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# Impaired transactivation of the glucocorticoid receptor cloned from the Guyanese squirrel monkey

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**Abstract**

Squirrel monkeys are among a diverse group of New World primates that demonstrate unusually high levels of circulating corticosteroids and glucocorticoid receptor (GR) insensitivity. Recent evidence suggests that overexpression of an immunophilin impairs dexamethasone binding to GR in the Bolivian squirrel monkey (*Saimiri boliviensis*). Here we describe the cloning, expression, and functional characterization of GR from the closely related Guyanese squirrel monkey (*S. sciureus*). The cloned Guyanese squirrel monkey GR (gsmGR) cDNA closely resembles human GR (hGR) cDNA, and yields a high affinity dexamethasone binding receptor when expressed in COS-1 cells. Transactivation analysis of hGR and gsmGR expressed in CV-1 cells and cultured squirrel monkey kidney (SMK) cells indicates that: (1) SMK cells elaborate a functional high activity GR from human GR cDNA; (2) gsmGR is an order of magnitude less efficient than hGR at transactivation in CV-1 and SMK cells; and (3) maximal transactivation by gsmGR is attenuated in both cell lines. Glucocorticoid resistance in *S. sciureus* is at least partly attributable to a naturally occurring mutation in the GR gene that results in impaired GR transactivation. 

*Keywords:* Glucocorticoid receptor; Complementary DNA sequence; Gene expression; Dexamethasone binding; Kidney primary cell culture; SMK-7 cells; COS-1 cells; CV-1 cells; Neonatal; Fetal; Transactivation reporter assay; New World primate

## 1. Introduction

Squirrel monkeys represent an extreme example of glucocorticoid receptor (GR) insensitivity. Cortisol levels are 20- to 50-fold higher than in humans [1,2], without the physiologic sequelae that accompany hypercortisolism [3,4]. Glucocorticoid biosynthetic drive is increased in squirrel monkeys [5], and corticosteroid binding globulin function is impaired [6]. As a consequence, 50% of cortisol in these New World primates circulates in the free state [7]. The squirrel monkey gene for the principal intracellular glucocorticoid catabolic enzyme, 11-β-hydroxysteroid dehydrogenase, is quantitatively and kinetically similar to the human ortholog [8], and does not protect against high circulating corticosteroid levels. Yet squirrel monkeys demonstrate effective negative-feedback regulation of the hypothalamic–pituitary–adrenal (HPA) axis, albeit at much higher glucocorticoid levels [1,9,10].

Until recently it was thought that a genetic alteration in the GR gene was responsible for low ligand binding affinity of GRs in the squirrel monkey [3]. Naturally occurring mutation related changes in steroid receptor affinities have been reported in a number of species, in tumors, and in familial syndromes of glucocorticoid resistance [11–14]. Nevertheless, cloning of the Bolivian squirrel monkey GR (bsmGR) failed to

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reveal an explanatory mutation, and when expressed in vitro, bsmGR yields a dexamethasone binding profile indistinguishable from human GR (hGR) [15]. A recent study suggests that overexpression of an immunophilin impairs dexamethasone binding to GR in the Bolivian squirrel monkey (*Saimiri boliviensis*) [16].

Here we describe the cloning, expression, and functional characterization of GR in the closely related Guyanese squirrel monkey (*S. sciureus*). The cloned Guyanese squirrel monkey GR (gsmGR) cDNA closely resembles hGR cDNA, is modestly different from bsmGR, and yields a high affinity dexamethasone binding receptor when expressed in cultured cells. Affinity for the endogenous ligand cortisol is only marginally decreased relative to hGR, but two significant differences emerge in reporter transactivation studies. Corticosteroid-dependent activation by gsmGR is an order of magnitude less efficient than hGR, and maximal activation is decreased as well. These results indicate that glucocorticoid resistance in the Guyanese squirrel monkey is at least partly attributable to a naturally occurring mutation in the GR gene that impairs GR transactivation.

