Allosteric role of the amino-terminal A/B domain on corticosteroid transactivation of gar and human glucocorticoid receptors

Kaori Oka[1], Andree Hoang[2], Daijiro Okada[1], Taisen Iguchi[3], Michael E. Baker[4, *], Yoshinao Katsu[1, 5, *]

[1]Graduate School of Life Science, Hokkaido University, Sapporo, Japan; [2]Department of Biology, University of California, San Diego, CA, USA; [3]National Institute for Basic Biology, Aichi, Japan; [4]Department of Medicine, University of California, San Diego, CA, USA; [5]Department of Biological Sciences, Hokkaido University, Sapporo, Japan

Correspondence:

mbaker@ucsd.edu

ykatsu@sci.hokudai.ac.jp

Abstract We studied the role of the A/B domain at the amino terminus of gar (*Atractosterus tropicus*) and human glucocorticoid receptors (GRs) on transcriptional activation by various glucocorticoids. In transient transfection assays, dexamethasone [DEX] and cortisol had a lower half-maximal response (EC50) for transcriptional activation of full length gar GR than of human GR. Both GRs had similar responses to corticosterone, while 11-deoxycortisol had a lower EC50 for gar GR than for human GR. In contrast, constructs of gar GR and human GR consisting of their hinge (D domain), ligand binding domain (LBD) (E domain) fused to a GAL4 DNA-binding domain (DBD) had a higher EC50 (weaker response) for all glucocorticoids. To study the role of the A/B domain, which contains an intrinsically disordered region, we investigated steroid activation of chimeric gar GR and human GR, in which their A/B domains were exchanged. Replacement of human A/B domains with the gar A/B domains yielded a chimeric GR with a lower EC50 for DEX and cortisol, while the EC50 increased for these steroids for the human A/B-gar C/E chimera, indicating that gar A/B domains contributes to the lower EC50 of gar GR for glucocorticoids. Our data suggests that allosteric signaling between the A/B domains and LBD influences transcriptional activation of human and gar GR by different steroids, and this allosteric mechanism

evolved over 400 million years before gar and mammals separated from a common ancestor.

Running Title: Amino Terminal Domain on Glucocorticoid Receptor

Keywords: Glucocorticoid Receptor, Evolution, Gar, Intrinsically Disordered Domain, Allosteric regulation of gene transcription

1. Introduction

In humans and other vertebrates, glucocorticoids regulate many physiological responses including carbohydrate and protein metabolism, immune function and the body's anti-inflammatory processes via transcriptional activation of the glucocorticoid receptor (GR) [1,2,3,4]. The GR belongs to the nuclear receptor family of transcription factors, which also contains receptors for sex steroids and other adrenal steroids, thyroid hormone and retinoids [5,6,7,8,9,10]. The GR and other steroid receptors have a characteristic modular structure with three functional domains [8,11,12,13,14] (Figure 1), an N-terminal domain (NTD) containing domains A and B, a central DNA-binding domain (DBD) (domain C), a hinge domain (domain D) and a C-terminal ligand-binding domain (LBD) (domain E). The NTD is intrinsically disordered, unlike the DBD and LBD [15]. The NTD contains an activation function 1 [AF1] domain that is important in transcriptional activation of the GR [15,16,17,18,19,20,21] and other nuclear receptors [22,23,24,25]. AF1 undergoes a conformational rearrangement during transcriptional activation of the GR [15,16,17]. Allosteric interactions between AF1 and other GR domains are important in transcriptional activation of the GR [15,19,26]. Deletion of the NTD in human GR and rat GR yields receptors, which are transcriptionally activated by dexamethasone

[DEX] at about 10% and 6%, respectively, of their full length GRs [27,28]. The strength of the transcriptional response of these deletion mutants to DEX depends on the gene that is transcribed.

The GR has been cloned from several teleost fish, including rainbow trout [*Onocorhynchus mykiss*] [29,30], cichlid [*Haplochromis burtoni*] [31], midshipman fish [*Poarichthys notatus*] [32], carp [*Cyprinus carpio*] [33] and zebrafish [*Danio rerio*] [34]. However, the only study of the NTD (A/B domains) in fish has been in rainbow trout GR1 and GR2 [35], in which the A/B domains were found to influence nuclear localization in the absence of hormone. That is, in the absence of hormone both trout GR1 and GR2 are partially localized in the nucleus and cytoplasm, in contrast to mammalian GR, which is found in the cytoplasm in the absence of hormone. In this respect, trout GR resembles mammalian MR. Studies with chimeras of trout GR and rat GR revealed that the E domain in both trout GR1 and GR2 was the primary determinant of transactivation sensitivity [35,36].

Our interest in the evolution of corticosteroid receptors [14,37,38] stimulated us to study the role of the A/B domains in transcriptional activation of the GR from tropical gar, *Atractosteus tropicus*, by different glucocorticoids. We chose gar because it is a basal fish (Semionotiformes), which evolved before a fish-specific genome

duplication in teleosts [39,40,41]. Thus, instead of two GRs as is found in teleosts, gar would be expected to contain one GR, as has been found for sturgeon (*Acipenser ruthenus*) GR [42]. To study the role of the A/B domains in transcriptional activation of gar GR, we compared transcriptional activation of full length gar GR and of a GAL4 DBD-GR D-E fusion by DEX, cortisol [F], corticosterone [B] and 11-deoxycortisol [S]. Here we report important differences in their response to these steroids, with full length gar GR having a lower half-maximal response (EC50) for steroids than does GAL4 DBD-gar GR D-E. Similar differences in the response to steroids were found for full length human GR and a GAL4 DBD-human GR D-E fusion. These data suggest that the A/B domain is important in transcriptional response of gar and human GR to glucocorticoids.

To investigate the allosteric role of the A/B domains on gar and human GR in their transcriptional response to steroids, we constructed chimeric GRs, in which we swapped the A/B domains on gar GR and human GR. Compared to full length human GR, the chimera of the gar A/B and human C-E domains displayed increased transcriptional activation (lower EC50) by DEX and F. Compared to full length gar GR, the chimera of the human A/B domains and gar C-E domains displayed decreased transcriptional activation by DEX and F. However, both chimeras had increased

transcriptional activity compared to fusions of their GR D-E with GAL4 DBD, supporting a role for an allosteric interaction between the A/B domains and LBD in human and gar GR in providing specificity for different steroids in transcriptional activation of gar and human GR. These data also indicate that this allosteric mechanism evolved over 400 million years ago before the separation of gar and mammals from a common ancestor of terrestrial vertebrates and ray-finned fish.

2. Experimental

2.1. Animals and chemical reagents

Tropical gar (*Atractosteus tropicus*) were obtained as described in the previous report [43]. Animals were anaesthetized in 0.1% (w/v) 3-amino benzoic acid ethyl ester (Sigma-Aldrich, St. Louis, MO, USA). After decapitation, tissues were removed, quickly frozen in liquid nitrogen, and kept at −80˚C. All animal experiments were conducted according to the Guideline for Care and Use of Animals approved by the committees of Hokkaido University. Dexamethasone [DEX], cortisol [F], corticosterone [B], and 11-deoxycortisol [S] were purchased from Sigma-Aldrich. For the reporter gene assays, all hormones were dissolved in dimethylsulfoxide (DMSO) and the final concentration of DMSO in the culture medium did not exceed 0.1%.

2.2. Molecular cloning of gar glucocorticoid receptor

First-strand cDNA was synthesized from 2 μg of total RNA isolated from the liver. Two conserved amino acid regions, GCHYGV located in the DBD and QNWQRFY located in the LBD of vertebrate GRs were selected and degenerate oligonucleotides were used as primers for PCR. After amplification, an additional primer set, CKVFFK located the internal region of GCHYGV and QNWQRFY that is the same region for first PCR, was used for the second PCR.

The amplified DNA fragments were subcloned with the TA-cloning plasmid pGEM-T Easy vector (Promega, Madison, WI), sequenced using a BigDye terminator Cycle Sequencing-kit (Applied Biosystems, Foster City, CA) with T7 and SP6 primers, and analyzed on the 3130 Genetic Analyzer (Applied Biosystems). The 5'- and 3'-ends of the GR cDNAs were amplified by rapid amplification of the cDNA end (RACE) using a SMART RACE cDNA Amplification kit (BD Biosciences Clontech, Palo Alto, CA).

