The Origin and Functions of Multiple Human Glucocorticoid Receptor Isoforms

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ABSTRACT: Glucocorticoid hormones are necessary for life and are essential in all aspects of human health and disease. The actions of glucocorticoids are mediated by the glucocorticoid receptor (GR), which binds glucocorticoid hormones and regulates gene expression, cell signaling, and homeostasis. Decades of research have focused on the mechanisms of action of one isoform of GR, GRα. However, in recent years, increasing numbers of human GR (hGR) isoforms have been reported. Evidence obtained from this and other laboratories indicates that multiple hGR isoforms are generated from one single hGR gene via mutations and/or polymorphisms, transcript alternative splicing, and alternative translation initiation. Each hGR protein, in turn, is subject to a variety of posttranslational modifications, and the nature and degree of posttranslational modification affect receptor function. We summarize here the processes that generate and modify various hGR isoforms with a focus on those that impact the ability of hGR to regulate target genes. We speculate that unique receptor compositions and relative receptor proportions within a cell determine the specific response to glucocorticoids. Unchecked expression of some isoforms, for example hGRβ, has been implicated in various diseases.

KEYWORDS: glucocorticoid receptor isoforms; alternative splicing; phosphorylation; ubiquitination; receptor mobility

INTRODUCTION

Glucocorticoids are essential for proper embryogenesis, development, growth, and survival.[1,2] In addition, glucocorticoids are broadly used as therapeutics in acute and chronic treatment of asthma,[3,4] rheumatoid arthritis,[5] degenerative osteoarthritis,[6] ulcerative colitis,[7] eosinophilic gastritis,[8] transplant rejection,[9] complications from acquired immunodeficiency syndromes,[10] as well as many other inflammatory and immune diseases. Furthermore, glucocorticoids have also been applied effectively as chemotherapeutic agents in the treatment of cancers, especially cancers of

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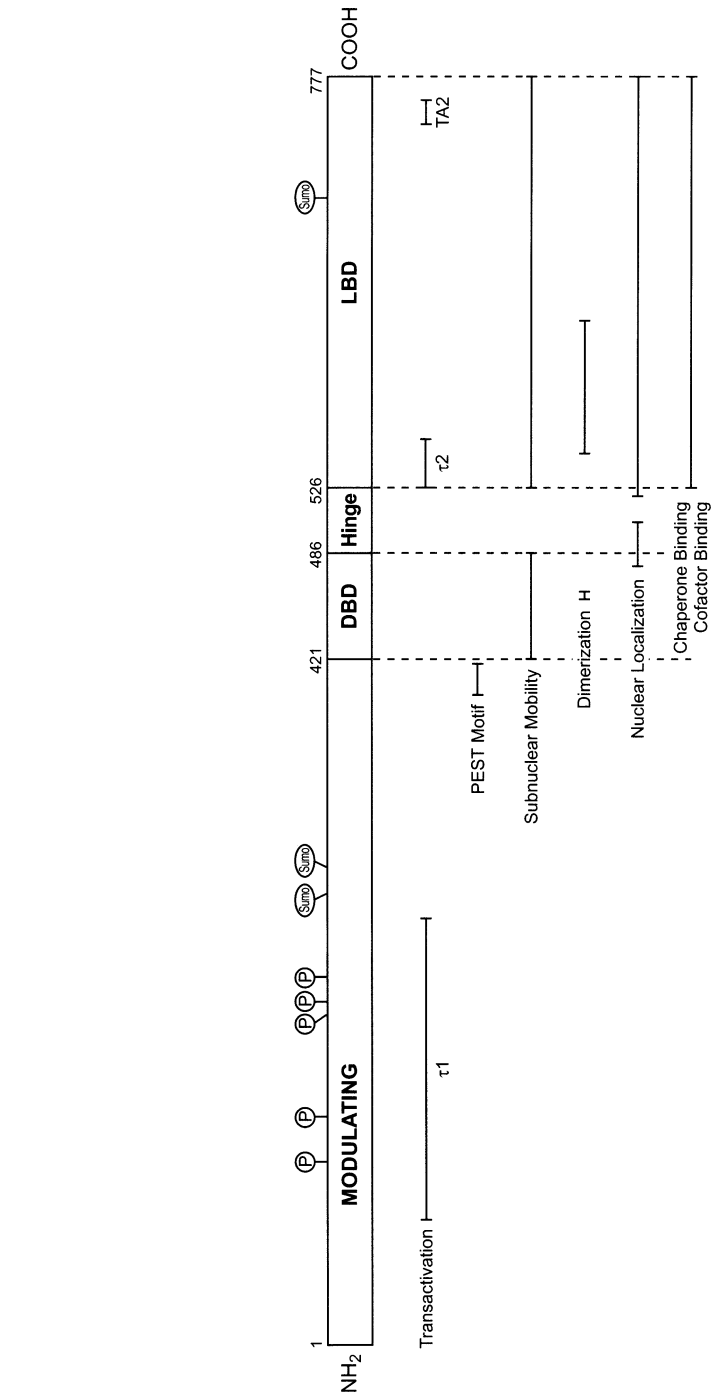

FIGURE 1. Domains and modified residues of the human glucocorticoid receptor (hGR). The N-terminal modulating domain contains the sequence for the main transactivation activity, τ1, while τ2 and an additional transactivation activity (TA2) reside in the ligand binding domain (LBD). Several functional domains of hGR overlap with each other. For example, portions of the DNA binding domain (DBD) are critical for ligand-dependent nuclear translocation of the receptor and receptor dimerization. P, phosphorylation sites; Sumo, sumoylation sites.

hematological origins,[1] including Hodgkin's lymphoma, acute lymphoblastic leukemia, and multiple myeloma. Despite the extensive clinical usage of glucocorticoids in the clinic, the mechanisms underlying the remarkable diversity of the glucocorticoid receptor (GR) function are poorly understood.

