Arsenic at Very Low Concentrations Alters Glucocorticoid Receptor (GR)-Mediated Gene Activation but Not GR-Mediated Gene Repression: Complex Dose−Response Effects Are Closely Correlated with Levels of Activated GR and Require a Functional GR DNA Binding Domain

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Arsenic (As) contamination of drinking water is considered a principal environmental health threat throughout the world. Chronic intake is associated with an increased risk of cancer, diabetes, and cardiovascular disease, and recent studies suggest increased health risks at levels as low as 5−10 ppb. We report here that 0.05−1 μM (6−120 ppb) As showed stimulatory effects on glucocorticoid receptor (GR)-mediated gene activation in rat EDR3 hepatoma cells of both the endogenous tyrosine aminotransferase (TAT) gene and the reporter genes containing TAT glucocorticoid response elements. At slightly higher concentrations (1−3 μM), the effects of As became inhibitory. Thus, over this narrow concentration range, the effects of As changed from a 2- to 4-fold stimulation to a greater than 2-fold suppression in activity. Interestingly, the inhibitory effect of GR on both AP1- and NF-κB-mediated gene activation was not affected by As. The magnitude of GR stimulation and inhibition by As was highly dependent on the cellular level of hormone-activated GR. Mutational deletion studies indicated that the central DNA binding domain (DBD) of GR is the minimal region required for the As effect and does not require free sulfhydryls. Point mutations located within the DBD that have known structural consequences significantly altered the GR response to As. In particular, point mutations in the DBD that confer a DNA-bound GR confirmation abolished the low dose As stimulatory effect but enhanced the inhibitory response, further indicating that the DBD is important for mediating these As effects.

Introduction

Human exposure to As[1] in occupational settings, at Superfund sites and other toxic waste sites, or from natural sources in the environment has become a major health concern (*1*, *2*). As is now considered the number one agent of concern on the Agency for Toxic Substances and Disease Registry's priority list of toxic chemicals in the environment and the top environmental chemical of concern by the World Health Organization (*1*, *2*). Of greatest concern is exposure to As in drinking water, primarily resulting from contamination of groundwater by certain natural underground geological sources containing As (*1*, *2*). The U.S. EPA, as a result of this concern, recently reduced the maximum allowable levels of As in public drinking water from 50 to 10 ppb (0.67 to 0.13 μM). In many areas of the country, As in drinking water is found naturally at levels greatly exceeding these guidelines. While all public water supplies must eventually comply with the new limits, millions of people in the United States continue to be exposed to elevated As from private, unregulated bedrock wells. In New Hampshire, for example, where as much as half the population obtains their drinking water from private wells, a recent study estimated that as many as one in five wells, representing 10% of the state's population, contain excess As (*3*). Thus, As exposure will continue to be a major environmental health concern.

The chronic ingestion of As has been linked to significant effects on human health. These risks include several types of cancer, type 2 diabetes, vascular disease, cardiovascular disease, and reproductive and developmental problems (*1*, *4*, *5*). As in drinking water is considered a probable human lung, skin, and bladder carcinogen based on strong human epidemiology data (*1*, *2*, *5*). As exposure has also been associated with an increased risk of liver, kidney, and other internal cancers (*1*, *2*, *5*). As may be unique in being the only known agent that increases lung and skin cancer following systemic rather than inhalation or dermal exposure, respectively. As exposure has also been strongly linked to the development of vascular and cardiovascular diseases (*1*, *2*, *5*).

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[1] Abbreviations: As, arsenic; Dex, dexamethasone; GR, glucocorticoid receptor; hGR, human GR; rGR, rat GR; mGR, mouse GR; LBD, ligand binding domain; DBD, DNA binding domain; GRE, glucocorticoid response element; C/EBP, CCAAT/enhancer binding protein; SP1, specificity protein 1; HNF3, hepatocyte nuclear factor 3; Ets, family of transcription factors binding to E26 transformation specific sequence; TAT, tyrosine aminotransferase; PMA, phorbol 12-myristate 13-acetate; TNFα, tumor necrosis factor-α.

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The mechanism(s) by which As is able to increase cancer risk or elicit these other various pathophysiological effects is not known, but several potential mechanisms have recently been proposed (*6*). In the environment, inorganic As predominates and is found in two forms, arsenite (As^{+3}), and arsenate (As^{+5}), which can be interconverted in the environment depending on redox conditions and other factors. Arsenite is approximately 2−4-fold more potent than arsenate in most biological systems, but they appear to have similar effects, since both forms can readily enter cells and can also be interconverted by redox pathways in vivo. The transport of As^{+3} (as arsenite) into the cell is similar to glycerol and enters through the same multifunctional aquaglyceroporin channels (*7*). Once inside the cell, As^{+3} prefers binding to sulfhydryl groups, with a strong preference for vicinal dithiols in proteins but with many other potential binding sites. On the other hand, As^{+5} (as arsenate) is isostructural with phosphate and sulfate and enters cells principally through the nonspecific anion transporter and possibly other sulfate and phosphate transport systems (*8*); it can then competitively interfere with phosphorylation reactions at enzyme active sites and target sites of phosphorylation. Inorganic As can also be metabolized in vivo in a series of sequential methylation steps that eventually lead to one or more highly methylated forms that are considered less toxic than the inorganic and intermediate forms and are readily excreted (*9*). However, several of the intermediates in this pathway are now recognized to have their own unique toxicities. In particular, the mono- and dimethylated As^{+3} forms (MMA^{+3}, DMA^{+3}) are more toxic than inorganic As and have been reported to be more carcinogenic in animals (*10*). Thus, the overall pathophysiological effects of As may result from a combination of effects from several different forms of As in vivo.

