Published in final edited form as:

Methods Mol Biol. 2009 ; 590: 33–60. doi:10.1007/978-1-60327-378-7_3.

Identification of Natural Human Glucocorticoid Receptor (hGR) Mutations or Polymorphisms and their Functional Consequences at the Hormone-Receptor Interaction Level

EVANGELIA CHARMANDARI, GEORGE P. CHROUSOS, and TOMOSHIGE KINO

Division of Endocrinology and Metabolism (E.C., G.P.C.), Biomedical Research Foundation of the Academy of Athens, Athens, 11527, Greece; Section on Pediatric Endocrinology (E.C., G.P.C., T.K.), Program in Reproductive and Adult Endocrinology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, U.S.A.

Abstract

Glucocorticoids regulate a broad spectrum of physiologic functions essential for life and play an important role in the maintenance of basal and stress-related homeostasis. At the cellular level, the actions of glucocorticoids are mediated by the human glucocorticoid receptor α (hGR α), a ligand-dependent transcription factor ubiquitously expressed in almost all tissues and cells. The molecular mechanisms of hGR α action involve (1) binding to glucocorticoids, (2) cytoplasmic to nuclear translocation, (3) binding/association to DNA/chromatin, (4) transcriptional activation or repression by interacting with cofactors and other transcription factors. Mutations or polymorphisms in the hGR gene may impair these molecular mechanisms of hGR α action, thereby altering tissue sensitivity to glucocorticoids. The latter may take the form of *glucocorticoid resistance* or *glucocorticoid hypersensitivity* and may be associated with significant morbidity. The identification of natural pathologic mutations in patients' hGR gene and the subsequent examination of the functional defects of the natural mutant hGR α receptors enhances our understanding of the molecular mechanisms of hGR α action and highlights the importance of integrated cellular and molecular signaling mechanisms for maintaining homeostasis and preserving normal physiology.

Keywords

cytoplasmic to nuclear translocation; dexamethasone binding assay; gene sequencing; fluorescent recovery after photobleaching; glucocorticoid receptor; glucocorticoid resistance; glucocorticoid hypersensitivity; green fluorescent protein; glutathione-S transferase pull-down assay; reporter assay; thymidine incorporation assay; transfection

1. Introduction

Glucocorticoids are steroid hormones synthesized and secreted by the adrenal cortices under the influence of hypothalamic-pituitary-adrenal (HPA) axis. Glucocorticoids regulate a broad spectrum of physiologic functions essential for life and play an important role in the maintenance of basal and stress-related homeostasis (1–3). At the cellular level, the actions of glucocorticoids are mediated by the human glucocorticoid receptor α (hGR α), a ligand-

Author for Correspondence: Tomoshige Kino, M.D., Ph.D. Section on Pediatric Endocrinology Program in Reproductive and Adult Endocrinology Eunice Kennedy Shriver National Institute of Child Health & Human Development National Institutes of Health Bldg. 10, Clinical Research Center, Rm. 1E-3140 10 Center Drive MSC 1109 Bethesda, MD 20892-1109, USA Phone: 301-496-6417 Fax: 301-402-0884 kinot@mail.nih.gov.

dependent transcription factor ubiquitously expressed in almost all tissues and cells (4,5). The gene encoding hGR α (hGR gene) is located in the long arm of chromosome 5 (q31.3), and consists of 9 exons, spanning over 150 kb (Figure 1 A). Expressed hGR α is a panel of 8 amino terminal translational isoforms of varying lengths, each of which consists of three subdomains, the N-terminal (NTD), the DNA-binding (DBD) and the ligand-binding (LBD) domains. In our expression and functional studies referred to here we have employed as representative the longest GR α isoform consisting of 777 amino acids. The GR gene also produces an equal number of hGR β isoforms by the use of an alternative 3' exon 9 β , which cannot bind glucocorticoids and function as dominant negative isoforms for hGR α (1).

In the absence of ligand, hGR α resides mostly in the cytoplasm of cells as part of a hetero-oligomeric complex, which contains chaperon heat shock proteins (HSPs) 90, 70, 23 and FKBP51, as well as other proteins (6) (Figure 1 B). Upon ligand-induced activation, the receptor dissociates from this multiprotein complex and translocates into the nucleus through the nuclear pore with the energy-dependent mechanism that includes importin α and β . Inside the nucleus, hGR α binds as a homodimer to glucocorticoid response elements (GREs) in the promoter regions of target genes and regulates their expression positively or negatively (6–9). The ligand-activated hGR α can also modulate gene expression independently of DNA-binding, by interacting, possibly as a monomer, with other transcription factors, such as activator protein-1 (AP-1), nuclear factor- κ B (NF- κ B), p53 and signal transducers and activators of transcription (STATs) (10–13).

To initiate transcription, hGR α uses its transcriptional activation domains, activation function (AF)-1 and AF-2, located in NTD and LBD, respectively, as surfaces to interact with the so called cofactors, such as nuclear receptor coactivators and chromatin-remodeling complexes (14–18). The p160 coactivators, including the steroid receptor coactivator 1 and the glucocorticoid receptor-interacting protein 1 (GRIP1), play an important role in the hGR α -mediated transactivation of glucocorticoid-responsive genes, interacting directly with both the AF-1 of hGR α through their carboxyl-terminal domain and the AF-2 through multiple amphipathic LXXLL signature motifs located in their nuclear receptor-binding (NRB) domain (19). They also have histone acetyltransferase activity through which they acetylate multiple lysine residues located in the N-terminal tail of chromatin-associated histones and facilitate access of other transcription factors, cofactors and the transcription initiation complex composing the RNA-polymerase II and its ancillary components to initiate and promote transcription (14–17) (Figure 1 C).