2.3. Construction of plasmid vectors

The full-coding regions of gar and human GR were amplified by PCR and the PCR products were gel-purified and ligated into the pcDNA3.1 vector (Life

Technologies, Carlsbad, CA). GAL4 DBD fusion constructs were prepared by PCR amplification of the D to E domains, which are amino acids 478-767 for gar GR and amino acids 488-777 for human GR (Figure 1). DNA fragments subcloned into pBIND vector (Progemga) [44]. To construct the chimeric GR expression plasmids, two fragments corresponding to the A-B domain, which are amino acids 1-410 in gar GR and amino acids 1-420 in human GR, and C to E domains, which are amino acids 411-767 in gar GR and amino acids 421-777 in human GR, were amplified by PCR, and assembled two fragments for creating chimeric GR using In-Fusin HD (Takara Bio, Tokyo, Japan). All constructs were verified by sequencing.

2.4. Transactivation Assay

Reporter gene assays were performed in human hepatoma cell line (HepG2) or Chinese hamster ovary cell (CHO-K1). Transfection and reporter gene assays were carried out as described previously [45]. HepG2 cells were seeded in 24-well plates at 5 x 104 cells/well in phenol-red free minimum essential medium (Sigma-Aldrich Co.) with 10% charcoal/dextran-treated fetal bovine serum (Hyclone, South Logan, UT). After 24 h, the cells were transfected with 400 ng of reporter construct, 100 ng of pRL-TK (as an internal control to normalize the variation in transfection efficiency;

contains the *Renilla reniformis* luciferase gene with the herpes simplex virus thymidine kinase promoter; Promega), and 200 ng of pcDNA3.1-GR using polyethylenimine (PEI) (Polysciences, Inc, PA). After 5 h of incubation, ligands were applied to the medium at various concentrations. After an additional 43 h, the cells were collected, and the luciferase activity of the cells was measured with the Dual-Luciferase Reporter Assay System (Promega). For GAL4-assays, CHO-K1 cells were transfected with 100 ng GAL4 DBD-GR D-E, 200 ng reporter pG5-luc vector. After 5 hour of incubation, ligands were applied to the medium at various concentrations. After 43 hours, the cells were collected, and the luciferase activity of the cells was measured with Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured using a Turner Designs Luminometer TD-20/20 (Promega). Promoter activity was calculated as firefly (*Photinus pyralis*)-luciferase activity/sea pansy (*Renilla reniformis*)-luciferase activity. All transfections were performed at least three times, employing triplicate sample points in each experiment. The values shown are mean ± SEM from three separate experiments, and dose-response data and EC50 were analyzed using GraphPad Prism (Graph Pad Software, Inc., San Diego, CA).

Preliminary assays with HepG2 cells and CHO-K1 cells determined that the fold response of the different GR constructs to corticosteroids was different in the two cell

lines. We continued our studies of the GR constructs using the cell lines that gave the optimal response to corticosteroids. Thus, we used HepG2 cells for studies with full-length and chimeric GRs and CHO-K1 cells for studies with GAL4 DBD-GR D-E. However, the pattern of the response to steroids was similar for full length GR and GAL4 DBD-GR D-E in both cell lines.

2.5. Statistical methods

Results are presented as mean ± SE (SEM) from three separate experiments. Comparisons between two groups were performed using *t*-test, and all multi-group comparisons were performed using one-way ANOVA followed by Bonferroni test. Dose-response data and EC_{50} were analyzed using GraphPad Prism (version 6.0b; Graph Pad Software, Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

2.6. Construction of a 3D model of Gar GR

The protein data bank (PDB) file for the ligand binding domain of human GR complexed with DEX [PDB: 1M2Z] [46] was download for use as a template to construct a 3D model of gar GR using the homology option in Insight II software [47,48]. The LBD of gar GR and human GR are 73% identical, which is well above

the 50% level required for construction of an accurate 3D model of gar GR. DEX was extracted from 1M2Z and inserted into the un-minimized 3D model of gar GR using the Biopolymer option. Then the gar GR-DEX complex was refined using Discover 3 for 10,000 iterations, using the CVFF force field with a distant-dependent dielectric constant of 2.

3. Results

3.1. Isolation of gar GR

We cloned the GR from gar using degenerate primers designed to match the most conserved parts in the DNA-binding and ligand-binding domains of vertebrate GRs. Rapid amplification of cDNA ends (RACE) was used to obtain the full-length cDNA, and we amplified gar GR cDNA (Genbank accession LC006087). Comparison of our gar GR sequence with human GR revealed that the steroid-binding domain of gar and human GR are 73% identical and the A/B domains are 34% identical. In Figure 1 we also show the comparison of gar GR with sturgeon (*A. ruthenus*) GR (Genbank accession: JQ781067), which like gar evolved prior to the teleost linage whole genome duplication [42]. The steroid-binding domain of gar and sturgeon GR are 90% identical and the A/B domains are 38% identical.

3.2. Phylogenetic analysis

We propose that there is only one gar GR because we could not find a second GR sequence after sequencing over 200 subcloned DNA fragments from PCR reactions. This hypothesis is consistent with the absence of a second GR in sturgeon [42], which like gar, split from the fish stem lineage before a whole genome duplication. Figure 2 shows the phylogeny of gar GR along with sturgeon GR and GR1 and GR2 from trout, flounder, carp and butterflyfish, as well as the human, chicken, alligator and frog GR.

3.3. 3D model of gar GR LBD is similar to human GR LBD.

To compare how gar GR and human GR contact DEX, we constructed a 3D model of gar GR with DEX. As shown in Figure 3, contacts between key residues on human GR and DEX [46] are conserved in our 3D model of gar GR with DEX. In gar GR (Figure 3A), Gln-560, Phe-623, Gln-632, Thr-729, Asn-564 have similar stabilizing contacts with DEX as the corresponding residues in human GR (Figure 3B). Thus, despite the phylogenetic distance between gar GR and human GR, the binding pocket of gar LBD is well conserved, which as discussed below is consistent with similar binding of glucocorticoids by these two GRs.

3.4. Different transcriptional response to glucocorticoids of full length gar GR and the gar LBD-GAL4 DBD fusion.

We constructed an MMTV-driven reporter construct [37], and examined corticosteroid-inducible transcriptional activation of gar GR. Full length gar GR was activated strongly by DEX at 1 nM, F at 10 nM and to a lesser extent by B and S at 100 nM (Figure 4A).

We used the LBD of gar GR fused to GAL4 DBD to investigate the role of the LBD on transcriptional activation by glucocorticoids (Figure 4B). There was a reduced transcriptional response to glucocorticoids by the gar LBD-GAL4 DBD fusion. Response to DEX and F at 1 nM was substantially reduced, and neither B nor S at 100 nM activated gar LBD-GAL4 DBD. We found this response in CHO-K1and HepG2, indicating it is independent in cell type.

3.5. Different transcriptional response to glucocorticoids for full length human GR and the human LBD-GAL4 DBD fusion.

Full length human GR was activated strongly by DEX at 10 nM and by F and B at 100 nM and weakly by S at 100 nM (Figure 5A). There was reduced activation of

human GR LBD-GAL4 DBD by DEX at 10 nM and weak activation by F at 100 nM and no activation by B and S at 100 nM (Figure 5B). These results indicated that the A/B domain also has a role in the response of human and gar GR to glucocorticoids.

Interestingly, full length gar GR (Figure 4A) is more sensitive to DEX, F and S than is full length human GR [Figure 5A] and Gar GR LBD-GAL4 DBD (Figure 4B) is more sensitive to DEX and F than is human GR LBD-GAL4 DBD (Figure 5B).

3.5. Swapping of A/B domains between human and gar GR

To investigate the role of the A/B domains in the corticosteroid activation of human and gar GRs, we constructed chimeric GRs in which their A/B domains, which are only 34% identical, were swapped. The EC50s of DEX and F for full length gar GR were $1.5x10^{-10}$M and $1.3x10^{-9}$M, respectively. For transcriptional activation by B and S of full length human GR and human GR A/B-gar GR C-E and of S for Gar GR A/B-human GR C-E we did not reach saturation of transcriptional activation at 1 μM (Figure 6, Tables 1 and 2). Nevertheless, the partial saturation curves for B and S are informative for investigating differences between the response to these steroids by full length gar and human GR and the two chimeras.

We found that a chimera containing domains C-E from gar GR and domains A/B

from human GR had an increased EC50 for DEX, F, B and S compared to wild type gar GR (Table 1, Figure 6). In contrast, the chimera containing human C-E domains with gar GR A/B domains had a decreased EC50 for DEX and F and a similar EC50 for B and S compared with wild type human GR (Table 2, Figure 6). These results indicate that the A/B domain on gar GR increases the transcriptional response to glucocorticoids and, in this regard, the gar A/B domains are more potent than the human A/B domains.

