Naturally occurring C-terminal splice variants of nuclear receptors

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Alternative mRNA splicing in the region encoding the C-terminus of nuclear receptors results in receptor variants lacking the entire ligand-binding domain (LBD), or a part of it, and instead contain a sequence of splice variant-specific C-terminal amino acids. A total of thirteen such splice variants have been shown to occur in vertebrates, and at least nine occur in humans. None of these receptor variants appear to be able to bind endogenous ligands and to induce transcription on promoters containing the response element for the respective canonical receptor variant. Interestingly, ten of these C-terminal splice variants have been shown to display dominant-negative activity on the transactivational properties of their canonical equivalent. Research on most of these splice variants has been limited, and the dominant-negative effect of these receptor variants has only been demonstrated in reporter assays *in vitro*, using transiently transfected receptors and reporter constructs. Therefore, the *in vivo* function and relevance of most C-terminal splice variants remains unclear. By reviewing the literature on the human glucocorticoid receptor β-isoform (hGRβ), we show that the dominant-negative effect of hGRβ is well established using more physiologically relevant readouts. The hGR β-isoform may alter gene transcription independent from the canonical receptor and increased hGRβ levels correlate with glucocorticoid resistance and the occurrence of several immune-related diseases. Thus, available data suggests that C-terminal splice variants of nuclear receptors act as dominant-negative inhibitors of receptor-mediated signaling *in vivo*, and that aberrant expression of these isoforms may be involved in the pathogenesis of a variety of diseases.

Received March 9th, 2009; Accepted May 27th, 2009; Published June 19th, 2009 | **Abbreviations: 3'UTR:** 3' untranslated region; **ACTR:** acetyltransferase; **AF-1:** activation function 1; **AF-2:** activation function 2; **AP-1:** activator protein 1; **BAL:** bronchoalveolar lavage; **CAR:** constitutive androstane receptor; **CAR(sv5):** constitutive androstane receptor (sv5); **CAR2:** constitutive androstane receptor 2; **CBP:** CREB-binding protein; **CD38:** cluster of differentiation 38; **DAX-1:** dosage-sensitive sex reversal-1; **DAX-1α:** dosage-sensitive sex reversal-1α; **DBD:** DNA-binding domain; **DRIP205:** vitamin D receptor-interacting protein 205; **ER:** estrogen receptor; **ERβcx:** estrogen receptor βcx; **GATA3:** GATA binding protein 3; **GCs:** glucocorticoids; **GILZ:** glucocorticoid-induced leucine zipper; **GPCRs:** G-protein-coupled receptors; **GRα:** glucocorticoid receptor α; **GRβ:** glucocorticoid receptor β; **GRβ:** glucocorticoid receptor β-isoform; **GRIP1:** glutamate receptor-interacting protein 1; **HNF-4:** hepatocyte nuclear factor 4; **HSP:** heat-shock protein; **IFN:** interferon; **IL:** interleukin; **LBD:** ligand-binding domain; **MAPK:** mitogen-activated protein kinase; **MKP-1:** MAP kinase phosphatase 1; **NF-κB:** nuclear factor κ-light-chain-enhancer of activated B cells; **NLS:** nuclear localisation signal; **NOR-1:** neuron-derived orphan receptor-1; **NOR-2:** neuron-derived orphan receptor-2; **NR:** nuclear receptor; **NRE:** nuclear receptor response element; **Nurr1:** nuclear receptor related1; **Nurr2:** nuclear receptor related2; **PKA:** cAMP-dependent protein kinase; **PBML:** peripheral blood mononuclear leukocytes; **PPARα:** peroxisome proliferator-activated receptor α; **PPARαtr:** peroxisome proliferator-activated receptor αtr; **PPARγ:** peroxisome proliferator-activated receptor γ; **PXR:** pregnane X receptor; **RXR:** retinoid X receptor; **RORα:** RAR-related orphan receptor α; **siRNA:** small interfering RNA; **SRC-1:** steroid receptor coactivator-1; **SRC-2:** steroid receptor coactivator-2; **SRC-3:** steroid receptor coactivator-3; **SRp30:** SF2-like pre-mRNA splicing factor; **T-cell:** thymus cell; **TIF2:** transcriptional intermediary factor 2; **TNF:** tumor necrosis factor; **TR:** thyroid hormone receptor; **TRα1:** thyroid hormone receptor α1; **VDR:** vitamin D receptor; **VDR1:** vitamin D receptor 1 | Copyright © 2009, van der Vaart and Schaaf. This is an open-access article distributed under the terms of the Creative Commons Non-Commercial Attribution License, which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.

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Introduction

Molecular mechanisms of nuclear receptor functioning

The nuclear receptor (NR) superfamily contains transcriptional regulators that are conserved throughout various metazoan species, such as nematodes, insects, and vertebrates [Sluder and Maina, 2001]. There are several molecular mechanisms via which NRs can influence transcription.

The first mechanism involves unliganded receptors that are bound in a complex with heat-shock proteins (HSPs). The interaction with heat-shock proteins prevents the NR from entering the nucleus and thus ensures its localization in the cytosol. The ligand-binding domain (LBD) of the receptor undergoes a conformational change upon ligand binding, which results in dissociation from the heat-shock protein complex. Furthermore, this conformational change exposes the nuclear localization signal (NLS) and activation function 2 (AF2) domain that are present in the LBD. After translocation of the ligand-receptor complex to the nucleus, it forms homodimers or heterodimers with other NRs and binds with high affinity to nuclear receptor response elements (NREs) in the promoter regions of target genes. Subsequent recruitment of cofactors creates a DNA-bound complex that is capable of remodeling the chromatin structure and influencing transcription. Usually, this influence is positive and results in an upregulation of target genes, but most NRs are also capable of transcriptional repression through this mechanism. The mechanism described above is the prevalent model for transcriptional activation by steroid receptors, like the glucocorticoid receptor (GR) and estrogen receptor (ER) [Glass and Ogawa, 2006; Mangelsdorf et al., 1995; Ribeiro et al., 1995].

Nuclear receptors that function via the second mechanism bind constitutively to their NRE, even in the absence of ligands. This group includes receptors for the thyroid hormone, vitamin D and retinoic acid, and they are known to form heterodimers with retinoid X receptors (RXRs). Without ligand present, this NR-RXR heterodimer is bound to the response element in a complex with corepressor proteins. Therefore, receptors from this group function as active transcriptional repressors in the absence of ligand. After ligand binding, receptors undergo a conformational change which removes the corepressor proteins and replaces them with coactivator proteins. The removal of the corepressor proteins is thought to be an active process, which requires ubiquitination- and proteasome-dependent mechanisms. Similar to the first NR transcriptional activation mechanism, the recruited coactivators can make the chromatin structure more accessible and facilitate the assembly of the general transcriptional machinery at the promoter [Glass and Ogawa, 2006; Mangelsdorf et al., 1995; Ribeiro et al., 1995].

The majority of the so-called orphan receptors (receptors for which no ligand has been identified (yet)) bind to DNA as monomers. The methods via which orphan receptors regulate transcription are not as thoroughly investigated as for other nuclear receptors. However, orphan receptors might affect transcription through changes in their expression or via posttranslational modifications. These modifications include the phosphorylation or sumoylation of receptors via GPCRs (G-protein-coupled receptors), MAPKs (mitogen-activated protein kinases), and PKA (cAMP-dependent protein kinase). It is also possible that orphan receptors are activated by ligands that are yet to be discovered.

Besides activating or repressing genes via binding to NREs present in promoter regions, some NRs can also influence transcription via a mechanism called transrepression, which does not require DNA-binding of the receptor. Instead, this form of negative regulation seems to be mediated by inhibiting the activity of other transcription factors via direct physical interaction. Examples of transcription factors that are affected by transrepression are members of the nuclear factor-κB (NF-κB) and activator protein 1 (AP1) families. Both these families of transcription factors play crucial roles in pro-inflammatory signaling pathways and are of great interest in the treatment of immunity-related diseases [Glass and Ogawa, 2006; Mangelsdorf et al., 1995; Ribeiro et al., 1995].

