Journal of Steroid Biochemistry & Molecular Biology 117 (2009) 67–73

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Identification of the functional domain of glucocorticoid receptor involved in RU486 antagonism

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ARTICLE INFO

Article history:
Received 2 November 2008
Received in revised form 16 July 2009
Accepted 17 July 2009

Keywords:
Mifepristone (RU486)
Glucocorticoid receptor
Antagonism
Transactivation
Guyanese squirrel monkey

ABSTRACT

Mifepristone, also known as RU486, is a potent glucocorticoid receptor (GR) antagonist that inhibits GR-mediated transactivation. As an alternative to existing antidepressants, RU486 has been shown to rapidly reverse psychotic depression, most likely by blocking GR. Although a number of studies have demonstrated RU486-induced GR antagonism, the precise mechanism of action still remains unclear. To identify the GR domain involved in RU486-induced suppression, GR transactivation and nuclear translocation were examined using cells transfected with human GR (hGR), Guyanese squirrel monkey GR (gsmGR), and GR chimeras into COS-1 cells. RU486 showed a much more potent suppressive effect in gsmGR-expressing cells versus hGR-expressing cells, without significant cortisol- or RU486-induced changes in nuclear translocation. A GR chimera containing the gsmGR AF1 domain (amino acids 132–428) showed a marked decrease in luciferase activity, suggesting that this domain plays an important role in RU486-induced GR antagonism. Furthermore, fluorescence recovery after photobleaching (FRAP) analysis indicated that, in the presence of RU486, gsmGR AF1 domain contributes to GR mobility in living COS-1 cells. Taken together, these results demonstrate, for the first time, that the antagonistic effects of RU486 on GR transactivation involve a specific GR domain.

1. Introduction

The synthetic steroid RU486, also known as mifepristone (17β-hydroxy-11β-(4-dimethylaminophenyl)-17-(1-propynyl)estra-4,9-dien-3-one), is an effective antagonist of glucocorticosteroid action both *in vitro* and *in vivo* at high concentrations [2,14,15,25,26,35,36]. Numerous studies have demonstrated a significant reduction in psychotic symptoms in patients treated with RU486 [4,5,12,15,16,20]. These data support the notion that GR antagonists may be useful as antidepressants in treating psychotic major depression (PMD) through the regulation of the hypothalamic–pituitary–adrenal (HPA) axis.

The molecular structure of GR consists of three functional domains: (1) an amino (N)-terminal domain (NTD) containing a major transactivation domain termed activation function-1 (AF1), which is functionally important because it is required for full transcriptional activity of steroid hormone receptors and for many cell- and target gene-specific responses; (2) a DNA-binding domain (DBD), which binds to glucocorticoid response elements (GREs) in the promoter region of target genes; and (3) a ligand-binding domain (LBD) that contains a hormone-binding pocket, as well as sequences important for interacting with regulators such as coactivator (TIF2, GRIP1), corepressor (NCoR), and heat shock proteins (HSPs) [6,8,9,11,18,28]. Kauppi et al. [22] using X-ray crystallography studies on LBD showed that RU486 binding induces a conformational change, wherein helix 12 adopts a position that covers the coactivator pocket, preventing coactivation and recruiting corepressors, which leads to active antagonism.

Guyanese squirrel monkeys (gsmGR) closely resemble human GR (hGR) in structure with a 97% homologous amino acid sequence, but not GR function [10,27,29]. We previously identified a LBD domain for an agonist-induced GR nuclear translocation with GR chimeras replacing gsmGR LBD with hGR. Based on these observations, a specific domain is considered to be implicated in direct modulation of GR signaling such as RU486 antagonist action. In this study, we investigated the effect of specific GR domains on the antagonistic activity of RU486 using hGR, gsmGR, and various chimeras. Our results demonstrate that the carboxyl-terminal region of the AF1 domain (C-AF1) is associated with RU486 repression. Furthermore, using fluorescence recovery after photobleaching (FRAP) analysis, we showed that RU486 affects the dynamic mobility of gsmGR.

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0960-0760/$ – see front matter.
doi:10.1016/j.jsbmb.2009.07.005

2. Materials and methods

2.1. Cell culture and luciferase reporter assay

COS-1 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin under 5% CO_2 at 37 °C, as recommended by the supplier. Transfection was performed using SuperFect reagent (QIAGEN, Valencia, CA) according to the instruction manual. In brief, COS-1 cells were seeded in 24-well culture plates to 60% confluent in DMEM growth medium with 10% FBS at 37 °C and 5% CO_2 in an incubator 24 h prior to transfection. Cells were cotransfected with GR construct, pGRE-Luc harboring a luciferase reporter gene [27], and pCMV-β-gal-SPORT (Life Technologies, Inc., Gaithersburg, MD) as an internal control for transfection efficiency. For hormone treatment, the transfected cells were incubated for 36 h with cortisol (Sigma, St Louis, MO) and RU486 (Sigma) at the specified concentration mentioned in the legends of the figures. Cell extracts were assayed for luciferase activity using a commercially available kit (Promega, Madison, WI) according to the manufacturer's protocol. Data represent luciferase activity normalized against β-galactosidase activity within the same cotransfected cell culture [27].

