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Biochemistry

Dexamethasone negatively regulates the activity of a chimeric dihydrofolate reductase/glucocorticoid receptor protein

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ABSTRACT **A chimeric gene was constructed encoding the entire murine dihydrofolate reductase (DHFR) protein with a carboxyl-terminal extension encompassing amino acids 494–795 of the rat glucocorticoid receptor (GR). The chimeric DHFR/GR gene encoded a functional DHFR protein, as measured by the ability to transform DHFR-deficient Chinese hamster ovary (CHO) cells to a DHFR-positive phenotype. The DHFR/GR protein bound [^{3}H]dexamethasone with a similar affinity as wild-type GR. Selection of stable CHO transformants in increasing concentrations of methotrexate resulted in increased expression of DHFR/GR. Addition of dexamethasone, a synthetic glucocorticoid agonist, decreased the activity of the chimeric protein, as measured by colony formation in selective medium, binding of fluoresceinated methotrexate, and direct enzymatic assay for DHFR. Addition of RU486, a glucocorticoid antagonist, antagonized the effect of dexamethasone. In the absence of dexamethasone, the chimeric protein was primarily localized to the cytoplasm. In the presence of dexamethasone or RU486, DHFR/GR translocated into the nucleus. However, RU486 did not decrease DHFR activity, distinguishing subcellular location from functional activity. These results demonstrate that glucocorticoids negatively affect the function of DHFR/GR.**

The effects of steroid hormones are mediated by intracellular receptor proteins that modulate the expression of specific genes. The molecular cloning of genes encoding receptor proteins for many steroids (1–5) has allowed functional dissection of the receptors into discrete domains. For example, functional domains for nuclear translocation (6), DNA binding and transcriptional modulation (7–9), 90-kDa heat shock protein (hsp90) binding (10), and hormone binding (11) have been identified. Glucocorticoid receptor (GR) proteins with carboxyl-terminal deletions of the hormone-binding domain are constitutively active (9). Chimeric proteins made with the hormone-binding domains of various steroid receptors and E1A (12), Myc (13), and Fos (14) are activated by addition of hormone. These studies have led to a model in which the hormone-binding domains of steroid receptors act as repressors of protein function, possibly by virtue of their interaction with hsp90 (12), or by a hypothetical "unfoldase" or protein inactivation activity (14), and that the binding of an agonist steroid relieves this inhibition.

Dihydrofolate reductase (DHFR) is the target for the chemotherapeutic drug methotrexate (MTX). Resistance to MTX in tumors or cell lines is associated with gene amplification, resulting in DHFR enzyme levels that parallel the level of MTX resistance (15). Functional assays for DHFR, including enzymatic activity (16), growth of cells in selective medium (15), and binding of a fluorescent derivative of MTX (17), have been used to characterize this protein. Amplification of the DHFR gene also has practical utility for coamplifying heterologous genes to high levels (18).

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We have made a fusion protein between DHFR and the GR hormone-binding domain to study the mechanism of steroid hormone action by using assays for DHFR. Contrary to reports of global activation of steroid receptor function upon agonist binding (12–14), we report that the DHFR activity of the chimeric protein is negatively regulated by the synthetic glucocorticoid dexamethasone.

METHODS

DHFR/GR Gene and Expression Vector. The expression vector pMT2 (19) was digested with *Aha* II and *Hpa* I, thereby removing the 3′ end of the DHFR gene. A double-stranded oligodeoxynucleotide encoding the carboxyl-terminal 22 amino acids of DHFR from the *Aha* II site was synthesized. This oligonucleotide, which deleted the termination codon of DHFR and contained an overhang for the restriction enzyme *Sph* I, was ligated to the rat GR cDNA at the *Sph* I site (nucleotide 1552 in ref. 2). After digestion with *Sma* I (the *Sma* I site is within the SP64 polylinker of pRBal117), the 1.3-kb fragment was ligated into *Aha* II/*Hpa* I-digested pMT2. The resulting plasmid, pMT2D/G (Fig. 1), contained a chimeric gene encoding all of murine DHFR with a carboxyl-terminal extension of amino acids 494–795 of the rat GR, as confirmed by DNA sequencing.

