A Conserved Proline in the hsp90 Binding Region of the Glucocorticoid Receptor Is Required for hsp90 Heterocomplex Stabilization and Receptor Signaling*

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Studies of hsp90 in yeast have supported the notion that this chaperone plays a critical role in signaling by steroid receptors. One limitation to these studies is that yeast expressing hsp90 mutants may also be deficient in fundamental cellular functions of the chaperone required for steroid-dependent induction of transcription. In this work, we have prepared mutants of the glucocorticoid receptor (GR) that permit analysis of hsp90 binding and transcriptional activity in cells with normal chaperone function. Our previous data supported a model in which hsp90 binds to the receptor steroid binding domain according to a two-site model. By amino acid mutagenesis of these two sites, we have now generated three receptor mutants and analyzed their function. Upon their translation in vitro, all three mutants interacted with hsp90 similarly to the wild-type receptor. However, one mutant, P643A (GRo), was of particular interest because, although it showed normal steroid binding and transformation to a glucocorticoid response element-specific DNA binding form, it was remarkably deficient in nuclear translocation and transcriptional function at 37 °C. Furthermore, GRo-hsp90 heterocomplexes formed in vivo or assembled under cell-free conditions were much less stable than wild-type GR-hsp90 heterocomplexes. Our results demonstrate that Pro-643 plays a critical role in both stabilizing the receptor-hsp90 complex and in permitting an efficient nuclear translocation and, thus, support the concept that the chaperone is an integral component of the steroid-receptor signaling pathway.

The untransformed form of steroid receptors, such as the glucocorticoid receptor (GR), exists in cells as a large (9 S) heteromeric complex containing the 90-kDa heat-shock protein (hsp90) (1–5), p23, and one of several immunophins (for review of receptor heterocomplex structure, see Refs. 6–8). Upon steroid binding, hsp90 dissociates from the original component structure (3, 9), and the steroid-binding protein is transformed to its nuclear, DNA binding, and transcriptionally active form (9, 10). Given the correlation between hsp90 association and inactivity of the receptor, an inhibitory role for this association has been typically postulated (1, 2, 11). In apparent contradiction with the inhibitory model of hsp90, genetic analysis in yeast expressing low levels of hsp90 (12) or hsp90 point mutants (13, 14) indicates that hsp90 is required for steroid receptor signaling in vivo. Moreover, the drug geldanamycin, a benzoquinone ansamycin, specifically binds to hsp90 and, by interference with critical steps in the hsp90-assisted folding, causes significant functional defects in the substrate proteins for hsp90. In the case of GR, geldanamycin suppresses the steroid binding (15, 16), nuclear translocation (16), and transcriptional functions (15, 17) of the receptor. Also, the results of experiments where both the GR and the mineralocorticoid receptor are expressed in bacteria (18, 19) support a permissive role of hsp90. Although affinity-purified receptors produced in Escherichia coli are isolated in a nonfunctional form, they assume normal hydrodynamic properties, steroid and DNA binding characteristics, when assembled into complexes with eukaryotic hsp90, an assembly that is both ATP- and temperature-dependent.

Because hsp90 is required for cell viability, compromising hsp90 function by mutation or geldanamycin treatment can be expected to adversely affect a wide variety of general cellular functions. To the extent that these hsp90-dependent functions are required to support receptor-dependent activation of transcription, impairment of hsp90 function may not indicate a direct requirement for hsp90 in steroid receptor signaling. To obviate this problem, we have prepared mutations of selected conserved amino acids in a region of the steroid binding domain of the GR that is part of the hsp90 binding site. With this approach, any linkage between hsp90 binding and transcriptional activation activity of the receptor can be determined in cells with normal hsp90 function.

hsp90 binds to an extensive and rather hydrophobic portion of the steroid binding domain of the steroid receptors (20–24), and this interaction is required for the steroid binding conformation of both the glucocorticoid (25) and mineralocorticoid (18, 19) receptors. Thus, the body of evidence suggests a dual role for the chaperone proteins. It would appear that association with the hsp90 complex is critical for the appropriate folding of CMV, cytomegalovirus; LTR, long terminal repeat; GST, glutathione S-transferase; [1H]TA, N-[6,7-1H]triamcinolone acetonide; TBS, Tris-buffered saline.

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† The abbreviations used are: GR, glucocorticoid receptor; hsp, heat-shock protein; WT, wild-type; GRE, glucocorticoid response element; MMTV, mouse mammary tumor virus; CAT, chloramphenicol acetyltransferase; BES, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; [3H]TA, [3H]triamcinolone acetonide; LTR, long terminal repeat.
the receptor in a conformation that possesses high affinity ligand binding activity and that can eventually transform, translocate, and interact with DNA; yet, this very association with the hsp90 complex also restrains the receptor, thus requiring ligand for receptor transformation and nuclear translocation.

