0021-972X/07/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 92(10):3986–3990

doi: 10.1210/jc.2006-2830

A Novel Point Mutation in Helix 11 of the Ligand-Binding Domain of the Human Glucocorticoid Receptor Gene Causing Generalized Glucocorticoid Resistance

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Background: Generalized glucocorticoid resistance is a rare condition characterized by partial, end-organ insensitivity to glucocorticoids, compensatory elevations in adrenocorticotropic hormone and cortisol secretion, and increased production of adrenal steroids with androgenic and/or mineralocorticoid activity. We have identified a new case of glucocorticoid resistance caused by a novel mutation of the human glucocorticoid receptor (hGR) gene and studied the molecular mechanisms through which the mutant receptor impairs glucocorticoid signal transduction.

Methods and Results: We identified a novel, single, heterozygous nucleotide (T → C) substitution at position 2209 (exon 9α) of the hGR gene, which resulted in phenylalanine (F) to leucine (L) substitution at amino acid position 737 within helix 11 of the ligand-binding domain of the protein. Compared with the wild-type receptor, the mutant receptor hGRαF737L demonstrated a significant ligand-exposure time-dependent decrease in its ability to transactivate the glucocorticoid-inducible mouse mammary tumor virus promoter in response to dexamethasone and displayed a 2-fold reduction in the affinity for ligand, a 12-fold delay in nuclear translocation, and an abnormal interaction with the glucocorticoid receptor-interacting protein 1 coactivator. The mutant receptor preserved its ability to bind to DNA and exerted a dominant-negative effect on the wild-type hGRα only after a short duration of exposure to the ligand.

Conclusions: The mutant receptor hGRαF737L causes generalized glucocorticoid resistance because of decreased affinity for the ligand, marked delay in nuclear translocation, and/or abnormal interaction with the glucocorticoid receptor-interacting protein 1 coactivator. These findings confirm the importance of the C terminus of the ligand-binding domain of the receptor in conferring transactivational activity. **(*J Clin Endocrinol Metab* 92: 3986–3990, 2007)**

GENERALIZED GLUCOCORTICOID resistance is a rare condition characterized by partial, end-organ insensitivity to glucocorticoids, compensatory elevations in circulating cortisol and ACTH concentrations and resistance of the hypothalamic-pituitary-adrenal axis to dexamethasone suppression (1–4). The excess ACTH secretion results in increased production of adrenal steroids with mineralocorticoid and/or androgenic activity and the corresponding clinical phenotype(s). The molecular basis of the condition has been ascribed to mutations in the human glucocorticoid receptor (hGR) gene, which impair glucocorticoid signal transduction, thereby altering tissue sensitivity to glucocorticoids. Inactivating mutations within the ligand-binding (LBD) and DNA-binding domains of the receptor and a 4-bp deletion at the 3′-boundary of exon 6 of the gene have been described in five kindreds and four sporadic cases (5–15).

In the present study, we report a new case of generalized glucocorticoid resistance caused by a novel, heterozygous, point mutation of the hGR gene, and we present the molecular mechanisms through which the mutant receptor impairs glucocorticoid signal transduction.

First Published Online July 17, 2007

Abbreviations: AF, Activation function; BP, blood pressure; F, phenylalanine; GRIP1, glucocorticoid receptor-interacting protein 1; H, helix; hGR, human glucocorticoid receptor; L, leucine; LBD, ligand-binding domain; MMTV, mouse mammary tumor virus; SDS, SD score.