4. Discussion

Tropical gar belongs to the Semionotiformes, a lineage near the base of ray-finned or bony fishes [39,41]. Phylogenetic analysis of three nuclear genes (*fzd8*, *sox11* and *tyrosinase*) and a concatenated dataset from bony fishes supports the hypothesis that the fish-specific genome duplication occurred after the split of the Acipenseriformes (sturgeons) and the Semionotiformes (gars) from the lineage leading to teleost fish, but before the divergence of Osteoglossiformes [39]. This hypothesis is consistent with studies with sturgeon, *Acipenser ruthenus*, which evolved before fish-specific genome duplication, and which have single GR gene [42].

We find only a single GR in gar. Thus, our data support the hypothesis that the fish-specific genome duplication event occurred within the teleosts between the split of

the Semionotiformes from the fish stem lineage and the origin of the Osteoglossomorpha (335-404 Mya) [39,40,41,42,43,49]

Gar and human GRs diverged from a common ancestor of fish and terrestrial vertebrates over 450 million years ago, which makes comparisons of these GRs useful for understanding the origin and evolution of the roles of different domains in the response of the GR to different glucocorticoids. The sequences of the DBD and LBD of gar and human GR are highly conserved (Figure 1), while their A,B and D domains have diverged substantially. Consistent with the strong conservation of the LBD, a 3D model of the gar LBD with DEX (Figure 3) reveals conservation in gar GR of the amino acids that contact DEX in human GR [46]. Despite these similarities, compared to full length human GR, gar GR is more sensitive [lower EC50] to DEX, F and S (Figures 4A and 5A), which we suggest is due, in part, allosteric signaling between the E domain and A/B domains on the GR.

Analysis of the response to glucocorticoids of full length gar GR (Figure 4A) and human GR (Figure 5A) reveals that gar GR is more sensitive to DEX, F and S. Similarly, gar D-E fused to GAL4 DBD is more sensitive to DEX and F (Figure 4B) than is human D-E fused to GAL4 DBD (Figure 5B). For both gar GR and human GR, replacement of domains A-C with a GAL4 DBD led to substantial loss of sensitivity to

DEX, F, B and S (Figures 4and 5). These results indicate that gar GR D-E domains contribute to its increased sensitivity to DEX and F, compared to human GR and that domains A-C on gar GR are important in the increased sensitivity to glucocorticoids compared to human GR.

Analysis of chimeras in which A/B domains on gar GR and human GR were swapped revealed that the chimera of human A/B and gar domains C-E were less sensitive [higher EC50] to DEX, F, B and S than full length gar GR (Table 1, Figure 6 A and B). In contrast, the chimera of gar A/B and human domains C-E had a lower EC50 for DEX andF and an unchanged response to B compared to full length human GR (Table 2, Figure 6 C and D). These experiments extend previous studies on the role of the A/B domains on transcription by DEX of human and rat GR [27,28] to F and B, which are physiological glucocorticoids [2,3,4,14]. They also show that although the A/B domains of human and gar GR are only 34% identical, the gar A/B domains can increase the transcriptional response of human GR domains C-E to DEX and F. Considering the phylogenetic distance of fish and mammals, this indicates that the role of the A/B domains in transcriptional activation of the GR is ancient.

Allosteric signaling between protein domains on the GR [15,16,17,27,28] and other steroid receptors [22,23,24] is an important regulatory mechanism that is still not

well understood. Early studies identified a peptide in AF1 that contains the core transcriptional activity [50,51]. Recent studies, with a variety of techniques, have been employed to further elucidate the details of allosteric signaling between A/B domains and other domains on the GR and other nuclear receptors [11,13,16,19,52,53]. These studies indicate that interactions between regions in AF1 are important in allosteric signaling in the GR [50,51] Crystallographic analysis of the full length GR would advance our understanding of the interactions of AF1 and other domains on the GR, as has been accomplished for domains C-E on nuclear receptors [13,54,55]. At this time, an obstacle for understanding the mechanism of action of the intrinsically disordered region of the GR [15,16,17,19] is the difficulty in crystallizing it in an active conformation. However, as the amino terminal region of TIF2 binds to AF1 on the GR leading to a conformational reorganization of AF1 [16], it may be possible to co-crystalize TIF2 and GR and begin to understand the interaction of AF1 with TIF2 and other domains on the GR.

Interestingly, studies with human mineralocorticoid receptor [MR] [56,57,58] and zebrafish MR [59] also show an interaction between domains A and B and the hinge and LBD. This interaction is induced by the mineralocorticoid aldosterone (Aldo), and the interaction increases the response to Aldo. However, F is a weak inducer of this

interaction. The A/B domains on human and zebrafish MR can interact with each other's LBD, indicating that this is an ancient property of the MR [59]. The closely related GR and MR are descended from a common ancestor [14,38,60,61], which suggests that the steroid-mediated interaction between the A/B domains and LBD arose in their common ancestor in a basal vertebrate. Further studies of the A/B domains on the MR and GR may provide insights into the mechanism by which the intrinsically disordered region increases their response to corticosteroids.

Acknowledgments

We thank colleagues in our laboratories, especially Naho Yagi and Akira Sugimoto for their technical assistance. We also thank Drs. Arlette Hernandez-Franyutti and Mari Carmen for providing gar tissues.

Funding

K.O. was supported by the Japan Society for the Promotion of Science (JSPS) Research Fellowships for Young Scientists. This work was carried out under the auspices of the National Institute for Basic Biology Cooperative Research Program (11-322 and 12-326 to Y. K) and supported in part by Grants-in-Aid for Scientific Research 23570067 and 26440159 (YK) from the Ministry of Education, Culture, Sports, Science and

Technology of Japan.

References

[1] K. Chapman, M. Holmes, J. Seckl, 11beta-hydroxysteroid dehydrogenases: intracellular gate-keepers of tissue glucocorticoid action, Physiol Rev 93 (2013) 1139-1206.

[2] M. Kadmiel, J.A. Cidlowski, Glucocorticoid receptor signaling in health and disease, Trends Pharmacol Sci 34 (2013) 518-530.

[3] B.S. McEwen, Physiology and neurobiology of stress and adaptation: central role of the brain, Physiol Rev 87 (2007) 873-904.

[4] A.J. Rose, S. Herzig, Metabolic control through glucocorticoid hormones: an update, Mol Cell Endocrinol 380 (2013) 65-78.

[5] M.E. Baker, Origin and diversification of steroids: co-evolution of enzymes and nuclear receptors, Mol Cell Endocrinol 334 (2011) 14-20.

[6] J.T. Bridgham, G.N. Eick, C. Larroux, K. Deshpande, M.J. Harms, M.E. Gauthier, E.A. Ortlund, B.M. Degnan, J.W. Thornton, Protein evolution by molecular tinkering: diversification of the nuclear receptor superfamily from a ligand-dependent ancestor, PLoS Biol 8 (2010).

[7] G.V. Markov, R. Tavares, C. Dauphin-Villemant, B.A. Demeneix, M.E. Baker, V. Laudet, Independent elaboration of steroid hormone signaling pathways in metazoans, Proc Natl Acad Sci U S A 106 (2009) 11913-11918.

[8] F.M. Sladek, What are nuclear receptor ligands?, Molecular and Cellular Endocrinology 334 (2011) 3-13.

[9] S. Bertrand, M.R. Belgacem, H. Escriva, Nuclear hormone receptors in chordates, Molecular and cellular endocrinology 334 (2011) 67-75.

[10] M.E. Baker, Expanding the structural footprint of xenoestrogens, Endocrine Disruptors 2 (2014) e967138.

[11] P. Huang, V. Chandra, F. Rastinejad, Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics, Annual review of physiology 72 (2010) 247-272.

[12] J.M. Olefsky, Nuclear receptor minireview series, The Journal of biological chemistry 276 (2001) 36863-36864.

[13] F. Rastinejad, P. Huang, V. Chandra, S. Khorasanizadeh, Understanding nuclear receptor form and function using structural biology, Journal of molecular

endocrinology 51 (2013) T1-T21.

[14] M.E. Baker, J.W. Funder, S.R. Kattoula, Evolution of hormone selectivity in glucocorticoid and mineralocorticoid receptors, J Steroid Biochem Mol Biol 137 (2013) 57-70.

[15] R. Kumar, E.B. Thompson, Folding of the glucocorticoid receptor N-terminal transactivation function: dynamics and regulation, Mol Cell Endocrinol 348 (2012) 450-456.