Mutations or polymorphisms in the hGR gene impair one or more of the molecular mechanisms of hGR α action and alter tissue sensitivity to glucocorticoids (Figure 2). Alterations in tissue sensitivity to glucocorticoids may take the form of *glucocorticoid resistance* or *glucocorticoid hypersensitivity* and may be associated with significant morbidity (20–22). In previous studies, we have identified most of the known pathologic mutations in the hGR gene by sequencing the patients' genomic DNA, and have systematically investigated the molecular mechanisms through which various natural hGR α mutant receptors affect glucocorticoid signal transduction (Figure 2 A). The mechanisms studied included: i) the affinity of the mutant receptors for the ligand; ii) the *in vivo* sensitivity to glucocorticoids, using the thymidine incorporation assays as a method that evaluates the overall biologic effect of glucocorticoids in the patients' circulating mononuclear cells; iii) the subcellular localization of the mutant receptors and their nuclear translocation following exposure to the ligand; iv) the ability of the mutant receptors to bind to GREs; v) the transcriptional activity of the mutant receptors; vi) the ability of the mutant receptors to exert a dominant negative effect upon the wild-type receptor; vii) the interaction of the mutant receptors with the GRIP1 coactivator; and viii) the motility of the mutant receptors within the nucleus of living cells in order to evaluate their dynamic interactions to nuclear components (22).

2. Materials

2.1. Amplification and Sequencing of the hGR Gene

1. AmpliTaq DNA Polymerase (Invitrogen, Carlsbad, CA)
2. BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA)
3. Wizard Genomic DNA Purification Kit (Promega Corp. Madison, WI)
4. GeneAmp PCR System 9700 (Applied Biosystems)
5. ABI Prism 310 Genetic Analyzer (Applied Biosystems)

2.2. Cell Culture and Lysis

1. CV-1 and COS-7 embryonic African green monkey kidney cells, HeLa human cervical uterine carcinoma cells, and HCT-116 human colon carcinoma cells stably transfected with pMAM-neo-luc (Clontech, Palo Alto, CA), which contains the full-length mouse mammary tumor virus promoter and a neomycin (G418)-resistant cassette. All cells were purchased from American Type Culture Collection (Manassas, VA).
2. Phenol red-containing or -free Dulbecco's Modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS, HyClone, Ogden, UT) and antibiotics (Invitrogen).
3. McCoy's 5A medium (Invitrogen) supplemented with 10% FBS, neomycin (G418) and antibiotics.
4. RPMI1640 medium (Invitrogen) supplemented with or without 10% FBS and antibiotics.
5. Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ)
6. Trypsin EDTA 0.25% (Invitrogen).
7. Six-well plates, 75 cm² flasks, 35-mm-diameter dishes and 150-mm-diameter dishes (Corning Inc., Washington, DC).

2.3. Whole Cell Dexamethasone Binding Assays

1. [1,2,4,6,7-³H]-Dexamethasone, 70–110 Ci/mmol, 2.6–4.1 TBq/mmol (GE Healthcare).
2. Non-radioactive dexamethasone (Sigma Chemical Co., St Louis, MO).
3. β -Counter (Beckman LS6000IC counter, Beckman Coulter Inc., Fullerton, CA).

2.4. Thymidine Incorporation Assays

1. Thymidine, [Methyl-³H], 40–60 Ci/mmol; 1.48–2.22 TBq/mmol (MP Biologicals, Solon, OH)
2. Phytohaemagglutinin (PHA) (Sigma Chemical Co.)
3. β -Counter (Beckman LS6000IC counter).

2.5. Transient Transfection Assays

1. Lipofectin (Invitrogen).

2. FuGENE 6 (Roche Applied Science, Indianapolis, IN).
3. Opti-MEM (Invitrogen)

2.6. Western Blot Analyses

1. Lysis buffer consisting of 100 mM Tris-HCl [pH: 8.5], 250 mM NaCl, 1% Nonidet P-40 [pH: 7.2] and protease inhibitors (1 tablet/50 mL of lysate) (Complete™, Roche Applied Science).
2. Tris-Glycine SDS Sample Buffer (2x) (Invitrogen).
3. Molecular weight prestained markers (SeeBlue Plus 2 Prestained Protein Standard, Invitrogen).
4. 8% Tris-Glycine Gel (Invitrogen).
5. Hybond C nitrocellulose membranes (GE Healthcare).
6. TBS-T buffer (50 mM of 1M Tris-HCL [pH 8.5], 10 mM of 5M NaCl and 0.5% of Tween 20).
7. Blocking solution [5% dry milk powder/TBS-T buffer (50 mM of 1M Tris-HCL [pH 8.5], 10 mM of 5M NaCl and 0.5% of Tween 20)].
8. Purified specific rabbit polyclonal anti-hGR α antibody (Affinity BioReagents, Golden, CO).
9. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Affinity BioReagents).
10. ECL Plus Western Blotting Detection System (GE Healthcare).
11. High performance chemiluminescence film (Hyperfilm ECL, GE Healthcare).

2.7. Detection and Localization of Green Fluorescent Protein (GFP)-fused hGRs

1. The pF25GFP vector (a gift from Dr. G.N. Pavlakakis, National Institutes of Health, Frederick, MD), pEGFP-C1 vector (Clontech).
2. The green fluorescent protein (GFP)-fused plasmid expressing hGR α , which is constructed by subcloning the hGR α cDNA into the pF25GFP vector/pEGFP-C1, as previously described (23).
3. The GFP-fused plasmids expressing mutant hGR α receptors are constructed by introducing the corresponding mutations into the pF25GFP-hGR α using PCR-assisted site-directed mutagenesis.
4. 10% charcoal/dextran-treated FBS (Hyclone).
5. Inverted fluorescence microscope (Leica DM IRB, Wetzlar, Germany) with fluorescent source, or a confocal microscope (Zeiss LSM510 Inverted Meta/Zeiss Axiovert 200M microscope, Carl Zeiss, Thornwood, NY).
6. Digital charge-coupled device (CCD) camera (Hamamatsu Photonics K.K., Hamamatsu, Japan).
7. Openlab software (Improvision, Boston, MA).

