Distinct Interaction of Cortivazol with the Ligand Binding Domain Confers Glucocorticoid Receptor Specificity

CORTIVAZOL IS A SPECIFIC LIGAND FOR THE GLUCOCORTICOID RECEPTOR*

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Ligand-receptor coupling is one of the important constituents of signal transduction and is essential for physiological transmission of actions of endogenous substances including steroid hormones. However, molecular mechanisms of the redundancy between glucocorticoid and mineralocorticoid actions remain unknown because of complicated cross-talk among, for example, these adrenal steroids, their cognate receptors, and target genes. Receptor-specific ligand that can distinctly modulate target gene expression should be developed to overcome this issue. In this report, we showed that a pyrazolosteroid cortivazol (CVZ) does not induce either nuclear translocation or transactivation function of the mineralocorticoid receptor (MR) but does both for the glucocorticoid receptor (GR). Moreover, deletion analysis of the C-terminal end of the GR has revealed that CVZ interacts with the distinct portion of the ligand binding domain (LBD) and differentially modulates the ligand-dependent interaction between transcription intermediary factor 2 and the LBD when compared with cortisol, dexamethasone, and aldosterone. Thus, it is indicated that CVZ may not be only a molecular probe for the analysis of the redundancy between the GR and MR in vivo but also a useful reagent to clarify structure-function relationship of the GR LBD.

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1 The abbreviations used are: GR, glucocorticoid receptor; AF-1, activation function-1; ALD, aldosterone; AR, androgen receptor; 11β-hydroxysteroid dehydrogenase; CVZ, cortivazol; DDB, DNA binding domain; DCC, dextran-coated charcoal; DEX, dexamethasone; F, cortisol; FCS, fetal calf serum; GFP, green fluorescent protein; GRE, glucocorticoid response element; hsp90, heat shock protein 90; LBD, ligand binding domain; MR, mineralocorticoid receptor; MRE, mineralocorticoid response element; NID, nuclear receptor interaction domain; PMA, phorbol 12-myristate acetate; TIF2, transcriptional intermediary factor 2; TPS, phosphate-buffered saline; DTT, dithiothreitol; CHO, Chinese hamster ovary; MOPS, 4-morpholinepropanesulfonic acid; DMEM, Dulbecco’s modified Eagle’s medium; C, cytoplasmic; N, nuclear.

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lease (11). The receptors then translocate into the nucleus and act as a transcription factor, binding as a homodimer to the glucocorticoid response elements (GRE), and regulate transcription with the aid of those coactivators and mediators (6, 9). Recently, part of the glucocorticoid actions are not mediated by binding to DNA but by the interaction with other protein factors. For example, the GR represses activity of the transcription factor AP-1 and NF-kB, which is now considered to be a pharmacological basis of anti-inflammatory activity of glucocorticoids (often referred as transrepression) (12). Moreover, the concept of ligand-based modularity of the structure and function of the GR is now experimentally challenged, and so-called dissociated glucocorticoids or selective GR modifiers are being developed to separate untoward actions from therapeutic activities of glucocorticoids (12–14).

Secretion of a physiological glucocorticoid cortisol (F) is in general significantly greater than that of the other steroid hormone aldosterone (ALD) that is secreted as a mineralocorticoid from the adrenal cortex (4). Because glucocorticoids, under certain physiological and pharmacological conditions, can also cause mineralocorticoid-like sodium and fluid retention, the functional redundancy has been suggested between glucocorticoids and mineralocorticoids in the regulation of fluid and electrolyte homeostasis (15). It should be noted that the mineralocorticoid receptor (MR) is highly homologous with the GR (16), and these receptors are simultaneously expressed in several tissues (15, 17). Moreover, biochemical experiments have revealed that the GR binds not only glucocorticoids but also mineralocorticoid, and the MR binds not only mineralocorticoids but also glucocorticoids with high affinity (18–25). Although more complicated (26, 27), the GR and MR can bind common DNA sequences on the promoter region of some but not all of the target genes (26, 27). The enzyme type 2 11β-hydroxysteroid dehydrogenase (11β-HSD2) contributes to some extent in the functional distinction of these different classes of steroid hormones, because this enzyme inactivates endogenous glucocorticoids into 11-keto congeners (28). However, particularly in the brain and heart in which the GR and MR are simultaneously expressed but 11β-HSD2 is not (29), the biological significance of the redundancy between glucocorticoid and mineralocorticoid remains unknown (29–31). Several receptor-specific ligands that can distinctly regulate target gene expression have been developed and contribute to understanding this redundancy (19, 32, 33).