[16] S.H. Khan, S. Awasthi, C. Guo, D. Goswami, J. Ling, P.R. Griffin, S.S. Simons, Jr., R. Kumar, Binding of the N-terminal region of coactivator TIF2 to the intrinsically disordered AF1 domain of the glucocorticoid receptor is accompanied by conformational reorganizations, J Biol Chem 287 (2012) 44546-44560.

[17] S.S. Simons, Jr., R. Kumar, Variable steroid receptor responses: Intrinsically disordered AF1 is the key, Mol Cell Endocrinol 376 (2013) 81-84.

[18] P.J. Godowski, S. Rusconi, R. Miesfeld, K.R. Yamamoto, Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement, Nature 325 (1987) 365-368.

[19] J. Li, H.N. Motlagh, C. Chakuroff, E.B. Thompson, V.J. Hilser, Thermodynamic dissection of the intrinsically disordered N-terminal domain of human glucocorticoid receptor, J Biol Chem 287 (2012) 26777-26787.

[20] R. Rupprecht, J.L. Arriza, D. Spengler, J.M. Reul, R.M. Evans, F. Holsboer, K. Damm, Transactivation and synergistic properties of the mineralocorticoid receptor: relationship to the glucocorticoid receptor, Molecular endocrinology (Baltimore, Md) 7 (1993) 597-603.

[21] S.S. Lim-Tio, M.C. Keightley, P.J. Fuller, Determinants of specificity of transactivation by the mineralocorticoid or glucocorticoid receptor, Endocrinology 138 (1997) 2537-2543.

[22] L. Tora, H. Gronemeyer, B. Turcotte, M.P. Gaub, P. Chambon, The N-terminal region of the chicken progesterone receptor specifies target gene activation, Nature 333 (1988) 185-188.

[23] L. Tora, J. White, C. Brou, D. Tasset, N. Webster, E. Scheer, P. Chambon, The human estrogen receptor has two independent nonacidic transcriptional activation functions, Cell 59 (1989) 477-487.

[24] M.T. Bocquel, V. Kumar, C. Stricker, P. Chambon, H. Gronemeyer, The contribution of the N- and C-terminal regions of steroid receptors to activation of transcription is both receptor and cell-specific, Nucleic Acids Res 17 (1989) 2581-2595.

[25] R. Kumar, G. Litwack, Structural and functional relationships of the steroid hormone receptors' N-terminal transactivation domain, Steroids 74 (2009) 877-883.

[26] R. Kumar, E.B. Thompson, Influence of flanking sequences on signaling between the activation function AF1 and DNA-binding domain of the glucocorticoid receptor, Archives of biochemistry and biophysics 496 (2010) 140-145.

[27] S.M. Hollenberg, V. Giguere, P. Segui, R.M. Evans, Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor, Cell 49 (1987) 39-46.

[28] R. Miesfeld, P.J. Godowski, B.A. Maler, K.R. Yamamoto, Glucocorticoid receptor mutants that define a small region sufficient for enhancer activation, Science 236 (1987) 423-427.

[29] B. Ducouret, M. Tujague, J. Ashraf, N. Mouchel, N. Servel, Y. Valotaire, E.B. Thompson, Cloning of a teleost fish glucocorticoid receptor shows that it contains a deoxyribonucleic acid-binding domain different from that of mammals, Endocrinology 136 (1995) 3774-3783.

[30] N.R. Bury, A. Sturm, P. Le Rouzic, C. Lethimonier, B. Ducouret, Y. Guiguen, M. Robinson-Rechavi, V. Laudet, M.E. Rafestin-Oblin, P. Prunet, Evidence for two distinct functional glucocorticoid receptors in teleost fish, J Mol Endocrinol 31 (2003) 141-156.

[31] A.K. Greenwood, P.C. Butler, R.B. White, U. DeMarco, D. Pearce, R.D. Fernald, Multiple corticosteroid receptors in a teleost fish: distinct sequences, expression patterns, and transcriptional activities, Endocrinology 144 (2003) 4226-4236.

[32] A.S. Arterbery, D.J. Fergus, E.A. Fogarty, J. Mayberry, D.L. Deitcher, W. Lee Kraus, A.H. Bass, Evolution of ligand specificity in vertebrate corticosteroid receptors, BMC Evol Biol 11 (2011) 14.

[33] E.H. Stolte, A.F. de Mazon, K.M. Leon-Koosterziel, M. Jesiak, N.R. Bury, A. Sturm, H.F. Savelkoul, B.M. van Kemenade, G. Flik, Corticosteroid receptors involved in stress regulation in common carp, Cyprinus carpio, J Endocrinol 198 (2008) 403-417.

[34] M.J. Schaaf, D. Champagne, I.H. van Laanen, D.C. van Wijk, A.H. Meijer, O.C. Meijer, H.P. Spaink, M.K. Richardson, Discovery of a functional glucocorticoid receptor beta-isoform in zebrafish, Endocrinology 149 (2008) 1591-1599.

[35] H. Becker, A. Sturm, J.E. Bron, K. Schirmer, N.R. Bury, The A/B domain of the teleost glucocorticoid receptors influences partial nuclear localization in the absence of hormone, Endocrinology 149 (2008) 4567-4576.

[36] A. Sturm, L. Colliar, M.J. Leaver, N.R. Bury, Molecular determinants of hormone

sensitivity in rainbow trout glucocorticoid receptors 1 and 2, Mol Cell Endocrinol 333 (2011) 181-189.

[37] K. Oka, S. Kohno, H. Urushitani, L.J. Guillette, Jr., Y. Ohta, T. Iguchi, Y. Katsu, Molecular cloning and characterization of the corticoid receptors from the American alligator, Mol Cell Endocrinol 365 (2013) 153-161.

[38] M.E. Baker, C. Chandsawangbhuwana, N. Ollikainen, Structural analysis of the evolution of steroid specificity in the mineralocorticoid and glucocorticoid receptors, BMC Evol Biol 7 (2007) 24.

[39] S. Hoegg, H. Brinkmann, J.S. Taylor, A. Meyer, Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish, Journal of molecular evolution 59 (2004) 190-203.

[40] A. Meyer, Y. Van de Peer, From 2R to 3R: evidence for a fish-specific genome duplication (FSGD), Bioessays 27 (2005) 937-945.

[41] J. Postlethwait, A. Amores, W. Cresko, A. Singer, Y.L. Yan, Subfunction partitioning, the teleost radiation and the annotation of the human genome, Trends Genet 20 (2004) 481-490.

[42] Y. Li, A. Sturm, P. Cunningham, N.R. Bury, Evidence for a divergence in function between two glucocorticoid receptors from a basal teleost, BMC Evol Biol 12 (2012) 137.

[43] Y. Katsu, S. Kohno, S. Hyodo, S. Ijiri, S. Adachi, A. Hara, L.J. Guillette, Jr., T. Iguchi, Molecular cloning, characterization, and evolutionary analysis of estrogen receptors from phylogenetically ancient fish, Endocrinology 149 (2008) 6300-6310.

[44] Y. Katsu, S. Kohno, T. Oka, N. Mitsui, O. Tooi, N. Santo, H. Urushitani, Y. Fukumoto, K. Kuwabara, K. Ashikaga, S. Minami, S. Kato, Y. Ohta, L.J. Guillette, Jr., T. Iguchi, Molecular cloning of estrogen receptor alpha (ERalpha; ESR1) of the Japanese giant salamander, Andrias japonicus, Mol Cell Endocrinol 257-258 (2006) 84-94.

[45] Y. Katsu, K. Kubokawa, H. Urushitani, T. Iguchi, Estrogen-dependent transactivation of amphioxus steroid hormone receptor via both estrogen and androgen response elements, Endocrinology 151 (2010) 639-648.

[46] R.K. Bledsoe, V.G. Montana, T.B. Stanley, C.J. Delves, C.J. Apolito, D.D. McKee, T.G. Consler, D.J. Parks, E.L. Stewart, T.M. Willson, M.H. Lambert, J.T. Moore, K.H. Pearce, H.E. Xu, Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition, Cell 110 (2002) 93-105.

[47] M.E. Baker, P. Asnaashari, D.J. Chang, S. McDonnell, 3D models of lamprey

progesterone receptor complexed with progesterone, 7alpha-hydroxy-progesterone and 15alpha-hydroxy-progesterone, Steroids 76 (2011) 169-176.

