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Lysine 419 Targets Human Glucocorticoid Receptor for Proteasomal Degradation*

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Abstract

Glucocorticoid receptors (GRs) are members of a highly conserved family of ligand dependent transcription factors which following hormone binding undergo homologous down-regulation reducing the levels of receptor protein. This decline in human GR (hGR) is due in part to a decrease in protein receptor stability that may limit cellular responsiveness to ligand. To examine the role of the proteasome protein degradation pathway in steroid dependent hGR responsiveness, we utilized the proteasomal inhibitors MG-132, β -lactone, and epoxomicin. HeLa cells and COS cells were treated with proteasome inhibitors in the presence of the GR agonist dexamethasone (Dex), or were pretreated with proteasomal inhibitor and then Dex. Dexamethasone induced glucocorticoid responsive reporter activity significantly over untreated controls, whereas cells treated with proteasomal inhibitors and Dex together showed 2–3 fold increase in activity. Protein sequence analysis of the hGR protein identified several candidate protein degradation motifs including a PEST element. Mutagenesis of this element at lysine 419 was done and mutant K419A hGR failed to undergo ligand dependent downregulation. Mutant K419A hGR displayed 2–3 fold greater glucocorticoid responsive reporter activity in the presence of Dex than wild type hGR. These differences in transcriptional activity were not due to altered subcellular localization, since when the mutant K419A hGR was fused with the green fluorescent protein (GFP) it was found to move in and out of the nucleus similarly to wild type hGR. Together these results suggest that the proteasome and the identified PEST degradation motif limit steroid-dependent human glucocorticoid receptor signaling.

Four key terms

glucocorticoid receptor; proteasome inhibitor; dexamethasone; PEST

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1. Introduction

Glucocorticoids such as cortisol, prednisone and dexamethasone are steroid hormones widely used clinically for the treatment of inflammatory diseases and cancer. Immune cell disorders such as allergies, asthma, and rheumatoid arthritis are also successfully treated with glucocorticoids. In many cancer chemotherapeutic regimes, glucocorticoids are administered for their ability to kill cancer cells such as the case of leukemia and lymphomas, as well as their ability to lessen the edema, pain, and emesis associated with chemotherapy. The therapeutic effects of glucocorticoids result from specific binding and activation the glucocorticoid receptor (GR), a member of the superfamily of steroid/thyroid/retinoic acid proteins, which function as ligand dependent transcription factors. Activated GR can enhance the transcription of a number of primary target genes by binding to specific DNA elements termed glucocorticoid response elements (GREs). Activation of GR transcription occurs through the complex assembly of coactivators and corepressors [1,2]. Activated GR can also repress the transcription of target genes by interfering with the function of other transcription factors such as NF- κ B or activator protein (AP-1) by protein-protein interactions [3,4].

The ability of a cell to respond to glucocorticoid administration is dependent on the levels of the glucocorticoid receptor in the cell [5–7]. In the presence of ligand, the human glucocorticoid receptor (hGR) undergoes homologous down-regulation, which involves decreases in both messenger RNA and protein levels [8–11]. Loss of the hGR is clinically significant considering that without down-regulation, overstimulation of GR signaling pathways may occur in the presence of high levels of hormone. Alternatively, the effects of chronic glucocorticoid treatment may lead to such significant loss of GR protein levels that cells become unresponsive to further glucocorticoid treatment. In many cell types that have been investigated chronic treatment with glucocorticoids leads to dramatic decreases of hGR protein levels [8,10,12–14]. The significant cell and tissue-type specific differences in glucocorticoid receptor down-regulation may be the result of multiple mechanisms controlling GR levels. Thus loss of functional glucocorticoid receptor by down-regulation may leave a tissue unable to respond until translation of new receptor protein occurs.

The decrease in the levels of the glucocorticoid receptor in the presence of ligand is similar to the downregulation of other steroid receptors, and involves degradation by the proteasome-ubiquitination pathway. This pathway utilizes ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-ligase enzymes. Substrate protein recognition by E2/E3 molecules leads to addition of the 76 amino acid protein ubiquitin to the targeted protein. Once a protein is covalently modified by polyubiquitination it is recognized by the multisubunit protein complex known as the proteasome, which acts a protease to degrade the protein to its peptide and amino acid components. A number of receptors are ubiquitinated and degraded by the proteasome pathway including the androgen, estrogen, and progesterone receptors [15–18]. Interestingly, the interaction of steroid receptors with the proteasome-ubiquitination pathway may also be essential in the assembly and activity of transcription complexes [19,20]. Ligand dependent down-regulation of other steroid hormone receptors is blocked by proteasome inhibition. Unlike other receptors, proteasome inhibition leads to enhanced transcriptional activity of the rat and mouse glucocorticoid receptor [21,22]

Our previous investigation of ligand dependent mouse GR down-regulation implicated the proteasome in the degradation of GR and identified a degradation motif known as a PEST region. Analysis of the hGR identified a similar conserved PEST motif located in the amino terminal of the receptor before the DNA binding domain. We report here that the proteasome inhibitors MG-132, β -lactone, and epoxomicin enhance glucocorticoid

responsiveness in cells expressing the human glucocorticoid receptor. Site directed mutagenesis of the PEST degradation motif at lysine 419 was done to create a mutant form of the human glucocorticoid receptor. Mutant K419A hGR displayed greater reporter gene activity in the presence of Dex than wild type hGR and mutant K419A hGR did not undergo ligand dependent downregulation. Wild type hGR and mutant K419A hGR were fused with the green fluorescent protein (GFP) and both exhibit normal sub-cellular distribution in the presence or absence of ligand. Together these results suggest that the proteasome may limit steroid-dependent human glucocorticoid receptor signaling that may involve the identified GR PEST degradation motif.

