REVIEW

Evolution of glucocorticoid receptors with different glucocorticoid sensitivity

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Abstract

Glucocorticoids (GCs) are commonly used to treat a variety of immune diseases. However, the efficacy of treatment is greatly influenced by an individual variation in sensitivity to GCs, which is caused by differences in the glucocorticoid receptor (GR). The variable receptor profile results from variations in the GR gene, or alternative splicing of the gene coded. We investigated the evolution of the GR gene by comparing genomic GR sequences of vertebrates. Exon length and amino acid sequence are conserved among all classes of vertebrates studied, which indicates strong evolutionary pressure on conservation of this gene. Interestingly, teleostean fishes have two different GR proteins. One of the duplicate fish GR genes has a nine-amino-acid insert in the DNA binding region that results from alternative splicing. The duplicate GR genes and products of alternative splicing in teleostean fishes are differentially expressed *in vivo* and show different transactivation capacity *in vitro*. The presence of two GR genes appears to be a result of divergence of receptors rather than of ligands. Teleostean fishes express different, evolutionarily related, functional GR proteins within a single organism. Hereby, teleostean fishes present a model that facilitates investigation of the molecular basis of cortisol resistance and different regulatory functions of cortisol.

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Introduction

Glucocorticoids (GCs; cortisol and corticosterone) play a pivotal role in vertebrate physiology through a plethora of control mechanisms. The GC system, with a nuclear glucocorticoid receptor (GR), is found in all vertebrates, consistent with an evolutionary well-conserved stress response. GCs regulate cell growth, bone density, metabolism and cardiovascular system and influence behaviour (Charmandari *et al.* 2005). Moreover, *in vitro* studies with human, murine and rat immune cells showed the immunosuppressive effects of elevated GC levels following stressful circumstances. GCs suppress Th1 cellular immunity and mediate a Th2 shift by suppressing production of T helper cells type 1/(Th1; tumour necrosis factor-α, interferon-γ, interleukin (IL)-2 and IL-12) cytokines and inducing production of T helper cells type 1/ (Th2; IL-4, IL-10 and IL-13) cytokines (Elenkov 2004).

For this reason, GCs are used to treat a variety of immune diseases. Local anti-inflammatory properties of GCs make them a first-choice medication for asthma (Walsh 2005) and rheumatoid arthritis (Boers 2004). However, there is considerable individual variation in sensitivity to GCs (Hearing *et al.* 1999), which may affect the outcome of GC treatment. Mutations and splice variation in the GR gene and subsequent polymorphisms in the protein of humans and other vertebrates may explain the individual variation in sensitivity, rather than alteration in the consensus sequence of the GR-binding site in the DNA of target genes (Brandon *et al.* 1991, Keightley & Fuller 1994, Lamberts *et al.* 1996, DeRijk *et al.* 2002, van Rossum *et al.* 2002, Bray & Cotton 2003, Stevens *et al.* 2004*b*).

Recently it was shown that two teleostean (bony) fishes, the rainbow trout (*Oncorhynchus mykiss*; Bury *et al.* 2003) and Burtons' mouthbrooder (*Haplochromis burtoni*; Greenwood *et al.* 2003), each have two different GR genes. The two different receptors encoded by these genes display high amino acid sequence identity, especially in the part of the gene coding for the C-terminal part of the receptor protein. Rainbow trout GR1 and GR2 show 51% overall identity, and 86% identity in the C-terminal part (DNA-binding region, hinge region and ligand-binding region); for Burtons' mouthbrooder, these percentages are 49 and 80% respectively.

Moreover, alternative splicing occurs in these fish GR genes (Takeo *et al.* 1996, Greenwood *et al.* 2003), which leads to functional transcripts with different transactivation properties compared to wild-type. Both the duplicate GR genes as well as the splice variants of these GR genes in fish are differentially expressed in tissues and differ in their affinity for cortisol, the single dominant GC in fish (Bury *et al.* 2003, Greenwood *et al.* 2003).

We question whether differences among GRs affect the sensitivity to GCs and the potential impact of these differences for biological responses. We address the meaning of duplicate genes and splice variants. Genomic database information and review of the literature give evidence that duplicate GR genes and alternative splicing are common features of all teleostean fishes and set them apart from other vertebrate taxa.

Glucocorticoid receptor

The GR (Hollenberg *et al.* 1985) structure and function is well established in mammals and conserved among all vertebrate species analysed so far. The GR belongs to the nuclear receptor superfamily (Fuller 1991, Mangelsdorf *et al.* 1995, Kumar & Thompson 1999, Evans 2005). Its members act as ligand-dependent transcription factors. All receptors in this superfamily, which include those for steroid hormones, thyroid hormones, retinoic acid and vitamin D_3, share a similar domain structure, which was first predicted for the GR (Giguere *et al.* 1986) (Fig. 1). The N-terminal region varies greatly among different members of the superfamily, both in size and composition, and is involved in transactivation of downstream genes (hereafter transactivation). Mutations in this domain decrease transcriptional activity of

target genes without affecting ligand affinity (Giguere *et al.* 1986). The DNA-binding region (see below) is the central domain and binds to glucocorticoid responsive elements (GRE) in promoter regions to initiate transcription of a vast array of GC responsive genes. The amino acid sequence of this region is strictly conserved, both in different members of the superfamily and in virtually all vertebrate species (Fig. 1). The hinge region, involved in conformational changes during receptor–ligand binding, is quite variable in its length and amino acid sequence. The relatively well-conserved ligand-binding region (see below) is situated at the C-terminus. The marked similarity of amino acid sequences in the ligand-binding region explains why multiple receptors can bind the same ligand and, subsequently, elicit a similar response (activation of a downstream gene) in an *in vitro* transactivation assay. In these assays, a cell lacking endogenous corticoid receptors is co-transfected with a transcription vector with the corticoid receptor to be tested and a reporter plasmid bearing one or multiple GREs in its promoter. After addition of the ligand, the *in vitro* expressed corticoid receptor protein can bind the ligand and translocate to the nucleus, where it will subsequently bind to the GRE on the promoter of the reporter gene and induce transcription of this gene. These *in vitro* experiments showed that cortisol enhanced transcriptional activity of the mineralocorticoid receptor (MR) of Burtons' mouthbrooder (EC$_{50}$ 0·02 nmol) at a lower concentration than that required for transactivation via the GR (EC$_{50}$ 2–5 nmol) in one and the same assay. In rainbow trout, a similar result was found; the MR required a lower concentration of cortisol to induce transcriptional activity and a difference was seen for the duplicate GRs with EC$_{50}$s of ∼100 nmol, ∼10 nmol and ∼1 nmol, for GR1, GR2 and MR respectively (Greenwood *et al.* 2003, Sturm *et al.* 2004).

