Cysteines 638 and 665 in the Hormone Binding Domain of Human Glucocorticoid Receptor Define the Specificity to Glucocorticoids†

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ABSTRACT: To understand the function of cysteines, we have substituted cysteines 638, 643, and 665 by serine in the hormone-binding domain (HBD) of the human glucocorticoid receptor (hGR). In hormone-binding assays using [^{3}H]dexamethasone, hGR C643S and hGR C665S exhibited wild type receptor K_d of 2.5 nM and hGR C665SM666L displayed a K_d of 3.7 nM, while hGR C638S exhibited a K_d of 162 pM, a 15-fold higher affinity. The affinity of hGR C638S for RU486 was 10-fold higher, and the mutants C643S and C665S bound RU486 with a 10-fold lower affinity when compared to wild type GR. While C665S bound aldosterone with very high relative affinity, the double mutant C665SM666L failed to bind aldosterone. The expression of wild type, mutant, and truncated hGRs *in vitro* showed an identical level of expression of the cloned receptors. Similar levels of expression of the receptors were observed in transfected cells, both by immunoprecipitation and by Western blotting. Transcription activation of the chimeric reporter gene mouse mammary tumor virus−chloramphenicol acetyltransferase (MMTV-CAT) with hGR C638S was 4-fold higher than the level observed with wild type hGR in the presence of dexamethasone. In the presence of RU486, hGR C638S induced MMTV-CAT 25-fold compared to the highest levels observed with wild type hGR and RU486. Even though the hGR C665S stimulated transcription with aldosterone, hGR C665SM666L did not. DNA−receptor interaction analyses by gel mobility shift assay demonstrated that the increased transactivation potential of hGR C638S was due to its intense interaction with DNA. These findings suggest that C638 and C665 are involved in maintaining specificity to glucocorticoids.

Hormone-specific intracellular receptors mediate the biological functions of steroid hormones. In the absence of ligands, the physiologically inactive forms of these receptors exist as oligomeric complexes with heat shock proteins (hsp) 90, hsp70, and hsp56 (Housley & Pratt, 1983; Pratt et al., 1988, 1992; Pratt, 1987, 1990, Pratt, 1993). Ligand-induced conformational changes in the receptor protein result in the dissociation of these complexes (Scherrer et al., 1993; Bresnick et al., 1990; Scherrer et al., 1990; Cadepond et al., 1991), after which the activated receptor in the form of a dimer binds tightly to specific DNA recognition sequences of target gene promoters (Govindan et al., 1982; Kumar & Chambon, 1988; Tsai et al., 1988; Fawell et al., 1990).

Cloning of several members of the steroid receptor superfamily has facilitated the reconstitution of hormone-dependent transcription activation in heterologous cell systems (Evans, 1988; Green & Chambon, 1988). Subsequent *in vivo* and *in vitro* analyses with mutant and chimeric receptors have demonstrated the modular structure of steroid receptors and their organization into structurally and functionally defined domains (Danielsen et al., 1986; Kumar et al., 1986; Hollenberg et al., 1987; Rusconi & Yamamoto, 1987). The central DNA binding domain with defined 66 amino acids was studied in detail using both genetic and biochemical approaches. The hormone-binding domain (HBD), located in the carboxy-terminal half of the receptor, consists of 250 amino acids which fold into a complex tertiary structure, creating a specific hydrophobic pocket that surrounds the ligand. The HBD also contains sequences responsible for receptor dimerization, hsp interaction, and one of the two transcription activation functions. Extensive and systematic mutational analyses of this domain are essential to define the critical functional barriers.

The human glucocorticoid receptor (hGR)[1] is a regulatory protein that mediates the actions of corticosteroids in target cells. Although boundaries of the HBD have been identified through deletion analysis, a more detailed and refined analysis is necessary to identify amino acids important in binding of ligands as well as those implicated in modulating agonist and antagonist activities. Covalent affinity labeling and thiol-blocking agents were useful in studies with rat GR, which suggested that one of the methionines (Met-610) and multiple cysteines played important roles in the activation of the GR with agonists. Inactivation of cysteine has been previously shown to induce conformational changes in progesterone receptor and rat liver GR (Moudgil et al., 1989; Moudgil & Gunda, 1991). The HBD of the hGR, like that of the rat (Rusconi & Yamamoto, 1987) and mouse GRs

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[1] Abbreviations: Dexamethasone (DEX), 9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione; aldosterone (ALDO), 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al; dihydrotestosterone (DHT), 17β-hydroxy-5α-androstan-3-one; RU486, 11β-[4-(dimethylamino)-phenyl]-17β-hydroxy-17α-(prop-1-ynyl)estra-4,9-dien-3-one (generic name: mifepristone); hGR, human glucocorticoid receptor; hMR, human mineralocorticoid receptor; hPR, human progesterone receptor; hAR, human androgen receptor; hER, human estradiol receptor; MMTV, mouse mammary tumor virus; CAT, bacterial chloramphenicol acetyltransferase; GRE, glucocorticoid responsive element; PBS, phosphate buffered saline.

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(Danielsen et al., 1986), contains 13 methionine residues and 5 cysteine residues at positions 622, 638, 643, 665, and 736. Covalent labeling with dexamethasone 21-mesylate demonstrated a covalent labeling of cysteine 656 in rat GR (Simons, 1987), cysteine 644 in mouse GR (Smith et al., 1988), and cysteine 638 in hGR (Stromstedt et al., 1990). Surprisingly, substitution of any of cysteines 628, 644, or 649 in mouse GR did not affect agonist-dependent activation. However, substitution of cysteine 656 (equivalent to 638 in hGR) by either glycine or serine generated rat GR mutants which displayed increased affinity for glucocorticoids and resulted in the potentiation of hormone-mediated transcription activation function (Chakraborti et al., 1991; Opoku & Simons, 1994). Analyses with mouse GR cysteine substitution mutants (Chen & Stallcup, 1994) showed that cysteine 671 and 742 substitutions caused substantial reduction in function requiring 4−100-fold more dexamethasone for half-maximal activation of a glucocorticoid-responsive reporter gene.

We have used recombinant DNA techniques to address the importance of cysteines 638, 643, and 665 in the HBD of the hGR. Since the amino acids in other nuclear receptors at corresponding positions to C638 are not conserved, this cysteine may fulfill yet undefined functions important in facilitating the hormonal response. To understand the importance of the three cysteines in receptor function, we have characterized the nature of receptor−ligand and receptor−DNA interactions by hormone-binding, transcription activation, and gel shift analyses. We have analyzed the interactions between the various domains of the receptor by generating identical sets of internal deletions and subsequently expressing them *in vitro* by transcription and translation.

MATERIALS AND METHODS

Cell Culture Conditions and Transfections. Monkey kidney cells (CV-1), deficient in glucocorticoid receptor, were passaged in minimal essential medium (MEM, Sigma) supplemented with 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS), gentamicin (16 μg/mL), and Fungizone (1 μg/mL). The cells were grown to 70−80% confluency in 250 mL culture flasks and transfected as described (Warriar et al., 1994a−d). The cells were subjected to a 2 min glycerol shock [15% in Hepes buffered saline solution (HBS)] 4−6 h after transfection, washed twice as described, supplemented with 10 mL of fresh MEM, and grown overnight. The cells were dislocated the following day by trypsinization, resuspended in 36 mL of fresh medium, and distributed in 18 wells (25 mm). The transfectants were treated with hormones as indicated from a 1000× stock in ethanol. For hormone binding assays and gel shift studies, CV-1 cells were plated in 250 mL flasks and transfected with 15 μg of hGR expression plasmid (Warriar et al., 1994a,b) and 5 μg of β-galactosidase expression vector pCH 110 in 960 μL of precipitate. After glycerol shock and washing as described, 8 mL of fresh media supplemented with 10% DCC-FBS was added, and the cells were grown to confluency. For transactivation assays, cells were harvested after 24 h, and extracts were prepared in 100 μL of 250 mM Tris−PMSF (0.05 mM phenylmethanesulfonyl fluoride), pH 7.8. Lysis of the cells was conducted by three cycles of freeze−thaw. β-Galactosidase activity was measured in 10 μl of cell extract to normalize for transfection efficiency. Extracts containing 10 units of β-galactosidase were used to determine the CAT activity.

