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Genetic Analysis of the N-Terminal End of the Glucocorticoid Receptor Hormone Binding Domain

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Four site-directed missense mutations were constructed at the N-terminal end of the mouse glucocorticoid receptor (GR) hormone binding domain. This small subdomain is highly conserved among the steroid hormone receptors and is within a larger subregion believed to be important for hormone binding, transcriptional activation, and hsp90 binding. The ability of mutant and wild type GR to activate a reporter gene in response to various concentrations of dexamethasone was examined in transiently transfected COS-7 cells. Mutant GR species V544G (valine-544 changed to glycine) and V549G activated the reporter gene to approximately the same extent as wild type GR, but required approx. 7 and 23 times greater hormone concentrations, respectively. In contrast, double mutant LL541/2GG (leucines changed to glycines) could not activate transcription even at 10 μM dexamethasone or deacylcortivazol, while E543A (glutamic acid to alanine) was functionally indistinguishable from wild type GR. GR mutants LL541/2GG and V549G had reduced abilities to bind covalently to affinity label dexamethasone 21-mesylate. The partially and fully functional mutant GR species had no deficiency in transcriptional transactivation activity in the presence of saturating concentrations of agonist.

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INTRODUCTION

The glucocorticoid receptor (GR) is one of the best understood members of a large group of conditional transcriptional regulators known as the steroid/thyroid hormone receptor superfamily [1–3]. Like all members of this family the GR contains three functional domains required for the signal transduction pathway of glucocorticoid hormone [1, 4–6]. The N-terminal half of the receptor is the modulatory or transactivation domain which is required for full transcriptional activation, but is not required for DNA or hormone binding. A centrally located domain of 70 amino acids imparts DNA binding affinity and specificity to the receptor, and the C-terminal 250 amino acid region of the GR is responsible for hormone binding. The GR in its unliganded state is a cytosolic protein that is bound to hsp90. Upon binding hormone the GR dissociates from hsp90, forms homodimers, translocates to the nucleus, and binds to specific DNA enhancer elements that are associated with glucocorticoid regulated genes. Interaction of DNA-bound GR dimers with chromatin and/or transcription machinery causes increased or decreased transcription initiation from the associated promoter.

The focus of this study is the GR hormone binding domain (HBD). In addition to being responsible for binding hormone the C-terminal HBD is the location of one of two signals responsible for nuclear translocation [7], the putative hsp90 binding site [8–10], and two reported transactivation domains [11, 12]. The HBD and its association with hsp90 play a key role in regulating the function of the GR. Association with hsp90 is required for full GR function [13] including high affinity hormone binding [14, 15]. In addition, the unliganded cytosolic receptor binds DNA poorly, but receptors with the entire HBD deleted bind DNA with high affinity and specificity and activate transcription in the absence of hormone [16]. These findings suggest that hsp90 holds the GR in a conformation that is competent to bind hormone, but actively represses other receptor functions. Hormone binding appears to derepress the other functions of the receptor.

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The DNA binding domain has been extensively analyzed by site directed mutagenesis, and as a result many of the amino acids responsible for DNA binding affinity and specificity are known [6, 11, 17–19]. This genetic data, along with a crystallographic analysis of the 3-dimensional structure of the DNA binding domain [20], have provided a fairly detailed understanding of GR interactions with DNA. In contrast, despite the functional importance of the HBD, little is known of its structure or of specific amino acids that are important for its various functions.

A variety of genetic and biochemical studies have provided relevant clues about the hormone binding function. Deletion and insertion analyses defined the approximate boundaries of the HBD and suggested that sequences throughout the entire 250 amino acids of the HBD are required for high affinity ligand binding [21, 22]. However, protease digestion of the rat GR generated a 16-kDa fragment, representing the N-terminal half of the HBD, that retains hormone binding ability at a reduced affinity and is thus regarded as the core of the hormone binding function [23]. A limited number of specific point mutations that alter GR function have been reported in the HBD [12, 24–29]. In biochemical studies five amino acids (M610, C628, C644, C649, and C742 of the mouse GR) have been shown to be in the vicinity of the hormone binding site by covalent affinity labeling [30–32] or by interaction with thiol blocking reagents that inhibit steroid binding [33]. Thus, a very small number of functionally significant amino acids have been identified, but functional subdomains of the HBD have not been, and no comprehensive genetic or structural analyses have been performed to date.

