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Expression, purification, and characterization of multiple, multifunctional human glucocorticoid receptor proteins

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ABSTRACT

The glucocorticoid receptor (GR) is a nuclear receptor protein that plays a central role in glucose homeostasis, the stress response, control of the hypothalamic-pituitary-adrenal axis, and immuno-inflammatory processes via binding of the natural steroid, cortisol. GR is a well-validated drug target and continues to be an important target for new drug discovery efforts. Here, we describe a basic and simple method for *Escherichia coli* expression and purification of a variety of human GR proteins that contain all three of the functional domains of the protein: the activation function-1 domain, the DNA-binding domain, and the ligand-binding domain. We present characterization data to show that these purified, multifunctional GR proteins are active for ligand, coactivator, and DNA-binding. The work presented here should serve as a reference for future mechanistic, structural and drug discovery efforts that require purified, full or near full length, GR protein.

The glucocorticoid receptor (GR)[2] is perhaps one of the more heavily studied drug targets within the era of target-based drug discovery [1]. The natural ligand for GR is the adrenocorticosteroid, cortisol, and there are dozens of synthetic steroid ligands, such as prednisolone, dexamethasone, and fluticasone propionate that serve a critical function within the medical community [2]. For numerous conditions related to inflammatory, autoimmune, metabolic, and proliferative disorders, these GR-targeted drugs remain a first line therapy. However, it is well-known that prolonged oral treatment with these medicines can have serious and debilitating side-effects such as osteoporosis, fat redistribution, muscle loss, and psychosis. The holy grail of current and future pharmaceutical efforts involving GR is to identify GR-modulating ligands that retain the positive medical benefits with lower risk of side-effects.

GR is a member of the steroid receptor subfamily of nuclear hormone receptors (NRs) [3,4]. These receptors are multitasking proteins that contain an ensemble of smaller protein domains. The N-terminal region, which contains the activation function-1 (AF-1) subdomain, is quite variable in both sequence and length within the NR superfamily. For GR, this N-terminal region is approximately 400 amino acids in length and the AF-1 domain is roughly 300 amino acids. The N-terminal region contains substrate motifs for phosphorylating, sumoylating, ubiquitinylating, and acetylating enzymes [5–7]. This region of GR, more specifically the AF-1 domain, is also important in directing a non-ligand dependent transcriptional activity. The DNA-binding domain (DBD) resides in the middle of the receptor. This domain consists of two zinc fingers which specifically localize GR, typically as a receptor homodimer, to glucocorticoid DNA response elements (GREs) in the promoters of target genes. Following a short hinge region, the final C-terminal domain contains the ligand-binding capabilities of GR and this domain is aptly named the ligand-binding domain (LBD). Not only does this domain contain the binding pocket for small, lipophilic steroids and steroid analogues, but it also contains a shallow groove on its surface that is responsible for tethering the receptor to coactivator proteins, such as transcriptional intermediary factor 2 (TIF2) and peroxisome proliferator-activated receptor alpha (PGC-1α), via an activation function-2 (AF-2) activity. Structures for both the GR DBD and LBD have been solved by X-ray crystallography as independently produced separate domains [8,9].

Detailed biochemical studies on GR have been hampered by the lack of highly purified, active protein. The purpose of the present effort was to identify an *Escherichia coli* expression construct for obtaining appreciable quantities of near full length human GR which contains each of the functional modules. This reagent would be a valuable tool for studying the ligand-induced functional properties of GR and hopefully aid understanding of how these functional modules interplay with one another in response to ligand-

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[2] *Abbreviations used:* GR, glucocorticoid receptor; NRs, nuclear hormone receptors; AF, activation function; DBD, DNA-binding domain; GREs, glucocorticoid DNA response elements; LBD, ligand binding domain; TIF2, transcriptional intermediary factor 2; PGC-1α, proliferator-activated receptor alpha; FP, fluorescence polarization; PNMT, phenylethanolamine *N*-methyltransferase; NHS, *N*-hydroxysulfosuccinimide; F-DEX, fluorescein-labeled dexamethasone.