## 2. Materials and methods

### 2.1. Cloning of the glucocorticoid receptor cDNA from Saimiri sciureus

The squirrel monkey colony at Stanford University is derived from Guyanese animals imported in 1975, and maintained as a closed colony for the past 18 years. All animal handling followed NIH guidelines and was approved by Stanford University's Administrative Panel of Laboratory Animal Care. A maternally rejected 3-day-old male found on the cage floor during the 1996 breeding season was quickly euthanized, and the brain frozen on dry ice. Total RNA was extracted by the acid-phenol method and poly- (A) RNA was isolated with a kit using oligo- (dT) latex beads (Qiagen). First strand cDNA was synthesized by reverse transcriptase and second strand was synthesized according to the method of Gubler and Hoffman [17]. The cDNA was linkered (Promega) and inserted into lambda ZAP express (Stratagene). Packaging and lytic phage plating were achieved following manufacturer's instructions (Stratagene). Plaque lifts and low stringency screening [18] was performed with radiolabeled nick-translated probe to rat GR cDNA (2.5 kb SalI–SalI fragment). Positive clones were sequentially purified and the double stranded circular plasmid was isolated as described by the manufacturer (Stratagene). Sequencing was accomplished with a modified Taq polymerase kit (Promega) and florescence based automated sequencing at the Stanford Protein and Nucleic Acid Core Facility. All other DNA and RNA manipulations were carried out as previously described [18]. Sequence analysis was performed in the Wisconsin Package, v.9.0 (Genetics Computer Group) or the Lasergene package (DNASTAR).

### 2.2. Squirrel monkey kidney primary culture

Kidney tissue from a half-term squirrel monkey fetus and 34-day neonate, collected for unrelated research, was harvested under sterile conditions. Tissue was finely minced in phosphate buffered saline and treated with 0.25% trypsin for 9 h at 4°C. Following incubation at 37°C for 30 min, the tissue was disrupted by gentle pipetting, and the cell suspension plated in DMEM/50% fetal calf serum (Gibco) [19]. Cells were maintained in this medium until the time of transfection (10% serum) and 24 h prior to harvesting (10% serum, charcoal treated). Primary cells were immortalized by treatment with 150 μg/ml of ethyl-nitrosourea (Sigma) as previously described [20] and thereafter maintained in 10% serum/DMEM.

### 2.3. Steroid binding assay

[1,2,4,6,7-$^3$H]-Dexamethasone (82 Ci/mmol) and [1,2,6,7-$^3$H]-cortisol were purchased from Amersham Life Sciences. All other reagents were purchased from Sigma. The assay was performed essentially as previously described [21]. Sample extracts were prepared by Polytron disruption of tissue (liver from one week adrenalectomized, salt-replaced Sprague-Dawley rats, or tissues from intact squirrel monkeys) or sonication (Ultrasonic Inc., model W-220F) of cultured cells in TEGMD buffer (10 mM Tris buffer, pH 7.4, 1 mM EDTA, 10% glycerol, 1 mM sodium molybdate, fresh 1 mM dithiothreitol). The supernatant was collected after centrifugation at 100,000*g* for 1 h. A typical assay consisted of 50 μl of extract, 10 μl of tracer [$^3$H]-dexamethasone (or [$^3$H]-cortisol), and 5 μl of TEGMD buffer $\pm$1 μM unlabeled dexamethasone (or cortisol). After incubation overnight at 4°C, 100 μl of a 2% dextran coated charcoal (DCC) solution was added, incubation at 4°C was continued for an additional 10 min, and the charcoal was pelleted at 1000*g* for 10 min. One hundred microliters of the supernatant was mixed with UniverSol scintillation fluid (ICN) and measured on a Beckman LS3801 scintillation counter. The mixing studies were performed identically, except the total assay volume was doubled and test samples were introduced as 50 μl of each extract (or TEGMD buffer with 1 mg/ml bovine serum albumin). Steroid-free squirrel monkey liver cytosol was prepared by incubation of the 100,000*g* supernatant with 10% DCC for 30 min at 4°C, followed by centrifugation at 12,000*g*. Data

were analyzed in Excel (Microsoft) and plotted in Prism (GraphPad).

### 2.4. Northern and western analysis of hGR and gsmGR in transfected COS-1 cells

Standard protocols were used for formaldehyde-agarose Northern and discontinuous SDS-polyacrylamide gel Western analyses [18]. Total RNA from 10 cm plates of transfected COS-1 cells was prepared with TRIZOL reagent (Life Technologies). COS-1 protein samples were prepared from separate plates by extraction with 1 × SDS loading buffer and centrifugation at 12,000*g* for 10 min to remove debris. Bacterial cell extracts containing β-gal-GR fusion products were harvested as previously described [22]. The Northern analysis was probed with a [$^{32}$P]-riboprobe directed against the amplified gsmGR cDNA (SacI to BamHI) and washed under moderate stringency conditions (0.1 × SSC/0.1% SDS, 55°C). This condition gives identical results using hGR or gsmGR specific probes. The Western analysis was probed with the rabbit polyclonal "E-20" antibody to N-terminal hGR (Santa Cruz Biotechnology). This epitope contains two conservative substitutions in gsmGR: R11K and A11T. Secondary probing with HRP-linked anti-rabbit antibody and chemiluminescent detection were performed according to the manufacturer's instructions (Santa Cruz Biotechnology). X-ray film images were digitized and analyzed in Scion Image software for the PC.