2.8. Chromatin Immunoprecipitation (ChIP) Assays

1. 1% formaldehyde
2. Lysis buffer (10 mM Tris-HCl [pH 7.5], 3 mM CaCl₂, 2 mM MgCl₂).

3. Nonidet P-40 (NP-40) lysis buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40).
4. Buffer containing 50 mM Tris-HCl [pH 8.0], 25% glycerol, 5 mM (CH₃COO)₂Mg and 0.1 mM EDTA.
5. Chromatin Immunoprecipitation (ChIP) dilution buffer (16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, protease inhibitors).
6. Misonix sonicator 3000 (Misonix Inc., Farmingdale, NY).
7. Anti-hGR α antibody (Affinity Bioreagents).
8. Low salt wash buffer (20 mM Tris-HCl [pH 8.1], 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA).
9. High salt wash buffer (20 mM Tris-HCl [pH 8.1], 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA).
10. LiCl wash buffer (10 mM Tris-HCl [pH 8.1], 0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA).
11. TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).
12. Elution buffer (100 mM NaHCO₃, 1% SDS).
13. Primer set: forward primer: 5'-AACCTTGCGGTTCCCAG-3'; reverse primer: 5'-GCATTACATAAGATTTGG-3'.
14. 2% Agarose gel (?Company)
15. Ethidium bromide (?Company)

2.9. Reporter Assays

1. The pRShGR α plasmid, which expresses hGR α under the control of the Rous sarcoma virus (RSV) promoter (American Type Culture Collection).
2. The plasmids expressing various natural hGR α mutant receptors are constructed by introducing the corresponding mutations into the pRShGR α plasmid using PCR-assisted site-directed mutagenesis (Stratagene, La Jolla, CA).
3. The plasmid pRSV-erbA⁻¹, which contains a thyroid receptor cDNA in inverse orientation and is used as a negative control in the appropriate experiments (a gift from Dr. R.M. Evans, Salk Institute, La Jolla, CA).
4. The pMMTV-luc plasmid, which expresses luciferase under the control of the glucocorticoid-responsive mouse mammary tumor virus (MMTV) promoter (a gift from Dr. G.L. Hager, National Institutes of Health, Bethesda, MD).
5. The pSV40- β -gal plasmid, which encodes the β -galactosidase gene under the control of the simian virus (SV) 40 promoter (Promega Corp.).
6. Opti-MEM.
7. Dexamethasone (Sigma Chemical Co.).

2.10. Luciferase and β -Galactosidase Assays

1. Reporter lysis buffer (Promega Corp.).
2. Assay buffer solution (25 mM Gly-Gly (Sigma Chemical Co.), 10 mM ATP, 25 mM MgSO₄ and 1% Triton-X 100 [pH 8.0]).

3. Monolight 3010 Luminometer (BD PharMingen, San Diego, CA).
4. D-Luciferin sodium salt solution (BD PharMingen).
5. β -Galactosidase enzyme assay system (Galacto-Light Plus, Tropix, Bedford, MA).

2.11. Glutathione-S Transferase (GST) Pull-down Assays

1. The pGEX-4T3 vector (GE Healthcare).
2. The plasmids pGEX4T3-GRIP1(1–1462), pGEX4T3-GRIP1(596–774) and pGEX4T3-GRIP1(740–1217), which express GST-fused full-length-GRIP1, nuclear receptor-binding fragment of GRIP1 and carboxyl-terminal fragment of GRIP1, respectively, are constructed by subcloning the corresponding GRIP1 fragments of cDNA into the pGEX-4T3 vector.
3. E. coli, BL21 strain (Stratagene)
4. Isopropyl β -D-1-thiogalactopyranoside (IPTG) (GE Healthcare)
5. Glutathione Sepharose 4B Fast Flow beads (GE Healthcare)
6. Binding buffer (50 mM Tris-HCL [pH 8.0], 50 mM NaCl, 0.1% NP-40, 1 mM EDTA, 10% glycerol and 1mg/mL of bovine serum albumin fraction V (Sigma Chemical Co.) [pH 7.0]).
7. Coupled *in vitro* transcription/translation reactions (TNT Quick Coupled Transcription/Translation System, Promega Corp.).
8. [35 S] L-methionine, >400 Ci/mmol; >14.8 TBq/mmol Solution, *in vitro* translation grade (MP Biologicals)
9. The pBK-CMV vector (Stratagene).
10. The pBK/CMV-wild-type and mutant hGR α plasmids are constructed by subcloning the corresponding cDNA into the pBK-CMV vector (22).
11. 8% SDS-PAGE gel.
12. Enlightning buffer (NEN Life Science Products, Inc., Boston, MA).
13. Luria-Bertani (LB) broth
14. X-ray films

2.12. Confocal Microscopy and Fluorescent Recovery After Photobleaching (FRAP)

1. Plasmids expressing GFP-fused wild-type and mutant hGR α receptors.
2. African monkey kidney COS-7 cells.
3. Delta-T culture dishes (Bioprotechs, Butler, PA).
4. Zeiss LSM510 upright 2-photon meta/Zeiss Axioskop 2 microscope (Carl Zeiss).
5. Water immersion Zeiss Achroplan $\times$ 63, 0.9 NA, IR (working distance 2.2 mm) objective lens (Carl Zeiss).

3. Methods

3.1. Amplification and Sequencing of the hGR gene

Genomic DNA is extracted from peripheral mononuclear cells using the Wizard Genomic DNA Purification Kit, according to the instructions of the manufacturer (Promega Corp.). Eight

exonic sequences of the GR gene, which contain the coding region of the hGR α , are amplified by the polymerase chain reaction (PCR) using the AmpliTaq DNA polymerase in the GeneAmp PCR System 9700. The primers used for PCR amplification of genomic DNA are designed using the exon/intron junction sequences (Table 1). Initiation is performed at 94°C for 7 min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 55°C (exons 3–7, 9) or 60°C (exons 2, 8) for 30 sec and extension at 72°C for 1 min, and a final period of extension at 72°C for 7 min. Amplified DNAs are further sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and one of the primers is (?) used for the amplification or appropriate internal primers, according to the instructions of the manufacturer (Applied Biosystems). We use an automatic sequencer (ABI Prism 310 Genetic Analyzer) to read sequences.

