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Molecular Mechanisms Regulating Glucocorticoid Sensitivity and Resistance

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Abstract

Glucocorticoid receptor agonists are mainstays in the treatment of various malignancies of hematological origin. Glucocorticoids are included in therapeutic regimens for their ability to stimulate intracellular signal transduction cascades that culminate in alterations in the rate of transcription of genes involved in cell cycle progression and programmed cell death. Unfortunately, subpopulations of patients undergoing systemic glucocorticoid therapy for these diseases are or become insensitive to glucocorticoid-induced cell death, a phenomenon recognized as glucocorticoid resistance. Multiple factors contributing to glucocorticoid resistance have been identified. Here we summarize several of these mechanisms and describe the processes involved in generating a host of glucocorticoid receptor isoforms from one gene. The potential role of glucocorticoid receptor isoforms in determining cellular responsiveness to glucocorticoids is emphasized.

Keywords

glucocorticoid receptor; glucocorticoid resistance; hematological malignancy; alternative initiation of translation

Introduction

The nuclear receptor superfamily is comprised of a variety of ligand-dependent transcription factors. The steroid hormone receptors, such as the glucocorticoid, androgen, estrogen, mineralocorticoid, and progesterone receptors, are members of the nuclear receptor subfamily 3 (Nuclear Receptors Nomenclature Committee, 1999). These receptors are modular proteins that contain three distinct functional regions, the N-terminal transactivation domain (NTD), the central zinc-finger DNA-binding domain (DBD), and the C-terminal ligand-binding domain (LBD) (Figure 1A). The NTD is often referred to as the immunogenic region, as it is the most variable in length and primary sequence among steroid hormone receptors. Consistent with their evolution from a common ancestral precursor (Thornton, 2001), the DBD and LBD are highly conserved among members of the nuclear receptor subfamily 3 (Figure 1A). For example, the glucocorticoid (GR) and mineralocorticoid receptors (MR) share 94% and 56% amino acid identity in the DBD and LBD, respectively. Therefore, it is not surprising that these

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receptors regulate transcription from a common DNA element and exhibit overlapping hormone specificities. Given the degree of homology between functional domains within steroid hormone receptors and the promiscuous nature of steroid hormone receptors and ligands, it is remarkable that activated receptors specifically regulate a wide array of unrelated processes including, but not limited to, embryogenesis, reproduction, electrolyte homeostasis, glucose metabolism, inflammation, immunity, and cell proliferation and survival (Thompson, 1994; Lydon et al., 1995; Berger et al., 1998; Dupont et al., 2000; Cole et al., 2001; Chang et al., 2004; Schmidt et al., 2004; Rhen et al., 2005; Vihko et al., 2006). Multiple mechanisms, such as pre-receptor ligand metabolism, receptor isoform expression, and receptor-, tissue-, and cell type-specific factors, exist to generate diversity as well as specificity in the steroid hormone response (Vegeto et al., 1993; Wilson et al., 1994; Yeh et al., 1996; Nelson et al., 1999; Stewart et al., 1999; Flouriot et al., 2000; Quinkler et al., 2003; Rizner et al., 2003; Kobayashi et al., 2004; Pascual-Le Tallec et al., 2004; Lu et al., 2005; Pascual-Le Tallec et al., 2005; Chen et al., 2006; Grenier et al., 2006; Han et al., 2006; Heitzer et al., 2006; Blind et al., 2008).

The pleiotropic effects of ligand-activated steroid hormone receptors under normal and pathological conditions provide an abundance of opportunities for pharmacological manipulation. Indeed, steroid hormone receptors have been exploited in the treatment of a variety of pathologies including proliferative disorders of the prostate, breast, and blood. For example, androgen receptor (AR) antagonists are used in combination with androgen ablation therapy in the treatment of androgen-dependent prostate cancer and offer a survival advantage when compared to androgen ablation therapy alone (Klotz, 2006). Similarly, selective estrogen receptor (ER) modulators that act as ER antagonists in the breast are used in the treatment of ER-positive breast cancer and, in conjunction with screening mammography, significantly reduce the rate of patient death (Berry et al., 2005). In contrast, GR agonists are the cornerstone in the treatment of a number of hematological malignancies, including leukemia, lymphoma, and myeloma (Frankfurt et al., 2004; Schmidt et al., 2004), and induce remission in 60% of children with newly-diagnosed acute lymphoblastic leukemia (ALL) (Gaynon et al., 1995). Unfortunately, a subpopulation of patients either fail to respond to steroid hormone analogue-based therapy initially or fail to respond at relapse despite response at presentation (Klumper et al., 1995; Gaynon et al., 1999; Renner et al., 2003). This phenomenon, known as steroid hormone resistance, exemplifies the variability in the steroid hormone response among individuals as well as within the same individual and presents a challenge to scientists and clinicians in the treatment of malignancies. A considerable amount of research has been devoted to understanding the complex molecular determinants of the steroid hormone response, as this knowledge may well improve the efficacy of existing treatment regimens. GR agonists (glucocorticoids) are among the most widely prescribed drugs worldwide; therefore, in this review, we will focus on the mechanisms of action and resistance to glucocorticoids, with an emphasis on the recently identified GR isoforms.

