Proteasome-mediated Glucocorticoid Receptor Degradation Restricts Transcriptional Signaling by Glucocorticoids

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Ligand-dependent down-regulation of the glucocorticoid receptor (GR) has been shown to limit hormone responsiveness, but the mechanisms involved in this process are poorly understood. The glucocorticoid receptor is a phosphoprotein that upon ligand binding becomes hyperphosphorylated, and recent evidence indicates that phosphorylation status of the glucocorticoid receptor plays a prominent role in receptor protein turnover. Because phosphorylation is a key signal for ubiquitination and proteasomal catabolism of many proteins, we evaluated whether the ubiquitin-proteasomal pathway had a role in glucocorticoid receptor down-regulation and the subsequent transcriptional response to glucocorticoids. Pretreatment of COS-1 cells expressing mouse glucocorticoid receptor with the proteasome inhibitor MG-132 effectively blocks glucocorticoid receptor protein down-regulation by the glucocorticoid dexamethasone. Interestingly, both MG-132 and a second proteasome inhibitor β-lactone significantly enhanced hormone response of transfected mouse glucocorticoid receptor toward transcriptional activation of glucocorticoid receptor-mediated reporter gene expression. The transcriptional activity of the endogenous human glucocorticoid receptor in HeLa cells was also enhanced by MG-132. Direct evidence for ubiquitination of the glucocorticoid receptor was obtained by immunoprecipitation of cellular extracts from proteasome-impaired cells. Examination of the primary sequence of mouse, human, and rat glucocorticoid receptor has identified a candidate PEST degradation motif. Mutation of Lys-426 within this PEST element both abrogated ligand-dependent down-regulation of glucocorticoid receptor protein and simultaneously enhanced glucocorticoid receptor-induced transcriptional activation of gene expression. Unlike wild type GR, proteasomal inhibition failed to enhance significantly transcriptional activity of K426A mutant GR. Together these findings suggest a major role of the ubiquitin-proteasome pathway in regulating glucocorticoid receptor protein turnover, thereby providing a mechanism to terminate glucocorticoid responses.

Glucocorticoids are steroid hormones essential for life that are produced by the adrenal glands and affect all tissues of the body. Synthetic derivatives of glucocorticoids are widely used in the treatment of a number of inflammatory diseases. These hormones are necessary for normal growth and development, liver and immune functions, and in mediating stress responses (1–3). Glucocorticoids mediate their actions by binding to the glucocorticoid receptor (GR), a member of the superfamily of steroid/thyroid/retinoic acid proteins that function as ligand-dependent transcription factors. Like other members of this superfamily, GR contains a number of structure motifs including a DNA binding domain, ligand binding domain, and two transactivation motifs AF1 and AF2 (4). Upon ligand binding, the cytoplasmic GR becomes activated and translocates to the nucleus where it can bind to specific DNA elements termed glucocorticoid response elements (GREs), causing stimulation of transcription. Alternatively, activated GR can interact with other transcription factors such as NF-κB or AP-1 to repress gene expression activated by these pro-inflammatory transcription factors (5–8).

The sensitivity of cells to glucocorticoids has been shown to be dependent on the number of GRs found in the cell (9–11). Upon ligand binding the GR undergoes a process of homologous down-regulation that has been extensively studied (9–15). GR levels are regulated at multiple levels including mRNA and protein (16, 17). At the RNA level, several studies have shown that glucocorticoid treatment decreases GR mRNA levels by 50–80% in many different tissues (18–20). This occurs via transcriptional mechanisms requiring an intragenic element within the GR gene itself (20). Additionally, early studies showed that hormone treatment leads to a subsequent decrease in the ability of the receptor to bind hormone, but the mechanism for this phenomena was poorly understood. Dense amino acid labeling of 3H-labeled steroid-bound receptor suggested that hormone treatment influenced receptor protein turnover (12). Generation of GR-specific antibodies permitted analysis of GR protein and revealed that ligand binding of GR led to a reduction in receptor protein levels (10, 19). Studies examining the half-life of the GR protein, separate from any ligand effects on RNA, have shown that ligand occupation of the receptor significantly decreases GR protein half-life from 18 to 9 h. Recently, the phosphorylation state of the receptor has been shown to be an important determinant of receptor stability, and a phospho-deficient mutant of mouse GR does not undergo ligand-dependent down-regulation (21).