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Subject and Methods

Case report

A 7-yr-old boy presented with severe hypertension and hypokalemia. He had been treated with β-blockers, calcium channel blockers, and large doses of potassium supplements with no improvement. The past medical history and family history were unremarkable. On examination, he had elevated blood pressure (BP) [systolic BP 180 mm Hg (+11 SD score [SDS]), diastolic BP 120 mm Hg (+8.62 SDS)] but no evidence of hyperandrogenism and no signs suggestive of Cushing's syndrome. His weight was 31.0 kg (+2.58 SDS), his height 133.8 cm (+2.56 SDS), and his body mass index 17.3 kg/m^2 (+0.9 SDS). Biochemical and endocrinologic evaluation at presentation revealed hypokalemia (2.6 mmol/liter); elevated serum cortisol concentrations, which maintained circadian rhythmicity (0800 h cortisol: 160 μg/dl, 1700 h cortisol: 50 μg/dl; normal range 8–19 μg/dl); and elevated 0800 h plasma ACTH (425 pg/ml; normal range 10–60 pg/ml) concentrations. A low (1 mg) and high (8 mg) dose overnight dexamethasone suppression test revealed resistance of the hypothalamic-pituitary-adrenal axis to dexamethasone suppression (0800 h serum cortisol: 29 and 15 μg/dl, respectively). Abdominal computed tomography scan confirmed bilateral adrenal hyperplasia. Chest x-rays and magnetic resonance scans of the pituitary and hypothalamic regions did not reveal any pathology. Written informed consent was obtained from the parents of the patient for further molecular studies.

Sequencing of the hGR gene

Genomic DNA was extracted from peripheral lymphocytes, and the entire coding region of the hGR gene was amplified by the PCR and sequenced as previously described (14).

Plasmids

The plasmids used in this study included pRShGRα, pF25GFP-hGRα, pBK/CMV-hGRα, pMMTV-luc, pSV40-β-gal, pRSV-erbA^{-1}, pGEX4T3-GRIP1(1–1462), pGEX4T3-GRIP1 (596–774), and pGEX4T3-GRIP1(740–1217) (12–15). The plasmids pRShGRαF737L, pF25GFP-hGRαF737L, and pBK/CMV-hGRαF737L were constructed by introducing the F737L mutation into pRShGRα, pF25GFP-hGRα, and pBK/CMV-hGRα, respectively, using PCR-assisted site-directed mutagenesis (12–15).

Transactivation assays

CV-1 cells were cotransfected with pRShGRα, pRShGRαF737L, or pRSV-erbA^{-1} (0.05 μg/well), pMMTV-luc (0.5 μg/well), and pSV40-β-gal (0.1 μg/well) using lipofectin. In further experiments, cells were cotransfected with pMMTV-luc, pSV40-β-gal, a constant amount of pRShGRα (0.05 μg/well), and progressively increasing concentrations of pRShGRαF737L. Forty-eight hours after transfection, cells were exposed to dexamethasone for 3–24 h. Luciferase and β-galactosidase activities were determined in the cell lysates as previously described (12–15).

Western blot analyses

CV-1 and COS-7 cells were transfected with pRShGRα or pRShGRαF737L (15 μg/flask) using lipofectin. Western blot analyses were performed as previously described (13–15).

Dexamethasone-binding assays

COS-7 cells were transfected with pRShGRα or pRShGRαF737L (1.5 μg/well) using lipofectin. Confluent cells were incubated with six different concentrations of [^{3}H]dexamethasone at 37 C in the presence or absence of a 500-fold molar excess of nonradioactive dexamethasone for 1 h. Dexamethasone-binding assays were performed as previously described (13–15).

Nuclear translocation studies

HeLa cells were transfected with pF25GFP-hGRα or pF25GFP-hGRαF737L (2 μg/dish) using FuGENE 6 according to the instructions of the manufacturer (Roche Diagnostics Corp., Indianapolis, IN). In further experiments, cells were transfected with pF25GFP-hGRα and pRShGRαF737L (1.5 μg/dish). Nuclear translocation studies were performed as previously described (13–15).

Chromatin immunoprecipitation assays

HCT-116 cells, in which the mouse mammary tumor virus (MMTV) promoter was stably integrated within chromatin, were transiently transfected with pRShGRα or pRShGRαF737L (10 μg/dish). Chromatin immunoprecipitation assays were performed as previously described (14–16).