GR, along with related steroid receptors such as estrogen receptor (ER), progestin receptor (PR), androgen receptor (AR), and mineralocorticoid receptor (MR), likely emerged through a series of gene duplication events from a common ancestral receptor some 400 million years ago.[11] Similar to other steroid receptors, GR protein has a modular structure.[12–16] From amino terminus to carboxyl terminus are the amino acid sequences for the transactivation domain 1 (τ1 or TA1), DNA binding domain (DBD), hinge region, and ligand binding domain (LBD, FIG. 1). Additional transactivation domains embedded in the LBD, the τ2 and TA2, are less potent in autonomous transactivation activity than the τ1 domain. Correctly folded GR presents "pockets" for cognate hormone recognition[17] and motifs for recognizing specific DNA sequences termed glucocorticoid response element (GREs) on target genes.[18] Somewhat overlapping with the aforementioned major domains are additional regions that may allow interdomain interactions, e.g., between TA1 and DBD.[19] These regions also facilitate interactions between GR and other proteins, including chaperones that are involved in the compartmentalization and trafficking of the receptor[20–22] and coregulators that control the efficacy of the receptor function.[23–29] In addition, these regions may also facilitate the heterodimerization of GR isoforms[17,30] and direct interactions between GR and other transcription factors that may expand the potential gene targets of GR.[31–41]

In this article, we describe the processes that generate multiple human GR (hGR) isoforms from a single gene, including alternative RNA splicing, alternative translation initiation, and gene mutations. Also summarized is recent evidence for post-translational modifications of GR proteins, with the emphasis on phosphorylation and ubiquitination as well as the consequences of these modification processes on receptor function. Finally, we present novel observations on the intranuclear movement of hGR as a result of selective ligand binding.

COMPLEXITIES WITHIN THE hGR GENE

Only one GR gene has been identified in every species examined to date. The hGR gene is located on chromosome 5q31-32 and comprises over 140 kb of nucleotides, less than 2% (~2.5 kb) of which are exons.[42–47] There are 9 exons in the hGR gene (FIG. 2): exon 1 (~116–981 bp) is a leader sequence; exon 2 (1,197 bp) contains the coding sequence for τ1 at the amino terminal; exons 3 (167 bp) and 4 (117 bp) code for the first and second zinc-finger motif in the DBD, respectively; exons 5 (280 bp), 6 (145 bp), 7 (131 bp), and 8 (158 bp) code for τ2 and a large portion of the LBD; and exon 9 (4,108 bp) contains coding sequences for the two alternative carboxyl termini of the LBD, α and β, and their respective 3′ untranslated regions. Remarkable homology has been found within the splice junctions of exons for the DBD and LBD among GR and related steroid hormone receptors, such as PR, AR, and ER.[45] Divergence, however, exists between GR and less-related nuclear receptors, such as thyroid hormone receptors (TR) and vitamin D receptors. These findings suggest that, evolutionarily, three parallel branches of receptors for steroids,