Because phosphorylation has been shown to act as a signal for protein recognition by the ubiquitin and proteasome pathways for protein degradation, we considered the possibility that

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† The abbreviations used are: GR, glucocorticoid receptor; GRE, glucocorticoid response element(s); CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis; Dex, dexamethasone (1,4-pregnadien-9α-fluoro-16α-methyl-11β,17,21-triol-3,20-dione); E2, estrogen-17β; PBS, phosphate-buffered saline; ER, estrogen receptor; AhR, aryl hydrocarbon receptor.
the proteasome mediates GR down-regulation (22–25). Proteins such as IκBα, Fos, and the tumor suppressor p53 have been shown to be regulated by this pathway with phosphorylation playing a critical role (26–28). Phosphorylation is thought to be the signal to allow substrate recognition by enzymes in the ubiquitination pathway. The ubiquitin pathway consists of ubiquitin-activating enzymes (UBAs), E2 ubiquitin-conjugating enzymes (UBCs), and E3 ubiquitin-ligase enzymes. Phosphorylation allows recognition by E2 and/or E3 molecules that covalently add the 76-amino acid protein ubiquitin to lysine residue(s) of the targeted protein (26, 29, 30). Poly-ubiquitination of a protein allows it to be recognized by the multisubunit protein complex known as the proteasome, which degrades the protein into small peptides and amino acids. Recent development of specific inhibitors of the proteasome has permitted identification of its central role in the turnover of both short lived and long lived proteins (31–34).

The glucocorticoid receptor has been shown recently to interact with an E2-conjugating protein and two E3-ligase proteins found within the ubiquitin enzymatic pathway, but the functional consequences of GR interaction with these proteins remain unclear. We report here that inhibition of the proteasome by MG-132 abolishes dexamethasone-induced mouse glucocorticoid receptor protein down-regulation. By using the proteasome inhibitors MG-132 and β-lactone, we demonstrate enhanced hormone responsiveness by use of a glucocorticoid response element-containing reporter construct. By immunoprecipitation of GR protein, we have visualized higher molecular weight forms of the glucocorticoid receptor that we have identified as ubiquitinated forms of GR. Mutagenesis of lysine 426, found in a candidate PEST degradation motif within the glucocorticoid receptor, abrogated ligand-dependent down-regulation, enhanced hormone responsiveness, and was found to be unresponsive to proteasome inhibition. Together these findings suggest a mechanism for termination of glucocorticoid-dependent glucocorticoid receptor signaling via protein degradation through the proteasome pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dexamethasone (1,4-pregnadien-9α-fluoro-16α-methyl-11β,17,21-triol-3,20-dione) (Dex) was purchased from Steleraloids (Wilton, NH). MG-132 (benzoylarginyl-Leu-Leu-aldehyde) and clasto-lactacytin β-lactone were purchased from Calbiochem. N-Ethylmaleimide was purchased from Pierce. [14C]Chloramphenicol (40–60 mCi/mmol) was obtained from PerkinElmer Life Sciences. Acetyl coenzyme A, ATP, and 0.1 μM of [14C]Chloramphenicol. Reactions were terminated by ethanol extraction, and CAT activities were determined by thin layer chromatography (TLC) separation of acetylated products from substrate chloramphenicol. Plates were visualized by PhosphorImager (Molecular Dynamics, Inc. Sunnyvale, CA) and analyzed using ImageQuant software.

**Cell Culture and Transfection**—COS-1 (African Green monkey kidney) cells were grown in Dulbecco’s modified Eagle medium and harvested with dextran-charcoal stripped serum were added. Plasma DNA was prepared using Qiangen Maxiprep kits (Valencia, CA).

**Luciferase Assays**—Cells were transfected as described above. Following transfections, media were changed to media containing dextran-charcoal-stripped serum, and cells were allowed to recover for 12–16 h and then incubated with vehicle or dexamethasone for the times indicated. Cells were harvested and lysates prepared, and luciferase assay was performed using an Enhanced Luciferase Assay Kit from BD PharMingen (Franklin Lakes, NJ). Luciferase activity was measured using a Microtiter Plate Luminometer and Revelation 4.06 Software (Dynex Technologies). Luciferase activity was calculated per μg of protein for each sample.

**Chloramphenicol Acetyltransferase (CAT) Assays**—Cells were transfected as described above. HeLa cells were washed with ice-cold 1× PBS and incubated with Versene at 37 °C for 15 min. Cells were spun down for 5 min and then taken up in 0.25 × EDTA (pH 8.0). Lysis were prepared by tip sonication, and endogenous CAT activity was inactivated by heating to 65 °C for 15 min. Protein concentration was determined by the method of Bradford, using a reagent kit from Bio-Rad. Equal amounts of protein were incubated in reaction mixture A and 0.1 μCi of [14C]Chloramphenicol. Reactions were terminated by ethanol extraction, and CAT activities were determined by thin layer chromatography (TLC) separation of acetylated products from substrate chloramphenicol. Plates were visualized by PhosphorImager and analyzed using ImageQuant software. Activity was expressed as percent chloramphenicol acetylation. In some experiments CAT activity was determined by terminating reactions by the mixed xylene method as described by Pro-mega (Madison, WI) and utilized a standard curve of CAT activity from supplied protein.