Transient Expression of DHFR/GR in COS-1 Cells. COS-1 monkey cells were transfected with pMT2 (19), pMT2T-GR (20), or pMT2D/G (21). At 60 hr posttransfection, cells were analyzed for expression of DHFR/GR protein.

Stable Expression of DHFR/GR in CHO Cells. DHFR-Deficient CHO DUKX-B11 cells were electroporated (200 V, 1250 μF) with 100 μg of circular plasmid (22). Two days later, cells were switched to selective α minimum essential medium (α-MEM; GIBCO) containing 10% dialyzed fetal bovine serum (Flow Laboratories) and lacking nucleosides (18). The cell line D/GM2 was established from a single colony selected in the absence of hormone or MTX and was subsequently selected for amplification by growth in increasing concentrations of MTX (18). Other independently isolated cell lines yielded results similar to those obtained with D/GM2. For colony assays of CHO DUKX cells transfected with pMT2D/G, hormones were added (1 μM) immediately after transfection and were present throughout the selection period. For colony assays of D/GM2 cells selected for resistance to 20 nM MTX, 100 cells were plated in medium containing 20 nM MTX in the presence of the indicated hormones at 1 μM each. After 9–11 days, colonies were fixed and stained in methanol with methylene blue and counted.

Analysis of Metabolically Labeled DHFR/GR. Cells were incubated in methionine-free Eagle's MEM (GIBCO) for 15 min, and then pulse-labeled with [^{35}S]methionine (New England Nuclear) at 200 μCi (7.4 MBq)/ml for 15 min. For the chase period, labeling medium was removed and complete

Abbreviations: DHFR, dihydrofolate reductase; GR, glucocorticoid receptor; hsp, heat shock protein; MTX, methotrexate; Flu-MTX, fluoresceinated MTX.

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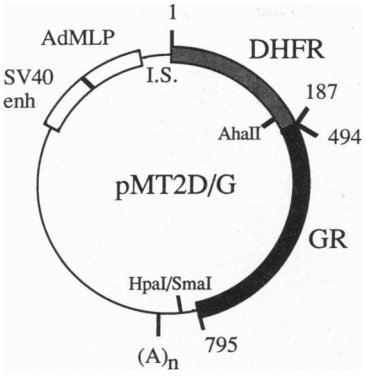


FIG. 1. Expression vector pMT2D/G encodes a fusion protein consisting of the entire DHFR protein with a carboxyl-terminal extension of the hormone-binding domain of the GR (see *Methods*). The simian virus 40 (SV40) enhancer (enh), adenovirus major late promoter (AdMLP), intervening sequence (I.S.), and SV40 polyadenylylation signal [$(A)_n$] allow efficient expression of this vector in both COS-1 and CHO cells (19). Numbers indicate the amino acids of DHFR and GR encoded by the fusion gene.

medium was added for various times. In cells treated with dexamethasone (Sigma) or RU486 (Roussel–Uclaf), hormone was added after the pulse period. Cells were lysed in Laemmli sample buffer (23) containing 2-mercaptoethanol, electrophoresed through 15% polyacrylamide gels, and analyzed by autoradiography and laser densitometry (Pharmacia LKB UltroScan XL).

Labeling of Cells with Fluoresceinated MTX (Flu-MTX). Cells were labeled for 16–20 hr with 10 μM Flu-MTX (Molecular Probes) in serum-free α-MEM (17) supplemented with adenosine, deoxyadenosine, and thymidine (each at 10 μg/ml) in the absence or presence of dexamethasone or RU486. Cell fluorescence was analyzed by flow cytometry (FACScan, Becton Dickinson). For microscopy, cells were plated on glass coverslips, labeled with Flu-MTX as above, and were extensively washed in phosphate-buffered saline. Fluorescence microscopy (Axiophot, Zeiss) was immediately performed on unfixed, viable cells.