	AB	C	D	E
Human GR	420 aa	66	41	250 aa
Mouse GR	84%	98%	81	94%
Chicken GR	60%	100%	63	88%
African clawed frog GR	47%	100%	56	75%
Tetraodon GR 1a	17%	85%	34	70%
Tetraodon GR 1b	17%	96%	34	**70%**
Tetraodon GR 2	21%	95%	39	74%
Human MR	14%	93%	26	55%
Human AR	14%	78%	17	47%

Figure 1 Percentage amino acid identity of the different receptor regions of several members of the nuclear receptor superfamily of vertebrate species. Length of the boxes represents lengths of particular regions. MR, mineralocorticoid receptor; AR, androgen receptor. AB, N-terminal region; C, DNA-binding region; D, hinge region; E, ligand-binding region.

DNA-binding region

The 3D structure of the DNA-binding region of the human GR was confirmed by nuclear magnetic resonance imaging and it shows two sub-domains (CI and CII), each consisting of a zinc finger (Hard *et al.* 1990).

The most N-terminally located zinc finger is involved in binding of the receptor to DNA. Site-specific DNA recognition depends on the amino acids glycine, serine and valine in the proximal (P) box that bind to the hormone responsive element (GRE) of target genes (Umesono & Evans 1989, Luisi *et al.* 1991). The main loop of this N-terminal zinc finger is a short segment of antiparallel β-sheet, whereas the second pair of cysteines is at the start of an α-helix that provides the contact with the major groove of the GRE. This α-helix between Ser^{459} and Glu^{469} (in rat) is referred to as the DNA recognition helix (Hard *et al.* 1990, Luisi *et al.* 1991). The more C-terminal zinc finger is involved in receptor homodimerisation at the GRE and stabilises binding of the GR to the GRE, which is mediated by amino acids AGRND in the distal (D) box (Luisi *et al.* 1991, Kumar & Thompson 1999, 2005). The zinc fingers together with the conserved amino acids in the two sub-domains result in the 3D structure required for specific DNA binding and thereby for the downstream effects of GCs. This is demonstrated by insertional mutagenesis, where disruption of the C-x-x-C motif silences transcriptional activity of the receptor without interfering with the ligand-binding ability (Giguere *et al.* 1986, Wickert & Selbig 2002).

Ligand-binding region and heat shock proteins (Hsps)

To allow binding of the ligand, the receptor conformation has to change to give the ligand access to a hydrophobic cleft in the ligand-binding region (Pratt & Toft 2003). Heat shock proteins Hsp90 and Hsp70 are both essential as chaperones for the opening of this cleft (Morishima *et al.* 2000*b*). In formation of the hormone-receptor complex, the chaperone Hsp70 first binds directly to the ligand-binding region, which induces a conformational change in the GR that initiates the opening of the steroid-binding cleft, an ATP-consuming process (Morishima *et al.* 2000*a*). Hsp90 binds to this primed GR★Hsp70 complex and completely opens the cleft. As soon as the steroid-binding cleft is opened, cortisol enters and binds to the hydrophobic pocket of the ligand-binding region (Morishima *et al.* 2000*a*, Kanelakis *et al.* 2002, Pratt & Toft 2003). After binding of the hormone with the GR★Hsp70★Hsp90 (GR-hetero) complex, the chaperone complex dissociates. The receptor conformation returns from a labile open structure into a stable compact DNA-binding state that can exist independently of chaperone complexes (Pratt & Toft 1997). Subsequently, the receptor is translocated into the nucleus, where it will homodimerise and bind as a homodimer to the specific DNA motifs (GREs) which in turn will activate transcription of the downstream genes (Schoneveld *et al.* 2004).

However, translocation of the receptor to the nucleus depends on phosphorylation of the receptor (Ismaili & Garabedian 2004). Furthermore, phosphorylation is involved in the half-life of GR and in downregulation following ligand treatment (Webster *et al.* 1997). Thus, GR phosphorylation has an essential effect on the ultimate receptor function (Ismaili & Garabedian 2004).

Although details of the formation of the GR heterocomplex are yet to be established in teleostean fishes, we assume, based on sequence conservation of Hsp70, Hsp90 and the GR ligand-binding region, that comparable processes take place in all fish and thus in vertebrates. At present, data on phosphorylation of the teleostean fish GR proteins are not available. However, we will make a prediction based on conserved phosphorylation consensus motifs in alignments of the N-terminal region of fish and mammalian sequences.

Stress and the immune system

The stress response of all vertebrates, including teleostean fishes, is comparable. In fish, stress signals are conveyed from the hypothalamus via corticotrophin-releasing hormone to the pituitary, where adrenocorticotrophic hormone (ACTH) is released into the circulation. ACTH in turn activates the cortisol-producing cells of the head kidney, the functional equivalent of the adrenal gland. In fish under acute stress, cortisol levels can easily increase more than tenfold, from 10–20 ng/ml to over 350 ng/ml (Wendelaar Bonga 1997). Both basal levels as well as increases in cortisol level during stress are in the same range as those in the mammalian stress response (Van Cauter *et al.* 1996, Hennessy *et al.* 1997, Piekarzewska *et al.* 2000).

High cortisol levels significantly affect the immune system (Sapolsky *et al.* 2000, Franchimont 2004, Charmandari *et al.* 2005). However, the resulting response depends on the parameter studied and type of immune cell involved. *In vitro* studies showed that, in common carp, high levels of cortisol that occur during stress rescue neutrophilic granulocytes from apoptosis by neglecting them (Weyts *et al.* 1998*b*). Activated B-lymphocytes, however, show increased apoptosis when exposed to high levels of cortisol (Weyts *et al.* 1998*a*). Thus, in stressed fish, the innate immune system becomes activated. This is further illustrated by the redistribution of neutrophilic granulocytes from the head kidney into circulation after immersion vaccination (Huising *et al.* 2003). This stress-induced neutrophilia is considered to improve peripheral surveillance and is also reported in mammals (Dhabhar *et al.* 1996). Meanwhile, the adaptive immune system becomes temporarily repressed (Franchimont 2004).