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binding. Further, a full length, multifunctional GR protein could be used to develop new assays and produce new crystal structures to help discover and characterize novel GR-modulating ligands.

Materials and methods

DNA constructs for GR expression testing

The human GR gene, containing the F602S mutation, was codon-optimized for *E. coli* expression by GenScript (GenScript Corporation, Piscatway, NJ, USA; www.genscript.com) and the gene was supplied in the vector pUC57FLGR with BamHI and HindIII cloning sites. The pUC57FLGR was digested with BamHI and HindIII and the insert was subcloned into pET24a (Novagen) that was engineered to contain six histidines at the N-terminus (6× His tag) followed by a thrombin cleavage site (MKKGHHHHHHGLVPRGS–) [9]. Constructs for GR truncation proteins (see Fig. 1) were made by PCR from pUC57FLGR with oligonucleotides containing BamHI and HindIII sites in the overhang segments. The PCR products were gel purified, cleaved with BamHI and HindIII, and ligated into the 6× His-modified pET24a vector using the Rapid Ligation Kit (Roche Diagnostics). Ligation products were transformed into DH5α cells for selection of clones. All constructs were confirmed by sequencing the entire insert in both directions.

Small scale expression testing of GR constructs in E. coli

BL21(DE3) cells were transformed with pET24a (modified to contain an N-terminal 6× His tag) containing the appropriate GR insert as described above. Typically, 5 ml of LB (plus 50 μg/ml kanamycin) was inoculated with a single colony and grown overnight at 30 °C with shaking at 250 rpm. The next day, 50 ml LB (plus 50 μg/ml kanamycin, 10 μM $ZnCl_2$, and 1 μM ligand of interest) was inoculated with 0.5 ml of the overnight starter culture and grown at 37 °C shaking 250 rpm for 2–3 h until OD_{550} was ~0.7. The temperature was reduced to 18 °C for 1 h, then IPTG was added to a final concentration of 250 μM and the culture was grown at

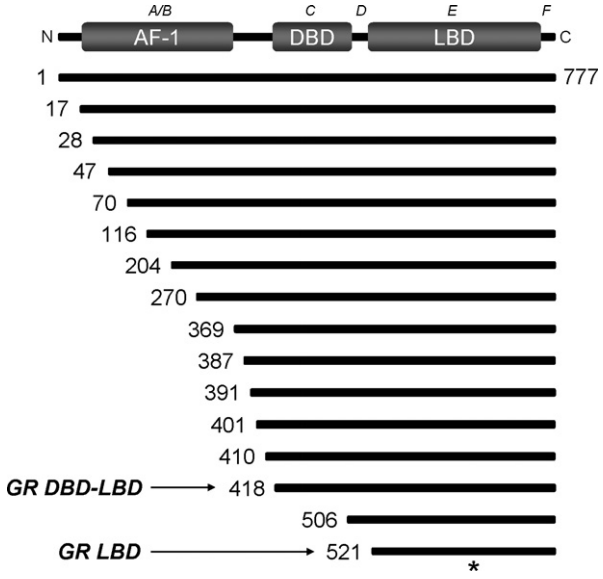

Fig. 1. Linear representation of GR protein and constructs made for expression testing. The GR protein is shown with each functional domain labeled: the activation function-1 (AF-1), the DNA-binding domain (DBD), and the ligand-binding domain (LBD). Start sites were chosen based on criteria as discussed in the text. Each construct was made as an N-terminal 6× His tagged fusion protein for expression in *E. coli* via a modified pET24a vector. The asterisk indicates the location of the F602S mutant used in each of the constructs.