DHFR Assays. An enzymatic assay for DHFR measuring the reduction of [3′,5′,7,9-^{3}H]folic acid (Amersham, 37 Ci/mmol) was performed (24). Enzyme properties were determined by using Lineweaver–Burk reciprocal plots (25) from 60-min assays performed at 37°C with 2–50 μM folic acid and 1–2 mg of total cell protein per ml.

Dexamethasone Binding. The binding of [^{3}H]dexamethasone (New England Nuclear, 39.4 Ci/mmol) was assayed as described (20) and analyzed by the method of Scatchard (26).

RESULTS

Expression of DHFR/GR Protein in COS-1 and CHO Cells. DHFR-deficient CHO DUKX-B11 cells were transfected with pMT2D/G (Fig. 1), a vector encoding the DHFR/GR fusion protein, and selected for growth in nucleoside-free medium. In the absence of hormone, many colonies survived the selective conditions (Table 1, line A), suggesting that DHFR/GR is a functional protein. Further, when transfected cells were selected in the presence of dexamethasone, colony formation was reduced by a factor of 3–5. The dexamethasone-induced decrease in colony formation was prevented by RU486, a compound with known glucocorticoid antagonist activity (27). There was no cell survival under selective conditions in mock-transfected cells (data not shown).

Several clonal cell lines were established from colonies growing in nucleoside-free medium and were subjected to stepwise selection for MTX resistance (18). In several of these lines, dexamethasone induced a decrease in the binding of Flu-MTX (data not shown), suggesting that the DHFR/GR protein was responsive to hormone. A cell line demonstrating pronounced dexamethasone responsiveness, D/GM2(X) [where X is the level of MTX-resistance (μM) at the time of analysis], was further characterized.

Table 1. Effect of dexamethasone (Dex) and RU486 on the functional activity of DHFR/GR in transfected CHO cells

Exp.	No hormone	Dex	RU486	Dex + RU486
A*	13.2 ± 4.0	4.2 ± 0.7	13.0 ± 2.4	8.7 ± 1.4
B†	62.5 ± 2.9	27.8 ± 2.9	62.0 ± 12.5	53.5 ± 9.0

*Effect on transformation to a DHFR-positive phenotype. CHO DUKX cells were electroporated with the vector pMT2D/G and selected as described in *Methods*. The transformation frequency is expressed as colonies per 10^4 electroporated cells. The data represent the mean ± SD from duplicate plates. The experiment was performed three times, with consistent results.

†Effect on colony formation of D/GM2(0.02) cells. The D/GM2 cell line was selected for growth in 20 nM MTX. Approximately 100 cells were plated in medium containing 20 nM MTX and the indicated hormone(s). The number of colonies represents the mean ± SD from four plates under each condition. The addition of nucleosides, which relieves the requirement for DHFR for cell survival, increased the colony count of cells plated with dexamethasone to the levels obtained in the absence of hormone (data not shown). In addition to reduced numbers, the colonies surviving growth in dexamethasone were significantly smaller. The experiment was performed three times, with consistent results.

Upon addition of 20 nM MTX to the medium, a stringent selection occurred to yield a stable resistant subpopulation of the D/GM2 cell line. Typically, cells selected in this fashion have amplified the DHFR gene, providing just enough enzyme to overcome MTX inhibition (28). Addition of dexamethasone to D/GM2(0.02) cells resulted in a further selection against the growth of colonies in 20 nM MTX (Table 1, line B). This effect was antagonized by RU486. The results in Table 1 suggest that the amount of functional DHFR activity in cell lines expressing the DHFR/GR fusion protein is reduced by dexamethasone.