Evolution of vertebrate GRs

GR proteins of teleostean fishes and other vertebrates were compared based on genomic sequences and gene structure of

representatives of mammals, birds, amphibians and bony fishes. Moreover, the duplicate amino acid sequences of Burtons' mouthbrooder and rainbow trout GR proteins and other fish species, for which only a single GR protein has been identified to date, were investigated. From this information, we dare to make general predictions about the similarities and, more interestingly, the differences of fish GR sequences compared to other vertebrate GR sequences.

In this review, we obey the present rainbow trout GR nomenclature with subscripts 'a' and 'b' for splice variants. (Bury *et al*. 2003). In this nomenclature, GR1 has alternative splicing, in which $GR1_a$ has a nine-amino-acid insert and $GR1_b$ does not. In GR2, alternative splicing has so far not been reported. Unfortunately, the published nomenclature of Burtons' mouthbrooder is deviant; $GR2_b$ has the nine-amino-acid insert as a result of alternative splicing, and $GR2_a$ does not have this insert. The GR1 gene does not show alternative splicing (Greenwood *et al*. 2003).

Conservation of sequence identity, but two different GR genes

There are over 23 000 species of teleostean fishes compared to 'only' 14 000 species of birds and mammals taken together (Nelson 1994). Species of teleostean fishes are vastly different and live in very diverse ecosystems, but still use the same basic regulatory mechanisms.

As mentioned earlier, it has been shown that both rainbow trout as well as Burtons' mouthbrooder have duplicated GR genes (Bury *et al*. 2003, Greenwood *et al*. 2003). It was then investigated whether other teleostean fishes also have duplicate copies of their GR gene. Using the Ensembl genome database, the gene structures of both tetraodon (*Tetraodon nigroviridis*) and fugu (*Takifugu rubripes*) GR genes were predicted. Both these teleostean fishes were considered ideal for sequencing as their genomes are extremely compact due to a major lack of intronic DNA and this enabled efficient comparisons with other vertebrate genomes (Hedges & Kumar 2002). As predicted, in the genome of both these species, duplicate copies of the GR gene were found. These two different proteins are encoded on different scaffolds in fugu and on different chromosomes in tetraodon (GR1 on chromosome 7 and GR2 on chromosome 1). The duplicate copies of the GR genes have conserved amino acid sequences, but are distinctly different. Both teleostean fish GR genes are conserved in exon length (Fig. 3) and amino acid sequences (Fig. 1). The zebrafish (*Danio rerio*) genome also has been sequenced. To date, however, only one zebrafish GR gene has been identified.

A phylogenetic tree was constructed, comparing the GR sequences of different vertebrate species using the neighbour-joining method (Saitou & Nei 1987, Kumar *et al*. 2004) (Fig. 2). This phylogenetic tree clearly shows a cluster of tetrapod GRs and a cluster of teleost fish GRs. This cluster of teleost GRs is subdivided into two different clades, representing two different GR genes. The duplicate GR genes of teleostean fishes appear to be the result of the

genome duplication that occurred early in the evolution of actinopterygii, i.e. before the radiation of the teleostean fishes and after the divergence of tetrapods from the fish lineages, which occurred around 450 million years ago (Hedges & Kumar 2002). Duplicate GR genes are seen in both salmonids (rainbow trout) and percomorphs (fugu, tetraodon and Burtons' mouthbrooder), which are two lineages of fish species that diverted very early in teleostean fish evolution. Therefore, it is reasonable to assume that the presence of duplicate GR genes throughout fish species relates to the fish-specific genome duplication that occurred approximately 300 million years ago (Hoegg *et al*. 2004, Nelson 1994, Volff 2005).

A 'corticoid receptor' of a sea lamprey (*Petromyzon marinus*) was included in our analysis. This receptor was named corticoid receptor as it resembled both the vertebrate corticoid receptors, MR and GR. Sea lamprey is an agnathan (jawless) fish of an evolutionary lineage that preceded the teleostean fish lineage. Moreover, it was predicted that evolution of the vertebrate steroid receptors took place from an ancestral gene through serial genome expansions. In this hypothesis, a common corticoid receptor gene was the ancestral entity for both the GR and the MR (Thornton 2001). Indeed, our analysis shows that the lamprey corticoid receptor seems to cluster with the outgroup MR, not with any of the GR genes. Based on this phylogenetic tree, we therefore cannot predict which of the duplicate GR genes is more reminiscent of the ancestral GR.

If the duplication of the GR gene is indeed a result of the fish-specific genome duplication, we predict that many more teleostean fishes will have duplicate copies of their GR gene. In fact, from the genome point of view, all teleostean fishes should have at least duplicate GR genes, but one of the copies could of course be secondarily lost due to mutational events if one copy was sufficient for biological function.

Phosphorylation sites in N-terminal region of fish GR

As mentioned earlier, phosphorylation has significant effects on GR function. By comparing the mouse, human and rat phosphorylation sites, Ismaili and co-workers constructed an instructive scheme of consensus sequences for phosphorylation and the putative protein kinases that are predicted to target certain motifs (Ismaili & Garabedian 2004). By alignment of the GR gene sequence of several vertebrate species (both mammalian and fish), predictions can be made about presence and number of phosphorylation sites in teleostean fishes (Table 1).

