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ORIGINAL ARTICLE

Further characterization of human glucocorticoid receptor mutants, R477H and G679S, associated with primary generalized glucocorticoid resistanceMINI RUIZ¹, ERIK HEDMAN², MATS GÅFVELS³, GÖSTA EGGERTSEN³, SIGBRITT WERNER¹, HANS WAHRENBERG¹ & ANN-CHARLOTTE WIKSTRÖM²¹Department of Medicine, Karolinska Institutet, Stockholm, Sweden, ²Department of Biosciences & Nutrition, Karolinska Institutet, Stockholm, Sweden and ³Division of Clinical Chemistry, Karolinska University Hospital, Huddinge, Stockholm, Sweden**Abstract**

Objective. Primary generalized glucocorticoid resistance is a rare condition characterized by a generalized insensitivity to glucocorticoids, to some extent due to an impaired function of the glucocorticoid receptor. Our earlier genetic analysis of the human glucocorticoid receptor (hGR) in 12 unrelated patients with primary generalized glucocorticoid resistance revealed two new mutations, R477H in exon 4 and G679S in exon 8 in two patients. In order to further study the molecular mechanisms underlying the phenotype of these mutations we have investigated their effect on glucocorticoid signal transduction. **Methods.** We have studied the DNA-binding ability of the R477H mutant with an electrophoretic mobility shift assay (EMSA). The ability of the R477H and the G679S mutants to affect TNF α induced NF- κ B activity and wild-type GR signalling was studied in transient transfection assays. **Results.** In EMSA the R477H mutation showed a reduced ability to bind to a glucocorticoid-response element compared to the wild-type GR. In transient transfection assays both the R477H mutant and the G679S mutant showed a dominant negative effect on co-transfected wild-type GR in Cos 7 cells. However, both mutants showed full capacity to repress TNF α -induced NF- κ B activity. **Conclusion.** The impaired DNA-binding of the hGR, R477H mutant may explain the severe phenotype of cortisol resistance seen with this mutation. The dominant negative effects of both mutants on wild-type GR signalling probably contribute to the patients' cortisol resistance.

Key Words: Hormones of the adrenal cortex, medical biochemistry, adrenal, endocrine physiology, medical physiology, endocrinology, internal medicine, receptors glucocorticoid/genetics, humans, mutation

Introduction

Glucocorticoids exert their effects mainly through the GR isoform α (GR α), a ligand-dependent transcription factor. In its non-liganded form, GR is mainly localized in the cytoplasm in a complex together with chaperone proteins such as heat shock protein (hsp) 90 and 70, the co-chaperones p23 and the immunophilin FKBP51 [1]. Upon binding of glucocorticoids to GR α , the receptor becomes hyperphosphorylated and several proteins in the complex dissociate and domains responsible for dimerization, nuclear translocation, DNA-binding and transactivation are exposed [2]. The liganded complex then translocates into the nucleus with the assistance of two nuclear localization (NL) signals, NL1 and NL2. NL1 is

located in the junction between the DNA-binding domain (DBD) and ligand-binding domain (LBD) and catalyzes rapid transport of the GR through the nuclear pore via the classic importin pathway. NL2 is located throughout the LBD and contributes to a slower traffic to the nucleus via a yet unknown mechanism [3]. Following nuclear translocation, the receptor regulates gene transcription by binding as a homodimer to glucocorticoid responsive elements (GREs) in promoter regions of target genes [4]. GR α also influences the activities of other transcription factors, such as NF- κ B, via protein-protein interaction, i.e. crosstalking. The NF- κ B dependent gene expression has been suggested to be repressed by GR in a GRE independent manner [5]. The DBD of the

GR seems to participate in this cross-talk with other signalling pathways [6].

The rare syndrome of primary generalized glucocorticoid resistance is a disorder characterized by biochemical hypercortisolism without the clinical stigmata of Cushing's syndrome. Only partial or incomplete resistance has been reported. Abnormalities of the intracellular GR α concentration, stability and affinity for glucocorticoids have been reported [7–9]. Glucocorticoid resistance due to mutations in the gene for the glucocorticoid receptor has been the case in ten patients, where five are kindred, with each patient having their own causative mutation. A total of 12 mutations, one 2-bp deletion and one splice site deletion have hitherto been found, one in the DBD and the others in the LBD of the receptor [10–22].