**Western Blot Analysis**—In protein half-life studies using the mouse GR vector pSV2Rec and mutant GR GRK426A cellular extracts were prepared from transiently transfected COS-1 cells pretreated with 1 μM chloroethylmaleimide for 1 h and then treated with vehicle or 100 nM dexamethasone. Cells were washed with cold 1× PBS and harvested by scraping with a rubber spatula into PBS, and cells were centrifuged. The cell pellet was taken up in RIPA buffer (20 mM Tris (pH 7.5), 2 mM EDTA, 150 mM NaCl, 0.05% Triton X-100 (pH 7.5)) containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, and 1 μM leupeptin). Membranes were placed in a blocking solution of 10% nonfat milk (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05%Tween 20) for 1 h at room temperature or overnight in a cold room. Membranes were then washed twice for 15 min with 1% nonfat milk buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) and then incubated for 1 h with anti-GR or anti-apoptosis antibodies and Pabflav and Mini Complete protease inhibitor tablet from Roche Molecular Biochemicals. Protein levels were quantitated using the Lowry method (Bio-Rad). Approximately 100 μg of protein per sample was resolved by 8% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and stained with Ponceau S (0.5% in 1% acetic acid) to evaluate loading equivalency and transfer efficiency. BenchMark Prestained Protein Ladders from Life Technolo-gies were used as molecular size standards. Membranes were probed with antibodies to mouse GR or N-ethylmaleimide. Cells were harvested and lysates prepared using an Enhanced Luciferase Assay Kit from BD PharMingen (Franklin Lakes, NJ). Luciferase activity was measured using a Microtiter Plate Luminometer and Revelation 4.06 Software. Luciferase activity was calculated per μg of protein for each sample.

**Immunoprecipitations**—Cellular lysates from COS-1 cells were prepared in immunoprecipitation buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, and 0.5% Nonidet P-40) containing protease inhibitors 1 μg/ml aprotinin and Pabflav, Mini Complete protease inhibitor tablet from Roche Molecular Biochemicals, and 5 mM N-ethylmaleimide (26). Addition of N-ethylmaleimide to isolated cellular extracts inhibits isopeptidase activities that may otherwise affect the detection of ubiquitinated proteins (26). To immunoprecipitate GR, a 1:1 mixture of antibodies 57 and 59, epitope-specific rabbit polyclonal antibodies, were added to 200 μg of cellular extract, and samples were rotated at 4 °C overnight (41, 42). Protein A-Sepharose was added and samples were rotated for 2 h. Sepharose was pelleted, and samples were washed 5× in a large volume of IP buffer.

**Recombinant Plasmids**—Wild-type and mutant mouse GR were expressed in the pSV2Rec vector under control of the SV40 promoter (35), pCMV5-mGR was also used in some experiments, and detailed descriptions of this expression vector are available (36). Mutation of mouse glucocorticoid receptor cDNA from Lys-426 to K426A was done by polymerase chain reaction site-directed mutagenesis using the QuickChange Site-directed mutagenesis kit from Stratagene (Cedar Creek, TX). Primers used were 5′-ACG GGA CCA CCT CCC GCA CTC TGC CTT GTG TGC-3′ and 5′-GCA CAC GAG CAC GAG TGG TGC TTC CGT-3′. GRE-ΔTATA-CAT and GRE-ΔTATA-Luc reporter plasmids have been previously described (37, 38). The c-Myc-tagged ubiquitin expression vector (wt-H6M-Ub) was a kind gift of Ron Kopito (39).