### 2.5. Expression of GRs and GRE-luciferase in cultured cells

Vectors for mammalian GR expression were constructed by PCR amplification of hGR or gsmGR cDNA with common primers flanking the open reading frame (CCAGAGCTCATATTCACTGATGGAC and CCGGATCCTTTAAGGCAACCATT), and the SacI/BamHI fragment inserted into the corresponding sites of pBKCMV (Stratagene). The resulting constructs place the complete GR open reading frame 62 nucleotides downstream from the bacterial β-galactosidase translation start site, followed by 10 nucleotides of GR 5′-untranslated sequence. The constructs were verified by sequencing the ligated regions. β-Galactosidase control vector was pCMV-β-gal-SPORT (Life Technologies). GRE-luciferase reporter was constructed by subcloning the PvuII/BamHI fragment containing the 5 × GRE enhancer and adenovirus intermediate-early promoter from pGRE-CAT (US Biochemicals) into the polylinker of pGL3basic (Promega). Plasmids were prepared with Qiagen "Maxi" kits.

On the day prior to transfection, 7–8 × $10^5$ cells were plated on 10 cm disposable culture dishes in 10 ml of DMEM containing 10% fetal calf serum. Cells were incubated overnight at 37°C in 5% $CO_2$, after which plates were 60–70% confluent. Transfection was accomplished with the "Superfect" transfection reagent (Qiagen) at a ratio of 4:1 test vector:β-galactosidase vector (and 4:1:1 for GRE-luciferase). Five micrograms of DNA and 30 μl of reagent (SMK-7 cells) or 10 μg of DNA and 60 μl of reagent (COS-1 and CV-1 cells) were mixed with 300 μl of DMEM and incubated at room temperature for 10 min. Culture plates were washed once with 10 ml of PBS. Three milliliters of growth medium was added to the transfection complex and the solution was gently pipetted onto the cells. After incubation for 3 h at 37°C (5% $CO_2$), the suspension was aspirated, cells were washed again with PBS, covered with growth medium containing 50 μg/ml gentamycin sulfate, and incubated for 1–2 days at 37°C prior to harvesting. Steroid-defined media was exchanged at 24 h. Lysates for luciferase or β-galactosidase activity were prepared with Reporter Lysis Buffer (Promega).

### 2.6. β-Galactosidase and luciferase assays

*O*-Nitro-phenyl-β-D-galactopyranoside (ONPG) was purchased from Sigma. In vivo and in vitro assays were performed essentially as described previously [23]. Briefly, 20 μl of tissue/cell extract in TEGMD buffer, prepared for binding assay, was combined with 500 μl of reaction buffer (58 mM $Na_2HPO_4$, 42 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, 0.8 mg/ml ONPG). Incubation was continued at 37°C until the yellow color fell within the linear range of the assay. Reaction was terminated by addition of 500 μl of 1 M $Na_2CO_3$ and absorption was read at 420 nm. Assay of luciferase activity was performed according to the supplier's protocol (Promega), and measured on a luminometer (Turner Designs).

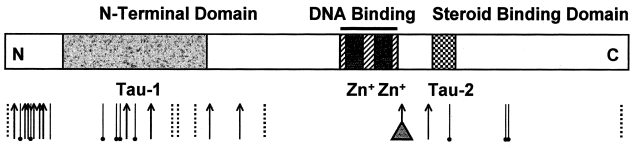


Fig. 1. Comparison of deduced squirrel monkey GR protein with human GR. Schematic of amino acid differences between gsmGR and hGR. Dashed lines represent conservative substitutions, lines with a single dumbbell represent related substitutions, and arrows represent significant changes (per the "bestfit" algorithm in GCG software). A triangle marks the insertion of an isoleucine residue. Functional domain boundaries are estimated by relatedness to human GR [31]. "Zn+" is equal to zinc fingers.