2.2. Plasmid constructs and mutagenesis

In this study, we used four GR chimeras (A–D) constructed in a previous study [17], and two additional chimeras (F and G) that we constructed using SalI/ClaI restriction enzyme sites for chimeras A and B, respectively. For analysis of the 132–465 domain, we constructed six chimeras (H, I, J, K, L, and M) from chimera F using BglI/BglII restriction sites, which generated three segments in this domain. The chimera constructs used in the study were confirmed by restriction enzyme mapping (in agarose gels) and by sequencing the ligated regions and contains 777 amino acids full sequence of GR.

2.3. GR nuclear translocation

COS-1 cells were cultured on glass coverslips to express the appropriate GFP-GR protein for 36 h after transfection before cortisol or RU486 application. The cells were fixed with 4% paraformaldehyde in 1× PBS for 5 min. Coverslips were then mounted on slides with mounting solution (2% propyl gallate and 80% glycerol in 1× PBS) containing Hoechst 33342 (bisbenzimide, Hoechst; Sigma) for nuclear staining. Cells were imaged using a Zeiss LSM 510 Meta scanning confocal microscope system (Carl Zeiss, Wetzlar, Germany). Quantification of subcellular GR location analysis was performed using Zeiss LSM software and expressed as the ratio between the fluorescence intensity of nuclear and total cell area guided by Hoechst staining. Expression and location of GR were achieved by counting at least 50 cells from triplicate experiments; the results are presented as the mean ± SEM.

2.4. FRAP analysis

COS-1 cells transfected with pEGFP-hGR or pEGFP-gsmGR were treated for 1 h with 100 nM cortisol or RU486. Before photobleaching, the medium was replaced with Hanks balanced salt solution (HBSS) containing 10% FBS, and FRAP analysis was performed immediately afterward using a Zeiss LSM 510 Meta scanning confocal microscope system (Carl Zeiss). Three dishes were analyzed for fluorescence recovery in each treatment group using three visual areas per dish and four to six cells per area. Image analysis was performed using the Zeiss LSM 510 analysis software, and FRAP recovery curves were generated using LSM software and Microsoft Excel. Using this FRAP curve, the $t_{1/2}$ of maximal recovery was determined, which is defined as the time point after bleaching at which the normalized fluorescence intensity has increased to half that of maximal recovery.

2.5. Statistical analysis

Dose–response curve fitting and data analysis were performed by nonlinear regression using the Graph Pad Software Prism 4 (GraphPad Software Inc., San Diego, CA). Significant differences were detected via paired *t*-tests in all cases. Each result is represented as the mean ± SEM. n.s.: non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$.

3. Results

3.1. Inhibition of GR transactivation by RU486

To examine differences in RU486 antagonism against hGR versus gsmGR, we transfected pGRE-Luc reporter constructs into COS-1 cells, and treated the cells with the indicated doses of RU486 before stimulation with 250 nM cortisol (Fig. 1). The EC_{50} values for the transactivation of gsmGR and hGR by cortisol were 9.82 and 24.81 nM, respectively, and the doses required for maximal luciferase activity were 53.50 and 48.51 nM (Supplemental Fig. 1), respectively; thus, 250 nM cortisol was deemed sufficient to activate both hGR and gsmGR. As shown by other groups [1,3,13], the addition of RU486 to hGR-transfected cells decreased luciferase reporter activity in a concentration-dependent manner. The incubation of gsmGR-expressing cells with RU486 also showed concentration-dependent transrepression of luciferase reporter activity. At a concentration of 100 nM, RU486 showed a 14-fold greater transrepressive effect on gsmGR-transfected cells compared to hGR-transfected cells (relative luciferase activity of hGR = 0.59 ± 0.047; gsmGR = 0.042 ± 0.004, $p < 0.01$). Similarly, dose-dependent transrepression was detected in the presence of 50 nM cortisol (Supplemental Fig. 2). Note that in hGR-transfected cells, a mild agonistic effect was observed upon treatment with 10 nM RU486 (relative luciferase activity of hGR = 1.26 ± 0.045 compared to non-treatment in hGR-transfected cells, $p < 0.01$).