**Cell Culture and Transfection**—COS-1 (African Green monkey kidney) cells were grown in Dulbecco’s modification Eagle’s medium with high glucose and nonessential amino acids, with 10% fetal bovine serum. HeLa cells were grown in monolayer cultures in Joklik modified essential medium and harvested with Versene as described previously (40). All cultures were maintained in 5% CO2 humified atmosphere at 37 °C and passed every 3–4 days. For transfection of cells, cells were plated 24 h prior to transfection and allowed to grow to 50–70% confluency. Cells were washed with Opti-MEM (Life Technologies, Inc.) and transfected using DMLIEN (Life Technologies, Inc.) or TransIT transfection reagent from Panvera Corp. (Madison, WI) according to the manufacturers’ recommendations. The precipitates were added to cells in Opti-MEM and media containing dextran-charcoal-stripped serum were added. Plasmid DNA was prepared using Qiangen Maxiprep kits (Valencia, CA).
FIG. 1. Glucocorticoid receptor-dependent down-regulation is blocked by the proteasome inhibitor MG-132 in COS-1 cells. COS-1 cells were transiently transfected with GR expression vector pSV2Rec. Cells were treated with vehicle or 1 μM MG-132 for 1 h and then treated with dexamethasone (Dex) for 12 h. Whole cell lysates were prepared, and 100 μg was separated by SDS-PAGE electrophoresis. Blots were analyzed by Western blot using antipeptide hGR antibody 57. Western blot shown is representative of typical results. Densitometry was determined using NIH IMAGE 1.6 software, and the results of three independent experiments are shown (*, p < 0.05 versus basal).

Samples were taken up in Laemmli sample buffer and boiled for 5 min to dissociate immune complexes from Sepharose. Equal volumes of samples were run on SDS-PAGE and BenchMark Prestained Protein Ladders from Life Technologies, Inc., were used to determine molecular size of visualized bands. Immunoprecipitated proteins were visualized using GR-specific peptide antibody 18S7, as described above. Blots were also probed with anti-c-Myc antibody from Babco (Richmond, CA) to detect Myc-tagged ubiquitin GR conjugates. Immunoprecipitated GR was run on SDS-PAGE gels, and blots were probed with an anti-ubiquitin antibody that was the generous gift of Dr. Arthur Haas (43).

Statistical Analyses—Statistical significance was determined using a *t* test with a confidence level of 0.05. Western blot data was determined for significance by using pixel data from NIH Image Software, and CAT assay data were visualized using PhosphorImager technology and ImageQuant software from Molecular Dynamics, Inc. (Sunnyvale, CA).

RESULTS

Dexamethasone-induced Mouse GR Protein Down-regulation Is Blocked by Proteasome Inhibition in Transiently Transfected COS-1 Cells—Glucocorticoid receptor ligand-dependent down-regulation is thought to be a complex process involving regulation of both GR receptor gene expression and receptor protein. Regulation at the RNA and the protein levels occurs simultaneously and has been demonstrated in numerous studies using transcriptional or translational inhibitors. With the development of GR-specific antibodies, it has been shown in a number of tissues and cell types that in the presence of ligand GR protein becomes destabilized and receptor levels decrease (10, 13, 14, 19). Interestingly, disruption of GR interaction with heat shock protein 90 (Hsp90) also leads to GR protein down-regulation, and inhibition of the proteasome function has recently been shown to block this down-regulation (44).

Thus we wished to determine the role of the proteasome in ligand-dependent down-regulation of the glucocorticoid receptor. To address this issue the COS-1 cell line (which is devoid of endogenous GR) was transfected with pSV2Rec, a mouse GR expression vector. Following transfection cells were pretreated with the proteasomal inhibitor MG-132 (benzoyloxy carbonyl-Leu-Leu-Leu-aldehyde) or vehicle for 1 h and then were treated with vehicle or dexamethasone. Cellular extracts were isolated, and Western blot analysis of GR was performed with peptide-specific antibodies. Glucocorticoid treatment caused a profound decrease in the levels of GR protein (Fig. 1, 1st versus 2nd lanes) that was inhibited by the inclusion of MG-132 (Fig. 1, 2nd versus 4th lanes). Densitometric analysis of three separate experiments revealed that MG-132 abrogated the repressive effect of dexamethasone on GR protein levels. Dexamethasone treatment for 12 h caused GR protein levels to fall on average to 42% of control, whereas cells pretreated with MG-132 maintained normal receptor levels. The ability of the proteasome inhibitors to block ligand-dependent glucocorticoid receptor protein down-regulation suggests that the GR is degraded by the ubiquitin-proteasome pathway.

Proteasome Inhibition Enhances GR Transcriptional Activity in Transiently Transfected COS-1 Cells—To ascertain if the observed receptors preserved by proteasome inhibition were functional, assays were performed using glucocorticoid-responsive reporter gene vectors. COS-1 cells were transiently transfected with pSV2Rec GR expression vector and hormonally responsive GRE2-TATA-CAT. Transfected cells were pretreated with MG-132 and then treated with various doses of dexamethasone. Dexamethasone treatment induced reporter activity, whereas MG-132 alone had little effect on activity (Fig. 2A). In contrast cells treated with MG-132 and dexamethasone showed significantly increased reporter activity compared with cells treated with Dex alone. Percent CAT conversion levels increased 3- to 4-fold in cells treated with MG-132 and Dex. Similar observations were made when cells were treated with clasto-lactacyclin β-lactone (β-lactone), a second more specific inhibitor of the proteasome (Fig. 2B). These data suggest the receptors maintained by proteasome inhibition during glucocorticoid treatment are functional and further suggest that hormone responsiveness may be limited by glucocorticoid receptor protein down-regulation via the proteasome pathway.