Mechanism of Glucocorticoid Action

Endogenous glucocorticoids are stress-induced hormones synthesized under the control of the hypothalamic-pituitary-adrenal axis. In response to a variety of stressors, the hypothalamic factor corticotropin-releasing hormone stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary, and ACTH induces glucocorticoid synthesis from cells within the *zona fasciculata* of the adrenal cortex. Glucocorticoids are highly lipophilic and are transported in the blood predominantly bound to corticosteroid-binding globulin (CBG). In many tissues, CBG-free glucocorticoids readily diffuse across the plasma membrane to exert their effects. Upon encountering a cytosolic, mature GR heterocomplex, the multi-step GR signal transduction pathway is initiated. GR heterocomplexes are formed through a series of dynamic but coordinated associations with molecular chaperones, such as heat shock proteins (hsp) 40,

70, and 90, and co-chaperones including hsp70-interacting protein (hip) and hsp70 / hsp90-organizing protein (hop) (Pratt et al., 1997; Cheung et al., 2000). The mature GR heterocomplex is made up of GR, an hsp90 dimer, a stabilizing protein called p23, and at least one of the following co-chaperones: FKBP52, FKBP51, cyclophilin 40, or protein phosphatase 5 (Pratt et al., 1997; Cheung et al., 2000). In the classic signal transduction pathway, ligand binding induces molecular rearrangement of the GR heterocomplex and promotes GR nuclear localization, homodimerization, and DNA-binding (Davies et al., 2002; Wochnik et al., 2005). The regions of the GR important for these activities are highlighted in Figure 1B. The activated GR homodimer exerts its genomic effect by interacting with specific DNA elements, termed glucocorticoid response elements (GRE), located within regulatory regions of glucocorticoid-responsive genes. After organized recruitment of coactivators or corepressors and the basal transcriptional machinery, the GR modulates the rate of gene transcription. The transcriptional response depends on the integrity of the activation function (AF) domains embedded within the NTD and LBD (Figure 1B). AF-1 is a strong transactivation domain and is able to modulate transcription in a hormone-independent fashion, whereas the relatively weak transcriptional activity of AF-2 requires hormone binding (Hollenberg et al., 1988). The direction of the transcriptional response depends in part on the nature of the GRE. Positive GREs mediate transcriptional up-regulation, whereas negative GREs (nGRE) mediate transcriptional down-regulation (Sakai et al., 1988; Drouin et al., 1989; Dostert et al., 2004). Activated GR can also influence transcription independent of a classical GRE. For example, the GR physically interacts with other transcription factors, such as nuclear factor κ B (NF κ B) (Ray et al., 1994), activator protein-1 (AP-1) (Yang-Yen et al., 1990), and signal transducers and activators of transcription (STAT) (Stocklin et al., 1996), and modulates their action at target genes. Deciphering the molecular mechanisms of glucocorticoid action is further complicated by the realization that glucocorticoids can induce rapid, non-genomic effects within the cytoplasm. Croxtall *et al.* showed that glucocorticoids stimulate the release of Src kinase from GR heterocomplexes, resulting in lipocortin 1 activation and inhibition of arachidonic acid release (Croxtall et al., 2000). Thus, the GR influences cellular processes through multiple genomic and non-genomic mechanisms.

Glucocorticoid Resistance

The glucocorticoids dexamethasone and prednisone are efficacious in the treatment of hematological malignancies presumably for their ability to regulate the expression of genes involved in cell cycle progression and programmed cell death (Renner et al., 2003; Frankfurt et al., 2004; Schmidt et al., 2004). The net effect of glucocorticoids in these disorders is induction of apoptosis, thereby inhibiting the expansion of cells that have escaped the normal constraints of the cell cycle. Glucocorticoid resistance or insensitivity is defined here as the inability of malignant cells to initiate the apoptotic program in response to a GR agonist. Remission failure or relapse in cancer patients undergoing systemic glucocorticoid therapy is characteristic of glucocorticoid resistance or acquired glucocorticoid resistance, respectively. As insensitivity to glucocorticoids limits the therapeutic benefit of GR agonists in disorders of proliferation and is positively correlated with poor prognosis (Dordelmann et al., 1999; Felice et al., 2001), it is of clinical importance to elucidate molecular mechanisms of glucocorticoid resistance.