Glutathione-S-transferase pull-down assay

In vitro transcription/translation reactions were used to produce ^{35}S-labeled hGRα and hGRαF737L in rabbit reticulocyte lysate by using pBK/CMV-hGRα and pBK/CMV-hGRαF737L, respectively, as templates. The *in vitro* interaction between hGRα-related plasmids and glutathione-S-transferase-fused glucocorticoid receptor-interacting protein 1 (GRIP1) proteins was tested as previously described (13–15).

Results

Sequencing of the hGR gene

A single, heterozygous thymine to cytosine (T → C) substitution was identified at nucleotide position 2209 in exon 9α of the gene, which resulted in phenylalanine (F) to leucine (L) substitution at amino acid position 737 in the LBD of the receptor. Using the three-dimensional crystal structure of the LBD of hGRα, we determined that the F737L mutation is located in helix 11 of this domain (17) (Fig. 1A).

The mutant receptor hGRαF737L displays decreased transcriptional activity and exerts a dominant negative effect upon the wild-type hGRα only after short-duration exposure to the ligand

Compared with hGRα, hGRαF737L displayed a 2-fold reduction in its ability to transactivate glucocorticoid-responsive genes (Fig. 1B). The decreased transcriptional activity of hGRαF737L was ligand-exposure time-dependent and progressively more pronounced at shorter exposure times. Cotransfection with a constant amount of hGRα and increasing concentrations of hGRαF737L did not result in a dose-dependent inhibition of hGRα-mediated transactivation of the MMTV promoter, suggesting that hGRαF737L does not exert a dominant-negative effect on the wild-type hGRα (Fig. 1C). The latter does not preclude a partial dominant-negative effect in more complex transcriptional systems or in different cell lines. Indeed, exposure to dexamethasone for shorter periods of time (3 and 6 h) revealed a dominant-negative effect of hGRαF737L on hGRα (Fig. 1D).

The mutant receptor hGRαF737L demonstrates decreased affinity for the ligand

The apparent dissociation constant of hGRαF737L was significantly higher than that of hGRα (13.9 ± 1.7 *vs.* 7.8 ± 0.3 nM, $P = 0.006$), indicating that hGRαF737L had a 2-fold lower affinity for the ligand than hGRα. No difference in the number of dexamethasone-binding sites was noted between hGRα and hGRαF737L. Western blot analyses demonstrated no differences in the expression of hGRα and hGRαF737L proteins in CV-1 or COS-7 cells, indicating that the above-described findings did not reflect differences at the protein expression level.

The mutant receptor hGRαF737L demonstrates marked delay in nuclear translocation

In the absence of ligand, hGRα was primarily localized in the cytoplasm of cells. Exposure to dexamethasone resulted in nuclear translocation of the receptor within 15 min (14.75 ± 0.25 min) (Fig. 2A). The mutant receptor hGRαF737L was observed in both the cytoplasm and nucleus of cells in the absence of ligand, whereas exposure to the same concentration of dexamethasone induced a 12-fold delay in nuclear translocation (175.00 ± 5.00 min) (Fig. 2B). Coexpression of hGRα and hGRαF737L at a 1:1 ratio had no apparent effect on the nuclear translocation of hGRα (Fig. 2C).

The mutant receptor hGRαF737L preserves its ability to bind to DNA

Both hGRα and hGRαF737L coprecipitated with MMTV glucocorticoid-response elements similarly, in a ligand-