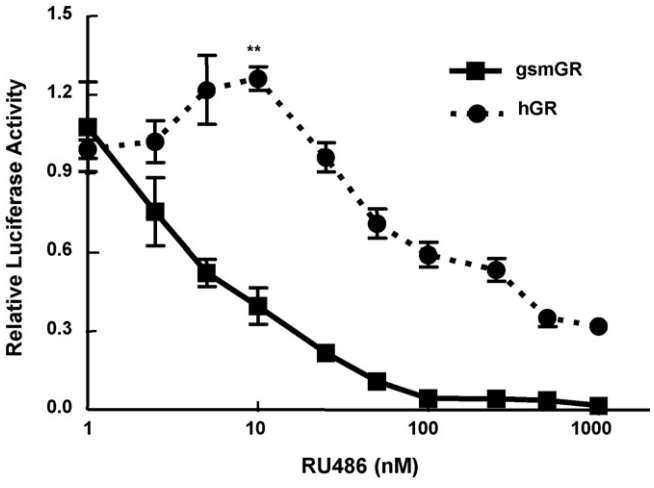

Fig. 1. RU486 differentially inhibits GR transactivation in hGR-expressing cells versus gsmGR-expressing cells. COS-1 cells were cotransfected with a pGRE-Luc reporter plasmid and pCMV-β-gal-SPORT harboring an EGFP-GR construct. After incubation with the indicated concentrations of RU486, COS-1 cells were incubated with 250 nM cortisol. Each point represents the relative luciferase activity normalized against β-galactosidase activity in triplicate samples from three independent transfections. The error bars represent the means ± standard error of the mean (SEM). **Significantly different from non-treated hGR ($p < 0.01$).

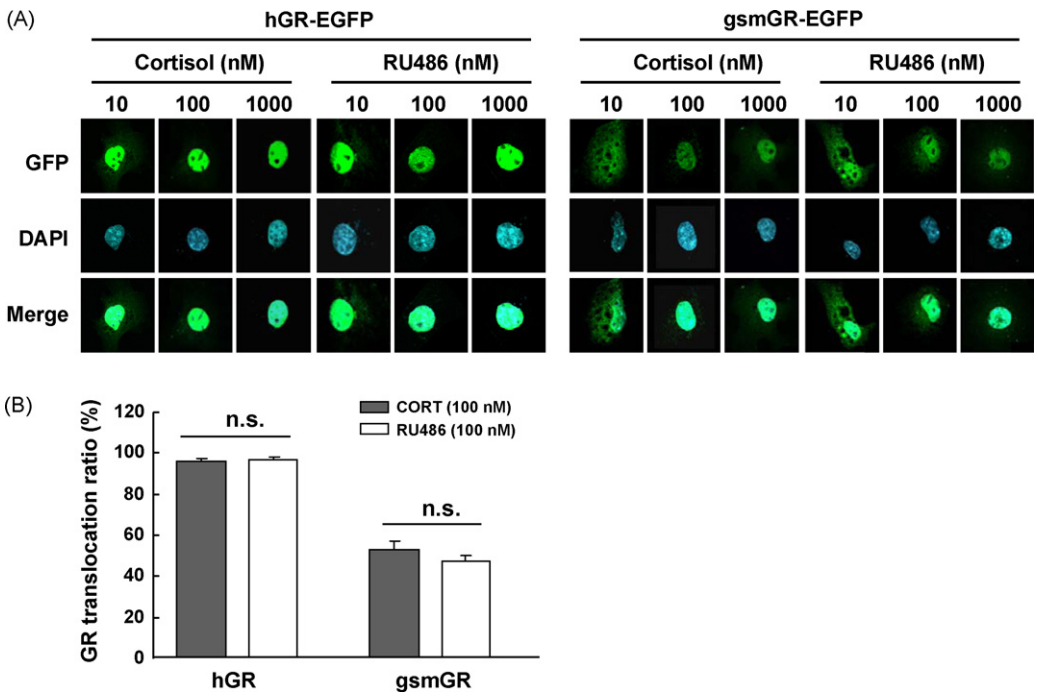

Fig. 2. Effects of cortisol and RU486 on GR translocalization in hGR- and gsmGR-expressing cells. (A) Representative fluorescent images of cells expressing EGFP-tagged GR. GR translocation was induced by cortisol and RU486 treatment. (B) Each bar represents the ratio of the fluorescence intensity of the nucleus to the total area (cytosol plus nucleus). GR translocation was examined in at least 50 cells from triplicate experiments and the results are expressed as the means ± SEM. n.s. means non-significant.