Transfection of COS-1 cells with pMT2D/G directed the production of an abundant 55-kDa intracellular protein (Fig. 2, lane 3) that was absent from mock-transfected cells (lane 1). This is the size predicted from the cDNA fragments used to construct the hybrid gene and is distinct from wild-type DHFR (lane 2) or wild-type GR (lane 4). Similar analysis of D/GM2(0.5) cells revealed a high rate of synthesis of 55-kDa DHFR/GR that was absent from CHO DUKX cells (lanes 5 and 6) and comigrated with the protein produced in COS cells (lane 3). DHFR/GR was labile, exhibiting a half-life of 3.2 hr in the absence of hormone (lanes 7–11) and 2.4 hr in the presence of either dexamethasone (lanes 12–16) or RU486 (lanes 17–21). This small decrease in the stability of the protein in the presence of hormone does not quantitatively account for the much larger dexamethasone-induced decreases in other properties of DHFR/GR described below. In addition, the rate of synthesis of DHFR/GR in D/GM2 cells was not affected by addition of dexamethasone or RU486 (data not shown).

Effect of Dexamethasone on Flu-MTX Binding. Flu-MTX labeling is a sensitive and quantitative probe for the levels of DHFR in intact viable cells (17). Treatment of D/GM2(0.5) cells with dexamethasone induced a 5- to 10-fold decrease in the fluorescence intensity of cells labeled with Flu-MTX (Fig. 3). In contrast, binding of Flu-MTX did not decrease in cells treated with RU486 (data not shown). We used this assay to measure other parameters associated with DHFR/GR in D/GM2 cells. In D/GM2 cells selected for increasing levels of MTX resistance, there was a corresponding increase in the amount of Flu-MTX binding (data not shown), consistent with the gene amplification observed with wild-type DHFR (18). The threshold response to dexamethasone was 3 nM,

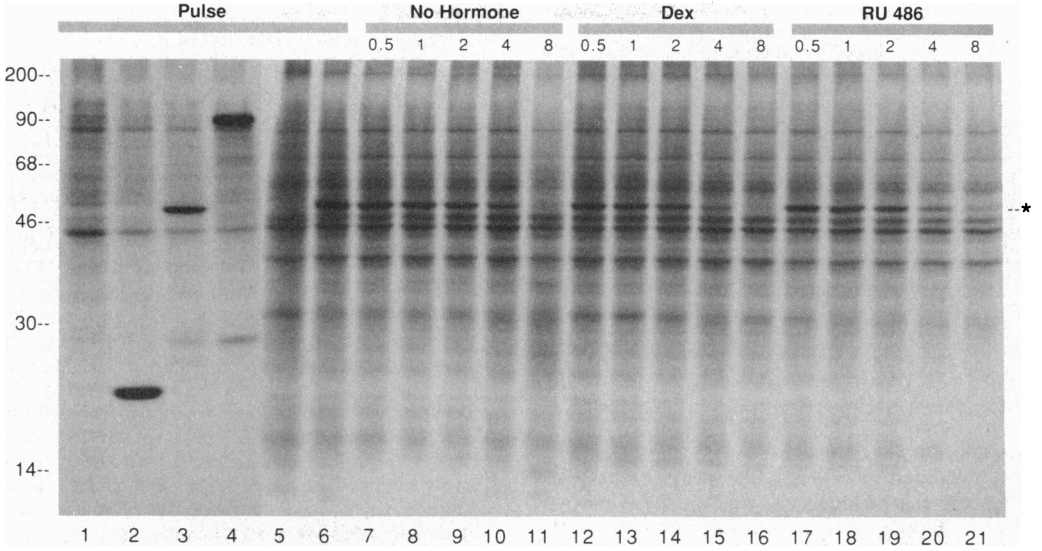


FIG. 2. Expression of DHFR/GR in COS-1 and CHO cells. COS-1 cells were transfected with the vectors pMT2, pMT2T-GR, and pMT2D/G. Cells were metabolically labeled for 15 min with [^{35}S]methionine 60 hr posttransfection. Lane 1, mock-transfected cells; lane 2, transfection with pMT2; lane 3, transfection with pMT2D/G; lane 4, transfection with pMT2T-GR. The D/GM2 cell line, selected for growth in 0.5 μM MTX, was pulse labeled as described in *Methods* and then was incubated in complete medium in the absence of hormone (lanes 7–11) or in the presence of dexamethasone (Dex, lanes 12–16) or RU486 (17–21). Lane 6, 15-min pulse, no chase; lanes 7, 12, and 17, 30-min chase; lanes 8, 13, and 18, 1-hr chase; lanes 9, 14, and 19, 2-hr chase; lanes 10, 15, and 20, 4-hr chase; lanes 11, 16, and 21, 8-hr chase. Total cell lysate was analyzed by SDS/PAGE. For comparison, cell lysate from pulse-labeled CHO DUKX cells is shown (lane 5). Chase time (hr) and hormone treatments are indicated at top. Size markers (kDa) are indicated at left. Location of DHFR/GR is denoted by a star.