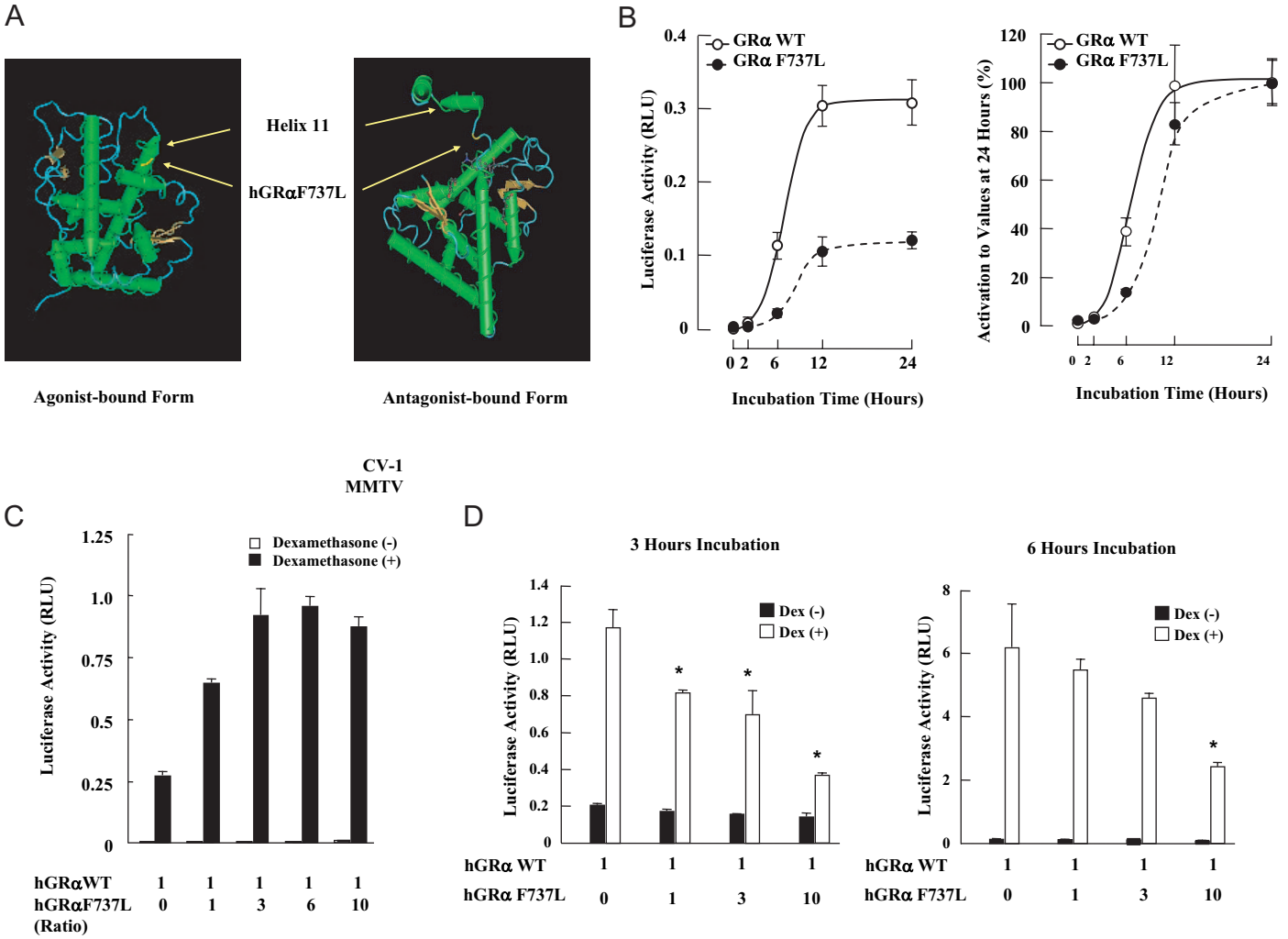

FIG. 1. A, Crystal structure of the agonist-bound (*left panel*) and antagonist-bound (*right panel*) ligand-binding domain of hGRα. The *upper arrows* indicate the position of helix 11 of the receptor. The *lower arrows* indicate the position of the F737L mutation (in *yellow*) identified in our patient. B, Transcriptional activity of the wild-type hGRα and mutant receptor hGRαF737L. Compared with the wild-type receptor, the mutant receptor demonstrated a 2-fold reduction in its ability to transactivate the MMTV promoter in response to dexamethasone. C, Absence of a dominant-negative effect of the mutant receptor hGRαF737L on the wild-type hGRα after exposure to the ligand for 24 h. D, Dominant-negative effect of the mutant receptor hGRαF737L on the wild-type hGRα after exposure to the ligand for 3 and 6 h. Cotransfection with a constant amount of hGRα and progressively increasing concentrations of hGRαF737L revealed a dose-dependent inhibition of the hGRα-mediated transactivation of the MMTV promoter only after a shorter duration (up to 6 h) of exposure to dexamethasone. *Bars* represent mean ± SE of at least five independent experiments.

dependent fashion, suggesting that hGRαF737L preserves its ability to bind to DNA.