2. Experimental

2.1. Materials

Dexamethasone (1,4 pregnadien-9 α -fluoro-16 α -methyl-11 β , 17, 21-triol-3, 20-dione) (Dex) was purchased from Steraloids (Wilton, NH). MG-132 (Z-Leu-Leu-Leu-CHO), clasto-latacystin β -lactone, and epoxomicin were purchased from CalBiochem (La Jolla, CA). N-ethylmaleimide was purchased from Pierce (Rockford, IL). [14 C] chloramphenicol (40–60 mCi/mmol) was obtained from NEN Life Science Products. Acetyl coenzyme A, Mini complete tablets, Pefabloc, and aprotinin protease inhibitors were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Peroxidase-labeled anti-rabbit and anti-mouse antibodies and the ECL chemiluminescent detection reagents were purchased from Amersham Pharmacia Biotech. All other reagents were obtained from Sigma (St. Louis, MO).

2.2. Recombinant Plasmids

Wild type and human glucocorticoid receptor (GR) were expressed in the pCMV5 vector under the control of the CMV promoter. Mutation of human glucocorticoid receptor cDNA from K419 to K419A was done by PCR site-directed mutagenesis using the Quick Change Site directed mutagenesis kit from Stratagene (Cedar Creek, TX). Primers used were 5'-ACG GGA CCA CCT CCC GCA CTC TGC CTG GTG TGC-3' and 5'-GCA CAC CAG GCA GAG TGC GGG AGG TGG TCC CGT -3'. GRE₂-TATA-CAT and GRE₂-TATA-Luc reporter plasmids have been previously described [23,24].

2.3. Cell Culture and Transfections

COS-1 (African Green monkey kidney) cells were grown in DMEM with high glucose (DMEM-H) medium supplemented with 2 mM glutamine, (v/v) of a 1:1 mixture of heat-inactivated fetal calf/calf serum (Summit Biotechnology, Ft. Collins, CO), and 100 IU/ml penicillin and 100 ug/ml streptomycin. HeLa cells were grown in monolayer cultures in Joklik's minimum essential medium (JMEM) and harvested with Versene as previously described [25]. All cultures were maintained in 5% CO₂ humidified atmosphere at 37 °C and passaged every 3–4 days. For transfection of cells, cells were plated 24 hours prior to transfection and were allowed to grow to 50–70% confluency. Cells were washed with Opti-MEM (Invitrogen) and transfected using DMRIEC (Invitrogen) or TransIT-LT1 transfection reagent from Panvera Corp. (Madison, WI) according to the manufacturer recommendations. The Opti-MEM media was removed after transfection and media containing dextran-charcoal stripped serum was added. Plasmid DNA was prepared using Qiagen Midiprep kits (Valencia, CA).

2.4. Luciferase Assays

Cells were transfected as described above. Following transfections, media was changed to media containing dextran-charcoal stripped serum. Subsequently, cells were allowed to recover for 12–16 hours and then were treated with vehicle or dexamethasone for times

indicated. Cells were harvested, lysates prepared and luciferase assay was performed using an Enhanced Luciferase Assay Kit from BD PharMigen (Franklin Lakes, NJ) or Promega's luciferase assay system (Promega Corp., Madison, WI). Luciferase activity was measured using a Microtiter Plate Luminometer and Revelation 4.06 Software (Dynex Technologies). Luciferase activity was calculated per ug of protein for each sample.

2.5. Chloramphenicol Acetyl Transferase (CAT) Assays

Cells were transfected as described above. Cells were washed with ice-cold 1XPBS and spun down for 5 minutes and taken up in 0.25 M EDTA (pH=8.0). Lysates were prepared by tip sonication and endogenous CAT activity was inactivated by heating to 65°C for 15 minutes. Protein concentration was determined by the method of Bradford, using a reagent kit from Bio-Rad Laboratories, Inc. (Hercules, CA). Equal amounts of protein were incubated in reactions with acetyl coenzyme A and 0.1 uCi [¹⁴C] chloramphenicol. Reactions were terminated and assayed using the by the mixed xylene method as described by Promega (Madison, WI) and a standard curve of CAT activity from supplied protein was utilized.

2.6. Western Blot Analysis

In protein half-life studies using the expression vectors pCMV5-hGR and mutant pCMV5-hGRK419A, cellular extracts were prepared from transiently transfected COS-1 cells. Cells were pre-treated with 1 μM cycloheximide for 1 hour and then treated with vehicle or 100 nM dexamethasone. Cells were washed with ice-cold 1xPBS, harvested by scraping with a rubber spatula into PBS, and cells were centrifuged. The cell pellet was taken up in RIPA buffer (20mM Tris (pH=7.5), 2MM EDTA, 150 mM NaCl, 0.05% Triton X-100, pH to 7.5) containing a Mini Complete protease inhibitor tablet from Boehringer Mannheim. Protein levels were quantitated using the Lowry method (BioRad). Western blots were prepared and visualized as previously described using the anti-GR epitope purified polyclonal antibody 1857 [22]. Studies were also done using an anti-rabbit IRDye800 fluorescently labeled secondary antibody (LI-COR Biosciences, Lincoln, NE) and results were visualized using a LiCOR Odyssey Infrared Imaging System. The integrated intensity of GR protein and β-actin levels was assessed by LiCOR Odyssey Imaging Software (Lincoln, NE).