Proteasome Inhibition Enhances Endogenous hGR Transactivation in HeLa Cells—Because studies in transfected cells...
overexpressing glucocorticoid receptor suggested that proteasome inhibition blocked glucocorticoid-induced down-regulation and maintained functional responsiveness, we wished to determine whether the proteasome plays a similar role in cells that contain endogenous human GR. The HeLa cell line was utilized to address this question because these cells express hGR endogenously, and the down-regulation of glucocorticoid receptor protein by treatment with dexamethasone has been well documented in this cell line (9, 14). Down-regulation of GR protein levels has been proposed to be the limiting factor in HeLa cells responsiveness to glucocorticoids (11, 15). To examine if inhibiting the proteasome protein degradation pathway affected GR responsiveness in HeLa cells, we again used the proteasome inhibitor MG-132 and analyzed the effects of proteasome inhibition on hormone responsiveness in HeLa cells transfected with a hormone-responsive dimer GRE driving the CAT reporter gene. Following transfection, cells were pretreated with MG-132 and then treated with dexamethasone. Cells treated with MG-132 demonstrated only a slightly higher percent CAT conversion than control, whereas dexamethasone markedly induced activity over the control (Fig. 3). In contrast, HeLa cells treated with a combination of MG-132 and dexamethasone showed significantly induced reporter activity as compared with 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 process of GR degradation involving the proteasome pathway.

Immunoprecipitation of Mouse Glucocorticoid Receptor Identified Higher Molecular Weight Ubiquitinated Species of GR—The process of protein degradation by the proteasome involves enzymes of the ubiquitination pathway, which add ubiquitin molecule(s) to a protein and which effectively target that protein for degradation by the proteasome. Because our studies indicated that proteasome inhibition blunted dexamethasone-induced GR protein down-regulation and enhanced transcriptional response, we wished to determine whether GR is subject to ubiquitination. To determine whether GR is ubiquitinated, COS-1 cells were co-transfected with pCMV-GR and the Myc-tagged wild type ubiquitin expression vector. Transfected cells were treated with vehicle or dexamethasone in the absence and presence of MG-132; cellular extracts were prepared, and samples were immunoprecipitated using a mixture of epitope-specific GR antibodies. Immunoprecipitated samples were analyzed by Western blot using antibodies to GR and to the c-Myc-tagged ubiquitin. By using GR-specific antibody 57, the ~97-kDa band of GR was seen. It should be noted that under these experimental conditions dexamethasone not only alters GR turnover but also decreases transcription of the GR gene and promotes GR mRNA degradation (13, 20). Additionally, longer film exposures revealed higher molecular mass forms of GR, at ~180 kDa and greater, that were consistently observed in a striated pattern suggesting the addition of multiple ubiquitin molecules (Fig. 4). The higher molecular weight proteins were more prominent in extracts from MG-132-treated cells. When the same blots were stripped and then probed with anti-c-Myc antibody to detect the tagged c-Myc-ubiquitin, corresponding proteins were seen at 180 kDa in a striated pattern that was particularly apparent in cells treated with MG-132. Because immunoprecipitations with antibodies specific to GR were used as the initial selection, these higher molecular weight forms of GR probably represent forms of GR that have a number of lysines modified with mono-ubiquitin or specific lysines that are poly-ubiquitinated as seen for other proteins (39, 45, 46). These findings suggest that GR is targeted for degradation by ubiquitination and then are recognized and degraded by the proteasome complex. We conclude that the GR is ubiquitinated. To determine how GR is targeted for degradation, we undertook analysis of GR for any degradation motifs.