DNA Constructs. Cloning was performed by standard procedures (Maniatis et al., 1982). The human GR_{1-777} devoid of internal *Eco*R1 sites was inserted at the *Eco*R1 site of M13mp18 (Govindan et al., 1991), and single-stranded DNA templates were isolated in both orientations. The construction of MMTV-CAT and the eukaryotic expression plasmids for various steroid receptors was as described previously (Govindan, 1990).

In Vitro Mutagenesis. Oligonucleotides corresponding to various coding regions of hGR (15−20 nucleotides) containing a single mismatch (C638S: 5′CTACCCAGCATGTAC3′; C643S: 5′GACCAATCTAAACAC3′; C665S: 5′GAGTATCTCTCTATGAAAAC3′) or a double mismatch (C665SM666L, 5′GAGTATCTCTCTCTGAAAAC3′) were synthesized by a Biosearch DNA synthesizer and used without purification. Single-stranded template was amplified in CJ236 superinfected with M13 phage and grown in medium containing 0.25 μg/mL uridine (Kunkel, 1985). The mutant plaques were identified by screening with labeled mutant oligonucleotide probes and differential washing. The substitution mutants were identified by sequencing the single-stranded DNA. The mutant hGR cDNA fragments were isolated by restriction digestion of the replicative form with *Eco*R1, followed by sucrose gradient centrifugation, and ligated at the *Eco*R1 site of pcDNA1. The internal deletion of amino acids 76−262 was performed by digestion of the pcDNA1 expression vectors with *Bgl*II, removal of the *Bgl*II fragments by 5−20% sucrose density gradient, and religation of the vector. The recombinants were grown in superbroth and purified by two cesium chloride gradient centrifugations.

Receptor Binding Assays in Vitro. Cells transfected with 15 μg of the expression vectors were collected by scraping with a rubber policeman, washed with PBS, resuspended in 2 mL of homogenization buffer (50 mM KCl, 25 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 10% glycerol, 2.5 mM β-mercaptoethanol, and 0.1 mM PMSF), and homogenized in a glass Dounce homogenizer (50 strokes with a B pestle). A high speed supernatant of the homogenate was prepared by centrifugation at 150000*g* at 4 °C for 1 h. Saturation analyses were performed with 50 μL of cytosol aliquots (protein concentration adjusted to 2.5 mg/mL) by incubation in the presence of increasing concentrations of [1,2,4(n)-^{3}H] dexamethasone (specific activity 2.6−4.1 TBq/mmol, Amersham Corp.) in the absence (total binding) and in the presence of 200-fold molar excess of radioinert dexamethasone (nonspecific binding) overnight at 4 °C. Incubation was terminated by the addition of an equal volume of dextran-coated charcoal (0.5% Norit A charcoal, 0.05% dextran T-70) in the homogenization buffer. Specific binding was between 8900 and 14 300 cpm in 50 μL of cytosol with 62.3% counting efficiency in a Wallac, LKB 1411 scintillation counter. Specific binding (total − nonspecific binding) was plotted by Scatchard analysis, and the equilibrium dissociation constant was calculated by linear regression.

In Vitro Transcription and Translation of hGR Wild Type, Mutant, and Truncated Molecules. The cDNAs cloned in pcDNA1 expression vector were linearized with *Xba*1, deproteinized, and purified by ethanol precipitation. The template (1 μg) in a final volume of 50 μL was used for the

synthesis of mRNA using T7 coupled Reticulocyte Lysate System L4610 from Promega Corp. The mRNAs were purified by phenol extraction and ethanol precipitation. The purified mRNA (1 μg) was used in translation assays *in vitro* with [^{35}S]methionine (37 Tbq/mmol, NEN-Dupont). Aliquots (5 μL) of the *in vitro* translation products were solubilized in 20 μL SDS sample buffer and resolved on a 7.5% SDS−polyacrylamide gel by electrophoresis. The gels were treated with EnHance (NEN-Dupont), dried, and autoradiographed using Kodak X'Omat films.

Labeling of Transfected Cells with [^{35}S]Methionine and Immunoprecipitation. Transfected CV-1 cells were incubated overnight with [^{35}S]methionine in Dulbecco's modified Eagle's Medium (DMEM) devoid of methionine at 37 °C. The cells were washed with PBS, harvested in PBS, resuspended in 500 μL of homogenization buffer containing 400 mM KCl, and homogenized (50 strokes with a B pestle). The homogenates were centrifuged at 105000*g* to yield soluble cellular extracts. The hGR was immunoprecipitated from extracts containing 50 units of β-galactosidase (total protein ~150 μg) as previously described (Warriar et al., 1994a; Govindan & Gronemeyer, 1984), with 5 μL of polyclonal antibodies raised in rabbits against the synthetic polypeptide of hGR (H_2N-Phe^{383}-Pro-Gly-Arg-Thr-Val-Phe-Ser-Asn-Gly-Tyr^{393}-COOH) coupled to keyhole hemocyanin. Fifty microliters of these antibodies at 1:1000 dilution recognized 12 pg of the receptor polypeptide and precipitated 78% of [^{3}H]dexamethasone-bound hGR present in 100 μl of cytosol. The immune complexes were precipitated using protein A−Sepharose (Pharmacia, Canada) as described previously (Govindan & Gronemeyer, 1984), and the GR was analyzed on a 7.5% SDS−polyacrylamide gel by electrophoresis. The gels were treated with EnHance (Dupont, U.S.A.), dried, and subjected to autoradiography.

Western Blotting. Extracts prepared from transiently transfected CV-1 cells (150 μg total proteins) were resolved on a 7.5% SDS−polyacrylamide gel by electrophoresis. The proteins were electroblotted from the gel onto an Immobilon-P membrane, and the marker proteins (high molecular weight protein markers from Bio-Rad) were stained with Coomassie blue. The membranes were blocked with BLOTTO [5% w/v nonfat dry milk, 0.01% Antifoam A (Sigma), and 0.0001% merthiolate in PBS] (Johnson et al., 1984) at room temperature for 2 h and incubated with a 1:100 dilution of anti-hGR antipeptide antiserum or preimmune serum in BLOTTO for 3 h, washed three times over a period of 1 h with 50 volumes of Tris−EDTA−NaCl (TEN: 20 mM Tris-HCl, pH 7.8, 1 mM EDTA, and 140 mM NaCl), TEN + 0.05% Tween 20, and TEN. Finally, the filters were incubated with ^{125}I-labeled donkey anti-rabbit immunoglobulin (Amersham; catalog no. IM-134) in BLOTTO for 1 h. Membranes were washed as described above and autoradiographed with Kodak X'OMAT films and intensifying screens.

Analysis of Receptor−DNA Interaction by Gel Mobility Shift. Cells were collected by scraping, washed twice with PBS, resuspended in extraction buffer A (10 mM Hepes−KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM PMSF) with or without 1×10^{-6} M hormones for 15 min on ice, and then vortexed for 10 s. The samples were centrifuged, and the pellets were resuspended in 100 μL of ice-cold extraction buffer B (20 mM Hepes−KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM PMSF) with or without 1×10^{-6} M hormones and incubated on ice for high salt extraction. Cellular debris was removed by centrifugation, and the supernatant fractions were stored in aliquots at −70 °C (Andrews & Faller, 1991). β-Galactosidase activity was determined in an aliquot of the extract. The yield was 300−350 μg of protein/5 $\times$ 10^{6}cells. Extracts containing 3 μg of protein equivalent to 1 unit of β-galactosidase activity were incubated with poly(dI·dC) on ice for 15 min in a final volume of 20 μL of DNA binding buffer. Radiolabeled probe (5000 cpm) was added and incubated at room temperature. The GR−DNA complexes and free probe were then separated on a 4% polyacrylamide gel run for 100 min at room temperature, dried, and autoradiographed using intensifying screens.

