (Dunker et al. 2002). Double arrows and grey bars indicate the extension of the AF1 domain and its core in hGR (Hollenberg et al. 1987, Dahlman-Wright et al. 1994), or regions aligning with these regions in rtGR1 and rtGR2.
study from our laboratory had found that, unlike mammalian GRs, both rtGR1 and rtGR2 are partially nuclear in the absence of hormone (Becker et al. 2008). In this study, a first experiment assessed the subcellular localisation of rtGR1 or mutant GR1Ndel234 in transfected COS-7 cells using ICC with an anti-rtGR1 antibody (Fig. 7). Micrographs were double blindly encrypted before rating the subcellular distribution of staining by comparing with a panel of example images (Fig. 7, top panel). In the absence of hormone, about 60% of cells expressing rtGR1 showed a predominantly nuclear localisation of staining (N>C), but few cells had an exclusively nuclear signal (N) (Fig. 7, bottom panel). After dexamethasone treatment, a shift of rtGR1 towards a more nuclear localisation upon dexamethasone treatment (Fig. 7).

We next compared the subcellular distribution of GR1delA/B and GR2delA/B with that of the wild-type receptors rtGR1 and rtGR2, using N-terminally GFP-tagged derivatives of the receptors. When GFP-tagged versions of rtGR1, rtGR2, GR1delA/B and GR2delA/B were transiently transfected into COS-7 cells, immunoblot analysis using an antibody recognising GFP confirmed expression of protein fractions of roughly the expected molecular masses (Fig. 8A). The mutant receptors lacking the A/B domain, particularly GFP-GR1delA/B, appeared to be expressed at higher levels than the GFP-tagged wild-type GRs (Fig. 8A). Following transfection, 36 h of incubation with complete media, and serum withdrawal for 16 h to synchronise cells in the G0 cycle (Sackey et al. 1996), cells grown on glass coverslips were treated with dexamethasone or ethanol (controls), fixed, stained with the nuclear dye Hoechst 33342, mounted onto microscope slides and subjected to confocal microscopy. Digital micrographs were double blindly encrypted before being compared with a panel of example images (Fig. 8B). Cells transiently expressing the GFP-tagged rat GR mutant C656G were included as internal controls (data not shown). While few cells (3-2%) expressing rat GR C656G showed a predominantly or exclusively nuclear localisation of signal in the absence of hormone, all cells transfected with this receptor exhibited such a localisation of fluorescence after dexamethasone treatment (10\(^{-7}\) M, 2 h), demonstrating suitability of the experimental conditions to detect the hormone-dependent nuclear transfer of GR. As reported previously (Becker et al. 2008), GFP-tagged rtGR1 and rtGR2 expressed in COS-7 showed a different degree of nuclear localisation in the absence of hormone. In this study, the rtGR1 signal

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Figure 6 Transactivation activities of rtGR2-derived GR mutants lacking the parts of the A/B domain. Left panel: graphical representation of mutants, the pattern-filled part of the bars representing the A/B domain. Right panel: transactivation activities of receptors at an optimal dexamethasone concentration (10\(^{-6}\) M). Symbols denote a statistically significant (P<0.05) difference of activities to those of rtGR2 (†) or GR2delA/B (§).

The graphical representation of mutants, the pattern-filled part of the bars representing the A/B domain. Right panel: transactivation activities of receptors at an optimal dexamethasone concentration (10\(^{-6}\) M). Symbols denote a statistically significant (P<0.05) difference of activities to those of rtGR2 (†) or GR2delA/B (§). Transfection, hormone treatment and calculation of transactivation activities followed the procedures described in the legend of Fig. 1. Each point is the mean and S.E.M. of at least three separate experiments.