We had initially observed that As (administered as arsenite) has profound but highly selective effects on gene expression in vivo at very low doses (*11*). In subsequent studies aimed at determining the underlying mechanism for these effects, we demonstrated that As can act as a potent endocrine disruptor, altering hormone-activated gene transcription mediated by the GR at very low doses both in cell culture (rat hepatoma cells) and in a whole animal model (the chick embryo) (*11*, *12*). Interestingly, unlike most organic chemicals that have previously been characterized as endocrine disruptors, As did not activate GR by acting as a hormone mimetic nor did it interfere with hormone binding or hormone activation of GR by acting as a competitive antagonist. In fact, although As was shown to preferentially bind to GR at very low (nM) concentrations, As also did not appear to affect the ability of hormone to activate GR nor did it alter the translocation of activated GR to the nucleus from the cytoplasm (*12*). As also did not appear to alter the binding of activated GR to DNA, at least as determined by semiquantitative assays such as gel mobility shift (Kaltreider, R. C., Bodwell, J. E., et al. Unpublished results). These results suggested that As interferes with the ability of GR to act as a transcription factor once it is bound to DNA in a hormone-activated form. The goal of the current study was to examine in greater detail the structural basis for the effects of As on GR-mediated gene transcription using site-directed mutagenesis of rGR and hGR.

Experimental Procedures

Caution: *As is classified as a probable human carcinogen* (*1*, *2*, *5*). *All arsenicals should be handled as potentially highly toxic compounds*.

Reagents and Buffers. Dex (Steroids, Newport, RI) was dissolved in ethanol as a 1 mM stock and stored at −20 °C. Iron-supplemented calf serum was purchased from Sigma. DMEM/F12 was purchased from Gibco (Gaithersburg, MD). Charcoal-stripped serum was prepared as previously described (*13*). D-Luciferin potassium salt was from PharMingen (San Diego, CA). All other reagents were from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Cell Lines and Vectors. EDR3, a generous gift from Dr. Gary Firestone, is a minimal hepatoma cell line derived from the Fu5 cell line (*14*). It contains no detectable GR. The cell line 10.1.11.14 is based on the EDR3 cell line and stably expresses ∼10 000 mGRs/cell. COS-7 is an African green monkey kidney fibroblast line containing ∼10 000 GRs/cell that are transcriptionally inactive, and it was obtained from the American Type Culture Collection (Rockville, MD). The culturing of these lines is described by Bodwell et al. (*15*).

The plasmid pSV2 WT2X encodes for the mGR (*15*). Drs. Chris Jewel and John Cidlowski kindly supplied PCMV5mGR (*15*) and pCMV5hGR (*16*), which express the mGR and hGR, respectively. Dr. David Pearce generously provided the rGR encoding plasmid p6rGR. NT− and LBD− were constructed from pSV2 WT2X (mGR) using standard cloning techniques. Amino acid residues 5−379 were deleted from the wild-type (WT) mGR to make the NT− mutant while residues 514−780 were eliminated to produce the LBD− mutant. Point mutations used in this study were made with the Quickchange Mutagensis kit (Stratgene, La Jolla, CA) according to the manufacturers instructions and were confirmed by DNA sequencing.

The G2T reporter gene (*15*) has two tandem GREs from the TAT gene controlling luciferase expression. PLUC2, a generous gift from Dr. Constance Brinkerhoff, is a luciferase reporter gene containing 1.8 kb of the collagenase promoter region encompassing well-defined response elements for AP1 (*17*). NF-κB-mediated transcription was monitored by the NF-κB-INFb-LF reporter gene (*18*, *19*) (graciously donated by Aaron Barchowsky) and had three tandem repeats of the NF-κB recognition sequences controlling luciferase expression.

Transfection and Harvesting of Cells. Long duration electroporation was used to transfect cells with the appropriate constructs and was described in detail (*15*). In some experiments, a fixed amount of reporter gene was transfected with different amounts of GR encoding DNA while in other experiments the total amount of DNA was kept constant with vector DNA. There was no discernible difference in the response to As between either approach.

After all of the cells were electroporated, they were distributed to culture containers using media containing charcoal-stripped serum. Early experiments used six well plates, but as more treatment conditions were needed, we switched to 24 well plates. Each treatment had three replicates for six well plates and six replicates for 24 well plates. The culture containers were incubated overnight at 37 °C, and the medium was replaced 24−28 h posttransfection with DMEM/F12 containing 5% 2× stripped serum with the indicated concentrations of Dex and other reagents (see Results). Equivalent volumes of vehicle (ethanol and/or DMSO) were added to control cells. The cells were incubated for 16−20 h and then harvested, and the cell lysates were assayed as described below.

Assays. Transfected cells in culture containers were rinsed twice with saline at room temperature and then chilled on ice. The cells were lysed with 300 μL/well (24 well plate; 1 mL/well for six well plates) of HNE buffer (0.020 M HEPES, 0.05 M NaCl, 10% glycerol, and 0.001 M EDTA) containing 10 mM Chaps {3-([(3-chloamidopropyl)dimethyammonio]-1-propanesulfonate} for 12 min at 4 °C with mixing (Orbitron, Boekel Scientific, Feasterville, PA). Cell lysates were either centrifuged for 10 min at 14000*g* or were vacuum filtered through a 96 well