3.2. Cell Culture and Lysis

Mononuclear cells in whole blood are separated by using Ficoll Paque PLUS, and are cultured in RPMI1640 supplemented with 10% FBS and antibiotics. CV-1 and COS-7 embryonic African green monkey kidney cells, and HeLa human cervical uterine carcinoma cells are grown in DMEM supplemented with 10% FBS and antibiotics. HCT-116 human colon carcinoma cells stably transfected with pMAM-neo-luc are grown in McCoy's 5A medium supplemented with 10% FBS, G418 (0.4 mg/mL) and antibiotics. Cells are incubated in a humidified atmosphere of 5% CO₂ at 37°C and passaged every 3 to 4 days. Twenty-four hours before transfection, subconfluent cells are removed from their flasks by trypsinization, resuspended in supplemented medium and plated in six-well plates (CV-1, COS-7), 75 cm² flasks (CV-1, COS-7), 35-mm-diameter dishes (HeLa) or 150-mm-diameter dishes (HCT-116) at a concentration of 1.5×10^5 cells/well, 1×10^6 cells/flask, 1.5×10^5 cells/35-mm dish and 2.5×10^6 cells/150-mm dish, respectively.

3.3. Whole Cell Dexamethasone Binding Assays

COS-7 cells are seeded in six-well plates (1.5×10^5 cells/well) and transfected with the wild-type or mutant hGR α (1.5 μ g/well) using lipofectin (24). Six hours later, the transfection medium is replaced with DMEM supplemented with 10% FBS and antibiotics. Confluent cells are incubated in plain DMEM with six different concentrations (1.56nM, 3.125nM, 6.25nM, 12.5nM, 25nM and 50nM) of [1,2,4,6,7-³H]-Dexamethasone at 37°C in the presence or absence of a 500-fold molar excess of non-radioactive dexamethasone for 1 hour. After incubation, cells are washed with PBS (3 mL/well) twice to remove free steroid. Cells are harvested, centrifuged at 1,300 rpm for 15 min and washed with PBS twice. Cells are transferred to scintillation vials, and the radioactivity is measured using a β -counter. Specific binding is calculated by subtracting nonspecific from total binding, and these data are analyzed using the Scatchard method. Binding capacity is expressed as femtomoles per 10^6 cells, and the apparent dissociation constant (K_d) is expressed in nanomolar concentrations (nM).

3.4. Thymidine Incorporation Assays

Mononuclear leukocytes isolated from patients and control subjects are seeded in 24-well plates (10^5 cells/well) with 0.5 ml of RPMI1640 supplemented with 10% FBS and antibiotics, and are incubated at 37°C for 2 hours. They are then stimulated with PHA (5 μ g/well) and exposed to seven different concentrations (0 nM, 2.5 nM, 10 nM, 25 nM, 100 nM, 250 nM, 1000 nM) of dexamethasone. Following incubation at 37°C for 72 hours, [³H]-thymidine is added to each well (0.1 μ Ci/well) and incubation is continued for a further 4 hours. Subsequently, cells are transferred to eppendorf tubes, centrifuged for 1–2 min at 3000rpm, and 0.5 ml of 10% trichloroacetic acid is added to each tube after removing the supernatant. Cell lysates are transferred to scintillation vials and the radioactivity is measured using a β -counter.

3.5. Transient Transfection Assays

CV-1, COS-7 and HCT-116 cells are transfected using the lipofectin method, as previously described (24). HeLa cells are transfected using FuGENE 6 reagent according to the instructions of the manufacturer (Roche Applied Science). The FuGENE 6 to transfected DNA ratio is 2:1.

3.6. Western Blot Analyses

CV-1 and COS-7 cells are seeded in 75 cm² flasks at a concentration of 1×10^6 cells/flask and grown in supplemented DMEM. Subconfluent cells are transfected with wild-type or mutant hGR α (15 μ g/flask) using the lipofectin method (24). Twenty-four hours (CV-1) or six hours (COS-7) after transfection, the transfection medium is replaced with supplemented DMEM. Twenty-four hours later, cells are washed with ice-cold PBS three times, gently scraped from their flasks, centrifuged briefly and lysed using a lysis buffer consisting of 100 mM Tris-HCl [pH: 8.5], 250 mM NaCl, 1% Nonidet P-40 [pH: 7.2] and protease inhibitors (1 tablet/50 mL of lysate). The homogenates are centrifuged (500xg at 4°C) for 5 min to obtain whole cell extracts. Whole cell extracts are mixed with an equal amount of Tris-Glycine SDS Sample Buffer (2x), heated to 95°C for 3 min, and electrophoresed alongside molecular weight prestained markers through 8% Tris-Glycine Gel. Following electroblotting (25V/0.8 mA/cm²) onto Hybond C nitrocellulose membranes, proteins are incubated with blocking solution for 4 hours. Immunoblotting is performed at 4°C overnight, using purified specific rabbit polyclonal anti-hGR α antibody at 10 μ g/mL. After washing with TBS-T three times, membranes are incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG at 1:1000 dilution at room temperature for 1 hour. The wild-type and mutant hGR α receptors are visualized using the ECL Plus Western Blotting Detection System and exposed to high performance chemiluminescence film.