2.7. Cellular localization of GFP-hGR and GFP-hGRK419A mutant GFP-GR

Human GR and mutant GR K419A were subcloned into the pEGFP-C1 vector (BD Biosciences Clontech). COS-1 cells were transfected as previously described with 0.25 ug wild type GFP-hGR or mutant GFP-hGRK419A. Transfected cells were maintained in stripped serum media, pretreated with cycloheximide to block new protein production, and were then treated with vehicle or 1 uM corticosterone for 12 hours. Nuclear localization of both wild type and mutant K419A was observed using a SpotRT slider camera (Diagnostic Instruments, Sterling Hts, MI) on a Nikon LaboPHOTO-2 microscope equipped with EGFP filter set (Chroma Tech. Corp, Brattleboro, VT). Image analysis was done using SpotRT software supplied by the manufacturer. Cells were then washed 5X with stripped serum media to remove hormone and maintained in cycloheximide. After 12 hours both wild type and mutant GR were visualized again to determine the cellular localization of GR after washout of hormone. Corticosterone was then re-administered to media and cellular localization of GR was then assessed again after 4 hours of hormone treatment.

2.8. Statistical Analyses

Statistical analysis was performed using JMP software, version 7.0.0 (SAS Institute, Inc., Cary, NC). Analysis was done using ANOVA followed by Tukey-Kramer Honestly Significant Difference (HSD) test with a significance level of 0.05.

3. Results

3.1. Glucocorticoid receptor protein levels after glucocorticoid and proteasome inhibitor treatment

Upon ligand binding the glucocorticoid receptor undergoes a rapid decline of receptor levels that has been well demonstrated in a number of glucocorticoid receptor target tissues and diminishes cellular responsiveness to ligand. We wished to determine the role of the proteasome in human glucocorticoid receptor ligand dependent down-regulation of the glucocorticoid receptor. The COS-1 cell line (which does not express endogenous GR) was transiently transfected with the pCMV5-human GR expression vector. After transfection, cells were pretreated with cycloheximide and then with the proteasome inhibitor MG-132 or vehicle for 1 hour and then dosed with Dexamethasone (Dex). Cellular extracts were prepared and Western blot analysis of GR was done with GR specific antibodies. MG-132 caused an increase in the levels of GR (Fig. 1) and glucocorticoid treatment led to a decrease in GR levels. Glucocorticoid downregulation of GR levels was inhibited by the co-administration of MG-132 (Fig 1. 3rd vs. 4th lanes). These data using the proteasome inhibitor MG-132 suggests that ligand dependent human GR degradation occurs by the proteasome pathway.

3.2. Glucocorticoid responsiveness after glucocorticoid and proteasome inhibitor treatment

To establish if the elevated levels of human GR in the presence of proteasome inhibitors altered GR functional activity assays were done using the glucocorticoid responsive reporter vector GRE₂CAT. The expression vector pCMV5-hGR and GRE₂CAT were transiently transfected into COS-1 cells. In these experiments, proteasome inhibitors that were utilized include the reversible peptide aldehyde MG-132, and the irreversible inhibitors β -lactone, and epoxomicin. Transfected cells were pretreated with proteasome inhibitors and then were treated with Dex. Proteasome inhibitors in the absence of Dex had little effect on reporter activity (Fig. 2). In the presence of Dex and proteasome inhibitors, reporter activity was significantly increased over Dex alone. The ability of proteasome inhibitors to enhance the functional activity of GR in the presence of Dex suggests that the proteasome may function to limit glucocorticoid responsiveness.

To investigate whether the proteasome plays a similar role to limit glucocorticoid responsiveness in cells that contain endogenous human GR a HeLa cell line was utilized. The HeLa cell line, which expresses endogenous GR, undergoes ligand dependent downregulation, and the levels of GR has been proposed to limit glucocorticoid responsiveness [5,7,26]. To determine if inhibiting the proteasome degradation pathway affected GR responsiveness in HeLa cells, we evaluated the proteasome inhibitors MG-132 and epoxomicin for effects of proteasome inhibition on hormone responsiveness in HeLa cells. First, HeLa cells were transfected with a hormone responsive dimer GRE driving the CAT reporter gene. Following transfection, cells were pre-treated with proteasome inhibitors and then were treated with dexamethasone. Cells treated with proteasome inhibitors demonstrated only slightly higher percent CAT conversion than control, whereas dexamethasone induced activity over the control (Fig. 3). In contrast, HeLa cells treated with a combination of inhibitors and dexamethasone showed significantly induced reporter activity as compared to control. Thus, inhibition of the proteasome was also effective in enhancing glucocorticoid responsiveness in HeLa cells harboring endogenous glucocorticoid receptor. These findings suggest that glucocorticoid responsiveness in HeLa cells is limited by the proteasome pathway.

3.3. Mutant human glucocorticoid receptor protein levels after dexamethasone treatment

The glucocorticoid receptor and a number of other steroid receptors have been identified as being ubiquitinated and targeted for degradation by the proteasome in a process involving receptor binding of ligand. Previously, we had determined that the mouse glucocorticoid receptor is ubiquitinated, and others have also demonstrated ubiquitination of the human glucocorticoid receptor [27,28]. To identify possible protein degradation pathways involved in the process of ligand dependent down-regulation, we analyzed the GR protein for candidate degradation elements. Using the PEST-FIND computer program we identified a conserved PEST element found in the human, mouse, and rat GR (Fig. 4A). This region in the human GR from amino acids 399-419 has a PEST-FIND score of +16.1, and on a scale from -50 to +50 a value above +5 is thought to be indicative of possible functional PEST motif. The potential PEST motif in GR could help explain the rapid destabilization of GR after ligand binding. The identified region in the human glucocorticoid receptor contains a lysine (K) residue at 419, a possible site of ubiquitination.