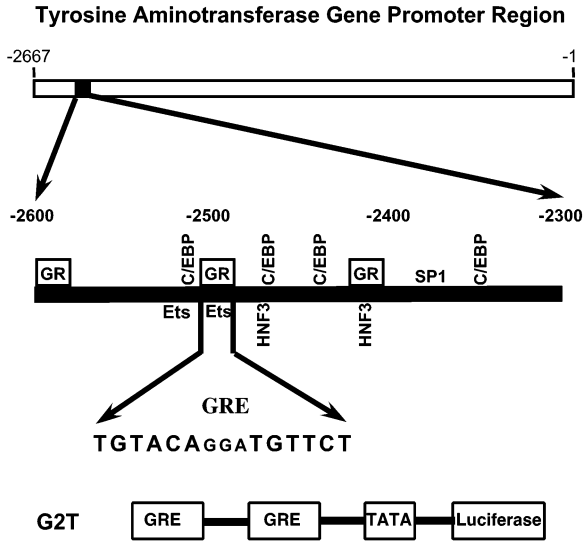

Figure 1. Diagrammatical representation of the TAT gene promoter region and derived reporter gene. The TAT promoter region is shown at the top of the figure. The region from −2300 to −2600 from the transcriptional start site is expanded in the middle of the figure to show the approximate location of interactions with known transcription factors. The GR binding sites are in blocks, and the sequence of the middle GRE is shown. It was used to construct the G2T reporter gene shown in the bottom of the figure. Modified from ref *59*.

1.2 μm multiscreen filter plate (Millipore, Milford, MA). Aliquots of the cell lysate were assayed for protein and luciferase activity (*20*) or TAT activity (*21*). A whole cell binding assay was sometimes performed on additional plates 24 h after transfection to determine the number of GRs/cell and to ensure equivalent GR content between treatments (*15*).

To compare GR types with different transcriptional activities (either because of different GR or hormone levels or the effect of mutation), data were normalized to the no As control for each series of As treatments. These values are included in the figure legends.

Results

Similar Response of Endogenous TAT Gene and Transfected Reporter Gene to Dex and As. The present study utilized EDR3 rat hepatoma cells (*14*) to probe the molecular mechanisms involved with As modulation of GR-mediated gene expression. EDR3 cells are one of the few cell lines that are truly GR negative (neither protein nor mRNA are detectable). However, the introduction of functional GR restores glucocorticoid induction of genes normally regulated by GR (*14*). Thus, the necessary machinery for GR function and modulation of regulated genes remains intact in these cells and requires only the restoration of GR expression.

The TAT gene is a classic glucocorticoid-regulated gene with a basal level of expression that can be enhanced by glucocorticoids (Figure 2A). The overall regulation of the TAT gene is influenced by numerous transcription factors that can bind to the promoter region of this gene. This is represented schematically in Figure 1, which also shows GRE locations (the middle GRE is absolutely necessary for glucocorticoid-induced activity) (*22*). Our initial studies investigated the role of As on the endogenous TAT gene. For this, we utilized a stable line (designated as 10.1.11.14) derived from EDR3 cells containing stably integrated WT mGR (~10 000 GRs/cell). This line was chosen in lieu of transiently transfecting GR into EDR3,

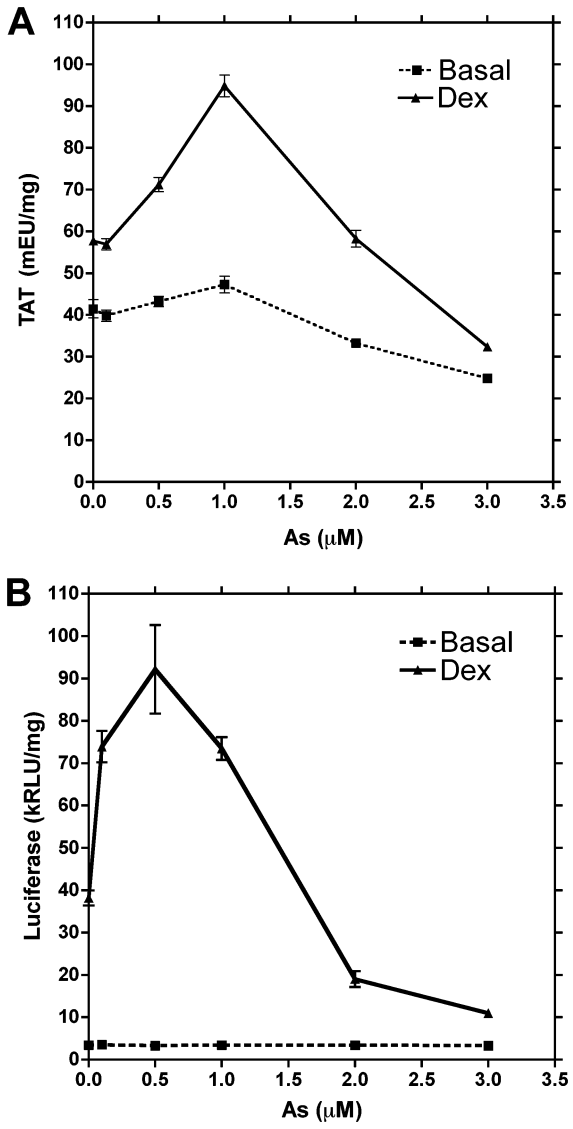

Figure 2. Effect of As on TAT and reporter gene activity. (A) 10.1.11.14 cells were treated with media or media containing 50 nM Dex for ~18 h in the presence or absence of 0.1, 0.5, 1.0, 2.0, or 3.0 μM As. Cytosols were assayed for TAT activity and protein as described in the Experimental Procedures. (B) EDR3 cells were transfected with DNA encoding for the mGR (pSV2 WT2X, 15 μg/10^7 cells) and a GRE containing luciferase reporter gene (G2T, 13 μg/10^7 cells). The next day, the cells were treated as in panel A and cytosols were assayed for luciferase and protein as described in the Experimental Procedures. All figures in these studies are representative of at least three experiments, and each point is the mean of replicates (Figures 2, 3, and 5 were done in triplicate; the remaining figures have six replicates per point). Variation is presented as ±SEM.

to ensure that all cells contained receptors and in order to prevent masking of GR effects by the basal TAT activity that would have occurred in nontransfected cells.

