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Functional analysis reveals no transcriptional role for the glucocorticoid receptor β-isoform in zebrafish

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In humans, two splice variants of the glucocorticoid receptor (GR) exist: the canonical α-isoform, and the β-isoform, which has been shown to have a dominant-negative effect on hGRα. Previously, we have established the occurrence of a GR β-isoform in zebrafish, and in the present study we have investigated the functional role of the zebrafish GRβ (zGRβ). Reporter assays in COS-1 cells demonstrated a dominant-negative effect of zGRβ but no such effect was observed in zebrafish PAC2 cells using induction of the *fk506 binding protein 5 (fkbp5)* gene as readout. Subsequently, we generated a transgenic fish line with inducible expression of zGRβ. Transcriptome analysis suggested transcriptional regulation of genes by zGRβ in this line, but further validation failed to confirm this role. Based on these results, its low expression level and its poor evolutionary conservation, we suggest that the zebrafish GR β-isoform does not have a functional role in transcriptional regulation.

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1. Introduction

The glucocorticoid receptor (GR) is expressed throughout the human body and regulates a wide variety of biological processes, like our metabolism, growth, reproduction, vascular tone, bone formation, immune response and brain function (Chrousos and Kino, 2005; de Kloet et al., 2005; Heitzer et al., 2007; Revollo and Cidlowski, 2009; Sapolsky et al., 2000; Schoneveld et al., 2004). The GR is activated upon binding to glucocorticoid (GC) ligands, and acts as a transcription factor, orchestrating gene expression via DNA-binding-dependent and -independent mechanisms (Beato and Klug, 2000, Buckingham, 2006; De Bosscher and Haegeman, 2009, Heitzer et al., 2007; Nicolaides et al., 2010; Schoneveld et al., 2004; van der Laan and Meijer, 2008). Cloning of the human GR gene revealed the occurrence of two splice variants, named as hGRα and hGRβ, which derive from alternative usage of an acceptor splice site within the last coding exon (exon 9, see Suppl. Fig.1) (Encio and Detera-Wadleigh, 1991; Hollenberg et al., 1985). The GR α-isoform (777 amino acids) is able to interact with GCs, and represents the canonical receptor. The hGR β-isoform (742 amino acids) has a shorter LBD with a unique C-terminal 15 amino acid sequence, which renders it unable to respond to GCs (Encio and Detera-Wadleigh, 1991; Hollenberg et al., 1985; Kino et al., 2009a,b).

Almost ten years after the discovery of hGRβ, it was shown using *in vitro* reporter assays that this isoform had a pronounced dominant-negative inhibitory effect on hGRα's transcriptional properties on GRE-containing promoters (Bamberger et al., 1995; Oakley et al., 1996, 1999). Moreover, hGRα-mediated repression of NF-κB activity in *in vitro* reporter assays was also reported to be inhibited by hGRβ (Oakley et al., 1999). These findings were soon coupled to clinical data demonstrating a positive correlation between high expression levels of the β-isoform and GC resistance of patients suffering from immune-related disorders, like asthma (Christodoulopoulos et al., 2000; Goleva et al., 2006; Hamid et al., 1999; Hamilos et al., 2001; Leung et al., 1997; Sousa et al., 2000), ulcerative colitis (Fujishima et al., 2009; Orii et al., 2002; Zhang et al., 2005), leukemia (Koga et al., 2005; Longui et al., 2000; Shahidi et al., 1999) and rheumatoid arthritis (Derijk et al., 2001; Goecke and Guerrero, 2006). Further cell-based research has supported an inhibitory role for hGRβ on both hGRα-induced activation and repression of endogenous genes (MKP-1, myocilin, fibronectin, TNFα and IL6 (Goleva et al., 2006; Li, 2006; Zhang, 2005)), as well as on hGRα-mediated regulation of cell death, proliferation and

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phagocytosis (Hauk et al., 2002; Strickland et al., 2001; Zhang et al., 2007). Moreover, recent data support the notion that hGRβ can have its own intrinsic transcriptional activity. Overexpression of zGRβ has been demonstrated to attenuate NF-κB and AP-1 induction of luciferase reporter constructs (Gougat et al., 2002), as well as GATA3-mediated activation of IL5 and IL13 promoters of luciferase genes (Kelly et al., 2008). Additionally, transcriptome analyses of cultured cells showed that hGRβ can direct gene transcription independently of hGRα activation (Kino et al., 2009a,b; Lewis-Tuffin et al., 2007). In a recent study, overexpression of hGRβ in the liver of mice showed both GRα-dependent and -independent regulation of gene expression by hGRβ (He et al., 2016).

Despite the available data described above, the physiological relevance of hGRβ is still under debate. First, in many studies the dominant-negative role of hGRβ on hGRα's transcriptional properties could not be confirmed (Bamberger et al., 1997; Brogan et al., 1999; Carlstedt-Duke, 1999; Gougat et al., 2002; Hecht et al., 1997; Kelly et al., 2008; Kim et al., 2009; Taniguchi et al., 2010). Second, hGRβ's expression levels are significantly lower compared to those of hGRα (Bamberger et al., 1995; de Castro et al., 1996; Oakley et al., 1996, 1997; Pujols et al., 2002; Strickland et al., 2001). This raises doubts about its in-vivo dominant-negative effect, since in most studies this required transfection of a 10-M excess of hGRβ expression vector compared to hGRα (Bamberger et al., 1995; Oakley et al., 1996, 1999). Third, the evolutionary conservation appears to be poor. Previously, we showed that the GRβ protein sequence is only conserved between primates, and that the gene organization required for GRβ expression is present in a limited group of placental mammals (Schaaf et al., 2008). For example, rodents, cats, dogs and hedgehogs contain a mutation in the splice acceptor site required for GRβ expression (Otto et al., 1997; Schaaf et al., 2008). As a result, until recently no animal model was available for functional studies on GRβ.