RESULTS

Expression of Intact hGR Cloned in pcDNA1 by Coupled in Vitro Transcription and Translation. In vitro transcription and translation of the cloned products was performed to analyze the expression of intact and truncated GR cDNAs cloned in pcDNA1 vectors. *In vitro* transcription and translation with linearized control pcDNA1 vector showed the absence of methionine-labeled protein bands (not shown). The coupled transcription and translation of wild type and mutants hGRs *in vitro* showed a major labeled product with an apparent molecular mass of 90 000 Da (Figure 1A) and a 68 kDa translation product which was often thought to be a degradation product of 90 kDa hGR. However, expression of deletion hGRΔ_{76-262} mutants produced not only identical 78 kDa proteins but also the 68 kDa receptor fragment (Figure 1A). The expression of receptor mutants C638S, C643S, C665S, and C665SM666L reached identical levels with the same quantity of linearized cDNA.

To verify the level of receptor expression following transient transfection, immunoprecipitation was performed with [^{35}S]methionine-labeled total cell extracts prepared from transfected CV-1 cells. The immune complexes were precipitated with protein A−Sepharose and analyzed by SDS−polyacrylamide gel electrophoresis and autoradiography. The hGR wild type extract treated with the preimmune serum failed to react with the 90 kDa hGR band (Figure 1B, lane 1) demonstrating the specificity of the anti-hGR antiserum. Treatment of the extracts containing hGR wild type (Figure 1B, lane 2), hGR C638S (lane 3), hGR C643S (lane 4), hGR C665S (lane 5), and hGR C665SM666L (lane 6) with GR-specific antiserum showed a labeled band of 90 kDa. To correct for variations in transfection efficiencies, cotransfection of the β-galactosidase expression vector pCH 110 was performed. The subsequent measure of enzyme activity assured the use of an identical quantity of cell extract. Immunoprecipitation of [^{35}S]methionine-labeled receptors expressed in CV-1 cells with hGR-specific polyclonal antibodies (Figure 1B) showed that wild type and mutant receptor expression levels were identical.

Extracts prepared from CV-1 cells were separated on a SDS−polyacrylamide gel, blotted onto membrane, and treated with preimmune serum (Figure 1C, lane 1) or with anti-GR antiserum (Figure 1C, lanes 2−6). The preimmune serum-treated blot did not contain any specific radioactive band, thus demonstrating the specificity of anti-receptor antiserum generated against synthetic receptor polypeptide

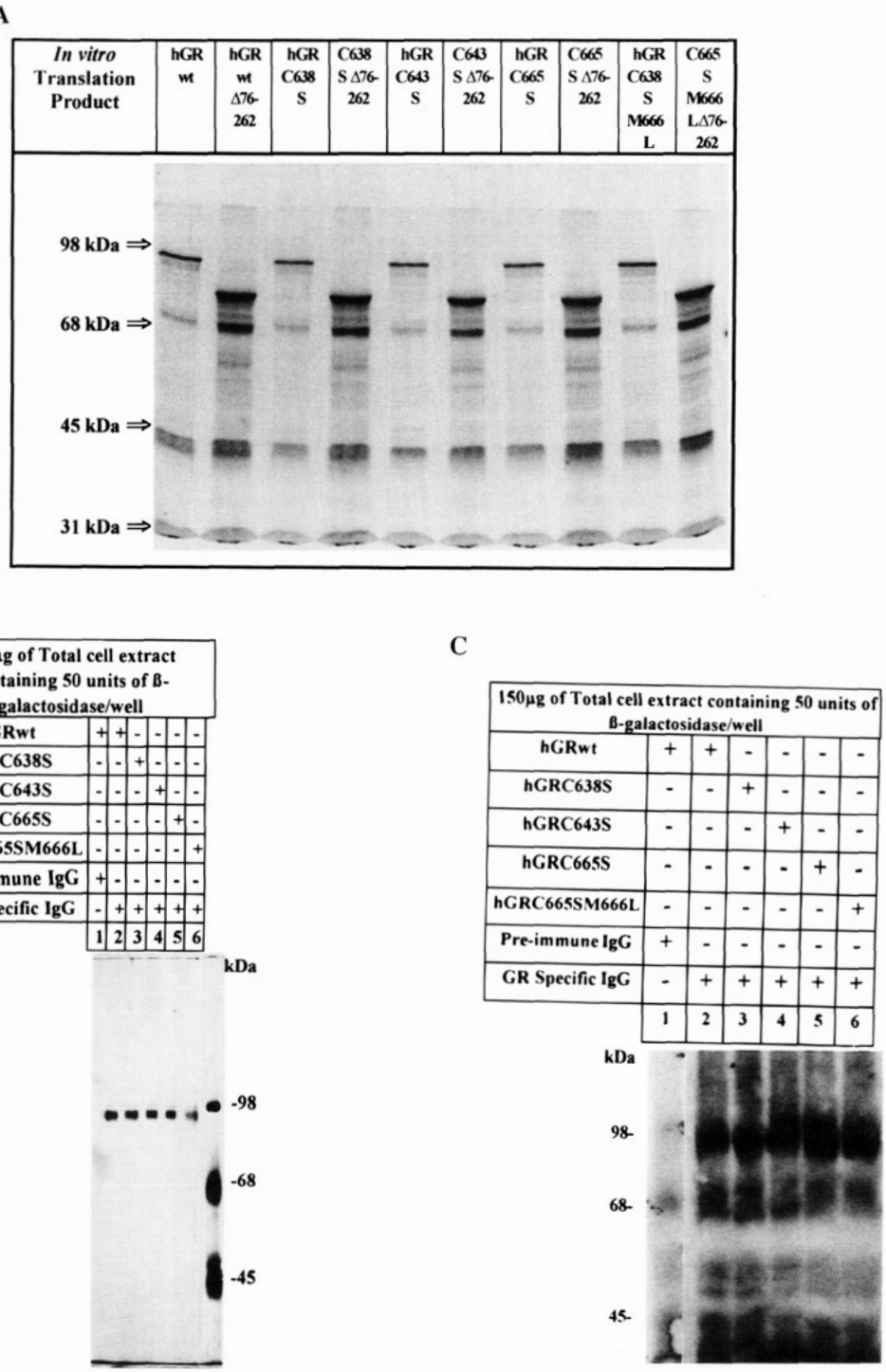

FIGURE 1: (A) *In vitro* transcription and translation of wild type and mutant hGRs. 1 μg of purified mRNAs was used in *in vitro* translation assays with [^{35}S]methionine (37 Tbq/mmol, NEN-Dupont). The wild type hGR_{1-777} migrated with a molecular mass of 90 kDa (lane hGRwt). The calculated molecular mass of the truncated $hGR\Delta_{76-262}$ is 65 000 Da (lane $hGR\Delta_{76-262}$), but it migrated with a higher apparent molecular mass in SDS−polyacrylamide gels. The templates used in *in vitro* transcription and the *in vitro* translation experiments are indicated in the figure. Molecular masses in kDa correspond to the positions of standards stained with Coomassie blue (Bio-Rad). (B) Immunoprecipitation of hGR(s) expressed in CV-1 cells with hGR-specific antibodies. An aliquot of 150 μg of total [^{35}S]methionine-labeled proteins (equivalent to 50 units of β-galactosidase activity), extracted from CV-1 cells transiently transfected with hGR expression vector(s), was incubated with 5 μL of hGR-specific antiserum or preimmune serum as described in Materials and Methods. The immunoreactive products were precipitated with protein A−Sepharose, analyzed by SDS−polyacrylamide gel electrophoresis, and visualized by autoradiography. The control containing hGR wild type extract treated with preimmune serum (lane 1) demonstrates the specificity of the hGR-specific antibodies. The ^{14}C-labeled protein size markers were from Gibco-BRL. (C) Immunoblot of hGR. Aliquots of proteins were resolved on a 7.5% SDS−polyacrylamide gel by electrophoresis. The proteins were electroblotted from gel onto Immobilon-P membrane, and marker proteins were stained with Coomassie blue. Relative positions are indicated in kilodaltons. Membranes were treated as described in Materials and Methods, and the antigen−antibody complexes were visualized by autoradiography.

