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# A hotspot in the glucocorticoid receptor DNA-binding domain susceptible to loss of function mutation

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ABSTRACT

Glucocorticoids (GCs) are used to treat a variety of inflammatory disorders and certain cancers. However, GC resistance occurs in subsets of patients. We found that EL4 cells, a GC-resistant mouse thymoma cell line, harbored a point mutation in their GC receptor (GR) gene, resulting in the substitution of arginine 493 by a cysteine in the second zinc finger of the DNA-binding domain. Allelic discrimination analyses revealed that the R493C mutation occurred on both alleles. In the absence of GCs, the GR in EL4 cells localized predominantly in the cytoplasm and upon dexamethasone treatment underwent nuclear translocation, suggesting that the ligand binding ability of the GR in EL4 cells was intact. In transient transfection assays, the R493C mutant could not transactivate the MMTV-luciferase reporter. Site-directed mutagenesis to revert the R493C mutation restored the transactivation activity. Cotransfection experiments showed that the R493C mutant did not inhibit the transcriptional activities of the wild-type GR. In addition, the R493C mutant did not repress either the AP-1 or NF-κB reporters as effectively as WT GR. Furthermore, stable expression of the WT GR in the EL4 cells enabled GC-mediated gene regulation, specifically upregulation of IκBα and downregulation of interferon γ and interleukin 17A. Arginine 493 is conserved among multiple species and all human nuclear receptors and its mutation has also been found in the human GR, androgen receptor, and mineralocorticoid receptor. Thus, R493 is necessary for the transcriptional activity of the GR and a hotspot for mutations that result in GC resistance.



## 1. Introduction

Glucocorticoids (GCs) are widely prescribed for inflammatory disorders and lymphoid malignancies. However, severe side effects and resistance to treatment limit their use [1]. GCs signal through the glucocorticoid receptor (GR), a ligand-dependent nuclear receptor. The GR, like other steroid receptors, the mineralocorticoid receptor (MR), progestin receptor (PR), androgen receptor (AR), and estrogen receptor α and β (ERα and β), contains three main functional domains: an N-terminal activation domain, a DNA-binding domain, and a C-terminal ligand-binding domain [2]. Upon ligand binding, activation of the GR leads to receptor dimerization and translocation from cytoplasm to nucleus where regulation of target gene expression occurs. On the promoter region of a large number of target genes, direct DNA-binding by the GR leads to gene activation (transactivation) whereas protein–protein interactions between GR and other transcription factors result in gene suppression (transrepression) [1]. GR mutations can lead to generalized primary GC resistance, characterized by elevated adrenocorticotropic hormone, hypertension, hypokalaemic alkalosis, and other symptoms [3]. GR mutations in lymphoid malignancies decrease the effectiveness of GC therapy [3]. For example, a point mutation in the second zinc finger of the GR at position 477 (arginine to histidine) impaired the transactivation and transrepression abilities of the GR, resulting in GC resistance [4,5].

Multiple cell lines have been used to study how cancer cells respond to GC treatment and how GR mutations affect GC signaling. The EL4 murine thymoma cells have been useful in understanding GR signaling in cytokine production. GC studies in EL4

*Abbreviations:* AP-1, activator protein 1; AR, androgen receptor; DAPI, 4′, 6-diamidino-2-phenylindole; Dex, dexamethasone; ER, estrogen receptor; GC, glucocorticoid; GR, glucocorticoid receptor; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha; IFNγ, interferon γ; IL-17A, interleukin 17A; MR, mineralocorticoid receptor; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PR, progestin receptor; Rpl23, ribosomal protein L23; WT, wild-type.

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cells, however, require transfection of WT GR since untransfected EL4 cells are unresponsive to GCs [6–8]. The mechanism underlying the resistance of EL4 cells to GCs is unknown, although previous work has shown EL4 cells have a defect in the binding of the GR to DNA-cellulose [7,8]. In this study, we describe a homozygous mutation (R493C) in the second zinc finger of the DNA-binding domain of the GR in EL4 cells. Both transactivation and transrepression activities of the mutant GR were impaired whereas the ligand-induced nuclear translocation appeared intact. Stably expressing the WT GR enabled GCs to regulate endogenous genes such as nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha (IκBα), interferon γ (IFNγ), and interleukin 17A (IL-17A) in EL4 cells. Since R493 is conserved among steroid receptors and its mutation has been found in both human and mouse GR and in the AR and MR [4,5,9–17], our findings support that this arginine is a hotspot for mutations that cause steroid resistance.

## 2. Experimental

### 2.1. Reagents and antibodies

Dexamethasone (Dex, 1,4-pregnadien-9α-fluoro-16α-methyl-11β,17,21-triol-3,20-dione) was purchased from Steraloids (Newport, RI). Rabbit anti-GR antibodies were from ThermoFisher (Waltham, MA). Goat anti-rabbit antibodies conjugated with horseradish peroxidase (HRP) were from Jackson Immunoresearch (West Grove, PA). All other reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified.

### 2.2. Cell culture

EL4 cells (ATCC, Manassas, VA) were maintained in RPMI medium (Life Technologies/Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT), 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin in a 5% $CO_2$ atmosphere at 37 °C. Cos-1 cells (ATCC) were maintained in DMEM (Invitrogen) supplemented as above.