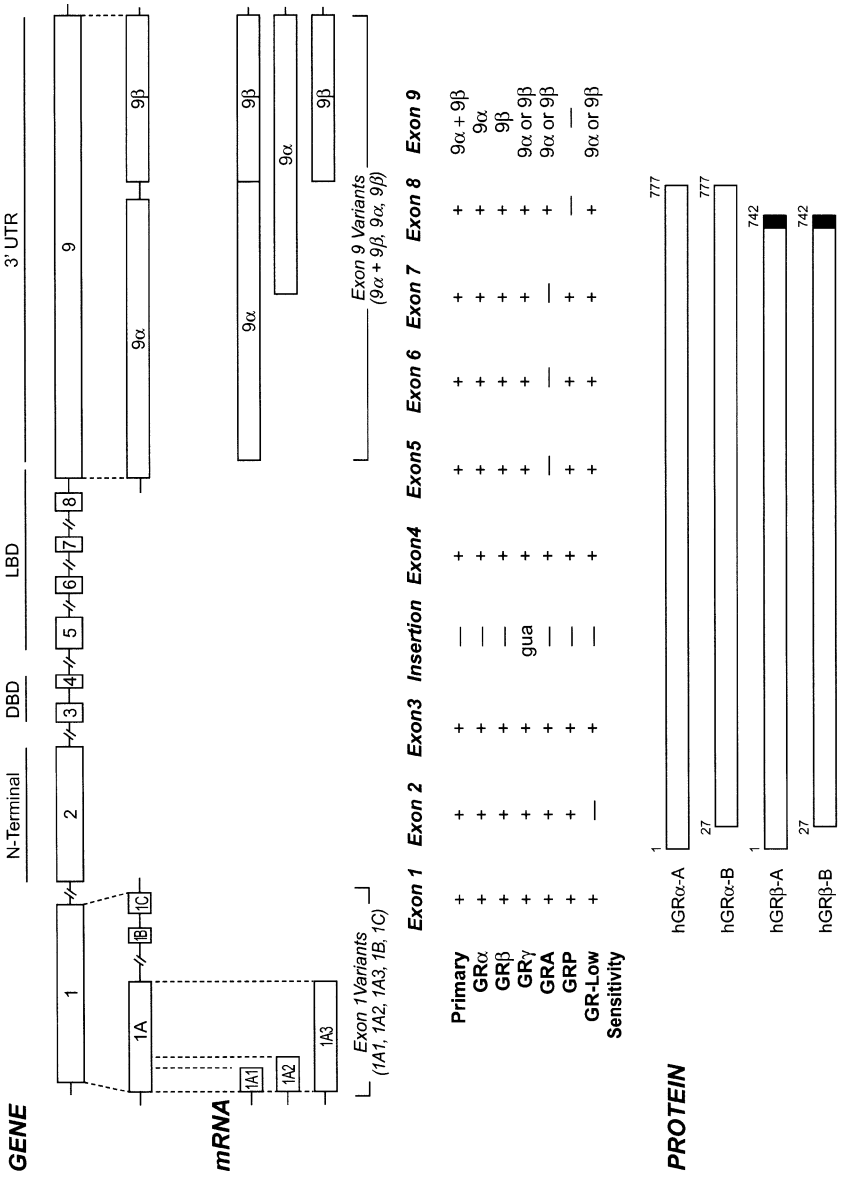

FIGURE 2. *See following page for legend.*

vitamin D, and thyroid hormones split early from a common ancestral receptor. The least homologous sequences and intron-exon organization among steroid receptors are found in exons 1 and 2.

Exon 1 of hGR exists in three forms, 1A, 1B, and 1C, each of which is driven by a distinct proximal promoter.[47–49] Thus, promoter 1A, approximately 31 kb upstream of the first start codon, drives the expression of exon 1A (~212–981 bp); promoter 1B drives the expression of exon 1B (~116 bp) approximately 5 kb upstream of the first start codon; whereas promoter 1C drives the expression of exon 1C (184 bp) approximately 4 kb upstream of the first start codon. None of the promoter regions of the hGR gene contain a consensus TATA or CAAT box, but all contain multiple GC islands, reflecting the necessity for constitutive expression of hGR. Multiple transcription factors have been reported to bind to various sites on the hGR promoters, including four Sp1 and three YY1 binding sites in promoter 1B[50,51] and six Sp1, one AP-2, one NF-κB and one YY1 sites in promoter 1C.[47–52] This vast array of transcription factors seems to ensure constitutive expression of hGR under a variety of physiological conditions. However, the expression level of hGR transcripts containing each species of exon 1 may also be regulated in a cell type–specific as well as developmental state–dependent manner. For example, exons 1B and 1C are ubiquitous although expression levels differ in various cells and tissues.[47] The hGR transcripts containing exon 1A3, one of the three 1A exons (see below), are more abundant in cancer cells of hematopoietic lineage than in cancer cells from the bone, liver, lung, or breast. In addition, hormonal factors also may regulate hGR promoter usage. For example, promoter 1A, but not 1B or 1C, contains an element identified as a noncanonical GRE, through which the expression of the exon 1A3 is upregulated in CEM-C7 T cells but, interestingly, downregulated in IM-9 B-lymphoma cells by dexamethasone.[47]

Although the expression level of exon 1 is highly regulated, the first exon of the hGR transcript is not a component of the coding region due to an in-frame stop codon at the very beginning of exon 2, only 9 bp upstream of the first start codon. However, the terminology of "5′ untranslated region" may be a misnomer since, in the mouse GR exon 1, at least one potential open reading frame exists in the leader sequence. This 5′ open reading frame may be translated into a small peptide of

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FIGURE 2. Organization of the human glucocorticoid receptor (hGR) gene and diversification of hGR transcripts and proteins. The hGR gene contains nine exons (numbered in boxes), coding various regions of the receptor, such as the DNA binding domain (DBD), ligand binding domain (LBD), and untranslated regions (UTR). Alternative promoter usage and mRNA alternative splicing generate multiple hGR transcripts. For example, at least five exon 1 variants, 1A1, 1A2, 1A3, 1B, and 1C, can join exon 2, yielding transcripts containing various 5′ leader sequences. In addition, alternative splicing of exon 9 generates mRNAs coding for hGRα or hGRβ. Furthermore, alternative splicing can also result in the insertion of an additional codon (GRγ), exon skipping (GRA), or exon deletion (GRP), generating receptor isoforms with blunted activity. All mRNA variants, except GR–low sensitivity (mouse lymphoma cells), have been detected in human cells and tissues. Compositions of exon 9 have been confirmed experimentally for primary transcript of hGR, hGRα, and hGRβ, and predicted for the other mRNA variants. During translation of transcripts containing both AUG^{1} and AUG^{27}, the number of GR proteins is doubled by alternative translation initiation. The labels on hGR proteins refer to amino acids in the full-length hGRα receptor and are from references listed in the text.

8.5 kDa,[53] which is thought to regulate the translation efficiency of the full-length mouse GR protein.[54] Interestingly, transcripts of many potent regulatory proteins, such as cytokines, growth factors, kinases, and transcription factors, similar to GR, often contain a 5′ leader sequence.[55] The length of the leader sequence has been correlated with the translational efficiency for some transcripts; however, such information about hGR is lacking.