3.2. Effect of RU486 on GR translocation

Although previous studies have shown that RU486 enhances the nuclear translocation of hGR, it is unknown whether RU486 has similar effects on gsmGR translocation. We examined the direct effects of RU486 treatment on the subcellular location of gsmGR using COS-1 cells expressing an EGFP-tagged gsmGR. Images of pEGFP-GR-transfected COS-1 cells taken after exposure to cortisol or RU486 are shown in Fig. 2A. Regarding hGR, fluorescent intensity increased in the nucleus compared to the cytoplasm even at low cortisol and RU486 concentrations, whereas gsmGR was present predominantly in the cytoplasm even at a high concentration of RU486 dose. Subcellular GR location was quantified as the ratio of green fluorescence in the nucleus to total fluorescence (nucleus plus cytosol). No significant difference was observed in terms of nuclear translocation after cortisol or RU486 treatment in pEGFP-hGR- or pEGFP-gsmGR-transfected cells (Fig. 2B, $p > 0.05$). Although these results indicate that the antagonistic effect of RU486 on GR does not involve the disruption of nuclear translocation, nuclear translocation by RU486 could be explained as a partial agonist effect, as others have suggested [19,32].

3.3. Effect of AF domain substitutions on RU486 antagonism

In a previous study, we reported that mutations in the LBD impaired nuclear translocation and thus reduced GR transactivation [17]. Identifying the GR domain that is involved in RU486-induced transrepression is critical to understand the antagonistic mechanism of RU486. To identify this domain, we used six GR chimeras, four of which were developed in a previous study (chimeras A–D) [17] and two new constructs (chimeras F and G) with SalI/ClaI restriction enzyme sites. Chimeras A, C, and F contained the C-terminal region of AF1 (C-AF1, amino acids 132–428) of gsmGR, and chimeras B, D, and G contained partial C-AF1 of hGR (Fig. 3A). First, to evaluate whether the replacement of the GR domain in the chimeras affected the agonistic activity, we performed a promoter activity assay of the chimeras with 250 nM cortisol treatment. As shown in Fig. 3B, chimeras A, C and F showed mild reductions of the agonistic activity compared to hGR (Fig. 3B). However, transfection with chimeras A, C, F and wild-type gsmGR, which commonly contain fragment II of gsmGR, strongly enhanced the RU486-induced antagonism. These results show that the replacement of hGR C-AF1 with that of gsmGR enhanced RU486-induced antagonism (Fig. 3C), indicating that RU486-induced antagonism involves the C-AF1 domain in which 11 amino acids are substituted. In an attempt to pinpoint the site involved in transrepression, we constructed six sub-chimeras in which smaller segments replaced partly gsmGR with hGR in the domain of amino acids from 132 to 465 in the chimera F (Fig. 4A). In cells transfected with chimeras J, L, and M, which commonly contain fragment VI of gsmGR, RU486 treatment decreased luciferase activity to 41.55, 29.79, and 33.86%, respectively, of that observed in cells transfected with wild-type hGR. In contrast, cells expressing chimeras H, I, and K showed little change in luciferase activity compared to cells expressing wild-type hGR (Fig. 4B). We also confirmed that the cortisol-induced transactivation of chimera J was abolished in the presence RU486 ($p < 0.01$, Fig. 4C). These results suggest that the C-terminal region of the AF1 domain (amino acids 264–465) is involved in RU486-induced transrepression.

3.4. Effect of RU486 on the nuclear mobility of GR in living cells

The FRAP procedure is used to examine the mobilization of steroid hormone receptors with respect to DNA and other nuclear structures. Because RU486 treatment elicited different effects in cells transfected with hGR versus gsmGR, we examined the effects of RU486 on the nuclear dynamics of each GR. Representative images of cells expressing EGFP-tagged gsmGR or hGR and quantitative FRAP curve analyses are shown in Fig. 5. In both pEGFP-hGR- and pEGFP-gsmGR-transfected cells, FRAP analysis showed that the agonist cortisol facilitated a more rapid recovery after photobleaching than did the antagonist RU486. No significant difference was observed in the degree of recovery between human and monkey cytoplasmic GR ($p > 0.05$) in the