Naturally occurring C-terminal splice variants of nuclear receptors

Alternative mRNA splicing in the region encoding the C-terminal end of nuclear receptors results in receptor variants that lack one or more helices of the LBD, which are replaced by an alternative amino acid sequence unique to the splice variant. In this review, we present a literature study on these LBD splice variants, which reveals that out of forty-eight nuclear receptor genes encoded by the human genome, at least nine display this type of alternative splicing, and that in other species, several different C-terminal splice variants occur, which may occur in humans, as well. None of these receptor variants appear to be able to bind ligand and to induce transcription on promoters containing the response element for the respective canonical receptor variant. Interestingly, almost all LBD splice variants have been shown to display dominant-negative activity on the transactivational properties of their canonical equivalent.

Although these receptor variants are highly comparable in structure and function, the available literature on these proteins is very scattered. They have never been described as a group before, and articles on one C-terminal splice variant hardly ever cite work on a similar splice variant of another receptor. Therefore, we would like to provide a broader perspective on C-terminal splice variants, by considering them as a group of proteins, rather than individually. The aim of this review is to provide a structural and functional comparison of these potential dominant-negative nuclear receptors, which could lead to a better understanding of their function *in vivo* and the molecular mechanisms underlying their activity. Unfortunately, most of these splice variants have been poorly studied, so only limited data are available. The member of this group that has been studied in most detail is the β-isoform of the glucocorticoid receptor. By reviewing the literature on this splice variant, we would like to shed light on the possible *in vivo* function and relevance of nuclear receptor C-terminal splice variants in general.

The ligand-binding domain of nuclear receptors

The LBD of nuclear receptors is involved in several processes required for transcription initiation, like ligand binding, binding to coactivator proteins, and dimerization. Most nuclear receptor LBDs are comprised of 12 α helices, which are arranged in a three-layer sandwich [Bourguet et al., 2000; Li et al., 2003; Wurtz et al., 1996]. Helices 1-3 form one outer layer of the sandwich and helices 6, 7 and 10 form the other outer layer. Helices 4, 5, 8, and 9 form the middle layer, which is absent in the bottom half of the sandwich, thereby creating a cavity for ligand binding in most receptors. Upon ligand binding, a conformational change occurs, which depends on the nature of the ligand. When an agonist is bound, helix 12, which contains the AF-2 domain, is packed against helices 3 and 10 and becomes an integral part of the LBD. Thereby, it closes the binding pocket and forms, together with helix 3, 4 and 5, a binding surface for coactivator proteins [Weatherman et al., 1999]. Coactivators that contain an LXXLL amino acid motif can interact with this surface [Savkur and Burris, 2004], enabling optimal transcription initiation by the nuclear receptor. In most nuclear receptors, LBD-dependent receptor dimerization is mediated by the N-terminal half of helix 10, which packs against the same domain in its dimerization partner. In the resulting dimer, the two helix 10 subunits form a coiled-coil structure. Finally, the (non-helical) most C-terminal part of the LBD is variable in length and sequence for different receptors and is called the F-domain. The precise function of this domain

remains unclear [Mangelsdorf et al., 1995], but it has been suggested that it stabilizes the conformational state of the LBD when ligand is bound [Koide et al., 2007; Ribeiro et al., 1995].

The occurrence of C-terminal splice variants of nuclear receptors

In this section, thirteen C-terminal splice variants of nuclear receptors will be described, which are listed in Table 1. Nine splice variants have been demonstrated to occur in humans: the glucocorticoid receptor β (GRβ), estrogen receptor βcx (ERβcx), thyroid hormone receptor α1 (TRα1), constitutive androstane receptor (sv5) (CAR(sv5)), dosage-sensitive sex reversal-1α (DAX-1α), nuclear receptor related 2 (Nurr2), neuron-derived orphan receptor-2 (NOR-2), peroxisome proliferator-activated receptorα_{tr} (PPARα_{tr}), and the PPARγ isoform γORF4. One splice variant has been shown to occur in rats (vitamin D receptor 1 (VDR1)), one in mouse (CAR2), and in fish two splice variants named identical to their human equivalents exist (GRβ in zebrafish and TRα1 in goldfish), but most likely, they have evolved independently.

NR gene	C-terminal splice variant(s)	Splicing mechanism[4]	# amino acids lacking from canonical receptor	# amino acids specific for splice variant
GR	hGRβ[1] / zGRβ	ER/IR	50/49	15/40
ERβ	hERβcx	ER	62	27
TRα	hTRα2[3] / gTRα2	-/?[5]	40/142	120/53
VDR	rVDR1[2]	IR	86	19
CAR	hCAR(sv5) / mCAR2[2]	AA/ER	42/78	7/6
DAX-1	hDAX-1α	ER	81	12
Nurr1	hNurr2[3]	AA	208	1
NOR-1	hNOR-2[3]	AA	207	25
PPARα	hPPARα_{tr}	IR	298	4
PPARγ	hγORF4	IR	232	21

Table 1. Overview of naturally occurring C-terminal splice variants of nuclear receptors. All thirteen C-terminal splice variants described in this review are listed, as well as the splicing mechanism generating them (see also Figure 1 and Supplementary File 1), and the number of amino acids lacking from the canonical receptor and the number of variant-specific amino acids in their C-terminus. [1] Absence in mouse demonstrated experimentally. [2] Occurrence only demonstrated experimentally in rat/mouse, not in humans, but most likely conserved between humans and rodents, based on comparison of genomic sequence. [3] Presence in mouse/rat demonstrated experimentally. [4] ER: exon replacement; IR: intron retention; AA: alternative acceptor site usage (see Figure 1). [5] Could not be determined, due to lack of goldfish genomic sequence data.

There are three mechanisms which are commonly used for the generation of C-terminal splice variants of nuclear receptors (Figure 1). With all three mechanisms, most of the resulting protein sequence is identical between the canonical receptor and the splice variant, but a C-terminal sequence is different. This difference can be a result of an alternative exon replacing the most 3' exon(s) of the mRNA encoding the canonical receptor. This mechanism is called exon replacement (Figure 1A). Alternatively, an intronic sequence may be retained as an exon which replaces the most 3' exon (Figure 1B). Finally, an alternative splice acceptor site inside an exon may be used, which results in a frame shift and an alternative C-terminal end of the protein (Figure 1C). In all three cases, the sequence replacing the final exon(s) of the canonical receptor contains a sequence encoding a unique C-terminus with no homology to the C-terminus of the canonical receptor. The generation of TRα2 is more complicated and involves a combination of alternative splice donor site usage and exon replacement. The precise splicing mechanisms for all variants are depicted in Supplementary File 1.

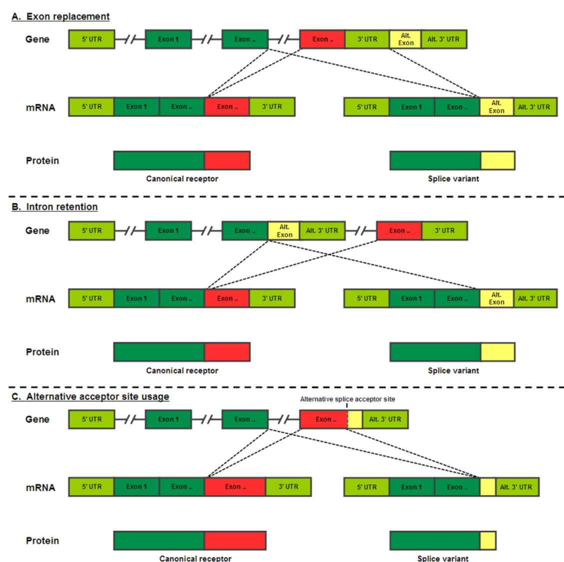

Figure 1. Alternative splicing in the C-terminal region of nuclear receptors. A schematic view of the three commonly used alternative splicing mechanisms for the generation of C-terminal splice variants of nuclear receptors. A: exon replacement. Replacing the final exon(s) of the canonical receptor with an alternative exon and 3'UTR results in a unique protein ending. B: Intron retention. An intronic sequence is not spliced out when producing the mRNA, resulting in a premature stop codon and a unique C-terminal ending. C. Alternative acceptor site usage. An alternative splice acceptor site inside an exon is used, which results in a frame shift and an alternative C-terminal end of the protein.