The structural bases for the complex interplay between hsp90 and the glucocorticoid receptor are unknown. We have previously mapped a minimal hsp90 binding site of the GR steroid binding domain by peptide competition (21). Our data supported a potential two-site model for hsp90 binding in which one critical contact site involves the region encompassing amino acids 639–671 of the rat GR sequence. This region contains two features that are present only in members of the steroid receptor superfamily that stably bind hsp90. First, there is a stretch of nine hydrophobic amino acids (residues 639–647) with a central conserved proline (residue 643). We reasoned that mutation of this proline might disrupt the secondary structure of the region and likely affect the hsp90-receptor interaction. Accordingly, we mutated proline 643 to alanine (mutant GrRo). The second feature is a charge dipole consisting of a positively charged and a negatively charged residue separated by a single amino acid. In all steroid receptors, this dipole is never more than 10 amino acids away from a cysteine residue. We have speculated (21) that the proximity of this dipole to cysteine residues could affect the acidity of the sulfhydryl group and thereby influence sulfhydryl-dependent receptor-protein interactions. We therefore mutated glutamate 649 to glutamine (mutant Gr1) to test whether the disruption of this dipole would affect hsp90 binding. Furthermore, in our peptide competition experiments, a second hsp90 contact site was predicted in the region between amino acids 586 and 644 in the rat GR, which contains the only highly conserved amino acid sequence in the superfamily outside of the DNA binding domain (21). Therefore, we produced a third receptor mutant (Gr8) within this region by changing the conserved lysine and proline in positions 597 and 600 for isoleucine and leucine, respectively. To gain more insight into the functional relevance of this hsp90 binding region of the GR, we have assessed the impact of the mutations described above at various levels of receptor function, including hsp90 binding, steroid binding affinity and specificity, activation to the DNA binding form, and transcriptional activity.

In this report we show that the conserved proline at position 643 plays a critical role in that it appears to be required for the efficient coupling of GR to hsp90; thus, it is also critical to the appropriate responsiveness of the receptor to ligand. When this residue is mutated, Gr-hsp90 heterocomplexes assembled in vitro are less stable, and steroid binding causes a more rapid dissociation of hsp90 from receptor heterocomplexes assembled in vitro or in vivo, even in the presence of molybdate. Furthermore, this mutation significantly affected the nuclear translocation capacity of the receptor at physiological temperature. Importantly, the transcriptional activation function of this mutant receptor is severely affected, whereas the rest of the biochemical properties evaluated were not. This study provides structural evidence supporting the importance of stable GR-hsp90 heterocomplex assembly for receptor signaling in cells with normal chaperone function.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmid pCMV-Neo was provided by Dr. Michael uhler (Mental heath Research Institute, University of Michigan, Ann Arbor, MI). pCMV-Neo is a pUC 13 derivative that contains the immediate early promoter of human cytoemalgavirus (CMV) upstream from the human growth hormone polyadenylation signal. The CMV promoter and the human growth hormone transcription termination sequences are separated by a unique Bgl II site that was used for construction of GR and GR mutant expression plasmids (see below). Plasmid pRoII17 (26), containing the cDNA encoding for the full-length rat GR, was obtained from Dr. Keith Yamamoto (Department of Biochemistry and Biophysics, University of California, San Francisco, CA). This plasmid contains a 2.8-kilobase pair BamHI insert including the complete coding region of the rat glucocorticoid receptor cDNA 24 nucleotides upstream of the AUG start codon and 360 nucleotides downstream of the translational termination codon. Plasmid pGEX-KG (27) was provided by Dr. Jack Dixon (Department of Biochemistry, University of Michigan, Ann Arbor, MI). Plasmid pCMV-fgal is a β-galactosidase expression vector derived from the vector pCMV-Neo. The reporter gene p-MMTV-CAT, which contains 750 base pairs of the long terminal repeat of murine mammary tumor virus (MMTV-LTR) promoter (H Authority) fused to the bacterial chloramphenicol acetyl transferase (CAT) gene, was kindly provided by Dr. Audrey Seasholtz (Mental Health Research Institute, University of Michigan, Ann Arbor, MI). Plasmid pGEX5-CAT was purchased from U. S. Biochemical Corp. This vector features the GRES promoter (28), which is composed of five glucocorticoid responsive elements (GREs) placed upstream of the TATA region of the adenovirus2 major late promoter and is also fused to the CAT gene. All the plasmids were purified using Qiagen kits (Santa Clarita, CA).