coupled to keyhole hemocyanin. The intensity of the anti-receptor antiserum-treated blots reveals that the levels of expression of the wild type hGR and the cysteine mutants following transfection in CV-1 cells were identical.

Hormone Binding and Affinity Studies with the in Vitro Generated Mutant Receptors. The expression of wild type

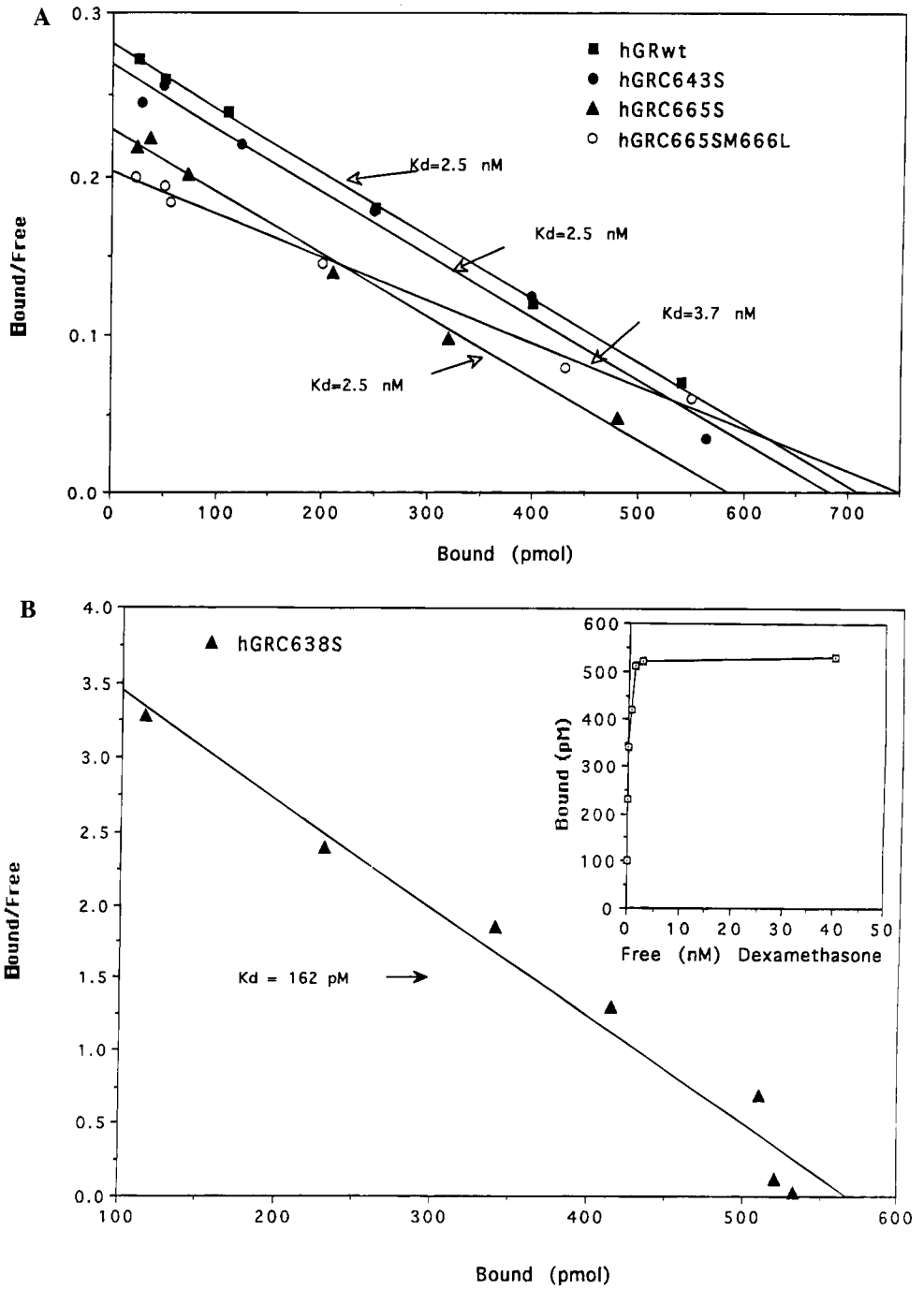

FIGURE 2: Determination of dissociation constant of dexamethasone binding to wild type GR, hGR C638S, hGR C643S, and hGR C665S by Scatchard analysis. For hormone binding analysis, aliquots of cytosol prepared from CV-1 cells transfected with wild type and mutant hGR expression vectors were incubated with 10 nM [^{3}H]cortisol in the presence of increasing concentrations of the radioinert competitor (10^{-12}–10^{-6} M) DEX. Each point represents the average of duplicate samples and is expressed as a percentage of the control incubates in the absence of radioinert competitor.

and mutant hGRs in cytosolic extracts of cells transfected with the wild type GR and mutant expression vectors was analyzed for glucocorticoid binding by a dextran-coated charcoal assay. The specifically bound radioactivity, which varied between 96% and 98%, was determined in parallel incubations containing 200-fold molar excess of radioinert ligand.

To investigate the possibility that the GR mutants could be inactive in transcription activation due to a loss in the ability to bind ligands efficiently, we determined their affinities for dexamethasone by saturation analysis. hGR C643S, hGR C665S, and hGR C665SM666L exhibited an essentially wild type hGR K_d of 2.5 nM (Figure 2A), while hGR C638S exhibited a K_d of 162 pM, a 15-fold higher affinity (Figure 2B). The analysis of the relative affinities of hGR wild type and mutants for ALDO and RU486 showed that the mutations affected the ability of the mutants to interact with various ligands.

To further characterize the interaction of agonist and antagonist with the substitution mutants, we performed the competition assays with RU486 in the presence of 10 nM [^{3}H]dexamethasone (Figure 3A). The competition curve of hGR C643S and C665S for RU486 shifted to the right when compared to that of the wild type hGR. While the half-