3.7. Detection and Localization of Green Fluorescent Protein (GFP)-fused hGRs

HeLa cells are plated on coated 35-mm-diameter dishes (1.5×10^5 cells/well) in supplemented DMEM. Twenty-four hours later, cells are transfected with GFP-fused wild-type or mutant hGR α expressing plasmids (2 μ g/dish) using FuGENE 6. In further experiments, and in order to determine the effect of the mutant receptors upon the nuclear translocation of the wild-type receptor, HeLa cells are transfected with equal amounts of GFP-fused wild-type and mutant hGR α (1 μ g/dish). Forty-eight hours after transfection, the medium is replaced by phenol red-free DMEM supplemented with 10% charcoal/dextran-treated FBS and antibiotics. Sixteen hours later, cells are exposed to dexamethasone (10^{-6} M) and fluorescence is detected sequentially by an inverted fluorescence microscope or a confocal microscope, as previously described (26). Twelve-bit black-and-white images are captured using a digital CCD camera. Image analysis and presentation are performed using the Openlab software. Representative images of the nuclear translocation of GFP-hGR α are shown in Figure 3.

3.8. Chromatin Immunoprecipitation (ChIP) Assays

HCT-116 human colon carcinoma cells stably transfected with pMAM-neo-luc are seeded in 150-mm-diameter dishes (2.5×10^6 cells/dish) and grown in supplemented McCoy's 5A medium. Subconfluent cells are transiently transfected with wild-type or mutant pRShGR α (15 μ g/dish) using lipofectin (24). Three hours later, the transfection medium is replaced with supplemented McCoy's 5A medium. Sixteen hours after transfection, cells are treated with dexamethasone (10^{-6} M) or vehicle (100% ethanol) for 3–6 hours. Cells are fixed using 1% formaldehyde at 37°C for 10 min, harvested by using cell-scrapers, and re-suspended in ice-cold lysis buffer (10 mM Tris-HCl [pH 7.5], 3 mM CaCl₂, 2 mM MgCl₂). Swollen cells are resuspended with equal volumes of lysis buffer and Nonidet P-40 (NP-40) lysis buffer, homogenized in a Dounce homogenizer, and centrifuged at 1,400 rpm at 4°C for 5 min. Nuclear

pellets are stored in a buffer containing 50 mM Tris-HCl [pH 8.0], 25% glycerol, 5 mM $(\text{CH}_3\text{COO})_2\text{Mg}$ and 0.1 mM EDTA at -80°C (27).

Equal amounts of nuclei (100 μg) are used for each immunoprecipitation experiment. Nuclei are diluted in 500 μL of ChIP dilution buffer, sonicated 8 times for 10 sec (at 10 sec intervals) and centrifuged at 14,000 rpm for 5 minutes. Supernatants are precleared and the amount of DNA-protein complexes present in each sample is measured at 260 nm. Equal amounts of chromatinosomes are immunoprecipitated with anti-hGR α antibody at 4°C for 14 hours. Immunocomplexes are captured on protein A-agarose, washed sequentially with low salt wash buffer, high salt wash buffer, LiCl wash buffer and twice with TE buffer. Samples are eluted with freshly prepared elution buffer at room temperature for 15 min twice, and after adding 20 μL of 5M NaCl, cross-linking is reverted at 65°C for 4 hours. Following treatment with 0.5 M EDTA, 1M Tris-HCL [pH 6.5] and proteinase K (10 mg/mL) at 45°C for 1 hour, genomic DNA fragments are extracted with phenol/chloroform and precipitated with ethanol.

Primer sets are designed to amplify the MMTV promoter region, which contains two GREs and is located approximately 250 bps upstream of the transcription initiation site (forward primer: 5'-AACCTTGCGGTTCCAG-3'; reverse primer: 5'-GCATTACATAAGATTTGG-3').

Equal volumes of DNA are used for PCR amplification of the MMTV promoter region. Initiation is performed at 94°C for 7 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min, and a final period of extension at 72°C for 7 min. PCR-amplified products (173 bp) are electrophoresed on 2% agarose gel and visualized by ethidium bromide staining.

3.9. Reporter Assays

CV-1 cells are seeded in six-well plates at a concentration of 1.5×10^5 cells/well. Twenty-four hours later, cells are cotransfected with wild-type hGR α , mutant hGR α or a control plasmid (pRSV-erbA $^{-1}$) (0.05 μg /well), pMMTV-luc (0.5 μg /well) and pSV40- β -gal (0.1 μg /well). For experiments designed to determine whether the mutant receptor exerts a dominant negative or positive effect upon the wild-type receptor, CV-1 cells are cotransfected with pMMTV-luc (0.5 μg /well), pSV40- β -gal (0.1 μg /well), a constant amount of wild-type hGR α (0.05 μg /well) and five, progressively increasing concentrations of the mutant receptor, so that the ratio between the wild-type and mutant receptor would range from 1:0 to 1:10 (1:0, 1:1, 1:3, 1:6, 1:10). pRSV-erbA $^{-1}$ is added in appropriate quantities to maintain a constant amount of DNA in each well. Twenty-four hours later, the transfection medium is replaced with supplemented DMEM. Forty-eight hours after transfection, dexamethasone or vehicle (100% ethanol) is added to the medium at a concentration of 10^{-6} M.

3.10. Luciferase and β -Galactosidase Assays

Seventy-two hours after transfection, cells are washed with PBS twice, and lysed at room temperature using a reporter lysis buffer. 350 μL of assay buffer solution (25 mM Gly-Gly, 10 mM ATP, 25 mM MgSO_4 and 1% Triton-X 100 [pH 8.0]) are added to 50 μL of cell lysates. Luciferase activity in the cell lysates is determined in a Monolight 3010 Luminometer using as substrate 100 μL of 1 mM D-Luciferin sodium salt solution, as previously described (25). β -Galactosidase activity is determined in the same samples using a β -galactosidase enzyme assay system according to the instructions of the manufacturer (Tropix). Luciferase activity is divided by β -galactosidase activity to account for transfection efficiency.