The mutant receptor hGRαF737L interacts with the GRIP1 coactivator in vitro only through its activation function (AF)-1

Both hGRα and hGRαF737L bound to full-length GRIP1; however, hGRαF737L did not demonstrate a ligand-dependent increase in its interaction with GRIP1. Also, although hGRα interacted with the amino-terminal fragment of GRIP1 in a ligand-dependent fashion, there was no interaction between hGRαF737L and this fragment of GRIP1. Both hGRα and hGRαF737L bound to the carboxyl-terminal fragment of GRIP1 in a ligand-independent fashion. These results suggest that hGRαF737L interacts with the GRIP1 coactivator *in vitro* only through its AF-1.

Discussion

We have identified a novel, heterozygous point mutation in exon 9α of the hGR gene and studied the molecular mechanisms through which the mutant receptor impairs glucocorticoid signal transduction. We showed that hGRαF737L demonstrated ligand-exposure time-dependent decreased transcriptional activity, reduced affinity for the ligand, and a marked delay in nuclear translocation. The mutant receptor preserved its ability to bind to glucocorticoid-response elements, interacted with the GRIP1 coactivator *in vitro* only through its AF-1 domain, and exerted a dominant-negative effect on the transcriptional activity of hGRα only after up to 6 h of exposure to dexamethasone. These findings suggest that hGRαF737L causes generalized glucocorticoid resistance by affecting multiple steps in the cascade of hGRα action, and further

