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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 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- κ B 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 I κ B α 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.

Keywords

Glucocorticoid; glucocorticoid receptor; glucocorticoid receptor mutation; glucocorticoid resistance

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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), progesterone receptor (PR), androgen receptor (AR), and estrogen receptor α and β (ER α 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 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 (I κ B α), interferon γ (IFN γ), 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-9 α -fluoro-16 α -methyl-11 β ,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% CO₂ 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 pMHCI-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 minutes. 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 minutes. 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 40-60X 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'-AGTGGAAAGGACAGCACAATTA, reverse 5'-TCGAGCTTCCAGGTTTCATTC, 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 plasmid, and R493C mutant GR plasmid. The zygosity was determined using the scatter plot of the Sequence Detection Systems (SDS) software.

2.9 Real-time RT-PCR

RNA samples were extracted from cells using Quick-RNA MiniPrep kits (Zymo Research, Irvine, CA) and treated with DNase according to the manufacturer's protocol. The level of mRNA in each sample was measured using the one-step RT-PCR procedure on a Prism 7500HT thermocycler (Applied Biosystems). Quantification was achieved using the Sequence Detection Software 2.0 Absolute Level subroutine. Mouse IκBα was measured by using primers 5'-TGGCCTTCCTCAACTTCCAGAACA and 5'-TCAGGATCACAGCCAGCTTTCAGA and probe 5'-ATCACCAACCAGCCAGGAATTGCTGA. For mouse IFNγ primers were 5'-GGCCATCAGCAACAACATAAGCGT and 5'-TGGGTTGTTGACCTCAAACCTTGGC and probe was 5'-ACCTTCTTCAGCAACAGCAAGGCGAA. Mouse IL-17A primers were 5'-ATCATCCCTCAAAGCTCAGCGTGT and 5'-TATCAGGGTCTTCATTGCGGTGGA

and probe was 5'-AGGCCAAGGACTTCCTCCAGAATGTGAA. Mouse ribosomal protein L23 (Rpl23) primers were 5'-AAAGGCTCTGCTATCACAGGTCCA and 5'-ACTGGAGAATCATGCAATGCTGCC and probe was 5'-TGCAGACTTGTGGCCCAGAATTGCAT. RT-PCR probes were labeled with 6-carboxyfluorescein (6-FAM) reporter at the 5' end and Iowa Black (IDT, Coralville, IA) quencher at the 3' end. Rpl23 levels were used for normalization.

2.10 Statistics

Averages $\pm$ SEM are presented. One-way ANOVA was performed when comparing three or more treatment groups followed by Tukey post hoc comparison using Prism software (GraphPad Software, La Jolla, CA). Two-way ANOVA was performed when comparing WT GR to MUT GR or EL4 cells to EL4-hGRA cells followed by Bonferroni test. Student t tests were performed to compare two groups. A p value < 0.05 was considered significant.

3. Results

3.1 EL4 cells carry a homozygous mutation in the GR allele

To determine whether a mutation in the GR of EL4 cells was underlying the GC insensitivity, we cloned the full-length EL4 GR cDNA. Sequence analysis revealed a point mutation at nucleotide +1477 (C to T) that results in a substitution of an arginine by a cysteine at amino acid position 493 (Fig. 1A and B). This mutation is located in the second zinc finger of the DNA-binding domain of the GR and is conserved among all human nuclear receptors. The conservation of this arginine among steroid receptors is shown in Fig. 1C. The R493 in mouse GR is also mutated in certain clones of S49 cells and it corresponds to R477 in human GR, the mutation of which has been observed in Jurkat cells (Fig. 1D) and in a patient with generalized GC resistance [4, 5, 17]. EL4 cells have two copies of chromosome 18 where the GR is located [22]. To determine the zygosity of R493C mutation in EL4 GR, we performed allelic discrimination assays using plasmids for the WT, mutant GR, or a mixture of both as controls. Figure 1E shows that EL4 GR was detected by probes for mutant, but not WT, GR indicating R493C mutation in EL4 GR is a homozygous mutation.

A defect in EL4 GR nuclear translocation has been suggested [7] although R477H mutant in Jurkat cells undergo GC-induced nuclear translocation [4]. We, therefore, determined the ability of GC to induce nuclear translocation of GR in EL4 cells. EL4 cells were cultured in the presence or absence of 30 nM Dex for 3 h and GR immunohistochemistry analyses were performed. In the absence of Dex, the GR is localized in the cytoplasm and upon Dex treatment, the GR translocated into the nucleus and overlapped with nuclear DAPI staining in the majority of the cells (Supplementary Fig. 1A), indicating that GR signaling downstream of ligand binding may underlie the GC resistance in EL4 cells. The ability of the GR to translocate into the nucleus was also examined in Cos-1 cells transfected with WT or R493C GR. Supplementary Fig. 1B shows that both WT and EL4-GR underwent steroid-dependent nuclear translocation. Thus, R493C mutation did not impair the ability of the receptor to translocate into the nucleus.