To evaluate the significance of this region, the identified PEST region of GR at amino acids 399-419 was next targeted for site directed mutagenesis. The candidate site for ubiquitination at K419 was mutated to alanine and the mutation confirmed by DNA sequencing. Previous half-life studies have shown that GR protein levels fall to 50% in the presence of 100 nM dexamethasone in about 9 hours, while in the absence of ligand GR protein levels fall to 50% in approximately 18 hours [29]. COS-1 cells were transiently transfected with wild type GR or mutant K419A GR expression vector and treated with cycloheximide prior to hormone administration. This generates an intracellular pool of GR protein and allows us to examine changes in GR protein levels without interference by the effects of hormone on the repression of GR gene transcription. Cells were then treated with dexamethasone and cellular extracts were prepared for Western blot analysis (Fig. 4B). K419A GR protein levels at the beginning of the experiment were 80% greater than wild type GR. Wild type GR levels were quantified using the integrated intensity of GR and β -actin and were significantly lower due to ligand dependent down-regulation (Fig. 4C). However, mutant K419A GR did not undergo ligand dependent down-regulation and receptor levels remained relatively unchanged by treatment with dexamethasone for 12 or 24 hours. The failure of K419A GR to undergo ligand dependent down-regulation suggests that the lysine within the identified PEST region plays a prominent role in GR turnover. We next considered if mutagenesis of K419 had any effects on GR function.

3.4. Glucocorticoid responsiveness of mutant K419A hGR using simple and complex promoters

The maintenance of normal levels of K419A GR protein observed in western blot studies prompted us to examine whether this mutant receptor incapable of down-regulation at the protein level displayed an altered response to hormone. In these studies, glucocorticoid responsiveness was investigated using a simple GRE containing promoter and the more complex mouse mammary tumor virus (MMTV) glucocorticoid responsive promoter. The GRE₂-Luc contains two GREs and a TATA box in front of the luciferase reporter. The complex MMTV promoter contains two different GREs, two GRE half-sites, and binds sites for the transcription factors Oct-1 and NF-1. The ability wild type and mutant K419A GR were investigated by transiently transfection into COS-1 cells with the simple GRE and complex MMTV luciferase reporter plasmids. Mutant K419A GR displayed enhanced reporter activity over that of wild type GR in experiments using both the simple and complex promoters (Fig. 5). These findings illustrated the mutagenesis of Lysine-419 leads to enhanced glucocorticoid responsiveness, and this response was not limited to simple or complex glucocorticoid promoters. The observed enhanced glucocorticoid responsiveness is

presumably due to the Lysine-419 GR not undergoing protein degradation in the presence of Dex, and not a change in the intrinsic transcriptional activity of GR by the K419A mutation.

3.5. Glucocorticoid responsiveness of mutant K419A hGR

To further investigate the enhanced glucocorticoid responsiveness of COS-1 cells transfected with K419A GR mutant as compared to wild type GR, transfection studies were done with varying amount of plasmid expression vectors. Glucocorticoid responsiveness of the GRE₂-Luc plasmid was greater in cells transfected with K419A GR over that of wild type GR at each GR concentration and wild type GR failed to reach the maximum level of luciferase activity achieved by the K419A GR mutant (Fig. 6A). To determine if glucocorticoid responsiveness of the K419A GR mutant or wild type GR varied over time, COS-1 cells were transfected and treated with Dex for 12, 24, and 48 hours. The K419A mutant GR displayed greater glucocorticoid responsiveness than wild type GR at each time point (Fig. 6B).

3.6. Subcellular localization of mutant K419A hGR

To determine if the mutant K419A glucocorticoid receptor was in the appropriate subcellular location in the presence and absence of ligand, translocation experiments were done. Wild type hGR and mutant K419A hGR and their ability to translocate to the nucleus were investigated in COS1 cells by fusing these receptors with the green fluorescent protein (GFP). As expected, both wild type GFP-hGR and GFP-hGRK419A were located in cytoplasm. Cells were treated with cycloheximide to block new protein production and upon treatment with the glucocorticoid corticosterone, wild type GFP-hGR and mutant GFP-hGRK419A both entered the nucleus in equal numbers (Fig. 7). Cells were rinsed with media and upon wash out of hormone both GFP-hGR and GFP-hGRK419A exited the nucleus and redistributed in the cytoplasm. These results confirm that the changes in transcriptional activity observed in previous transient transfections were not caused by differences in subcellular localization of mutant K419A GR. Together these results suggest a mechanism for termination of steroid-dependent human GR signaling by protein degradation through the proteasome pathway.

4. Discussion

Our previous work has demonstrated that the mouse glucocorticoid receptor protein undergoes down-regulation in response to hormone binding via the proteasome protein degradation pathway [22]. Similarly, proteasome degradation of the rat GR was also shown [21]. In both cases, inhibition of GR degradation by the proteasome was demonstrated to elevate GR proteins levels, and in the presence of proteasome inhibitors, GR glucocorticoid responsiveness was enhanced. In these studies we also observed that proteasome inhibition by MG-132 inhibited human glucocorticoid receptor protein degradation in the presence of ligand. Proteasome degradation of the glucocorticoid receptor protein has also been demonstrated to occur with disruption of the GR heterocomplex with heat shock proteins (Hsps) [30,31]. Two E3 ligases have been implicated in GR degradation and include the protein carboxyl terminus of hsc70-interacting protein (CHIP) and murine double mutant 2 (mdm2) [32–34]. The proteasome degradation pathway also has a role in the degradation of other steroid receptors and inhibition of the proteasome leads to altered transcriptional responses. Proteasome inhibition leads to steroid receptor transcriptional repression in most cases, even when levels of receptors increase [20,35]. It has been suggested that the steroid transcriptional process is dynamic and requires the proteasome to maintain the appropriate interaction of coactivators and receptors [17,19].