Mutations in the GR Gene

The GR is the protein product of one gene that has been mapped to chromosome 5 (5q31-32) (Francke et al., 1989; Theriault et al., 1989). Although GR gene mutations are the primary cause of an inherited form of generalized glucocorticoid resistance and *in vitro* acquisition of glucocorticoid resistance in various malignant cell lines (Danielsen et al., 1986; Ashraf et al., 1993; Powers et al., 1993; Lee et al., 1995; Strasser-Wozak et al., 1995; Hala et al., 1996; Riml

et al., 2004; Schmidt et al., 2006), GR mutations in primary cells from glucocorticoid-resistant cancer patients had not been identified until recently (Soufi et al., 1995). Hillmann *et al.* were the first to discover a transactivation-deficient GR mutant (L753F) in cells from an individual with glucocorticoid-resistant ALL (Figure 2A) (Hillmann et al., 2000). Irving *et al.* subsequently identified a LBD-deficient GR mutant ($\Delta 702$) in cells from another individual with glucocorticoid-resistant ALL at relapse (Figure 2A) (Irving et al., 2005). The $\Delta 702$ mutation was not apparent at presentation but was evident in a day 28 remission sample, indicating that the subpopulation of cells harboring this mutation emerged as the dominant population at relapse (Irving et al., 2005). The acquisition of the GR gene mutation occurred without mention of generalized glucocorticoid resistance and has been attributed to either spontaneous mutation or mutation induced by DNA damaging agents that were used in conjunction with glucocorticoid therapy. Indeed, the chemotherapeutic agent bleomycin has been reported to induce deletions and/or chromosomal breaks in the GR gene resulting in the emergence of glucocorticoid-resistant cell lines (Palmer et al., 1992). The two cases described above are the only known examples of resistance to glucocorticoid therapy in malignancy attributable to acquired GR mutations *in vivo*. However, the sensitivity of conventional assays for identification of GR gene mutations may be inadequate for detection of aberrant GR in heterogeneous cell populations. Therefore, acquisition of GR mutations *in vivo* may be underrepresented as a mechanism of glucocorticoid resistance.

GR Expression Levels

The GR is a ubiquitously expressed protein, found in almost all human cell types and tissues at appreciable levels. It is well-established that the level of GR expression is closely correlated with the magnitude of the glucocorticoid response (Vanderbilt et al., 1987); therefore, it is not surprising that the sensitivity of malignant cells to glucocorticoids is partially dependent on the number of receptors found within the cell. Several groups have shown that reduced GR expression in primary ALL cells is associated with initial resistance to glucocorticoid therapy, relapse, and poor prognosis (Bloomfield et al., 1981; Wells et al., 1981; Ho et al., 1982; Pui et al., 1984; Federico et al., 1986; Sakurai et al., 1986). GR levels in cells are dynamic and are regulated in a cell-type specific manner by the surrounding concentration of ligand (Cidlowski et al., 1981; Hoeck et al., 1989; Silva et al., 1994; Alarid, 2006). In several cell lines and tissues, administration of GR agonists results in down-regulation of the GR (Cidlowski et al., 1981; Svec et al., 1981; Schlechte et al., 1982; Tornello et al., 1982; Lacroix et al., 1984; Sapolsky et al., 1984; Hoeck et al., 1989; Silva et al., 1994). The mechanism of glucocorticoid-induced GR down-regulation has been attributed to reduced transcription of the GR gene as well as decreased stability of the GR mRNA and protein (Dong et al., 1988; Rosewicz et al., 1988; Vedeckis et al., 1989). Promoter-dependent (Govindan et al., 1991) and -independent (Okret et al., 1986; Burnstein et al., 1990; Burnstein et al., 1994) DNA elements are likely involved in down-regulation of GR mRNA expression, whereas proteasome-mediated GR degradation contributes to increased turnover of the GR protein (Wallace et al., 2001). While hormone-induced down-regulation of GR represents a mechanism for maintaining glucocorticoid homeostasis in normal cells, it has the potential to limit therapeutic responses to glucocorticoids in malignant cells. For example, glucocorticoid-induced reductions in GR content in primary ALL cells between diagnosis and relapse have been observed and are associated with resistance to glucocorticoid therapy (Bloomfield et al., 1981; Pui et al., 1984; Pui et al., 1986). In contrast, hormone-induced up-regulation of GR (autoinduction) is associated with glucocorticoid sensitivity. For example, T-cell lines that exhibit GR autoinduction at the protein and/or mRNA level are sensitive to glucocorticoid-induced apoptosis (Eisen et al., 1988; Antakly et al., 1989; Gruol et al., 1989; Gomi et al., 1990; Ashraf et al., 1991; Denton et al., 1993), whereas T-cell lines that fail to autoinduce GR are resistant to this effect (Gomi et al., 1990; Ramdas et al., 1999; Pedersen et al., 2003; Riml et al., 2004; Schmidt et al., 2006). The mechanism of autoinduction is likely to be transcriptional as GREs have been identified within specific