Mutant Generation—The rat GR cDNA was excised from pRoII17 with BamHI and ligated into the BamHI site of M13MP19 in an orientation placing the antisense strand of GR cDNA in the (+) strand of M13. New M13 clones were then screened by colony hybridization using oligonucleotides coding for the desired amino acid mutation. Finally, the representative form of M13 carrying the mutation was used to transform E. coli JM101 with the oligonucleotide-directed mutagenesis system, following the protocol provided by the manufacturer (Amersham Pharmacia Biotech). Mutations of the wild-type sequence to the desired one was confirmed by chain termination DNA sequencing (Sequenase 2.0; U. S. Biochemicals) using the sequencing primer GAGAATTATGACCAC. Mutants were excised from M13 with BamHI and subcloned into pGEM3z (Promega, Madison, WI) and pCMV-Neo for in vitro translation and COS-1 cell expression, respectively. To provide wild-type controls, the BamHI fragment from pRoII17 was also subcloned into pCMV-Neo and pGEM3z. Furthermore, to express the GR derivatives as C-terminal fusion proteins of glutathione S-transferase (GST) in bacteria, Avii1/Avii1 fragments were isolated from the corresponding pGEM3z vectors. The fragments were ligated to BamHI linkers (Promega, Madison, WI) and then inserted into BamHI-digested pGEX-KG vectors (29). The resulting constructs have open reading frames coding for a fusion protein, including the complete GST sequence, a fragment containing a potential thrombin cleavage site, and the rat GR sequence from position 1368 to 2733 of the cDNA. A total of three of these mutants were prepared. Plasmid designation, followed by the encoded wild-type residue and the resulting residue between parenthesis, is the following: pGEM3zGrRo, pCMVGor and pGEX-KGrRo (Pro 643 to Ala); pGEM3zGr1, pCMVGor1 and pGEX-KGr1 (Glu 649 to Gln); pGEM3zGr1, pCMVGor8 and pGEX-KGr8 (Lys 597 and Pro 600 to Ile and Leu, respectively).

In Vitro Transcription and Translation—E. coli TG-1 cells, made competent by calcium phosphate transfection, were transformed with template containing ampicillin according to standard protocols. Plasmids were isolated from genomic DNA by the alkaline lysis protocol. These preparations were treated with RNase extracted with phenol/chloroform, and templates were linearized with XhoI for transcription. T7 RNA polymerase was used to produce the RNA transcripts, and subsequent transcription and translation were carried out as described previously (30). Immunoadsorption of the in vitro translation products with an anti-hsp90 antibody, separation by SDS-polyacrylamide gel electrophoresis, and autoradiography were performed as described previously (31). The monoclonal antibody 8D3 against hsp90 (32) was kindly provided by Dr. Gary Perdew (Department of Veterinary Medicine, Pennsylvania State University).

Expression, Purification, and in Vitro Assembly of Fusion Proteins—Overnight cultures of E. coli (HB101 strain) transformed with wild-type or mutant pGEX-KG GR and affinity purification of the corresponding GST-GR fusion proteins with a suspension of glutathione-agarose beads was as described previously (18). Typically, untreated rabbit reticuloocytes (Promega, Madison, WI) was added to the glutathione-agarose beads and the GST-GR (1:1 volume ratio) and incubated at 30°C for 60 min in the presence of 1 mM ATP, 1 mM MgCl2, and an ATP-regenerating system (3 mM phosphocreatine and 5 units/ml creatine phosphokinase). The effect of the incubation time (0–70 min) and temperature (20–40°C) on GST-GR-hsp90 heterocomplex assembly was evaluated in other experiments.