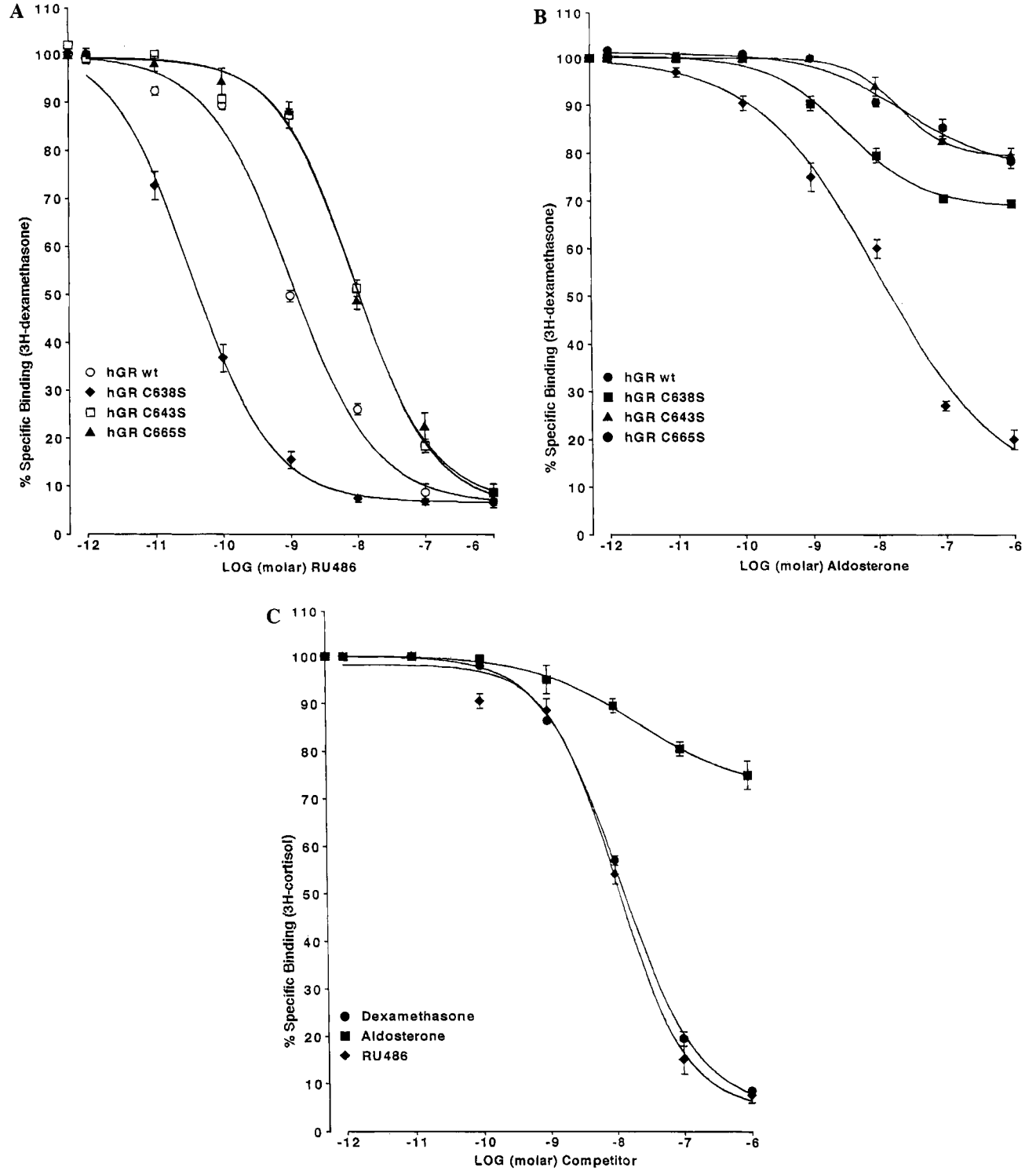

FIGURE 3: (A) Determination of relative binding affinities of wild type GR, hGR C638S, hGR C643S, and hGR C665S for RU486. Identical experiment as described in Figure 2A except that the binding assays were performed with 10 nM [^{3}H]DEX in the presence of increasing concentrations of the radioinert competitor (10^{-12}–10^{-6} M) RU486. (B) Determination of relative binding affinities of wild type GR, hGR C638S, hGR C643S, and hGR C665S for aldosterone (ALDO). Identical experiment as described in Figure 2B except that the binding assays were performed with 10 nM [^{3}H]DEX in the presence of increasing concentrations of the radioinert competitor (10^{-12}–10^{-6} M) ALDO. (C) Determination of relative binding affinities of hGR C665SM666L for dexamethasone (DEX), aldosterone (ALDO), and RU486. For hormone binding analysis, aliquots of cytosol prepared from CV-1 cells transfected with hGR C665SM666L expression vector were incubated with 10 nM [^{3}H]cortisol in the presence of increasing concentrations of radioinert competitor (10^{-12}–10^{-6} M) DEX, ALDO, or RU486. Each point represents the average of duplicate samples and is expressed as a percentage of the control incubates in the absence of radioinert competitor.

maximal saturation of wild type receptor with RU486 was achieved with 1 nM, the half-maximal saturation point with C643S and C665S was at 10 nM RU486. The half-maximal RU486 competition with hGR C638S was reached at 50 pM.

Additional competition assays were performed to detect any change in binding specificities introduced by these substitutions. In a competition assay, the extracts prepared from CV-1 transfected hGR wild type and substitution

mutants were challenged with increasing concentrations of ALDO in the presence of 10 nM [^{3}H]dexamethasone (Figure 3B). The wild type hGR, hGR C638S, and C643S showed a relatively weak affinity for ALDO. However, ALDO competed efficiently for the binding of [^{3}H]dexamethasone to C665S. Specific binding of dexamethasone to this mutant was reduced by 80% in the presence of 1 μM aldosterone.

Competition assays with the double mutant hGR C665SM666L were conducted to determine if a change in an adjacent amino acid would interfere with the hormone binding process. The receptor mutant was challenged with a series of increasing concentrations of the competitors dexamethasone, ALDO, and RU486 in the presence of 10 nM [^{3}H]cortisol (Figure 3C). Half-maximal competition for RU486 with hGR C665SM666L was achieved at 10 nM, whereas this mutant failed to bind ALDO.

Transcriptional Activation of MMTV-CAT by Wild Type and Mutant GRs. The transcriptional activation function of the wild type and mutant receptors was analyzed by measuring CAT activity in CV-1 cells cotransfected with the glucocorticoid-responsive reporter MMTV-CAT. The wild type hGR showed the highest response to 10 nM dexamethasone (Figure 4A). When compared to wild type receptor, CAT activity with mutant C638S was almost 4-fold higher at saturating dexamethasone concentrations, which paralleled the higher affinity of this receptor mutant for dexamethasone, and the half-maximal level of dexamethasone required was 10 pM. With hGR C665S and C643S, the maximum CAT induction was at 1 nM dexamethasone.

We then examined the effects of the cysteine mutations on the inducibility of the reporter gene in the presence of RU486. While transcription activation of MMTV-CAT was suppressed with RU486 and wild type hGR or C643S (Figure 4B), treatment of the hGR mutants C638S and C665S cotransfectants with RU486 stimulated the CAT activity. The hGR C638S mediated a 25-fold induction of CAT activity with RU486, a response which was half-maximal with 100 pM RU486. Even though the substitution of C665S did not affect dexamethasone and RU486 interactions with the mutant receptor, MMTV-CAT was induced by treatment of the transfectants with RU486 in a dose-dependent fashion (Figure 4B). By comparing the transcription activation of MMTV-CAT by hGR C665S in the presence of RU486 (Figure 4B) and dexamethasone (Figure 4A), we conclude that RU486 is a full agonist here.

To compare the hormone-responsive transcription activation function, we treated hGR wild type and mutant transfectants with 10 nM dexamethasone (Figure 5, lanes 2, 8, 14, 20, and 26), ALDO (lanes 3, 9, 15, 21, and 27), progesterone (lanes 5, 11, 17, 23, and 29), dihydrotestosterone (lanes 6, 12, 18, 24, and 30), and 1 μM RU486 (lanes 4, 10, 16, 22, and 28). Transactivation of MMTV-CAT by wild type, C643S, and the double mutant hGR C665SM666L was not substantially affected with 10 nM ALDO (Figure 5, lanes 3, 15, and 27, respectively). However, the mutants C638S (lane 9) and C665S (lane 21) displayed a dramatic ALDO-dependent transcription activation of MMTV-CAT in transfected CV-1 cells. The levels of transactivation by hGR C665S with ALDO reached similar high levels as with dexamethasone (lanes 21 and 20).