Analysis of GR for Potential Degradation Motifs Identified a PEST Element—Knowledge of the mechanism involved in protein recognition by the enzymes of ubiquitin pathway remains unclear, but a number of motifs have been shown to be important in substrate recognition by E2/E3 proteins (25). Furthermore, current research suggests that phosphorylation is a critical signal for the rapid degradation of a number of proteins. A number of rapidly degraded proteins contain PEST regions that consist of the amino acids Pro (P), Glu (E), Ser (S), and Thr (T). PEST regions contain phosphorylation sites, are regions of hydrophilic amino acids, and are flanked by Lys, Arg, and His (47, 48). Analysis of the mouse glucocorticoid receptor using a PEST-FIND program allowed identification of a PEST motif from amino acids 407–426 (Fig. 5). This region has a PEST-FIND score of +18.3 and on a scale from ~50 to +50 a value above +5 is thought to be indicative of possible functional PEST motif (48). For example two proteins, IKBα and Fos, are known to be degraded by the proteasome have PEST scores of 5.9 and 10.1, respectively (27, 49, 50). The potential PEST motif in GR could help explain the rapid destabilization of GR after ligand binding. Interestingly, both the rat and human glucocorticoid receptor also contain this destabilizing motif (Fig. 5).
also been shown to alter the protein turnover rates of GR turnover. Mutagenesis of one or more lysine residues has suggested that the lysine within the identified PEST region of K426A GR to undergo ligand-dependent down-regulation. In a separate transfection mutant K426A GR, but the K426A mutant displayed enhanced responsiveness.

Mutagenesis of Lysine Residue Abrogates GR Protein Ligand-dependent Down-regulation—The identified PEST region of GR at amino acids 407–426 was next targeted for site-directed mutagenesis. The candidate site for ubiquitination at Lys-426 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 h, whereas in the absence of ligand levels fall to 50% in 18 h (21). To determine the effect of this lysine mutation on GR ligand-dependent down-regulation similar half-life studies were performed. COS-1 cells were transiently transfected with wild type GR or mutant K426A 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 analyzing the effects of glucocorticoid on the repression of GR gene transcription. Cells were then treated with dexamethasone, and cellular extracts were prepared for Western blot analysis (Fig. 6A). Wild type GR levels were lower due to ligand-dependent down-regulation. In a separate transfection mutant K426A GR did not undergo ligand-dependent down-regulation, and receptor levels remained relatively unchanged by treatment with dexamethasone. Densitometry of three independent experiments determined that wild type GR levels fell to an average of \(-50\%\) of control levels (Fig. 6B), whereas the K426A GR levels remained constant despite the presence of hormone. The failure of K426A GR to undergo ligand-dependent down-regulation suggests that the lysine within the identified PEST region serves as an acceptor ubiquitin site that plays a prominent role in GR turnover. Mutagenesis of one or more lysine residues has also been shown to alter the protein turnover rates of IκBα and yeast α-factor receptor (29, 51). We next considered if mutagenesis of Lys-426 had any effects on GR function.

Transcriptional Activation by Lysine 426 Mutant Is Enhanced—The maintenance of normal levels of K426A GR protein observed in the half-life studies prompted us to examine whether this mutant receptor incapable of down-regulation at the protein level had altered transcriptional activation in response to hormone. In this study a glucocorticoid-responsive reporter was transiently transfected with either wild type or mutant K426A GR and treated with dexamethasone for 12, 24, 48, and 72 h. Cells expressing K426A displayed increased reporter activity over wild type mouse GR at all times tested (Fig. 7A). Levels of luciferase activity for mutant K426A were consistently 2–3.6-fold higher than that of wild type, with fold activity increasing as the duration of the experiment increased from 12 to 48 h. Western blot analysis indicated that after 12 or 24 h of dexamethasone treatment, K426A mutant GR protein levels were similar to those of the untreated controls (Fig. 7B), probably reflecting the fact that K426A mutant is not undergoing down-regulation at these early time points. In contrast, wild type GR protein levels treated with dexamethasone were undergoing ligand-dependent down-regulation after 12 and 24 h when compared with untreated controls. With extended hormone treatment we do see a reduction in both wild type and mutant GR reflecting the transcriptional component of ligand down-regulation described previously (15–16). Also as expected in these transient transfection experiments, we observed in the controls a time-dependent decrease in protein expression from transfected plasmids. Thus, mutagenesis of Lys-426 enhanced both glucocorticoid receptor levels, and hormone responsiveness suggests lysine 426 plays a role in limiting GR hormone responsiveness.

Proteasome Inhibition Failed to Enhance the Transcriptional Activity of the K426A Mutant GR—To ascertain if proteasome inhibition enhanced K426A mutant GR transcriptional activity as seen in previous experiments with wild type GR, CAT assays were performed using a glucocorticoid-responsive reporter. COS-1 cells were transiently transfected with wild type GR or K426A mutant GR expression vectors and hormone-responsive GRE\(_2\)-TATA-CAT. Transfected cells were pretreated with MG-132 and then treated with dexamethasone. Dexamethasone treatment induced reporter activity of both wild type and mutant K426A GR, but the K426A mutant displayed enhanced
activity over wild type GR (Fig. 8). When cells were pretreated with MG-132 and then treated with dexamethasone, wild type GR displayed enhanced activity as seen previously. In contrast, cells expressing K426A GR and treated with MG-132 and dexamethasone failed to significantly enhance transcription, although some small increases were seen in each of the experiments performed. These data demonstrate that Lys-426 is a critical amino acid involved in maintaining GR hormone responsiveness. Mutagenesis of this site leads to enhanced hormone responsiveness and also blocks the additional induction of transcriptional activity seen in the presence of the proteasomal inhibitor MG-132.