The phenylpyrazolo glucocorticoid cortivazol (CVZ) is a synthetic glucocorticoid agonist, which has been reported to have two dissociation constants for the GR and be 40-fold more potent than the synthetic glucocorticoid dexamethasone (DEX) in inducing tyrosine aminotransferase in HTC cells (34, 35). Furthermore, CVZ has been shown more effective in raising blood pressure than other natural and several synthetic glucocorticoid from the adrenal cortex (4). Because glucocorticoids, unless otherwise specified. Monoclonal anti-IgG antibodies (IgG and IgM) were obtained from Affinity Bioreagents, Inc. (Golden, CO). Goat anti-mouse IgM and control mouse IgM, TRPC18, were obtained from Sigma. Monoclonal anti-GFP antibody was obtained from CLONTECH Laboratories (Palo Alto, CA). Polyclonal anti-GRIP1/TIF2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Culture and Heat Shock Treatment**—COS7, CV-1, CHO, F9, and HeLa cells were obtained from the RIKEN Cell Bank (Tsukuba Science City, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) and medium supplemented with 10% fetal calf serum (FCS) and antibiotics. In all experiments, serum steroids were stripped with dextran-coated charcoal (DCC), and cells were cultured in a humidified atmosphere at 37°C with 5% CO₂. Heat shock treatment for COS7 cells was achieved by shifting flasks to another 5% CO₂ incubator set at 43°C.

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**Experimental Procedures**

**Reagents and Antibodies**—F, DEX, ALD, and phorbol 12-myristate acetate (PMA) were purchased from Sigma. CVZ was a gift from Merck. Other chemicals were from Wako Pure Chemical (Osaka, Japan) unless otherwise specified. Monoclonal anti-IgG and IgM antibodies were obtained from Affinity Bioreagents, Inc. (Golden, CO). Goat anti-mouse IgM and control mouse IgM, TRPC18, were obtained from Sigma. Monoclonal anti-GFP antibody was obtained from CLONTECH Laboratories (Palo Alto, CA). Polyclonal anti-GRIP1/TIF2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Whole Cell Ligand Binding Assay**—COS7 cells transfected with pShRGr or pShMR were cultured in DMEM supplemented with 2% DCC-treated FCS in 12-well flat-bottom plastic plates (Iwaki Glass) to confluence. The cells were then transfected with the pCMX-GFP as an internal control for transfection efficiency when appropriate.

**Plasmids**—The expression plasmids for the wild-type human GR, pShRGr, and wild-type human MR, pShMR, were the kind gifts from Dr. R. M. Evans (Salk Institute, La Jolla, CA). Another expression plasmid for the wild-type human GR, pCMX-GR, was constructed by cutting out a KpnI-XhoI fragment including the entire human GR-coding sequence and the 5′- and 3′-untranslated regions from pShRGr, and this fragment was inserted into parent pCMX (37). The expression plasmids for chimeric protein of green fluorescent protein (GFP) and the wild-type human GR or MR, pCMX-GFP-GR (38) and pCMX-GFP-MR (39), respectively, were described previously. The expression plasmids for chimeric protein of GAL4-DBD and the LBD of the human MR (Glu-489 to Lys-777), pCMX-GAL-L-MrGR (pCMX-GAL4-GR(LBD)), was a kind gift from Dr. K. Umesono (University of Kyoto, Japan). To construct an expression plasmid for chimeric protein of GAL4-DBD and a reporter activation domain and nuclear receptor interaction domain (NID) of the TIF2, the DNA fragment encoding 173 amino acids (Glu-594 to Leu-766) of the human TIF2 were amplified by PCR using pSG5-TIF2 (the kind gift from Dr. P. Chambon, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France) as a template with appropriate flanking sequences and inserted into the parent pCMX-PV16 (37), resulting in pCMX-PV-TIF2NID. To construct the expression plasmids for the wild-type human GR, pCMX-GR (1–774), pCMX-GR-(1–750), pCMX-GR-(1–750), pCMX-GR-(1–750), the DNA fragments encoding corresponding amino acids (Leu-596 to Phe-774, Ser-765, or Pro-750) of the human GR were amplified by PCR with appropriate flanking sequences and inserted into pCMX-GAL-L-MrGR or pCMX-GAL4-GR(LBD) (pCMX-GAL4-GR-LBD), respectively. The expression plasmids pCMX-GAL4-GR(LBD)-His, pCMX-GAL4-GR-LBD, and pCMX-GAL4-GR(LBD) were constructed by inserting a ClaI-opened parent pCMX-GR-(1–774) into the NcoI-PstI fragment of pCMX-GAL4-GR(LBD) via a NotI linker. The expression plasmid for the wild-type human GR, pCMX-GR-(1–774), pCMX-GR-(1–750), pCMX-GR-(1–750), pCMX-GR-(1–750), and pCMX-GAL4-GR(LBD)-His were constructed by inserting suitable DNA fragments encoding residues 1–774, 1–750, and 1–750, respectively, into pCMX-GAL4-GR(LBD), pCMX-GAL4-GR(LBD)-His, and pCMX-GAL4-GR(LBD)-His, respectively. The expression plasmids were transfected into COS7 cells using Lipofectamine (Invitrogen) and then cultured with 20 nM [3H]DEX (70 Ci/mmol, 85 Ci/mmol) and medium was replaced with Opti-MEM medium (Invitrogen) and then cultured with 20 nM [3H]DEX (70–110 Ci/mmol, Amersham Biosciences) in GR-expressing cells or [3H]ALD (50–85 Ci/mmol, Amersham Biosciences) in the MR-expressing cells in the presence or absence of various concentrations of radioactive ligands for 4 h at 37°C. The monolayer was washed three times with PBS and lysed in the whole cell extract buffer (20 mM HEPES, pH 7.9, 350 mM NaCl, 1
**Cortivazol Is a Selective Ligand for GR**

Cortivazol is a selective ligand for glucocorticoids. While many steroids can bind to the glucocorticoid receptors (GR) and mineralocorticoid receptors (MR), cortivazol (CVZ) binds with high specificity to the GR, allowing for more selective activation of the GR in comparison to MR. This selectivity is crucial for the clinical use of glucocorticoids, as MR activation can contribute to side effects such as sodium retention and potassium loss.