Figure 5 Transactivation activities of rtGR1-derived GR mutants lacking the parts of the A/B domain. (A) Graphical representation of mutants, the pattern-filled part of the bars representing the A/B domain. (B) Transactivation activities of receptors at an optimal dexamethasone concentration (10\(^{-6}\) M). Symbols denote a statistically significant (P<0.05) difference of activities to those of rtGR1 (†) or GR1delA/B (§). (C) Immunoblot on lysates of COS-7 cells transiently expressing receptors; 30 μg protein was loaded per lane. 1: mock-transfected cells; 2: rtGR1; 3: GR1Ndel63; 4: GR1Ndel166; and 5: GR1Ndel234. Bars and numbers on the left of the panel indicate the position of molecular mass markers. (D) Dexamethasone concentration dependency of transactivation activity. Transfection, hormone treatment and calculation of transactivation activities followed the procedures described in the legend of Fig. 1. Each point is the mean and S.E.M. of at least three separate experiments.
in untreated control cells was equally distributed between the nucleus and the cytoplasm (C=N) in about 50% of cells, and predominantly (N>C) or exclusively nuclear (N) in the remaining 50% of the cells (Fig. 8C). Dexamethasone treatment caused the GFP-rtGR1 signal to become exclusively nuclear in more than 80% of the cells (Fig. 8C). With GFP-rtGR2, already predominantly nuclear in the absence of hormone, dexamethasone caused only a minor further shift of signal to the nucleus (Fig. 8C). With GFP-GR1delA/B, a predominantly NR localisation was observed both in untreated cells and after dexamethasone treatment (Fig. 8C). GFP-GR2delA/B resembled GFP-rtGR2 in subcellular localisation both in the absence of hormone and after dexamethasone treatment (Fig. 8C).

Discussion

Following the DNA binding of NRs, AFs of the receptor serve to recruit coactivators to the preinitiation complex, which in turn facilitate the binding of components of the general transcriptional machinery. In NRs, a ligand-independent AF1 is located in the
N-terminal A/B domain, whereas a ligand-dependent AF2 resides in the C-terminal E-domain (Wärmmark et al. 2003, Bain et al. 2007). AF1 and AF2 cooperate synergistically to realise the receptor’s full transcriptional potential; however, they differ markedly in structure and function. AF2 is highly structured and involves a conserved segment in the C-terminus of the receptor (Danielian et al. 1992). Coactivators binding to AF2 are characterised by conserved leucine-rich motifs that interact with the AF2 surface, which is formed by helix 12 and other parts of the agonist-bound NR ligand-binding domain (Heery et al. 1997, Moras & Gronemeyer 1998, Bledsoe et al. 2002). In contrast, AF1 is highly variable between NRs and intrinsically unstructured. No common motifs have been identified among coactivators binding to AF1 (Wärmmark et al. 2003, Lavery & McEwan 2005). The selective disruption of hGR AF1 or AF2 had differential, gene-specific effects on the transcriptional activation of different glucocorticoid-responsive genes in a single cell line, demonstrating that the two AFs of GR provide an additional level through which specificity of hormone action is achieved (Rogatsky et al. 2003).

The aim of this study was to characterise AF1 in duplicated GRs from a teleost fish, rainbow trout. We have shown elsewhere, using domain-swap mutants, that the A/B domains can be exchanged between rtGR1 and rtGR2 (A Sturm & NR Bury, unpublished data), or between either trout GR and rat GR (Becker et al. 2008), with little if any effect on transactivation properties. This suggests the functional equivalency of AF1 among these receptors, despite their limited sequence homology in the A/B domain. The hypothesis that AF1 of rtGR1 and rtGR2 functionally resemble those of other GRs is further supported by observations on domain deletion mutants in this study, which paralleled the behaviour of analogous hGR mutants described in early pioneering studies on GR activation functions. Similar to what has previously been shown in hGR (Hollenberg et al. 1987, Hollenberg & Evans 1988), ablation of the A/B domain in rtGR1 or rtGR2 resulted in receptors showing unchanged dexamethasone-binding affinity, but greatly reduced hormone-dependent transactivation activity when compared to the wild-type receptors. Conversely, and again paralleling earlier studies of hGR (Hollenberg et al. 1987, Hollenberg & Evans 1988), removal of the E-domain from rtGR1 or rtGR2 resulted in constitutively active receptors.