3.11. Glutathione-S Transferase (GST) Pull-down Assays

E. coli (B21 strain) are transformed with GST-fusion protein expression vectors [GST-fused GRIP1(1–1462), GRIP1(559–774) and GRIP1(740–1217)], and are grown in LB broth overnight. On the day of purifying GST-fused proteins, transformed bacteria are cultured at 37°C for 2–3 hours by adding 1/10 volume of its confluent solution into LB broth. When OD_{600nm} of the culture reaches to 0.6–0.8, IPTG (0.5 mM) is added to express GST fusion proteins, and cells are further cultured for 2 hours. They are then collected by centrifugation, re-suspended in 900 µL of PBS, and cell-extracts are generated by pulse sonication (Misonix Sonicator 3000) on ice (10 sec). Cell debris is then pelleted at 14,000 rpm at 4°C for 10 min. Fifty percent slurry of Glutathione Sepharose 4B Fast Flow beads (60 µL) is added to the cell extracts and allowed to bind by gentle agitation at 4°C for 2 hours in binding buffer (50 mM Tris-HCL [pH 8.0], 50 mM NaCl, 0.1% NP-40, 1 mM EDTA, 10% glycerol and 1mg/mL of albumin bovine fraction [pH 7.0]). The beads with the bound GST fusion protein are then pelleted, washed three times with 1 mL of the same buffer, and analyzed by SDS-PAGE for the amount of protein bound to the GST-beads (28).

Coupled *in vitro* transcription/translation reactions are used to produce ³⁵S-labelled wild-type (WT) and mutant hGRα in rabbit reticulocyte lysate by using pBK/CMV-hGRαWT and pBK/CMV-hGRαMutant, respectively, as templates. ³⁵S-labelled hGRαWT and pBK/CMV-hGRαMutant (4–20 µL of crude translated protein) are incubated with GST fusion proteins bound to Glutathione Sepharose 4B beads, washed, eluted and fractionated by SDS-PAGE. Samples are loaded and electrophoresed on an 8% SDS-PAGE gel. The percent of starting material loaded in input lanes is 3 or 5%. The gel is fixed, treated with Enlightning buffer and dried. Radioactivity is detected by exposing a film on the gel.

3.12. Confocal Microscopy and Fluorescent Recovery After Photobleaching (FRAP)

COS-7 cells, cultured in Delta-T culture dishes and transfected with pF25-hGRs are treated for 4 hours with 10^{−6} M dexamethasone 24 hours after transfection. Before photobleaching, the media are replaced with Hanks balanced salt solution (HBSS) containing 10% FBS, and the cells are examined under a microscope. Emitted signals are recorded at 37 ± 0.5°C with the Zeiss LSM510 upright 2-photon meta/Zeiss Axioskop 2 microscope (29). A water immersion Zeiss Achroplan × 63, 0.9 NA, IR (working distance 2.2 mm) objective lens is used for image acquisition. Confocal images are built point by point by collecting the intensities from the photo-multiplier tube using Zeiss LSM 5 software version 3.2 (Carl Zeiss). For FRAP, GFP is excited with an argon laser at 488 nm, and emission is collected using the Meta detector with custom emission range from 495 to 590 nm. Images are taken every 63 ms at zoom factor 3 and resolution 128 by 128 pixels (pixel size, 0.38 by 0.38 µm; pixel time, 3.84 s). After the first 2 images, a selected rectangular region of fixed size (11.02 by 2.28 µm) in the nucleus is bleached at a set laser power of 15 mW for 50 iterations. Fluorescence in the bleached region is measured as a function of time using the LSM software. To account for bleaching to laser scanning, the intensity of an identical area in a distant nuclear area is also measured with time.

To correct for differences in the expression level between individual cells, fluorescence data for the bleached and control areas in the nucleus are normalized as fractional recovery: $R = (F - F_0)/(F_{\text{infinite}} - F_0)$. In addition, results obtained from the bleached area are normalized to those obtained from the control area to account for attenuation of fluorescence due to laser scanning, as previously described (30). Using the obtained FRAP curve, the $t_{1/2}$ of maximal recovery is determined, which is defined as the time point after bleaching at which the normalized fluorescence has increased to half the amount of the maximal recovery. A representative FRAP curve is shown in Figure 4, with analysis (A) and photobleaching images (B) (31). Recovery $t_{1/2}$ values obtained from a population of cells for each procedure are statistically analyzed.

4. Notes

1. For all methods using live cells, the cell viability is very important to obtain reproducible results. Keep cells in a good condition by replating them every 3–4 days, depending on their growth rates.
2. Dexamethasone and other steroids are usually dissolved easily in 100% ethanol. Add them directly into culture media or reactions with 1000x higher solutions of the final concentrations. These concentrated solutions can be kept at –20 and –80°C over 6 months.
3. **Amplification and Sequencing of the hGR Gene:** Concentrations of MgCl₂ should be adjusted in the PCR for appropriate amplification of the hGRα exons from the patients' genomic DNA. If several non-specific bands are amplified with the target DNA, gel-purification of the band may be required for obtaining clear sequence results. Ready Reaction Mixture of the BigDye Terminator v3.1 Cycle Sequencing Kit can be used with ½ or ¼ volumes of the original reaction by adding the 5X buffer supplied by the Kit. After PCR reactions using the sequencing Kit, samples should be further purified with ethanol precipitation or appropriate columns to remove free fluorescent dyes.
4. **Whole Cell Dexamethasone Binding Assays:** Radioactivity of the culture media should be counted to estimate the concentrations of free radioactive dexamethasone for the Scatchard method.
5. **Thymidine Incorporation Assays:** Some of the cells are tightly attached on the bottom of the plates after 3-day culture. Trypsinization may be required for harvesting them.
6. **Western Blot Analyses:** Multiple protein bands usually appear with anti-GR antibody due to the presence of N-terminal translational isoforms of the hGRα (32).
7. **Detection and Localization of GFP-fused hGRs and FRAP Analyses:** GFP-fused hGRα easily leaks from the cytoplasm into the nucleus in cells with over-loaded expression. Such cells sometimes show delayed nuclear translocation of the GFP-hGRα. Thus, cells with its appropriate expression should be selected for the analyses. At least 5–10 different cells should be tested to evaluate the time required for cytoplasmic to nuclear translocation of GFP-hGRα. Exposure to the laser beam can damage cells and easily bleach GFP. Thus, exposure of the cells with laser should be kept as short as possible.
8. **ChIP Assays:** Since PCR can detect trace amounts of the MMTV GREs, contamination of GR-unbound DNA could cause false-positive results. Thus, the washing steps are crucial for successful reactions in ChIP assays. Quantitative real-time PCRs using the TaqMAN or the SYBR Green-based system represent an alternative to the regular PCR and subsequent gel analyses.
9. **GST Pull-down Assays:** If bacteria express low levels of GST-fused proteins, culture at 30°C with extended incubation may improve their production. Large-scale purification using the Glutathione Sepharose 4B Pre-packed Columns (bed volume: 1 or 5 ml) and the automated injection pump/fraction collector system (GE Healthcare) may be convenient for making stock of the GST-fused proteins. Cell lysis with sonication is complete when the cloudy cell suspension becomes translucent. The frequency and intensity of sonication should be adjusted so that complete lysis will occur in 10 seconds, without frothing (frothing may denature proteins). Before treating with Enlightning, gels should be fixed by incubating them with a buffer containing 10% methanol and 10% glacial acetic acid for 20 min, followed by washing with distilled water for an additional 20 min.