**Visualization of Intracellular Trafficking of GFP Fusion Proteins in Living Cells**

For analysis of subcellular localization of the GR and MR in living cells, we transiently expressed GFP-tagged receptors in COS7 cells and visualized their trafficking. GFP fusion proteins were efficiently precipitated not only by the anti-GFP antibodies but also by anti-hsp90 antibodies, indicating their association with the GR and MR.

**Immunocytofluorescence Assays**

GFP-tagged chimeric GR and TIF2-expressing COS7 cells were grown on 8-chambered sterile glass slides and further cultured for 24 h in the presence of various steroid ligands. For immunostaining of TIF2, the cells were fixed in cold acetone for 2 min on ice and air-dried. After fixation, the cells were washed three times with PBS at room temperature and incubated with anti-mouse GRIP1/ TIF2 polyclonal goat antibody at a dilution of 1:50 in PBS containing 0.1% Triton X-100 for 1 h at room temperature. The cells were then washed three times with PBS and incubated with rhodamine-conjugated anti-goat IgG (Santa Cruz Biotechnology) at a dilution of 1:100 in PBS containing 0.1% Triton X-100 for 1 h at room temperature. The cells were finally washed three times with PBS and mounted with GEL/MOUNT™ (Biomeda Corp.) for examination on a confocal laser scanning microscope IX70. Dual excitation was achieved from krypton-argon laser, and digital images were analyzed on FLUOVIEW FV 500 systems (Olympus).

**RESULTS**

**Receptor Selectivity of Corticosteroids**

As described in the Introduction, both the GR and MR, at least in part, bind to the common palindromic DNA sequence and transactivate gene expression. To test the effect of natural and synthetic glucocorticoids and mineralocorticoid on transactivation function of the receptors, we first performed transient transfection assay using GRE/MRE-luciferase as a reporter in COS7 cells. In the absence of expression plasmids for the receptors, any of F, DEX, ALD, and CVZ did not induce significant luciferase activity (Fig. 1A). When the human GR expression plasmid was cotransfected, not only DEX, and CVZ but also ALD as well as F, DEX, and CVZ did not induce significant luciferase activity (Fig. 1A). However, MR-dependent reporter gene activation was not observed in the presence of 100 nM CVZ (Fig. 1A).

Because the results of this assay might artificially be influenced by, for example, the levels of expressed GR and MR, arbitrary specificity of ligand was calculated. As shown at the top of Fig. 1, we confirmed receptor-specific action of these ligands; DEX and ALD are relatively specific to the GR and MR, respectively, but CVZ is extremely specific for the GR when compared with F, DEX, and ALD (Fig. 1A). Next we examined the binding affinity of these corticosteroids on the GR and MR. For that purpose, we determined the effect of these ligands on [3H]DEX binding to the GR and on [3H]ALD binding to the MR (Fig. 1B). Addition of radioinert ligand not only F, DEX, and CVZ but also ALD inhibited the [3H]DEX binding to the GR in a dose-dependent manner, and DEX and CVZ appeared to be equally efficient when compared with F and ALD (Fig. 1B). On the other hand, [3H]ALD binding to the MR was inhibited by the radioinert F, DEX, and ALD, but not by CVZ (Fig. 1B). These results suggest that CVZ is a selective ligand for the GR.