We have previously described new mutations in two patients within a group of 12 unrelated patients with generalized glucocorticoid resistance. One is located in the DNA-binding domain and another in the ligand-binding domain of GR, R477H and G679S respectively [15]. The two mutations show *in vitro* alterations in function correlating to the *in vivo* changes. The R477H mutation is located in exon four in the second zinc finger of the DBD, close to the NLI sequence. When tested *in vitro* this mutation showed a negligible transactivation capacity but intact ligand binding capacity. The patient having this mutation suffered from obesity (BMI 35), hirsutism requiring daily shavings and hypertension. The G679S mutation is located in exon nine in the LBD. When tested *in vitro* G679S showed an impaired transactivation and ligand binding capacity. The patient having this mutation suffered from hirsutism and was alleviated from her symptoms when treated with Dexamethasone.

To further elucidate if the *in vitro* alterations, that we originally demonstrated, correlate to the *in vivo* changes, we have studied whether the mutant receptors had a dominant negative effect on the wild-type receptor, and also if the mutations had any effect on glucocorticoid repression of NF- κ B mediated transactivation.

Materials and methods

Plasmids and *in vitro* mutagenesis

The plasmid pCMVhGR, expressing the wild type human GR, and the reporter vectors p19tk-luc and (NF- κ B) β -ITK-luc have been previously described [15]. Site-directed mutagenesis of pCMVhGR according to the refined method of Kunkel [23,24] was used as previously described [15]. The PSGstop and RelA plasmids were kind gifts from Dr L-G. Bladh.

Cell culture, transient transfection and luciferase assay

Cos7 cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ in Dulbecco's modified

Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (0.1 mg/ml). All media, serum and antibiotics were from Invitrogen, Stockholm, Sweden. In the transient transfection reporter gene experiments with mutants or GR wild type and NF- κ B, cells were seeded at a density of 30,000 cells/well, in 24-well plates 24 hours before transfection. In the case of transient transfection studies in the GC driven reporter gene assay, cells were transfected with a constant amount (20 ng) wild-type GR plasmid (pCMVhGR) and increasing amount of either mutant R477H or G679S plasmids respectively (0–30 ng). A silent vector, PSGstop, was also added in decreasing amount to maintain the transfected DNA at a constant level. Finally, 200 ng of the GC driven reporter gene, p19tkluc using FuGENE™-6 transfection reagent (Roche Diagnostics Scandinavia AB, Stockholm, Sweden) at a ratio of 3 μ l/ μ g DNA, according to the manufacturer's protocol, was added to the transfection mixture. When studying the mutants effects on NF- κ B activity, cells were transfected with a constant amount of RelA (10 ng) with either pCMVhGR, mutant R477H or G679S, respectively, together with 200 ng p(NF κ B) β -ITK-luc, also using FuGENE™-6. Then, 24 hours after transfection, 10 nM Triamcinolone acetone (TA) (Sigma Aldrich, Stockholm, Sweden) was added to the GR transfected cells, and, 24 hours after hormone treatment, the NF- κ B reporter gene system was induced by tumour necrosis factor TNF α (200 IU/ml) (Roche Diagnostics Scandinavia AB, Bromma, Sweden) for 1 hour. The cells were then harvested in 100 μ L lysis buffer (25 mM Trisacetate pH 7.8, 2 mM dithiothreitol, 1.5 mM EDTA, 10% glycerol and 1% Triton X-100). Luciferase activity was monitored with the GenGlow luciferase kit (Bio Orbit, Turku, Finland), using 50 μ L of extract in a Lucy 3 luminometer (Anthos, Eugendorf, Austria). All assays were performed in triplicate using three separate plates of transfected cells. We have also transfected all the cells with β -GAL, as an internal control in the luciferase assay, as a measure of transfection efficiency.