The mammals in this alignment show good similarity. Similarity decreased when chicken, African clawed frog and teleostean fishes were compared. The serine residues corresponding to Ser^{122} and Ser^{234} in mouse are conserved in mammals, chicken and African clawed frog. The serine corresponding to Ser^{315} in mouse is conserved throughout all the species but, as there is no consensus motif, it is hard to
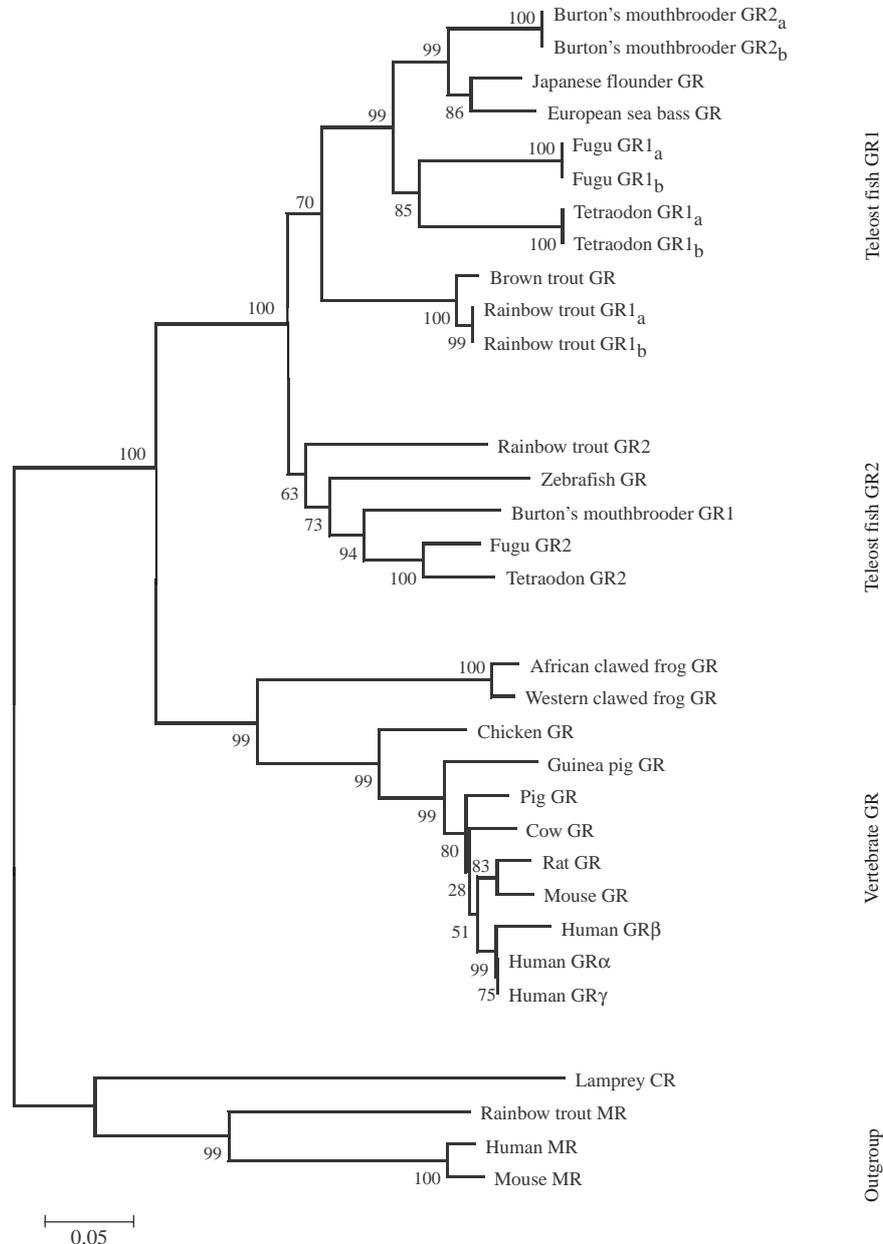
Figure 2 Phylogenetic tree; comparison of the amino acid sequences of the vertebrate glucocorticoid receptors (GRs) and corticoid receptor (CR). This tree was generated with MEGA version 3.1 software (Institute of Molecular Evolutionary Genetics, Pennsylvania State University, PA, USA) using the neighbour-joining method. Reliability of this tree was assessed by bootstrapping using 1000 bootstrap replications (values in percentage are indicated at branch nodes). Mineralocorticoid receptor (MR) sequences were used as outgroup. Human (*Homo sapiens*) GRα, P04150; human GRβ, NP_001018661; human GRγ, NP_001019265; mouse (*Mus musculus*) GR, P06537; rat (*Rattus norvegicus*) GR, NP_036708; guinea pig (*Cavia porcellus*) GR, P49115; pig (*Sus scrofa*) GR, AY779185; cow (*Bos taurus*) GR, AY238475; chicken (*Gallus gallus*) GR, Q8JHA4; African clawed frog (*Xenopus laevis*) GR, P49844; western clawed frog (*Xenopus tropicalis*) GR, NM_001016967; zebrafish (*Danio rerio*) GR, ENSDARESTT00000005443 (Chr.14); rainbow trout (*Oncorhynchus mykiss*) GR1, P49843, GR2, AY4953720; Burton's mouthbrooder (*Haplochromis burtoni*) GR1, AF263738, GR2$_a$, AF263739, GR2$_b$, AF263740; Japanese flounder (*Paralichthys olivaceus*) GR, O73673; European sea bass (*Dicentrarchus labrax*) GR, AY549305; brown trout (*Salmo trutta*) GR, AY863149; sea lamprey (*Petromyzon marinus*) (ancestral) CR, AY028457; human MR, M16801; mouse MR, XP_356093; rainbow trout MR, AY495584.

predict if this serine is indeed part of a true phosphorylation site in all the species.

The alignment suggests that the consensus motif for the casein kinase II, (CKII; S/T(P)–x–x–E/D) is widely present in the AB region of teleostean fishes and found at conserved locations in a series of fish species. Moreover, all fish species examined show one or more mitogen-activated protein kinases motifs (non–polar–x–S/T(P)–P) in their AB domain, yet these motifs do not correspond to conserved locations. Furthermore, the consensus motif for cyclin–dependent kinase (CDK) (S/T(P)–P–x–R/K) could not be found in any of the fish species examined. This indicates that fish exploit a different consensus motif for the CDKs, or that different kinases are used to phosphorylate the GR. Indeed, at the location corresponding to Ser^{212} in mouse, the fish species have a different consensus motif (S/T(P)–x–x–E/D) than mammals.

Whether the conserved serines and consensus motifs for the different kinases do indeed correspond with phosphorylation sites needs to be confirmed by peptide mapping, mutagenesis and phosphorylation studies.