COS-1 Cell Transformation and Cytoskeleton Preparation—COS-1 cells were
cultured in Dulbecco’s modified Eagle’s medium containing 10% charcoal-stripped fetal bovine serum (Hyclone Labs, Inc., Logan, UT). Transfection was performed by the calcium phosphate precipitation method under low CO₂ conditions, as described elsewhere (33). Briefly, cells were plated into 10-cm tissue culture dishes (1 × 10⁶ cells/dish) and allowed to grow until 80% SDS-PAGE. The transfection mixture contained the plasmid DNAs in 125 mM CaCl₂, 30 mM BES, 140 mM NaCl, 0.75 mM Na₂HPO₄, pH 9.65. The amount of plasmid DNA transfected varied according to the experiment. For GR-stereoid binding, DNA-cellulose binding, specific GRE binding, and sucrose gradient analysis, 2–10 μg of pCMVGR, pCMVRo, or pCMVGR8 were cotransfected with 3 μg of pCMVMTV-LTR-LS. The transfected cell extracts were prepared by scraping with a rubber policeman and then washed three times with phosphate-buffered saline. The pellets were resuspended in 500 μl of the binding buffer (TEDGM: 20 mM sodium molybdate, 20 mM Tris-HCl, 1 mM EDTA, 5 mM diethiothreitol, and 10% glycerol, pH 7.6, at 0 °C), homogenized at 0 °C by 30 strokes in a Dounce homogenizer, and centrifuged at 10,000 × g for 1 h at 2 °C. For saturation binding assays, aliquots (50 μl) of the reticulocyte-treated GST-GR preparations or COS-1 cell cytosols were incubated for 16 h at 0–2 °C with 0.1–60 nM [1,2,6,7-³H]corticosterone (90 Ci/mmol; Amersham) in the presence or absence of radioinert corticosterone. The apparent equilibrium dissociation constant (∙Kₛ, 2 °C) and the maximal binding capacity (∙Bₘₐₓ) were calculated by Scatchard analysis (43). For competition assays, incubations in GST-GR preparations were performed as described above with 2 μM [³H]corticosterone in the presence or absence of 0.5–10 μM unlabeled steroids. After 16 h, bound and free steroids were separated by the addition of a charcoal/dextran suspension (1% Norit A, 0.1% dextran in TEDGM buffer) as described previously (18). In all cases, the specific binding (B) was calculated as the difference between the radioactivity bound in the presence and in the presence of 1 μM unlabeled corticosterone (Sigma) before harvesting and resuspension in 100 μl of 250 mM Tris-HCl, pH 7.8. Cells were then disrupted by three cycles of freezing in dry ice and thawing at 37 °C. After centrifugation, the supernatants were used for the determination of CAT activity (30 μl). In the dose-response experiments, the transfected cells were incubated with or without 1 μM [³H]corticosterone (Sigma) for 16–20 h. At the end of the incubation period, the cell lysates were prepared by scraping the cells into the TEDGM buffer and centrifuged at 10,000 × g for 1 h at 0–2 °C with 30 μl of [³H]corticosterone (10 μl of a 0.1 M solution in TEDGM buffer) for 1 h at 2 °C. After washing three times with phosphate-buffered saline, 200 μl of 0.1 x Tris-HCl, pH 8.0, 0.1% Triton X-100 was added to each well. After 2 h at 70 °C, the plates were washed at 37 °C, and the cell lysates were centrifuged. CAT activity was measured as the change in absorbance at 240 nm of the cell lysates, the method of diffusion of reaction products into water-insoluble scintillation fluid (Econofluor-2; Amersham) (37). To control the efficiency of each transfection, β-galactosidase was determined as described previously (37) in 10-μl aliquots of the cell extracts. Protein concentration was determined by the method of Bradford (38) using bovine serum albumin as standard. Labeling of Wild-type and Mutant GRs with [3H]Triamcinolone Acetonide—The cotransfection of COS-1 cells with 200 nM [³H]Triamcinolone Acetonide (30%) was performed according to a previously reported method was used (39). Briefly, COS-1 cells transfected with pCMVGR and pCMVRo were harvested by scraping with a rubber policeman into fresh Dulbecco’s modified Eagle’s medium containing 10% charcoal-stripped fetal bovine serum and diluted to a concentration of 1 x 10⁵ cells/ml of suspension. Aliquots (1 ml) of the cell suspensions were incubated for 1 h at 0–2 °C with 30 μl of [³H]corticosterone (Sigma) in the presence or absence of a 200-fold molar excess of radioinert dexamethasone. Subsequently, the cell suspensions were incubated at 0–2 or 37 °C for 30 min. Each condition was performed in triplicate. At the end of the incubations, cells were washed three times with phosphate-buffered saline, resuspended in 50 μl of HE buffer (10 mM HEPES, pH 7.4, 1 mM EDTA), homogenized at 0 °C by 30 strokes in a Dounce homogenizer, and centrifuged at 3,600 × g for 20 min to yield crude nuclear and cytosolic fractions. To obtain the nuclear-free cytosols, the supernatants were centrifuged at 100,000 × g for 1 h. The nuclear pellets were resuspended in 50 μl of 1 mM HEPES, pH 7.6, 1 mM EGTA, 3 mM MgCl₂, 0.3 x sucrose and centrifuged again. The pellets were then resuspended in 400 μl of 2.5 mM Tris, pH 7.6, 12.5 mM NaCl, 1 mM EDTA containing 2.5% glycerol and 2.5% SDS and boiled for 3 min. COS-1 cell lysates were used for radioactive protein assay and the specific binding was calculated as the difference between the radioactivity bound in the absence and in the presence of dexamethasone.