Proteasome inhibition has been shown enhance glucocorticoid responsiveness of cells expressing mouse and rat GR [21,22,36]. In our studies of the human GR, enhanced glucocorticoid responsiveness was demonstrated in COS cells treated with the proteasome inhibitors MG-132, β -lactone, and epoxomicin. Proteasome inhibition is also known to affect the cellular mobility of GR and other steroid receptors, but in the presence of GR ligands, effects on mobility may be lost [37]. In experiments using fluorescent recovery after photobleaching (FRAP) analysis glucocorticoid receptors have been shown to be highly mobile [38,39]. The mechanisms of liganded GR binding to DNA and initiation of the transcription complex involves interaction with chaperones and the proteasome [40]. The effects of proteasome inhibition on the glucocorticoid receptor was initially thought to cause longer GR residence time which was associated with greater transcriptional activity [41]. Additional studies indicate that while proteasome inhibitors may reduce receptor mobility, there is not a clear correlation with enhanced transcriptional activity [21,42]. In the HT22 mouse hippocampal cell line, the mouse glucocorticoid receptor was shown to not undergo ligand dependent down-regulation, and CHIP overexpression was shown to restore GR down-regulation [36,43]. These findings by Wang and DeFranco suggests the cellular level of proteasome related proteins, in this case CHIP targeting GR to the proteasome, may have critical roles in the cellular glucocorticoid responsiveness and the effects of proteasome inhibition.

Having shown that proteasome inhibition alters cellular glucocorticoid responsiveness, we investigated the human glucocorticoid receptor protein to identify degradation motifs. Analysis of the human GR identified a PEST motif located in the amino terminal of the receptor before the DNA binding domain. Site directed mutagenesis of this element at lysine 419 was done to create a mutant form of the human glucocorticoid receptor, which we observed by Western blot was resistant to ligand dependent down-regulation. The mutant K419A hGR displayed greater reporter activity in the presence of Dex than wild type hGR using both simple GRE₂ and complex MMTV promoters. This was mostly likely due to the elevated level of K419A GR protein and not any intrinsic change in GR transcriptional activity due to this mutation. Enhanced glucocorticoid responsiveness of the K419A mutant over wild type was also seen in plasmid dose response experiments and time course experiments. Together these findings indicate the enhanced glucocorticoid responsiveness of the K419A mutant over wild type GR is consistent regardless of promoter context, plasmid dose, and time points after glucocorticoid treatment. The increase in glucocorticoid responsiveness after proteasome inhibition was greater than that observed in studies using the K419A mutant. It has been previously shown by others that proteasome inhibitors also stabilize important receptor coactivators and thus enhance responsiveness [17,44]. As seen in Fig 4, K419A GR protein did not undergo ligand dependent down-regulation in the presence of cycloheximide after 12 or 24 hours.

To determine if mutant K419A, in the absence of ligand, had a normal cytoplasmic distribution, moved into the nucleus after hormone treatment, and exited the nucleus after hormone removal, wild type hGR and mutant K419A hGR were fused with the green fluorescent protein. Both GFP-hGR and mutant GFP-hGRK419A had normal sub-cellular distribution in the presence or absence of ligand. After hormone removal, both wild type hGR and mutant hGRK419A exited the nucleus. These findings indicate that the K419A mutant GR enhanced transcriptional activity and resistant to ligand dependent degradation was not due to altered cellular localizations as it moved within the cell like wild type GR.

Recent development of proteasome inhibitors for use as chemotherapeutic agents in the treatment of multiple myeloma (MM) has been demonstrated to be an effective for relapsed/refractory MM [45,46]. Proteasome inhibitor PS-341/bortezomib (Velcade™), a dipeptidyl boronic acid, is thought to activate the apoptosis pathways in cancer cells and was approved

by the U. S. Food and Drug Administration for MM treatment [46]. Understanding the effects of proteasome inhibition on glucocorticoid responsiveness has clinical significance as some therapies using the proteasome inhibitor bortezomib include glucocorticoids such as dexamethasone [47,48]. Together our results suggest that the proteasome limits steroid-dependent human glucocorticoid receptor signaling and that GR ligand dependent down-regulation involves the identified GR PEST degradation motif.