Here we report a convenient system for producing and functionally analyzing site directed mutations in the HBD of the mouse GR cDNA. The new experimental system was used to investigate the functional significance of a small subdomain at the extreme N-terminal end of the HBD (amino acids 541–549 of the mGR). The potential importance of this region was suggested by its high degree of sequence conservation among steroid receptors and by the fact that two previously reported random mutations that alter GR function [24, 25] were found there. This subdomain also falls within the 16-kDa peptide fragment that binds hormone [23], a putative transactivation domain [11], and the putative hsp90 binding domain [9, 10].

MATERIALS AND METHODS

Materials

COS-7 cells [34] were grown in Dulbecco's modified Eagle's medium (DMEM; high glucose) with 10% horse serum and 5% fetal bovine serum (FBS). Powdered cell culture medium was obtained from Irvine Scientific (Santa Ana, CA) and sera were obtained from Whittaker Bioproducts (Walkersville, MD).

The glucocorticoid inducible choramphenicol acetyltransferase (CAT) expression vector pMMTV-CAT has been described previously [24]. All vectors and their derivatives were propagated in *E. coli* DH5α. Restriction endonucleases, T4 DNA ligase, and acetyl coenzyme-A were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Dexamethasone (DEX) was obtained from Sigma Chemical Co. (St Louis, MO). Deacylcortivazol was kindly provided by Dr S. Simons (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). [α-^{35}S]dATP for DNA sequencing, [^{3}H]dexamethasone 21-mesylate (DM), [^{14}C]chloramphenicol, and En^{3}Hance Autoradiography Enhancer were purchased from DuPont/New England Nuclear (Wilmington, DE). Thin layer silica gel plates were purchased from Brinkmann Instruments (Westbury, NY).

Oligonucleotide primers were obtained from University of Southern California Comprehensive Cancer Center Microchemical Core Facility. The oligonucleotides used to create the three new restriction endonuclease sites in the mouse GR cDNA were: primer No. 11, 1457-GTCCAGCATGCCGGTACCGAAAAT-GTC for *Kpn*I; primer No. 12, 1775-CGATACC-CGGGTTCAGAAACTTACACC for *Xma*I (*Sma*I); and primer No. 14, 2044-CTGCTTCTGAGCTC-AGTTCC for *Sac*I. All three represent the plus strand of the cDNA, and the number gives the location of the first (5′) nucleotide in the mouse GR cDNA sequence [24]. Underlined nucleotides in the primers indicate changes from the wild type sequence. Oligonucleotides used to make the missense mutations in this study were 20–24 nucleotides in length and contained the minimum number of nucleotide substitutions required to make the desired amino acid coding changes. Other primers used for DNA sequencing or polymerase chain reaction (PCR) are represented below by their plus or minus strand designations and 5′ and 3′ nucleotide position numbers: primer No. 2, −2518 to −2499; primer No. 3, +1540 to +1560; primer No. 5, +1378 to +1397.

Construction of pSV2Krec

PCR assisted site directed mutagenesis was used to develop a GR expression vector with a new unique KpnI restriction site in the C-terminal portion of the DNA binding domain [Fig. 1(A)]. The new site facilitated subcloning of the mutant DNA fragments into the HBD region. Although the nearby *Sph*I site is unique in the GR cDNA, it is not unique in the pSV2Wrec expression vector [24], since *Sph*I sites also occur in the SV40 promoter region. A pUC12 vector called pRec containing the mouse GR cDNA was used as template in a PCR reaction with primers 2 and 11: primer 2 (minus-strand) contains the *Xba*I site present