Figure 2A shows that the treatment of 10.1.11.14 cells with 50 nM Dex in the absence of As (zero concentration point on graph) stimulates TAT activity by about 50% over basal levels (this value can fluctuate depending on the individual experiment). When cells were treated with increasing concentrations of As, the magnitude of Dex-induced activity was increased, with the maximal effect occurring at 1 μM As at which Dex-induced activity was approximately 2-fold higher than basal activity. Conversely, at 3 μM As, there was a substantial reduction in Dex-induced activity, which was only slightly higher

than basal level.

These results replicated earlier studies showing a similar biphasic effect of As on Dex-induced PEPCK gene expression (*11*, *12*). The native TAT gene promoter with its multiple transcription factor binding sites and complex regulation is a difficult system in which to study specific effects of As on the GR-mediated response. To simplify our analysis of GR specific effects, we next employed a transient transfection system using EDR3 cells. This allowed us to transfect in WT or mutant GR to these GR negative cells and also assay the more sensitive luciferase reporter gene, regulated in these constructs solely by two tandem GREs (G2T) having the same sequence as the GREs found in the native TAT promoter (*22*).

Results from both the endogenous TAT gene and the transfected G2T reporter gene were very similar, indicating that the primary effect of As is on GR-mediated transcription and also validating the use of this transient transfection system as a model for the effects on native nuclear gene expression. The treatment of transfected cells with 50 nM Dex and increasing As concentrations (Figure 2B) showed that Dex stimulation of luciferase was markedly stimulated in the presence of 0.1−1 μM As, whereas at 2−3 μM As there was significant repression. In the absence of Dex, there was very little basal expression from the reporter gene, consistent with previous results indicating that GR normally supports both basal and hormone inducible expression of many GRE-regulated genes. Subsequent experiments showed that removal of the GREs from the G2T vector eliminated luciferase activity in response to either Dex or As (data not shown), similar to what had been observed in preceding studies. We had previously demonstrated using site-directed mutagenesis of the GREs or the accessory factor binding sites within the 0.6 kb promoter region of the rat PEPCK promoter that this construct would support basal expression of luciferase but was refractory to Dex and/or As treatments (*11*). Likewise, a basal pGL3 vector with an active minimal promoter but lacking the two GREs was shown to express basal luciferase activity but was also refractory to Dex, As, or RU486 (*12*). These results suggest that any observed luciferase activity with the G2T construct under control conditions (i.e., no Dex) in the current experiments was probably due to low levels of cortisol that were not completely extracted by the dextran−charcoal stripping of the serum (see Experimental Procedures). It should also be noted that the dual stimulatory/inhibitory response is also seen if the origin of the GR is endogenous (C6 cells, data not shown), stably integrated (10.1.11.14 cells), or transiently transfected (EDR3 cells). Previous studies examining the effects of As on Dex induction of endogenous GR-mediated PEPCK gene expression in chick embryo liver in vivo also demonstrated a biphasic response, with a significant enhancement of Dex induction in the first few hours immediately after As treatment, followed by an almost complete suppression of Dex inducible PEPCK expression several hours later (*11*). Thus, the effect of As on GR-mediated gene expression in the transient transfection system and on the endogenous TAT gene mirror one another, validating the use of the transient transfection system to study the effects of As on GR.

Transcriptional Repression by GR Is Not Affected by As. Because the effects of As on GR-mediated positive gene transcription were quite pronounced, we were interested in whether the well-known repressive effects of GR on gene transcription could also be affected by As. Two well-characterized systems are the GR-mediated inhibition of gene transcription by AP1 (Jun and Fos) and by NF-κB. EDR3 cells are missing some of the components for these pathways. Because both of these pathways are intact in COS-7 cells, these cells were used to examine this mechanism. COS-7 cells were transfected with GR along with either an AP1 reporter gene (pPLUC2; *17*, *23*) or an NF-αB reporter gene (NF-αB-INFb-LF; *24*). The phorbol ester, PMA, has been shown to activate AP1 transcription by stimulating the phospholipase C pathway, which leads to phosphorylation of Jun and Fos (*25*). Phosphorylated Jun and Fos interact with specific response elements and the basic transcription machinery to increase gene transcription. PLUC2 is a luciferase reporter gene controlled by the promoter region of the collagenase gene that shows a strong response to AP1 stimulation (*17*). Figure 3A shows the effect of three different concentrations of PMA on expression from PLUC2 (black bars) in COS-7 cells and also shows that Dex inhibited this expression to a similar extent (about 2-fold) at each PMA concentration. As alone had no significant effect on PMA stimulation from PLUC2 (data not shown). Data from experiments utilizing five different PMA levels (1×10^{-11} to 1×10^{-8} M) were pooled, and the inhibition by Dex was expressed as a function of As concentration. These results are presented in Figure 3B and show that As (0.1−3 uM) had no effect on the ability of hormone-activated GR to inhibit AP1 expression.