18 °C for an additional 2.5–4 h. Cells were harvested by centrifugation then resuspended in 1 ml lysis buffer (50 mM NaH_2P0_4 pH 8.0, 300 mM NaCl, 2 M urea, 1 mM DTT, 2 mM $MgCl_2$, and 50 μM ligand of interest). The cells were pulse sonicated and debris was cleared by centrifugation. Ni-chelating resin (50 μl pre-washed, pre-equilibrated 50% slurry; GE Healthcare HP Ni) was added and slowly rotated at 4 °C for 1 h. The resin was washed 3× with wash buffer (20 mM NaH_2P0_4 pH 8.0, 300 mM NaCl, 10% glycerol, 50 mM imidazole, 1 mM DTT, 50 μM ligand) and eluted with 75 μl of elution buffer (wash buffer + 500 mM imidazole). Samples were analyzed on a 4–12% NuPage gel (Invitrogen).

Purification of GR protein

Transformed BL21(DE3) cells were grown as above but scaled up to produce 12 L culture per protein preparation. Cell pellets were resuspended in 5 ml lysis buffer (20 mM NaH_2P0_4 pH 8.0, 500 mM NaCl, 2 M urea, 1 mM DTT, 2 mM $MgCl_2$, and 50 μM ligand) per gram of cells. Benzonase (Novagen) was added at a ratio of 10 U/ml lysate. The lysate was stirred in the cold (4 °C) for 1.5 h, passed through a Rannie cell homogenizer 2×, then centrifuged in an SLA3000 at 12 K rpm for 30 min. The supernatant was removed and buffered imidazole was added to 50 mM final concentration. The cleared lysate was loaded onto an His-Trap HP (GE Healthcare; 5 ml) column then gradient equilibrated to running buffer (20 mM NaH_2PO_4 pH 8.0, 500 mM NaCl, 50 mM imidazole, 10% glycerol, 1 mM DTT, and 10 μM ligand). Protein was eluted with a gradient to 500 mM imidazole in buffer containing 150 mM NaCl. The pool containing GR protein (typically a wide, diffuse elution peak) was diluted 2× with 25 mM Hepes pH 8.5, 10% glycerol, 1 mM MgCl2, 1.0 U/ml benzonase, 1 mM DTT, 10 μM ligand, 0.5 mM EDTA) and incubated at 4 °C for 30 min. The protein was passed through a Hi-Trap Q HP anion exchange column (GE Healthcare; 5 ml) and loaded onto an HS cation exchange column (either Applied Biosystems Poros HS or GE Healthcare HiTrap SP HP). Protein was eluted from the cation exchange column with a linear gradient to buffer containing 1 M NaCl (25 mM Hepes pH 8.5, 1 M NaCl, 10% glycerol, 1 mM DTT, 1 or 10 μM ligand). For some of the preparations, we also employed a tandem Superose 6 10/300 (GE Healthcare) size exclusion column. Concentration of the pooled protein was determined by molar absorptivity ε_{280} using a molar extinction coefficient determined using Lasergene Protean (DNA STAR, Inc.). Concentrations were also confirmed by Bradford analysis compared to a bovine serum albumin standard. Each protein sample was also confirmed by mass spectrometry.

Ligand-binding assay using fluorescence polarization

Fluorescein-labeled dexamethasone (Sigma; F-DEX; 5 nM) was titrated with purified GR proteins 28, 116, 369, or 418 using buffer 10 mM Hepes pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.005% Tween-20 in a black polystyrene 384-well microtiter plate (Corning Inc., Corning, NY). Immediately prior to the assay, GR proteins were exchanged into assay buffer using protein desalting spin columns (Pierce Biotechnology). Samples were incubated for 20 min then read (ex 484 nm, em 520 nm) using a Perkin Elmer Envision.

Cofactor peptide interaction assay via fluorescence polarization

A cofactor peptide binding assay was conducted using a C-terminal fluorescein-labeled (5-carboxyfluorescein; 5-FAM) PGC-1α peptide (5 nM) and 3 μM of respective purified GR protein: 28, 116, 369, and 418. The PGC-1α peptide represents the second NR box of the cofactor and contains amino acids 137–155 EAEEPSLLKKLLLAPANTQC with an added C-terminal 5-FAM-la-