in the wild type cDNA, and primer 11 (plus-strand) contains the *Sph*I site encoded in the wild type cDNA and two base mismatches that produce a novel *Kpn*I site by silent mutation [see Materials and Fig. 1(A)]. PCR reactions were carried out with the Perkin–Elmer Cetus (Norwalk, CT) GeneAmp DNA Amplification Kit used as directed with 0.1 ng purified plasmid template, 1 μM primers, and 2.5 U Taq DNA polymerase in a 100-μl reaction. Reactions were overlaid with mineral oil and carried through 20 cycles. Each cycle consisted of 1 min at 92°C, 2 min at 45°C, and 3 min at 72°C. Resulting products were purified by agarose gel electrophoresis and extracted from agarose with the Gene Clean Kit (Bio101, La Jolla, CA). The purified DNA was digested with *Sph*I and *Xba*I; the resulting 1042-bp fragment with the new *Kpn*I site was gel-purified and substituted into pRec in place of the analogous wild type *Sph*I–*Xba*I fragment. The resulting vector pKrec2 was digested with *Sal*I and *Xba*I, and the 2070-bp fragment was substituted into the mouse expression vector pSV2Wrec [24] to produce a GR expression vector with a novel *Kpn*I site called pSV2Krec.

Site directed mutagenesis by a four primer method

A two-step PCR assisted site directed mutagenesis procedure [35] using complementary primers containing the desired mutation was used in situations where the desired mutation was not near an existing unique restriction site. In step one purified plasmid DNA was used as template in two separate reactions: primer 2 and the plus-strand mutant primer were paired in one reaction, while primer 5 and the complementary minus-strand mutant primer were paired in the other [see Materials and Fig. 1(A)]. In the second step 10 ng of gel-purified product from each of the first reactions were mixed in a 100-μl PCR reaction with 1 μM each of primers 2 and 5 and carried through 20 cycles. Second round product was gel purified, digested with *Kpn*I and *Xba*I, and substituted into pSV2Krec to replace the analogous fragment. This technique was initially used to create two additional unique restriction sites in pSV2Krec by silent mutation. First, a *Sac*I site was engineered with complementary mutant primers 14 and 15, resulting in a vector called pSV2KSrec. Next a novel *Xma*I (*Sma*I) site was created with

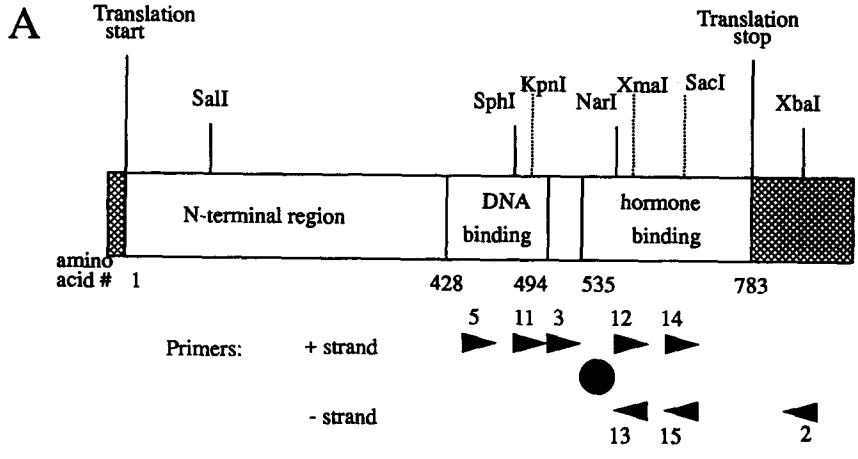

Fig. 1. The mouse GR cDNA coding region. (A) the unshaded coding region and the hatched noncoding region of the mGR cDNA are shown. The major functional domains are indicated along with some unique restriction endonuclease sites found in the wild type cDNA (indicated with solid lines). Unique restriction endonuclease sites added by silent mutations to construct pSV2KSXrec are indicated with dotted lines. Key oligonucleotide primers used in this study are indicated by arrowheads pointing in the 5′ to 3′ direction and are labeled by identification numbers. Primers used to make four missense mutations in GR were in the area indicated by the large dot. (B) The amino acid sequence at the N-terminal end of the mouse GR HBD is aligned with the homologous amino acid sequences of the four other human steroid receptors: mGR, mouse GR [24]; hMR, human mineralocorticoid receptor [48]; hPR, human progesterone receptor [49]; hAR, human androgen receptor [50]; hER, human estrogen receptor [51]. The numbers indicate the first amino acid shown. Dashes indicate sequence identity with the mouse GR and dots represent spaces introduced to insure optimum alignment. Arrows pointing downward indicate the two previously described missense mutations in this region [24, 25], whereas arrows pointing upward indicate the missense mutations reported here. Underlining indicates that L541G and L542G were constructed together as a double mutant. Alignment was performed with Genetics Computer Group software [52].

complementary mutant primers 12 and 13 to make expression vector pSV2KSXrec, which contains three new unique restriction sites [Fig. 1(A)] without altering the amino acid sequence of the expressed protein.