[48] M.E. Baker, K.Y. Uh, P. Asnaashari, 3D models of lamprey corticoid receptor complexed with 11-deoxycortisol and deoxycorticosterone, Steroids 76 (2011) 1451-1457.

[49] N.R. Bury, A. Sturm, Evolution of the corticosteroid receptor signalling pathway in fish, Gen Comp Endocrinol 153 (2007) 47-56.

[50] K. Dahlman-Wright, H. Baumann, I.J. McEwan, T. Almlof, A.P. Wright, J.A. Gustafsson, T. Hard, Structural characterization of a minimal functional transactivation domain from the human glucocorticoid receptor, Proceedings of the National Academy of Sciences of the United States of America 92 (1995) 1699-1703.

[51] T. Almlof, J.A. Gustafsson, A.P. Wright, Role of hydrophobic amino acid clusters in the transactivation activity of the human glucocorticoid receptor, Mol Cell Biol 17 (1997) 934-945.

[52] J. Liu, N.B. Perumal, C.J. Oldfield, E.W. Su, V.N. Uversky, A.K. Dunker, Intrinsic disorder in transcription factors, Biochemistry 45 (2006) 6873-6888.

[53] A. Christopoulos, J.P. Changeux, W.A. Catterall, D. Fabbro, T.P. Burris, J.A. Cidlowski, R.W. Olsen, J.A. Peters, R.R. Neubig, J.P. Pin, P.M. Sexton, T.P. Kenakin, F.J. Ehlert, M. Spedding, C.J. Langmead, International union of basic and clinical pharmacology. XC. multisite pharmacology: recommendations for the nomenclature of receptor allosterism and allosteric ligands, Pharmacol Rev 66 (2014) 918-947.

[54] F. Rastinejad, V. Ollendorff, I. Polikarpov, Nuclear receptor full-length architectures: confronting myth and illusion with high resolution, Trends Biochem Sci 40 (2015) 16-24.

[55] X. Lou, G. Toresson, C. Benod, J.H. Suh, K.J. Philips, P. Webb, J.A. Gustafsson, Structure of the retinoid X receptor alpha-liver X receptor beta (RXRalpha-LXRbeta) heterodimer on DNA, Nat Struct Mol Biol 21 (2014) 277-281.

[56] P.J. Fuller, Y. Yao, J. Yang, M.J. Young, Mechanisms of ligand specificity of the mineralocorticoid receptor, J Endocrinol 213 (2012) 15-24.

[57] F.M. Rogerson, P.J. Fuller, Interdomain interactions in the mineralocorticoid receptor, Mol Cell Endocrinol 200 (2003) 45-55.

[58] J.B. Pippal, Y. Yao, F.M. Rogerson, P.J. Fuller, Structural and functional characterization of the interdomain interaction in the mineralocorticoid receptor,

Mol Endocrinol 23 (2009) 1360-1370.
[59] J.B. Pippal, C.M. Cheung, Y.Z. Yao, F.E. Brennan, P.J. Fuller, Characterization of the zebrafish (Danio rerio) mineralocorticoid receptor, Mol Cell Endocrinol 332 (2011) 58-66.
[60] J.T. Bridgham, S.M. Carroll, J.W. Thornton, Evolution of hormone-receptor complexity by molecular exploitation, Science 312 (2006) 97-101.
[61] M.E. Baker, D.R. Nelson, R.A. Studer, Origin of the response to adrenal and sex steroids: Roles of promiscuity and co-evolution of enzymes and steroid receptors, J Steroid Biochem Mol Biol (2014).
[62] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, Mol Biol Evol 28 (2011) 2731-2739.

Figure Legends

Figure 1. Comparison of gar (tropical gar, *A. tropicus*) GR with human GR and sturgeon (*A. ruthenus*) GR.

The functional A to E domains are represented schematically with the numbers of amino acid residues indicated. The values within each box indicate the percentage sequence identity of the domain relative to the tropical gar GR.

Figure 2. Phylogenetic analysis of fish and terrestrial vertebrate GRs.

The phylogenetic tree was constructed using the maximum likelihood with JTT+G+I model [62] with 1,000 bootstrap replications, which are shown as percentages at the nodes of the tree. Accession IDs are shown for each GR. Teleosts contain two GRs,

GR1 and GR2, while only one GR has been found in sturgeon [42] and gar.

Figure 3. Comparison of 3D structure of human GR with a 3D model of gar GR.

In gar GR, Gln-560, Arg-601, Phe-613 have stabilizing contacts with the A ring on DEX; Gln-632, Phe-725 contact the 17α-hydroxyl and 16α-methyl, respectively, and Thr-729, Phe-739 and Asn-554 stabilize the C17-side chain. In human GR there are similar stabilizing contacts between Gln-570, Arg-611, Phe-623, Gln-642, Tyr-735, Thr-739, Phe-749 and Asn-564 and DEX. The predicted helix location of each amino acid is shown in parentheses.

Figure 4. Ligand-dependent transactivation of full length gar GR and the ligand binding domain of gar GR

Full length gar GR was transiently transfected into HepG2 cells (A) and the ligand-binding domain the ligand-binding domain of human GR was transiently transfected into CHO-K1 cells (B). Cells were incubated with 1nM, 10nM, and 100nM of DEX, F, B, S, or vehicle (DMSO) alone. Each bar represents the mean of the triplicate determinations, and vertical bars present the mean ± SEM. The y-axis indicates fold activation as measured by luciferase expression compared with the

activity of DMSO treatment alone.

Figure 5. Ligand-dependent transactivation of full length human GR and the ligand binding domain of human GR

Full length human GR was transiently transfected into HepG2 cells (A) and the ligand-binding domain of human GR was transiently transfected into CHO-K1 cells (B). Cells were incubated with 1nM, 10nM, and 100nM of DEX, F, B, S, or vehicle (DMSO). Each bar represents the mean of the triplicate determinations, and vertical bars present the mean ± SEM. The y-axis indicates fold activation as measured by luciferase expression compared with the activity of vehicle (DMSO) treatment alone.

Figure 6. Ligand-dependent transactivation of chimeric gar and human GRs

Concentration-response profiles for chimeras in which the A/B domains were swapped between human and gar GR. Full length gar GR (A), A/B domains of gar GR replaced with human GR A/B domains (B), full length human GR (C) A/B domains of human GR was replaced with gar GR A/B domains (D) were transiently transfected into HepG2 cells. Cells were incubated with increasing concentrations of DEX, F, B, and S. Each bar represents the mean of the triplicate determinations, and vertical bars present the mean ± SEM. The y-axis indicates fold activation as measured by luciferase

expression compared with the activity of vehicle (DMSO) treatment alone.

Table 1. Transcriptional activity of corticosteroids mediated by gar GR and chimera GR (human GR A/B-gar GR C-E).

	DEX	F	B	S
full length gar GR (EC50, [M])	1.5×10^{-10}	1.3×10^{-9}	6.7×10^{-8}	4.1×10^{-8}
95% Confidence Interval ([M])	1.1×10^{-10} to 2.1×10^{-10}	8.5×10^{-10} to 2.0×10^{-9}	4.3×10^{-8} to 1.1×10^{-7}	3.0×10^{-8} to 5.6×10^{-8}
human GR A/B-gar GR C-E (EC50, [M])	4.4×10^{-10}	3.2×10^{-9}	ND[b]	ND[b]
95% Confidence Interval ([M])	3.5×10^{-10} to 5.5×10^{-10}	2.2×10^{-9} to 4.5×10^{-9}		
RP[a] (%)	34	41		

[a] RP, Relative potency; EC50 of gar GR = 100%

[b] ND, Not determined due to lack of saturation at 1µM

Table 2. Transcriptional activity of corticosteroids mediated by human GR and chimera GR (gar GR A/B-human GR C-E).