**DISCUSSION**

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 (9, 12, 19, 52). The rapid decrease in receptor levels is due to a dramatic decrease in GR protein half-life in the presence of ligand that is thought to diminish cellular responsiveness to ligand (10–15). Glucocorticoid receptor protein levels have also been shown to decline when cells are treated by other agents that disrupt interactions with heat shock proteins by either heat shock-mediated degradation of the glucocorticoid receptor, it was shown to be an ATP-dependent process. Unlike other proteases, the need for ATP is a hallmark of proteasome activity suggesting that the ubiquitin-dependent pathway of proteolysis may be involved (53). Whitesell and Cook (44) were able to block geldanamycin-induced degradation by use of the proteasome inhibitor lactacystin. These researchers suggested that GR is targeted for destruction by the ubiquitin pathway. In this study we demonstrate that inhibition of the proteasome by the peptide aldehyde MG-132 blocked GR ligand-dependent down-regulation in COS-1 cells transfected with GR cDNA. Similar results have been demonstrated in experiments with transfected estrogen receptor (ER), where proteasome inhibition by MG-132 was observed to block estrogen-induced degradation of ER although ER does not have an obvious PEST element. Ligand-dependent down-regulation was also shown to be blocked by MG-132 in studies of the progesterone receptor and the aryl hydrocarbon receptor (AhR) (54, 55).

Our study of COS-1 cells transiently transfected with GR and HeLa cells, containing endogenous hGR, demonstrated that proteasome inhibition by MG-132 or β-lactone leads to enhanced GR transactivation in reporter assays. These experiments reveal that the elevated levels of GR protein seen by Western blot, in the presence of MG-132 and dexamethasone, induced greater reporter activity than cells treated with either agent alone. The elevation of hormone-induced reporter expression suggests that hormone responsiveness can be significantly affected by inhibition of GR degradation. Proteasome inhibition elevates reporter activity of other transcription factors such as the AhR, Sp1, and p53 (56–58). Like GR, the AhR is a cytoplasmic protein that upon ligand binding enters the nucleus. The enhanced transcriptional activity of AhR in the presence of MG-132 was found to be consistent with the elevated levels of AhR in the nucleus (58). Similar to AhR, the enhancement of GR transcriptional activity observed in the presence of dexamethasone and MG-132 may be due to a greater fraction of total cellular GR being in the nucleus, as well as the decrease in GR degradation. In contrast, in transcriptional activity assays of the estrogen and thyroid receptors, it was found that proteasomal inhibition decreased ligand-induced transcriptional activity (56, 59). The reasons for this discrepancy are not clear, but it has been suggested that the ERα co-activator complex formation may be disrupted by proteasome inhibition (56). In the case of the thyroid receptor, it was suggested that proteasomal activity may be necessary to modify thyroid receptor by proteolysis to produce a transcriptionally active form of the receptor (59). Whereas AhR, Sp1, and p53 do contain PEST elements with scores greater than 10.0, the significance of the lower scoring PEST element of the thyroid receptor and the lack of an element in the estrogen α is unclear. This suggests that additional mechanisms perhaps involving the degradation of receptor-associated co-activators or co-repressors may prevail.
A number of steroid receptors have now been identified as being ubiquitinated and targeted for destruction by the proteasome in a process involving ligand binding by the receptor (56, 60, 61). We present evidence that the GR is ubiquitinated, although previously published attempts (44) to detect ubiquitinated forms of GR had been unsuccessful. When COS-1 cells were transfected with GR cDNA and treated with MG-132, higher molecular weight forms of immunoprecipitated GR protein were seen by Western blot analysis. These data suggest a mechanism of ligand-dependent down-regulation for GR involving targeting for degradation by the proteasome. To identify the possible phosphorylation and ubiquitination sites involved in this mechanism, we analyzed the GR protein for candidate degradation motifs. By using the PEST-FIND computer program, we identified a conserved PEST element found in the mouse, rat, and human GR. PEST elements have been shown to be important in the turnover of a number of proteins including IκBα, β-cyclins, and c-Fos (27, 62–64). These elements contain phosphorylation sites and are usually flanked by a lysine residue, a candidate site for ubiquitination. It remains unclear whether these regions allow recognition by kinases or members of the ubiquitination pathway (48). The identified region in the mouse glucocorticoid receptor contains the identified phosphorylation site Ser-412 or peptide 23 (65–67) and is flanked by a lysine residue at position 426, a possible site of ubiquitination. To determine the significance of this region, Lys-426 was mutated to alanine by site-directed mutagenesis. In half-life studies using the protein synthesis inhibitor cycloheximide, we observed by Western blot that wild type GR underwent dexamethasone-induced down-regulation, but the mutant K426A GR did not. These data suggest that residue Lys-426 is critical in the ligand-dependent regulation of GR. In the case of IκBα, mutagenesis of two critical lysine residues caused the protein to be resistant to signal-regulated degradation (29). The K426A glucocorticoid receptor mutant also displayed enhanced ability to transactivate the hormone-responsive GRE-luciferase reporter when compared with wild type glucocorticoid receptor. Western blot revealed that dexamethasone treatment of the mutant GR K426A-expressing cells did not lead to protein down-regulation when compared with untreated controls at 12 and 24 h. In contrast, wild type GR-expressing cells treated with dexamethasone were observed undergoing ligand-dependent down-regulation at 12 and 24 h when compared with vehicle-treated controls. With extended hormone treatment we did observe down-regulation of wild type and mutant GR probably reflecting the transcriptional component of ligand down-regulation previously studied (13–16). As expected in these transient transfection experiments, we also observed a time-dependent decrease in protein expression from both transfected plasmids and the growth of non-transfected cells during the extended experimental protocol.