**Effect of Corticosteroids on the Subcellular Localization of the GR and MR**

We next investigated the role of these ligands on the subcellular localization of the GR and MR, using the GFP-tagged chimeric receptor expression system. Because association of hsp90 is believed to be essential for steroid binding and determination of subcellular localization of the GR and MR, we addressed whether these chimeric receptor proteins could bind hsp90 in situ. For this purpose, COS7 cells were transfected with these expression plasmids and cellular lysates were prepared. After immunoprecipitation with anti-hsp90 antibodies, proteins were blotted, and immunoreactivities of the GFP-GR and GFP-MR were examined using anti-GFP antibodies. As shown in Fig. 2A, control immunoglobulin did not show GFP-specific immunoreactivities but anti-hsp90 antibodies efficiently precipitated not only the GFP-GR but also GFP-MR.
from the GFP-GR- and GFP-MR-transfected extracts, respectively (Fig. 2A). Together, we concluded that these chimeric receptors were shown to be associated with hsp90 in the absence of ligand. After treatment with cognate ligands for each receptor, green fluorescence derived from these chimeric receptors showed apparent nuclear condensation, indicating that these chimeric receptors appeared to be competent for ligand binding and translocate into the nucleus in a ligand-dependent manner (Fig. 2B). As shown in Table I, these chimeric receptors showed distinct subcellular localization response when exposed to various steroid ligands. For example, F and DEX promoted nuclear translocation of not only the GFP-GR but also GFP-MR. Of note, ALD showed significant induction of the nuclear localization of the GFP-GR as well as GFP-MR. In clear contrast, CVZ did not influence the subcellular localization of the
MR, but preferentially promoted nuclear translocation of the GR. Either at extremely high concentration (i.e. 1 μM) or in other cells (e.g. HeLa cells, CHO cells, and F9 cells), CVZ still remains GR specific with regard to receptor translocation activity (Table I and data not shown). Thus, GR specificity of CVZ and redundancy of the other steroid ligands strongly indicate that those ligands affect the GR in a distinct manner. Fig. 3A shows the time course of nuclear import of the GFP-GR in the presence of the indicated concentrations of those ligands. Every ligand revealed a time- and concentration-dependent effect on the GR nuclear import (Fig. 3A). The effects of F and ALD were weak when compared with those of DEX and CVZ. When DEX and CVZ were compared, the rate of nuclear import of the GFP-GR in the presence of DEX appeared to be more rapid than that in the presence of CVZ at 1 nM. However, the difference of the rate of nuclear import was not clear at higher concentrations (100 nM and 1 μM), and maximum levels of import were identical between these ligands. Concerning nuclear export, the GFP-GR was rapidly washed out from the nucleus after removal of F or ALD (Fig. 3B). However, treatment with DEX and CVZ resulted in longer retention of the receptor in the nucleus (Fig. 3B). Of note, the effect of CVZ was stronger than that of DEX; after 24 h of treatment with CVZ, more than 90% of the cells still showed exclusively nuclear fluorescence of the GFP-GR (Fig. 3B). These results indicate that ligand differentially modulates nuclear import and export of the GR. DNA binding is suggested to be one of the important determinants for localization in and tight binding to the nucleus of the GR. To address this issue, we used the ligand binding-competent but DNA binding-deficient mutant of the GR, GFP-D4X (40). DEX and CVZ again promoted nuclear import of GFP-D4X, with slower rate of import than the wild-type GFP-GR (Fig. 3C). In clear contrast to wild-type GR, the difference in nuclear harboring of GFP-D4X was not discernible between DEX and CVZ in the nuclear export assay (Fig. 3C).

Distinct Interaction of Corticosteroids with the C-terminal End of the GR LBD—Because the LBD of the GR is critical for the determination of ligand specificity, we constructed several mutant GR with amino acid deletion or substitution in the LBD and studied the effect of CVZ with reference to other steroid ligands. Fig. 4A schematically illustrates the structure of wild-type GR and the mutant GR used in the present study. Because mutation in the LBD might affect binding of hsp90, we performed immunoprecipitation assay before using them in subsequent experiments. For this purpose, COS7 cells were transfected with these expression plasmids, and cellular lysates were prepared. After immunoprecipitation with anti-hsp90 antibodies, GR immunoreactivity was examined using anti-GFP antibodies as described before. As shown in Fig. 4B, these GFP-GR mutants associated with hsp90 (upper part), without changing the cellular hsp90 levels (bottom part). Moreover, functional significance of association of GR with hsp90 was confirmed in heat-shock experiments; treatment of transfected cells at 43°C for 2 h promoted nuclear translocation of every GFP-GR mutant used as well as the wild-type GFP-GR (Fig. 4C). Therefore, we concluded that these mutations did not affect association of hsp90 with the LBD of the receptor in the absence of ligand. Given these results, we tested the effect of ligand on subcellular localization of these mutants, and the results are summarized in Table II. Notably, F and ALD were extremely sensitive to even subtle deletion of the C-terminal end of the LBD, because only a 3-amino acid deletion significantly abrogated receptor movement after treatment with these ligands. In contrast, DEX could promote nuclear translocation of the GFP-GR (1–774) but not GFP-GR (1–765), and CVZ still promoted nuclear translocation of not only the GFP-GR (1–774) but also GFP-GR (1–765). However, when the GFP-GR (1–750) was used, the effect of CVZ was totally diminished (Table II). These results indicate that each ligand induces nuclear translocation through distinct interaction with the LBD of the GR, and its C-terminal end may be critical for ligand discrimination.