	DEX	F	B	S
full length human GR (EC50, [M])	1.8×10^{-9}	3.9×10^{-8}	4.6×10^{-8}	ND[b]
95% Confidence Interval ([M])	9.9×10^{-10} to 3.1×10^{-9}	2.7×10^{-8} to 5.6×10^{-8}	2.4×10^{-8} to 8.7×10^{-8}	
gar GR A/B-human GR C-E (EC50, [M])	1.1×10^{-9}	2.3×10^{-8}	4.7×10^{-8}	ND[b]
95% Confidence Interval ([M])	8.2×10^{-10} to 1.6×10^{-9}	1.6×10^{-8} to 3.4×10^{-8}	2.5×10^{-8} to 8.5×10^{-8}	
RP[a] (%)	164	170	98	

[a] RP, Relative potency; EC50 of human GR = 100%

[b] ND, Not determined due to lack of saturation at 1µM

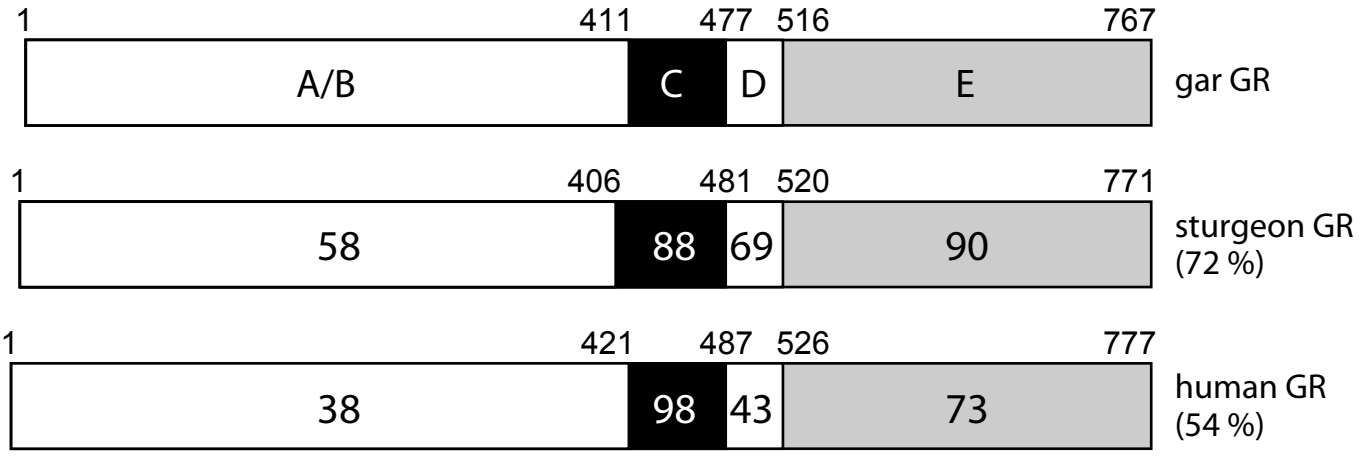

1
411
477
516
767
A/B
C
D
E
gar GR
1
406
481
520
771
58
88
69
90
sturgeon GR
(72 %)
1
421
487
526
777
38
98
43
73
human GR
(54 %)

Figure 1

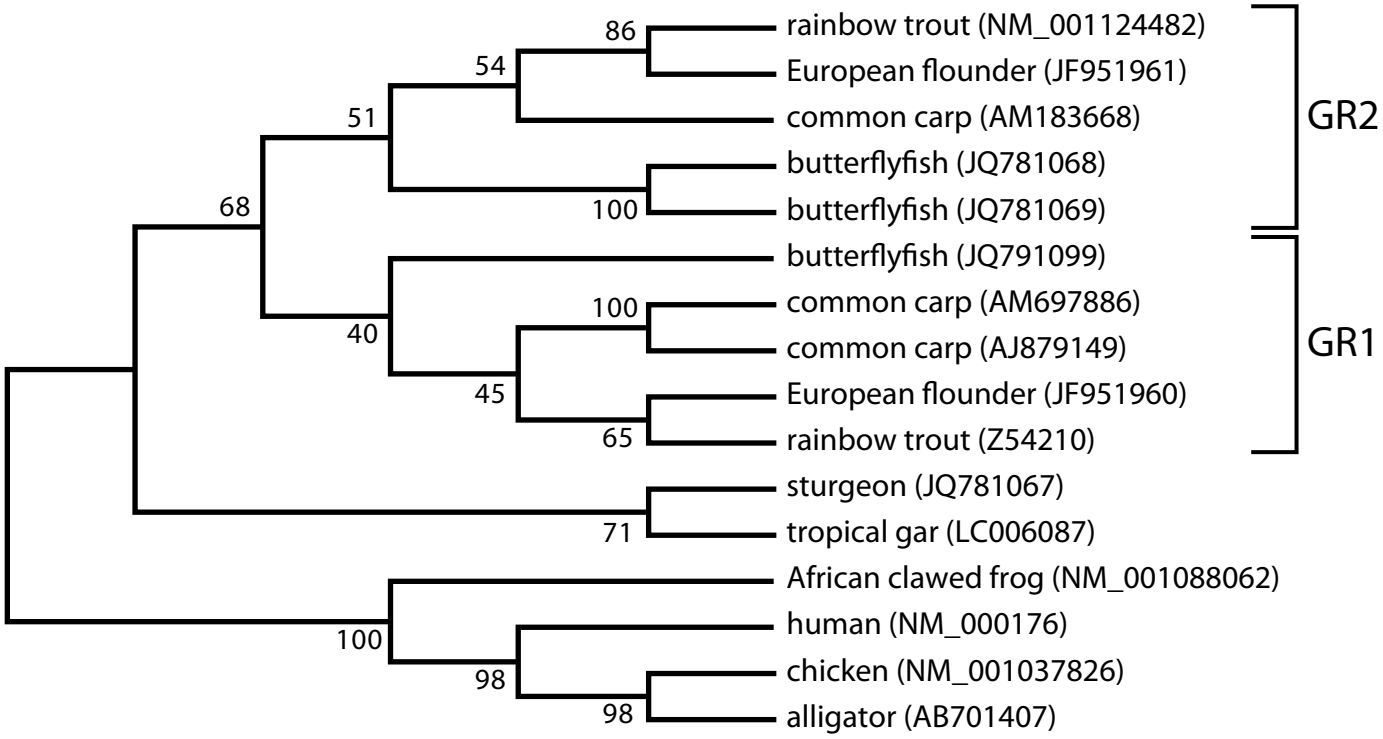

rainbow trout (NM_001124482)
European flounder (JF951961)
common carp (AM183668)
butterflyfish (JQ781068)
butterflyfish (JQ781069)
butterflyfish (JQ791099)
common carp (AM697886)
common carp (AJ879149)
European flounder (JF951960)
rainbow trout (Z54210)
sturgeon (JQ781067)
tropical gar (LC006087)
African clawed frog (NM_001088062)
human (NM_000176)
chicken (NM_001037826)
alligator (AB701407)
86
54
51
68
100
100
40
45
65
71
100
98
98
GR2
GR1