## 3. Results

### 3.1. Cloned Guyanese squirrel monkey GR is structurally similar to human GR

A neonatal *S. sciureus* whole brain cDNA library was constructed, and a clone for GR was isolated by hybridization to probes directed against the rat GR cDNA [24]. Cloned gsmGR cDNA (Genbank entry AF041834) is 3.5 kilobases long, and has an open reading frame of 2334 nucleotides that is overall 97% similar to the hGR ortholog. The cDNA codes for a protein of 778 aa. that bears 97% similarity to the hGR primary sequence [25], and is identical through the DNA binding domain (Fig. 1). The steroid binding domain is almost identical, except for a number of conservative substitutions: $Gly^{516}$ to $Ala^{517}$, $Ser^{551}$ to $Thr^{552}$, $Ser^{616}$ to $Ala^{617}$, $Ala^{618}$ to $Ser^{619}$, and $Ile^{761}$ to $Leu^{762}$ (human to squirrel monkey). Guyanese squirrel monkey GR is closely related to bsmGR [15], with two notable differences. A three base insertion at nucleotides 1859–1861, yields one additional isoleucine after aa. 497, and $Ser^{176}$ is substituted for $Thr^{176}$. Guyanese squirrel monkey GR 5′-untranslated sequence is 92% homologous to a fragment of Genbank entry M32284 (human GR exon 1) and represents transcription initiation at least 170 bp farther upstream than that mapped in humans [26].

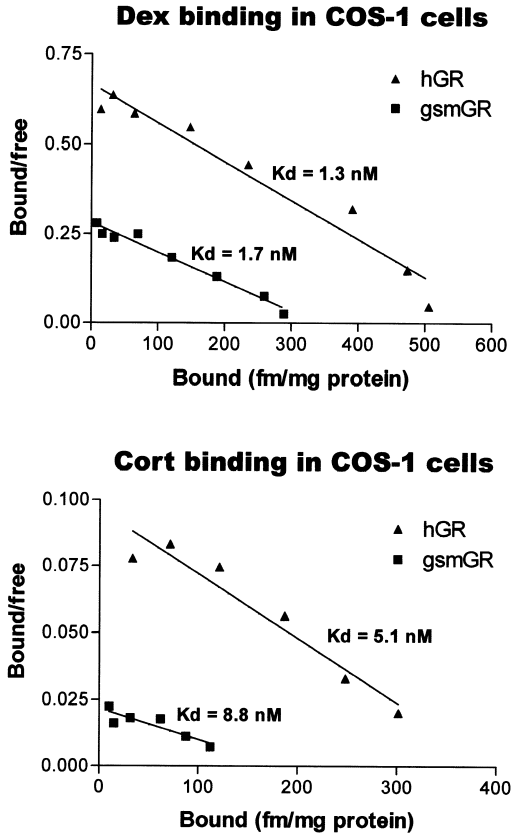


Fig. 2. Squirrel monkey GR and hGR expressed in COS-1 cells yield indistinguishable, high affinity dexamethasone binding receptors. (a) Scatchard of [$^3$H]-dexamethasone (Dex) binding in COS-1 cells transfected with gsmGR or hGR expression constructs. Calculated dissociation constants are shown above each line. (b) [$^3$H]-Cortisol (Cort) binding to similar preparations. Affinity differences based on cortisol binding reach statistical significance (see text for details). Abscissa is expressed in femtomoles (fm)/mg protein. No ligand binding is seen in cells transfected with the control vector (pBKCMV).

### 3.2. Guyanese squirrel monkey GR expressed in COS-1 cells yields high affinity ligand binding

In light of the discrepancy between poor binding of dexamethasone to squirrel monkey GR in tissues [3] and the structurally preserved steroid binding domain of the deduced protein, we expressed both gsmGR and hGR in COS-1 cells and measured ligand binding in cytosolic extracts. PCR primers to identical sequences in gsmGR and hGR 5′- and 3′-untranslated regions were used to generate orthologous constructs in