Differential Effects of Corticosteroids on Transcriptional Function of the GR—Next we studied the effect of these steroid ligands on transactivation and transrepression function of the GR and these mutants. For transactivation assay, the expression plasmids of these mutant GRs and NF-κB-responsive luciferase reporter plasmids were cotransfected, and cells were cultured in the presence of these ligands. As shown in Fig. 5, the transactivation effect of F or ALD was abrogated when the C-terminal part of the LBD was deleted at the amino acid position 774, and DEX could not induce transactivation of the GR (1–765). However, CVZ still induced reporter gene expression when the GR (1–765) was used, despite the slightly lesser extent when compared with the wild-type GR. Further deletion of the LBD resulted in complete loss of transactivation function of the GR even in the presence of any ligands (Fig. 5). For transrepression assay, the expression plasmids of these mutant GRs and NF-κB-responsive luciferase reporter plasmids were cotransfected, and cells were cultured in the presence of PMA and these ligands. Transrepression effect of F or ALD was abolished when the C-terminal end of the LBD was deleted at the amino acid position 774, and DEX could not induce transrepression of the GR (1–765). Moreover, CVZ again induced transrepression effect of the GR (1–774) and GR (1–765) but not the GR (1–750). Together, the effects of these ligands on the GR and GR mutants were comparable between transactivation and transrepression, reflecting that nuclear translocation might be a key determinant for eliciting these receptor functions by these ligands. When the GR mutants GRI747T and GRL753F with amino acid substitution at positions 747 (Ile to Thr) and 753 (Leu to Phe) in the C-terminal end of the LBD, respectively, were used, treatment with either F or ALD did not induce significant nuclear translocation. DEX and CVZ showed almost identical effects on nuclear import, although with a slower rate when compared with that of the wild-type GFP-GR (Fig. 6A, compare with Fig. 3A). When using the GRI747T, DEX and CVZ were equally efficient as a switch for not only nuclear translocation but also for induction of reporter gene expression (Fig. 6A and B). In contrast to GRI747T, when GRL753F was used, DEX was shown to be a weaker inducer of expression of the reporter gene than CVZ (Fig. 6B). This uncoupling (DEX)
and coupling (CVZ) between nuclear localization and transactivation clearly highlighted the multifarious effects of ligand on the architecture of the LBD. On the other hand, both DEX and CVZ preserved the anti-NF-H9260 effect of these mutants at the same level when compared with that of the wild-type GR (Fig. 6C). Together, we may consider that the LBD discriminates ligand via a variable interaction with distinct regions of the LBD and elicits differential modulation of the receptor functions. Moreover, these results also indicate that transrepression of the NF-H9260 activity does not essentially require the same modulation of the LBD as that for transactivation.

Differential Effect of DEX and CVZ on GR-TIF2 Interaction—As described in the Introduction, the interaction of the LBD with coactivators plays a critical role in controlling gene expression. Given a possible modularity of ligand-LBD interaction, we studied the effect of ligand on this receptor-coactivator interaction with focusing on DEX and CVZ. For this purpose, we employed mammalian two-hybrid assays using the expression plasmids for the wild-type and mutant LBD of GR expressed as a fusion protein with the DNA binding domain of GAL4 and VP16-NID of a coactivator TIF2 fusion protein (Fig. 7A). After transfection of these expression plasmids with GAL4-responsive reporter plasmid, CV-1 cells were cultured in the presence of DEX and CVZ, and luciferase activity was determined. When GAL4-GRLBD was expressed, reporter gene expression was induced after treatment with either DEX or CVZ, indicating that the transactivation function of the LBD (AF-2) was equally provoked in the presence of these agonistic ligands (Fig. 7B). Increasing amounts of VP-TIF2NID expression resulted in a dose-dependent increase in reporter gene expression in the presence of each ligand (Fig. 7B), demonstrating that this assay could successfully monitor the ligand-dependent interaction between the LBD and TIF2NID. When the GAL4-GRLBDL753F was expressed, however, not only ligand-dependent activation of AF-2 but also ligand-dependent interaction between the LBD and TIF2NID was not demonstrated in the presence of DEX but in the presence of CVZ (Fig. 7B). These results suggest that the interaction between the GR and TIF2 may be under the strict control of ligand, and usage of mutant GR including the GRL753F highlights the modularity of such interaction. To visualize the effects of ligand on the interaction between the GR and TIF2, we performed the fluorescence colocalization assay using the expression plasmids for the GFP-tagged GR and TIF2 (Fig. 8). In COS7 cells, we could not detect endogenous immunogenic activity of either GR or TIF2 so far as our assay conditions were used (Fig. 8 and data not shown). In the case of the wild-type GFP-GR, the nuclei showed diffuse green fluorescence after treatment with either DEX or CVZ in the

![Image](https://i.imgur.com/3.png)
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Absence of the TIF2 overexpression. The fluorescence of overexpressed TIF2 appeared to be condensed in discrete nuclear foci. Of note, when the GFP-GR and TIF2 were coexpressed, GFP fluorescence overlapped with dot-like appearance derived from the TIF2, indicating the colocalization of these molecules. The GFP-GRL753F was docked in the cytoplasm in the absence of ligand and was mainly distributed in the nucleus after treatment with either DEX or CVZ. When the GFP-GRL753F and TIF2 were coexpressed, however, discrete dot formation was not observed by DEX but by CVZ (Fig. 8).