Creating these new restriction sites facilitated subsequent site directed mutagenesis of the HBD in several ways. Use of these sites allowed smaller mutant PCR fragments to be substituted; this decreased the number of bases that required sequencing and the possibility of polymerase infidelity. In addition, as described below these sites provided a convenient means of screening for newly generated mutant plasmids.

Construction of the four missense mutants described in this paper employed the same complementary mutant primer procedure described above. In each case pSV2KSrec was the template for the first round of PCR, and the resulting second round fragments were cloned into pKSX with the *Kpn*I and *Sac*I sites. Because the *Xma*I (*Sma*I) site is located between the *Kpn*I and *Sac*I sites in pKSX, but is absent in pSV2KSrec, the new mutant GR expression vectors could be screened for the absence of a *Xma*I site. The *Kpn*I–*Sac*I fragments were sequenced directly in the mutant expression vectors using the chain termination method [36] with the Sequenase 2.0 kit obtained from U.S. Biochemical Corp. (Cleveland, OH) and primers 3, 5 and 12.

Transient expression assay for GR function

CsCl-purified plasmids [37] were used for transfections. COS-7 cells were transfected in 60-mm culture dishes with 1.5 μg pMMTV-CAT and 0.5 μg GR expression vector by the low-pH calcium phosphate method [38]. Two days after transfection cells were induced with DEX and harvested the following day; cell extracts were prepared and assayed for CAT activity as described previously [39]. Quantification of thin layer chromatograms was performed on an Ambis Radioanalytical Scanner Model 100 (San Diego, CA). In some transfection experiments pCMV-βgal [40], a constitutive β-galactosidase expression vector, was included as an internal control; β-galactosidase activity was measured in the transfected cell extracts as described by Liu and Lee [41].

Western blot and [^{3}H]DM binding analysis of transfected COS-7 cells

COS-7 cells were transfected with 8 μg GR expression vector per 60-mm culture dish. Two days after transfection media was replaced with 1 ml DMEM containing 2.5% FBS and 20 nM [^{3}H]DM. Cells were incubated for 1 h at 37°C, washed 2 times in phosphate-buffered saline, harvested in phosphate-buffered saline with 2 mM EDTA, and extracted as described previously [42]. Protein concentrations were determined by the BioRad (Richmond, CA) Protein Assay, and equal amounts of protein (20–100 μg) from all samples to be compared were loaded on two identical 8% SDS–polyacylamide gels. Immunoblot analysis was performed on one of these gels as described previously [42] with an ascites preparation of monoclonal antibody 49 [43] or BuGR2 [44] against rat GR. The secondary antibody was HRP-conjugated goat anti-mouse Ig purchased from Promega (Madison, WI). Immunoblots were visualized with an Enhanced Chemiluminescence kit from Amersham (Arlington Heights, IL). The second gel was impregnated with En^{3}Hance Autoradiography Enhancer to visualize [^{3}H]DM binding.

RESULTS

Strategy for site directed mutagenesis of the GR HBD

The mouse GR expression vector pSV2KSXrec (pKSX) was engineered to contain three new unique restriction endonuclease sites (*Kpn*I, *Sac*I, and *Xma*I) [Fig. 1(A)] by addition of silent mutations via PCR assisted site directed mutagenesis. These sites greatly simplified the process of introducing further mutations throughout the HBD. The *Kpn*I site made it possible to insert mutant DNA fragments directly into the GR expression vector without going through intermediate constructions. The *Sac*I and *Xma*I (*Sma*I) sites made it possible to subclone smaller mutant PCR fragments; this decreased the amount of sequencing required and the chances of introducing undesirable mutations through PCR infidelity. Reports on Taq DNA polymerase infidelity vary, but a range of 10^{-5} to 10^{-6} mismatches per nucleotide per cycle has been reported [45], which coincides with our experience (data not shown). Due to this potential problem all PCR-generated fragments were completely sequenced after insertion into the expression vectors. All amino acid position numbers cited in this paper are those of the mouse GR [24].