The mouse GR gene also has three promoter regions homologous to hGR,[56] and in cancer cells, such as S-49 lymphoma cells, four or five promoters maybe active.[57] In humans, the versatility of the GR gene in directing the expression of hGR receptors can be demonstrated not only by alternative promoter usage, but also by numerous polymorphisms within the gene. Over a hundred natural single nucleotide polymorphisms have been documented in the hGR database (http://www.ncbi.nlm.nih.gov:80/SNP/snp_ref.cgi?locusId=2908). Although few of these polymorphic loci are correlated with human diseases, individual differences in glucocorticoid responses may very well be attributed to sequence substitution within the hGR gene.[58] Furthermore, scores of additional natural mutations in the hGR gene have been recorded in instances of glucocorticoid resistance, as defined by the decreased therapeutic effects of glucocorticoid drugs in patients after prolonged usage or the absence of ACTH suppression by dexamethasone challenge. The former type of glucocorticoid resistance frequently occurs selectively in tissues exposed to glucocorticoids (upper airways, for example, when inhaling agents are used for asthma) while the latter usually indicates a generalized dysfunction. In either scenario, mutations within the hGR gene are often the cause. Key amino acid changes in the τ1 region, LBD, DBD, or mutations leading to truncated proteins have all been identified to cause glucocorticoid resistance.[58]

ALTERNATIVE SPLICING OF hGR TRANSCRIPTS

As described above, three promoters drive the expression of at least three exons 1 (1A, 1B, or 1C). Exon 1A, through alternative splicing, produces three additional isoforms,[47] 1A1 (~212 bp), 1A2 (~308 bp), and 1A3 (981 bp, FIG. 2). Sequences of exons 1A1, 1A2, and 1A3 are identical towards the 5′ end whereas three distinct splicing donor sites at the 3′ end join with the common acceptor sites on exon 2, increasing the potential number of hGR transcripts to five, i.e., 1A1, 1A2, 1A3, 1B, and 1C. Additional alternative splicing events exist and affect the coding region of hGR as well. For example, at the carboxyl terminus of the hGR primary transcript, exon 9 comprises the originally defined exon 9α (2,475 bp), an intron of 155 bp, and exon 9β (1,478 bp).[45,46] This large exon can be alternatively spliced to join exon 8, generating hGRα and hGRβ (FIG. 2).

*hGR*β

Amino acid sequence analysis revealed that hGRα and hGRβ isoforms are identical from the amino terminus through amino acid 727 but diverge beyond this position, with hGRα having an additional 50 amino acids and hGRβ having an additional, non homologous, 15 amino acids. The existence of the hGRβ isoform was predicted ever since the cloning of the hGR cDNA;[42] however, only hGRα appeared to bind hormone

and induce the expression of a glucocorticoid-responsive reporter gene in a hormone-dependent manner.[42,59] In contrast, the hGRβ isoform has been largely ignored because early studies reported that the recombinant hGRβ does not bind hormones and does not activate glucocorticoid-responsive promoters.[42,59] Recent years have seen a spur of interest in hGRβ since this isoform was found to have widespread tissue expression.[46,60,61] hGRβ acts as a dominant negative inhibitor for hGRα transcriptional regulation and, importantly,[46,60] increased hGRβ expression has been correlated with several diseases related to glucocorticoid resistance.[41,62–67]

Using Northern blot or reverse transcription PCR analyses, both hGRβ and hGRα mRNAs have been detected in multiple adult and fetal tissues, including the heart, brain, placenta, lung, liver, skeletal muscle, and pancreas.[46,60] To investigate the expression of hGRβ protein, we have produced an antipeptide, hGRβ-specific antibody termed BShGR.[61] This antibody has been made against the unique 15-amino acid peptide at the carboxyl terminus of hGRβ and recognizes both the native and denatured conformations of hGRβ, but it does not crossreact with hGRα. Using BShGR on Western blots and in immunoprecipitation experiments, we have also detected hGRβ protein in a variety of human cell lines and tissues. A second hGRβ antibody has been recently produced in a separate laboratory and has also been used to confirm the wide distribution pattern of hGRβ.[62]

In the absence of ligand, hGRα resides in the cytoplasmic compartment, forming a complex with molecular chaperones like hsp90. When treated with cognate hormones, it is released from the molecular complex in the cytoplasm and translocates to the nucleus. In support of this two-step translocation model, we previously have shown that, in HeLa-S3 cells, hGRα translocates from the cytoplasm to the nucleus in a hormone-dependent manner.[46,68] However, in marked contrast to hGRα, hGRβ has been found largely in the nucleus, independent of glucocorticoid treatment.[46] Further detailed analysis has demonstrated that within tissues, hGRβ is expressed at high levels in a cell type–specific manner.[61] For example, hGRβ protein is expressed abundantly in the epithelial cells lining the terminal bronchiole of the lung, forming the outer layer of Hassall's corpuscle in the thymus, and lining the bile duct in the liver. In contrast, thymic lymphocytes and other epithelial cells in these tissues show very little immunoreactivity. Moderate immunoreactivity has also been observed in hepatocytes. These studies indicate that relative levels of hGRα and hGRβ may vary considerably among different cells. Thus, ratios of hGRα and hGRβ proteins determined from whole tissues or organs do not necessarily reflect the ratio of hGRα and hGRβ within an individual cell.

The molecular difference between hGRα and hGRβ lies within the 3′ end of the LBD. The 50 amino acids at the carboxyl terminus of hGRα are replaced with 15 unique amino acids in hGRβ. With few exceptions,[69,70] amino acid changes in the hGRα LBD often result in a reduction or complete loss of hormone binding.[59,71–73] In agreement with previous reports,[42,59,60] we observed that this natural carboxyl terminus modification prevents agonist binding to hGRβ protein.[46] Similar observations have been reported for other steroid receptors. For example, the truncated version of the human PR-B, missing the carboxyl terminal 42 amino acids, does not bind progesterone or the synthetic agonist R5020 but does bind the antiprogestin RU486.[74] This finding suggests that amino acids at the extreme carboxyl terminus of the human PR are critical for agonist but not antagonist binding. To date, we have not found ligands that bind to hGRβ.