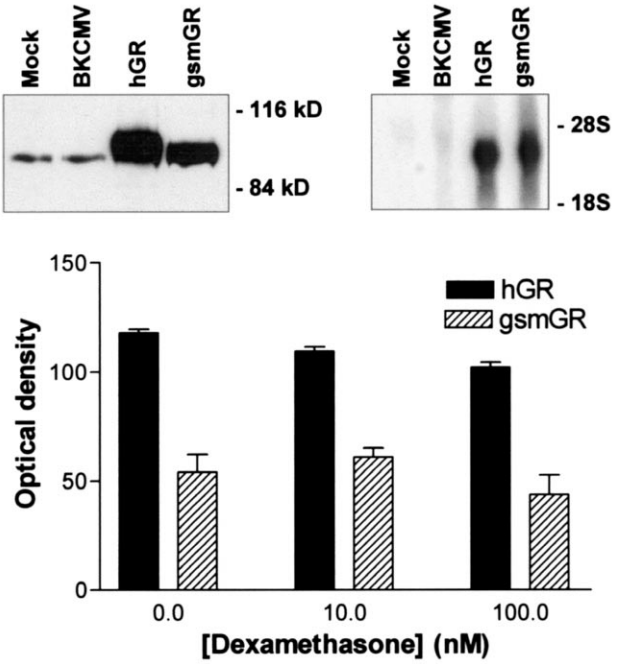


Fig. 3. GR protein and mRNA expression in COS-1 cells. Top left: Representative Western analysis of hGR and smGR expressed in COS-1 cells. Twenty milligrams of protein were loaded in each lane. "Mock" is transfection without DNA and "BKCMV" is transfection of control vector lacking insert. Top right: Northern analysis in collateral samples probed for smGR mRNA. Bottom: Densitometric results from Western analysis of COS-1 cells transfected with GRE-luciferase reporter and the hGR or smGR. Three representative dexamethasone concentrations were assayed in triplicate. Results are shown as mean ± SEM. Optical density is corrected for β-gal expression (transfection efficiency). There is an effect of receptor type ($P < 0.0001$) but not dexamethasone concentration ($P = 0.06$) by two-way ANOVA.

pBKCMV. The resulting vectors constitutively transcribe gsmGR and hGR mRNA, respectively, under the control of the CMV promoter. In a series of three replicates of the same study, we confirmed that these two receptors are indistinguishable based on affinity for [$^3$H]-dexamethasone (Fig. 2). Hyperbolic regression (one-site) kinetic and Scatchard analyses reveal dissociation constants of 1.3 nM for hGR (95% C.I. = 1.1–1.5 nM) and 1.7 nM for gsmGR (95% C.I. = 1.5–1.9 nM). For binding to [$^3$H]-cortisol, two replicates yielded dissociation constants of 5.1 nM for hGR (95% C.I. = 4.3–5.8 nM) and 8.8 nM for gsmGR (95% C.I. = 6.5–11 nM). In both binding assays, the maximal binding ($B_{max}$) for gsmGR (dexamethasone binding: 340 fm/mg protein; cortisol binding: 187 fm/mg protein) is consistently diminished in comparison to hGR (dexamethasone binding: 619 fm/mg protein; cortisol binding: 401 fm/mg protein).

### *3.3. Decreased maximal binding is related to post-transcriptional alteration of receptor content*

Since both vectors are identical except with respect to the nucleotide differences between the two receptors, and similar results were obtained from two separate plasmid preparations, we hypothesized that the $B_{max}$ difference is secondary to post-transcriptional changes. To investigate this possibility, we examined GR mRNA (by Northern analysis) and GR immunoreactivity (by Western analysis) in extracts from COS-1 cells transfected with hGR or with gsmGR (Fig. 3, top panels). In two separate studies, gsmGR mRNA levels were comparable to hGR mRNA levels (93 and 128%), but gsmGR protein levels were significantly reduced (53 and 67% of hGR). To test if the difference in immunoreactivity was due to a difference in the target sequence, we also probed β-gal-hGR and β-gal-gsmGR fusion proteins expressed in bacterial cells under control of the lacZ promoter. β-gal-gsmGR immunoreactivity was 106% of β-gal-hGR immunoreactivity, suggesting that the difference in GR immunoreactivity in transfected COS-1 cells was not the result of antibody selectivity.

### *3.4. Cell lines developed from Guyanese squirrel monkey kidney lack GR ligand binding*

To test GR ligand binding in squirrel monkey cells thoroughly free of endogenous cortisol, and to develop a system to further investigate GR function, we isolated and cultured kidney cells from a half-term Guyanese squirrel monkey fetus. Similar lines from a 34-day neonate were also tested, with identical results. Cells from the fetal line were immortalized at passage #7 and designated SMK-7. The fetal and immortalized cell lines behaved similarly in all parameters measured.