A number of different cytokines stimulate gene expression through NF-κB. NF-κB (a heterodimer of p50 and p65) exists in the cytoplasm as a complex with a family of inhibitors such as IαB. Upon hormone stimulation, the IαB is phosphorylated and subsequently degraded. NF-κB can then migrate into the nucleus, interact with specific response elements, and mediate gene expression. It is well-established that hormone-activated GR is able to interact with NF-κB and inhibit transcription (*26*). As shown in Figure 3C, a basal level of luciferase expression was observed from the NF-κB reporter gene, likely a result of being supported by serum factors in the culture medium. When Dex was added, this basal expression was decreased by 2-fold, indicating a GR-dependent suppression in NF-κB-mediated expression. Likewise, when NF-αB-mediated expression of the reporter gene was stimulated by TNFα, there was a large increase in luciferase expression, and the addition of Dex decreased this level of expression by approximately 2-fold. The addition of As (0.1−3 μM) had no effect on the ability of GR to inhibit NF-κB-mediated gene expression (Figure 3D). Therefore, while As has a marked effect on GR-mediated gene activation, it does not affect the inhibition of these other transcription factors by activated GR.

Functional Domains of GR Associated with As Response. Studies of mGR, rGR, and hGRs reveal three modular domains (*27*): the LBD comprising approximately 200 amino acids at the C terminus, a segment of ∼66 amino acids near the middle containing the DBD, and the remaining portion of the GR, which contains the N terminus (see Figure 4). The DBD contains two zinc fingers (*28*) with each zinc molecule coordinated by four cysteine residues. There are two additional free cysteines located within the DBD that are not involved in zinc coordination. The DBD bestows the specificity for the GR

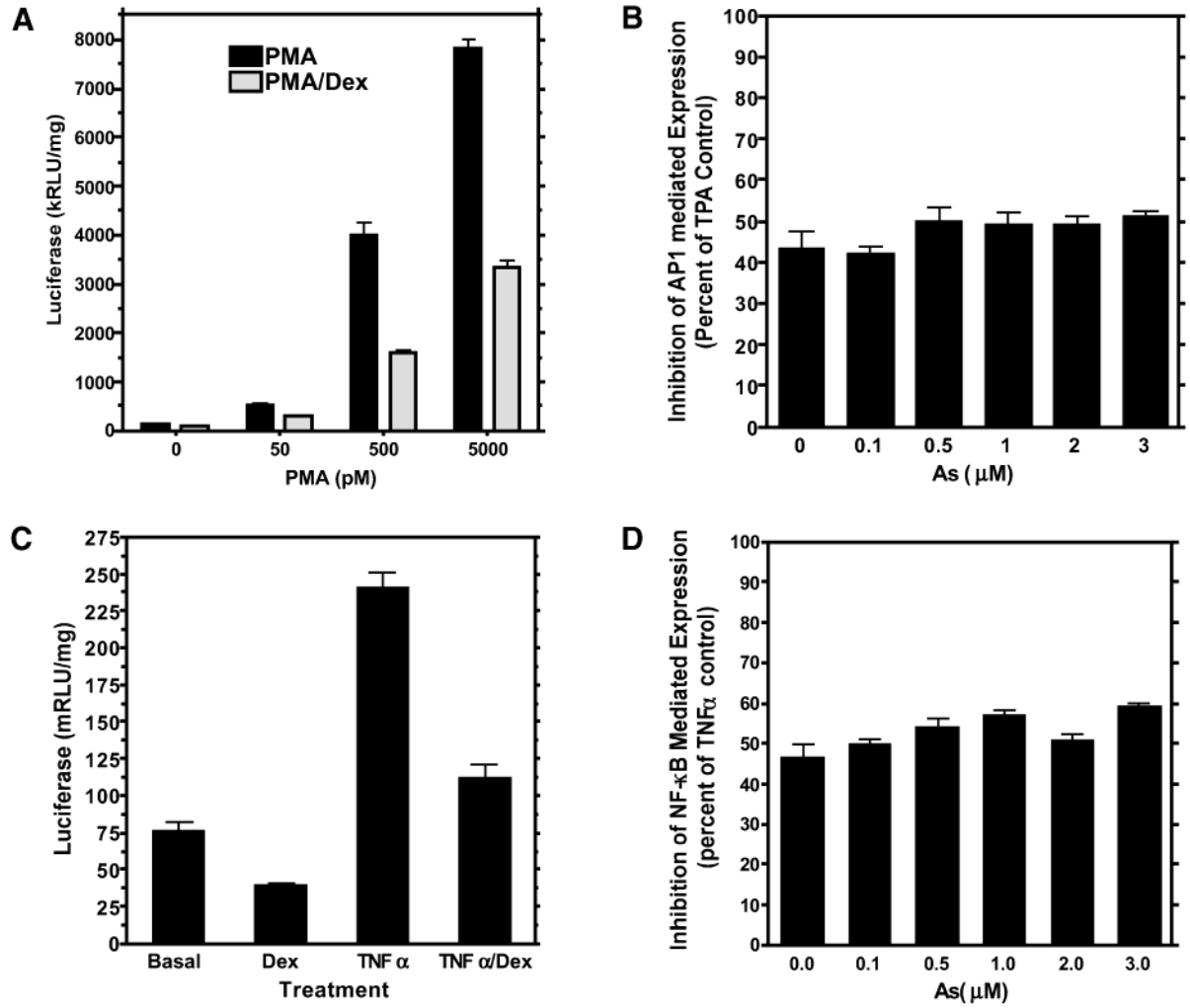

Figure 3. As does not affect repression by GR. (A) COS-7 cells were transfected with DNA encoding mGR (pSV2 WT2x, 2.5 μg/10^7 cells) and an AP1 responsive reporter gene (pLUC2, 15 μg/10^7 cells). The next day, the cells were treated for ~18 h with the indicated amount of PMA with and without 50 nM Dex. Cytosols were assayed for luciferase and protein as described in the Experimental Procedures. (B) Cells treated as in panel A were also treated with and without the indicated amount of As. Results from five different PMA treatments, ranging from 1×10^{-11} to 1×10^{-8} M, were pooled, and the inhibition by GR is plotted as a percentage of the PMA plus As control (mean ± SEM). (C) COS-7 cells were transfected like those in panel A but with substitution of a NF-κB responsive reporter gene (NF-κB-INFb-LF, 15 μg/10^7 cells) for the AP1 gene. The cells were treated with and without Dex (50 nM) and TNFα (3 pg/mL) and processed as described for panel A. (D) Cells treated with TNFα and TNFα plus Dex were also treated with the indicated concentration of As, and the inhibition by GR was plotted as a percentage of the TNFα plus As control.