regions of the GR promoter (Breslin et al., 2001; Presul et al., 2007). Importantly, Tissing *et al.* showed no correlation between autoinduction of GR mRNA and glucocorticoid responsiveness in primary childhood T-cell ALL or precursor B-cell ALL (Tissing et al., 2006). It should be noted, however, that in this study GR protein level was not analyzed (Tissing et al., 2006). Thus, the precise role of GR autoinduction in determining glucocorticoid sensitivity in patients warrants further investigation. Taken together, these data suggest that GR expression level may be an important determinant of the glucocorticoid response in malignant cells. Moreover, glucocorticoid sensitivity may critically depend on the effect of GR agonists on the number of intracellular GR, *i.e.* whether glucocorticoid-induced down-regulation or autoinduction predominates.

Alternative Processing of the GR Gene

Experiments conducted in our laboratory and others have revealed multiple mechanisms for generating a host of GR proteins from the single GR gene (Hollenberg et al., 1985; Moalli et al., 1993; Rivers et al., 1999; Yudt et al., 2001; Geng et al., 2005; Lu et al., 2005; Turner et al., 2007). These isoforms exhibit tissue-specific patterns of expression as well as differences in subcellular localization and transcriptional activity (Oakley et al., 1997; Oakley et al., 1999; Rivers et al., 1999; Yudt et al., 2001; Geng et al., 2005; Lu et al., 2005; Turner et al., 2007). Therefore, the repertoire of GR subtypes expressed by a particular cell may contribute to the ability of the cell to respond to glucocorticoids. Below, we describe transcriptional, post-transcriptional, and translational mechanisms involved in controlling GR expression, and the potential roles for specific GR isoforms in glucocorticoid resistance are discussed.

Alternative promoter usage—The human GR cDNA was first cloned in 1985 (Hollenberg et al., 1985), and elucidation of genomic structure revealed the presence of 9 exons that span an 80 kB region (Encio et al., 1991; Oakley et al., 1996). Exon 1 contains the 5' untranslated region, and recent studies have identified 9 alternative exon 1s (1A, 1B, 1C, 1D, 1E, 1F, 1H, 1I, and 1J) that are generated from unique promoters (Breslin et al., 2001; Turner et al., 2005; Presul et al., 2007). Additional variability in exon 1 is accomplished through alternative splicing of exon 1A into 1A1, 1A2, or 1A3 and exon 1C into 1C1, 1C2, or 1C3 (Breslin et al., 2001; Turner et al., 2005). Even though the heterogeneity in exon 1 does not affect the sequence of the GR protein *per se*, cell type-selective promoter usage exists and may serve to regulate GR protein levels (see section on GR expression levels) (Breslin et al., 2001; Presul et al., 2007), alternative splicing that occurs elsewhere in the primary GR transcript (see section on alternative splicing) (Russcher et al., 2007), and/or alternative initiation of translation of mature GR mRNA (see section on alternative translation initiation) (Pedersen et al., 2004), all of which are important factors in determining glucocorticoid sensitivity.

Alternative Splicing—The GR protein is encoded by exons 2 – 9 of the GR gene (Encio et al., 1991) (Figure 2A), and alternative splicing of GR precursor mRNA gives rise to 5 GR protein subtypes that have been termed GR α , GR β , GR γ , GR-A, and GR-P (Figures 2A and 2B). The ubiquitously expressed GR α is the classic, functionally active receptor and is generated through splicing of exon 8 to the proximal end of exon 9 (9 α), whereas GR β is produced through splicing of exon 8 to the distal end of exon 9 (9 β). GR α is made up of 777 amino acids, including 50 C-terminal residues derived from exon 9 α . In contrast, GR β is a shorter protein of 742 residues, the final 15 of which are encoded by exon 9 β . Thus, GR α and GR β proteins share identical N-termini encoded by exons 2-8 and are distinguished only by their C-termini (Hollenberg et al., 1985). The presence of the 15 unique C-terminal amino acids exerts a profound influence on GR β function (Yudt et al., 2003). GR β has been reported not to bind glucocorticoid agonists (Oakley et al., 1999) and was originally thought to control transcription only through a dominant-negative effect on GR α -induced gene expression (Bamberger et al., 1995; Oakley et al., 1999). Recently however, Lewis-Tuffin *et al.*

demonstrated that GR β regulates gene expression even in the absence of GR α and interacts with the GR α antagonist/partial agonist RU-486, which diminishes its transcriptional capacity (Lewis-Tuffin et al., 2007). Furthermore, in a manner similar to GR α , GR β represses basal activity of the interleukin 5 (IL-5) and IL-13 cytokine promoters through recruitment of histone deacetylase 1 (Kelly et al., 2008). These recent studies indicate that GR β may have a previously unappreciated role in cell signaling.