To characterize the response, similar experiments were conducted with hGR wild type or mutants and MMTV-CAT and transfectants were treated with progesterone and DHT.

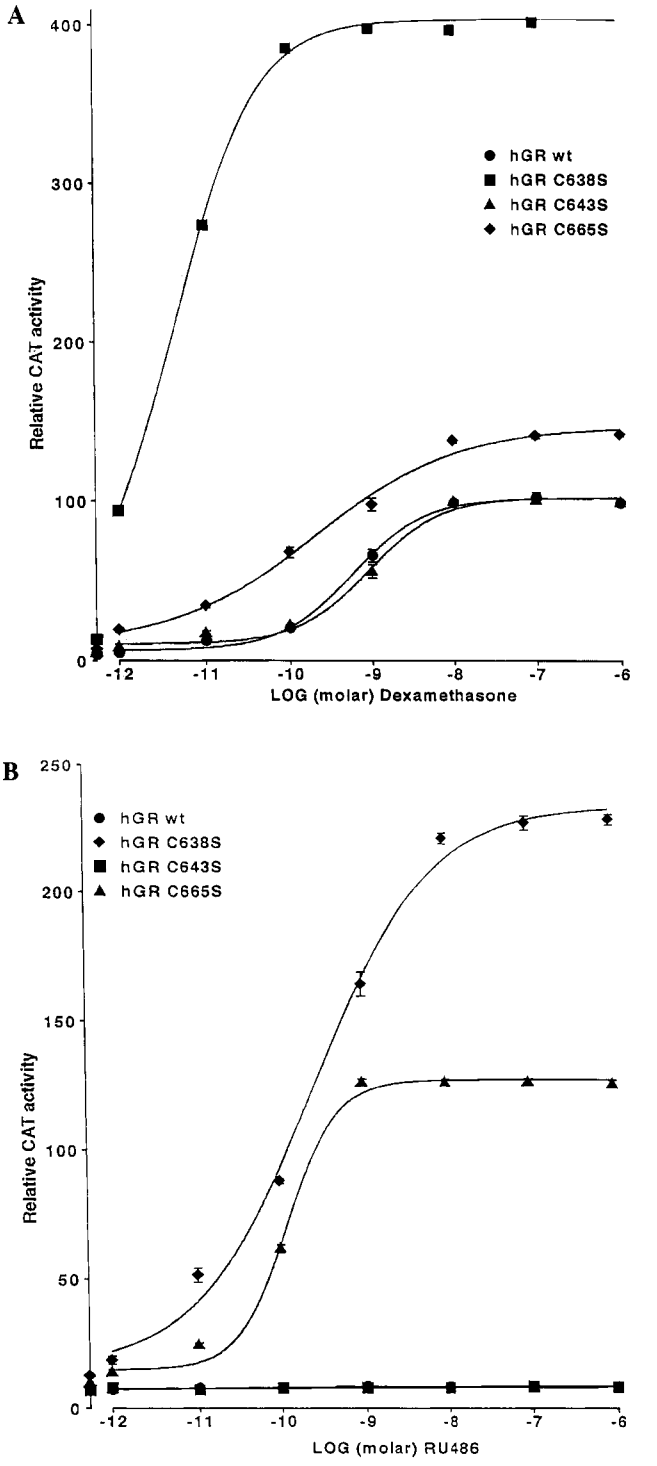

FIGURE 4: Transcription activation and dose response to dexamethasone. (A) CV-1 cells transiently cotransfected with MMTV-CAT and CH110 were collected and distributed in 18 wells as described previously (Warriar et al., 1994a–d). The cells in wells were treated with DEX (10^{-12}–10^{-6} M) for 24 h and collected, and CAT activity was determined as described in Materials and Methods. (B) Identical experiments as described for panel A except that the cells were treated with the glucocorticoid antagonist RU486.

Incubation of the hGR wild type and MMTV-CAT cotransfectants with progesterone (lane 5) or dihydrotestosterone (lane 6) did not elevate transcription above the basal levels. The mutant hGR C638S produced measurable levels of CAT activity following treatment both with progesterone (lane 11) and with dihydrotestosterone (lane 12). Even though C665S affected the response to progesterone slightly (lane 23), no