Electromobility shift assay (EMSA)

Double stranded oligonucleotides (50 ng) were end labelled with [γ -³²P]ATP (6000 Ci/mmol) by using T4 polynucleotide kinase. Unincorporated nucleotides were removed by chromatography on a Sephadex G50 Nick Column (GE Healthcare, Uppsala, Sweden). A total of 9 μ g of total protein from whole cells extracts from *Escherichia coli* expressing wild-type hGR-DBD (w3), corresponding to aa 418–503 of human GR) or mutant R477H-DBD was incubated with 2 μ l 10 \times binding buffer (10 mM Tris HCl pH 7.6, 0.5% (v/v) NP-40, 500 mM KCl, 50 mM MgCl₂, 10 mM EDTA, 25% (v/v) glycerol), 2 μ l poly [dIdC], dithiothreitol and water up to 20 μ l for 10 min at room

temperature. A total of 50,000 cpm of ^{32}P labelled oligonucleotides was added in the presence or absence of 100-fold excess of non-labelled specific and non-specific oligonucleotides. The protein-DNA complexes were further analyzed on a 4% polyacrylamide Tris-glycin-EDTA gel. The following oligonucleotides were used for analysis of hGR-DBD interaction with $1 \times \text{GRE}$, Nonspecific competitor (AP-1 binding site) 5'-CTAGGTGTCTGACTCATGCTT-3' and GRE, 5'AGCTTAGAACACAGTGTCTCTAGAG3'.

SDS-PAGE, Western blotting and immunostaining

A total of 9 μg of total protein from *Escherichia coli* extracts transfected with the w3 or the R477H forms of hGR-DBD, respectively, were boiled in SDS sample buffer and resolved on 12% (v/v) SDS-PAGE gels under reducing conditions at 30 mA/gel for 50 min with the mini-Protean III gel system (Bio-Rad, Stockholm, Sweden). Western blotting and immunostaining proceeded as in [19] using mouse monoclonal antibody (3.25 $\mu\text{g}/\text{ml}$), clone 29:3, raised against hGR-DBD in our laboratory. Horseradish peroxidase (HRP) conjugated sheep anti-mouse (GE Healthcare, Uppsala, Sweden) was used as secondary antibody at a 1:50,000 dilution. The membranes were finally incubated with ECLTM Advanced Western Blotting Detection Kit (GE Healthcare, Uppsala, Sweden) according to the instructions from the manufacturer.

Statistical analysis

Statistical significance was evaluated using one- or two-way analysis of variance for repeated measurements when appropriate. Holm-Sidak's and Bonferroni's methods were used for multiple comparisons between groups. Values of $p < 0.05$ in two-tailed tests were considered significant. Results are presented as mean + SD.

Results

Expression of the wt and the mutant R477H allowed us to study the DNA, i.e. GRE, binding capability of R477H in comparison to wt with electrophoretic mobility shift assay (EMSA). The R477H mutant showed a reduced ability to bind to DNA, see Figure 1A lane 5 compared to the wt (w3) in Figure 1A lane 2, when similar amounts of protein were loaded, as shown in Figure 1B.

Due to our previous observation of an impaired transactivation capacity of the G679S mutant and the negligible transactivation capacity of the R477H mutant, we tested the repressive activity of these mutants. Figure 2A shows the profile of the wild-type GR-induced transactivation and the effect of over-expression of the R477H and G679S mutants respectively in absolute luciferase activity values. Both