3.2 R493C mutant GR lost transcriptional activity

The GR DNA-binding domain is involved in transactivation and transrepression of the GR. We sought to determine the effect of the R493C mutation on GR activity. Cos-1 cells were transfected with WT or mutant GR and were examined for the ability of Dex to activate the MMTV-luciferase reporter. WT GR, but not the R493C mutant, induced MMTV-luciferase activity (Fig. 2A). To further determine the role of this arginine residue, we generated two additional mutants: alanine (R493A) or lysine (R493K). R493A, but not R493K, lost transactivation activities. To determine whether the R493C mutant acts as a dominant-negative receptor, Cos-1 cells were transfected with WT and R493C GR at a 1:1 ratio. R493C mutant did not interfere with the ability of WT GR to activate the MMTV-luciferase reporters (Fig. 2B). Since another mutation in the DNA-binding domain (R488, rat GR) impaired GR transrepression of NF- κ B but not AP-1 [23, 24], we determined the transrepression capacity of the R493C, R493A, and R493K mutants for both NF- κ B and AP-1. Cos-1 cells were transfected with WT or mutant GR and either a p65 expression plasmid with MHCII-luciferase reporters or c-fos/c-jun expression plasmids with AP-1-luciferase reporters. WT GR suppressed both NF- κ B and AP-1 activities whereas the mutant GRs had little or significantly impaired transrepression activity (Fig. 2C). These observations indicate that the R493C mutation leads to defects in both transactivation and transrepression activity of the receptor.

3.3 WT GR restores the GC sensitivity of EL4 cells

To determine whether WT GR could restore GC sensitivity, EL4 cells were transiently transfected with WT GR and GC responsiveness was measured using MMTV-luciferase reporters. Dex in EL4 cells expressing WT GR, but not in cells transfected with vector control, induced MMTV-luciferase activity (Fig. 3A). To further examine the ability of EL4 cells to respond to GC treatment, EL4 cells stably expressing WT human GR-A (Fig. 3B) were generated and the regulation of several endogenous genes were determined. In EL4-hGR-A cells, Dex was able to induce I κ B α and repress IFN γ and IL-17A gene expression (Fig. 3C). However, EL4 cells, parental or those stably expressing WT GR, did not undergo GC-induced apoptosis (data not shown), possibly due to elevated anti-apoptotic mechanisms in these transformed cells.

4. Discussion

In this study, we found in the EL4 murine T cell line a homozygous point mutation (C to T) in the DNA-binding domain of the GR resulting in the substitution of an arginine by a cysteine that renders the receptor inactive. The R493C mutant was able to undergo ligand-induced nuclear translocation suggesting the mechanisms underlying EL4 GC resistance are downstream of ligand binding and receptor-chaperone interactions. In contrast, DNA-binding of the R493C mutant is impaired [8] likely due to that the mutated residue resides within the second zinc finger of the DNA-binding domain. Modeling of the DNA-binding domain of the GR reveals that replacing this critical arginine residue disrupts the interaction between GR with phosphate groups of target DNA [5]. Since R493A, but not R493K, lost transactivation activity, positive charge on the arginine or lysine residue may be needed for proper interaction with DNA backbone. However, the R477H mutation in the human GR

also impairs transactivation activity [5], suggesting that in addition to the positive charge, the receptor conformation afforded by arginine or lysine at this position favors DNA binding. We also found that the R493C mutant did not have dominant negative activity, possibly due to lack of heterodimerization between R493C and WT GR. EL4 cells produce pro-inflammatory cytokines, such as IFN γ and IL-17A, which were suppressed by ectopic expression of WT GR.

While the impaired transactivation activity of the R493C mutant is likely due to impaired DNA-binding, the blunted transrepression activity of the R493C, R493A, and R493K mutants supports that R493 and, likely, the conformation sustained by R493 play a role in GR interactions with other transcription factors as well. Transrepression by the GR involves protein-protein interactions between the GR and partner transcription factors [1]. The zinc finger region of the GR has been shown to be critical for interacting with and inhibiting AP-1 and NF- κ B activity [20, 23-25]. The GR directly interacts with the p50, p65, and c-rel subunits of NF- κ B and this interaction leads to diminished activity of NF- κ B [20, 23]. This inhibition is dependent on the zinc fingers of the GR [20, 23, 24]. Specifically, mutation of zinc finger residues (R488 or K490 in rat GR) prevents transrepression of NF- κ B [23]. For transrepression of AP-1, the GR acts via direct interaction with the c-Jun subunit [24]. Deletion of either the N- or C-terminus of the zinc finger region abolishes the transrepression of AP-1 by the GR [24]. However, not all DNA-binding domain mutations result in impaired transrepression. The GR Dim mutant (A458T) or Dim4 mutant (A458T, R460D, D462C, and N454D) has limited receptor dimerization and DNA-binding ability but intact transrepression ability [26, 27]. Our data suggest that the R493 residue of the DNA-binding domain is not only pivotal for GR transactivation but also indispensable for transrepression of AP-1 and NF- κ B.