to bind its DNA recognition element, the GRE (*29*−*31*). The DBD also provides GR with a dimerization interface (*32*) and nuclear localization signal (*33*).

Steroid binding to the LBD produces a conformational change in GR involving a major movement of helix 12 to help form a new surface (*34*). This surface is believed to have a transcriptional activation function by virtue of interaction with other molecules, notably coactivator proteins (*35*). This activation function, termed AF2 (*36*), is ligand-dependent and differs from another activation region near the N-terminal end of the LBD, termed τ 2 (*36*), which is ligand-independent. If the LBD is removed, the DBD becomes constitutively active (*37*, *38*).

The remaining portion of the GR contains the N-terminal domain, which is necessary for full activity of the GR (*36*, *39*, *40*). Regions within this domain appear to have transactivation activity (AF1) (*36*, *39*−*41*). The transcriptional activation by AF1 is ligand-independent.

Each of the three domains is essentially modular, retaining their functions when isolated or used in other contexts. The modular nature of GR allows one to use deletion and point mutations to investigate which domains and regions are important for the As response. To investigate which domains were essential for the As response of GR, we either deleted the LBD (LBD−), which eliminated the AF2 and τ 2 activation domains, we deleted the N terminus (NT−), which eliminated the AF1 activation domain, or we eliminated both the N terminus and the LBD retaining only the DBD. These constructs are shown schematically in Figure 4.

Transfecting these mutants into the GR negative EDR3 cells, we compared the responses of mutant and WT GRs to As. The removal of the LBD did not greatly alter the activity of GR, and the response to As was very similar to that of WT GR (Figure 5A). There was clear stimulation of GR-mediated expression at 0.1−1 μM As and inhibition at 2−3 μM. The LBD− is constitutively active so there is no difference in activity with or without Dex. This also demonstrates that Dex itself is not directly involved in the As response through some nonspecific effect on the EDR3 cells, since the As effect was evident in the constitutively active LBD− GR even in the complete absence of Dex (Figure 5A).

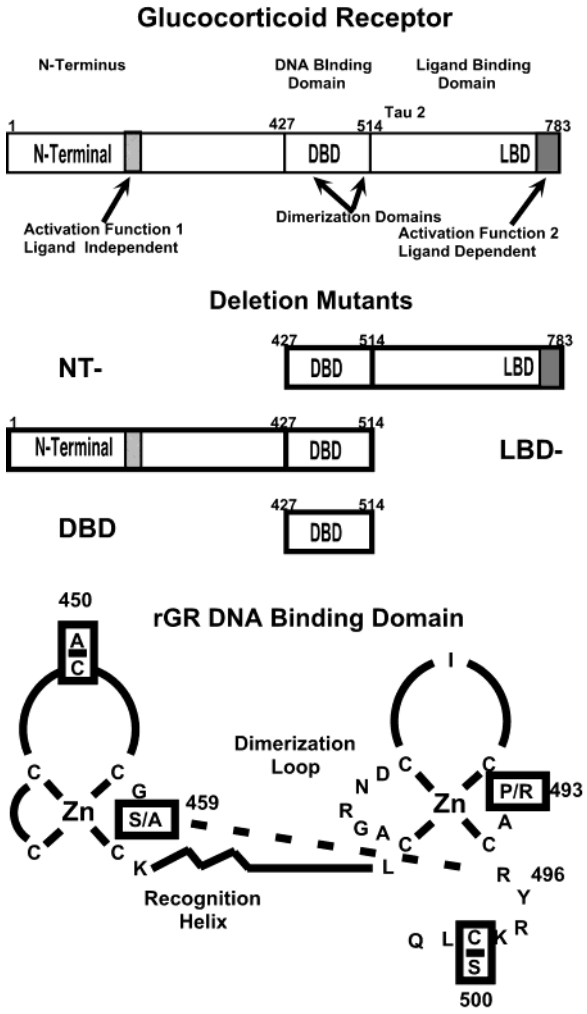

Figure 4. Structure of GR. The upper portion of the figure shows the three major domains of GR as well as regions important for dimerization and transcriptional activation. The middle portion of the figure shows the construction of the various deletion mutants used in this study. The bottom section is a schematic representation of the rGR DBD indicating the two zinc finger structures, the DNA recognition helix, and the dimerization loop. Amino acid positions referred to in the text are numbered according to the rat structure. Point mutations producing the DNA bound conformation are in horizontal boxes. Vertical boxes show mutations of the two free cysteines in the DBD. The H-bond between S459 and P493 is indicated by a dashed line.