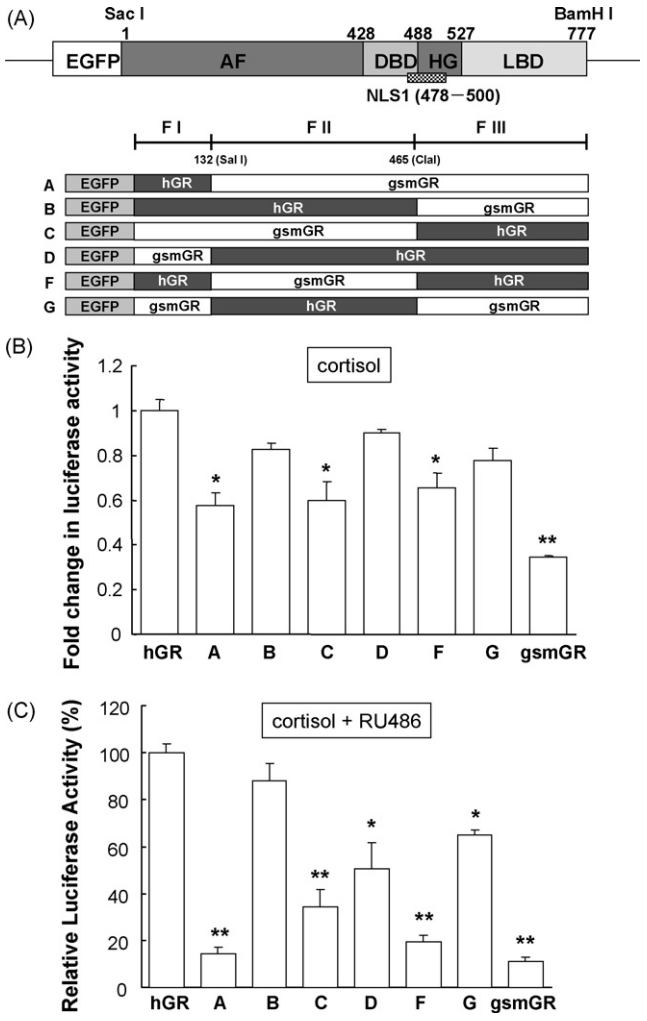

Fig. 3. RU486 antagonism is enhanced by inserting the AF domain (amino acids 132–465) from gsmGR. (A) A schematic diagram of the functional domains of GR and chimeras in which a given hGR domain was replaced by the corresponding gsmGR domain using SalI/ClaI restriction enzyme sites. AF, activation domain; DBD, DNA-binding domain; NLS, nuclear localization signal. F I, fragment containing amino acids 1–132 with 11 amino acid substitutions; F II, fragment containing amino acids 133–465 with 11 amino acid substitutions; F III, fragment containing amino acids 466–777 with seven amino acid substitutions. All constructs in this figure were GFP-tagged. (B) Agonist activity of the chimeras. Transfected COS-1 cells were measured for luciferase activity after treatment with 250 nM cortisol for 36 h at 37 °C. The error bars represent the means ± SEM. *Significantly different from hGR ($p < 0.05$). (C) Quantitative assay of GR promoter activity. Transfected COS-1 cells were measured for luciferase activity after treatment with 250 nM cortisol and 100 nM RU486 for 36 h at 37 °C. Each bar represents the mean ± SEM of the luciferase activity normalized against the β-galactosidase activity in triplicate samples from three independent transfections. *Significantly different from hGR ($p < 0.05$); **$p < 0.01$.