Acknowledgments

This book chapter was created based on the work supported in part by the Intramural Research Program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD.

References

1. Kino, T.; Chrousos, GP. Glucocorticoid effects on gene expression. In: Steckler, T.; Kalin, NH.; Reul, JMHM., editors. *Handbook of Stress and the Brain*. Amsterdam; Elsevier: 2005. p. 295-311.
2. Chrousos GP, Charmandari E, Kino T. Glucocorticoid action networks—an introduction to systems biology. *J. Clin. Endocrinol. Metab* 2004;89:563–564. [PubMed: 14764762]
3. Chrousos GP. The glucocorticoid receptor gene, longevity, and the complex disorders of Western societies. *Am. J. Med* 2004;117:204–207. [PubMed: 15300973]
4. Zhou J, Cidlowski JA. The human glucocorticoid receptor: one gene, multiple proteins and diverse responses. *Steroids* 2005;70(5–7):407–17. [PubMed: 15862824]
5. Duma D, Jewell CM, Cidlowski JA. Multiple glucocorticoid receptor isoforms and mechanisms of post-translational modification. *J Steroid Biochem Mol Biol* 2006;102(1–5):11–21. [PubMed: 17070034]
6. Pratt WB. The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. *J Biol Chem* 1993;268(29):21455–8. [PubMed: 8407992]
7. Terry LJ, Shows EB, Wentz SR. Crossing the nuclear envelope: hierarchical regulation of nucleocytoplasmic transport. *Science* 2007;318(5855):1412–6. [PubMed: 18048681]
8. Bamberger CM, Schulte HM, Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocr Rev* 1996;17(3):245–61. [PubMed: 8771358]
9. Schaaf MJ, Cidlowski JA. Molecular mechanisms of glucocorticoid action and resistance. *J Steroid Biochem Mol Biol* 2002;83(1–5):37–48. [PubMed: 12650700]
10. Jonat C, Rahmsdorf HJ, Park KK, Cato AC, Gebel S, Ponta H, Herrlich P. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* 1990;62(6):1189–204. [PubMed: 2169351]
11. Scheinman RI, Gualberto A, Jewell CM, Cidlowski JA, Baldwin AS Jr. Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. *Mol Cell Biol* 1995;15(2):943–53. [PubMed: 7823959]
12. Chrousos GP, Kino T. Intracellular glucocorticoid signaling: a formerly simple system turns stochastic. *Sci STKE* 2005;2005(304):pe48. [PubMed: 16204701]
13. Kino T, Chrousos GP. Tissue-specific glucocorticoid resistance-hypersensitivity syndromes: multifactorial states of clinical importance. *J Allergy Clin Immunol* 2002;109(4):609–13. [PubMed: 11941307]
14. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* 1999;20(3):321–44. [PubMed: 10368774]
15. McKenna NJ, Xu J, Nawaz Z, Tsai SY, Tsai MJ, O'Malley BW. Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions. *J Steroid Biochem Mol Biol* 1999;69:3–12. [PubMed: 10418975]
16. McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* 2002;108:465–474. [PubMed: 11909518]
17. Auboeuf D, Honig A, Berget SM, O'Malley BW. Coordinate regulation of transcription and splicing by steroid receptor coregulators. *Science* 2002;298:416–419. [PubMed: 12376702]
18. Hittelman AB, Burakov D, Iniguez-Lluhi JA, Freedman LP, Garabedian MJ. Differential regulation of glucocorticoid receptor transcriptional activation via AF-1-associated proteins. *EMBO J* 1999;18(19):5380–8. [PubMed: 10508170]
19. Heery DM, Kalkhoven E, Hoare S, Parker MG. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* 1997;387(6634):733–6. [PubMed: 9192902]

