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# Glucocorticoid receptor concentration and the ability to dimerize influence nuclear translocation and distribution

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ABSTRACT

Glucocorticoid receptor (GR) concentrations and the ability of the GR to dimerize are factors which influence sensitivity to glucocorticoids. Upon glucocorticoid binding, the GR is actively transported into the nucleus, a crucial step in determining GR function. We examined the effects of GR concentration and the ability to dimerize on GR nuclear import, export and nuclear distribution using both live cell microscopy of GFP-tagged GR and immunofluorescence of untagged GR, with both wild type GR (GRwt) and dimerization deficient GR (GRdim). We found that the observed rate of GR nuclear import increases significantly at higher GR concentrations, at saturating concentrations of dexamethasone ($10^{-6}$ M) using GFP-tagged GR, while with untagged GR it is only discernable at sub-saturating ligand concentrations ($10^{-10}$–$10^{-9}$ M). Loss of dimerization results in a slower observed rate of nuclear import (2.5- to 3.3-fold decrease for GFP-GRdim) as well as a decreased extent of GR nuclear localization (18–27% decrease for untagged GRdim). These results were linked to an increased rate of GR export at low GR concentrations (1.4- to 1.6-fold increase for untagged GR) and where GR dimerization is abrogated (1.5- to 1.7-fold increase for GFP-GRdim). Furthermore, GR dimerization was shown to be required for the appearance of discrete GC-dependent GR nuclear foci, the loss of which may explain the increased rate of GR export for the GRdim. The reduction in the observed rate of nuclear import and increased rate of nuclear export displayed at low GR concentrations and by the GRdim could explain the lowered glucocorticoid response under these conditions.

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## 1. Introduction

The glucocorticoid receptor (GR) mediates the effects of endogenous glucocorticoids (GCs) [37,49,73], as well as natural or synthetic GCs used to treat inflammatory diseases [18,24,50]. The GR is a ubiquitous ligand dependent transcription factor [50] and essential for life [17,54]. In the absence of ligand the GR occurs primarily in the cytoplasm in the form of a heteromeric complex consisting of a heat shock protein (Hsp) 90 dimer, Hsp70, the small acidic protein, p23, and one of the tertratricopeptide repeat (TPR)-domain proteins [59]. Binding of a GC to the GR produces a conformational change in the GR resulting in a change in the proteins making up the heteromeric complex [4], GR dimerization [66] and active import into the nucleus [26,72].

Nuclear import of the GR occurs quickly [39] and relies on the association with Hsp90 [10,57], the TPR FK506-binding protein 52 (FKBP52) [23] and importin-α [77]. This complex is actively shuttled into the nucleus by dynein [41] along the cytoskeleton [35] through the nuclear pore complex [30,31,57]. Two nuclear localization (NL) sequences have been identified in the human GR, the NL1 sequence, which is situated within amino acids 479–506 [33,46,65], and the NL2 domain situated within amino acids 526–777 [33,65]. The unliganded GR, although mostly cytoplasmic, does exist in a dynamic equilibrium where a small proportion of the population is actively shuttled into the nucleus and allowed to diffuse back into the cytoplasm. Upon ligand activation this equilibrium shifts toward a predominantly import driven state, which results in a primarily nuclear localization of the GR [27,65]. Thus, the degree of nuclear localization reflects both the rate of nuclear import as well as the rate of nuclear export [53]. There is clear evidence that the nuclear import rate is ligand dependent [82] and that the degree of GR nuclear localization is a critical factor in determining the level of GR function [40,42].

After ligand withdrawal the unliganded GR remains nuclear for a considerable amount of time [85]. The retention of the GR in the

*Abbreviations:* CpdA, compound A; DEX, dexamethasone; F, cortisol; FISH, fluorescence in situ hybridization; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; MPA, medroxyprogesterone; NFκB, nuclear factor-κB; Prog, progesterone; RU486, mifepristone.

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nucleus is linked to GR association with Hsp90 in the nucleus [76] and relies on the nuclear retention signal found within the hinge region of the GR [11]. GR dissociation from DNA following ligand withdrawal occurs rapidly [61] and is followed by the subsequent localization of the GR to transcriptionally inactive areas of the nucleus [85], prior to export of the GR from the nucleus or degradation of the GR by the proteasome [47]. It has been demonstrated that nuclear export of the GR is independent of the exportin 1/CRM1-directed nuclear export pathway [47], is an inactive process, which occurs independently of ATP [85], and relies on the nuclear export signal within amino acids 442–456 of the human GR [7]. Considering the slow rate of nuclear export and the fact that ATP is not required for export, it is most likely that nuclear export of the GR occurs through passive diffusion [67,76].

The nuclear import of GR has a half-time ($t_{½}$) of 4 to 5 min following $10^{-6}$ M DEX stimulation [39,82] and has been shown to be cell type [55], ligand [87] and ligand concentration dependent [45]. In addition, previous research demonstrated that once imported, nuclear mobility [39] and the pattern of GR distribution [68] in the nucleus are differentially affected by ligands and ligand concentration. Induction with the potent GR agonist, DEX, results in discrete nuclear foci, while induction with the GR antagonist, RU486, leads to diffuse nuclear localization of the activated GR [45,67,82]. RNA fluorescence in situ hybridization (FISH) studies have demonstrated active transcription close to receptor nuclear foci [74,81,83]. GR export from the nucleus following the washout of $10^{-6}$ M cortisol (F) shows a $t_{½}$ of 8–9 h [39] and is ligand [39] as well as ligand concentration dependent [11]. Thus, although ligand type and ligand concentration have been investigated, the influence of GR concentration or the ability of GR to dimerize on the rate of GR nuclear import and export or nuclear distribution has not been previously examined.