with a half-maximal response at 10 nM. Maximal response occurred at 30 nM and higher concentrations and was antagonized by RU486. Addition of 100 nM dexamethasone to cells prelabeled with Flu-MTX produced no visible response after 15 min. By 30 min, a small decrease was seen in the fluorescence of cells. A half-maximal response occurred 1.5–3 hr after addition of dexamethasone, and by 5 hr the maximal response was observed (data not shown). The decrease in Flu-MTX binding produced by dexamethasone therefore occurs rapidly and at a similar concentration of hormone as is required to produce effects via the wild-type GR (29, 30).

Subcellular Distribution of DHFR/GR in Intact Cells. We determined the subcellular location of DHFR/GR in the absence or presence of hormone by labeling cells with Flu-MTX and performing fluorescence microscopy on intact viable cells. In the absence of hormone, DHFR/GR was predominantly a cytoplasmic protein (Fig. 4*A*). In the presence of dexamethasone, the predominant fluorescence was found in the nucleus (Fig. 4*B*). These results suggest that dexamethasone induced nuclear translocation of DHFR/GR, similar to the behavior of wild-type GR (31). Wild-type DHFR in stable CHO cell lines selected for MTX resistance was present primarily in the nucleus but was also found in the cytoplasm (Fig. 4*D*). In D/GM2 cells incubated with both Flu-MTX and RU486, the predominant fluorescent signal occurred in the nucleus (Fig. 4*C*), similar to the results obtained with dexamethasone. Since both hormones induced nuclear translocation of DHFR/GR but only dexamethasone produced a decrease in functional activity (Table 1), the reduced activity in response to dexamethasone was not simply a consequence of subcellular redistribution.

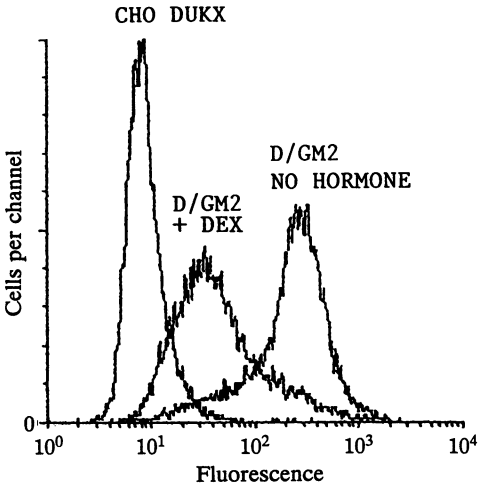


FIG. 3. Effect of dexamethasone on Flu-MTX-binding activity in D/GM2 cells. D/GM2(0.5) cells were labeled with 10 μM Flu-MTX in the absence or presence of dexamethasone (Dex) and analyzed for fluorescence by flow cytometry. DHFR-deficient CHO DUKX cells were analyzed in parallel and define the background fluorescence of the assay. Each histogram represents the analysis of 10,000 cells.

Biochemical Characterization of DHFR/GR. We measured both the dexamethasone-binding properties and the enzymatic characteristics of DHFR/GR by using assays previously developed for the wild-type proteins (24, 29). Scatchard analysis demonstrated that binding of [^{3}H]dexamethasone occurred with a K_d of 3.4 nM (Fig. 5), a value that is virtually identical to the binding affinity of wild-type GR (20, 29). The binding capacity of D/GM2(0.5) cells was 7.4 pmol/mg of cytoplasmic protein (Fig. 5). Similar analyses at various levels of MTX resistance demonstrated that the dexamethasone-binding capacity changed in parallel with the level of MTX resistance and occurred with no significant change in K_d (data not shown), consistent with coamplification of both dexamethasone binding and DHFR activity.