R493 is highly conserved in the GR among mammalian species. This arginine is also conserved among nuclear receptors including the AR, ER α and β , MR, and PR. Our data provide new evidence supporting this position as a mutation hotspot. In S49 cells, a mouse lymphoma cell line, an arginine to histidine mutation at position 493 resulted in reduced DNA-binding and decreased transactivation activity [17]. In human studies, a mutation at a position corresponding to R493 in murine GR, R477H, was found in a patient with general GC insensitivity and in Jurkat cells, a GC-resistance cell line [4, 5]. In the AR, mutations of R616, equivalent to R493 in the GR, to a histidine, a proline, or a glycine have been found to inactivate the AR and cause androgen insensitivity in patients [10-13, 15, 16]. In the MR, a corresponding mutation (R659S) renders the MR inactive and gives rise to mineralocorticoid resistance [9].

Mutation hotspots are sites where high mutation frequency occurs and they have been found in a variety of proteins including p53, Ras, Fms-like tyrosine kinase 3, CD117, and epidermal growth factor receptor [28-30]. Evolutionarily conserved regions are positively correlated with mutation hotspots [29, 30]. The DNA-binding domain of steroid receptors is highly conserved and this may help to explain the frequent detection of mutations at R493. Mutations at hotspots depend on the nucleotide context or the expansion of mutants with high fitness [31]. Our findings add to the growing body of evidence supporting that R493 mutation is an effective means for a cell to evade steroid regulation. Hotspot mutations can

be induced or spontaneous. The R493C mutation may be an induced mutation since EL4 cells is derived from a thymoma from C57BL/6N mice treated with the mutagen 9,10-dimethyl-1,2-benzanthracene. Spontaneous mutations occur due to errors in DNA replication or a basal level of endogenous DNA damage. While the R477H mutation in Jurkat cells could be induced by carcinogenic stimuli, the cause of the mutation in the GC resistance R477H carrier may be spontaneous since the patient did not have any malignancies [5]. In summary, our findings indicate that mutations at the critical arginine residue in the GR cause loss of receptor function. In patients, mutation at this position results in hormone insensitivity. Further investigation of GR mutations and how they affect GR signaling may help to improve GC efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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	progesterin receptor
Rpl23	ribosomal protein L23
WT	wild-type

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Highlights

- The glucocorticoid receptor in EL4 cells has an arginine to cysteine mutation.
- The mutation occurs in the second zinc finger of the DNA-binding domain.
- The mutant receptor lacks both transactivation and transrepression activity.
- The mutant receptor is not a dominant negative receptor.
- The same arginine is a mutation hotspot in multiple steroid receptors.

A) GR DNA Binding Domain DNA Sequence (partial)

WT 1441 ATTGATAAAATTCGAAGAAAAAAGTCCAGCATGCGCTATCGAAAATGTCTTCAAGCT 1500
 EL4 1441 ATTGATAAAATTCGAAGAAAAAAGTCCAGCATGCTGCTATCGAAAATGTCTTCAAGCT 1500

B) GR DNA Binding Domain AA Sequence

Mus musculus 433 PPKLCLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICIIDKIRRNCPACRYRKCLQAGMNLEARKTK 510
 EL4 433 PPKLCLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICIIDKIRRNCPACRYRKCLQAGMNLEARKTK 510
Homo sapiens 417 PPKLCLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICIIDKIRRNCPACRYRKCLQAGMNLEARKTK 494
Rattus norvegicus 435 PPKLCLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICIIDKIRRNCPACRYRKCLQAGMNLEARKTK 512
Macaca mulatta 417 PPKLCLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICIIDKIRRNCPACRYRKCLQAGMNLEARKTK 494