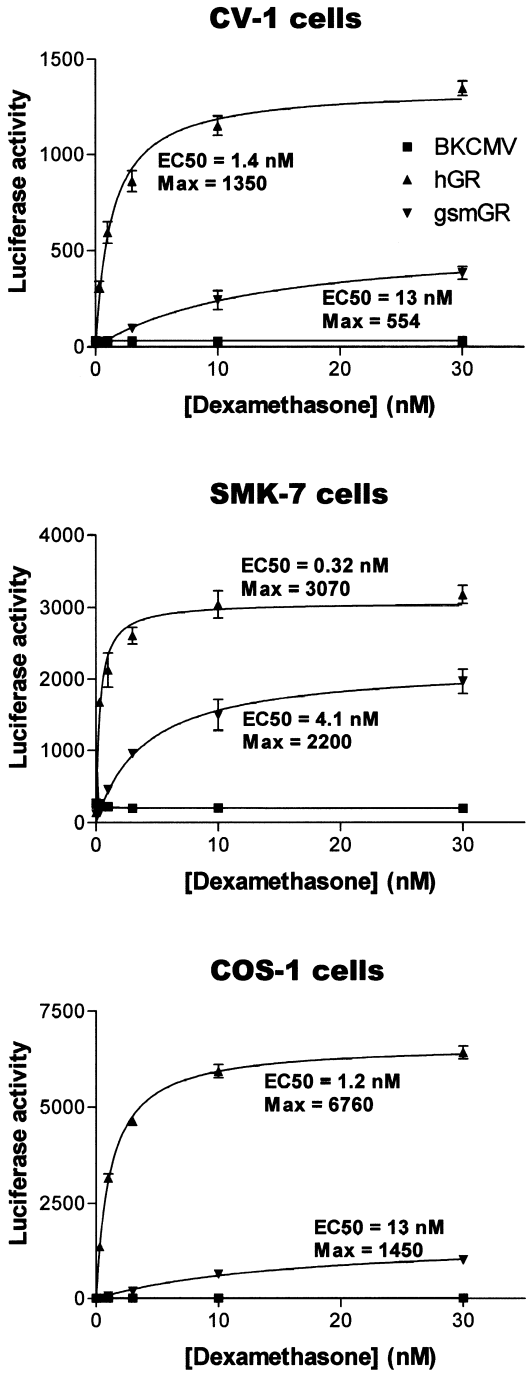


Fig. 4. Human GR and gsmGR are distinguished by transactivation function in all cell types. Human GR, gsmGR, or control vector (pBKCMV) were expressed in CV-1 (top), SMK-7 (middle) and COS-1 cells (bottom) along with GRE-luciferase and β-galactosidase control vector. After 24 h, charcoal-stripped media containing indicated concentrations of dexamethasone was substituted. Cells were harvested after 48 h. Luciferase activity is normalized to β-galactosidase activity. Each point is plotted as the mean ± standard deviation of triplicate samples from one study. The legend for all three panels is shown in the top graph. Hyperbolic regression by Prizm software (GraphPad) was used to generate the kinetic data. $EC_{50}$ is effective concentration for 50% maximal (Max) activation. See text for statistical details. Transactivation studies were carried out to 300 nM dexamethasone (not shown) without further activation of the reporter.

Even after culture in steroid-free media, we could detect only trace levels of binding to [$^3$H]-dexamethasone in either transformed SMK-7 or primary SMK cell extracts. This binding was not of sufficient magnitude to convincingly determine dissociation constant. Consistent with the binding data, no GR immunoreactivity by Western analysis was observed in extracts prepared from these cells. Lack of GR ligand binding in these cells permitted their use in subsequent mixing studies.

### *3.5. Lack of GR ligand binding in Guyanese squirrel monkey cells is not due to an inhibitory factor*

To investigate the possibility that ligand binding was impaired by an endogenous inhibitor, we tested the binding of [$^3$H]-dexamethasone (11.5 nM) to liver cytosol preparations from adrenalectomized rats pre-mixed with cytosolic extract prepared from SMK-7 cells cultured in charcoal-treated serum. There was no inhibition of [$^3$H]-dexamethasone binding to rat GR by extracts from SMK-7 cells. Addition of SMK-7 extract produced 113% (not statistically significant) of the binding of rat extract alone. A 10-min pre-incubation of the mixture at 30°C decreased the overall binding, but did not uncover an inhibitory effect. Insofar as SMK-7 cells might not faithfully reflect in vivo biology, we also tested extracts from squirrel monkey liver, pre-absorbed with dextran-coated charcoal to remove endogenous cortisol. This preparation contained no intrinsic binding activity, and failed to inhibit dexamethasone binding in liver extract from adrenalectomized rats.