We also measured the enzymatic properties of the fusion protein, using an assay for folate reductase activity (24). The K_m for folate was not altered by treating cells with either dexamethasone or RU486 (Fig. 6) and was similar to the value obtained for wild-type DHFR measured here (data not shown) and previously (28). The functional effects of dexamethasone therefore do not result from a significant change in the affinity of the fusion protein for folate. In the presence of dexamethasone the V_{max} was decreased by a factor of 2–4

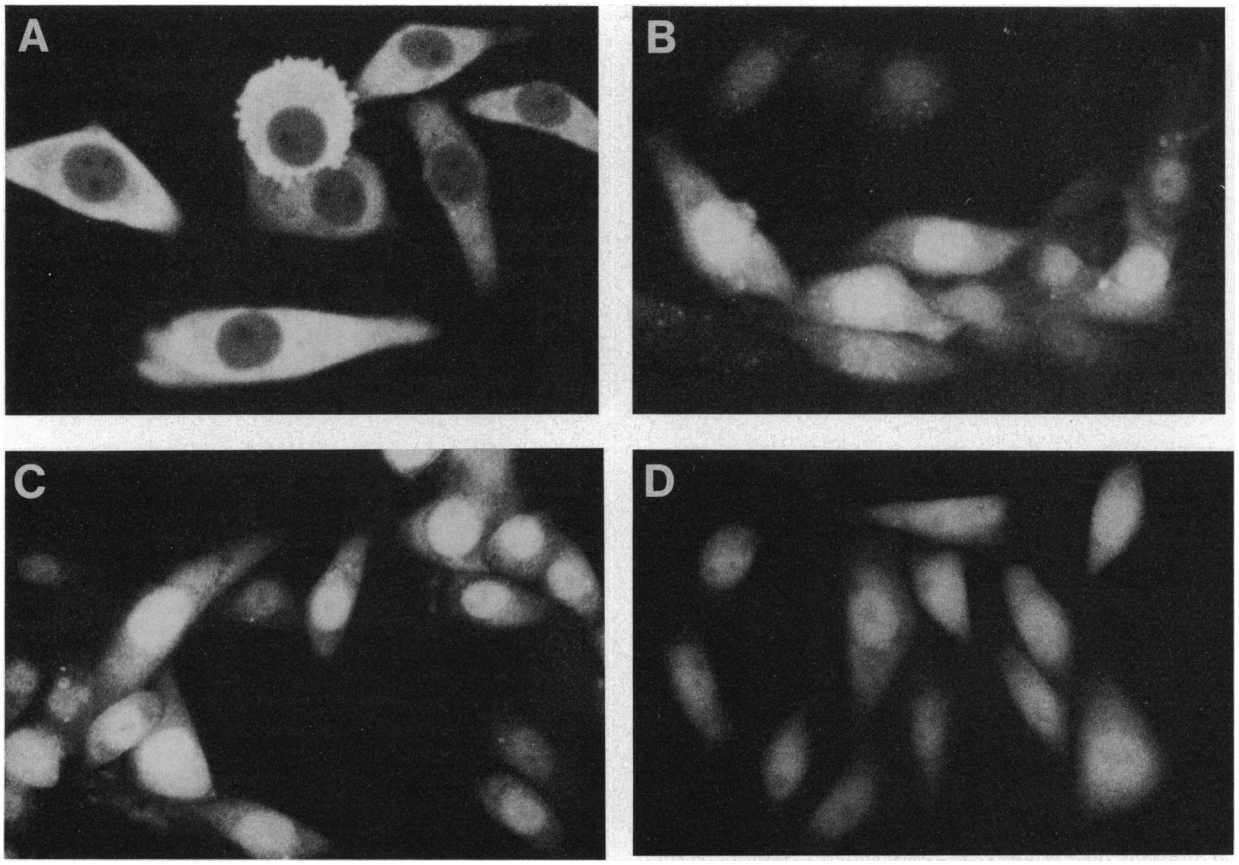


FIG. 4. Subcellular location of DHFR/GR and wild-type DHFR. D/GM2(2.0) cells were labeled with Flu-MTX in the absence of hormone (*A*) or in the presence of 100 nM dexamethasone (*B*) or 1 μM RU486 (*C*) and analyzed by fluorescence microscopy. For comparison, Flu-MTX-labeled ΔVA(2.0) cells, a stable derivative of CHO DUKX cells transfected with a wild-type DHFR expression vector and selected for resistance to 2.0 μM MTX, are shown (*D*).

(Fig. 6). Since dexamethasone did not change the rate of synthesis (data not shown) and had only a small effect on the rate of degradation of the fusion protein (Fig. 2), we conclude that the hormone decreased the catalytic efficiency of DHFR/GR. This can in part account for the functional consequences of hormone on DHFR activity in the colony-formation assays (Table 1).