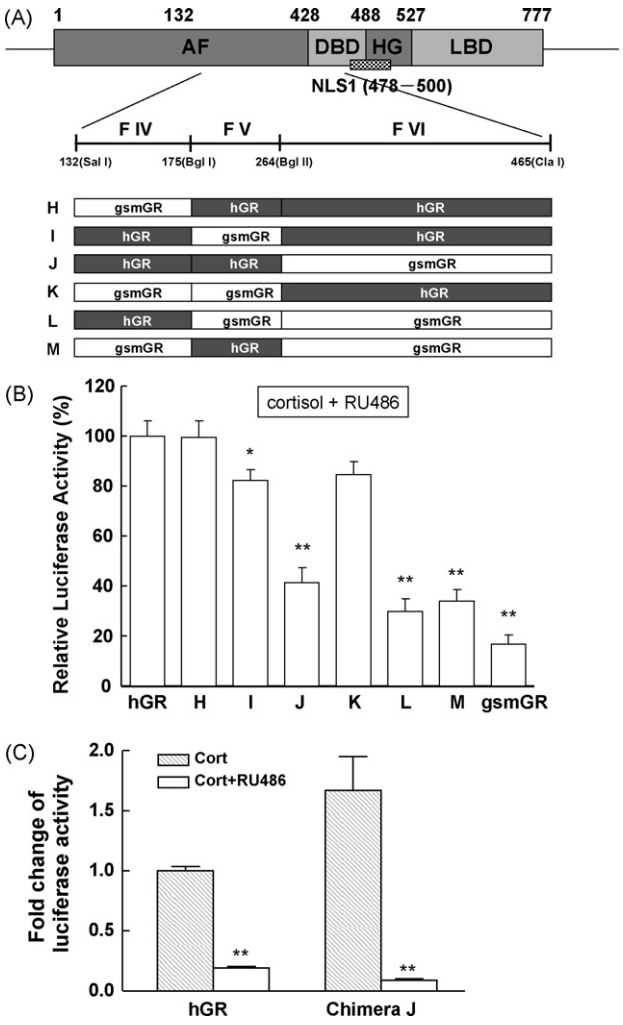

Fig. 4. RU486 antagonism is enhanced by replacement of an even smaller segment (amino acids 264–465) of constructs from chimera F. (A) Schematic diagram of the chimera constructs containing amino acids 1–777 without GFP, in which a given gsmGR segment was replaced with the corresponding hGR segment using the BglI/BglII restriction enzyme sites. AF, activation domain; DBD, DNA-binding domain; NLS, nuclear localization signal. F IV, fragment containing amino acids 132–175 with four amino acid substitutions; F V, fragment containing amino acids 176–264 with four amino acid substitutions; F VI, fragment containing amino acids 265–46 with three amino acid substitutions. (B) Quantitative assay of GR promoter activity. The luciferase activity of transfected COS-1 cells was measured after treatment with 250 nM cortisol and 100 nM RU486 for 36 h at 37 °C. Each bar represents the means ± SEM of the luciferase activity normalized against the β-galactosidase activity in triplicate samples from three independent transfections. *Significantly different from hGR ($p < 0.05$); **$p < 0.01$. (C) Agonist and antagonistic activity of chimera J. Each point represents the relative luciferase activity normalized against the β-galactosidase activity in triplicate samples from three independent transfections. The error bars represent the means ± SEM. **Significantly different from cortisol treatment in each group ($p < 0.01$).

absence of a ligand (data not shown). In the nucleus, consistent with our previous study showing that hGR transactivation was higher than gsmGR transactivation, cortisol treatment led to a slower recovery in hGR fluorescence, with a half-maximal time ($t_{1/2} = 1.533 \pm 0.084$) greater than that observed for gsmGR fluorescence recovery ($t_{1/2} = 1.268 \pm 0.049$). However, RU486 treatment showed no significant difference between hGR and gsmGR fluorescence recovery ($p > 0.05$). The ratio of the cortisol- to RU486-induced fluorescence recovery rate was higher in gsmGR-expressing cells (1.38, $p < 0.01$) than in hGR-expressing cells (1.13). Moreover, RU486-induced fluorescence recovery rate was significantly higher ($t_{1/2} = 1.346 \pm 0.057$, $p < 0.05$) than cortisol-induced fluorescence recovery rate in chimera J, which contained the C-terminal region of the AF1 domain (amino acids 264–465). These results demonstrate that both the type of ligand, whether agonist or antagonist, and nature of GR strongly affect the dynamic mobility.

4. Discussion

Our data reveal a differential RU486-induced antagonistic effect between hGR and gsmGR functionality via a specific GR domain. The promoter assay demonstrated that treatment with 100 nM RU486 led to a 14-fold greater inhibition of gsmGR transactivation than hGR transactivation, and that the AF1 domain (amino acids 264–465) plays an important role in RU486 antagonistic func-