Discussion

Redundancy of signal transduction occurs at multiple levels. Particularly in the case of the GR and MR, the situation is extremely complicated as described in the Introduction. Specifically, any single assay cannot precisely predict the role of ligand for the complicated interplay between ligand, receptor, and target DNA transcription in given cells or tissues. In the case of the GR, for example, RU486 binds to and promotes nuclear translocation of the GR but does not elicit transactivation function of the GR, acting as an anti-glucocorticoid (44). Given the fact that both GR and MR finally act as a transcription factor, we first characterized the effects of ligand on the transactivation function of the GR and MR in reporter gene assay, using F, DEX, CVZ, and ALD as model ligands. As shown in Fig. 1A, each of four corticosteroid ligands so far studied has distinct function on the GR and MR as follows: F, DEX, and ALD show redundant effects, but CVZ is extremely selective for the GR. Although part of these results were anticipated from the results of ligand binding assays (see Ref. 18–25 and Fig. 1B), GR selectivity of CVZ and its underlying mechanism has not yet been clearly documented. Nuclear translocation assay further supported GR selectivity of CVZ, and subcellular localization of the GFP-MR was not influenced by CVZ. Although it has been shown that COS7 cells contain 11β-HSD2 (45), accelerated metabolism of CVZ is not likely to be the reason for GR specificity of CVZ, because CVZ efficiently elicits transactivation function of the GR in COS7 cells. Moreover, this selectivity of CVZ was also observed in HeLa, F9, and CHO cells (data not shown), in which 11β-HSD2 is reported not to be expressed (46). We thus favor such an idea that this receptor selectivity principally originates from the difference in the interaction of CVZ with the GR and MR. On the other hand, 11β-HSD1 is shown to potentiate glucocorticoid action (47). Therefore, we cannot completely exclude the possibility that differential affinity of those steroid ligands to 11β-HSD1 may alter intracellular availability of the ligands.

Subcellular compartmentalization is considered as one of the important processes for determination of nuclear receptor function in situ (48). Our kinetic analysis of subcellular localization of the GR showed that nuclear import of the GR occurs as a function of the concentration of ligand but that the rate of import extremely varies among ligands; DEX and CVZ promoted rapid nuclear import of the GR. Surprisingly, nuclear export of the GR appeared to be more distinctly controlled by ligand; the GR rapidly redistributes to the cytoplasm after washout of F and ALD, while showing a prolonged stay in the nucleus after washout of CVZ and DEX. Such longer retention of the GR in the nucleus has also been reported in the case of RU486-treated GR (49). Of note, when DNA binding-deficient GR mutant D4X was used, its nuclear import slowed down, and moreover, the differences in nuclear import and export between CVZ and DEX were diminished. We do not yet know the reason why the rate of nuclear import of D4X was slower than that of wild-type GR. It has been shown that the rate of nuclear import of the GR may be determined by that of dissociation of hsp90 (50). Although D4X mutant has intact LBD, mutation in the DDB might alter receptor conformation after ligand binding in a way that hsp90 release becomes retarded. On the other hand, the fact that nuclear export of D4X mutant GR was comparable between DEX and CVZ may suggest the D4X mutation and resultant loss of DNA binding abrogates the difference between DEX and CVZ. Recently, it is reported that the DNA binding and r2 transactivation domains of the rat GR constitute a nuclear matrix targeting signal, which, most possibly via in-
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Effect of corticosteroids on the nuclear translocation of C-terminal truncated mutants of the GR

TABLE II

| Receptor       | % N | % N + % N > C |
|----------------|-----|---------------|
| GFP-GR(1-774)  | 0   | 0             |
| GFP-GR(1-765)  | 0   | 0             |
| GFP-GR(1-750)  | 0   | 0             |

**Fig. 5. Effect of corticosteroids on the transactivation and transrepression function of the GR mutants.** Left, transactivation. COS7 cells were transfected with 3 μg of pGREG/MRE-Luc reporter plasmid and 10 ng of each receptor expression plasmid as indicated (see also Fig. 4A). The cells were further cultured in the presence or absence of 100 nM of either cortisol (F), dexamethasone (DEX), cortivazol (CVZ), or aldosterone (ALD) for 24 h, and cellular luciferase activities were determined as described under “Experimental Procedures.” Results are expressed as fold induction serving the luciferase activity from non-ligand-treated cells as control, and means ± S.D. of 3 independent experiments are shown. Right, transrepression. HeLa cells were transfected with 2 μg of pNFeBHL reporter plasmid and 1 μg of each receptor expression plasmid as indicated. The cells were further cultured and treated with 10 nM PMA in the presence or absence of 100 nM of various steroid ligands for 24 h, and cellular luciferase activities were determined. Results represent % of maximum induction that is given relative to the maximal luciferase activity obtained from the cells treated with PMA alone, and means ± S.D. of 3 independent experiments are shown.

interaction with heterogeneous nuclear ribonucleoprotein U, tightly bridges with nuclear matrix (51). It was recently reported (52) that the conformation of the DNA binding domain of the transcription factor Ikaros is essential for the pericentromeric nuclear localization. Given this, we might speculate that DEX and CVZ bind to the LBD and induce conformational change of the DBD of the GR in a distinct manner, thereby differentially modulating the duration of nuclear retention.