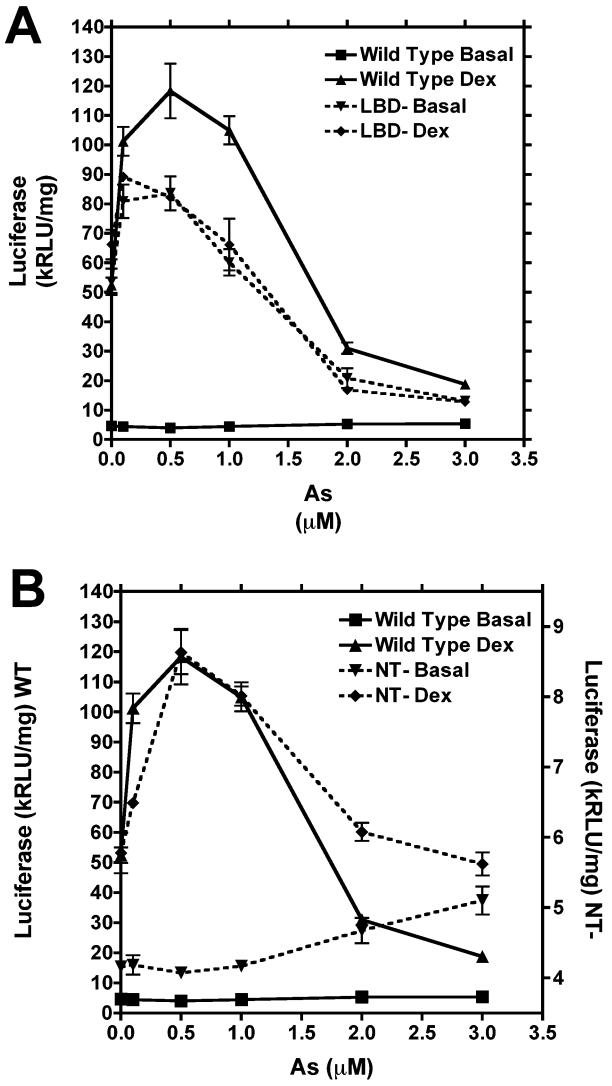

Figure 5. GR deletion mutants still response to As. EDR3 cells were transfected and treated as described in Figure 2B but also included treatments with DNA encoding for NT− (right-hand scale) and LBD− mutants.

Removal of the N terminus had a much greater effect on GR activity, since the NT− mutant had only about 10% of WT activity (Figure 5B, note right-hand scale). However, low concentrations of As (0.1−1 μM) clearly stimulated GR-mediated activity while at 3 μM the hormone-activated GR expression was suppressed almost to baseline values.

Because WT GR and mutant GRs missing either the N-terminal domain or the LBD had a similar response to As, these results imply that the effect of As is mediated principally through the central DBD. While we tested the DBD in our system, unfortunately, removing all of the activation domains essentially makes this construct inactive. However, as discussed in subsequent sections, an alternative approach of generating specific point mutations within the DBD provided further support for the idea that this central DBD domain is critically important to the As response.

Cellular Level of Activated GR Influences the Response to As. During the course of these studies, it became apparent that the amount of GR transfected into EDR3 cells had a profound effect on the shape of the As dose−response curve in two ways. First, the amount of GR changed the magnitude of the stimulatory response to As at low concentrations. Second, there appeared to be much more detail in the stimulatory portion of the As dose−response curve, necessitating a much finer titration of As concentrations in the 0.05−1 μM range.

To evaluate the effect of GR levels on the response to As, we determined the average number of GR molecules/cell resulting from transfecting EDR3 cells with different amounts of GR-encoding DNA. A typical dose−response curve is shown for hGR as an insert in Figure 6A, which demonstrates that within the range that we were using, the amount of DNA transfected was directly proportional to the average number of GRs expressed per cell. Similar linear responses were obtained for mGR and rGR (data not shown).

Figure 6A−C shows the results of altering the levels of hGR, rGR, or mGR, respectively, in combination with a finer and more detailed titration of As concentrations.