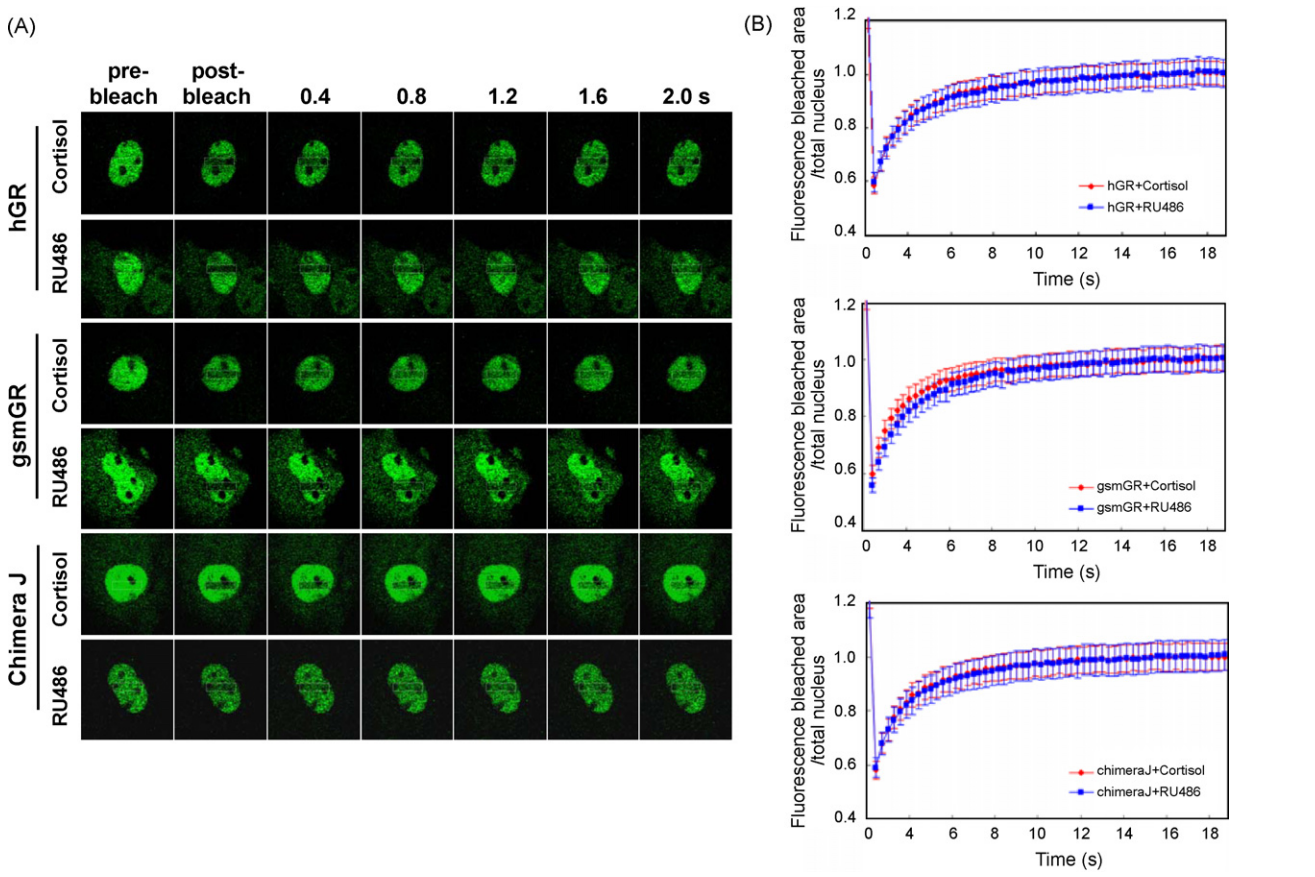

Fig. 5. RU486 treatment influences GR mobility in the nuclei of gsmGR-expressing cells. (A) COS-1 cells were transfected with pEGFP-hGR, pEGFP-gsmGR, or pEGFP-chimer J, and 100 nM cortisol or RU486 was added 48 h later. A region (indicated by the rectangles) in the nucleus of a cell containing GFP was bleached. The cell was imaged at the indicated times after bleaching. (B) FRAP recovery curves for pEGFP-GRs after cortisol or RU486 treatment. The fluorescence intensity was followed temporally, and the time until half-maximal recovery ($t_{1/2}$) was estimated from the recovery curve.

tion. Furthermore, FRAP analysis showed that gsmGR fluorescence recovered at a significantly higher rate in response to cortisol compared to hGR, whereas RU486 treatment showed no significant difference in terms of fluorescence recovery between hGR- and gsmGR-expressing cells, suggesting that the modulation of dynamic mobility may be related to GR antagonism.

Many studies examining the LBD of GR have demonstrated that ligand binding affects the stability of the active receptor protein and induces conformational changes, which dictate cofactor binding and subsequent transactivation or transrepression [6,18,24]. Other studies have shown that a domain region including the LBD is important for antagonism. An X-ray crystallography study showed that the binding of RU486 to GR induces a conformation change in which helix 12 of the LBD covers the coactivator pocket, preventing coactivator binding [22]. In our results, the N-terminal region of GR (AF1 domain) was identified as a major determinant of RU486-induced GR transrepression. Fine mapping of the AF1 domain revealed that the C-terminal region (amino acids 264–465) is involved in RU486-induced hGR transrepression. Using glutathione S-transferase pull-down assays, mammalian two-hybrid assays, and coimmunoprecipitation methods, Renkawitz and coworkers [32] also demonstrated that the GR N-terminus is involved in antagonism via the direct binding of NCoR to RU486-bound GR.