Hormone dependent transactivation of a reporter gene by mutant GR

A subdomain in the N-terminal end of the mouse GR HBD bounded by amino acids 541 and 561 is highly conserved among the steroid hormone receptors [Fig. 1(B)], suggesting functional importance. Furthermore, two of the first four reported random point mutations in the GR HBD are in this region: randomly generated missense mutations E546G (glu-546 changed to gly) and P547A (pro-547 changed to ala) severely diminished hormone binding by the GR [24, 25]. Therefore, we decided to extend the genetic analysis of this subdomain by site directed mutagenesis. Amino acids in this region were replaced with either alanine or glycine.

Four mutant GR were constructed: LL541/2GG, E543A, V544G, and V549G. Each mutant codes for a single amino acid substitution except LL541/2GG in which both L541 and L542 were changed to glycines. Wild type and mutant expression vectors were cotrans-

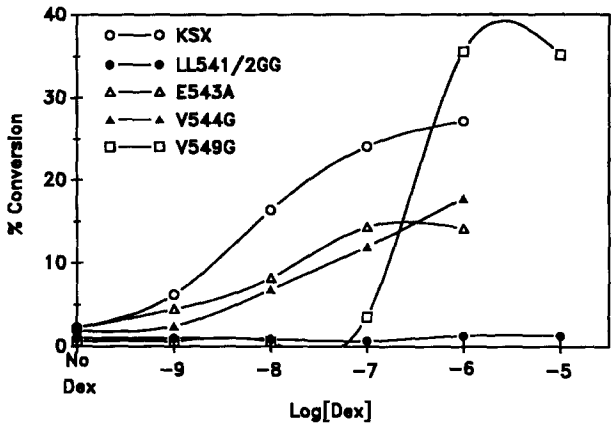

Fig. 2. Transcriptional activation of the pMMTV-CAT gene by mutant and wild type GR in response to DEX. COS-7 cells were contransfected with wild type (KSX) or mutant GR expression vector and the pMMTV-CAT reporter gene vector and exposed to the indicated DEX concentrations. CAT activity is shown as % chloramphenicol converted to acetylated product. Each data point represents the mean from two independent experiments, each with duplicate samples; data were not adjusted for transfection efficiency.

fected into COS-7 cells along with a CAT reporter gene under the control of the glucocorticoid responsive mouse mammary tumor virus promoter, pMMTV-CAT. In transfected cells the GR was constitutively expressed from a SV40 promoter; upon addition of hormone, activated GR bound to glucocorticoid response elements on the reporter gene promoter to induce its expression. Dose–response curves were generated by adding increasing concentrations of the synthetic glucocorticoid DEX until a maximal response was reached (Fig. 2). From multiple dose–response curves for each mutant GR the hormone concentrations that caused half maximal stimulation were determined (Table 1).

The half maximal response for wild type GR produced by pKSX was observed with approx. 7 nM DEX; for mutants E543A, V544G, and V549G the half maximal concentrations were approx. 13, 54, and 235 nM DEX, respectively (Table 1, column 2). Thus, mutant GR V544G and V549G could activate transcription of the reporter gene, but required approx. 7 and 23 times more DEX, respectively, than the wild type GR to achieve half maximal activation (Table 1, column 3). The half maximal concentration for E543A was less than 2-fold greater than for wild type GR, and this difference was not significant. In contrast, LL541/2GG did not respond even to 10 μM DEX. Deacylcortivazol [46] is a powerful glucocorticoid analog that stimulates wild type GR with a half maximal concentration of approx. 0.1 nM; yet with LL541/2GG even 10 μM deacylcortivazol did not induce reporter gene expression (data not shown). We also observed no DEX activation of the reporter gene in COS cells transfected with pMMTV-CAT alone, confirming that these cells lack detectable GR in this assay (data not shown).