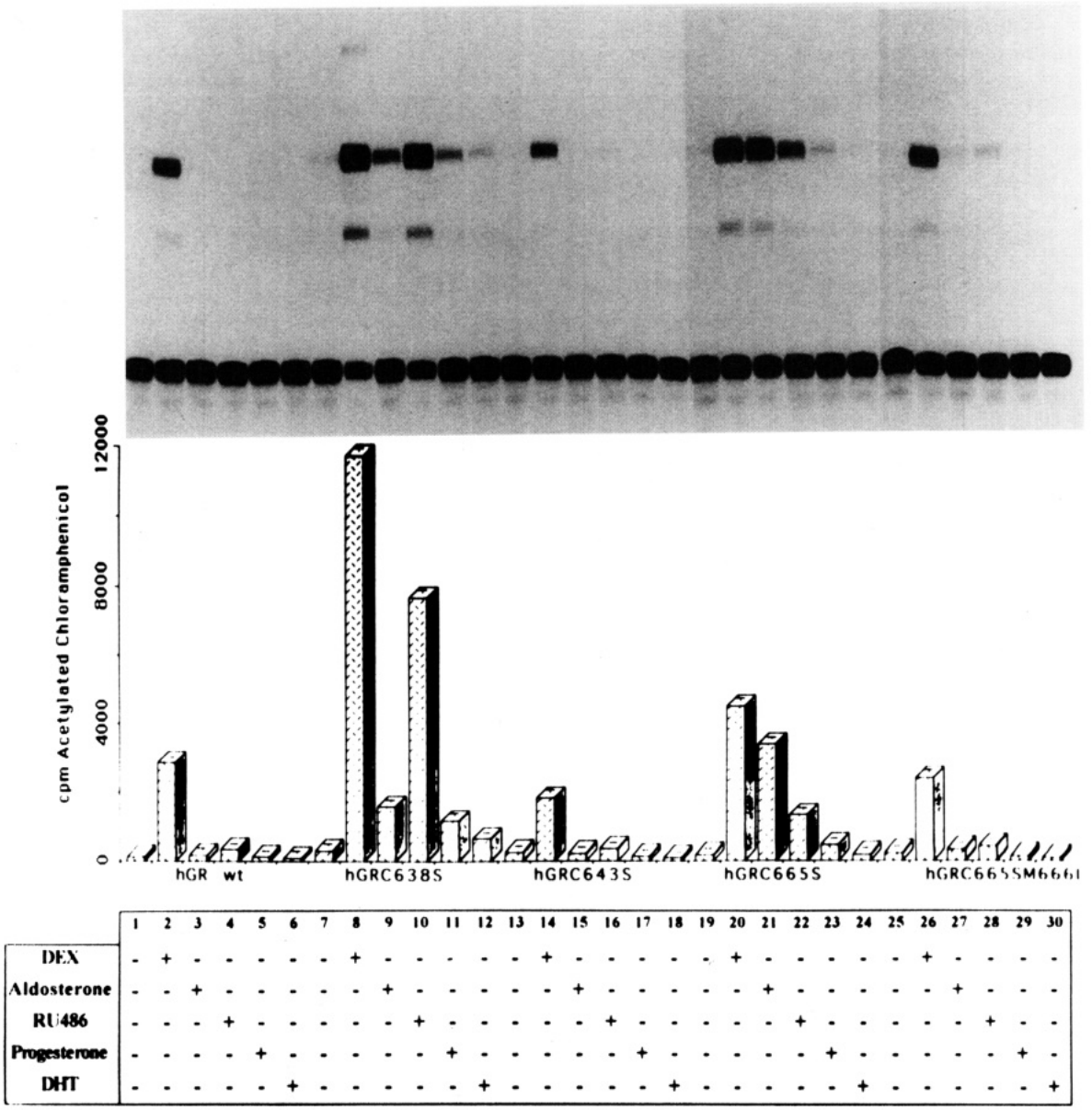

FIGURE 5: Comparison of transcription activation by wild type and mutant hGRs. CV-1 cells were cotransfected as described under Materials and Methods and subjected to the treatments as indicated. The cotransfectants were treated with 10 nM DEX (lanes 2, 8, 14, 20, and 26), 10 nM ALDO (lanes 3, 9, 15, 21, and 27), 1 μM antiglucocorticoid RU486 (lanes 4, 10, 16, 22, and 28), 10 nM progesterone (lanes 5, 11, 17, 23, and 29), and 10 nM DHT (lanes 6, 12, 18, 24, and 30), respectively. The cells used in hormone treatments were from a single pool of transiently transfected CV-1 cells. The induced CAT activity was determined in an aliquot of the lysate containing 10 units of β-galactosidase. The acetylated chloramphenicol derivatives were visualized by autoradiography, and the radioactivity was determined by scintillation counting. The values are the average of triplicate experiments, and shown are the results of transactivation by hGR wild type (lanes 1−6), hGR C638S (lanes 7−12), hGR C643S (lanes 13−18), hGR C665S (lanes 19−24), and hGR C665SM666L (lanes 25−30).

activation of CAT was observed with C643S or with the double mutant C665SM666S in the presence of progesterone.

Interaction of hGR Wild Type and Mutant Receptors with ^{32}P-Labeled GRE. We prepared cell extracts from CV-1 cells transfected with receptor expression vectors to overexpress the encoded proteins. The extracts used in protein−DNA interactions were initially normalized for transfection efficiency by determining the β-galactosidase activity. A 3 μg amount of the extract used in DNA−protein interactions contained 1 unit of β-galactosidase activity. This procedure excludes the possibility that the differences observed in complex formation were the result of differential receptor expression in transfected CV-1 cells. CV-1 nuclear protein extract, containing hGRΔ_{76-262} bound with dexamethasone and incubated with [^{32}P]GRE, showed that the complexes were smaller (Figure 6, panels A and B, lanes 2−4) than the DNA complexes observed with wild type hGR bound with dexamethasone (Figure 6A, lanes 5−10). The wild type hGR in the absence of dexamethasone bound to GRE less efficiently than in the presence of dexamethasone (compare Figure 6A, lanes 5−7 with lanes 8−10, respectively). The specificity of interaction with DNA was characterized by the addition of 10-fold (Figure 6A, lanes 6 and 9) and 100-fold (lanes 7 and 10) molar excess of competitor GRE in parallel incubations. hGR C638S, in the presence of dexamethasone, formed a more intensely complexed band than the wild type hGR (Figure 6A, lanes 11−13). The DNA−hGR C643S complexes (lanes 14 and 15) were similar in size to that observed with hGR wild type. Similar DNA−protein interaction analyses demonstrated the specificity and hormonal dependency with hGR C665S and hGR C665SM666L (Figure 6B, lanes 5−15). In the absence of

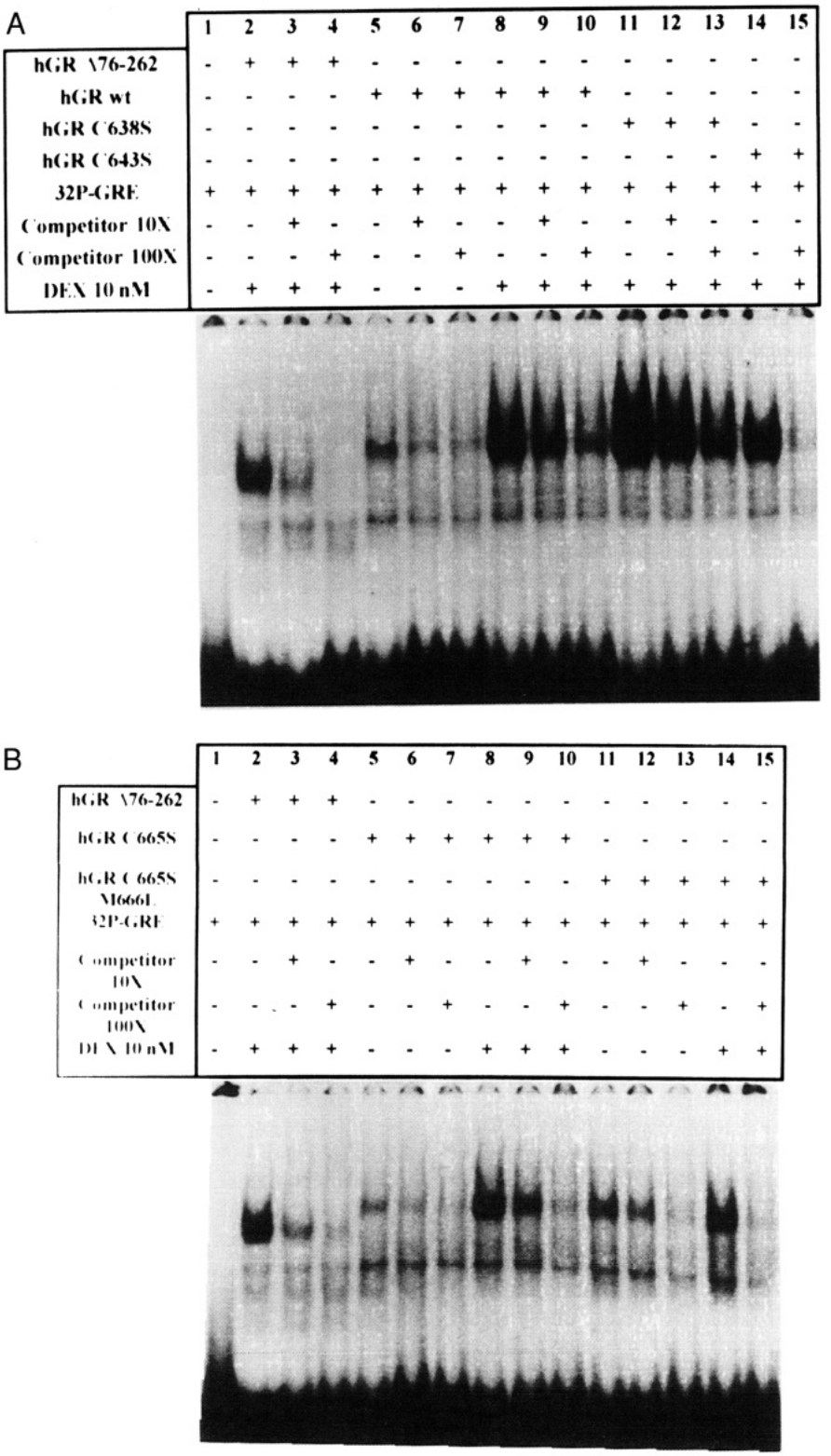

FIGURE 6: Anaysis of specific interaction of hGR overexpressed in CV-1 cells with labeled GRE by mobility shift assay. Total cell extracts were prepared and incubated with labeled GRE in the presence of the indicated concentrations of competitor. Extracts from $hGR\Delta_{76-262}$ in the presence of DEX (panels A and B, lanes 2−4) show a faster migrating GRE−receptor complex. The complexes formed with wild type hGR in the absence (panel A, lanes 5−7) and in the presence of DEX (panel A, lanes 8−10) are shown for comparison. The complexes formed with mutant hGR C638S (panel A, lanes 11−13) and mutant hGR C643S (panel A, lanes 14 and 15) are in the presence of DEX. The interaction of hGR C665S with GRE in the absence of DEX (panel B, lanes 5−7) and in the presence of DEX (panel B, lanes 8−10) demonstrates the hormonal dependency of DNA−protein interactions. Identical experiments performed with hGR C665SM666L expressed in CV-1 cells with GRE in the absence (panel B, lanes 11−13) and in the presence of DEX (panel B, lanes 14 and 15) also demonstrate the hormonal dependency of receptor−DNA interactions. Additional specificity of the GRE−hGR interaction was performed by including 10-fold (panels A and B, lanes 3, 6, 9, and 12, respectively) and 100-fold (panels A and B, lanes 4, 7, 10, 13, and 15, respectively) molar excess of unlabeled GRE as competitor in parallel incubations. The gels were dried, and the radioactive complexes were visualized by autoradiography.

hormone, the receptor−DNA complexes were less abundant (Figure 6B, lanes 5−7 and 11−13, respectively) and the addition of hormone facilitated a more intense interaction between the mutant hGRs and labeled GRE (Figure 6B, lanes 8−10 and 14−15, respectively).