DNA-binding region in fish GR

Another interesting difference between GR proteins in teleostean fishes and other vertebrates is seen in the DNA-binding region. This C-domain is the best-conserved region of the GR protein (98, 100, 100 and 85% amino acid identity between human GR and mouse, chicken, xenopus and tetraodon GR1$_a$ sequences respectively). However, some fish GR1 genes show a 27-nucleotide insert that encodes nine additional amino acids. Apart from this insert, the C-domain of teleostean fishes is almost identical to that in other vertebrates: 96 versus 85% amino acid identity between human GR and tetraodon GR1$_b$ and GR1$_a$ respectively; Fig. 1). These nine amino acid inserts between the two zinc fingers are also remarkably conserved among teleostean fish species. In rainbow trout (Ducouret *et al.* 1995, Takeo *et al.* 1996), Japanese eel (*Anguilla japonica*) (Todo & Nagahama 1998), Japanese flounder (*Paralichthys olivaceus*) (Tokuda *et al.* 1999), Burtons' mouthbrooder (Greenwood *et al.* 2003), brown trout (*Salmo trutta*; AY863149), European sea bass (*Dicentrarchus labrax*; AY549305) and fugu (*T. rubripes*) a **WRARQNTDG** insert is present. A slightly different insert is found in tetraodon (*T. nigroviridis*): **WRARQNT*VC***. Especially, the D to V substitution is interesting, as aspartic acid is hydrophilic and negatively charged, whereas valine is hydrophobic. Glycine and cysteine are also substantially different with regard to size and biochemical properties.

The nine amino acid inserts appear to be the result of alternative splicing and are encoded by an extra exon. This extra exon is 'hidden' in the intron that separates exons 3 and 4 (Fig. 3). This is evident from the genomic sequences of fugu and tetraodon and was confirmed experimentally by the amplification of a genomic DNA fragment in rainbow trout (Lethimonier *et al.* 2002).

Duplicate GRs; expression and transactivation capacities

The presence of duplicate GR genes which also show this particular alternative splicing gives reason to hypothesise that the resulting duplicate receptor proteins have separated functions. This hypothesis is strengthened as teleostean fishes do not possess the mineralocorticoid aldosterone and use cortisol instead of osmoregulation (Dean *et al.* 2003, Metz *et al.* 2003). This adds another important function to the long list of different functions to be regulated by one hormone. To be able to regulate all these different functions with a single hormone, one can assume that differentiation occurred in the hormone receptor.

Differences in receptor protein function could be indicated by differential expression of the duplicate GR genes in different organs. A literature comparison of different species of fish reveals that both genes are expressed in all organs examined. In all these species, GR1 (the gene showing the nine-amino-acid insert) is more strongly expressed than the GR2 gene (Bury *et al.* 2003, Greenwood *et al.* 2003). Moreover, most first-reported teleostean fish GR sequences have the nine-amino-acid insert and would therefore be classified as GR1, such as in European sea bass and brown trout (Ducouret *et al.* 1995, Takeo *et al.* 1996, Todo & Nagahama 1998, Tokuda *et al.* 1999). Whether this reflects relative abundance of the GR1 messenger or preferential primer design is unclear.

Separate biological functions of the duplicate GR forms may further result from different affinity for the ligand or from different transactivation properties. Information about ligand binding is scarce, but in rainbow trout it was reported that duplicate GR genes have similar dexamethasone-binding affinities that are comparable to the affinity of human GR for dexamethasone (Ducouret *et al.* 1995, Ray *et al.* 1999, Bury *et al.* 2003). However, the resulting biological effect, as measured with transactivation assays, is quite different for the duplicate GR genes.

In rainbow trout, concentrations of cortisol required to induce activation of downstream genes is significantly different between the duplicate GR proteins. Rainbow trout (rt)GR2 requires lower concentrations of cortisol (EC$_{50}$ of 0·7 nM) than rtGR1$_a$ (with nine-amino-acid insert; EC$_{50}$ of 46 nM) to induce transactivation (Bury *et al.* 2003).

Variation in transactivation properties of the receptor after stimulation with a ligand is not the only result of the GR protein involved. Variation in transactivation also depends greatly on the ligand used. In *in vitro* experiments in rainbow trout, the GR agonist dexamethasone activated the tested downstream gene at lower concentrations than cortisol in the same experiment: EC$_{50}$ for GR1 stimulated with cortisol was 46 nM while it was 4·3 nM with dexamethasone treatment; for GR2, this was 0·7 nM for cortisol and 0·35 nM for dexamethasone (Bury *et al.* 2003). In other experiments, it was shown that, with the same concentration of ligand, dexamethasone gave a stronger activation of the reporter gene

than cortisol (Takeo *et al*. 1996, Tokuda *et al*. 1999, Greenwood *et al*. 2003).

It is crucial to keep this difference between cortisol and dexamethasone in mind when testing newly found GR genes or GR variants. Indeed, experiments with dexamethasone might slightly (rainbow trout GR2; twofold) or severely (rainbow trout GR1; tenfold) overestimate the cortisol-induced transactivation capacity.

GR splice variants; expression and transactivation capacities

Several different splice variants are described for the GR gene (Oakley *et al*. 1999, Geng *et al*. 2005). However, in this review, we focus only on the splice variations in the DNA-binding region of the GR gene.