The maximum reporter gene activation by mutant and wild type GR was determined by cotransfecting GR expression vectors, pMMTV-CAT, and constitutive β-galactosidase expression vector pCMV-βgal, and growing the transfected cells with 10 μM DEX. This saturating concentration of DEX caused similar reporter gene activation by wild type, E543A, V544G, and V549G GR, indicating that the transactivating potential of GR is unaffected by each of these three mutations (Table 1, column 4). Similar levels of β-galactosidase expression in all transfected cells indicated that comparable transfection efficiences were achieved.

DM binding and immunoblot analysis

Wild type and mutant GR were expressed transiently in COS-7 cells, and the cells were incubated at 37°C with [^{3}H]DM, a modified form of DEX with a reactive group that covalently binds GR at Cys-644 [30, 31]. The binding affinity of GR for DM is similar to that for DEX [47]. Extracts of DM-labeled cells were analyzed by denaturing polyacrylamide gel electrophoresis. On autofluorographs of the polyacrylamide gels [Fig. 3(A)] wild type GR (labeled KSX) appeared as a pair of specific bands (lanes 3–4) that flanked a nonspecific band seen in the mock transfected control (lanes 1–2). Comparison with molecular weight mark-

Table 1. *Reporter gene activation by mutant GR: half maximal DEX concentrations and maximum activity with saturating DEX*

GR isoform	EC_{50} for DEX[a] (nM)	Relative EC_{50} for DEX[b]	Reporter gene act. at saturating DEX[c]
KSX	7.1 ± 2 (7)	1	1 ± 0.1 (2)
LL541/2GG	NR[d] (4)	NR (4)	0 (2)
E543A	13 ± 3 (4)	1.7 ± 0.4 (4)	1.6 ± 0.3 (2)
V544G	54 ± 20 (3)	6.8 ± 2 (4)	1.9 ± 0.4 (2)
V549G	235 ± 60 (2)	23 ± 12 (2)	1.4 ± 0.1 (2)

[a]Concentrations of DEX that produced half maximal MMTV-CAT reporter gene activation were determined from dose–response curves including those in Fig. 2. The mean and standard error of the mean are for the number of independent experiments shown in parentheses. *P* values from Student's *t*-test for comparing KSX with each mutant were: E543A, 0.14; V544G, 0.04; V549G, 0.002.

[b]The half maximal DEX concentration value determined from each experiment was divided by that obtained for wild type GR (KSX) in the same experiment. The mean and standard error of the mean are for the number of independent experiments shown in parentheses.

[c]COS cells were cotransfected with the indicated GR expression vector, pMMTV-CAT, and pCMV-βgal and exposed to 10 μM DEX. The β-galactosidase activity in each sample was used as an internal control to correct the amount of extract assayed for CAT activity. The CAT activity is expressed relative to that for wild type GR (KSX) as the mean and standard deviation from 2 transfected dishes. The experiment was repeated again with similar results.

[d]No Response, i.e. no increase in CAT activity caused by DEX.