Consequently, independent of steroid treatment, hGRβ seems to be transcriptionally inactive on reporter genes studied thus far. However, hGRβ can bind GREs in the promoter regions of target genes.[60] In addition, hGRβ binds GRE-containing DNA with a greater capacity than hGRα in the absence of glucocorticoids.[75] Glucocorticoid treatment enhances hGRα binding, but not hGRβ, to DNA. hGRβ interacts with hsp90, which, in the nucleus, may facilitate the release of chromatin-bound hGR.[76] Remarkably, hGRβ inhibits the hGRα-mediated activation of several reporter genes in a dose-dependent fashion.[46,60] Furthermore, hGRβ represses the activity of endogenous hGRα.[75] In contrast, the ability of the PR or AR to activate reporter genes is only weakly affected by hGRβ, demonstrating that the dominant negative activity of hGRβ is specific for hGRα. In addition, hGRβ also inhibits hGRα-mediated repression of NF-κB– and AP-1–responsive promoters.[46,77]

The molecular basis for the dominant negative activity of hGRβ has been recently elucidated.[78] Molecular modeling of the wild type and mutant hGRα and hGRβ has delineated a possible structural basis for the lack of hormone binding and the dominant negative actions of hGRβ. The absence of helix 12 in the LBD is neither necessary nor sufficient for the dominant negative phenotype of hGRβ. Using a series of truncated hGRα mutants and sequential mutagenesis, our laboratory has generated a series of hGRα/β hybrids. We have demonstrated that two residues within the unique 15 amino acids of hGRβ are responsible for the dominant negative activity. In addition, hGRα and hGRβ have been found to physically associate with each other as heterodimers,[75] which may hinder the formation of the transcriptionally active hGRα homodimers. Thus, the physiological significance of hGRβ may reside in its ability to antagonize the function of hGRα. High levels of hGRβ would confer glucocorticoid resistance, and low levels of hGRβ would confer hypersensitivity to glucocorticoids.

Thus, it is of great interest to determine the factors that regulate the relative expression levels of hGRα and hGRβ: the identification of such factors would elucidate potential treatment targets for hGR-related diseases. Webster and colleagues have recently reported that in HeLa-S3 cells TNFα treatment selectively enhances the steady-state levels of the hGRβ protein isoform, making hGRβ the predominant endogenous receptor isoform over hGRα.[52] Similar results have also been observed following treatment of human CEM-C7 lymphoid cells with TNFα or IL-1. TNFα and IL-1 are both pro-inflammatory agents whose actions can be counteracted by glucocorticoids. The increase in hGRβ protein expression correlates with the development of glucocorticoid resistance. For example, increases of hGRβ levels have been reported in T cells in the airway, peripheral blood mononuclear cells, and in tuberculin-induced inflammatory lesions in glucocorticoid-insensitive asthmatics.[41,62–65] Elevated expression of hGRβ in peripheral blood mononuclear cells from patients with rheumatoid arthritis has also been correlated with glucocorticoid resistance.[66] In another report, high levels of hGRβ are found in 10 of 12 patients with glucocorticoid-resistant colitis.[67] Additionally, Hauk and colleagues have demonstrated that isolated peripheral blood mononuclear cells, when stimulated with various superantigens, become insensitive to glucocorticoids: this insensitivity is believed to be the result of an increased expression of hGRβ.[63] In a separate study, incubation of myoblasts with 50–1,000 nM of cortisol resulted in a dose-dependent decline in hGRα expression and a dose-dependent increase in hGRβ expression.[79] These studies underscore the importance of hGRβ in causing diseases and suggest

that a strong correlation exists between the expression level of hGRβ, relative to hGRα, and resistance to glucocorticoids.

Glucocorticoid insensitivity is observed not only in disease states but also during normal physiological processes. For example, we and others have shown that genes that are positively regulated by hGRα are unresponsive to glucocorticoids during the G2 phase of the cell cycle.[80,81] During development, the chicken retina is resistant to circulating glucocorticoids before embryonic day 6, but thereafter becomes progressively more sensitive even though the level of hGRα does not change significantly over this time period.[82] In each case, cell cycle or developmentally regulated induction of hGRβ might account for the temporary resistance. Indeed, alternative splicing is often regulated in a cell type– and developmental state–specific fashion, or in response to specific cellular signals. Information on the regulation of hGR alternative splicing events is scarce, although a recent report suggests that serine arginine-rich protein p30 is involved in directing alternative splicing of hGR pre-mRNA to hGRβ in neutrophils.[83]

Other members of the nuclear receptor superfamily, TRα for example, bear resemblance to the alternative splicing pattern of hGR. Through alternative splicing of the last exon, TRα generates two receptor isoforms, TRα1 and TRα2, that differ at the carboxyl terminus.[84,85] The TRα2 isoform does not bind thyroid hormones and represses the transcriptional activity of TRα1 by competing with TRα1 for binding to the thyroid hormone responsive elements.[85] These data imply that a wide range of hormone responses can be achieved by varying the ratio of receptor isoforms within a cell. The carboxyl terminal sequence of rat GR is homologous to hGR with both α and β isoforms being produced.[86] In contrast, mouse GR has exon 9α but no 9β.[87]