Discrimination of ligand is considered to be mainly ascribed to the ligand binding pocket in the LBD (10). Recent structural studies (10, 53) indicated that the ligand binding pocket of the nuclear receptor mainly consists of H3, H5, H7, H11, H12, and β-sheet, but the precise contacts are unique for each receptor-cognate ligand pair. Our results indicate that CVZ interacts with the LBD of the GR in a different manner when compared with not only F and ALD but also DEX. Structurally, F, DEX, and ALD have in common the A-ring bearing a C-3-ketone and the bulky phenylpyrazol structure at the C-3 position (35). It has been shown that this C-3-ketone structure closely associates with several amino acids located in H3 and H5 of the GR but also the MR (25, 54, 55). These contact amino acids are completely conserved between the GR and MR (24, 55, 56). Structural analyses have proposed that three hydroxyl groups at C-11, C-17, and C-18 in addition to the C-21 hydroxyl group relatively decrease mineralocorticoid activity, because the interaction between C-20 carboxyl group and Cys-942 of the MR is sterically hampered by these structures (25, 57). Therefore, it may be speculated that CVZ cannot stably interact with the LBD of the MR because of the space-occupying effect of not only the C-17 hydroxyl group but also its phenylpyrazol structure in the A-ring at C-3. On the other hand, our results also support the notion that substitution of C-3-ketone to the bulky phenylpyrazol structure still allows certain class of ligand including CVZ to bind the GR with high affinity, and again indicate that CVZ may interact with the GR LBD in a distinct fashion. Therefore, we were prompted to study the interaction between CVZ and the GR with various mutations in the LBD. First, deletion of 12 amino acids from the C-terminal end of the LBD, which results in GR(1-765), easily abrogates the activity of F, ALD, and DEX on the GR, whereas CVZ still promotes its nuclear entry and transactivation. This was also the case when transrepression activity was tested, indicating that the C-terminal end may be critical for discrimination of these ligands on the GR. Indeed, it has already been shown that the C-terminal deletion abrogates specific binding of DEX and F in classical ligand binding assays, which has also been predicted by a homology model obtained from x-ray crystal structure of the progesterone receptor (56, 58). Next, we used GRI747T and L753F mutants, in which Ile and Leu at positions 747 and 753 were substituted to Thr and Phe, respectively. It has been shown that GRI747T, which did not respond to F, showed slightly reduced DEX binding but that ligand-dependent nuclear translocation and transactivation of GRI747T increased in concert with increasing concentrations of DEX (59). In the present study, however, the differences between these parameters for receptor function were not discernible. Although we do not yet know the reason, it is likely that relatively high concentrations of DEX used in the present study (i.e. 100 nM) might diminish such differences (59). In the
case of GRL753F, ligand-binding parameters are strikingly different between DEX and CVZ; DEX showed single high affinity binding site, but curvilinear binding data favor a two-site model having low and high affinity sites (60). However, the biochemical nature of this characteristic interaction between GRL753F and CVZ remains unknown. In the present study, CVZ promoted nuclear translocation of GRI747T and GRL753F with almost similar rates as compared with wild-type GR. In agreement with this, DEX and CVZ showed equal effects on both transactivation and transrepression function of GRI747T. However, GRL753F-mediated transactivation was significantly reduced in DEX-treated cells, whereas CVZ-treated cells revealed a comparable response to wild-type GR, despite the fact that nuclear translocation was equally promoted by these ligands. Of note similar results have been reported in the androgen receptor (AR); N727K mutation in the AR does not alter ligand binding characteristics, nonetheless ARN727K displayed only half the transactivation capacity when exposed to androgen. The reason for this discrepancy is ascribed to deficiency in ligand-dependent recruitment of TIF2 by this mutant AR. Interestingly, a synthetic androgen analog mesterolone restores TIF2 recruitment and transactivation of ARN727K (61). As described in the Introduction, ligand-dependent recruitment of coactivators is an essential step for the nuclear receptor to regulate target gene transcription. After ligand binding, the repositioning of H12, together with additional structural changes such as bending H3–H5, brings it into a distinct receptor environment, creating an interface suitable for nuclear receptor coactivator binding. Together with the fact that amino acid Leu-753 is inside H12 of the GR LBD (Ile-747

![Figure 6](image.png)