The first group (consisting of hGRβ, hERβcx, rVDR1, mCAR2/hCAR(sv5), and hDAX-1α) has a point of divergence between the splice variant and the canonical receptor in the region close to helix 10. The transcripts encoding these receptor variants lack only the final exon of the canonical receptor mRNA (apart from the mCAR2 mRNA, which lacks two exons), which encodes in most cases between 50 and 100 amino acids. In the second group (consisting of hNurr2, hNOR-2, hPPARα_{tr} and hγORF4), a significant part of the LBD (at least 200 amino acids), which in most cases spans almost the entire LBD, is replaced by an alternative sequence. The mRNA encoding these splice variants lacks two to four exons of the canonical messenger. In Figure 2 and Figure 3, a schematic representation of the structures of all splice variants is shown, indicating the divergence points between the canonical receptor and the splice variant and their predicted secondary structures. The amino acid sequences of the different variant-specific C-termini are shown in Table 2.

Splice Variant	Specific C-terminal sequence
hGRβ	NVMWLKPESTSHTLI
zGRβ	MMLLLNNAYRKPCLSLLQSPSELSGCLLPLDGCFQRQFSN
hERβcx	RAEKASQTLTSFGMKMETLLPEATMEQ
hTRα2	EREVQSSILYKGAAAEGRPGGSLGVHPEGQQLLGMHVVQGPQVRQLE QQLGEAGSLQGPVLQHQSPKSPQQRLLELLHRSGILHARAVCGEDDS SEADSPSSSEEEPEVCEDLAGNAASP
gTRα2	GINTQHSFTFQPVVELAELAQEPIIGPDLASLSHRGHSSSDIHPLLQ HQVCDD
rVDR1	GTEPGREELRDLGHVGDCE
hCAR(sv5)	TSRACLP
mCAR2	GFCMQS
hDAX-1α	GRKMIAIIINII
hNurr2	I
hNOR-2	VSFMISCFQMNDQGLYLWLLVIRVD
hPPARα_{tr}	FCHT
hγORF4	VSSLLLSSLGEAGSCFGFLFL

Table 2. Splice variant-specific C-terminal amino acid sequences. The table shows the variant-specific amino acid sequences in the C-termini of all thirteen nuclear receptor C-terminal splice variants described in this review.

Variants of the glucocorticoid receptor (GR)

Glucocorticoids (GCs) are a class of steroid hormones that are essential in the maintenance of homeostasis and enable the organism to respond to both physical and emotional stress. GCs are capable of stimulating metabolism, while they attenuate immune and inflammatory processes [Buckingham, 2006]. In 1985, two human GR (hGR) isoforms were identified and named hGRα and hGRβ. The canonical receptor, hGRα only differs from the hGR β-isoform at its C-terminal end. The first 727 amino acids are identical for hGRα and hGRβ, after which hGRα has an additional 50 amino acids and hGRβ has an additional, non-homologous 15 amino acids [Bamberger et al., 1995; Oakley et al., 1996]. The *hGR* gene contains 10 exons: exons 1-8, followed by two copies of exon 9 (exon 9α and 9β for hGRα and hGRβ, respectively). Alternative splicing between the eighth and the ninth exon replaces the canonical exon 9α with exon 9β, which leads to the generation of a mRNA encoding hGRβ [Bamberger et al., 1995; Oakley et al., 1996]. This splicing event results in the absence of helix 12 and part of helix 11 of the LBD in hGRβ, which is replaced by a unique C-terminal end [Yudt et al., 2003].

The hGR β-isoform has been detected in a variety of human cell lines and tissues [Oakley et al., 1996]. At the mRNA level, hGRβ expression is significantly lower than the expression of hGRα [Dahia et al., 1997; Mu et al., 1998; Oakley et al., 1996]. However, this difference is not observed at the protein level, at which a higher hGRβ expression level is detected in some cells (e.g., human neutrophils), as compared to hGRα [de Castro et al., 1996; Strickland et al., 2001]. Experiments concerning the expression levels of hGRα and hGRβ revealed that mRNA transcripts for both hGR isoforms are present at a ratio of at least 200:1 in different tissues [Oakley et al., 1996]. Nevertheless, regional or cellular ratios can differ dramatically from this overall ratio. Furthermore, hGRβ shows a different subcellular localization than hGRα. While unliganded hGRα resides in the cytoplasm and only translocates to the nucleus upon ligand binding, hGRβ is constitutively present in the nucleus [Oakley et al., 1997].

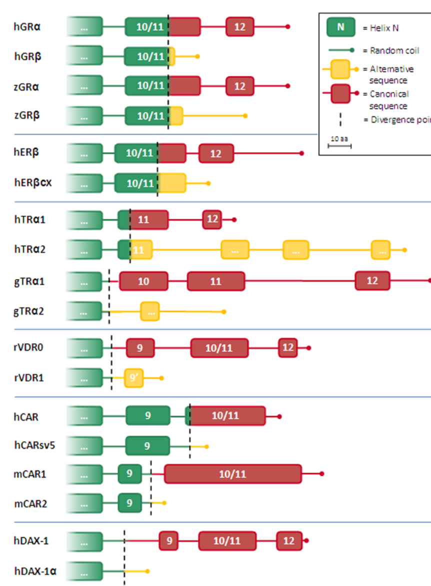

Figure 2. Comparison of the secondary structure at the C-terminal end of canonical receptors and their splice variants. Predictions of the secondary structure of canonical NRs and their respective splice variants are shown at the C-terminal end of the LBD. Helices are indicated by boxes (with addition of the helix number), while random coil (or beta sheet) is indicated by a line. The divergence points are marked with a dotted line, while color differences indicate sequential differences between the canonical receptor and its splice variant.

The alternatively spliced GR β-isoform is not present in rodents, due to the absence of the required splice site [Otto et al., 1997]. So far, the zebrafish is the only other organism in which the occurrence of a GR β-isoform has been demonstrated [Schaaf et al., 2008]. Like humans, the zebrafish genome contains one *GR* gene, and the pre-mRNA can be alternatively spliced into a zGRα (the canonical receptor) and a zGRβ messenger. The structure and the expression levels of zGRβ are strikingly similar to its human equivalent [Schaaf et al., 2008]. The zGRβ protein is identical to zGRα for the first 697 amino acids, after which it contains 40 unique amino acids. Protein alignment revealed that the divergence point between zGRα and zGRβ is located at the exact same position as for hGRα and hGRβ. The two divergence points are located in helix 11 of the LBD, resulting in an alternative ending of helix 11 and the absence of helix 12 for both hGRβ and zGRβ [Goleva et al., 2006; Hauk et al., 2002]. The 40 unique C-terminal amino acids of zGRβ show little homology to the additional 15 amino acids present in hGRβ [Schaaf et al., 2008]. As for hGRβ, zGRβ is expressed at a much lower level than the canonical receptor isoform, with ratios of zGRα:zGRβ varying from 20:1 to 146:1 [Schaaf et al., 2008]. The subcellular localization of zGRα and zGRβ is similar to the localization of their human homologs. Unliganded zGRα is located in the cytosol, while zGRβ is constantly present in the nucleus [Schaaf et al., 2008].

Although the protein structure of zGRβ closely resembles that of hGRβ, the splicing event leading to the formation of the GRβ isoforms is different for humans and zebrafish. For the human β isoform, its unique C-terminal sequence is encoded in an alternative version of exon 9, called exon

9β, which is located downstream of exon 9α. In the zebrafish genome, the unique sequence of GRβ is located in intron 8. Therefore, hGRβ is created by alternative exon usage, while zGRβ results from retention of part of intron 8 [Schaaf et al., 2008]. It is likely that zGRβ evolved independently of hGRβ, since the genetic organization and β-specific C-terminus of the human and zebrafish isoforms are different. Therefore, the presence of GRβ in humans and zebrafish is most likely a case of convergent evolution, which suggests a significant role for this isoform in these species.

Variants of the estrogen receptor (ER)

Estrogens are steroid hormones that play key roles in development and maintenance of normal sexual and reproductive functions in both men and women [Heldring et al., 2007]. They also exert a vast range of biological effects in the cardiovascular, musculoskeletal, immune, and central nervous system. The most potent estrogen produced *in vivo* is 17β-estradiol [Heldring et al., 2007]. The two nuclear receptors via which estrogens signal are named ERα and ERβ. Multiple isoforms exist for both ERα and ERβ as a result of alternative splicing [Leung et al., 2006]. One of the ERβ isoforms is the C-terminally truncated ERβcx (also known as ERβ2) [Ogawa et al., 1998]. ERβcx is identical to ERβ for the first 469 amino acids, but the final 61 amino acids of ERβ are replaced by a unique 26 amino acid sequence in Erβcx [Ogawa et al., 1998]. The canonical ERβ is encoded by 9 exons. Alternative splicing replaces the final *ERβ* exon (named L4) by an exon which is specific for ERβcx (named Lcx). The splicing mechanism used to create ERβcx is exon replacement (exon L4 is replaced by exon Lcx [Ogawa et al., 1998]).