GR β is detected in most tissues and cell lines, but it is generally expressed at low levels when compared to GR α (Bamberger et al., 1995; de Castro et al., 1996; Oakley et al., 1996; Oakley et al., 1997; Pujols et al., 2002). However, in support of a dominant-negative function for GR β , resistance to glucocorticoid therapy in leukemia has been associated with high cellular levels of GR β when compared to GR α (Shahidi et al., 1999; Longui et al., 2000; Koga et al., 2005). It is important to note that this relationship is controversial, as it has not been observed by other investigators (Haarman et al., 2004; Tissing et al., 2005a). Therefore, the contribution of relative GR β levels in determining cellular sensitivity to glucocorticoids in individuals undergoing glucocorticoid therapy for malignancies remains unclear.

In addition to the GR α and GR β isoforms, GR γ , GR-A, and GR-P splice variants have been described (Figure 2B). The GR γ isoform contains a three base insertion in the DBD between exons 3 and 4 that results in addition of arginine between the two zinc fingers of the DBD (Rivers et al., 1999). Analysis of various tissues revealed that GR γ is widely expressed, representing 3.8 to 8.7% of the total GR mRNA (Rivers et al., 1999). Since GR γ exhibits decreased transcriptional activity when compared to GR α (Kasai, 1990; Ray et al., 1996), it is not surprising that elevated levels of GR γ are associated with glucocorticoid resistance in childhood leukemia (Beger et al., 2003; Haarman et al., 2004). The GR-A and GR-P splice variants lack portions of the LBD due to the absence of exons 5 through 7 and exons 8 through 9, respectively (Moalli et al., 1993). The GR-P isoform is preferentially derived from transcripts containing exon 1B and is widely expressed (de Lange et al., 2001; Russcher et al., 2007). Studies from a number of laboratories have revealed a correlation between elevated levels of GR-P or GR-A and glucocorticoid resistance in myeloma and leukemia (Moalli et al., 1993; Krett et al., 1995; de Lange et al., 2001). However, more recent studies directly contradict these earlier findings (Tissing et al., 2005a). Interestingly, even though the GR-P variant exhibits lower transactivation potential when compared to GR α , GR-P is capable of stimulating GR α -mediated transcription in certain cell lines (de Lange et al., 2001). Therefore, GR-P may act in a cell type-specific manner to enhance glucocorticoid responsiveness. Taken together, these data suggest that alternative splicing of a common precursor GR mRNA gives rise to multiple GR isoforms. Moreover, up-regulation of the GR- β , - γ , -A, or -P isoform may represent one of many mechanisms for modulating cellular responsiveness to glucocorticoids.

Alternative Translation Initiation—Recently, our laboratory has identified alternative translation initiation as yet another mechanism for generating diversity in GR protein expression. Yudit *et al.* first reported that GR α cDNA expressed either *in vitro* or transiently in COS-1 cells, which are known to be void of detectable endogenous GR, consistently appeared as a doublet of 94 kD and 91 kD in Western blots (Yudit et al., 2001; Lu et al., 2005). Although partial proteolysis or phosphorylation could potentially explain the appearance of the doublet, neither protease inhibitor nor phosphatase inhibitor treatment affected the GR α expression pattern (Yudit et al., 2001). Thus, another mechanism must account for generation of the two GR α forms. Since alternative translational start site usage is an important mechanism for producing isoforms of other steroid hormone receptors (see below), the potential for initiation of GR translation at internal AUG codons was investigated. The GR α cDNA was first examined for additional translational start sites downstream of the initial AUG codon corresponding to methionine 1. Indeed, an in-frame AUG codon (methionine 27) that is conserved among the human, rat and mouse was identified (Figures 1A and 1B) and is indispensable for expression

of the rapidly migrating species within the GR doublet (Yudt et al., 2001). The 94 kD protein is the classic GR α that is now referred to as GR α -A, and the 91 kD protein has been termed GR α -B (Yudt et al., 2001). Subsequent analysis revealed that, in addition to GR α -A and GR α -B, proteins of 82-84 and 53-56 kD are also expressed from a single GR α mRNA species (Lu et al., 2005). AUG codons corresponding to methionines 86, 90, and 98 were required for formation of the 82-84 kD proteins, whereas methionines 316, 331, and 336 were necessary for expression of the 53-56 kD proteins (Lu et al., 2005). These AUG codons are also conserved among the human, rat, and mouse and initiate the expression of GR α isoforms designated GR α -C1, C2, C3, D1, D2, and D3, respectively (Figures 1A and 1B). The mechanism for generating the translational isoforms involves leaky 5' ribosomal scanning and/or ribosomal shunting (Lu et al., 2005), which refers to discontinuous scanning of the mRNA by the translation initiation complex (Touriol et al., 2003). Both ribosomal scanning and shunting are likely controlled by mRNA-specific elements. For example, Pederson *et al.* reported that GR α -B is preferentially expressed from GR α transcripts containing exon 1A3 (Pedersen et al., 2004). Although all of the GR α translational variants were detected in rat and mouse tissues, the expression levels differed (Lu et al., 2005). These data indicate that alternative initiation of translation operates *in vivo* and gives rise to tissue-specific GR α isoform expression patterns. Additional complexity and diversity in GR isoform expression is implicated by the potential for alternative translation initiation of GR- β transcripts, as GR α and GR β mRNA contain identical 5' coding regions.