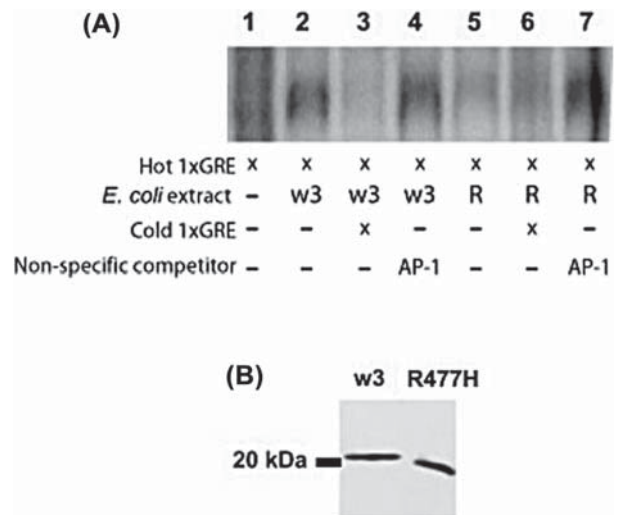


Figure 1. EMSA with hGR-DBD and the R477H-DBD mutant. (A) The EMSA was run on a 4% SDS-PAGE gel with the following additions: hot probe, ^{32}P -end-labelled oligomer, containing one classical GRE in all lanes, 1–7; in lanes 2–4; *Escherichia coli* cell extract containing expressed wt GR DNA-binding domain (DBD) (w3); in lanes 5–7 GR mutant R477H (R) DBD; cold probe in lane 3 & 6 and oligomer with an AP1 consensus sequence as a non-specific competitor in lane 4 & 7. The experiment shows one representative experiment out of two. (B) Loading control, 9 μg of total protein from *Escherichia coli* extracts containing the wt (w3) or the R477H forms of DBD respectively was loaded onto a 12% SDS-PAGE gel and immuno-stained with the mouse monoclonal antibody, clone 29:3, raised against hGR-DBD.

mutants inhibited transactivation of the wild-type GR equally in a dose-dependent fashion ($p < 0.001$). These findings suggest that both mutant receptors exert a dominant negative effect on the glucocorticoid-dependent wild-type GR transcriptional activity.

The cross-talk between the transcription factor NF- κB and GR was studied with transactivation studies where a constant amount of RelA was co-transfected with wild-type receptor together with either mutant R477H or G679S, respectively, together with the (NF κB)3-ITK-luc reporter gene. Figure 2B shows the ability of the three different forms of GR, pCMVhGR, R477H and G679S to repress TNF α -induced NF- κB reporter gene activity. The mutants R477H and G679S showed a similar capacity to repress NF- κB -mediated gene transcription as the wt did.

Discussion

We have previously identified two novel mutations in the hGR gene; R477H and G679S [15]. Our original findings of the disability of these two mutations to bind to ligands and to transactivate were later on confirmed by Charmandari et al. [25].

Our findings that the expressed DBD of the R477H mutant showed a decreased capacity to bind to DNA are also in accordance with the findings of Charmandari et al. As the DBD is the domain being affected by this mutation it is most likely to have an impact for the R477H protein as well. The ability of