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References

1. Jenkins BD, Pullen CB, Darimont BD. Novel glucocorticoid receptor coactivator effector mechanisms. *Trends Endocrinol Metab* 2001;12(3):122–6. [PubMed: 11306337]
2. Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr Rev* 2004;25(1):45–71. [PubMed: 14769827]
3. Adcock IM, Ito K, Barnes PJ. Glucocorticoids: effects on gene transcription. *Proc Am Thorac Soc* 2004;1(3):247–54. [PubMed: 16113442]
4. McKay LI, Cidlowski JA. CBP (CREB binding protein) integrates NF-kappaB (nuclear factor-kappaB) and glucocorticoid receptor physical interactions and antagonism. *Mol Endocrinol* 2000;14(8):1222–34. [PubMed: 10935546]
5. Cidlowski JA, Cidlowski NB. Regulation of glucocorticoid receptors by glucocorticoids in cultured HeLa S3 cells. *Endocrinology* 1981;109(6):1975–82. [PubMed: 7308136]
6. Vanderbilt JN, Miesfeld R, Maler BA, Yamamoto KR. Intracellular receptor concentration limits glucocorticoid-dependent enhancer activity. *Mol Endocrinol* 1987;1(1):68–74. [PubMed: 2842660]
7. Silva CM, Powell-Oliver FE, Jewell CM, Sar M, Allgood VE, Cidlowski JA. Regulation of the human glucocorticoid receptor by long-term and chronic treatment with glucocorticoid. *Steroids* 1994;59(7):436–42. [PubMed: 7974528]
8. Dong Y, Poellinger L, Gustafsson JA, Okret S. Regulation of glucocorticoid receptor expression: evidence for transcriptional and posttranslational mechanisms. *Mol Endocrinol* 1988;2(12):1256–64. [PubMed: 3216865]
9. Burnstein KL, Jewell CM, Sar M, Cidlowski JA. Intragenic sequences of the human glucocorticoid receptor complementary DNA mediate hormone-inducible receptor messenger RNA down-regulation through multiple mechanisms. *Mol Endocrinol* 1994;8(12):1764–73. [PubMed: 7708063]
10. Hoeck W, Rusconi S, Groner B. Down-regulation and phosphorylation of glucocorticoid receptors in cultured cells. Investigations with a monospecific antiserum against a bacterially expressed receptor fragment. *J Biol Chem* 1989;264(24):14396–402. [PubMed: 2668288]
11. Okret S, Poellinger L, Dong Y, Gustafsson JA. Down-regulation of glucocorticoid receptor mRNA by glucocorticoid hormones and recognition by the receptor of a specific binding sequence within a receptor cDNA clone. *Proc Natl Acad Sci U S A* 1986;83(16):5899–903. [PubMed: 3016728]
12. Waxman DJ, Attisano C, Guengerich FP, Lapenson DP. Human liver microsomal steroid metabolism: identification of the major microsomal steroid hormone 6 beta-hydroxylase cytochrome P-450 enzyme. *Arch Biochem Biophys* 1988;263(2):424–36. [PubMed: 3259858]
13. Vedeckis WV, Ali M, Allen HR. Regulation of glucocorticoid receptor protein and mRNA levels. *Cancer Res* 1989;49(8 Suppl):2295s–2302s. [PubMed: 2702670]
14. McIntyre WR, Samuels HH. Triamcinolone acetonide regulates glucocorticoid-receptor levels by decreasing the half-life of the activated nuclear-receptor form. *J Biol Chem* 1985;260(1):418–27. [PubMed: 3965455]
15. Sheflin L, Keegan B, Zhang W, Spaulding SW. Inhibiting proteasomes in human HepG2 and LNCaP cells increases endogenous androgen receptor levels. *Biochem Biophys Res Commun* 2000;276(1):144–50. [PubMed: 11006097]

16. Alarid ET, Bakopoulos N, Solodin N. Proteasome-mediated proteolysis of estrogen receptor: a novel component in autologous down-regulation. *Mol Endocrinol* 1999;13(9):1522–34. [PubMed: 10478843]
17. Lonard DM, Nawaz Z, Smith CL, O'Malley BW. The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. *Mol Cell* 2000;5(6):939–48. [PubMed: 10911988]
18. Lange CA, Shen T, Horwitz KB. Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. *Proc Natl Acad Sci U S A* 2000;97(3):1032–7. [PubMed: 10655479]
19. Reid G, Hubner MR, Metivier R, Brand H, Denger S, Manu D, et al. Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. *Mol Cell* 2003;11(3):695–707. [PubMed: 12667452]
20. Kinyamu HK, Chen J, Archer TK. Linking the ubiquitin-proteasome pathway to chromatin remodeling/modification by nuclear receptors. *J Mol Endocrinol* 2005;34(2):281–297. [PubMed: 15821097]
21. Deroo BJ, Rentsch C, Sampath S, Young J, DeFranco DB, Archer TK. Proteasomal inhibition enhances glucocorticoid receptor transactivation and alters its subnuclear trafficking. *Mol Cell Biol* 2002;22(12):4113–23. [PubMed: 12024025]
22. Wallace AD, Cidlowski JA. Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. *J Biol Chem* 2001;276(46):42714–21. [PubMed: 11555652]
23. Allgood VE, Powell-Oliver FE, Cidlowski JA. Vitamin B6 influences glucocorticoid receptor-dependent gene expression. *J Biol Chem* 1990;265(21):12424–33. [PubMed: 2373699]
24. Allgood VE, Oakley RH, Cidlowski JA. Modulation by vitamin B6 of glucocorticoid receptor-mediated gene expression requires transcription factors in addition to the glucocorticoid receptor. *J Biol Chem* 1993;268(28):20870–6. [PubMed: 8407919]
25. Scheible PP, DeLorenzo TM, Cidlowski JA. Analysis of the glucocorticoid antagonist action of dexamethasone 21- mesylate in HeLa S3 cells. *J Steroid Biochem* 1987;26(2):181–7. [PubMed: 3560935]
26. Burnstein KL, Bellingham DL, Jewell CM, Powell-Oliver FE, Cidlowski JA. Autoregulation of glucocorticoid receptor gene expression. *Steroids* 1991;56(2):52–8. [PubMed: 2020978]
27. Sengupta S, Wasylyk B. Ligand-dependent interaction of the glucocorticoid receptor with p53 enhances their degradation by Hdm2. *Genes Dev* 2001;15(18):2367–80. [PubMed: 11562347]
28. Dvorak Z, Modriansky M, Ulrichova J, Maurel P, Vilarem MJ, Pascucci JM. Disruption of microtubules leads to glucocorticoid receptor degradation in HeLa cell line. *Cell Signal* 2005;17(2):187–96. [PubMed: 15494210]
29. Webster JC, Jewell CM, Bodwell JE, Munck A, Sar M, Cidlowski JA. Mouse glucocorticoid receptor phosphorylation status influences multiple functions of the receptor protein. *J Biol Chem* 1997;272(14):9287–93. [PubMed: 9083064]
30. Whitesell L, Cook P. Stable and specific binding of heat shock protein 90 by geldanamycin disrupts glucocorticoid receptor function in intact cells. *Mol Endocrinol* 1996;10(6):705–12. [PubMed: 8776730]
31. Segnitz B, Gehring U. The function of steroid hormone receptors is inhibited by the hsp90-specific compound geldanamycin. *J Biol Chem* 1997;272(30):18694–701. [PubMed: 9228040]
32. Connell P, Ballinger CA, Jiang J, Wu Y, Thompson LJ, Hohfeld J, et al. The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nat Cell Biol* 2001;3(1):93–6. [PubMed: 11146632]
33. Demand J, Alberti S, Patterson C, Hohfeld J. Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. *Curr Biol* 2001;11(20):1569–77. [PubMed: 11676916]
34. Kinyamu HK, Archer TK. Estrogen receptor-dependent proteasomal degradation of the glucocorticoid receptor is coupled to an increase in mdm2 protein expression. *Mol Cell Biol* 2003;23(16):5867–81. [PubMed: 12897156]