Since the translational isoforms are distinguished only by the length of their N-terminus, it is not surprising that they possess common as well as unique properties. All 8 isoforms exhibit a similar affinity for glucocorticoids and most undergo hormone-induced nuclear localization followed by GRE binding and transcriptional regulation (Lu et al., 2005; Lu et al., 2007). The GR α -D3 variant is peculiar in that it localizes to the nucleus and can bind certain GREs even in the absence of hormone (Lu et al., 2005; Lu et al., 2007). The translational isoforms are further differentiated by their transcriptional activity in reporter assays. The GR α -C3 variant is the most active, whereas the GR α -D proteins were the least active (Lu et al., 2005). Furthermore, GR α -C3 enhanced the ligand-dependent transcriptional activity of GR α -A, but GR α -D3 did not (Lu et al., 2005). These data suggest that the glucocorticoid-induced transcriptional response reflects the composite actions of GR α isoforms and that the specific intracellular pool of GR α subtypes may determine cellular sensitivity to glucocorticoids. Microarray analysis revealed a similar hierarchy with respect to transcriptional capability (Lu et al., 2005; Lu et al., 2007). Moreover, in addition to regulating a common subset of endogenous genes, the translational isoforms also regulate unique, subtype-specific genes (Lu et al., 2005). The difference in gene regulatory profiles likely contributes to the distinct apoptotic phenotype of U2-OS osteosarcoma cells stably expressing the individual translational forms. Expression of the more active GR α -C3 correlated with increased sensitivity to glucocorticoid-induced apoptosis, and expression of the relatively inactive GR α -D3 was associated with resistance to glucocorticoid-induced apoptosis (Lu et al., 2007). Additional studies are needed to determine whether the relative expression levels of translational isoforms, particularly GR α -D3, correlate with resistance to glucocorticoid-induced apoptosis in malignancies.

Interestingly, other nuclear receptor subfamily 3 members have the potential for alternative translation initiation at internal AUG codons. Multiple methionines are found in the N-terminal regions of steroid hormone receptors, and these residues are conserved among the human, rat, and mouse (Figure 1A). In fact, some of these AUG codons have been confirmed as translational start sites. For example, the AUG codon corresponding to methionine 15 gives rise to MR-B (Pascual-Le Tallec et al., 2004), whereas alternative splicing followed by usage of a translation start site corresponding to methionine 174 gives rise to ER α -46 (Flourirot et al., 2000). In addition, it has been suggested that methionine 189 gives rise to the A isoform of the

AR (Wilson et al., 1994; Wilson et al., 1996), although controversy exists (Gregory et al., 2001). The N-terminal variants of ER and AR have been shown to oppose the actions of the corresponding full length receptor (Flouriot et al., 2000; Liegibel et al., 2003). These data suggest that alternative translational initiation may represent a common mechanism for controlling cellular responses to steroid hormones. However, the relative expression levels of ER and AR isoforms in steroid hormone analogue-resistant prostate and breast cancer are unknown.

Polymorphisms in the GR Gene

Genetic polymorphisms are defined as variations in DNA that are observed in 1% or more of the population. Since polymorphisms in the GR gene have been associated with variations in GR function (Lamberts et al., 1996; Huizenga et al., 1998; van Rossum et al., 2002; van Rossum et al., 2003; Russcher et al., 2005a), it has been proposed that genetic alterations in the GR gene may account for the variability in the glucocorticoid response in individuals undergoing glucocorticoid therapy for hematological malignancies.