Remarkably, several years ago we discovered the occurrence of a GR β-isoform in zebrafish (Schaaf et al., 2008). Like its human equivalent, the zebrafish GR β-isoform differs from the α-isoform at the C-terminus and diverges from the GRα sequence at the same point as the human GRβ. Both the human and zebrafish GR β-isoforms exhibit the same predominantly nuclear localization, and zGRβ also acts as a dominant-negative inhibitor of zGRα-mediated transactivation in *in vitro* reporter assays (Schaaf et al., 2008). However, differences exist between these GR β-isoforms, which demonstrates that they have evolved independently. First, the GRβ-specific C-terminal sequences are very different between zebrafish and human GRβ (Schaaf et al., 2008; Yudt et al., 2003). Second, in the human GR gene the GRβ-specific sequence is located in exon 9, whereas in the zebrafish GR gene it is found in the intronic sequence immediately downstream of exon 8 (Suppl. Fig.1). Thus, human and zebrafish GRβ mRNA are generated using different alternative splicing mechanisms (exon replacement and intron retention, respectively (van der Vaart and Schaaf, 2009)). A few years after our discovery of the zebrafish GRβ, a GR β-isoform generated by intron retention was observed in mice as well, and this isoform appears to play a role in metabolic regulation (Hinds et al., 2010).

The scope of this study was to investigate the role of zGRβ in gene transcription either as a dominant-negative inhibitor of zGRα or as a transcription factor independent of zGRα. We have utilized both *in vitro* and *in vivo* approaches. While a dominant-negative effect of zGRβ was observed in reporter assays in cultured cells, our data did not reveal any dominant-negative activity of zGRβ on endogenous genes in either cultured cells or zebrafish larvae. Furthermore, we found no convincing evidence for an intrinsic transcriptional activity of zGRβ.

2. Materials and methods

2.1. Cell cultures

COS-1 cells were cultured in DMEM (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin and streptomycin (Invitrogen). Cells were grown at 37 °C and 5% CO2. PAC2 zebrafish cells were cultured in Leibovitz's medium (Invitrogen), supplemented with 15% fetal bovine serum (Invitrogen) and 1% penicillin and streptomycin (Invitrogen). Cells were grown at 28 °C.

2.2. Luciferase reporter assays in COS-1 cells

COS-1 cells were seeded into 24-well plates (3×10^4 cells/well). Transfection was performed 24 h later using the TransIT®–COS Transfection Kit (Mirus Bio) according to the manufacturer's instructions. MMTV:luciferase reporter construct (200 ng) was transfected, with a range of PCS + zGRα plasmid concentrations (1–300 ng) and/or 100 ng pCS2+zGRβ expression vector (Schaaf et al., 2008), together with 2 ng pCMV:renilla (Promega). In a second set of experiments, cells were transfected with 50 ng of a κB:luciferase reporter construct (Stratagene) and 50 ng of a human p65 expression vector (pCMV4-p65 (Ruben et al., 1991)), together with a range of pCS2+zGRα concentrations (0–1000 ng) in the presence and absence of 100 ng pCS2+zGRβ. The total amount of transfected DNA was always kept equal among groups by transfecting empty pCS2+ vector. Twenty-four hours after transfection, cells were treated with 100 nM dexamethasone (Sigma) and 24 h later, they were assayed for luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega). Bioluminescence was detected using a Wallac 1450 MicroBeta Luminometer. For each sample, the luciferase activity was normalized to the renilla activity. Per sample, measurements were performed in duplicate, and data shown are averages ± s. e.m. of three experiments.

2.3. Gene expression analysis using the PAC2-zGRβ cell line

The pCS2+zGRβ plasmid (Schaaf et al., 2008) and a neomycin resistance plasmid were transfected into PAC2 zebrafish cells using the Amaxa® Cell line Nucleofector kit V and the Nucleofector™ II device (Lonza). Four days after transfection, cells were subjected to selection for antibiotic resistance by supplementing their culture medium with 500 μg/ml of geneticin (G418, Invitrogen). Resistant cells were propagated to establish the PAC2-zGRβ cell line. For gene expression analysis, PAC2 wild type and PAC2-zGRβ cells were seeded in 6-well plates (2×10^5 cells/well) the day before treatment with 10 μM betamethasone 17-valerate (Sigma) or vehicle (2% DMSO) for 3 h. Samples were collected in TRIzol® reagent (Invitrogen) and total RNA was isolated following the manufacturer's instructions (Invitrogen).

2.4. RNA isolation & cDNA synthesis

Total RNA was extracted using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. RNA was dissolved in water and denatured for 5 min at 60 °C. Samples were treated with DNase using the DNA-free™ kit (Ambion). For microarray analysis, RNA was further purified using the RNeasy MinEluteTM Cleanup kit from Qiagen and its integrity was checked with a lab-on-a-chip analysis using the 2100 Bioanalyzer (Agilent Technologies). For subsequent cDNA synthesis, at least 200 ng of total RNA was added as a template for reverse transcription using the iSCRIPTTM cDNA Synthesis Kit (Biorad).

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