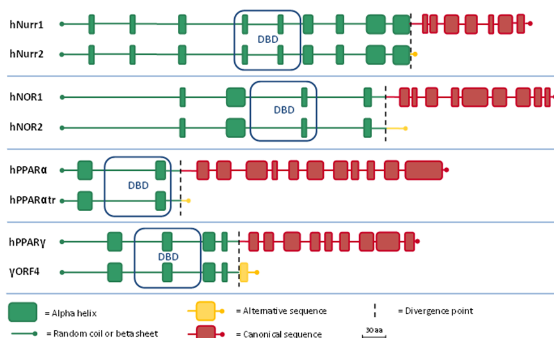

Figure 3. Comparison of the secondary structure of canonical receptors and their splice variants Predictions of the secondary structure of canonical NRs and their respective splice variants are shown at whole protein level. The region of the DBD is indicated as a reference point. Helices are indicated by boxes, while random coil (or beta sheet) is indicated by a line. The divergence points are marked with a dotted line, while color differences indicate sequential differences between the canonical receptor and its splice variant.

ERβcx is primarily expressed in testis, ovary, prostate, and thymus. The relative expression levels of ERβcx and ERβ vary per tissue [Leung et al., 2006]. ERβcx is expressed at higher levels than ERβ in testis, while ERβ expression exceeds ERβcx expression in ovary [Leung et al., 2006]. Ogawa et al. [Ogawa et al., 1998] predict that a homolog for ERβcx might also exist for ERα. However, this type of ERα isoform has yet to be discovered.

Variants of the thyroid hormone receptor (TR)

Thyroid hormones are regulators of physiological functions, such as the control of body temperature and heart rate. Following the secretion of thyroid stimulating hormone, circulating T_4 is converted into T_3, which is the active form of thyroid hormone [Yen, 2001]. Thyroid hormones signal via thyroid hormone receptors (TRs) present in the nucleus of cells. The two receptors that mediate T_3-regulated gene expression are named TRα and TRβ [Yen, 2001]. The human *TRα* and *TRβ* genes both encode several receptor variants due to alternative promoter usage and alternative splicing. The different TRα and TRβ variants are highly homologous in human, rat and mouse [Yen, 2000]. One of the TRα isoforms that is generated by alternative splicing was named hTRα2 when it was isolated from a human cDNA library by Benbrook and Pfahl [Benbrook and Pfahl, 1987]. It differs from the canonical TRα receptor, hTRα1, at its C-terminal end. The human *TRα* gene contains a total of 10 exons. Usage of a splice donor site in exon 9 (approximately 128 bp downstream of the start of this exon) and an acceptor site at the 5' end of exon 10 results in a protein in which the 40 most C-terminal amino acids of hTRα1 are replaced by 120 amino acids specific for hTRα2 [Thijssen-Timmer et al., 2006].

Mitsuhashi et al. [Mitsuhashi et al., 1988] demonstrated that an isoform generated in a similar fashion as hTRα2 also exists in rats. They identified two transcripts that differ from the 410 amino acids long canonical rTRα receptor in their C-terminal region. Both these isoforms, 452 and 372 amino acids long, respectively, are generated by alternative splicing of the rat *TRα* gene. The longest isoform is spliced at the same location as hTRα2, and also replaces the final 40 amino acids of the canonical receptor with a unique C-terminal end [Mitsuhashi et al., 1988].

C-terminal splice variants of TRα have also been identified in goldfish. Nelson and Habibi [Nelson and Habibi, 2006] analyzed goldfish cDNA for the occurrence of alternative TR transcripts and found two alternatively spliced TRα isoforms. The two truncated goldfish TRα (gTRα) isoforms were named TRα2 and TRα-truncated. gTRα-truncated is only 206 amino acids long, and similar to the canonical gTRα1 (466 amino acids) until the hinge region [Nelson and Habibi, 2006]. gTRα2 is similar to gTRα1 for the first 324 amino acids, after which the final 142 amino acids of gTRα1 are replaced by 54 variant-specific amino acids in gTRα2 [Nelson and Habibi, 2006]. The hTRα2 and gTRα2 isoforms are similar in that they both lose homology with their respective TRα1 counterparts in the C-terminal region of the LBD. Nevertheless, hTRα2 and gTRα2 are spliced in different regions of the *TRα* gene, and have different C-terminal endings. Unfortunately, the complete genomic sequence of the goldfish *TRα* gene is unknown, which makes it

difficult to describe the splicing event leading to gTRα2 in detail.

Variants of the vitamin D receptor (VDR)

The biologically-active metabolite of vitamin D is called 1,25-dihydroxyvitamin D_3 (further referred to as vitamin D_3). Vitamin D_3 is important in the metabolism of minerals, and in the formation and growth of bone [Demay, 2006]. The biological actions of vitamin D_3 are mediated by the vitamin D receptor (VDR). In the rat, a VDR isoform has been identified which differs from the canonical receptor at its C-terminal end [Ebihara et al., 1996]. Alternative splicing generates an isoform which has retained intron 8 of the canonical rat vitamin D receptor (rVDR0). A stop codon within intron 8 truncates the C-terminal region of this isoform (named rVDR1). As a result, rVDR1 lacks the most C-terminal 86 amino acids of rVDR0. These amino acids are replaced by 19 variant-specific amino acids. Analysis of the genomic structure and sequence of the *rVDR* gene demonstrated that the inserted sequence corresponds to the entire intron between exon 8 and 9 [Ebihara et al., 1996]. The rVDR1 transcript is expressed at 1/15th to 1/20th of the level of rVDR0 expression [Ebihara et al., 1996]. Both rVDR0 and rVDR1 are primarily expressed in the intestines and the kidney, and rVDR1 was only detected in mature rats, not in embryos [Ebihara et al., 1996].

To date, a dominant-negative VDR isoform has only been identified in rats. However, allelic variations in intron 8 of the *hVDR* gene can be used to predict the density of bone [Ebihara et al., 1996]. Intron 8 of the *hVDR* gene corresponds to the intron that is retained in the *rVDR* gene for the formation of rVDR1. Therefore, a similar splicing mechanism may exist for the *hVDR* gene.

Variants of the constitutive androstane receptor (CAR)

The constitutive androstane receptor (CAR) was originally identified as an orphan receptor. Later, CAR was found to act as a receptor for foreign compounds, known as xenobiotics [Timsit and Negishi, 2007]. Together with the pregnane X receptor (PXR), CAR performs a pivotal role in hepatic drug metabolism. To date, no definitive physiological ligand has been identified for CAR. Instead, CAR responds to a range of toxic by-products of endogenous metabolism and to exogenous chemicals [Timsit and Negishi, 2007]. The genetic organization of CAR has been studied in humans and mice. Both genes contain 9 exons and share a high degree of sequence similarity throughout the DNA-binding and ligand-binding domain [Choi et al., 1997]. The human *CAR* gene can generate a C-terminally spliced receptor isoform. The canonical hCAR is 352 amino acids long, while the truncated hCAR splice variant 5 (hCAR(sv5)) isoform is only 317 amino acids long [Arnold et al., 2004]. Alternative splice acceptor site usage in exon 9 leads to a deletion of the first 76 base pairs of this exon. Consequently, the final 42 amino acids of hCAR are replaced by 7 amino acids, which are specific for hCAR(sv5) due to a frame shift [Arnold et al., 2004].

The canonical human CAR (hCAR) is expressed in the liver, kidney, spleen, testis, and small intestines. In contrast, the hCAR(sv5) isoform was shown to be present in every tissue studied, with the exception of the small intestine [Arnold et al., 2004]. Therefore, most tissues only contain the truncated hCAR(sv5) isoform. The subcellular localization of hCAR(sv5) is similar to that of hCAR [Arnold et al., 2004].

In mouse, the canonical CAR is named mCAR1. A C-terminal splice variant, named mCAR2, is identical to mCAR1, except for the most C-terminal 78 amino acids present in mCAR1 [Choi et al., 1997]. These amino acids are replaced by 6 variant-specific amino acids in mCAR2 [Choi et al., 1997]. The mCAR2 isoform is generated by replacement of exon 8 and 9 with an alternative exon that is located between these exons [Choi et al., 1997]. The mouse CAR1 and CAR2 are primarily expressed in the liver. Compared to the expression of mCAR1, the mCAR2 variant is expressed at relatively low levels [Choi et al., 1997].