The ER22/23EK GR polymorphism that results in an arginine to lysine change (R23K) within the NTD (Figure 2A) is associated with decreased GR transcriptional activity in reporter assays and decreased expression of endogenous genes when compared to wild type GR (van Rossum et al., 2002; Russcher et al., 2005a). Upon further analysis, Russcher *et al.* discovered that the ER22/23EK GR polymorphism facilitated the expression of GR α -A, but had no effect on the expression of the GR α -B translational isoform (Russcher et al., 2005b). Since some studies have shown that GR α -A is transcriptionally less active when compared to GR α -B (Yudt et al., 2001), these molecular data suggest that the relative inactivity of ER22/23EK results from decreased relative levels of GR α -B and that ER22/23EK may correlate with glucocorticoid insensitivity (Russcher et al., 2005b). Although ER22/23EK was not associated with differences in responsiveness to exogenous glucocorticoids in ALL (Tissing et al., 2005c), adult carriers of the ER22/23EK polymorphism were shown to have decreased incidence of type 2 diabetes and decreased risk for cardiovascular disease (van Rossum et al., 2004a; van Rossum et al., 2004b). Similarly, the GR β polymorphism A3669G located in the 3' untranslated region (Figure 2A) results in enhanced expression of the dominant negative GR β and is associated with favorable metabolic parameters (Syed et al., 2006). These data imply that ER22/23EK and A3669G carriers have a more favorable metabolic profile due to a relative insensitivity to endogenous glucocorticoids.

In contrast to the polymorphisms described above, two particular polymorphisms in the GR gene, *Bcl* I and N363S, are associated with generalized increases in glucocorticoid sensitivity (Huizenga et al., 1998; van Rossum et al., 2003; Russcher et al., 2005a) and metabolic disorders (Buemann et al., 1997; Dobson et al., 2001; Di Blasio et al., 2003; Lin et al., 2003). The *Bcl* I variant is a restriction fragment length polymorphism (RFLP) located in intron 2, whereas the N363S polymorphism occurs within the NTD and results in an asparagine to serine substitution (Figure 2A). Interestingly, microarray analysis revealed a unique, polymorphism-specific pattern of gene regulation for N363S when compared to GR α wild type (Jewell et al., 2007). Moreover, some reports suggest that N363S is not only associated with glucocorticoid hypersensitivity but also with decreased bone mineral density (van Rossum et al., 2004b). Although individuals harboring *Bcl* I or N363S have no identifiable therapeutic advantage when undergoing systemic glucocorticoid therapy for hematological malignancies (Tissing et al., 2005c), it is plausible to speculate that these variants may be associated with an increased sensitivity to dose-limiting side effects of glucocorticoid therapy, such as avascular necrosis of bone (Lamberts et al., 1996). Taken together, these data imply that polymorphisms in the GR may be an indicator of disease. Furthermore, the primary sequence of the GR may prove

to be of prognostic value in glucocorticoid-managed proliferative disorders and a predictor of adverse reactions.

The GR Heterocomplex

Koper *et al.* discovered that approximately 9% of individuals failed to respond adequately to dexamethasone suppression tests by efficiently down-regulating endogenous glucocorticoid production (Koper *et al.*, 1997). As these subjects exhibit an underlying glucocorticoid resistance that was not associated with mutations or polymorphisms in the GR gene, it has been suggested that mechanisms independent of the GR gene itself may contribute to the development of glucocorticoid resistance in a subpopulation of individuals challenged with systemic glucocorticoid therapy (Koper *et al.*, 1997). Since the integrity of the mature GR heterocomplex is required for optimal ligand-binding and subsequent activation of the transcriptional response, abnormalities in the chaperones and co-chaperones that make up the heterocomplex may contribute to decreased glucocorticoid responsiveness. Kojika *et al.* showed that alterations in hsp90 and hsp70 were associated with decreased cellular sensitivity to glucocorticoids (Kojika *et al.*, 1996). Furthermore, aberrant hsp90 protein and low hsp70 levels were identified in two out of nine glucocorticoid-resistant human leukemic cell lines (Kojika *et al.*, 1996). In addition, altered levels of hsp90 were found in peripheral blood mononuclear cells from individuals with steroid-resistant forms of asthma (Qian *et al.*, 2001), multiple sclerosis (Matysiak *et al.*, 2008), and idiopathic nephrotic syndrome (Ouyang *et al.*, 2006). However, Lauten *et al.* found no relationship between hsp90 mRNA or protein levels and resistance to glucocorticoids in primary cells from individuals with ALL (Lauten *et al.*, 2003). In agreement with these data, Tissing *et al.* found no significant differences in hsp90 in addition to hsp70 mRNA expression in mononuclear cells from glucocorticoid-resistant individuals with ALL when compared to glucocorticoid-sensitive individuals (Tissing *et al.*, 2005b). Therefore, the role of hsp90 and hsp70 proteins in glucocorticoid-resistant malignancies remains unclear.