The profile of antagonistic activity was similar in both gsmGR- and hGR-expressing cells; however, RU486 had a much more potent effect on gsmGR-expressing cells. Interestingly, RU486 showed partial agonistic activity in hGR-expressing cells, which was enhanced over its ability to prevent cortisol-mediated transactivation at the low RU486 dose (Fig. 1). This observation may reflect a differential mechanism for hGR versus gsmGR functionality via the RU486 ligand. Little is known regarding the mechanisms by which steroid antagonists inappropriately activate transcription, although several models have been proposed [7,21] and two existing hypotheses explaining a "partial antagonism" molecular mechanism could be used to describe the hGR–RU486 interaction [32]. First, RU486 binds to the hGR with an affinity roughly 18-fold that of cortisol [30]. This relatively high binding affinity may promote more efficient translocation of cytosolic hGR into the nucleus. Thus, at a low ligand dose, it is possible that the higher affinity of RU486 for hGR drives GR into the nucleus, thereby positively activating GR function by enhanced binding to nuclear DNA, resulting in slightly agonistic activity [17]. The second mechanism may involve cross-talk between antagonist-occupied steroid receptors and coactivators. Antagonist-occupied steroid receptors are targets for the actions of both corepressors and coactivators, bringing these coregulatory proteins to the transcriptional machinery complex [19]. As suggested by Jackson et al. [19], the sum of the combined effects of these coregulatory proteins, which are determined by the nature of GR and the type of ligand, will control the direction of transcription by a particular ligand. However, our data showed enhanced antagonism in gsmGR-expressing cells versus hGR-expressing cells, implying that the lack of a partial agonistic effect contributes to total antagonism. This insight into the mechanism of gsmGR antagonism may be useful for developing candidate receptor antagonist screening methods.

The FRAP procedure is useful for investigating the diffusion and motion of biological macromolecules [33]. Kino and Chrousos [23] used this technique to demonstrate that pathologic mutant GRs show a remarkable increase in nuclear motility and a decrease

Table 1
Half-maximal recovery times ($t_{1/2}$) for fluorescence in pEGFP-hGR- and pEGFP-gsmGR-expressing cells in the presence of 100 nM cortisol or RU486.

Receptor	Treatment	$t_{1/2}$ (s)[a]	
hGR	Cortisol	1.533 ± 0.084 (n = 51)	n.s.
	RU486	1.728 ± 0.061 (n = 49)	
gsmGR	Cortisol	1.268 ± 0.049 (n = 47)	**
	RU486	1.748 ± 0.053 (n = 49)	
Chimera J	Cortisol	1.346 ± 0.057 (n = 49)	*
	RU486	1.582 ± 0.080 (n = 53)	

* $p < 0.05$, ** $p < 0.01$, n.s. means non-significant.
[a] A values are means ± SEM of $t_{1/2}$ after photobleaching.

in chromatin retention, which was correlated impaired transcriptional activity. To investigate the relationship between GR dynamics and antagonistic action, we performed FRAP assays using cells transfected with pEGFP-hGR or pEGFP-gsmGR. In the absence of a ligand, EGFP-hGR and EGFP-gsmGR were localized predominantly in the cytoplasm and showed relatively high cytosolic mobility. The addition of cortisol markedly decreased the mobility of hGR compared to that of gsmGR in the nucleus, which is consistent with our previous report in which cortisol-induced gsmGR transactivation was an order of magnitude less efficient than cortisol-induced hGR transactivation [17]. Schaaf and Cidlowski [31] showed that the difference in GR mobility was ligand-dependent and that ligand affinity was an important determinant of receptor mobility. However, although the affinity of gmsGR was only slightly decreased relative to hGR [27], our results (Table 1) demonstrate that the difference in GR mobility was due to a specific domain. Thus, we suggest a model in which RU486-induced GR antagonism is dependent upon the relative mobility induced by RU486 and cortisol, because this discrepancy in relative mobility is affected by the nature of GR. It seems likely that changes in GR mobility are involved in multiple remodeling complexes, and therefore the dynamic motility of GR in the nucleus plays an important role in RU486-induced GR antagonism.

Initially, glucocorticoid resistance in squirrel monkeys and a few other New World primates was thought to reflect diminished GR ligand-binding affinities [34]. RU486-induced transrepression by gsmGR is an order of magnitude more efficient than hGR in COS-1 cells as shown in Fig. 1. However, we likewise found that the binding affinity for cortisol is approximately 40% less with gsmGR relative to hGR [27], and cortisol- or RU486-induced translocations were no different in gsmGR and hGR. A possible mechanistic explanation for the enhanced antagonistic effect could be exclusive and differential binding affinities, which indicates that mutations could conceivably alter other aspects of ligand binding and thereby enhance antagonistic action.

In conclusion, this study demonstrated that the C-terminal region of the AF1 domain plays an important role in RU486-induced GR antagonism. However, a more detailed study is required to determine whether three mutations (Pro268Ser, Gly298Ser and Val321Ile) in this region are responsible for the observed decrease in transactivation and/or receptor stability by RU486.

Acknowledgments

This study was supported by a grant from the Korean Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (A060075).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2009.07.005

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