35. Nawaz Z, O'Malley BW. Urban renewal in the nucleus: is protein turnover by proteasomes absolutely required for nuclear receptor-regulated transcription? *Mol Endocrinol* 2004;18(3):493–9. [PubMed: 14673136]
36. Wang X, Pongrac JL, DeFranco DB. Glucocorticoid Receptors in Hippocampal Neurons that Do Not Engage Proteasomes Escape from Hormone-Dependent Down-Regulation but Maintain Transactivation Activity. *Mol Endocrinol* 2002;16(9):1987–98. [PubMed: 12198236]
37. Schaaf MJ, Cidlowski JA. Molecular determinants of glucocorticoid receptor mobility in living cells: the importance of ligand affinity. *Mol Cell Biol* 2003;23(6):1922–34. [PubMed: 12612067]
38. Schaaf MJ, Lewis-Tuffin LJ, Cidlowski JA. Ligand-selective targeting of the glucocorticoid receptor to nuclear subdomains is associated with decreased receptor mobility. *Mol Endocrinol* 2005;19(6):1501–15. [PubMed: 15705660]
39. McNally JG, Muller WG, Walker D, Wolford R, Hager GL. The glucocorticoid receptor: rapid exchange with regulatory sites in living cells. *Science* 2000;287(5456):1262–5. [PubMed: 10678832]
40. Freeman BC, Yamamoto KR. Disassembly of transcriptional regulatory complexes by molecular chaperones. *Science* 2002;296(5576):2232–5. [PubMed: 12077419]
41. Stavreva DA, McNally JG. Fluorescence recovery after photobleaching (FRAP) methods for visualizing protein dynamics in living mammalian cell nuclei. *Methods Enzymol* 2004;375:443–55. [PubMed: 14870682]
42. Stavreva DA, McNally JG. Role of H1 phosphorylation in rapid GR exchange and function at the MMTV promoter. *Histochem Cell Biol* 2006;125(1–2):83–9. [PubMed: 16397795]
43. Wang X, DeFranco DB. Alternative effects of the ubiquitin-proteasome pathway on glucocorticoid receptor down-regulation and transactivation are mediated by CHIP, an E3 ligase. *Mol Endocrinol* 2005;19(6):1474–82. [PubMed: 15761032]
44. Li C, Wu RC, Amazit L, Tsai SY, Tsai MJ, O'Malley BW. Specific amino acid residues in the basic helix-loop-helix domain of SRC-3 are essential for its nuclear localization and proteasome-dependent turnover. *Mol Cell Biol* 2007;27(4):1296–308. [PubMed: 17158932]
45. Chauhan D, Hideshima T, Anderson KC. Proteasome inhibition in multiple myeloma: therapeutic implication. *Annu Rev Pharmacol Toxicol* 2005;45:465–76. [PubMed: 15822185]
46. Mani A, Gelmann EP. The ubiquitin-proteasome pathway and its role in cancer. *J Clin Oncol* 2005;23(21):4776–89. [PubMed: 16034054]
47. Jagannath S, Durie BG, Wolf J, Camacho E, Irwin D, Lutzky J, et al. Bortezomib therapy alone and in combination with dexamethasone for previously untreated symptomatic multiple myeloma. *Br J Haematol* 2005;129(6):776–83. [PubMed: 15953004]
48. Schmielau J, Teschendorf C, Konig M, Schmiegel W, Graeven U. Combination of bortezomib, thalidomide, and dexamethasone in the treatment of relapsed, refractory IgD multiple myeloma. *Leuk Lymphoma* 2005;46(4):567–9. [PubMed: 16019485]

Fig. 1.

Human glucocorticoid receptor ligand dependent downregulation is inhibited by the proteasome inhibitor MG-132. COS-1 cells were transiently transfected with human glucocorticoid receptor expression vector pCMV5-hGR. Cells were treated with 1 μ M cycloheximide for 1 hour, pretreated with DMSO vehicle or 1 μ M MG-132 for 1 hour, and then dosed with either vehicle or dexamethasone (Dex) at 100 nM for 12 hours. Whole cell lysates were prepared and Western blots were probed with anti-peptide hGR antibody and β -actin. Densitometry was done and GR protein levels were determined relative to β -actin control. The results of three independent experiments and a representative Western blot are shown.