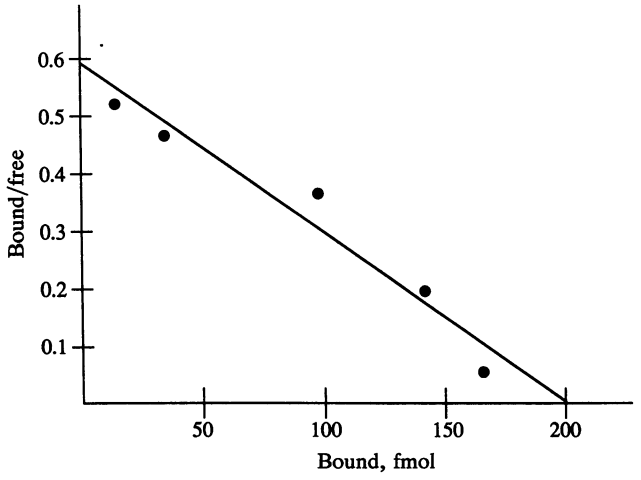
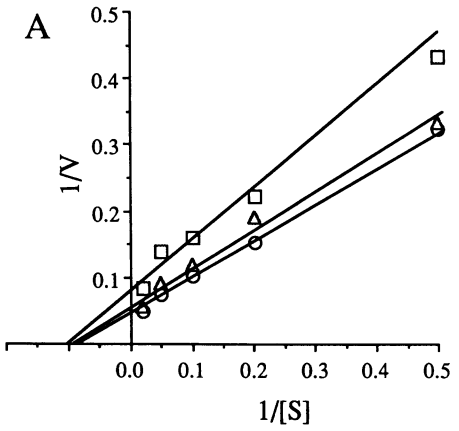


FIG. 5. Scatchard analysis of [^{3}H]dexamethasone-binding activity in the D/GM2(0.5) cell line. The maximum number of binding sites (B_{max}) is 7.4 pmol/mg and the dissociation constant (K_d) is 3.4 × 10^{-9} M. Negligible binding was observed with extracts of control CHO DUKX cells.

DISCUSSION

The activity of the DHFR/GR fusion protein decreased in response to dexamethasone. This decrease occurred in intact cells with a Flu-MTX binding assay, in functional assays for cell growth under selective conditions in which DHFR activity is required for survival, and in cell extracts assayed for DHFR activity. Although dexamethasone induced a redistribution of DHFR/GR into the nucleus, we do not believe that this explains the decrease in activity, since RU486 induced nuclear translocation without any detectable effect on Flu-MTX binding or activity of the fusion protein. The reduced Flu-MTX binding in dexamethasone-treated cells most likely reflects a decrease in binding capacity, rather than in affinity, since Flu-MTX labeling was performed under saturating conditions (17). Our *in vitro* data suggest that dexamethasone also decreased the V_{max} of DHFR/GR. The results lead us to believe that dexamethasone inactivates a portion of DHFR/GR, accounting for changes in the functional properties of the protein.