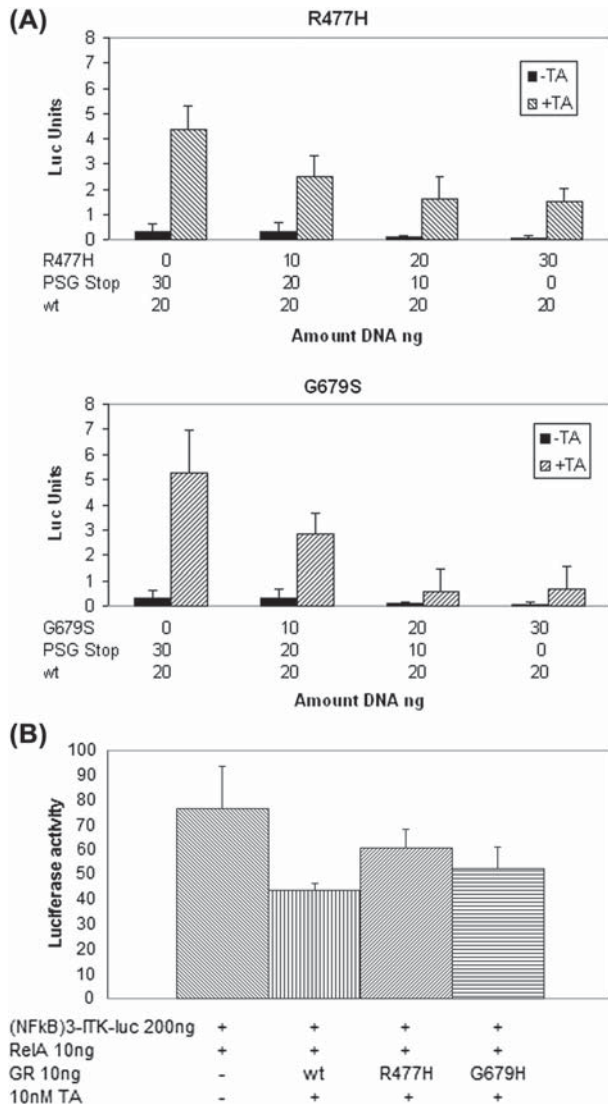


Figure 2. Transient transfection studies of the R477H mutant and the G679S mutant. (A) Negative transdominant activity of the R477H and the G679S mutant on wild-type GR respectively. Cos7 cells were transiently transfected with a constant amount of wild-type GR together with an increasing amount of R477H or G679S mutant, respectively. After transfection the cells were stimulated with 10 nM TA for 24 hours before luciferase activity was measured. Absolute values of luciferase activity are shown on the y-bar. The figures show one representative experiment of three and all experiments were done in triplicate. There is no significant difference between the two mutants in their ability to act in a dominant negative way. However both mutants separately have a significance of $p < 0.001$ in their ability to act in a dominant negative way. (B) Repression of an NF- κ B reporter gene by wild-type human GR, mutant R477H and G679S. Cos7 cells were transfected with a constant amount of RelA and co-transfected with either pCMVhGR, mutant R477H or G679S together with the (NF κ B)3-ITK-luc reporter gene. The transfected cells were stimulated with 10 nM TA for 24 hours before NF- κ B activity was induced by TNF α for 1 hour. Absolute values of luciferase activity are shown on the y-bar. The figure shows one representative experiment of three and all experiments were done in triplicate. There were no significant differences between the three GR forms in their ability to repress NF- κ B regardless of the amount of DNA. Both mutants showed significant dose-dependent suppression for all steps, except between 20 and 30 ng.

the G679S mutant to bind to DNA was not tested, since the EMSA could only be performed with the *in vitro* expressed DBD and the DBD domain of this mutant receptor is identical to the wt DBD domain.

Furthermore, we have now shown that both the R477H and the G679S mutant receptors show a dominant negative effect on the transcriptional activity of wild-type GR, a result that differs from the one demonstrated by Charmandari et al. This difference may reflect the fact that various cell types and various sets of reporter genes were used; Cos7 cells and the p19tk-luc in our study, compared to CV1 cells and the MMTV reporter gene used in Charmandari et al. Our findings suggest that the R477H and the G679S mutants could exhibit various effects in various cell types also *in vivo*. Moreover, our results show that the two receptor mutations each have a dominant negative effect on the transactivating ability of wild-type GR. This could have a significant impact *in vivo* by diminishing the effect of the GR emanating from the normal allele in a GR heterozygous individual.

Being a nuclear receptor, as such, the receptor consists of three domains, the N-terminal domain, the DBD domain and the LBD domain. Studies of the different domains and the importance of specific amino acids are still being addressed. Studies of the N-terminal domain have for instance showed that the important GR phosphorylation mainly occurs in serine sites within this domain [26].

A mutation in the helix 10 within the LBD of the hGR causing generalized glucocorticoid resistance resulting in a replacement of arginine by glutamine at position 714 transmitted a conformational change to the LBD and the AF-2 transactivation surface, resulting in a decreased binding affinity to ligand [27].

Both our mutant receptors as well as the wild-type GR showed a similar capacity to repress NF- κ B mediated gene transcription. This indicates that the GRs with either the clinically severe R477H mutation or the clinically less severe G679S mutation are fully functional in their *in vivo* suppression of NF- κ B and that their ability to act on an inflammatory pathway is indeed functioning. This correlates to the previous findings of Lidén et al., showing that two amino acids in the second zinc finger of the rat GR-DBD, Arg-488 and Lys-490, corresponding to positions 469 and 471 in human GR are critical for the glucocorticoid-induced inhibition of NF- κ B. It also correlates to the findings of Ito et al. [28] showing that lack of ability to deacetylate K494 and K495 renders GR insensitive to NF- κ B while leaving other GR-associated functions such as nuclear translocation and DNA binding unaffected. All four of these amino acids are intact in the R477H and G679S mutations, which correlates well with the two patients clinical history, where there are no indications of a hyper-inflammatory condition [15]. We speculate that the intact ability to repress NF- κ B is a contributing factor to the actual survival of our two patients, as a complete lack of GR function is probably lethal.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Notice of correction

The version of this article published online ahead of print on 8 Feb 2013 contained an error on page 4. Figure 2b is part of Figure 2a, the correct Figure 2b was left out. The error has been corrected for this version.