In mammals, the unliganded GR locates to the cytoplasm where it is present in a large multiprotein complex. Following glucocorticoid binding, the nuclear transfer of hGR is a prerequisite to its transactivation of glucocorticoid target genes. In a previous study, we have shown that upon expression in the mammalian cell line COS-7 or the trout cell line RTG-2, rtGR1 and rtGR2 display a partially nuclear subcellular distribution in the absence of hormone that resembles that of mammalian MR (Becker et al. 2008). In this study, the deletion mutant GR1Ndel234 showed a complete disruption of transactivation activity, but resembled rtGR1 in subcellular distribution pattern both in the absence of hormone and after dexamethasone treatment. Similarly, GFP-GR2delA/B resembled GFP-rtGR2 in intracellular localisation. In contrast, GFP-GR1delA/B was shifted towards a more nuclear distribution than GFP-rtGR1 in the absence of hormone, but did not differ from the wild-type receptor after glucocorticoid treatment. The predominantly nuclear localisation of unliganded GFP-GR1delA/B could stem from the fact that its expression levels were higher than those of GFP-rtGR1, as overexpression of GFP-tagged proteins can affect their subcellular distribution (Walker et al. 1999). Alternatively, the difference between GR1delA/B and rtGR1 in localisation could potentially reflect a specific role of the rtGR1 A/B domain in nucleocytoplasmic trafficking. In a previous report, results from domain-exchange mutants between rtGR1/rtGR2 and rat GR G656G pointed to the possibility of such roles of the A/B domain in rtGR1 and rtGR2 (Becker et al. 2008). In the context of this study, the fact that after dexamethasone treatment both the mutants GR1Ndel234, GFP-GR1delA/B and GFP-GR2delA/B and the wild-type receptors rtGR1 and rtGR2 adopted a predominantly nuclear localisation suggested that the marked loss of transactivation activity in the mutants was not caused by an impairment of nuclear access by the mutants.