Our results contrast with those obtained with similar fusion proteins made between the hormone-binding domains of steroid receptors and various transcriptional activators such as E1A (12), Fos (14), and Myc (13). DHFR/GR differs from these other chimeric proteins in that it possesses a catalytic activity that provides an essential coenzyme involved in metabolic carbon transfer, whereas these other chimeric proteins are transcriptional regulators. Our results with DHFR/GR also differ from those reported for a β-galactosidase/GR fusion protein (13), where β-galactosidase activity was hormone-independent. We do not know whether dexamethasone induces dimerization of DHFR/GR. Our data are



B

	K_m	V_{max}
Exp. 1 - No hormone	15.4	16.7
Dex	22.2	4.4
RU486	16.7	10.0
Exp. 2 - No hormone	13.3	25.0
Dex	9.1	12.5
RU486	12.5	21.8
Exp. 3 - No hormone	12.0	8.0
Dex	13.5	4.1
RU486	n.d.	n.d.

FIG. 6. Enzymatic activity of DHFR/GR. Cell lysates from D/GM2(0.5) cells grown in the absence of hormone (○) or in the presence of dexamethasone (Dex) (□) or RU486 (△) were assayed for DHFR activity using various concentrations (2–50 μM) of [^{3}H]folate. Extracts prepared from CHO DUKX cells had no detectable enzyme activity. The data from a single experiment are presented graphically in *A*. The K_m (μM folate) and V_{max} (pmol of folate reduced per μg of protein per 60 min) values from three independent experiments are shown in *B*. V, velocity (units as for V_{max}); [S], folate concentration (μM); n.d., not determined.

inconsistent with a model in which the unliganded hormone binding domain is a global inhibitor of the function of other tethered domains (12, 14), but support one in which the conformation of the receptor protein is changed upon ligand binding. The consequence of such a conformational change may be specific to the protein domain that is covalently linked to the steroid-binding domain, rather than an intrinsic property of the steroid-binding domain itself.

In the absence of hormone, DHFR/GR was predominantly cytoplasmic, whereas wild-type DHFR was predominantly nuclear, suggesting that the hormone-binding domain can retain an otherwise nuclear protein within the cytoplasmic compartment. "Nuclear translocation" of steroid receptors may therefore reflect the relief of a cytoplasmic retention domain additive with the previously described nuclear localization domains (6, 32). Previous experiments demonstrating nuclear localization of GR mutants lacking the hormone-binding domain (6) and of wild-type receptor in cells treated with dinitrophenol (33) are consistent with the presence of an energy-dependent cytoplasmic retention domain.

Our data show that in intact viable cells, RU486 promotes the nuclear translocation of DHFR/GR. These data can be reconciled with reports that the RU486–GR complex is cytoplasmic (27, 34), if the complex is weakly associated within the nucleus and redistributes into the cytoplasm upon cell fractionation. Our data show that antagonism by RU486 is mediated by residues 494–795 of the GR and thus does not require the DNA-binding domain and occurs after receptor translocation into the nucleus. We propose that binding of agonist (dexamethasone) or antagonist (RU486) elicits different structural changes in the GR. While both steroids induce nuclear translocation, only dexamethasone induces an additional change(s) (e.g., in conformation, covalent modification, or interaction with other proteins) that alters the activity of other domains of the molecule.

The results show that steroids can be used to regulate the enzymatic activity of a chimeric protein. Modification of the assay conditions, such as inclusion of molybdate to stabilize the interaction of GR with hsp90 (35), might increase the magnitude of the dexamethasone response, thereby allowing steroid hormone action to be studied *in vitro* by using well-characterized assays for DHFR. Regulating the enzymatic activity of DHFR with glucocorticoids may permit gene amplification with dexamethasone in addition to MTX. Finally, similarly constructed DHFR fusion proteins stained with Flu-MTX should allow the analysis of intracellular protein trafficking in viable cells.

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