Figure 2

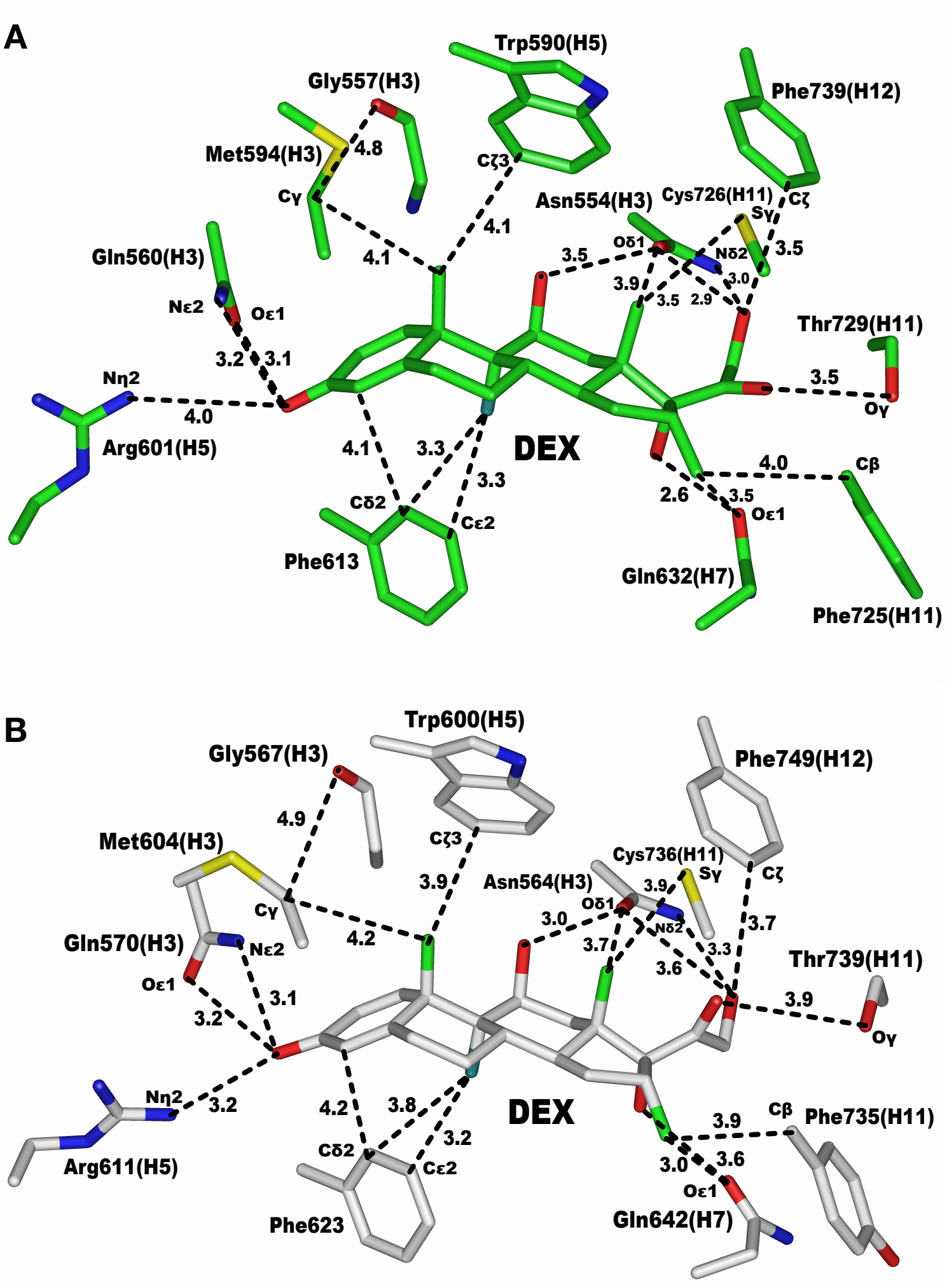
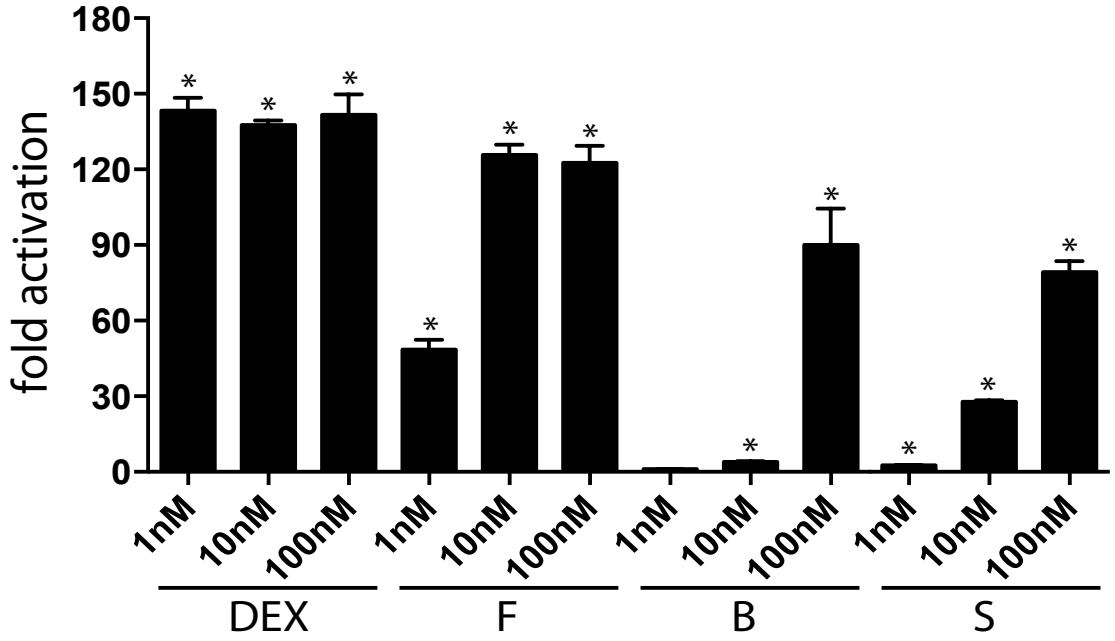

A
Trp590(H5)
Gly557(H3)
Phe739(H12)
Met594(H3)
4.8
Cγ
Cζ3
4.1
4.1
Asn554(H3)
Cys726(H11)
Cζ
Sγ
3.5
Oδ1
Nδ2
3.5
3.9
3.0
3.5
2.9
Gln560(H3)
Nε2
Oε1
3.2
3.1
Thr729(H11)
Nη2
4.0
3.5
Oγ
Arg601(H5)
4.1
3.3
DEX
3.3
4.0
Cβ
Cδ2
2.6
3.5
Cε2
Oε1
Phe613
Gln632(H7)
Phe725(H11)
B
Trp600(H5)
Gly567(H3)
Phe749(H12)
4.9
Met604(H3)
Cζ3
Cys736(H11)
Sγ
Cζ
3.9
Asn564(H3)
3.9
Cγ
Oδ1
3.0
3.7
4.2
Nδ2
3.7
Gln570(H3)
Nε2
3.3
Thr739(H11)
3.6
Oε1
3.1
3.9
3.2
Oγ
Nη2
3.2
4.2
3.8
DEX
3.9
Cβ
Phe735(H11)
3.2
Cδ2
3.0
3.6
Cε2
Oε1
Arg611(H5)
Phe623
Gln642(H7)

Figure 3

A: Full length gar GR



B: GAL4-DBD/gar GR-LBD

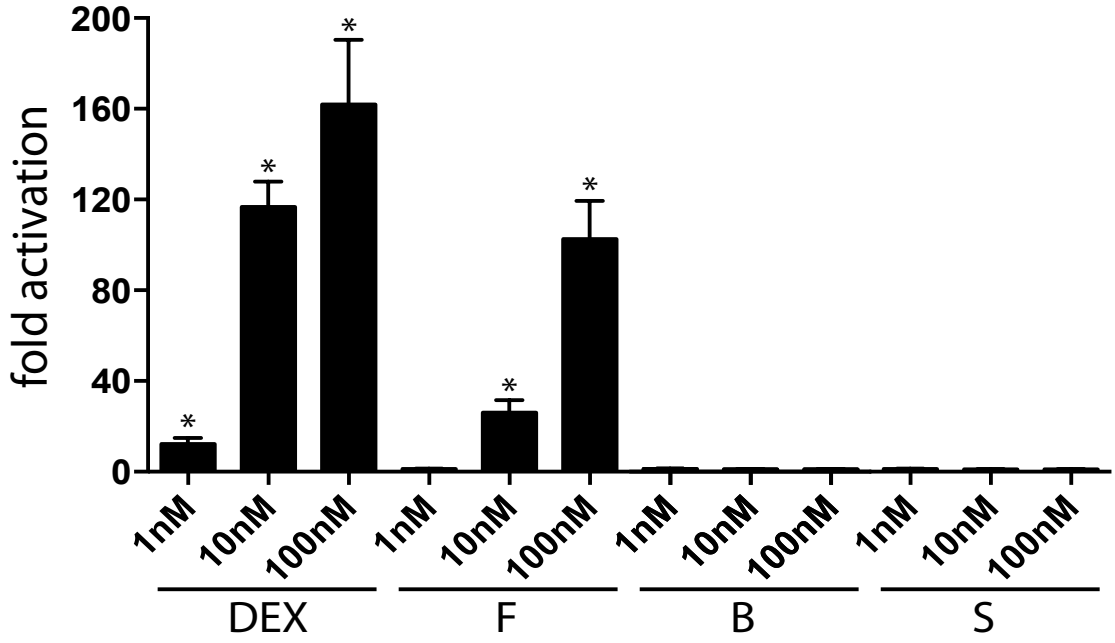
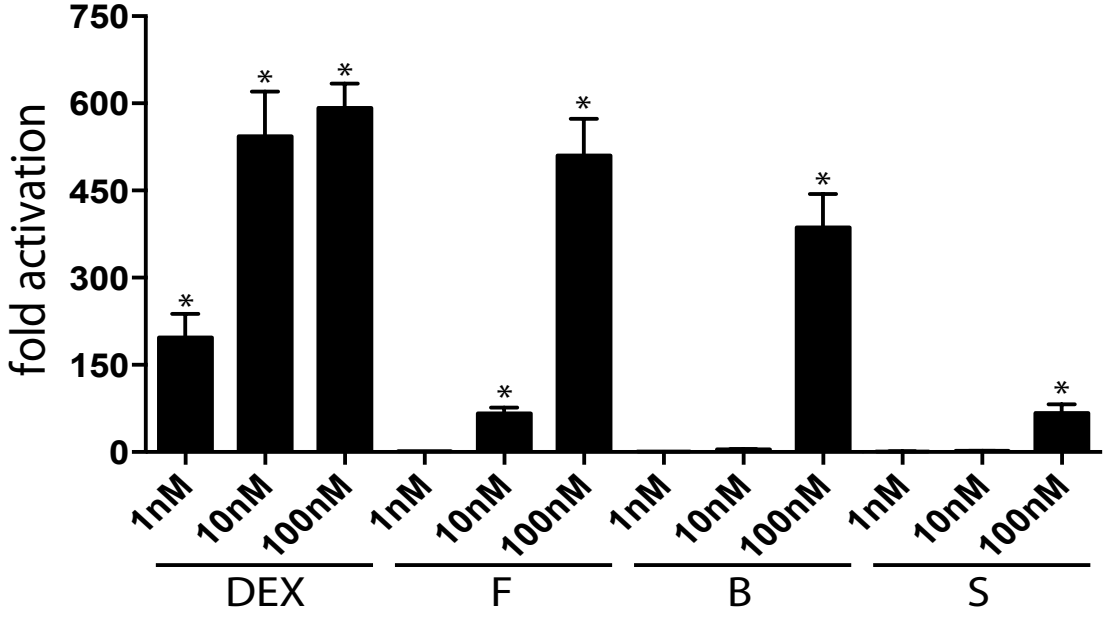