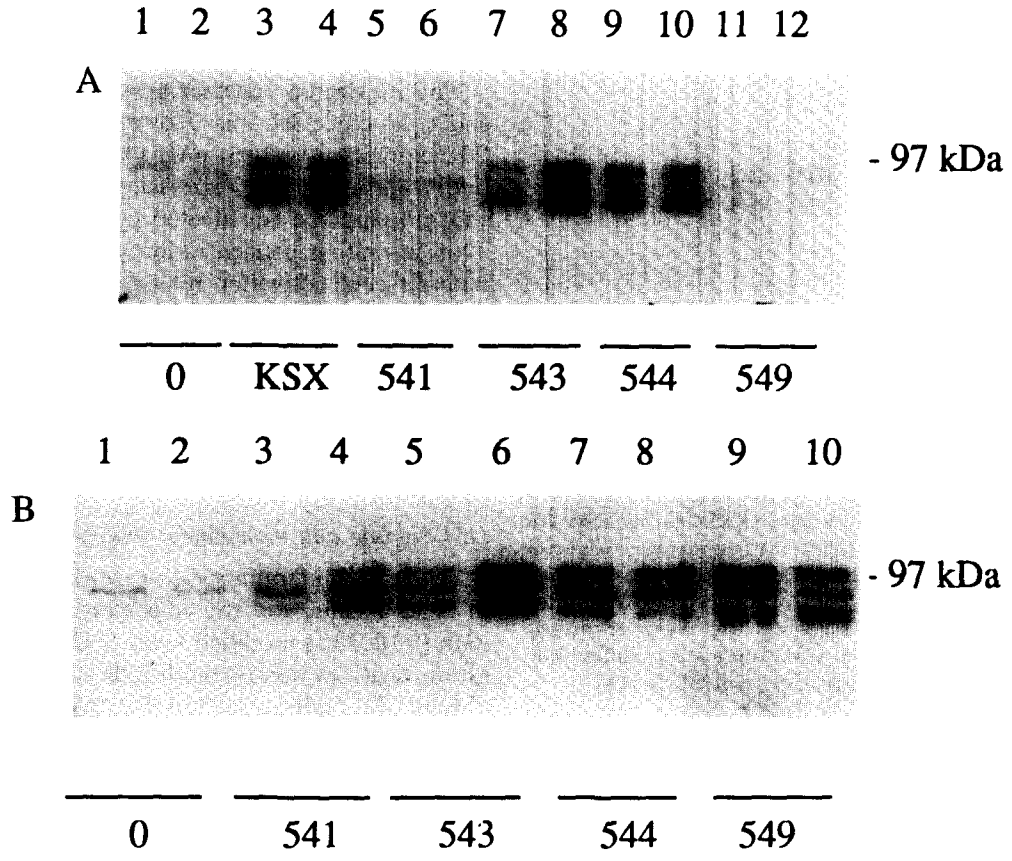

Fig. 3. [^{3}H]DM binding and immunoblot analysis. COS-7 cells were transfected with the indicated GR expression vector. (A) [^{3}H]DM binding. (B) Immunoblot analysis with monoclonal antibody BuGR2. Lanes 1–2 are mock-transfected COS cells. The position of the 97-kDa molecular weight marker is indicated at the right.

ers indicated that the larger specific species represents the full length 97-kDa GR; the smaller species may be a degradation product of the GR or a product of an alternative translation initiation site. At a concentration of 20 nM approximately equal amounts of DM bound to wild type (lanes 3–4), E543A (lanes 7–8), and V544G (lanes 9–10); there was much less DM labeling of V549G GR (lanes 11–12) and no detectable binding to LL541/2GG GR (lanes 5–6). Immunoblot analysis of the same extracts indicated that all mutant GR were present in transfected cells at approximately equal levels [Fig. 3(B)]. A nonspecific band was observed in the DM-binding and immunoblot experiments at approx. 90 kDa in untransfected COS cells [Fig. 3(A and B), lanes 1–2]. This species is not GR, since we observe it in both primate and rodent cell lines that lack GR RNA and protein (our unpublished results). We speculate that it is hsp90, because the high abundance of this protein may make it a good target for nonspecific binding of a variety of agents.

DISCUSSION

In this genetic analysis of the N-terminal end of the HBD of the mouse GR, amino acids normally found in the wild type GR were replaced with glycine or alanine. Substituting these two amino acids for others is equivalent to removing the amino acid side groups and replacing them with either a hydrogen atom in the case of glycine or a methyl group in the case of alanine. With this strategy conclusions can be made regarding the importance of individual amino acid side chains for GR function. In contrast, if bulky, charged, or reactive side groups were substituted for the original ones, any change in function may result not only from loss of the original side group but also from the addition of the new side group; this would not allow an unambiguous interpretation of the importance of the original side group.

The dose–response curves and corresponding half maximal concentrations indicate that E543A, V544G, and V549G all can effectively activate transcription of a reporter gene, but only E543A can accomplish this at a DEX concentration similar to that required by wild type GR. In contrast, LL541/2GG did not respond to DEX at all. The inactivity of LL541/2GG and the increased DEX concentrations required for a half maximal response with mutants V544G and V549G can be explained by a problem in hormone binding function. At saturating concentrations of DEX both V544G and V549G activated the reporter gene as well as wild type GR, indicating that they have no deficiency in their receptor activation, dimerization, DNA binding, or transactivation functions. Furthermore, the fact that

the half maximal DEX concentrations for V544G and V549G are considerably higher than the 5 nM K_d for DEX binding to wild type GR [27] can only be explained by a hormone binding deficiency. For example, GR V549G activated the reporter gene only slightly at 100 nM DEX; and reporter gene activity increased dramatically when the DEX concentration was increased to 1 μM DEX. GR with a wild type hormone binding function would be essentially saturated with hormone at 100 nM DEX, and addition of higher concentrations of DEX to hormone-saturated receptors would not elicit any further large increase in reporter gene transcription. Therefore, the V549G receptor must not be saturated at 100 nM DEX, and must therefore have a decreased ability to bind hormone. A similar argument can be made for V544G.