C) Steroid Receptor DNA Binding Domain AA Sequence

Homo sapiens 417 -PPKLCLVCSDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICIIDKIRRNCPACRYRKCLQAGMNLEARKTK--- 494
 GR 599 -PSKICLVCGDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICIIDKIRRNCPACRYRKCLQAGMNLEARKTK--- 673
 PR 563 -PQKICLVCGDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICIIDKIRRNCPACRYRKCLQAGMNLEARKTK--- 640
 AR 555 PPQKICLVCGDEASGCHYGVLTCGSKVFFKRAVEGQHNYLCAGRNDICIIDKIRRNCPACRYRKCLQAGMNLEARKTK--- 636
 ERα 180 KETRYCAVCNDYASGYHYGVWSCEGCAFFKRSIQGHNDYMCPTNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRG 261
 ERβ 144 RDAHFCAVCSYASGYHYGVWSCEGCAFFKRSIQGHNDYMCPTNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRG 225

D) R493 Mutations in GR DNA Binding Domain **E) Allelic Discrimination** **Figure 1.**

GR in EL4 cells contains a point mutation in codon 493. A) EL4 GR cDNA was cloned and sequenced. EL4 GR sequence was compared to WT GR (NCBI sequence reference number NM_008173.3). The point mutation is highlighted at position +1477 from start codon. The codon affected is underlined. B) Arginine 493 in the DNA-binding domain is conserved in GR protein sequences from multiple species including mouse (NP_032199.3), human (NP_000167.1), rat (NP_036708.2), and monkey (XP_001097126.1). C) Conservation of the arginine 493 in the DNA-binding domain of steroid receptors including the GR, MR (NP_000892.2), PR (NP_000917.3), AR (NP_000035.2), ERα (NP_000116.2), and β (NP_001035365.1). D) Schematic representation of the DNA-binding domain of the GR of EL4 cells and Jurkat cells. Arrows point to the mutated arginine in the second zinc finger. E) Allelic discrimination assay was performed to determine the zygosity of the GR gene in EL4 cells. VIC probe labels the 1477T mutation and FAM probe labels the 1477C WT sequence. Plasmids expressing the WT, mutant GR, or mixtures of the two were used as controls. Only VIC fluorescence was detected in EL4 samples. AA, amino acid; FI, Fluorescence intensity; NTC, No template control.

Figure 2.

Transactivation and transrepression activity of R493 mutant GR. A) Cos-1 cells were transiently transfected with MMTV-luciferase, pGL3-hRL, and pcDNA-WT or pcDNA-R493C, R493A, or R493K GR. After overnight culture, cells were treated with or without 100 nM Dex for 24 h. The means $\pm$ SEM of luciferase activity normalized to renilla activity of three to four independent experiments are shown. Student t test, *, $p < 0.05$. B) Cos-1 cells were transfected with MMTV-luciferase, pGL3-hRL, and pcDNA-WT or pcDNA-WT and pcDNA-R493C at a 1:1 ratio. After overnight culture, cells were treated with or without 100 nM Dex for 24 h. The means $\pm$ SEM of luciferase activity normalized to renilla activity of three independent experiments are shown. A representative western blot shows the expression level of transfected GR. Student t test, *, $p < 0.05$. C) Cos-1 cells were transiently transfected with pcDNA-WT or mutant GR together with pGL3-hRL and pGL2-MHCII-luciferase and pCMV-p65 or pGL2-AP-1-luciferase, pCMV-c-fos, and pCMV-c-jun. After overnight culture, cells were treated with 0, 1, 10, 100, or 1000 nM of Dex for 24 h. The means $\pm$ SEM of luciferase activity normalized to renilla activity of three independent experiments are shown. One-way ANOVA followed by Tukey post-hoc analysis was performed to compare Dex treated cells to control cells. *, $p < 0.05$. Two-way ANOVA followed by Bonferroni's post-hoc test was used to compare values from MUT GRs to WT GR. +, $p < 0.05$.

Figure 3.

Wild-type GR restores the GC sensitivity of EL4 cells. A) EL4 cells were transiently transfected with pMMTV-luciferase, pGL3-hRL, and pcDNA3.1 or pcDNA3.1-WT GR. After overnight culture, cells were treated with 100 nM Dex for 24 h. The means $\pm$ SEM of luciferase activity normalized to renilla activity of three independent experiments are shown. Student t test, *, $p < 0.05$. B) A representative Western blot analysis of GR proteins in EL4 and EL4 cells stably expressing the human GR-A. C) Real-time PCR analyses of gene regulation by Dex in EL4 cells stably expressing hGR-A. Cells were treated with vehicle (Con) or 100 nM Dex for 1 h and stimulated with vehicle or PMA (P, 20 ng/ml) and ionomycin (I, 250 ng/ml) for 5 h. RNA was harvested and realtime RT-PCR was used to measure $\text{IkB}\alpha$, $\text{IFN}\gamma$, and IL-17A gene expression. Two-way ANOVA followed by Bonferroni's post-hoc tests was performed. P/I vs. P/I Dex: *, $p < 0.05$. ***, $p < 0.001$.