Other hGR Splice Variants

Several other hGR splice variants have been detected in tissues and in certain cancer cell lines. For example, hGRγ transcripts (FIG. 2) have been detected where a 3-bp sequence from the intron separating exons 3 and 4 is retained, yielding an in-frame single amino acid insertion between the two zinc-fingers in DBD.[88] This isoform of hGR is also widely expressed and represents 4–8% of total hGR message in various tissues. Interestingly, however, hGRγ exhibits only about half of the transcriptional activity of hGRα. Recently, the level of hGRγ has been correlated with glucocorticoid resistance in childhood acute lymphoblastic leukemia.[89] A similar insertion at this splice junction has also been detected in the mouse GR,[90] rainbow trout GR,[91,92] as well as human MR.[93]

An additional splice variant of GR has been reported in glucocorticoid-resistant mouse lymphoma cells. This isoform lacks the entire exon 2 that encodes the amino terminal τ1 region, labeled as GR–low sensitivity in FIGURE 2.[94–96] One other splice variant of the GR, GRP, has retained the intron between exons 7 and 8, thus missing the appropriate exons 8 and 9.[97] The GRP variant is expressed at a high level in glucocorticoid-resistant myeloma patients.[98] In the same patient group, another splice variant missing the entire sequences of exons 5, 6, and 7, and thus a significant portion of the LBD, has also been identified and termed GRA.[97] Both GRP and GRA have been determined to contribute to glucocorticoid resistance.

ALTERNATIVE INITIATION OF hGR TRANSLATION

During translation of hGR transcripts, ribosome entry occurs at the 5′ end of the hGR message. Sequential addition of amino acids occurs after the recognition of the first start codon and concludes when the ribosomes encounter the stop codon at the 3′ end of hGR transcripts, yielding the full-length 777 amino acid peptide. However, translation reinitiation occurs at codon AUG^{27} in hGR transcripts, generating a receptor peptide of 751 amino acids that lacks the first 26 amino acids from the full-length hGR.[99] In the original paper that describes these two isoforms (FIG. 2), the 94 kDa full-length receptor translated from hGRα transcript is named hGRα-A and the 91 kDa protein is named hGRα-B.[99]

A survey of eukaryotic mRNAs has revealed that alternative start codon usage, also termed ribosomal leaky scanning, may occur in as many as 5% of the transcribed messages.[100] During translation, suboptimal nucleotide context in the proximity of the first start codon promotes weak ribosomal binding. Additional ribosomes are therefore permitted to scan for binding sites downstream of the first start codon. When the weak context at the AUG^{1} of hGRα was replaced with a consensus sequence that facilitates optimal interaction between ribosomes and mRNA, the production of hGRα-B can be diminished.[99]

hGRα-A and hGRα-B exhibit similar ligand-dependent translocation from the cytoplasm to the nucleus.[99] Interestingly, hGRα-B, in transient transfection experiments, activates reporter genes to a greater extent than hGRα-A. This is in agreement with the notion that ribosomal leaky scanning, instead of reflecting "sloppiness" of the translation machinery, deliberately produces potent regulators of cell function.[55] Whether hGRα-A and hGRα-B are differentially expressed in a tissue-specific manner and how their expressions are regulated are topics under investigation. Potentially, A and B isoforms derived from various hGR transcripts through alternative translation initiation may diversify the hGR receptor family exponentially. For example, the hGRβ transcript, which contains both AUG^{1} and AUG^{27}, produces both hGRβ-A and hGRβ-B isoforms (unpublished results).

POSTTRANSLATIONAL MODIFICATIONS OF hGR

Mature hGR proteins are covalently modified by various processes, which further modulate the transcription regulation activity of the receptors. For example, three consensus sumoylation sites (FIG. 1) have been identified within the hGR peptide sequence, and this modification process seems to affect receptor activity.[101–103] In addition, nitrosylation at cysteine residues on GR likely decreases ligand binding and may disable glucocorticoids from exerting anti-inflammatory effects during fatal septic shock.[104] In this section, we discuss in detail two other posttranslational modification processes that are directly linked with GRα-A function: phosphorylation and ubiquitination. The information on posttranslational modification of other GR isoforms is scarce.

Phosphorylation

Like most other nuclear receptors, mature GR proteins are phosphorylated. When activated by agonists, GR becomes hyperphosphorylated on several of the eight res-

idues at the amino terminus of the receptor. Identification of the phosphorylated residues on GR required the heroic efforts of several laboratories.[105] Receptor proteins were radiolabeled with [^{32}P] *in vivo*, purified, and subjected to digestion by trypsin at optimal conditions. Incomplete hydrolysis may introduce overlapping, thus confounding signals, whereas overdigestion may cut the peptide into fragments too small to be sufficiently purified, thereby increasing the number of misses. Tryptic peptides were then separated by HPLC, the phosphate content of each fraction measured, and the amino acids sequenced. Eight phosphorylated residues directly identified in the mouse GR are serines 122, 150, 212, 220, 234, 315, and 412, and threonine 159. Five corresponding amino acids in hGR are serines 113, 141, 203, 211, and 226 (FIG. 1). There are no counterparts in hGR for the other phosphorylated residues identified in the mouse GR, and this difference may underlie species-specific receptor functions.[106]