The binding of mutant and wild type GR species to a covalent affinity label, [^{3}H]DM was studied in living transfected cells at 37°C. This assay provided a direct semi-quantitative measure of the hormone binding affinity of GR under truly physiological conditions. The results from the DM binding experiments are consistent with the relative half maximal concentrations determined from the dose–response curves. Mutant E543A has a half maximal DEX concentration and DM binding indistinguishable from wild type GR. The binding of 20 nM DM to V544G and wild type GR were also indistinguishable, even though the half maximal DEX concentration for this mutant was approx. 7-fold higher than that for wild type GR. However, the kinetics of the covalent and non-covalent steps of DM binding are not known and could be responsible for this apparent discrepancy. The DM binding assay may be unable to discriminate between small differences in hormone binding abilities. The two most severely affected mutants, V549G and LL541/2GG, bound 20 nM DM poorly or not at all, confirming that their increased half maximal DEX concentrations in the reporter gene activation tests were due to a decreased hormone binding affinity. These results confirm the importance of mouse GR amino acids V549 and one or both of L541 and L542 in hormone binding. Since LL541/2GG is a double mutant, it is unclear if both mutations are required for this phenotype. Because LL541/2GG fails to respond even to 10 μM DEX and deacylcortivazol, we also could not determine whether L541 and/or L542 may be required for other GR functions (such as transactivation) in addition to hormone binding. Finally, E543 does not appear to play a significant role in hormone binding or other aspects of GR function.

The work reported here has provided a detailed functional map of a small subdomain (amino acids 541–549) in the mouse GR HBD. This is the first detailed single-amino acid structure–function analysis of a portion of the core hormone binding region [23] of the GR. The mutations reported here and two previously reported, randomly generated mutations found in this region [24, 25] all caused glycine and alanine substitutions for the original amino acids. As discussed above, substitution of alanine or glycine is equivalent to removing the bulk of the side chain of the original amino acid. In some cases, substitution of glycine for another amino acid can destabilize the functionally optimal conformation of the polypeptide backbone by allowing increased rotational freedom about the alpha-carbon atom of the glycine. However, all of the substitutions reported here resulted in stable and (except for the double mutant LL541/2GG) functional GR proteins, indicating that the structure of the mutant proteins was not drastically disrupted by the substitutions. Therefore, we conclude that the side chains of several amino acids in this region of the GR HBD are specifically involved in the function of hormone binding: Leu-541 and/or Leu-542; Val-544; Glu-546; Pro-547; and Val-549. Furthermore, our results indicate that E543, V544, and V549 are not required for any receptor function other than hormone binding. The exact role of these amino acids play in hormone binding is unclear. It is possible this region of the HBD forms part of the interior surface of the hormone binding pocket and directly interacts with hormone; alternatively this domain may be outside the hormone binding pocket but necessary for maintaining the proper tertiary structure of the pocket. Since most of the amino acids that were changed in this study are highly or partially conserved among the five steroid receptors, our findings on the GR will be relevant for understanding hormone binding by the other four steroid receptors as well.

Ultimately, both a three-dimensional structure and a thorough genetic analysis will be required to understand the function of the GR HBD. The three-dimensional structure will indicate which amino acid side groups face into the hormone binding pocket and which are outside the pocket; but this information alone cannot explain how hormone binding is accomplished. Genetic studies such as the current one will be required to show which specific amino acids within the pocket are important for affinity and specificity of hormone binding, and which amino acids that lie outside the hormone binding pocket are important for maintaining the proper shape of the pocket. The new pKSX vector described here for expression of mouse GR, with three unique restriction endonuclease sites in and around the HBD, will facilitate future mutational studies on this domain.

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