Data Analysis and Statistics—Data are expressed as means ± S.E. Analysis of variance was used to test overall differences between groups. Post hoc comparisons were made by using the Fisher protected least significant difference (probability value of less than 0.05).
GR1: 5.4

Experimental Procedures. In each type or mutant receptors (GR8, GRo, and GR1) were immunoabsorbed by the nonimmune antibody. Mr absorption, contains the total amount of translation products before immunoabsorption, and NI denotes the lane containing material immunoabsorbed by the nonimmune antibody. Mr, molecular masses.

RESULTS

Three mutants were designed in the hsp90 binding region of GR. In the GRo mutant, Pro-643, located within a conserved hydrophobic stretch of the C-terminal side of the region, was substituted by Ala. To disrupt a conserved putative dipole consisting of Glu-649 and Arg-651 in the GR1 mutant, the negatively charged Glu-649 was replaced by Gln. Finally, the N-terminal end of the hsp90 binding region was addressed with the double mutant GR in which Lys-597 and Pro-600, both of which are conserved in vitamin D, thyroid hormone, and retinoic acid receptors as well as in the steroid receptors were replaced by Ile and Leu, respectively. The following experiments were conducted to evaluate the effects of the above GR mutations on different aspects of receptor function.

Association with hsp90—When the GR is translated in vitro in rabbit reticulocyte lysate, it associates near or at termination of receptor translation with rabbit hsp90, generating a GR-hsp90 complex with the receptor in a high affinity binding conformation (30). To determine whether the expressed mutant receptors interact with hsp90, receptors were immuno precipitated from in vitro translation mixtures using the monoclonal antibody 8D3, which is specific for hsp90. Fig. 1 shows the autoradiogram of the translation products analyzed by SDS-polyacrylamide gel electrophoresis. Wild type (WT) and mutant receptors were all coprecipitated to a similar extent and were detected as species migrating at approximately 94 kDa. Thus, under the assayed conditions, none of the mutations prevented the steroid-free receptor from associating with hsp90.

Characterization of Steroid Binding—Due to the overlapping of the hsp90 binding region of the receptor with a significant segment of the steroid binding domain, the effect of each mutation on steroid binding was also investigated. Incubation of cytosols from transfected COS-1 cells with increasing concentrations of [3H]corticosterone revealed saturable steroid binding (Fig. 2). In each panel, T denotes the lane that contains the total amount of translation products before immunoabsorption, 8D3 denotes the lane containing the material immunoabsorbed by the 8D3 antibody, and NI denotes the lane containing material immunoabsorbed by the nonimmune antibody.

The specific binding (B) was calculated as indicated under “Experimental Procedures.” The data shown are from one experiment that is representative of four independent experiments. The inset is a Western blot showing expression levels of the mutant GRs. Cells transfected with pCMVGR, pCMVGRo, pCMVGR1, and pCMVGR8 were pelleted and ruptured by three consecutive cycles of freezing and thawing, and whole-cell extracts were prepared by directly boiling the lysates in SDS sample buffer. Samples were analyzed by gel electrophoresis and immunoblotting using the BuGR2 monoclonal antibody.

Ala resulted in a slight increase in the $K_d$ to 8.8 ± 2.8 nM, a steroid binding affinity that still lies within the physiological range of circulating corticosterone concentration in the rat. Although not statistically significant, the $B_{max}$ of GRo was consistently reduced to about 80% that of the $B_{max}$ of WTGR (332 ± 50 fmol/mg of protein). This reduction is not caused by a decrease in the steady-state level of expressed receptor, as determined by Western analysis of the extracts of transfected cells. As shown in the inset in Fig. 2, wild-type and mutant receptors were all expressed to a similar extent as a 94-kDa protein.

Similar corticosterone binding results were obtained with purified GST fusion receptors containing the DNA and the steroid binding domains of the wild-type and mutant receptors. Purified preparations of the fusion construct expressed in E. coli, a host that lacks the functional counterpart of the eukaryotic hsp90, show negligible steroid binding activity. However, after treatment with rabbit reticulocyte lysate at 30 °C for 60 min, which promotes receptor association with the rabbit hsp90, wild-type and mutant receptors were recovered in their functional, steroid binding, form ($K_d$, 4 °C: WTGR $= 4.6 ± 1.6$ nM; GRo: 9.4 ± 2.4 nM; GR1: 5.0 ± 2.3 nM; $B_{max}$, 4 °C: WTGR $= 83 ± 13$ pmol/mg of protein; GRo: 59 ± 10 pmol/mg of protein; GR1: 79 ± 14 pmol/mg of protein; n = 3 different experiments). Again, no binding was detected with the mutant GR5. In sum, there was a close agreement between the corticosterone binding characteristics of N-terminal fusion proteins that are produced in bacteria and assembled into heterocomplexes with hsp90 in vitro and those of full-length receptors expressed in COS-1 cells.