### 2.3. DNA constructs and site-directed mutagenesis

GR cDNA was reverse transcribed using EL4 RNA as template, cloned into pcDNA3.1 (Invitrogen) between *EcoRV* and *XhoI*. pcDNA3.1-WT GR(R493) and mutant GRs, R493A and R493K, were generated using site-directed mutagenesis with QuikChange kits (Stratagene, La Jolla, CA). DNA sequences were verified at the Genomics core at Northwestern University. The plasmids pCMV-p65, Fos, Jun, HMCII-Luc, AP-1-Luc, pMMTV-luc, pGL3-hRL, and pcDNA-hGR-A were previously described [18–21].

### 2.4. Luciferase reporter assays

To transfect Cos-1 cells at 80% confluency, Transit LT1 reagent (Mirus Corp, Madison, WI) was used at 3 μl/1 μg DNA in Opti-MEM (Invitrogen) according to the manufacturer's protocol. For transactivation assays, 500–1000 ng of pcDNA3.1-GR, 200 ng of pMMTV-Luc reporter, and 20 ng of pGL3-hRL were transfected into Cos-1 cells on 12-well plates. For activator protein 1 (AP-1) transrepression assays, 200 ng of pcDNA3.1-GR, 200 ng of pAP1-luc reporters, 183 ng of pCMV-Fos and pCMV-Jun, and 20 ng of pGL3-hRL were used. For nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transrepression assays, 200 ng of pcDNA3.1-GR, 327 ng of pMHCII-luc, 100 ng of pCMV-p65, and 20 ng of pGL3-hRL were used. Twenty-four h after transfection, cells were treated with vehicle or Dex (1–1000 nM, 24 h) and lysed. Ten μl of the lysates was used to measure luciferase activity using Promega (Madison, WI) Dual Luciferase reagents on a luminometer (BioTek Instruments, Winooski, VT). In each experiment, firefly luciferase activity was normalized to *Renilla* luciferase activity, measured in duplicate and averaged. Each experiment was repeated 3–4 times.

### 2.5. Transfection of EL4 cells

To transiently transfect EL4 cells, Amaxa (Lonza, program C-004) was used according to the manufacturer's protocol. To generate EL4 cells stably expressing WT GR, pCDNA-hGR-A was transfected into EL4 cells using Amaxa. Positive clones were selected using 1.5 mg/ml zeocin and maintained using 1 mg/ml zeocin. The expression of the WT GR was confirmed using Western blot analyses.

### 2.6. Western blot analysis

Cos-1 cells in 6-well plates were transfected with 170 ng of pcDNA3.1-GR or vector controls. Twenty-four h after transfection, lysates were prepared for Western blot analyses. EL4 lysates were prepared similarly. Lysates were resolved on 4–12% NuPage bis-tris gels (Invitrogen) and titers for antibodies were 1:400 (anti-GR antibodies) and 1:50,000 (anti-actin). Secondary antibodies were used at a 1:10,000 dilution for 30 min. The membranes were probed with ECL detection reagent (GE Amersham, Pittsburgh, PA) and exposed to ECL Hyperfilm (GE Amersham).

### 2.7. Immunofluorescent staining

EL4 cells were cultured in RPMI supplemented with 10% charcoal-stripped FBS, glutamine, penicillin, and streptomycin for 3 days before treatment with vehicle or Dex (30 nM, 3 h). Cells were cytospun and fixed with 4% paraformaldehyde. Cos-1 cells were grown in 4-well chamber slides. Twenty-four h after cells were transfected with WT or mutant GR as above. Twenty-four h after transfection, cells were treated with vehicle or Dex (30 nM, 3 h) and fixed with 4% paraformaldehyde. Slides were blocked using 5% normal goat serum in PBS containing 0.05% triton x-100 and incubated with anti-GR (1:200) in blocking solution overnight. After washing, slides were incubated with DyLight 549 conjugated goat anti-rabbit antibodies (1:200, Vector Laboratories, Burlingame, CA) in blocking solutions for 30 min. Slides were then incubated with 1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI), mounted with Fluormount, and imaged with a Nikon Eclipse E800 fluorescent microscope using 40–60× objectives. Slides processed without primary antibody were used as controls. GR signal was quantified using ImageJ. After areas of interest were selected, the area and integrated mean density for the whole cell and the nucleus were calculated. Values of the GR signal in cytoplasm were calculated by subtracting values of the nucleus from those of the whole cell. All values were corrected by mean fluorescence of the background in the area of interest.

### 2.8. Allelic discrimination assay

Allelic discrimination was performed using Custom TaqMan Assays for single nucleotide polymorphism (Life Technologies/Applied Biosystems). Real-time PCR was performed according to the manufacturer's protocol. The primer sequences were: forward 5′-AGTGGAAGGACAGCACAATTA, reverse 5′-TCGAGCTTCCAGGTTC ATTC, WT (1477C) probe 5′-AAACTGTCCAGCATGCCGCTATCGA, and 1477T probe 5′-AAACTGTCCAGCATGTCGCTATCGA. Thermocycling was performed using a Prism 7500HT thermocycler (Applied Biosystems). Controls included a no template control, WT GR