The relative levels of FKBP51 and FKBP52 have been shown to be important determinants of cellular sensitivity to glucocorticoids in various systems (Reynolds *et al.*, 1999; Denny *et al.*, 2000; Scammell *et al.*, 2001; Scammell *et al.*, 2002; Riggs *et al.*, 2003). For example, high levels of FKBP51 and low levels of FKBP52 were associated with glucocorticoid resistance in cell lines and tissues from several genera of New World primates (Reynolds *et al.*, 1999; Denny *et al.*, 2000; Scammell *et al.*, 2001; Scammell *et al.*, 2002). Furthermore, FKBP51 overexpression inhibits hormone-induced GR transactivation in mammalian cells (Denny *et al.*, 2000; Scammell *et al.*, 2001; Westberry *et al.*, 2006), and co-expression of FKBP52 blocks the inhibitory effect of FKBP51 (Denny *et al.*, 2005; Wochnik *et al.*, 2005). These data suggest that the relative levels of FKBP51 and FKBP52 may impact cellular sensitivity to systemic glucocorticoid therapy in malignancies. However, no significant differences in FKBP51 or FKBP52 mRNA levels were found between glucocorticoid-sensitive and glucocorticoid-resistant individuals with ALL (Tissing *et al.*, 2005b). Due to limited availability of patient material in the aforementioned studies, the hsp90, hsp70, FKBP51, and FKBP52 genes were not sequenced (Lauten *et al.*, 2003; Tissing *et al.*, 2005b). As mutated versions of chaperones and co-chaperones could alter signaling through the mature GR heterocomplex potentially leading to decreased cellular sensitivity to glucocorticoid-induced cell death, the role of these proteins in glucocorticoid-resistant malignancy cannot be ruled out (Denny *et al.*, 2000; Denny *et al.*, 2005).

Concluding Remarks

Glucocorticoids are used in the treatment of various malignant disorders of hematological origin for their ability to regulate the expression of genes involved in cell cycle progression and apoptosis, thereby inducing cell death. Glucocorticoids exert their effects through

activation of the GR signal transduction pathway, and resistance to glucocorticoid therapy can occur through multiple mechanisms. Reduced GR expression, GR down-regulation, and acquired GR mutations are factors that may potentially limit the therapeutic response to glucocorticoids. Although the impact of GR polymorphisms and altered chaperone or co-chaperone expression on glucocorticoid responsiveness in hematological malignancies is not well established, GR polymorphisms are emerging as an important risk factor for diseases of metabolic origin. Data from our laboratory and others indicate that multiple GR isoforms are derived from the single GR gene. In addition to differences in subcellular localization, transcriptional activity, and sensitivity to glucocorticoid-induced apoptosis, these isoforms exhibit tissue-specific expression patterns. These data suggest that cellular glucocorticoid responsiveness may reflect the intracellular composition of GR isoforms. Whether or not glucocorticoid-resistant malignant cells exhibit an altered pattern of GR isoform expression remains to be determined.

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Figure 1. Modular domain organization of steroid hormone receptors

A. Steroid hormone receptors contain three major functional regions, the N-terminal transactivation domain (NTD), the central DNA-binding domain (DBD), and the C-terminal ligand-binding domain (LBD). The region located between the DBD and LBD is known as the hinge region (H). The DBD and LBD are highly conserved among receptors, and percentages indicate amino acid identity between these domains when compared to the respective domains within GR α . The numbers correspond to amino acid positions, asterisks represent internal N-terminal methionine residues conserved between the human, rat, and mouse. B. Domain organization of the GR α translational isoforms. Alternative initiation of translation gives rise to the GR α translational isoforms GR α -A, GR α -B, GR α -C1, GR α -C2, GR α -C3, GR α -D1,

GR α -D2, and GR α -D3. The horizontal lines indicate functional regions embedded within the GR α protein. Due to deletions within the N-terminus, the translational isoforms lack portions of the NTD.

Figure 2. GR Variants

A. GR polymorphisms and acquired mutations. Exons within the GR precursor mRNA are denoted with a rectangle and shading indicates that the NTD is encoded by exon 2, the DBD is encoded by exons 3 and 4, and the C-terminal region is encoded by exons 5-9. The proximal and distal regions of exon 9 are denoted α and β , respectively. Introns are marked with hashed bars. The arrows in the top panel indicate polymorphisms that do not result in amino acid changes, whereas the arrows in the bottom panel indicate polymorphisms that do result in amino acid changes. Furthermore, the locations of the L753F and Δ 702 GR mutations that are associated with acquired glucocorticoid resistance to therapy *in vivo* are shown in the bottom panel. B. The GR splice variants. GR β mRNA is produced from splicing of exon 8 to exon

9 β , and the resulting protein contains 15 unique amino acids at the C-terminus (amino acids 728-742). GR γ mRNA is generated by alternative usage of a splice donor site at the exon 3 / exon 4 boundary and encodes for a protein containing an arginine insertion (R453) between the two zinc fingers of the DBD. The GR-A variant is produced from splicing of exon 4 to exon 8. Therefore, exons 5 – 7 are deleted. GR-P is formed by a failure to splice exon 7 to exon 8. Thus, GR-P contains the splice donor site of exon 7 followed by intact intronic sequences. Due to the deletion of 3' exons, GR-A and GR-P lack significant portions of the LBD.