Physiologically, the concentration of expressed GR varies considerably between tissues, ranging from 4.1 fmol GR per mg protein in PBMCs [15] to as high as 893 fmol GR per mg protein in the skin [38]. Considerable inter-individual variation, within the same tissue type, has also been reported, primarily in cancerous tissues [19,51]. Variations in GR concentration influence the response to GC treatment within the same tissue between individuals [44], as well as between different tissue types [52]. Clinical observations of patients broadly reveal hypersensitivity to GCs brought about by increased GR levels [44] or GC resistance at reduced GR levels [13,69].

The ligand bound GR may exist in equilibrium as either a monomer or dimer, although ligand binding shifts the equilibrium towards more dimer [29,71]. Two regions of the GR have been identified as influential in GR dimerization, the dimerization loop (D-loop) of the DNA binding domain (DBD) (amino acids 458–462 in the human GR) [22] and the ligand binding domain (LBD) [8]. Dimerization of the GR has been demonstrated in the cytoplasm following ligand binding in live cells [63,66] and through glycerol gradient centrifugation of purified GR [83]. Heck et al. [43] created a dimerization deficient human GR mutant, through the exchange of alanine to threonine at amino acid position 458, termed the GRdim. This GR mutant is widely used to elucidate the relevance of GR dimerization in GC signaling and has been shown to display low affinity binding of the receptor to DNA [22,43]. Furthermore, studies reveal that the GRdim generally has a reduced capacity for transactivation relative to the GRwt [34,43,60]. Although numerous other dimerization reduced GR mutants exist [1], the GRdim (hGRA458T) is the most widely characterized and as a result it is the one we will focus on. A recently characterized natural mutation in the C-terminal zinc finger of the DBD of the GR, referred to as $GR^{R477H}$, has been linked to primary cortisol resistance in patients [64]. This mutation is thought to affect GR homodimerization and results in a prolonged nuclear import time [12], an inability to bind directly to DNA [12] and a reduced transactivation efficacy of the GR [62].

Unlike the majority of GR agonists that induce GR dimerization, induction of GR by the selective GR agonist, CpdA [25,84,86], results in the abrogation of GR dimerization [28,63]. CpdA is a selective agonist in that it does not transactivate via the GR but retains the ability to repress via the GR to the same extent as a full agonist [25]. Recent findings by our group indicate that the action of DEX through the mouse GRdim is similar to that of CpdA through the mouse GRwt in immunofluorescent nuclear import and nuclear export assays [63] and piqued our interest in the influence of dimerization on nuclear translocation.

GR nuclear translocation and distribution are crucial factors in the behavior of GR and are known to be influenced by ligand type and concentration; however, it is not known whether GR concentration and the ability to dimerize affect these parameters. In order to address this we determined the influence of GR concentration and the ability of GR to dimerize on GR nuclear import, distribution and export, utilizing physiologically relevant and statistically different concentrations of GRwt and GRdim in parallel with induction by the dimerization-inducing agonist, DEX, and the dimerization-abrogating, selective GR agonist, CpdA. We evaluated nuclear import and export of untagged-GR in immunofluorescent studies as well as GFP-tagged GR in live cell assays and in addition investigated nuclear distribution of ligand activated GR.

## 2. Materials and methods

### 2.1. Reagents

Dexamethasone (11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione) (DEX), cortisol (11β,17α,21-trihydroxypregn-4-ene-3,20-dione or 17-hydroxycorticosterone) (F), progesterone (4-pregnene-3,20-dione) (Prog), medroxyprogesterone (6α-methyl-17αhydroxyprogesterone acetate) (MPA), mifepristone (11β-(4-dimethyl amino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one) (RU486), cycloheximide, DEAE–Dextran and chloroquine diphosphate salt (chloroquine) were purchased from Sigma–Aldrich. Compound A (2(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride) (CpdA) was synthesized as described previously [48]. The $[^3H]$-DEX (specific activity of 68–85 Ci/mmol) was obtained from AEC Amersham Biosciences.

### 2.2. Plasmids

The pGL2-basic (empty vector) was obtained from Promega. The pRS-hGRα (GRwt) was a gift from R. M. Evans [87] and pHisGRA458T (GRdim) from K. De Bosscher (University of Ghent, Belgium) [6]. The pEGFP-C2-GR (GFP-GRwt) was provided by S. Okret (Karolinska Institute, Sweden) [79]. The pEGFP-C2-GRA458T (GFP-GRdim) was cloned by excising the wild type GR from pEGFP-C2-GR with the restriction enzymes *Xma*I and *Sal*I and replacing it with the mutated GRdim sequence from pHisGRA458T. The presence of the mutation was confirmed through sequencing (primer, forward 5′-AGC TTC AGG ATG TCA TTA TGG AG-3′ and reverse 5′-CCC CCC CCG GGG TTT TGA TGA AAC AGA-3′). All plasmids were verified by restriction enzyme digest.

### 2.3. Cell culture and DEAE–dextran transfection

Monkey kidney fibroblast cells (COS-1) purchased from American Type Culture Collection (ATCC) were maintained in high glucose (4.5 g/ml) Dulbecco's modified Eagle's medium (DMEM) (Sigma) with 2 mM glutamine (Merck), 44 mM sodium bicarbonate

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