### *3.6. Guyanese squirrel monkey GR transactivation is impaired*

Despite normal gsmGR ligand binding in COS-1 cells, extraordinarily high levels of glucocorticoids are required to feedback regulate the squirrel monkey HPA-axis in vivo. In light of these findings, we tested gsmGR in a functional transactivation assay, and investigated whether SMK-7 cells were capable of elaborating a functional high activity receptor from hGR cDNA. A sensitive glucocorticoid response element (GRE)-luciferase reporter was constructed and co-transfected with gsmGR or with hGR into SMK-7 cells and CV-1 cells in a series of three replicates of the same experiment (Fig. 4). Unlike COS-1 cells, CV-1 cells lack the T-antigen which otherwise disproportionately amplifies the vector. CV-1 cells and SMK-7 cells share gross morphology (fibroblast type), and exhibit similar transfection efficiencies (~5–7%). In CV-1 cells, the $EC_{50}$ values for reporter activation by dexamethasone acting through gsmGR were nine-fold greater than those for hGR (gsmGR: 13 nM, 95% C.I. = 6.0–20 nM; hGR: 1.4 nM; 95% C.I. = 0.71–2.0 nM). A similar ratio was obtained for reporter activation in SMK-7 cells, although the absolute $EC_{50}$ values were significantly lower (gsmGR: 4.1 nM, 95% C.I. = 2.3–5.6 nM; hGR: 0.32 nM, 95% C.I. = 0.10–0.54 nM). These statistics are based on regression to a one-site hyperbolic model, with goodness-of-fit ($r^2$) ranging from 0.95 to 0.99. Guyanese squirrel monkey GR also failed to achieve the same maximal level of activation both in CV-1 cells (41% of hGR) and in SMK-7 cells (72% of hGR). These differences were confirmed using two different DNA preparations and careful quantitation of vector DNA. In COS-1 cells, $EC_{50}$ values for gsmGR were 11-fold greater than those for hGR, and maximal activation levels achieved by gsmGR were attenuated even further (21% of hGR). Western analysis for GR of the same extracts used in the transactivation assay, at three of the dexamethasone dose points (Fig. 3, bottom panel), confirmed approximately 50% reduction in protein.

## 4. Discussion

The cloned gsmGR cDNA closely resembles hGR cDNA, and yields a receptor that binds with high affinity to dexamethasone and cortisol when expressed in cultured cells. These findings concur with published results on dexamethasone binding for bsmGR expressed in reticulocyte lysate [15]. We further found that cultured Guyanese squirrel monkey cells are capable of assembling a functional high activity GR from human cDNA, and failed to demonstrate a cytosolic inhibitor as was recently reported in the Bolivian squirrel monkey [16]. Results from our experiments indicate that glucocorticoid resistance in the Guyanese squirrel monkey is at least partly attributable to a naturally occurring mutation in the GR gene that impairs GR transactivation.

The predicted amino acid sequence of gsmGR is not substantially different from human GR, and contains largely conservative substitutions (Fig. 1). Two differences are noteworthy with respect to bsmGR [15], though it is not yet clear whether these are attributable to different cloning methods (PCR vs. phage amplification) or true inter-species differences. The first difference is a conservative T176S substitution in the N-terminal domain. The second is an additional Ile at position 496 in gsmGR. The $Ile^{496}$ insertion differs from all mammalian GRs, was confirmed on bi-directional sequencing, and interrupts a basic amino acid region thought to associate with immunophilin. This association with immunophilin is reportedly involved in the nuclear translocation of GR [27]. While immunophilins are not necessary for reconstitution of high affinity ligand binding to mouse GR [28], overexpres-

sion of the immunophilin FKBP51 was recently proposed to impair dexamethasone binding to GR in the Bolivian squirrel monkey [16]. Our failure to detect a cytosolic inhibitor is in keeping with findings from at least one other neotropical hypercortisolemic primate [29], although the use of different methodologies hamper direct inter-species comparisons between Bolivian and Guyanese squirrel monkeys.

As previously reported for bsmGR expressed in reticulocyte lysate [15], we found high affinity dexamethasone binding to gsmGR expressed in COS-1 cells. Affinity for the endogenous ligand cortisol was only modestly decreased in gsmGR relative to hGR, and not of sufficient magnitude to account for marked glucocorticoid resistance in squirrel monkeys. In both dexamethasone and cortisol binding assays, $B_{max}$ for gsmGR was reduced by approximately 50%. This correlates with a similar reduction in gsmGR immunoreactivity, while mRNA levels for gsmGR and hGR were comparable in COS-1 cells. Since transcription and translation initiation was directed by identical sequences, these results suggest potential deficit(s) in post-transcriptional regulation of gsmGR. Either decreased translation efficiency or increased receptor turnover might account for decreased receptor content in the transfected COS-1 cells. Glucocorticoid receptor destabilization is typically associated with disruption of the GR:chaperone complex [30]. We tested this possibility in cells cultured from squirrel monkey kidney (SMK-7).