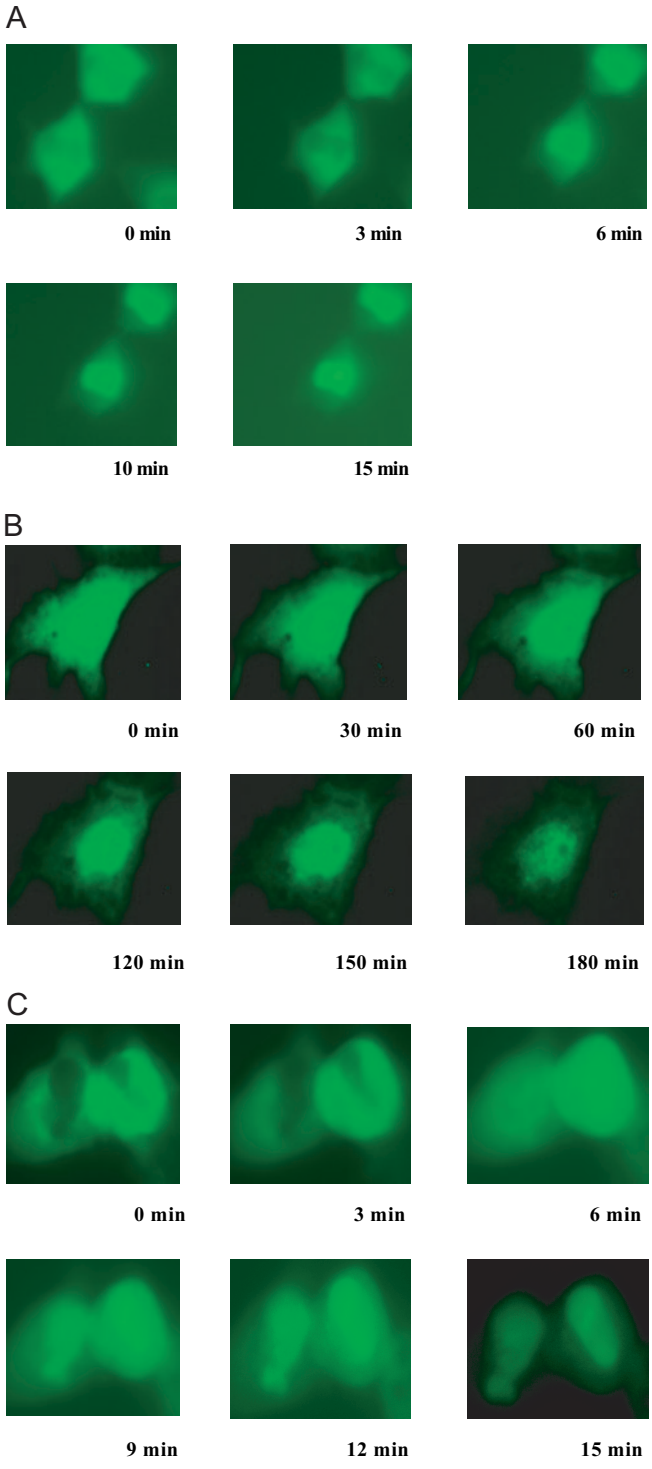

FIG. 2. Nuclear translocation of GFP-hGRα (A), GFP-hGRαF737L (B), and GFP-hGRα in the presence of pRShGRαF737L (C) before and after exposure to dexamethasone. HeLa cells transiently expressing GFP-hGRα or GFP-hGRαF737L were exposed to the same concentration of dexamethasone (10^{-6} M). Images of the same cells were obtained at the indicated time points.

underscore the importance of the C terminus of hGRα LBD in conferring transactivational activity.

The decreased affinity of the mutant receptor hGRαF737L for the ligand most likely reflects the location of the F737L mutation in helix (H) 11 of the LBD of hGRα. The structure of the hGRα LBD contains 12 α-helices and four small β-strands that fold into a three-layer helical domain (17) (Fig. 1). The ligand-binding pocket of hGRα has a similar architecture to that of other nuclear receptors and can be described as a cavity closed by a lid, which involves residues from H11 and H12. Divergent residues contributing to the ligand-binding pocket may determine ligand specificity. For example, the C742G mutation of the mouse glucocorticoid receptor, which corresponds to the C736G mutation of hGRα and is located in the lid region of the hGR ligand-binding pocket in H11, has been associated with dexamethasone-resistant lymphoma (18). Therefore, the presence of the F737L mutation in the LBD of the receptor may affect the affinity of the receptor for the ligand directly.

Upon ligand binding, the receptor undergoes major conformational changes, which alter the position of H11 and H12 and generate an interaction surface that allows coactivators to bind to the LBD through their LXXLL motifs. That hGRαF737L interacted with the GRIP1 coactivator *in vitro* only through its AF-1 highlights the importance of H11 of the LBD of the receptor in facilitating the formation of the AF-2 surface that interacts with coactivators (17, 18).

The mutant receptor hGRαF737L was localized in both the cytoplasm and nucleus of cells in the absence of ligand, whereas exposure to dexamethasone induced a markedly delayed nuclear translocation, which required up to 3 h. These findings suggest that the F737L mutation affects the nucleocytoplasmic shuttling of hGRα, probably through impairment of nuclear localization signal (NL) 1 and/or NL2 function (19). Given that the hGRβ isoform, which has a defective LBD, as well as hGRα mutants lacking their LBD, constitutively localize primarily in the nucleus (10), it is likely that the LBD of hGRα plays an important role in the cytoplasmic retention of the receptor in the absence of ligand. Therefore, a single-point mutation resulting in amino acid substitution within the LBD of hGRα might alter this activity of the LBD, resulting in nuclear retention of the receptor. Alternatively, defective mechanisms that relate to delayed nuclear export might account for the nuclear localization of the unliganded hGRαF737L (20), an effect that might be similar to the nuclear retention of hGRβ (10).

We conclude that hGRαF737L causes generalized glucocorticoid resistance by affecting multiple steps in the cascade of the glucocorticoid receptor signaling pathway. These findings confirm the importance of the C terminus of the LBD of hGRα in conferring transactivational activity.

Acknowledgments

Received December 20, 2006. Accepted July 10, 2007.

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This work was supported in part by the intramural program of the National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland.

Disclosure Statement: The authors have nothing to disclose.

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