The transcriptional activity of the K426A mutant GR also displayed enhanced responsiveness compared with wild type GR in studies using the hormone-responsive GRE−CAT reporter. In contrast to wild type GR, the K426A mutant did not display enhanced transcriptional activity in the presence of the proteasomal inhibitor MG-132. Preliminary studies of the K426A mutant GR have surprisingly indicated that this mutant is still ubiquitinated, but quantitation of the relative amounts occurring in cells has been difficult. These findings suggest that alternative ubiquitination sites besides K426A may be present on GR or that mutagenesis of Lys-426 could affect overall GR ubiquitination levels. Unidentified alternative ubiquitination sites also may have a role in ligand-independent turnover of GR. Nevertheless, our data clearly show that Lys-426 is an important amino acid involved in GR hormone responsiveness, and its mutagenesis leads to a more potent GR. Lys-426 is also a critical amino acid for the proteasomal inhibitor enhancement of GR transcriptional activation, and mutagenesis of this site successfully blocked the transcriptional enhancement of GR by proteasomal inhibition.

The identified PEST region of the glucocorticoid receptor also contains possible phosphorylation sites including the identified site Ser-412, first identified as peptide 23. Interestingly, Ser-412 was identified as a site of ligand-dependent phosphorylation (67). Phosphorylation status has also been shown to affect the half-life of GR in studies using a phospho-deficient GR, and a mutant containing all eight phosphorylation sites mutated to alanine did not undergo ligand-dependent down-regulation (21). Other members of the steroid receptor superfamily have been shown to be phosphoproteins (68). Recently, phosphorylation of a specific serine residue has been implicated in the degradation of the progesterone receptor, and proteasome inhibition blocked degradation by ligand-dependent and -independent mechanisms (60). The kinases involved in phosphorylation of ligand-bound GR, and the E2 and E3 ligases that recognize and ubiquitinate GR, are unknown. The proteins UBC9, E6AP, and RSP5 have been shown to interact with GR and have been shown to act as co-activators of GR, but their ability to ubiquitinate GR has not been demonstrated (65). The role of the Ser-412 phosphorylation site in ligand-dependent down-regulation, however, requires further investigation.

The clinical significance of glucocorticoid receptor protein down-regulation is evident when considering that without down-regulation there exists the potential for overstimulation when circulating levels of hormone are high. Conversely, the effects of long term glucocorticoid treatment could lead to GR down-regulation leaving cells unresponsive to hormone treatment (11). We provide evidence for the first time that the proteasome is involved in ligand-dependent GR down-regulation and that proteasomal inhibition enhances GR transcriptional activity. In the presence of proteasomal inhibitors, we visualized higher molecular weight forms of GR representing ubiquitinated GR. We identified a PEST degradation element (residues 407–426) in GR, and mutagenesis of lysine 426 effectively blocked ligand-dependent down-regulation. This mutant also demonstrated elevated transcriptional activity. Together these results suggest a mechanism for termination of steroid-dependent GR signaling by protein degradation through the proteasome pathway.

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REFERENCES

Proteasome-mediated GR Degradation Restricts Signaling