**A.** kinetic monitoring of subcellular localization of GFP-GRI747T and GFP-GRL753F. pCMX-GFP-GRI747T or pCMX-GFP-GRL753F was transiently transfected into COS7 cells, and 100 nM of either cortisol (F; filled triangle), dexmethasone (DEX; filled squares), cortivazol (CVZ; open circles), or aldosterone (ALD; open squares) was added to the culture media. Subcellular localization was determined as described under "Experimental Procedures." Experiments were repeated 3 times with almost identical results, and representative graphs are shown. **B.** effect of corticosteroids on the transactivation function of the GR mutants. For determination of GRE/MRE-dependent transactivation, COS7 cells were transfected with 3 μg of pGRE/MRE-luciferase reporter plasmid and 10 ng of either pCMX-GR, pCMX-GRI747T, or pCMX-GRL753F. The cells were further cultured in the presence or absence of 100 nM of either F, DEX, CVZ, or ALD for 24 h, and cellular luciferase activity was determined. All results are expressed as fold induction compared with the cellular luciferase levels cultured without ligand. Three independent experiments were performed, and means ± S.D. are shown. **C.** effect of corticosteroids on transrepression function of the GR mutants. For determination of transrepression effect, HeLa cells were transfected with 2 μg of pNFsBHL reporter plasmid and 1 μg of either pCMX-GR, pCMX-GRI747T, or pCMX-GRL753F. The cells were further cultured and treated with 10 nM PMA in the presence or absence of 100 nM of F, DEX, CVZ, or ALD for 24 h as indicated, and cellular luciferase activities were measured. Results represent % of maximum induction which is given relative to the maximal luciferase activity obtained from the cells treated with PMA alone, and means ± S.D. of 3 independent experiments are shown.
is within the loop between H11 and H12), we next studied the interaction between the GR LBD and coactivator TIF2 using this mutant GR. Mammalian two-hybrid assay revealed that not only wild-type GR but also GRL753F could interact with TIF2 NID in the presence of CVZ but not of DEX, further supporting the notion that CVZ-induced conformation of GRL753F could be different from a DEX-induced one and could efficiently recruit TIF2 to the LBD. This recruitment of TIF2 by GRL753F is also indicated in the fluorescence colocalization assay. It has already been reported that the GR evenly distributes in the cytoplasm (and partially in the nucleus), in the absence of ligand, and condenses in discrete regions exclusively in the nucleus after addition of ligand (62, 63). In our experiments, overexpression of TIF2 was necessary to produce a discrete dot-like appearance of the GR fluorescence in the presence of DEX and CVZ. Although the nature of the observed discrete speckles of the receptors has not yet been defined clearly, those sites are indicated to be related to chromatin architecture and involve coactivators including TIF2 as well as CBP/p300 (64, 65). Indeed, we could demonstrate colocalization of the GR and TIF2 in such dot regions after treatment with agonistic ligands. Corresponding to the results from two-hybrid assays, GRL753F still merges with TIF2 after treatment with CVZ. Recent photobleaching experiments have strongly suggested that ligand-dependent targeting of the GR to these sites is a rapidly exchangeable process (48). Together with the results from C-terminal deletion analysis of GR LBD, it is tempting to speculate that CVZ, via non-classical interaction with GR LBD, alters receptor conformation in such a way that is different from the other corticosteroids and modulates dynamic interaction between the GR, coactivators, and DNA-chromatin. This unique property of CVZ might also be related to prolongation of nuclear export of the GR. Recently, it has been reported that ligand activity on the GR and MR is determined in tissue- or cell type-specific context (23, 66) and, moreover, that the GR can heterodimerize with the MR in vivo (67). Although we showed GR specificity of CVZ in several cell lines originated from different tissues, more detailed analysis is necessary to characterize completely the molecular mechanism of specific interactions between corticosteroid ligands and the GR and/or MR.

From a pharmacological viewpoint, we raise the possibility that CVZ is exclusively specific for the GR, because CVZ, for example, promoted nuclear translocation of neither MR, AR, nor PR (Table I and data not shown). Several GR-specific ligands have already been developed including RU26988 and RU28362. These compounds have been shown to have extremely low affinity to the MR and are then classically considered as pure glucocorticoids (18, 19). However, this apparent GR selectivity of RU26988 and RU28362 has poorly been confirmed, especially concerning their effects on transactivation potential of the GR and MR. Moreover, their effect on Na+/K+ handling and regulation of body fluid also remains unclear. Again, it should be noted that treatment of sheep with CVZ...
raised blood pressure without significant alteration in either urinary K⁺ excretion or plasma K⁺ concentration (36, 68). It has been shown that increased activity of amiloride-sensitive apical epithelial sodium channel and basolateral Na⁺/K⁺-ATPase is theoretically sufficient to account for both ALD-induced Na⁺ reabsorption and K⁺ excretion (69). Recently, not only ALD but also glucocorticoids can regulate the activity of these channels, either directly or indirectly (70). The absence of an increase in K⁺ excretion in CVZ-treated sheep therefore may raise the possibility that CVZ activates the GR but does not affect the activity of these channels, most possibly due to alteration in the repertoire of target genes of the GR. In the present study, we showed that CVZ induced both GRE-dependent transactivation and repression of NF-κB transcription of GRL753F (Fig. 6, B and C). Originally, GRL753F was cloned from DEX-resistant leukemic cell ICR27TK.3, which undergoes apoptosis not with DEX but with CVZ (60). Although the precise mechanism of glucocorticoid-induced apoptosis of leukemic cells remains unknown, involvement of NF-κB inhibition is speculated (71). However, given the fact that NF-κB activity is suppressed not only by CVZ but also by DEX, we may indicate that GRE-dependent transactivation, at least in part, plays a certain role in induction of apoptosis in ICR27TK.3. Although further structural analysis of the LBD of the GR is essential, CVZ may be a useful compound not only as a GR-specific ligand but also for elucidation of the biological role of the GR.

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