Again, as with the duplicate genes, it is interesting to hypothesise about functional differences between the two splice variants. The expression level of the GR1 variant with
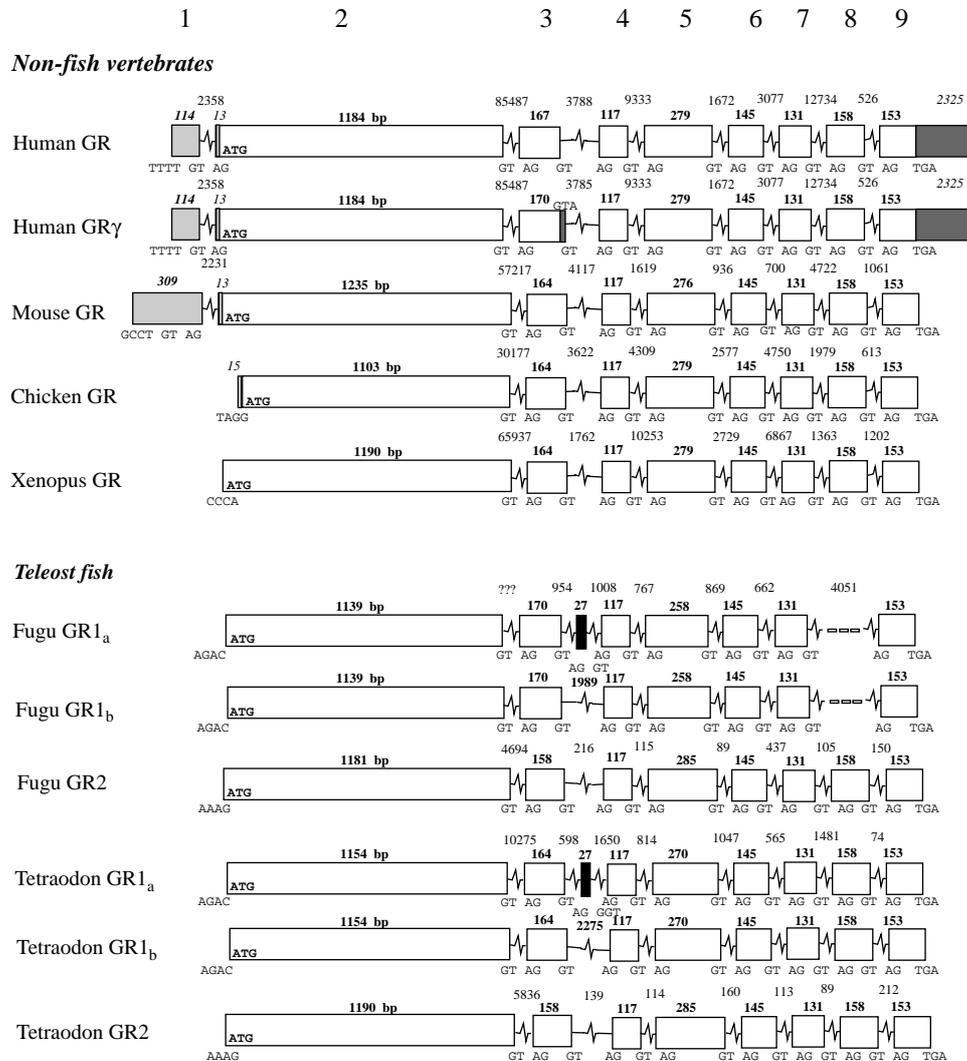

Figure 3 Gene structure of vertebrate GR receptors. Exon lengths are shown in bold and intron lengths in normal font. Splice sequences flanking the exons and nucleotide sequence preceding start codon are shown above blocks representing exons. Untranslated (parts of) exons are depicted in light grey. Elongation of exon 3 by alternative splicing in human GRγ is shown in dark grey. Extra exon, only present in splice variants in fish GR, is depicted in black. Exon 8 in Fugu GR1 could not be found in the Ensembl database. Human (*Homo sapiens*) GR, ENSG00000113580; mouse (*Mus musculus*) GR, ENSMUSG00000024431; chicken (*Gallus gallus*) GR, ENSGALG00000007394; western clawed frog (*Xenopus tropicalis*) GR, ENSXETT00000003968; fugu (*Takifugu rubripes*) GR1, GENSCAN00000003615 (scaffold 1264) and GENSCAN00000029451 (scaffold 4328), GR2, SINFRUG00000143550 (scaffold 59); tetraodon (*Tetraodon nigroviridis*) GR1, GIDT00024792001 (Chr. 7); GR2, GSTENG00017027001 (Chr. 1).

Table 1 Putative phosphorylation motifs in the glucocorticoid receptors of different species[a]

	Mammals	Chicken	African clawed frog	Teleostean fishes	Concensus mammals	Alternative concensus	Kinases mammals	Alternative kinases
Mouse								
S122	Not in guinea pig	Yes	Yes	No	S/T(P)-x-x-E/D		CKII	
S150	Not in guinea pig	No	No	No	None			
T171	Only in rat	No	No	No	Non-polar-x-S/T(P)		MAPK/GSK3	
S212	All	Yes	Different	Different	S/T(P)-P-x-R/K		CDK	
S220	Not in pig, not in cow	Yes	No	No	S/T(P)-P-x-R/K	S/T(P)-x-x-E/d	CDK	CKII
S234	All	Yes	Yes	No	Non-polar-x-S/T(P)		MAPK	
S315	Not in human	Yes	Yes	Yes	None			

[a]Depicted are the residues phosphorylated in mouse GR and a score if these residues are conserved in mammals, chicken, African clawed frog or teleostean fishes. The consensus motif for a particular phosphorylation site is mentioned for mammalian and teleostean fishes separately. The putative protein kinases expected to target these motifs are also shown separately and are based on mammalian literature. This table was constructed after analysis of a multiple sequence alignment of human (*Homo sapiens*) GRα, P04150; mouse (*Mus musculus*) GR, P06537; rat (*Rattus norvegicus*) GR, NP_036708; guinea pig (*Cavia porcellus*) GR, P49115; pig (*Sus scrofa*) GR, AY779185; cow (*Bos taurus*) GR, AY238475; chicken (*Gallus gallus*) GR, Q8JHA4; African clawed frog (*Xenopus laevis*) GR, P49844; zebrafish (*Danio rerio*) GR, ENSDARESTT00000005443 (Chr.14); rainbow trout (*Oncorhynchus mykiss*) GR1, P49843, R2, AY4953720; Burton's mouthbrooder (*Haplochromis burtoni*) GR1, AF263738, GR2$_b$, AF263740; Japanese flounder (*Paralichthys olivaceus*) GR, O73673; European sea bass (*Dicentrarchus labrax*) GR, AY549305; brown trout (*Salmo trutta*) GR, AY863149; fugu (*Takifugu rubripes*) GR1, GENSCAN00000003615 (scaffold 1264) and GENSCAN00000029451 (scaffold 4328), GR2, SINFRUG00000143550 (scaffold 59); tetraodon (*Tetraodon nigroviridis*) GR1, GIDT00024792001 (Chr. 7), GR2, GSTENG00017027001 (Chr. 1) (adapted from Ismaili & Garabedian 2004)

the nine-amino-acid insert exceeds that of the non-spliced form (Takeo *et al.* 1996, Bury *et al.* 2003, Greenwood *et al.* 2003). In Burtons' mouthbrooder, the variant without the insert was found in all organs tested in rainbow trout; however, it could only be detected in testis (Takeo *et al.* 1996, Greenwood *et al.* 2003). As mentioned before, most teleostean fish GR sequences that were found show this nine-amino-acid insert (Ducouret *et al.* 1995, Takeo *et al.* 1996, Todo & Nagahama 1998, Tokuda *et al.* 1999). It remains to be investigated if in all species mentioned a variant of the GR1 gene without the nine-amino-acid insert is also expressed. This variant can easily be overlooked as the difference between the two is so small that specific primers are required to detect them.