Since the phosphorylated residues of GR are concentrated in the τ1 region of the receptor, significant changes of receptor transactivation activity were anticipated when receptor phosphorylation was disrupted in receptors containing serine/threonine to alanine substitutions. However, it was not until after a series of target promoters were surveyed that the profile of transcription activity regulation by phosphorylation was revealed. For example, in COS-1 cells, replacing all eight phosphorylated residues in mouse GR does not alter the receptor's ability to induce a reporter gene driven by the mouse mammary tumor virus promoter.[107] In contrast, the non-phosphorylated receptor exhibits only 25–50% of the transactivation activity of the phosphorylated receptor on a simple GRE2-driven reporter. Thus, phosphorylation enhances the transactivation activity in a gene-specific manner. Different degrees of receptor phosphorylation, therefore, may extend the range of the gene regulatory capability of GR. Wang and colleagues, using antibodies that recognize phosphorylated Ser211 on hGR, have demonstrated a positive correlation between the amount of Ser211 phosphorylation and transactivation activity of hGR.[108] It is not known whether GR phosphorylation status affects GRE-independent regulation of gene expression.

Factors that facilitate GR phosphorylation include agonists such as dexamethasone and triamcinolone, but not antagonists, such as RU486.[109] In addition, low amount of basal phosphorylation on GR, observed during the DNA synthesis phase of the cell cycle but not during the mitotic phase, assists GR hyperphosphorylation.[109] Therefore, agonist treatment stimulates the degree of GR phosphorylation during S phase but not G2/M phase. Correspondingly, cells synchronized at S phase are glucocorticoid sensitive but cells synchronized at G2/M phase are glucocorticoid resistant.[110] This insight may assist with the designing of efficient chemotherapy regimens that could potentially overcome glucocorticoid resistance in some patients.

In the presence of agonists, phosphorylated GR has a half-life of 8–9 h whereas the half-life of non-phosphorylated GR is about 32 h.[107] This observation is in agreement with findings that agonist-activated GR has a shorter half-life than un-liganded receptors.[111–119] Thus, transcriptionally active GR exhibits a fast turnover rate. However, a slow turnover rate does not necessarily correlate with low receptor activity.

Ubiquitination

We recently reported that the mouse GR is degraded through the ubiquitin-proteasome pathway.[120] Protein phosphorylation facilitates E2 ubiquitin-conjugating

enzymes and/or E3 ubiquitin-ligase to recognize target proteins and covalently link the 76 amino acid ubiquitin to lysine residue(s).[121–123] Proteins tagged with poly-ubiquitin are trafficked primarily to the multiprotein complex known as the proteasome for degradation.[124–126] GR has been shown to interact with an E2-conjugating protein[127] and two E3-ligase proteins.[128]

A number of proteins rapidly degraded through the proteasome pathway contain PEST regions, which contain the amino acids Pro (P), Glu (E), Ser (S), and Thr (T). Hallmarks of PEST regions include phosphorylation sites, stretches of hydrophilic amino acids, and Lys, Arg, and His residues.[129,130] Analysis of mouse GR using a PEST-FIND program revealed a PEST motif from amino acids 407–426 (FIG. 1). This region has a PEST-FIND score of +18.3; on a scale from −50 to +50 a value above +5 is indicative of a possible functional PEST motif.[129] For example, two proteins known to be degraded by the proteasome, IκBα and FOS, have PEST scores of 5.9 and 10.1, respectively.[131–133] In addition, PEST-FIND analysis calculated a score of +18.3 for the rat GR and +16.1 for hGR, suggesting that PEST motifs in GR are conserved among mammals. Ser-412 within the mouse PEST region is a site of ligand-dependent phosphorylation.[105,134]

Pretreatment of COS-1 cells expressing mouse GR with proteasome inhibitor, MG-132, effectively blocks GR protein downregulation (degradation) by the glucocorticoid dexamethasone.[120] Furthermore, direct evidence for ubiquitination of the GR has been obtained by immunoprecipitation of cellular extracts from proteasome-impaired cells. MG-132 also blocks agonist-induced degradation of ERα, PR, as well as the aryl hydrocarbon receptor.[135,136] Interestingly, both MG-132 and a second proteasome inhibitor, β-lactone, significantly enhance the transactivation activity of transfected mouse GR as well as endogenous hGR in HeLa cells. Mutation of Lys426 within the PEST element abrogates ligand-dependent downregulation of the mouse GR protein and simultaneously enhances GR-induced transcriptional activation. MG-132 does not affect the receptor level or the transcriptional activity of K426A mutant mouse GR.[120]

Thus, when the turnover rate of GR is decreased by proteasomal inhibition, GR activity is increased. Inhibition of degradation also enhances the transcription regulatory activity of other transcription factors, such as the aryl hydrocarbon receptor, Sp1, and p53.[137–139] In contrast, proteasomal inhibition decreased ligand-induced transcriptional activity of ERα or TRα.[137,140] The causal features common to each category of relationship between turnover rate and activity are not known although it has been suggested that the formation of an ERα coactivator complex may be disrupted by proteasome inhibitors.[137] In addition, consensus PEST motifs are not present in either ERα or TRα and proteasomal activity may be necessary to produce a transcriptionally active form of TRα.[140] As discussed above, phosphorylation shortens the half-life but enhances the transactivation activity of GR. However, the long-lived GR proteins in the presence of MG132 exhibit increased transactivation activity. Together, these data support the notion that the amount of available phosphorylated GR, instead of receptor turnover rate, determines receptor activity. Recently, the phosphorylation status of Ser211 has been suggested as a biomarker for hGR activity *in vivo*.[108]

GR phosphorylation occurs within 5–10 min of hormone addition and the half-maximal rate ($t_{1/2}$) for GR dephosphorylation is 90–120 min.[109] Phosphorylated GR, in the presence of agonist, has a half-life of 9 h, before being trafficked to the pro-

teasome for degradation. Intriguing questions about the activation and degradation of GR remain to be answered. How does an activated GR molecule navigate through the cell nucleus where the genome resides? Furthermore, what signals terminate the usage of an individual receptor?