DISCUSSION

To assess the importance of amino acids C638, C643, and C665 in the hGR HBD (amino acids analogous to A844, C849, and I871 in the hMR), we have generated the substitution mutants C638S, C643S, C665S, and C665SM666L. Using rat GR cDNA, Chakraborti et al. (1991) and Opoku and Simons (1994) analyzed the formation of intramolecular disulfide bonds with *in vitro* generated receptor mutants. They came to the conclusion that the intramolecular disulfide bonds were of transitory importance and that the structural or functional changes of native GR associated with steroid binding, activation, and dissociation of heat shock protein 90 neither involve nor require the formation or reduction of stable intramolecular disulfide bridges. However, similar analyses by mutagenesis of cysteines in the HBD of the hER demonstrated alterations in binding and transcriptional activation by covalently and reversibly attaching ligands (Reese & Katzenellenbogen, 1991). To understand the mechanism of corticosteroid specificity and to characterize the importance of conserved cysteines in the HBD of hGR, we have generated a set of cysteine to serine substitution mutants. Our analyses included (a) expression of the wild type and mutant receptors by coupled in *vitro* transcription and translation, (b) determination of receptor affinity for dexamethasone by saturation analysis, (c) determination of receptor expression levels in transfected cells by immunoprecipitation and Western blotting with receptor-specific polyclonal antibodies, (d) expression of the hGRs in CV-1 cells by transient cotransfection and competition of the hGRs with glucocorticoids, mineralocorticoids, and antagonists, (e) analyses of dose response to dexamethasone and RU486 in transcription activation of MMTV-CAT, and (f) determination of DNA−receptor interaction by gel mobility shift assay.

We have focused on the functions of C638S and C665S in corticosteroid−hGR interaction in the HBD. In related steroid receptors such as hAR, hER, hGR, and hPR, homologous positions to C665 are occupied by cysteines, and in the hMR, this position is occupied by a hydrophobic isoleucine. With C665S we generated a receptor mutant with similar affinity for agonist dexamethasone as the wild type receptor, and competition analysis showed a 10-fold lower affinity of C665S for the antiglucocorticoid RU486 and an increased affinity for the mineralocorticoid ALDO. In transactivation analyses, RU486 had strong agonistic activity instead of the inhibitory effect observed with wild type hGR. This cysteine, while defining corticosteroid responsiveness and specificity, transformed the mutant hGR to mediate transactivation in the presence of ALDO, for which the wild type hGR had little or no affinity. Therefore, conservation of this cysteine is perhaps imperative to maintain receptor specificity in mineralocorticoid target tissues. A number of reports attribute a pivotal role of 11β-hydroxysteroid dehydrogenase in facilitating the discrimination between glucocorticoids and mineralocorticoids in target tissues (Funder et al., 1990). Our data show that C665 is also a good candidate for maintaining receptor specificity. The introduction of a serine at this position did not negatively influence glucocorticoid binding, but combined with the substitution of the adjacent methionine M666 by a comparably hydrophobic leucine, transcription activation in the presence of ALDO was abolished. With dexamethasone, CAT induction was similar to wild type receptor, and in the presence of other ligands, this double mutant did not activate the transcription of the model reporter gene.

Most internal deletions or substitutions of hGR HBD either eliminate or greatly decrease steroid binding (Danielsen et al., 1986; Kumar et al., 1986; Giguère et al., 1986; Hollenberg et al., 1989; Hurley et al., 1991). Among the related steroid receptors, C638 is not conserved and is analogous to hPR S793. hGR C638, analogous to rat GR C656, when replaced by a serine produced a receptor with a 15-fold higher affinity for ligand dexamethasone. We propose that C638 defines specificity to glucocorticoids because when serine is at this position, the receptor shows a remarkable increase in affinity for both agonist dexamethasone and antagonist RU486. As previously reported, rat GR C656S also demonstrated higher affinity for ligand, which is in complete agreement with our mutant C638S which induced MMTV-CAT at 1 pM dexamethasone. The analogous mouse receptor GR C644, on the other hand, when replaced by a serine, had no effect on affinity (Chen & Stallcup, 1994). We suggest that C638S in the HBD of the hGR generates a ligand-bound receptor that is more effective in displacing factors associated with the nonactivated form of the receptor, unmasking the DNA-binding domain and promoting a conformation more favorable to DNA interaction. Binding of the antagonist RU486 also stabilizes HBD−hsp90 binding (Denis et al., 1988; Fawell et al., 1990). A number of studies have established that the HBD of the GR is necessary for binding of the receptor to hsp90 (Kumar & Chambon, 1988; Fawell et al., 1990; Dalman et al., 1991; Cadepond et al., 1991; Scherrer et al., 1993). Except in the hER, positions corresponding to hGR C643 in the hAR, hMR, and hPR are all occupied by cysteines. The introduction of serine generated a receptor with wild type receptor properties except for affinity for RU486 which was 10-fold lower. These results indicate that though C638 is not crucial to the ligand-binding process, it is nevertheless involved in maintaining specificity to glucocorticoids. Rat GR C661S, analogous to hGR C643, showed a 4-fold loss in binding affinity, whereas hGR C643S maintained essentially wild type affinity and biological activity (Chakraborti et al., 1991; Opoku & Simons, 1994). In the mouse, GR C649, when substituted by a glycine, required 2−3-fold ligand concentration for eliciting a biological response (Chen & Stallcup, 1994).

In conclusion, the cysteines fulfill an important function in hGR by facilitating the interaction of specific ligands. This process may involve direct interaction with factors such as hsp90 and other uncharacterized factors by maintaining a tight hydrophobic pocket which prevents access of inappropriate ligands in circulation. Conservation of C665 regulates the interaction of glucocorticoids and mineralocorticoids with hGR, thus defining tissue and ligand specificity in corticosteroid action. The previously proposed cellular mechanism of aldosterone specificity conferred by the glucocorticoid inactivating enzyme 11β-HSD becomes obsolete when C665 is substituted by serine. Cysteine 638 plays a role in limiting the agonist and antagonist occupancy as well as in determining the level of transcription activation

induced by individual ligands. It is therefore important in the hGR to maintain optimal affinity for glucocorticoids. Subtle changes in the local interaction between residues in the HBD contribute to the contact between ligand and receptor where certain interactions result in the activation of the receptor at a lower ligand concentration as with hGR C638S. It will be interesting to assess the results of substitution of hMR A844, C849, and I871 with the corresponding residues found in hGR to evaluate the ligand affinity and transactivation potentials. Eventually, this process may provide a system and a method of analysis to differentially examine potentially important residues involved in achieving hormonal specificity, TAF-2 activity, and interaction with receptor activating factors.

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