Variants of dosage-sensitive sex reversal-1 (DAX-1)

The orphan nuclear receptor named dosage-sensitive sex reversal-1 (DAX-1) provides another interesting example of the generation of a C-terminal receptor splice variant. DAX-1 plays an important role in the development and functioning of the adrenal gland and the hypothalamic-pituitary-adrenal axis [Hossain et al., 2004]. Furthermore, DAX-1 is a transcriptional repressor of several genes involved in steroid hormone metabolism and acts as a corepressor of steroidogenic factor 1 (SF-1), ERα, and ERβ [Hossain et al., 2004]. DAX-1 is an unusual nuclear receptor, since it is encoded by only two exons and lacks the characteristic zinc-finger DNA-binding motif that is highly conserved in other nuclear receptors [Hossain et al., 2004]. The human *DAX-1* gene generates two distinct isoforms via the usage of an alternative version of the second exon [Hossain et al., 2004]. The mRNA encoding the canonical receptor, DAX-1, combines exon 1 with the normal version of exon 2 to generate a protein that contains 470 amino acids. Combining exon 1 with the alternative exon 2a results in the generation of DAX-1α (401 amino acids long), which contains a unique C-terminal sequence compared to DAX-1.

The canonical DAX-1 receptor is expressed in the adrenal gland, brain, fetal kidney, ovary, pancreas, and testis. DAX-1α is also present in these tissues, but was also detected in all other human tissues which were examined [Hossain et al., 2004]. DAX-1α is expressed at higher levels than DAX-1 in all tissues, except in the testis, where DAX-1 expression is higher [Hossain et al., 2004]. So far, this DAX-1 splice variant has only been discovered in humans.

Variants of the nuclear receptor related 1 (Nurr1)

The NGFI-B family of nuclear receptors consists of NGFI-B, Nurr1, and NOR1. All three members of the NGFI-B family are orphan receptors, since no ligands

have been identified yet [Ohkura et al., 1999]. These receptors have been implicated in many biological processes, such as neural differentiation, cell growth, and T-cell apoptosis [Ohkura et al., 1999]. Nevertheless, little is known about the molecular mechanisms via which they affect these processes [Ohkura et al., 1999].

The mouse *Nurr1* gene contains 8 exons, of which the first two are non-coding [Saucedo-Cardenas et al., 1997]. A C-terminally truncated variant of mouse Nurr1 is named Nurr2 [Ohkura et al., 1999]. The most C-terminal amino acids of Nurr2 are derived from alternative usage of a splice acceptor site in exon 6, ending in an in-frame stop-codon. In addition, Nurr2 is generated by alternative splicing in exon 1 and 2, resulting in a shorter N-terminal region, as well. The occurrence of Nurr2 is conserved in at least mouse, rat, and human [Ohkura et al., 1999]. Nurr1 and Nurr2 are expressed ubiquitously in all examined tissues in these species [Ohkura et al., 1999]. In each tissue, Nurr2 is expressed at significantly lower levels than Nurr1.

Variants of the neuron-derived orphan receptor-1 (NOR-1)

A C-terminal splice variant also exists for the NGFI-B family member NOR-1, which is named NOR-2 [Petropoulos et al., 1995]. NOR-2 has so far been found in humans [Ohkura et al., 1998], rat [Petropoulos et al., 1995] and mouse [Maltais and Labelle, 2000]. The full-length hNOR-1 is 626 amino acids long, while hNOR-2 only contains 443 amino acids. hNOR-2 is generated by retention of the intron between exon 5 and 6, which contains a premature stop-codon and the 3'UTR. The mRNA encoding hNOR-2 lacks exons 6, 7, and 8 [Maltais and Labelle, 2000]. The variant-specific amino acid sequence is 10 amino acids long in mouse and rat and 25 amino acids long in humans [Ohkura et al., 1998].

Variants of the peroxisome proliferator-activated receptor α (PPARα)

The peroxisome proliferator-activated receptors (PPARs) are ligand-dependent receptors involved in lipid homeostasis, energy metabolism, inflammation, and cellular differentiation and proliferation [Kim et al., 2006]. Endogenous ligands for the PPARs include free fatty acids and eicosanoids.

There are three PPARs: PPARα, PPARβ (also known as PPARδ), and PPARγ. A splice variant within the C-terminal region of the human PPARα has been described and named PPARα_{tr} [Gervois et al., 1999; Palmer et al., 1998]. PPARα is encoded by 7 exons and is 468 amino acids long, whereas PPARα_{tr} is only 174 amino acids long. PPARα_{tr} mRNA is generated by retention of the intronic sequence at the 3' end of exon 5. PPARα_{tr} mRNA lacks exon 6, 7 and 8 [Hamid et al., 1999; Mlodzik et al., 1990]. PPARα_{tr} mRNA is expressed in several human tissues and represents 20-50% of total PPARα mRNA [Gervois et al., 1999; Palmer et al., 1998]. PPARα_{tr} has only been identified in humans.

Variants of the peroxisome proliferator-activated receptor γ (PPARγ)

The *PPARκ* gene consists of 6 exons and a C-terminal splice variant, γORF4, is generated by retention of intronic sequence at the 3' end of exon 4 [Kim et al., 2006; Sabatino et al., 2005]. The final two exons of the mRNA encoding the canonical receptor, exons 5 and 6, are deleted from the transcript. This results in a protein that is identical to PPARγ for the first 243 amino acids and contains 21 additional amino acids coded for by intron 4 sequences [Kim et al., 2006; Sabatino et al., 2005]. Like PPARα_{tr}, γORF4 has only been identified in humans.

The function of C-terminal splice variants of nuclear receptors

Loss of ligand binding and transactivation activity

Binding to cognate ligand has not been reported for any of the described nuclear receptor splice variants. As is known from structural studies, the C-terminal region of the LBD that is lacking in these variants is required for ligand binding and apparently, the variant-specific C-terminal amino acids do not have the characteristics to fulfill this role.

In addition to the lack of ligand binding ability, all described splice variants lack the ability to transactivate gene transcription induced by a promoter containing their cognate response element. This has been shown in experiments with reporter assays using transient transfection of the reporter plasmid. For the non-orphan receptors, this obviously could be explained by the inability to bind ligand and become activated. However, all described receptor variants also lack the AF-2 containing C-terminal helix of the LBD, which is replaced by an alternative C-terminus. The inability to transactivate may therefore also be due to the variant-specific C-terminal sequence not having the characteristics to substitute for the function of the AF-2 domain, with the result that the surface required for the docking of LXXLL-motif-containing coactivators cannot be formed. Moreover, the alternative C-terminus apparently keeps the receptor variant in a conformation that does not allow the AF-1 to be functional, since this domain, together with an intact DNA-binding domain (DBD), can be sufficient for transactivation activity in reporter assays [Hollenberg et al., 1987].

Dominant-negative activity

Ten splice variants described have been shown to act as dominant-negative inhibitors of the activity of their respective canonical receptor. In most cases, this has been demonstrated in reporter assays in which the canonical receptor, the splice variant and a plasmid containing a reporter gene (CAT or luciferase) driven by a promoter containing the response element for the respective receptor, were transiently transfected into cultured cells. The only splice variant that fails to show dominant-negative activity in such an assay is the mouse CAR2 isoform, which does not inhibit the transactivation

properties of mCAR1 [Choi et al., 1997]. The possible dominant-negative activity of the human CAR splice variant hCAR(sv5) and the goldfish TRα2 was not studied, and the dominant-negative activity of DAX-1α was studied in a different assay. DAX-1α was demonstrated to inhibit the ability of the canonical receptor DAX-1 to repress SF-1-induced transactivation in transiently transfected reporter assays [Hossain et al., 2004].

The dominant-negative activity of hGRβ appears to depend on two amino acids (a lysine and a proline), which are present in the hGRβ-specific C-terminal 15 amino acid sequence, rather than on the absence of the AF-2 containing helix 12 [Yudt et al., 2003]. It has been shown that these two amino acids play a role in the nuclear localization of hGRβ [Yudt et al., 2003].