Since endogenous or transfected GR ligand binding in SMK-7 cells was below the detection limit, we employed a sensitive transactivation assay to characterize GR and chaperone function in these cells. Transactivation subsumes (a) ligand binding, (b) chaperone-complex reorganization, (c) nuclear translocation, (d) recognition/binding to target DNA, and (e) modification of transcription. A decrease in ligand binding affinity would be expected to cause a right shift in the dose-response curve for transactivation, but transactivation may be impaired in the face of high affinity ligand binding if dysfunction resides in one or more of the subsequent steps. We observed that the transactivation dose-response curve for hGR in SMK-7 cells is comparable, if not modestly left-shifted, as compared to that in COS-1 and CV-1 cells. This would suggest that the chaperone complex and translocation factors are fully functional in squirrel monkey tissues. Moreover, the dose-response relationship for transactivation of the reporter is in the same range as equilibrium ligand binding to hGR, suggesting that for this ortholog, transactivation is essentially coincident with receptor-ligand association.

The unusual finding raised by this report is that the above relationship does not prevail for gsmGR. Whereas gsmGR, expressed in COS-1 cells, yields an equilibrium dexamethasone binding affinity indistinguishable from hGR ($K_d$ = 1.7 vs. 1.3 nM), it is an order of magnitude less potent in activating the reporter ($EC_{50}$ = 13 vs. 1.4 nM). As with the binding data, maximal transactivation by gsmGR is also attenuated relative to hGR, consistent with less receptor immunoreactivity in these samples. The fact that SMK-7 cells effect high potency transactivation of hGR by dexamethasone argues that impairment of gsmGR is not due to a disturbance of SMK cell chaperones. The finding of a similar pattern of hGR vs. gsmGR transactivation in COS and CV-1 cells suggests that the difference is not due to a unique association between gsmGR and SMK cell chaperones. We conclude that sequence changes in gsmGR result in altered transactivation efficiency without a change in ligand binding affinity, and that the gsmGR protein is destabilized relative to hGR. Glucocorticoid resistance in *S. sciureus* may be, at least partly, attributable to these processes.

The exact alteration(s) in gsmGR primary structure responsible for impaired transactivation and destabilization is (are) as yet unidentified. A number of studies have demonstrated that the nature of the GR:chaperone complex affects the stability of the receptor protein [28] and that ligand binding is functionally dissociable from transactivation. Mutation of rat GR in the proposed hsp90 binding domain (P643A) results in normal binding but impaired nuclear translocation and transactivation [30]. In this case, the mutation is thought to destabilize association with hsp90. Insertional mutants in the "tau-1" domain spanning amino acids 78–262 of hGR (same in gsmGR) attenuate transactivation in the face of high affinity wild-type binding [31]. In particular, a 4-amino acid insertion at $Lys^{120}$, which is substituted by Gln in gsmGR, results in 2% of control transactivation through the mouse mammary tumor virus promoter despite 76% of control dexamethasone binding. More detailed studies have narrowed the tau-1 core to amino acids 187–244 [32]. This region contains several proline directed serine phosphorylation sites [33] that are conserved in gsmGR, but the substitutions E210Q and R214K in gsmGR, in an otherwise highly acidic region, are not found in other mammalian GRs, and flank a $Trp^{213}$ residue critical to transactivation [34]. Insertional mutagenesis at $Arg^{214}$ of hGR results in a binding vs. transactivation profile similar to that at $Lys^{120}$ described earlier [31].

A number of other phylogenetically conserved residues are substituted in gsmGR (Tyr30Cys, Val39Leu, Pro46Thr, Gly298Ser, and Val321Ile). Further study is needed to determine if any of these alterations are responsible for the observed decrease in transactivation and/or receptor stability. The insertion of Ile at residue 496 is of particular interest. It was not reported for the Bolivian squirrel monkey ortholog and would be con-

sistent with a different mechanism for glucocorticoid resistance in that species. $Ile^{496}$ is situated in a highly conserved domain thought to be critical for nuclear translocation of the liganded receptor. It is tempting to speculate that this mutation may be responsible for impaired nuclear translocation, and consequently transactivation, of gsmGR. Further study of this particular mutation is warranted.

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