To further characterize the steroid binding, the order of potency of a series of steroids in [3H]corticosterone competition assays of reticulocyte-treated bacterial preparations of GR and GRo was assessed. The GRo mutant exhibited the same order of potency as the wild-type GR, and similar results were obtained with the GR1 mutant (data not shown). Therefore, it is concluded that GRo and GR1 retain the steroid binding specificity of the wild-type receptor.

In Vitro Assembly of Functional Receptors—Generation of steroid binding activity when purified preparations of bacteri-
GST-GR at the same temperature). In all the cases, steroid binding was corresponding receptor at 30 °C) or as “b” (GST-GRo). Significant differences are indicated as “a” (p < 0.01 from the corresponding max) to increase at a similar rate for WTGR and GRo. In both cases, the maximal effect was reached by 30 min of incubation. Interestingly, when the incubation time with reticulocyte lysate was fixed at 30 min and the temperature was varied (Fig. 3B), a differential effect was observed. Although the optimal assembly temperature for both receptors was reached at approximately 30 °C, at sub-optimal temperatures, GRo binding activity was more adversely affected than the wild-type form. Considering the typical correspondence that has been observed between the thermal inactivation of GR steroid binding capacity and the destabilization of the receptor-hsp90 complexes (25), the most likely explanation for this observation is that although both receptors associate with hsp90, GRo is less stable in its steroid binding conformation than the wild-type receptor.

**Hydrodynamic Properties of the Steroid-bound Complexes**—The sedimentation coefficient of [3H]triamcinolone acetonide-bound GR complexes in cytosol was determined by analytical centrifugation in sucrose gradients containing 20 mM molybdate (Fig. 4). Under these assay conditions, the WTGR sediments as an hsp90-associated 9 S complex, whereas under the same conditions, the mutant GRo is recovered predominantly as a 4–5 S species (69 ± 11% after 12 h of centrifugation, n = 3 different experiments). The amount of 4–5 S species generated by GRo increased with increased centrifugation time (not shown). High salt concentration (0.3 M KCl) in the gradient caused the complete shift (activation) of 9 S to 4–5 S species of both receptors (data not shown). Similar results were obtained with bacterially produced receptors (wild type and mutant) that had been incubated with reticulocyte lysate (data not shown). These results show that the steroid-bound GRo-hsp90 heterocomplex is more susceptible to dissociation (activation) than WTGR-hsp90 heterocomplex. In contrast, under the same experimental conditions, the 9 S complex of GR1 was as stable as the WTGR heterocomplex.

**Transformation of the Receptors to a DNA Binding Form**—Given the fact that GRo shows different physical properties with respect to the WTGR, we investigated the binding of both receptors to DNA after their activation. The DNA-cellulose binding of [3H]corticosterone complexes generated in cytosolic and bacterial preparations was assayed after treatment with 0.3 M KCl. GR, GRo, and GR1 increased their binding to DNA-cellulose to a similar extent (data not shown), and transformation of all of the receptors was inhibited by molybdate.

The ability of cytosolic receptors to bind to a specific GRE was evaluated by electrophoretic mobility shift assays (Fig. 5). The WTGR and all three mutant receptors bound to a 5’P-labeled GRE to the same extent (Fig. 5A). Preincubation of the WTGR with BuGR2 monoclonal antibody completely shifted the upper band (1st lane), verifying GR as a component of the DNA-bound species. Fig. 5B shows the relative potency of unlabeled GRE to compete for GR and GRo binding. In both cases, a half-maximal effect was obtained with a 5-fold molar excess GRE, and complete competition was achieved with a 40-fold molar excess GRE. As shown in Fig. 5C, a 40-fold excess of GRE was also sufficient to fully compete out the binding of mutants GR1 and GR8, and an unlabeled MMTV construct was about 4 times more potent on a molar basis than the synthetic GRE. In contrast, a 40-fold excess of a synthetic OCT1 consensus oligonucleotide (GRE-unrelated element) was unable to compete, suggesting that the binding is GRE-specific. Thus, these experiments indicate that the capacity to bind to a specific DNA sequence is not significantly affected in any of the receptor mutants.
To evaluate the possible functional implications of the GR mutation, transcriptional activation assays were conducted in transiently transfected COS-1 cells. Using an MMTV-CAT reporter gene, corticosterone, the physiological ligand for rat GR, was tested for its ability to induce gene expression. As shown in Fig. 6, 1 mM corticosterone produced equivalent induction of the CAT level for GR and GR1. However, the maximal GRo-mediated response was only 21 ± 6% that of the maximal induction obtained for GR (n = 6 different experiments). As expected, the GR8 mutant, which lacks steroid binding activity, was not stimulated by the ligand.