The generation of further mutants of rtGR1 or rtGR2 in this study, with partial truncations of the A/B domain at its N- or C-terminus, generally confirmed the preliminary localisation of AF1 from the alignments of trout receptors with hGR. The AF1 of hGR, originally dubbed α1 (Giguere et al. 1986), has been mapped to amino acids 77 to 262 by deletion analysis (Hollenberg et al. 1987, Hollenberg & Evans 1988). Further studies have identified a 41-amino acid core region within AF1 essential for transactivation, and a 58-amino acid minimal transactivation domain containing the AF1 core retaining about 60% of AF1 activity (Dahlman-Wright et al. 1994). In-depth mutagenic studies have been carried out on the AF1 core in hGR and rat GR (Almlöf et al. 1995, 1997, Iniguez-Lluhi et al. 1997). In hGR, mutation of individual acidic amino acids in the AF1 core had little effect on transactivation (Almlöf et al. 1995). In contrast, most of the 15 hydrophobic amino acids, which are present in clusters in this receptor region, were required for optimal transactivation activity (Almlöf et al. 1997). The majority of these hydrophobic residues (14/15) are matched by identical or similar hydrophobic amino acids in rtGR1 and rtGR2 (data not shown). The most C-terminal hydrophobic cluster of the hGR AF1 core has been found to be
most important in transactivation (Almlof et al. 1997). This segment of hGR conforms to a consensus motif, composed of hydrophobic and acidic residues, shared with AFs of other transcription factors including retinoic acid receptor β2 and VP16 from herpes simplex virus (Almlof et al. 1997). At this site, hGR (194DLIQDLEF199), rGR1 (177DLIQDLEF183), and rGR2 (50DLIQDLEF58) sequences resemble one another closely, with hydrophobic amino acids (bold) at positions 2, 6 and 8. In the rat GR, a detailed mutagenesis study of the AF1 found that mutations negatively affecting transactivation clustered at the AF1 core, with the simultaneous mutation of three key amino acids (Glu219, Phe220, Trp234, corresponding to Glu198, Phe199, Trp213 of hGR) being sufficient to completely disrupt AF1 transactivation (Iniguez-Lluhi et al. 1997). While the first two of these amino acids correspond to the C-terminal end of the first hydrophobic cluster identified by Almlof et al. (1997), discussed above, rat GR Trp234 is not conserved in the trout GRs. In hGR, the minimal transactivation domain within AF1 was largely unstructured in water, but acquired a secondary structure characterised by three α-helical segments in the presence of trifluoroethanol, as evident from NMR spectroscopy (Dahlman-Wright et al. 1995). The formation of secondary structures in the isolated GR AF1 domain was further induced by the natural osmolyte trimethylamine oxide (Baskakov et al. 1999). Moreover, DNA binding of a recombinant protein comprising the combined A/B and C domains of GR induced the formation of additional helical structures (Kumar et al. 1999), whereas the TATA-box-binding protein bound by the recombinant GR AF1 domain coincided with an increase in helical content of AF1 (Kumar et al. 2004). Together, these data support a model of induced fit model of coactivator binding by AF1, in which allosteric interactions induce conformation changes that involve the emergence of secondary structures in the previously unstructured AF1 region, thereby forming interaction surfaces to auxiliary protein factors. Interestingly, mutations in AF1 of the rat GR disrupting transactivation had no effect on AF1-dependent transrepression (Iniguez-Lluhi et al. 1997), suggesting that distinct AF1 surfaces are involved. Within the AF1 core of hGR, segments with a propensity to the formation of α-helical structures have been shown to have a crucial role in transactivation and interaction with the coactivators (Dahlman-Wright et al. 1995, Almlof et al. 1997, 1998, Wärmmark et al. 2000). In line with these data, our bioinformatic analyses predicted α-helices in the putative AF1 core regions of rtGR1 and rtGR2. It has been previously observed that naturally disordered regions often overlap with AF1 in NRs (Lavery & McEwan 2005).

In this study, predicted naturally disordered regions were found in the A/B domains of rtGR1 and rtGR2. However, these regions are located outside the putative AF1 domains, and can be ablated without a complete disruption of transactivation activity, suggesting they are of lesser importance for transactivation by rtGR1 and rtGR2.

In summary, we have mapped and partially characterised AF1 in duplicated GRs from a teleost, rainbow trout. The data will provide the baseline for further studies of AF1 in duplicated teleost GRs. Many teleost fish possess two GRs, which most likely have a common origin. The retention of duplicated GRs in many teleosts suggests neofunctionalisation. The results of this and other studies from our group demonstrate an equivalent functionality of AF1 between rtGR1 and rtGR2 in a co-transfection transactivation assay using the mouse mammary tumour virus promoter (Becker et al. 2008, A Sturm & NR Bury, unpublished data). However, AF1 function might differ with other promoters, or on promoters integrated into the natural chromatin environment. More research is required to elucidate the relation of AF1 function to the duplication of GR in teleosts, and to test the hypothesis that these receptors have undergone neofunctionalisation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Biotechnology and Biological Sciences Research Council (Grant S18960).

Acknowledgements

We thank different colleagues for kindly placing plasmids at our disposal. Dr B Ducouret supplied pCMrtGR, Dr P Prunet provided pCMrtGR2, and pFC31Luc was a gift from Dr F Gouilleux. We are grateful to Prof. M-E Rafestin-Oblin, Dr J Fagart and one anonymous reviewer for their fruitful comments on the manuscript.

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Received in final form 1 September 2010
Accepted 4 October 2010
Made available online as an Accepted Preprint 4 October 2010