LIGAND-DEPENDENT GR TRAFFICKING IN THE NUCLEUS

In the absence of hormone, GRα resides predominantly in the cytoplasm of cells, forming a multiprotein complex with two molecules of hsp90 and several additional proteins.[141] Ligand binding induces conformational changes that are followed by the release of GRα from the chaperones and translocation of the receptor into the nucleus.[142] Most current experiments using transiently expressed fluorescent proteins (GFP or YFP) tagged receptors suggest that complete nuclear translocation of GFP-hGRα occurs within 30 min after ligand addition.[143] Similar data on nuclear translocation have been obtained for additional GFP-tagged receptors in the steroid receptor family as well.[144–148]

In the nucleus, the agonist-bound GFP-hGRα has been reported to be organized into discrete foci,[143] which is consistent with earlier results from immunocytochemical studies on endogenous GR.[149] A similar punctate distribution in the nucleus has also been found for agonist-bound GFP-tagged ERα,[150,151] AR,[144–146] MR,[147] vitamin D receptor,[148] and TRβ.[152] Treatment with an antagonist does not result in foci formation of GR,[143] AR,[144–146] or MR,[147] although additional studies are needed to determine whether this characteristic is strictly limited to certain antagonists. ERα antagonists induce a less pronounced punctate receptor distribution than agonists.[150,151] Nuclear GR foci take shape within 15 min of agonist treatment, but it is not completely understood whether all of these foci colocalize with transcription initiation sites.[153] Active transcription complexes are assembled in an orderly fashion where activated GR initiates the recruitment of RNA polymerase II, the cofactors GRIP-1,[154] SRC1, and CBP (which contains inherent acetyltransferase activity), BRG1 (a chromatin remodeler), and other transcription factors such as NFI and AP-2.[155]

Additional nuclear GR have been reported to colocalize with the nuclear matrix. The nuclear matrix is the non-chromatin elements of the nuclear structure readily observed under an electron microscope.[156,157] A main constituent protein, hnRNP U,[158,159] interacts with GR.[160] The rat GR τ2 region contains a nuclear matrix–targeting signal that facilitates the interaction between GR and hnRNP U.[161] Furthermore, overexpression of hnRNP U inhibits GR-induced transactivation.[161,162] This observation is consistent with the previous finding that ligand-bound GR is more resistant to high salt extraction from the nucleus than non-liganded GR.[163]

The relationship between chromatin-bound GR and nuclear matrix–bound GR was then examined in a series of elegant experiments, GR release from and redocking onto chromatin was visualized.[153] The recycling process is rather rapid with a half maximal rate ($t_{1/2}$) of 5 s. Our recent photobleaching experiments indicate that the mobility of nuclear hGR is highly dependent on the ligand that occupies the receptor.[164] For example, YFP-hGRα-A in the nucleus is less mobile when activated by triamcinolone acetonide ($t_{1/2} = 2.38$ s) than by cortisone ($t_{1/2} = 0.97$ s). The affinity of hGRα-A for the former ligand is more than 10-fold higher than that for the

latter. The positive correlation between ligand affinity and the ligand's ability to decelerate nuclear hGRα-A seems to be true when a panel of GR ligands was tested.[164] The structural determinants of hGRα-A mobility have been mapped as well.[164] Both the DBD and LBD of the receptor are required for the ligand-induced decrease in receptor mobility. Interestingly, the proteasome inhibitor MG132 immobilizes a subpopulation of non-liganded receptors.[165] This immobilization can be blocked by high- affinity dexamethasone but not by low-affinity cortisone. Thus, the range of GR mobility and function is extended further by a vast array of natural and synthetic ligands.

PERSPECTIVE

Efforts from many laboratories including ours provide convincing evidence that many forms of hGR exist in various physiological and pathological states. Gene regulation activities as well as expression levels of various hGR isoforms differ in various *in vitro* and *in vivo* systems. Different hGR isoforms may contribute to tissue specificity and differences of glucocorticoid responsivity among individuals. Similarly, changes in posttranslational modification status and in the relative proportion of receptor isoforms within a cell or tissue may result in dysfunction of GR-mediated physiology. The majority of our knowledge concerning how GR is modified and how modified GR regulates various gene targets has been generated from studies on the GRα isoform. GR activation requires binding of cognate ligands. About 5 to 10 min after agonist addition, GR phosphorylation is stimulated, followed by the assembly of the receptor-mediated transcription complex within target gene-containing chromatins. Before the receptors are directed to the proteasome for degradation, GR in the nucleus may be shuffled among multiple target sites rapidly (in seconds) to impact the genome. Posttranslational modification status of an individual receptor could very likely be a determinant of receptor usage. The latest development in methodology will greatly improve our understanding of the modification status and function of GR. For example, the number of phosphorylation residues on PR has been recently updated from seven to fourteen.[166] Although the discovery of multiple GR isoforms represents a step closer to understanding the pivotal roles of GR in health and disease, continuous endeavors from investigators in different laboratories will be needed to unveil how the diversity of GR relates to its function and how these mechanisms can be utilized in developing effective treatment regimens for GR-related diseases.

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