Differences in biological functions of the splice variants could also result from different affinity for the ligand or different transactivation properties related to this difference in affinity. As there is no difference between the splice variants in the ligand-binding region, it is unlikely that there is any effect on ligand-binding affinity.

The only differences between the splice variants are found in the DNA-binding region and, indeed, differences in DNA-binding affinity do occur. In an experiment with rainbow trout, two different promoters were used. One luciferase reporter plasmid contained one and the other two GREs from the rat tyrosine aminotransferase promoter. Interestingly, whereas the GR1$_a$ (with the nine-amino-acid insert) has a better binding affinity for single GRE than the GR1$_b$, the opposite was true for the double GRE sequence (Lethimonier *et al.* 2002).

Differences in the final transactivation capacity also occur. Quanta Protein Design computer (Accelrys, Cambridge, UK)

predictions of corticoid receptor DNA-binding domain function based on secondary structure analysis showed that insertions of one-, four- or nine-amino-acid residues are possible without destruction of the secondary structure. However, in all models predicted, the glutamic acid Glu[469] (in rat) was no longer included in forming the DNA recognition helix. The nine-amino-acid insert seen in teleostean fishes may form a loop that extends to the outside of the protein. Receptor function predicted by homology modelling of this peculiar receptor did not differ from functioning of wild-type receptor (Wickert & Selbig 2002). A Predict Protein software analysis (Cubic, Columbia University, New York, NY, USA) predicts that the nine-amino-acid insert would lengthen the α-helix involved in GRE recognition (Lethimonier *et al.* 2002). In rainbow trout, it was experimentally shown *in vitro* that trans-activation after stimulation of rtGR1$_a$ (with insert) with dexamethasone or cortisol was increased compared to GR1$_b$ (without the insert). These results were obtained by the use of RBCF-1 cells that are derived from goldfish fin tissue and a MSG-cat reporter plasmid (Takeo *et al.* 1996). In another experiment with rainbow trout GR using Chinese hamster ovary (CHO-K1) cells, it was again shown that the use of a single GRE luciferase reporter plasmid, the GR1$_a$, gave a stronger hormone-independent transactivation than GR1$_b$. However, with a double GRE reporter gene, this difference was abolished (Lethimonier *et al.* 2002). Moreover, in an *in vitro* co-transfection of Burtons' mouthbrooder with a triple GRE in the tat3-luciferase reporter plasmid, GR2$_b$ (with nine-amino-acid insert) showed only half maximal activation in response to cortisol compared to GR2$_a$ (without insert). These experiments were carried out with CV1b (corticoid receptor-deficient primate renal) cells (Greenwood

et al. 2003). Differences between triple, double and single GRE promoters therefore seem to substantially contribute to the results obtained and hamper a good comparison.

Receptor functionality has been shown for the GR splice variant with the nine-amino-acid insert in rainbow trout, Burtons' mouthbrooder and Japanese flounder (Tokuda *et al.* 1999). Therefore, it is assumed that other teleost GR proteins, with this particular nine-amino-acid insert (or a slight variation thereof) at exactly the same location in the DNA-binding region, will also be functionally active.

Alternative splicing has been reported in mammalian species as well. Human small lung cell carcinoma exhibits a GC-resistant phenotype, which was thought to be caused by a tri-nucleotide insertion in the GR gene; this insertion translates into an additional amino acid, Arg^{453} (Ray *et al.* 1996). This particular human GR splice variant was later named hGRγ (Rivers *et al.* 1999). Although the insertion found in hGRγ occurs at exactly the same location as the nine-amino-acid insert in the fish GR, it results from a different splice variation process. In the human GRγ, an alternative splice site is used that is located three nucleotides downstream of the original splice site at the 3′ end of coding exon 3. This leads to an insertion of one extra codon in exon 3 (Fig. 3) (Rivers *et al.* 1999). In teleostean fishes, in contrast, the original splice site of exon 3 is used, and a whole new exon is introduced between exons 3 and 4. The intron in between exons 3 and 4 contains this extra 27 nucleotide exon, which is flanked by common splice sequences AG at the 5′ end and GT at the 3′ end (Fig. 3). Human GRγ is ubiquitously expressed, but only accounts for 5% of all GR transcripts (Rivers *et al.* 1999, Stevens *et al.* 2004a)

The addition of an arginine as in the human GRγ or the marmoset monkey (see below) would create a new hydrogen bond and, therefore, allow a new 3-turn to be formed, which could affect affinity of receptor–DNA binding (Wickert & Selbig 2002). Indeed, the human GRγ (Arg^{453}) is half maximally activated compared to wild-type with a similar EC_{50} in an *in vitro* co-transfection assay with GR arg^{453} and pMMTV–luciferase reporter plasmid in COS7 (African green monkey kidney) cells (Ray *et al.* 1996, Wang & Fondell 2001). Alternative splicing comparable to that in human GRγ is also seen in the cotton-top marmoset (*Saguinus oedipus*) and thus is of wider occurrence (Brandon *et al.* 1991).

Collectively, these results show that the specificity of the alternatively spliced GRs for the specific target genes tested seems unaffected, but the differences in affinity of DNA binding or efficiency of dimerisation result in changes in transactivation capacity of the GR1 protein with the nine-amino-acid insert. Results of transactivation comparisons between insert and non-insert forms are likely to be affected by different experimental procedures (Lethimonier *et al.* 2002, Greenwood *et al.* 2003). Differences in cell type, reporter plasmid and the use of single or multiple GREs are paramount in the resulting transactivation capacity. It is thus essential to determine how the GR splice variants of different teleostean fish species perform in a standardised transactivation assay.