Fig. 2.

Glucocorticoid induction of reporter activity is enhanced by pretreatment with proteasome inhibitors in human GR transfected cells. COS-1 cells were transiently transfected with the pCMV-hGR human glucocorticoid receptor expression vector and the glucocorticoid responsive reporter GRE₂-CAT. Cells were treated with vehicle control (Con) or 1 μ M MG-132, β -lactone (Blact), or epoxomicin (Epox) for 1 hour and then treated with 100 nM dexamethasone (Dex) for 16 hours. Lysates were prepared for chloramphenicol acetyl transferase assay (CAT). Data are presented as CAT activity per ug of protein and represent the mean of three independent experiments. (* $p < 0.05$ vs. CPM/ug Protein).

Fig. 3.

Proteasome inhibition enhances endogenous human glucocorticoid receptor activity in HeLa cells treated with dexamethasone. HeLa cells were transiently transfected the glucocorticoid responsive reporter GRE₂-CAT. Cells were treated with vehicle control (Con) or 1 μ M MG-132 or epoxomicin (Epox) for 1 hour and then treated with 100 nM dexamethasone (Dex) for 16 hours. Lysates were prepared for chloramphenicol acetyl transferase assay (CAT). Equal μ g of extract were used in each reaction. Data are presented as CAT activity per μ g of protein and represent the mean of three independent experiments. (* $p < 0.05$ vs. CAT activity/ μ g Protein)

Figure 4A

PEST Regions	PEST-FIND Score
hGR RPDVS SPPSS SSTAT TGPPP K	+ 16.1
mGR RPDVS SPPSS SSTA. TGPPP K	+ 18.3
rGR RPDVS SPPSS SSAA. TGPPP K	+ 18.3

Figure 4B

Figure 4C

Fig. 4.

Human glucocorticoid receptor K419A mutant does not undergo ligand dependent down-regulation. **A**, To identify possible degradation motifs within the human GR the PEST-FIND program was run. The structure of the GR and where the identified PEST motif is found is indicated. Note the identified lysine (K) at amino acid 419 was targeted for site directed mutagenesis. Similar PEST sequences were found in the mouse and rat glucocorticoid receptors. **B**, COS-1 cells were transfected with wild type (WT), or in a separate experiment mutant K419A, and 16–24 hours later were treated with 1 μ M cycloheximide for 1 hour and then with 100 nM Dex or left untreated (Con) for 12 or 24 hours. Whole cell lysates were prepared and proteins were separated on 8% acrylamide gel and transferred to nitrocellulose membrane. GR was visualized using anti-GR 1857 antibody and LiCOR Odyssey Infrared Imaging System. A representative Western blot of three independent experiments is shown. **C**, The integrated intensity of GR and β -actin was determined by LiCOR Odyssey Imaging Software to quantitate the relative change in GR protein of wild type (WT) GR and K419A GR and is represented as a change relative to wild type GR control (T0) $\pm$ SD (n=3). Sample loading was normalized to β -actin protein levels. Statistical significance was determined by ANOVA, followed by comparisons of T0 control with Dex treatments using the Tukey–Kramer HSD test with a significance level of 0.05.

Fig. 5. K419A GR mutant displays enhanced transcriptional activation over that of wild type hGR after dexamethasone treatment. COS-1 cells were transiently transfected with wild type human GR or K419A mutant GR and GRE₂Luc or MMTV-Luc. Cells were then treated with 100 nM Dex or vehicle (Con) for 16 hours and cell extracts were prepared for luciferase activity determination. Luciferase activity was calculated per ug of protein for each sample and results are presented as RLU/ug protein. (* p<0.05 vs. RLU/ug protein.)

Figure 6A

Figure 6B

Fig. 6. GRE₂Luc reporter activation by K419A GR was enhanced over that of wild type GR regardless of the GR plasmid dose. **A**, COS-1 cells were transfected with increasing amounts of pCMV5-hGR or pCMV5-hGR K419A expression plasmids (50–800 ng) and the GRE₂Luc reporter plasmid. Cells were then treated with 100 nM Dex or vehicle (Con) for 16 hours. Cell extracts were prepared for luciferase activity determination. Luciferase activity was calculated per ug of protein for each sample and results are presented as RLU/ug protein. (* $p < 0.05$ Wt GR vs. K419A GR RLU/ug protein). **B**, COS-1 cells were transfected with pCMV5-hGR or pCMV5-hGR K419A expression plasmids (100 ng) and the GRE₂Luc reporter plasmid. Cells were then treated with vehicle or 100 nM Dex for 12, 24, or 48 hours. Cells were collected from untreated controls at the start (T₀) of the experiment. Cell extracts were prepared for luciferase activity determination as RLU per ug

of protein and fold induction vs. control was calculated. (* $p < 0.05$ Wt GR vs. K419A GR RLU/ug protein.)

Fig. 7.

Cellular localization of GFP-hGR and mutant GFP-hGRK419A after corticosterone administration and washout. COS-1 cells were transiently transfected with either GFP-human glucocorticoid receptor or mutant GFP-hGRK419A. Transfected cells were pre-treated with cycloheximide and then treated with vehicle or 1 μ M corticosterone for 12 hours and nuclear localization of both wild type GR and mutant K419A GR after hormone treatment was observed. Cells were washed 5x to remove hormone and maintained in cycloheximide. After 12 hours both wild type GR and K419A mutant exited the nucleus.