The dominant-negative activity of the splice variant is not always specifically affecting the activity of the respective canonical receptor, but may affect other members of the nuclear receptor family, as well. mNurr2 inhibits transactivation of all members of the NGFI-B family [Ohkura et al., 1999]. hTRα2 shows dominant-negative activity on hTRα1 and hTRβ, but also on hRARβ and hER-induced transactivation on the same (TRE-driven) promoter [Hermann et al., 1991]. hPPARα_{tr} shows inhibition of hPPARα, hPPARγ, hHNF-4, hRORα and hGRα [Gervois et al., 1999]. hERβcx shows dominant-negative activity on hERα (surprisingly, not on hERβ-induced transactivation [Ogawa et al., 1998]). In contrast, rVDR1 seems to specifically inhibit rVDR0, since it has been demonstrated not to inhibit transactivation induced by the closely related receptors TR and RAR on promoters containing their cognate response elements [Ebihara et al., 1996]. hGRβ shows dominant-negative activity over hGRα and hMR [Bamberger et al., 1995; Bamberger et al., 1997; Oakley et al., 1996], but not over hAR- and hPR-induced transactivation [Oakley et al., 1996], whereas all four receptors are able to induce transcription on the same response element.

Mechanism of action of dominant-negative activity

Three mechanisms via which receptor variants with dominant-negative activity can interfere with canonical receptor signaling have been proposed [Yen, 2000], and they are shown in Figure 4.

Occupation of response elements

The first mechanism involves competition for DNA binding on NREs by dominant-negative receptors. In this case, competition for a limited amount of NREs results in a reduced transcriptional activity of the canonical receptor. This competition can occur by both homodimerized dominant-negative receptors and by heterodimeric complexes consisting of a dominant-negative receptor and its canonical equivalent or another binding partner (e.g., the retinoid X receptor (RXR)). Indeed, rVDR1, hTRα2 [Hermann et al., 1991; Katz and Lazar, 1993], and hERβcx [Moore et al., 1998; Ogawa et al., 1998] inhibited the DNA-binding of their canonical receptors in gel shift assays, even though the affinity of the latter two splice variants is significantly reduced compared to the canonical receptors.

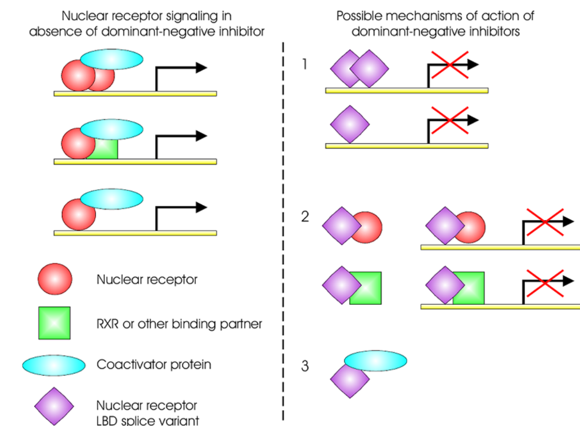

Figure 4. Three possible mechanisms for the dominant-negative activity of C-terminal splice variants of nuclear receptors. In the left panel, transactivation by nuclear receptors is shown in the absence of an inhibitor. Nuclear receptors can bind to their response elements as a homodimer, a heterodimer (e.g., with RXR), or as a monomer. In the right panel, transactivation is inhibited due to the presence of a dominant-negative inhibitor, that either occupies the response elements (1), forms transcriptionally-inactive heterodimers with the canonical receptor (2), or competes for binding with transcriptional coactivator proteins (3).

The affinity for the cognate response elements differs largely between splice variants, suggesting that this mechanism is not relevant for all splice variants. Rat NOR-2 [Petropoulos et al., 1995] and hDAX-1α [Hossain et al., 2004] bind to their response element with the same affinity as their cognate canonical receptor in gel shift assays. Other splice variants show significantly reduced DNA binding *in vitro* (hERβcx [Moore et al., 1998; Ogawa et al., 1998], hGRβ [Oakley et al., 1999], hTRα2 [Hermann et al., 1991; Katz and Lazar, 1993], hγORF4 [Kim et al., 2006], mNurr2 [Ohkura et al., 1999]), or do not bind to their response elements at all (hPPARα_{tr} [Gervois et al., 1999], mCAR2 [Choi et al., 1997], and hCAR(sv5) [Arnold et al., 2004]).

Heterodimerization

A second mechanism proposed is the formation of dimers between canonical receptors and the dominant-negative form, which are transcriptionally inactive and prevent the formation of active receptor complexes. Several techniques have been used to demonstrate these heterodimers, and in most studies dimerization was observed. Immunoprecipitation studies have shown that hGRβ-hGRα heterodimers occur in human neutrophils [Strickland et al., 2001] and after overexpression of hGRβ in mouse hybridoma cells [Hauk et al., 2002]. Using pull-down assays, hGRβ has been shown to interact with hGRα [Yudt et al., 2003], and hERβcx with hERα (and to a lesser extent with hERβ [Ogawa et al., 1998]). Using gel-shift assays, hERβcx has been demonstrated to dimerize with both hERα and hERβ on DNA [Moore et al., 1998], and similarly rVDR1 and rVDR0 [Ebihara et al., 1996], and hTRα2 and hTRα1 [Hermann et al., 1991] have been found to dimerize on DNA (although the latter

was shown not to occur in a study by Katz and Lazar [Katz and Lazar, 1993]).

For nuclear receptors of subfamily 1 (e.g., TR, VDR, CAR, PPAR), that depend on dimerization with RXR for their transactivational activity, splice variants may dimerize with RXR, thereby forming an inactive dimer. However, rVDR1 [Ebihara et al., 1996], hTRα2 [Katz and Lazar, 1993], mCAR2 [Choi et al., 1997], and hCAR(sv5) [Arnold et al., 2004] were shown to be unable to dimerize with RXR on NREs. Only hγORF4 was shown to dimerize with RXR on DNA [Kim et al., 2006].

Competition for coactivators

The third possible mechanism for dominant-negative activity is titration of cofactors by the transcriptionally-inactive receptors. Protein-protein interactions between the dominant-negative receptor isoforms and limited amounts of cofactors can interfere with canonical receptor activity, since these cofactors form the link between the liganded receptor and the basal transcription machinery. Several coactivators that interact with the AF-1 have been suggested to play a role. For example, overexpression of CBP has been shown to prevent the inhibition of hPPARα-induced transactivation by hPPARα_{tr} [Gervois et al., 1999], and overexpression of TIF2/GRIP1 attenuates the suppressive effect of hGRβ on transactivation by hGRα, through interaction with the AF-1 [Charmandari et al., 2005]. In contrast, since all described splice variants lack a functional AF-2 domain, it is unlikely that coactivators interacting with this region will bind to these splice variants. Indeed, TIF1α has been shown not to interact with hERβcx in a pull down assay [Ogawa et al., 1998], and the coactivators SRC-1, TIF2/GRIP1/SRC-2, ACTR/SRC-3, and DRIP205 have been shown not to interact with hCAR(sv5) in a mammalian two-hybrid assay [Arnold et al., 2004].

In vivo relevance; lessons from hGRβ

The dominant-negative role of hGRβ

Although evidence for a dominant-negative role for C-terminal splice variants of nuclear receptors is mounting, the physiological role of these receptors may still differ from what has been observed in the *in vitro* studies. All results regarding the repression of the activity of canonical receptors are obtained from studies based on transfection experiments, using vectors containing reporter assays as a measure for transcriptional activity. These experiments provide convincing evidence that the splice variants inhibit (part of) the transactivational activity of their canonical receptor in this experimental setup. However, these receptor variants do not necessarily behave similarly *in vivo*. For instance, reporter assays with vectors containing nuclear receptor response elements (NREs) lack the condense chromatin packaging in which genomic DNA is wrapped *in vivo*. Therefore, NREs in reporter assays are more accessible than promoter regions of endogenous NR target genes. The delicate mechanisms required for transcription initiation, like chromatin remodeling, histone acetylation and methylation are overlooked in this experimental setup.