20. Kino T, De Martino MU, Charmandari E, Mirani M, Chrousos GP. Tissue glucocorticoid resistance/hypersensitivity syndromes. *J Steroid Biochem Mol Biol* 2003;85(2–5):457–467. [PubMed: 12943736]
21. Chrousos GP, Kino T. Glucocorticoid action networks and complex psychiatric and/or somatic disorders. *Stress* 2007;10(2):213–9. [PubMed: 17514590]
22. Charmandari E, Kino T, Ichijo T, Chrousos GP. Generalized glucocorticoid resistance: clinical aspects, molecular mechanisms, and implications of a rare genetic disorder. *J Clin Endocrinol Metab* 2008;93(5):1563–72. [PubMed: 18319312]
23. Kino T, Stauber RH, Resau JH, Pavlakis GN, Chrousos GP. Pathologic human GR mutant has a transdominant negative effect on the wild-type GR by inhibiting its translocation into the nucleus: importance of the ligand-binding domain for intracellular GR trafficking. *J Clin Endocrinol Metab* 2001;86(11):5600–8. [PubMed: 11701741]
24. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A* 1987;84(21):7413–7. [PubMed: 2823261]
25. Brasier AR, Tate JE, Habener JF. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. *Biotechniques* 1989;7(10):1116–22. [PubMed: 2698191]
26. Stauber RH, Horie K, Carney P, Hudson EA, Tarasova NI, Gaitanaris GA, Pavlakis GN. Development and applications of enhanced green fluorescent protein mutants. *Biotechniques* 1998;24(3):462–6. 468–71. [PubMed: 9526659]
27. Bhattacharyya N, Dey A, Minucci S, Zimmer A, John S, Hager G, Ozato K. Retinoid-induced chromatin structure alterations in the retinoic acid receptor beta2 promoter. *Mol Cell Biol* 1997;17(11):6481–90. [PubMed: 9343411]
28. Kino T, Gragerov A, Kopp JB, Stauber RH, Pavlakis GN, Chrousos GP. The HIV-1 virion-associated protein Vpr is a coactivator of the human glucocorticoid receptor. *J Exp Med* 1999;189(1):51–62. [PubMed: 9874563]
29. Kino T, Tiulpakov A, Ichijo T, Chheng L, Kozasa T, Chrousos GP. G protein β interacts with the glucocorticoid receptor and suppresses its transcriptional activity in the nucleus. *J Cell Biol* 2005;169:885–96. [PubMed: 15955845]
30. Schaaf MJ, Cidlowski JA. Molecular determinants of glucocorticoid receptor mobility in living cells: the importance of ligand affinity. *Mol Cell Biol* 2003;23:1922–34. [PubMed: 12612067]
31. Kino T, Liou SH, Charmandari E, Chrousos GP. Glucocorticoid receptor mutants demonstrate increased motility inside the nucleus of living cells: time of fluorescence recovery after photobleaching (FRAP) is an integrated measure of receptor function. *Mol Med* 2004;10(7–12):80–8. [PubMed: 16307173]
32. Lu NZ, Cidlowski JA. Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. *Mol Cell* 2005;18(3):331–42. [PubMed: 15866175]

A **B**

Figure 1. Structures of the hGR gene and the hGR α protein, and hGR α -mediated transduction of the glucocorticoid signal

(A) Genomic, complementary DNA and protein structures of the hGR α . DBD: DNA-binding domain, LBD: ligand-binding domain, NTD: N-terminal domain

(B) Circulation of hGR α between the cytoplasm and the nucleus. Upon binding to ligand, hGR α dissociates from HSPs and translocates into the nucleus, where it binds to GREs in the promoter region of target genes or communicates with other transcription factors to modulate the transcription. After changing the transcriptional rates of glucocorticoid-responsive genes, hGR α is exported to the cytoplasm and is re-assembled into the complex with HSPs. HSP: heat shock protein

(C) Schematic representation of the interaction of hGR α with coactivators on a GRE-driven promoter. DRIP/TRAP: vitamin D receptor-interacting protein/thyroid hormone receptor-associated protein; GR: glucocorticoid receptor; GREs: glucocorticoid response elements; p/CAF: p300/CBP-associating factor; SWI/SNF: switching/sucrose non-fermenting;.

Figure 2. Location of the known mutations

(A) and polymorphisms **(B)** of the hGR gene. DBD: DNA-binding domain; GR: glucocorticoid receptor; GREs: glucocorticoid response elements; NTD: N-terminal domain; LBD: ligand-binding domain.

Figure 3. Nuclear translocation of GFP-fused hGR α

HeLa cells were transfected with pF25-hGR α and images were recorded under a confocal microscope every 3 minutes after addition of 10^{-6} M of dexamethasone.

Figure 4. Fluorescent recovery after photobleaching of GFP-hGR α

(A) Recovery of GFP-hGR α fluorescence intensity in the nucleus after photobleaching and calculation of $t_{1/2}$. COS-7 cells were transfected with pF25-hGR α , treated with 10^{-6} M dexamethasone and photo-bleached in the nucleus. Fluorescence intensity was traced time-sequentially, and the recovery $t_{1/2}$ was estimated from the recovery curve produced.

(B) Representative photobleaching (Adapted from Reference 31).

Table 1

Primer pairs for amplification of exonic sequences of the hGR gene

Exons amplified		Primer sequence
Exon 2	Forward	5'-GATTCGGAGTTAACTAAAAG-3'
	Reverse	5'-GTCCATTCTTAAGAAACAG-3'
Exon 3	Forward	5'-AGTTCAGTGTGAGCATTCTG-3'
	Reverse	5'-CGTGAGAAATAAAACCAAGT-3'
Exon 4	Forward	5'-GACAGAAGGCTGTCCTTATA-3'
	Reverse	5'-CATTATGCGTATCAAGCATA-3'
Exon 5	Forward	5'-GAATAAACTGTGTAGCGCAG-3'
	Reverse	5'-TAGTCCCCAGAACTAAGAGA-3'
Exon 6	Forward	5'-GATCTTCTGAAGAGTGTTC-3'
	Reverse	5'-GGGAAAATGACACACATACA-3'
Exon 7	Forward	5'-GAAAGTTCTCCAAAATTCTG-3'
	Reverse	5'-TTGGTGTCACTTACTGTGCC-3'
Exon 8	Forward	5'-GACACAGTGAGACCCTATCT-3'
	Reverse	5'-CACCAACATCCACAACTGG-3'
Exon 9	Forward	5'-GGAATTCCAGTGAGATTGGT-3'
	Reverse	5'-TATAAACCACATGTAGTGCG-3'

Exon 1 does not contain the coding sequence.