In view of the fact that the regulatory regions of glucocorticoid-responsive genes commonly contain clusters of GREs, the dose-response effect of corticosterone was studied on the expression of a reporter plasmid containing five consecutive GREs (Fig. 7). The GRo mutation causes a 40-fold increase in the ligand concentration required to attain the half-maximal response (ED50: WTGR 2.6 ± 0.9 nM; GRo = 103 ± 13 nM; GR1 = 2.9 ± 1.4 nM). As with the MMTV reporter plasmid, a saturating concentration of ligand only produced a limited transactivation with GRo (17.3 ± 2.7% that of the maximal wild-type-mediated induction).

Transactivation Activity—To evaluate the possible functional implications of the GR mutation, transcriptional activation assays were conducted in transiently transfected COS-1 cells. Using an MMTV-CAT reporter gene, corticosterone, the physiological ligand for rat GR, was tested for its ability to induce gene expression. As shown in Fig. 6, 1 μM corticosterone produced equivalent induction of the CAT level for GR and GR1. However, the maximal GRo-mediated response was only 21 ± 6% that of the maximal induction obtained for GR (n = 6 different experiments). As expected, the GR8 mutant, which lacks steroid binding activity, was not stimulated by the ligand.

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Nuclear Translocation of Ligand Prebound Receptors in Intact Cells—To study the effect of the GRo mutation on nuclear cytoplasmic translocation (Fig. 8), cells were suspended in growth medium and incubated at 0 °C with [3H]TA to form steroid-receptor complexes. The TA-dependent shift to the nucleus was then permitted by raising the temperature to 37 °C for 30 min. In cells kept at 0 °C, about 80% of WTGR and 95% of GRo steroid-bound complexes are recovered in the cytosolic fraction (Fig. 8A). After 30 min at 37 °C, about 60% of the WTGR complexes are recovered in the nuclear fraction, but only 20% of the GRo complexes are recovered in the same fraction (Fig. 8B). Thus, these results show that the mutation in GRo causes a significant inhibition of nuclear translocation.

DISCUSSION

The present mutational study is based on our previous findings from both peptide competition and deletion analysis, suggesting that hsp90 interacts (although probably not exclusively) with an ~86-amino acid region within the hormone binding domain of the receptor according to a two-site model (21). We have now focused on the functional role of specific
wild-type GR and GRo in intact cells. COS-1 cells were transfected with pCMVGR and pCMVGRo and incubated with [3H]triamcinolone acetonide as described under "Experimental Procedures." The graph shows the percentage of the total binding corresponding to the cytosolic (A) and nuclear (B) fractions at 0 and 37 °C. All data points were determined in triplicate (mean ± S.E.). The data shown correspond to one experiment that is representative of two independent experiments. * indicates p < 0.01 with respect to the corresponding receptor at 0 C.

amino acids present in both of these potential hsp90 contact sites.

After in vitro translation of full-length GR in reticulocyte lysate, we have found that all of the three mutants we prepared were recovered in association with hsp90 at levels that are similar to those of the wild-type receptor. In the highly conserved N-terminal portion of the hsp90 binding site, however, mutation of two completely conserved residues (GR8) abolished the steroid binding activity, thus precluding the characterization of ligand-mediated receptor activity. In contrast, both of the mutations in the more C-terminal hsp90 binding site (GRo and GR1) possessed steroid binding activity. Although GR1 behaved like the wild-type receptor, GRo showed several differences. Although GRo bound steroids at physiological concentrations in vitro and binding specificity was unaltered, there was a small reduction in both corticosterone binding affinity and steroid binding capacity. After cell-free assembly of hsp90 heterocomplexes with bacterially produced GRo, the complexes appeared to be less stable when the assembly reaction was performed at nonoptimal temperatures. Although steroid-bound complexes of GRo with hsp90 assembled either in vitro or in vivo possessed the same relative affinity and specificity of binding to a typical GRE construct, the GRo-hsp90 complexes were highly unstable and dissociated upon sedimentation analysis. Furthermore, the percentage of steroid-receptor complexes of GRo formed in intact cells that translocated to the nucleus at physiological temperature was reduced 3-fold in relationship to the wild-type receptor. Finally, the ligand-induced transactivation activity of GRo (corrected for steroid binding activity) was decreased in the maximal effect and required a 40-fold higher concentration of steroid to yield half-maximal effect.