In addition to these *in vitro* reporter assays, the transcription of endogenous genes has been used as readout in several studies on hGRβ. Li et al. [Li et al., 2006] have used the glucocorticoid-induced increase in the expression of MKP-1 mRNA and the decrease of TNFα and IL-6 mRNA expression as readout for the sensitivity to glucocorticoids [Li et al., 2006]. This sensitivity was significantly increased in monocytes after treating them with anti-hGRβ siRNA. A similar siRNA experiment was performed in BAL macrophages in which the glucocorticoid-induced increase in MKP-1 transcription was enhanced after knocking down the hGRβ expression [Goleva et al., 2006]. Overexpression of hGRβ in mouse DO-11.10 hybridoma cells resulted in a decreased induction of MKP-1 mRNA following treatment with the synthetic glucocorticoid dexamethasone [Goleva et al., 2006]. In cells derived from human eye tissue (trabecular meshwork cells), overexpression of hGRβ inhibited the dexamethasone-induced increase in the expression of the endogenous genes myocilin and fibronectin [Zhang et al., 2005b].

More cell-biological readouts have been used in several other studies on the dominant-negative effect of hGRβ. Increasing the expression of hGRβ by transfection of mouse neutrophils results in a decreased rate of dexamethasone-induced cell death [Strickland et al., 2001]. Overexpression of hGRβ in mouse DO-11.10 hybridoma by viral transduction reduced the inhibition of cell proliferation by several glucocorticoids [Hauk et al., 2002]. Transient overexpression of hGRβ prevented the glucocorticoid-induced inhibition of phagocytosis in human NTM-5 cells [Zhang et al., 2007].

In summary, the dominant-negative activity of hGRβ is well established. It has been shown to inhibit the activity of hGRα in reporter assays. In addition, it has been shown to inhibit glucocorticoid-induced changes in the expression of endogenous genes. Interestingly, this was observed for genes that are activated by glucocorticoids (MKP-1, myocilin, fibronectin) and for genes that are repressed in response to glucocorticoids (TNFα, IL-6), which occurs independently of the receptor's DNA binding ability. Finally, hGRβ has been demonstrated to inhibit glucocorticoid-induced changes in several physiological properties in a number of cell types.

hGRβ as a regulator of transcription independent from hGRα

Besides its role as a dominant-negative inhibitor, hGRβ may act as a regulator of transcription independent from hGRα. Three studies show such a role for hGRβ. First, stable overexpression of hGRβ in U-2 OS cells (which do not express hGRα or hGRβ endogenously) resulted in altered transcription of more than 5,000 genes in a microarray study, of which approximately half were increased and half decreased [Lewis-Tuffin et al., 2007]. Second, stable overexpression of green fluorescent protein (GFP)-tagged hGRβ in HeLa cells also showed that hGRβ has intrinsic transcriptional activity [Kino et al., 2009]. It was shown to regulate the expression of a large number of genes negatively or positively. Third, in

transiently transfected reporter assays hGRβ has been shown to, just like hGRα, transrepress the activity of other transcription factors like AP-1 and NF-κB. Overexpression of hGRβ attenuated NF-κB- and AP-1-induced activation in luciferase assays [Gougat et al., 2002] and the GATA3-induced activity of the IL-5 and IL-13 promoters driving a luciferase gene [Kelly et al., 2008]. Thus, although limited data are available, a role for hGRβ independent from hGRα is suggested.

Increased expression of hGRβ and glucocorticoid resistance *in vitro*

Treatment of cells with pro-inflammatory cytokines like IL-1 or TNFα [Webster et al., 2001], IL-2 and IL-4 [Leung et al., 1997], IL-8 [Strickland et al., 2001], IFNα and IFNγ [Tliba et al., 2008], and IL-7 or IL-18 [Orii et al., 2002] has been shown to induce the expression of hGRβ mRNA and/or protein in several studies. TNFα treatment changes the hGRα:hGRβ protein ratio in HeLa cells from 4:1 to 1:2, resulting from a 4-fold increase in hGRβ and a 2-fold increase in hGRα protein expression [Webster et al., 2001]. IL-8 was shown to induce the expression of hGRβ protein 2-fold (without affecting hGRα levels) in neutrophils, changing the already low hGRα:hGRβ protein ratio in these cells from 1:4 to 1:8 [Strickland et al., 2001]. The splice factor SRp30 has been suggested to be involved in this process, since it was shown to direct the alternative splicing to hGRβ in these cells [Xu et al., 2003].

These treatments also resulted in decreased sensitivity to glucocorticoids, as measured by reduced DNA binding of hGRα [Leung et al., 1997], reduced transactivational activity in reporter assays [Webster et al., 2001], and reduced transcriptional activity using CD38 repression as a readout [Tliba et al., 2008]. In addition, microbial super-antigens have been demonstrated to induce the expression of hGRβ and to induce glucocorticoid resistance, as demonstrated by a reduced inhibition of the proliferative response to glucocorticoids in human PBMCs [Hauk et al., 2002] and by the inhibition of glucocorticoid-induced IL-2 repression in a nasal explant model [Fakhri et al., 2004]. However, in none of the studies mentioned here, has it been demonstrated whether the decrease in sensitivity is due to the increased hGRβ expression, or to other effects of the cytokine treatment.

Several human cell types have been shown to have relatively high levels of hGRβ expression [Oakley et al., 1997; Pujols et al., 2002]. These high expression levels have been shown to correlate with insensitivity to glucocorticoids. For example, human monocytes have significantly higher hGRβ levels than T cells, and display a slower glucocorticoid induction of MKP-1 and glucocorticoids fail to repress the IL-6 expression in these cells [Li et al., 2006]. In addition, in monocytes GRα translocates to the nucleus more slowly in response to glucocorticoid treatment (an effect also seen after overexpression of hGRβ in mouse hybridoma cells [Goleva et al., 2006]).

Increased expression of hGRβ and glucocorticoid resistance *in vivo*

In a large number of studies, increased hGRβ expression has been shown to correlate with resistance to glucocorticoid treatment in patients suffering from several immune-related diseases, suggesting that hGRβ plays an important role in glucocorticoid resistance in these patients. An increased expression of hGRβ has been demonstrated in various immune cells, particularly T-cells, from glucocorticoid-resistant asthma patients [Goleva et al., 2006; Hamid et al., 1999; Leung et al., 1997; Sousa et al., 2000]. In addition, in patients suffering from nasal polyposis [Hamilos et al., 2001], Crohn's disease [Towers et al., 2005], ulcerative colitis [Honda et al., 2000; Zhang et al., 2005a], and leukemia [Shahidi et al., 1999], a similar correlation between increased hGRβ levels and glucocorticoid resistance was observed. Goleva et al. [Goleva et al., 2006] demonstrated a causal relationship between hGRβ expression levels and glucocorticoid resistance by decreasing the hGRβ expression level using specific RNA silencing in broncheoalveolar lavage cells from glucocorticoid-resistant asthma patients, which enhanced the glucocorticoid responsiveness in these cells. Finally, a correlation has been found between increased expression of hGRβ and the occurrence of several immune-related diseases. In various immune cells of severe asthma patients [Bergeron et al., 2006; Christodoulopoulos et al., 2000], and patients suffering from ulcerative colitis [Orii et al., 2002], leukemia [Shahidi et al., 1999], ankylosing spondylitis (Bechterew's disease [Lee et al., 2005]), nasal polyposis [Hamilos et al., 2001], or severe systemic lupus erythematosus [Piotrowski et al., 2007] increased expression levels of this receptor isoform were found.

A single-nucleotide polymorphism in the glucocorticoid receptor gene in the region encoding the 3'UTR of hGRβ mRNA results in a mRNA with increased stability *in vitro* [Derijk et al., 2001; Schaaf and Cidlowski, 2002] and this polymorphism is associated with the occurrence of rheumatoid arthritis [Derijk et al., 2001]. This polymorphism is therefore considered to increase the expression of hGRβ in carriers, although this has never been demonstrated. Interestingly, this polymorphism appears to affect only certain glucocorticoid-induced changes in gene expression. In PBMLs derived from carriers, the glucocorticoid-induced transrepression of IL-2 mRNA expression was reduced, but the induction of GILZ mRNA expression by glucocorticoids was not different from non-carriers [van den Akker et al., 2006b]. In addition, the risk of nasal carriage of *Staphylococcus aureus* is reduced [van den Akker et al., 2006a], whereas the risk of cardiovascular disease is increased [van den Akker et al., 2008].

Thus, relatively high expression levels of hGRβ have been found in cells under certain conditions *in vitro*, in specific cell types *in vivo*, and in patients suffering from a variety of immune-related diseases. In all cases, this increased expression appears to correlate with a decreased sensitivity to glucocorticoids, although the

involvement of hGRβ in the observed glucocorticoid resistance has not directly been established in any of these studies.