Figure 4

A: Full length human GR



B: GAL4-DBD/human GR-LBD

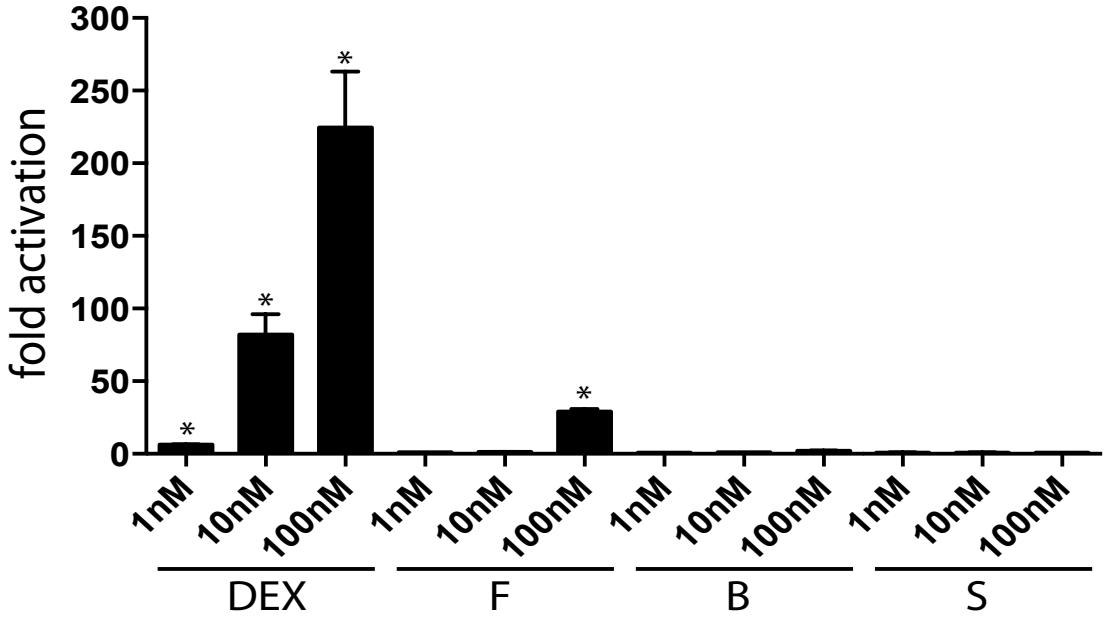
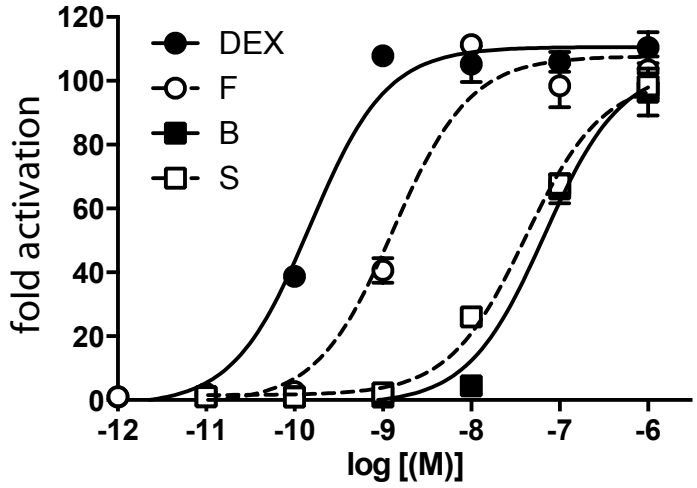
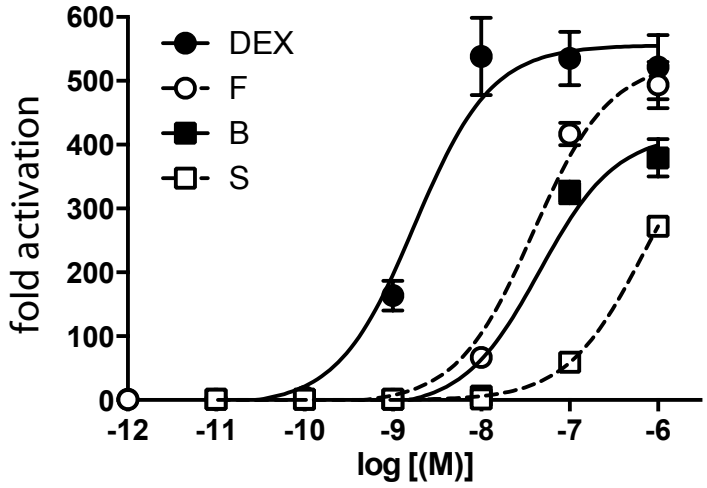


Figure 5

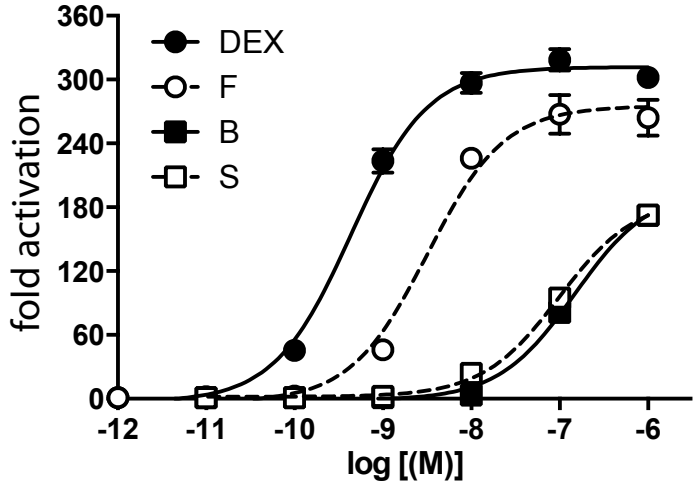
A: Full length gar GR



C: Full length human GR



B: Human GR A/B-gar GR C-E



D: Gar GR A/B-human GR C-E

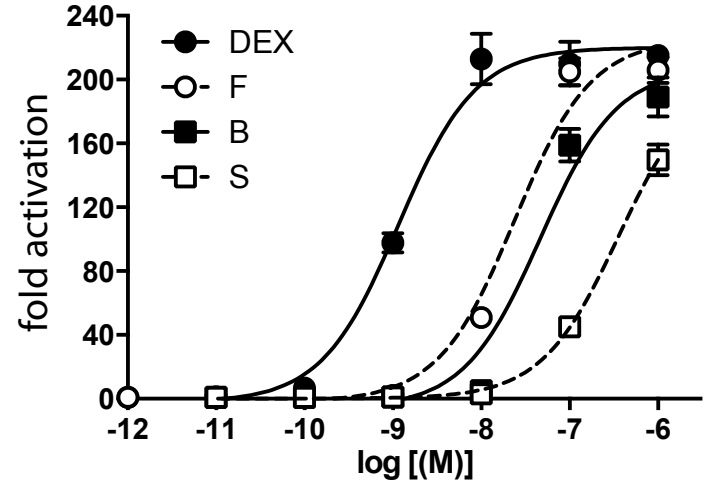


Figure 6