Given that the hsp90 binding region of the receptor on which we have focused encompasses a substantial portion of the steroid binding domain and that hsp90 is strictly required to maintain the receptor in a steroid binding form (18, 19, 25), it is not surprising to find that ligand binding is affected upon mutation within this region. However, with the GR8 mutant, steroid binding activity is lost without noticeable effect on the association of the hsp90 chaperone, which is required for proper folding of the steroid binding domain into a high affinity steroid binding conformation. This suggests that the proposed N-terminal hsp90 contact site that is mutated in GR8 is important for the proper opening of the steroid binding pocket by the hsp90-based chaperone system or that this conserved region is itself part of the steroid binding pocket.

Interestingly, neither of the mutations in the potential C-terminal hsp90 contact site (GRo and GR1) abolished steroid binding or altered the specificity of the steroid interaction. In the case of GR1, the substitution of positively charged Glu-649 by neutral Gln did not yield any appreciable changes in steroid binding and was indistinguishable from wild-type GR in the other characteristics evaluated. This suggests that the conserved dipole is not critical for receptor function. In contrast to GR1, mutation of the similarly conserved Pro-643 in GRo caused a minor reduction in corticosterone steroid binding affinity that still allows the binding of the ligand at physiological concentrations. Mutation of Pro-643, however, had profound effects on the stability of the heterocomplex with hsp90 and on transcriptional activation.

Under the experimental conditions of our steroid binding assay and subsequent sucrose gradient centrifugation, we typically observe that GR sediments almost exclusively as 9 S species associated with hsp90. This happens regardless of whether the receptors are produced as fusion constructs in E. coli and hsp90 heterocomplexes are subsequently assembled in vitro or the receptors are produced and heterocomplexes assembled in vivo in COS-1 cells. Interestingly, when GRo is assayed in both systems, approximately 70% of the complexes migrate as the smaller, hsp90-free, 4 S species. That this effect is caused by a reduced stability of the GRO-hsp90 interaction is supported by the fact that the percentage of complexes migrating at 4 S increased with increased centrifugation time. Importantly, the results obtained using the in vitro assembly approach further support the physiological relevance of this finding.

Perhaps the most interesting effect of the Pro-643 mutation is the substantial decrease in steroid-mediated transcriptional activation. This functional deficit not only significantly affects the response levels to an excess amount of the physiological steroid corticosterone but also the efficacy of lower concentrations of this ligand. Thus, the concentration of steroid required for half-maximal CAT induction by GRo was approximately 40 times higher than the corresponding concentration for the wild-type GR. Similar results were obtained using two different GR-regulated reporter plasmids, pMMTV-CAT and pGRE5-CAT, endowed with different promoters and different GRE structural and surrounding sequences (28). Therefore, the cause for the observed transcriptional defect relates to factors that are common to both reporter systems. Moreover, the markedly decreased transcriptional activity of GRo cannot be explained solely on the basis of the small decrease in steroid binding affinity detected in vitro. This impaired transcriptional
activation is still observed under ligand-saturating conditions and when the transcription data are normalized to the total amount of steroid-receptor complexes present in the samples. The possibility that the decreased ligand-mediated efficacy of GRo could arise from differences in the intrinsic capacity of the receptor to be activated to a DNA binding form was not supported by our results. Activation of GRo-hsp90 complexes by salt treatment of cytosol increased the DNA-cellulose binding to a similar extent as the wild-type receptor. Also, the results of mobility shift assays argue strongly against the possibility of changes in receptor affinity or specificity for glucocorticoid response elements. Thus, the impaired transcriptional activity of GRo in vivo is likely to relate to receptor properties different from those related to ligand-mediated activation and specific interaction with DNA. Our results demonstrate a defect in the ability of the liganded GRo to translocate to the nucleus at physiological temperature. This finding, coupled with the normal binding of GRo at low temperature and the great loss in the ability of the mutant to transactivate, reinforces the conclusion that this altered receptor exhibits a significant signaling defect, likely associated with a decreased stability of the receptor-hsp90 heterocomplex. Furthermore, our results strengthen previous hsp90-based mutational reports showing the chaperone as an integral component of the steroid-receptor signaling pathway. These earlier studies, which were based on the heterologous expression of GR in yeast with limiting or mutant (13, 14) hsp90, consistently agreed on the correspondence between hsp90 integrity and receptor function. However, mainly due to the broad involvement of hsp90 in diverse aspects of cell function and viability, the hsp90 mutational studies could not rule out the possibility of an indirect effect of those general factors on the observed functional deficit. In addition, the possibility of an indirect effect of mutation on hsp90 interactions with one or more of the additional proteins (other than GR) also present in the chaperone complex could not be discarded. This represents the first direct demonstration that GR-hsp90 heterocomplex stability is critical for the appropriate nuclear signaling of the glucocorticoid receptor.

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