Ligand binding of hGRβ

It has been shown that hGRβ is capable of binding the synthetic hGRα antagonist RU486 [Lewis-Tuffin et al., 2007]. Addition of RU486 increased the fraction of hGRβ residing in the nucleus in COS and U-2 OS cells, whereas a series of 57 related steroids did not affect subcellular localization of hGRβ. A similar effect of RU486 was found for zGRβ in COS and ZF4 (zebrafish fibroblast) cells (Schaaf, unpublished results). Furthermore, RU486 administration alters the regulation of approximately 1,000 genes in hGRβ-overexpressing U-2 OS cells (and not in wild type U-2 OS cells), of which the majority (~75%) were repressed. However, in a different study, no altered transcriptional activity or nuclear translocation of hGRβ was observed following addition of RU486 in HCT116 cells (derived from a human colon carcinoma) [Kino et al., 2009]. These studies suggest that RU486 might have cell-specific effects on the activity of hGRβ.

Discussion

In the present review, data on thirteen different C-terminal splice variants have been discussed. Nine human nuclear receptor genes have been demonstrated to show this type of alternative splicing, out of a total of forty-eight nuclear receptor genes in the human genome. The significant number of described C-terminal splice variants suggests that these splice variants play an important role in nuclear receptor functioning. Although many nuclear receptors have been studied in great detail, it is conceivable that several more nuclear receptor genes encode a C-terminal splice variant, but that this has not yet been described for these genes.

The most likely function of these splice variants is to act as a dominant-negative inhibitor of the canonical receptor, since this type of dominant-negative inhibition has been shown for many of the described receptor variants. Ten splice variants have been demonstrated to have dominant-negative activity on their cognate canonical receptor in reporter assays, and only one (mCAR2) failed to display this type of activity in these assays. The human GR β-isoform, the best studied member of this group, was not only studied in reporter assays, but it has also been shown to have dominant-negative activity on endogenous gene regulation by the canonical hGRα, and on glucocorticoid-induced alterations in cell physiology. These data suggest that the data from reporter assays can, at least in part, be extrapolated to the regulation of endogenous genes.

In addition, it is possible that the C-terminal splice variants described in this review function as transcription factors independent from their cognate canonical receptor, since it has been shown that hGRβ is able to regulate gene transcription independent from hGRα [Gougat et al., 2002; Kino et al., 2009; Lewis-Tuffin et al., 2007]. Although this latter activity of hGRβ has not been studied in detail yet, future studies on other C-terminal splice variants may uncover this type of functionality for these receptors, as well.

For the dominant-negative activity of these splice variants, three possible mechanisms of action have been suggested. First, occupation of response elements, although most receptor splice variants showed no or decreased DNA binding. Second, the formation of inactive heterodimers with the canonical receptor. For some C-terminal variants, such dimers with the canonical receptor have indeed been shown for some variants to occur in living cells. Furthermore, DNA binding of several of these dimers was demonstrated *in vitro* using gel shift assays. Third, competition for coactivators has been proposed. Although some variants have been shown to interact with specific coactivators, limited data are available to get a good overview of coactivator binding of the splice variants. Thus, none of the three proposed mechanisms could be excluded, and possibly all three mechanisms play a role. The level at which they contribute to this activity may differ between receptors, and this could be due to structural differences between the splice variants.

Due to their dominant-negative activity, the splice variants may play an important role in the regulation of the activity of their cognate canonical receptor *in vivo*. We hypothesize that these splice variants are capable of blunting (part of) the activity of their respective canonical receptors, which may occur in cells or tissues where the splice variant is expressed at high enough levels for dominant-negative regulation to take place. Since these canonical receptors play important physiological roles, aberrant expression of the splice variants may underlie several pathological conditions. For hGRβ, a role in glucocorticoid resistance and pathogenesis of several immune-related diseases is strongly suggested, as discussed in detail above. For other nuclear receptors, the *in vivo* relevance is less clear. For example, in some studies the presence of hERβcx and hERβ is correlated with better prognosis in breast cancer [Palmieri et al., 2004; Sugiura et al., 2007], while in others the presence of hERβcx is of no influence on the development of carcinomas [Yen, 2000]. In addition, allelic variations in intron 8 of the human VDR gene (which is the intron retained for the formation of rVDR1 mRNA) can be used to predict the density of bone [Ebihara et al., 1996]. Involvement in tumorigenesis in colorectal cancers has been suggested for the hPPARγ splice variant hγORF4 [Sabatino et al., 2005]. These interesting observations indicate that a better understanding of the role of nuclear receptor splice variants may help to elucidate the molecular mechanism of the pathogenesis of a variety of diseases. In addition, the observation that hGRβ is able to bind the synthetic ligand RU486, which alters its activity [Lewis-Tuffin et al., 2007], suggests that opportunities exist to exploit these splice variants as potential drug targets.

Comparison of the structure of these thirteen receptor isoforms yielded two interesting observations. First, two

main groups exist. The splice variants of the first group (hGRβ/zGRβ, hERβcx, hTRα1/gTRα1, rVDR1, mCAR2/hCAR(sv5), and hDAX-1α) have a splice-variant-specific tail that starts around helix 10. The variants of the second group (hNurr2, hNOR-2, hPPARα_{tr} and hγORF4) have almost the entire LBD replaced by an alternative sequence. Second, the splice variant-specific C-terminal amino acid sequences are highly heterogeneous (see Table 2). They do not appear to show any homology or to share common domains, suggesting that the splice variant-specific amino acid sequence is not important for the dominant-negative activity. Indeed, structure-function studies on hGRβ demonstrated that out of fifteen splice variant-specific amino acids, only two amino acids, that caused a distortion in the (helical) structure of the receptor's C-terminus, were required for the dominant-negative activity of this receptor isoform [Yudt et al., 2003]. These data, together with the lack of similarity between the different C-termini of the different splice variants, suggest that many alternative C-termini may result in dominant-negative activity, but that some specificity exists.

When we compared genomic sequences from human, mouse, chicken, clawed frog and zebrafish, it was shown that the splice sites required for the generation of C-terminal splice variants are relatively well conserved between human and mouse, but not between human and non-mammalian species (data not shown). Of the splice variants that occur in humans and rodents, only hGRβ and hERβcx appear not to be conserved between human and mouse. Interestingly, conservation of the amino acid sequence of the C-terminal tail of the splice variants is very poor, even between human and mouse. Only the sequences specific for TRα2 and CAR2 are more than 50% identical between human and mouse. Thus, similar to our findings when we compared sequences of the different splice variants, we found very little similarity in the C-terminal sequence of splice variants that are orthologs. Both findings suggest low selective pressure on most of these sequences.

In conclusion, many C-terminal splice variants of nuclear receptors exist, which probably play a role as dominant-negative inhibitors of the activity of the canonical receptor. Most of the sequences of their C-terminus may not be critical for this function. We suggest an important role for these receptor isoforms, controlling or fine-tuning the activity of nuclear receptors.

Supplementary Material

Supplementary File 1: Genetic organization of the NR LBD splice variant genes. The intron and exon structure of the NR genes and resulting mRNAs and proteins are shown. Splicing events are depicted with dotted lines. The numbers above the proteins indicate the first and final amino acid positions, as well as the divergence point. The location of the DNA-binding domain (DBD) in the proteins serves as a reference point. a.1: hGRβ is generated by replacing exon 9α with exon 9β. a.2: In contrast to hGRβ, zGRβ is created when intron 8β is retained. b: ERβcx is generated by replacing exon L4 with exon Lcx. c.1: hTRα1 is generated by using a cryptic splice donor site in exon 9 combined with an alternative exon 10. c.2: Goldfish TRα1 and gTRα2 proteins. Due to the lack of genomic sequence data, the splicing mechanism is unknown. d: rVDR1 is generated by retention of intron 8. e.1: hCAR(sv5) is generated using an alternative splice acceptor site in exon 9. e.2: mCAR2 is generated by replacing exon 8 by exon 8'. f: DAX-1α is generated by replacing exon 2 by exon 2a. g: Nurr2 is generated by alternative splice acceptor site usage in exon 6. Alternative acceptor site usage occurs in exon 1 and 2 as well, but this is not indicated in this figure since it is beyond the scope of this review. h: mNOR-1 is generated by alternative splice acceptor site usage in exon 5. i: PPARαtr is generated by retention of the intron between exon 5 and 6. j: γORF4 is generated by retention of the intron between exon 4 and 5.

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