Variation in the ligand-binding region of fishes and mammals

An important region in the GR gene that can influence cortisol sensitivity is the ligand-binding region. It has been shown that in rat a specific L-x-x-L-L motif (amino acids 547–553) at the N-terminal side of the ligand-binding region is essential for Hsp90 binding. This motif forms a hydrophobic patch that contributes to the stability of the tertiary structure of the ligand-binding region and thereby facilitates steroid binding. Mutations of Leu^{550} or Leu^{553} to Serine dramatically reduce steroid binding and biological activity, without altering the binding of Hsp90 (Giannoukos *et al.* 1999). Interestingly, although Leu^{553} is conserved throughout all vertebrate species, Leu^{550} is not found in any of the teleostean species. In fact, all fish GR sequences described to date have a Met at this location and duplicate GR genes also do not differ at this particular location.

Although there is little information about binding affinities of fish GRs, the affinity for dexamethasone of GR1 (K_d 5·5 nM) and GR2 (K_d 3·5 nM) were shown to be similar and comparable to human GR affinity (K_d 4·6) (Ducouret *et al.* 1995, Ray *et al.* 1999, Bury *et al.* 2003). In common carp (*Cyprinus carpio*) lymphocytes, affinity for dexamethasone was lower (K_d 16 nM) (Weyts *et al.* 1998c). In this experiment, only one or one class of carp GR was detected, although we now know from mRNA expression data that at least two different GRs, with comparable expression profiles, are present in this fish (E.H Stolte *et al.* in preparation). The biochemical analysis used to determine affinity, however, has insufficient resolution to confirm this. It is therefore predicted that the binding affinities of the duplicate GRs will be very comparable. Affinity for cortisol of these carp lymphocyte GRs was K_d 3·8 nM (Weyts *et al.* 1998c). This is comparable to the affinity for cortisol of Coho salmon (*Oncorhynchus kisutch*) gill GR receptors (K_d 2·2 nM) and Chinook salmon (*Oncorhynchus tshawytscha*) brain GR receptors (K_d 4·5 nM) (Maule & Schreck 1990, Knoebl *et al.* 1996).

While the L-x-x-L-L motif in guinea pig (*Cavia porcellus*) is exactly the same as in rat, it has been shown to exhibit resistance to GCs that results from lower sensitivity for the ligand (Kraft *et al.* 1979). Using a domain-swap approach, the guinea pig GR ligand-binding region was inserted in the human GR gene, replacing the human ligand-binding region. The chimeric GR gene product showed a decrease in hormone sensitivity of approximately 40-fold (Keightley & Fuller 1994). In further studies, the critical regions and/or residues of the ligand-binding region were identified and targeted for site-directed mutagenesis (Keightley *et al.* 1998). A similar approach is now used in rainbow trout research (Sturm & Bury 2005). In this case, domain-swap mutants were produced, not between GR genes of different species, but between the two different rainbow trout GR genes, rtGR1 and rtGR2. It was shown that the N-terminal region

of rtGR1 had a more potent transactivation function than rtGR2, whereas the opposite was observed for the ligand-binding region. By combinations of rtGR1 and rtGR2 domains, therefore, hypersensitive and hyposensitive recombinant receptors could be obtained (Sturm & Bury 2005).

In this way, both the guinea pig and the rainbow trout provide naturally 'altered', but functional GRs (Keightley & Fuller 1994). Domain swaps and site-directed mutagenesis provide the opportunity to investigate the molecular basis of cortisol resistance. GR domain-swap research within one species, however, can only be performed on teleostean fishes.

Perspectives

To fully comprehend the functional implications of the different teleostean GRs, we now search for (immune) cells that differentially express GRs. *In situ* hybridisation is probably the most powerful tool. To objectively compare the mammalian and the teleostean GRs, they have to be tested in the same *in vitro* system. This will yield only approximations of fish receptor function as the protein is expressed in a mammalian cell with its mammalian responsive machinery. An important advantage of studies with fish is that we can study receptor function at different biologically relevant ambient temperatures as fishes are poikilotherms. This is especially interesting as it is known from studies with mammals that GR function is critically dependent on Hsps/stress proteins, the expression of which is temperature dependent. However, to perform transactivation studies with a teleostean GR at different temperatures, one would need a cell type that functions properly at low temperature, preferably a fish cell type. The major drawback is that, as yet, no fish cell types/lines comparable to their mammalian counterparts and lacking endogenous corticoid receptors have been described.

Summary and conclusion

Teleostean fishes have duplicate GR proteins where other vertebrates only have one. This likely results from the fish-specific genome duplication event. One of these two GR genes has two different transcripts that are generated by alternative splicing. The most abundant of these two splice variant transcripts has a nine-amino-acid insert in its DNA-binding region. This insertion is the result of transcription of an extra exon, which is located in the intron between exons 3 and 4, a feature that is unique for the GRs of teleostean fishes. Most interestingly, the three different GR forms in fish are differentially expressed *in vivo* and show different transactivational capacities, but only slightly different affinity for their ligand. As both genes and different splice variants are transcriptionally active, it is suggested that they both play an important and probably different role in the fishes' physiology.

The splice variation found in the DNA-binding region of the GR gene of teleostean fishes, occurring at exactly the same location as the DNA-binding region splice variation in mammals, but resulting from a different mechanism, is associated with different affinity for DNA binding and different transactivation capacity. Moreover, fish with two different GR genes exhibit intraspecies genetic variation that gives the opportunity to investigate which region of the GR gene and which residues within these regions are involved in ligand affinity and resulting transactivation activity. Since transactivation capacity following cortisol binding in different GR gene products does not necessarily correlate with transactivation capacity after dexamethasone binding, it is of paramount importance to check biological activity of both these ligands when a novel GR sequence is discovered. The multiple corticoid receptors in fish should provide a stimulating model to test the hypothesis of ligand exploitation (Thornton 2001). In fish, a major function is ascribed to cortisol, but also significant levels of 11-deoxycortisol and 11-deoxycorticosterone occur (Sturm *et al.* 2004) and these ligands bind to GRs (and MRs). The notion of a far more complex signalling by related steroids and a subtle repertoire of adjusted/adjusting receptors may eventually greatly improve our understanding of fish physiology. The same notion may give rise to a better understanding of variation in GC sensitivity in man.

Acknowledgements

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