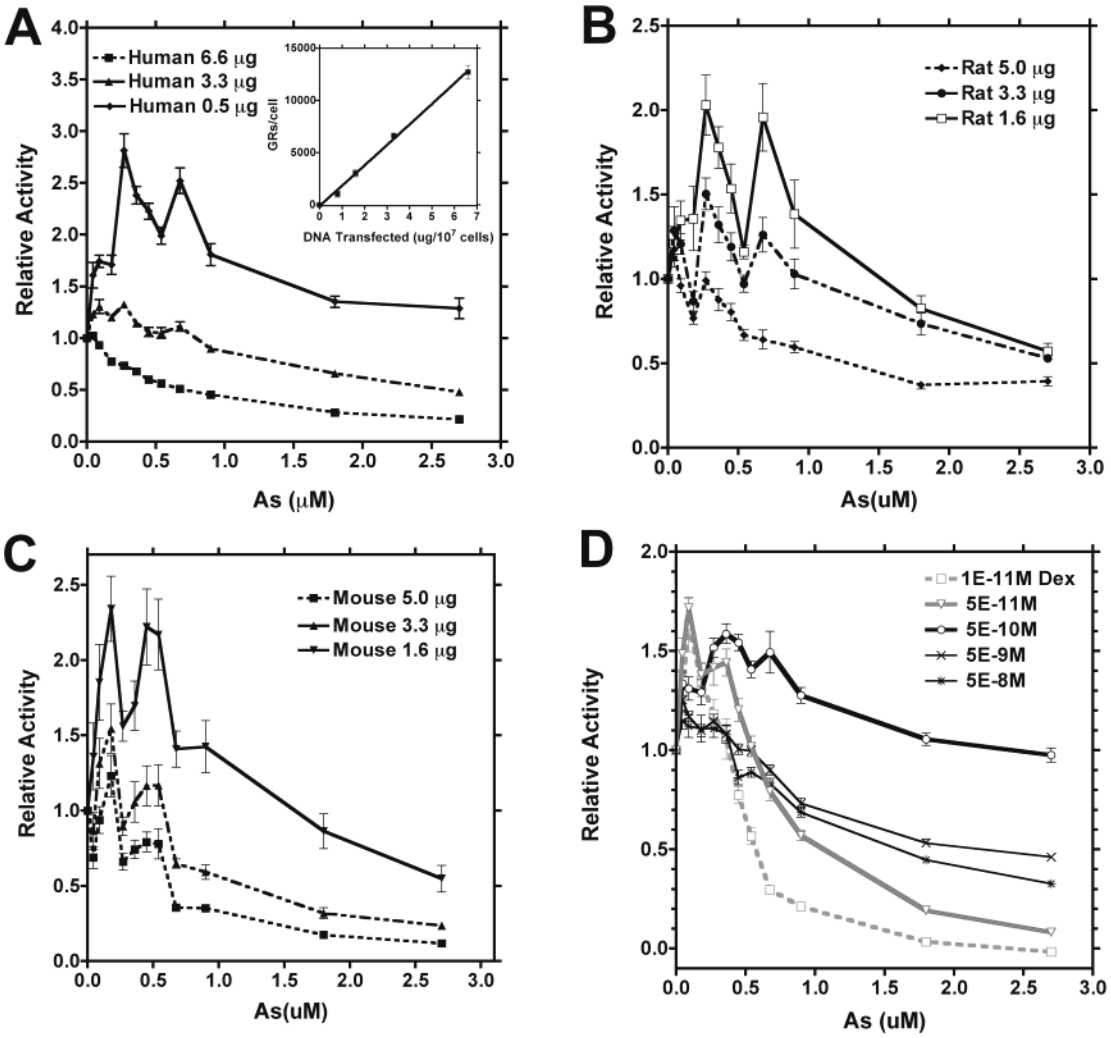

Figure 6. Changes in cellular GR levels alter the response to As. Different amounts of DNA encoding hGR (pCMV5 hGR; A), rGR (p6GR; B), or mGR (pCMV5 mGR; C) were transfected into EDR3 cells with the G2T reporter gene as described in Figure 2. The cells were treated with 50 nM Dex with and without As (see text for concentrations) for ~18 h and processed as described in Figure 2. For each GR level, individual treatments were divided by the value of the no As control. This normalization to relative activity allows comparison of As effects on treatments that have different GR-mediated transcriptional activities. Control values (kRLU/mg) for (most to least transfected DNA) (A) 426.6, 249.3, and 57.8; (B) 66.6, 45.7, and 29.3; and (C) 339.8, 260.6, and 65.3. The insert in panel A shows the linear response of hGRs/cell relative to the amount of DNA transfected. Similar curves were obtained for rGR and mGR (data not shown). (D) Changes in activated GR levels were made by altering hormone levels. DNA encoding hGR (6.6 μg/10^7-cells) and the G2T reporter gene were transfected into EDR3 and processed as described in Figure 2. The cells were treated with the indicated amount of Dex for ~18 h and normalized as described above. No As control values (from highest to lowest Dex concentrations) were 238.1, 229.8, 169.5, 46.9, and 9.9 kRLU/mg.

These data are expressed as relative luciferase activity, in which the expression levels for a given receptor level were normalized to the zero-As control value for each experiment. This normalization aids in the comparison of GR treatments with inherently different activities in their response to As. Increasing the number of different concentrations of As tested (0.045, 0.09, 0.18, 0.27, 0.36, 0.45, 0.54, 0.675, 0.9, 1.8, and 2.7 μM) revealed that there were multiple peaks of stimulation within this range. We observed major peaks of stimulation at ~0.3 and ~0.7 μM As with a minor peak around 0.1 μM for the rGR and hGR (Figure 6A,B), while peaks for the mGR occurred at slightly lower As concentrations (Figure 6C). mGR, rGR, and hGR all showed the same response to increasing the level of GR within the cell, i.e., a marked decrease in the magnitude of the stimulatory peaks. The higher levels of GR typically resulted in diminution of the stimulatory phase to the point where mainly inhibition at the higher doses was the only remaining As effect (for example, compare hGR at 6.5 vs 0.5 μg in Figure 6A). In the course of this transition, typically, the stimulatory peaks were eliminated or greatly reduced, and this occurred progressively from the 0.7 μM peak to those occurring at lower As concentrations (for example, compare the rGR peaks from 3 to 0.5 μg in Figure 6B).

In these experiments, we were changing the levels of activated GR by altering the amount of GR expressed in the cell and then stimulating essentially all of the receptors using a saturating concentration of Dex (50 nM). If the amount of activated GR is governing the response to As, then one should also be able to control the levels of activated GR by altering the levels of hormone and to see similar effects of As. To test this, we transfected EDR3 cells with enough hGR-encoding DNA to result in ~12 000 hGRs/cell and then treated the cells with different concentrations of Dex from 10 pM to 50 nM (1×10^{-11} to 5×10^{-8} M). These results are shown in Figure 6D. At a Dex treatment of 5×10^{-8} and $5 \times$

10^{-9} M, in which virtually all of the GRs should be ligand-activated, there was very little stimulation by lower concentrations of As but there was inhibition at the higher As concentrations. However, at 5×10^{-10} M Dex, where there should be fewer activated GRs, there were multiple peaks of As stimulation at the lower concentrations of As, similar to the previous DNA titration experiments. Decreasing the concentration of Dex further resulted in the sequential decrease in the 0.7 μM peak and then the 0.3 μM peak and a concomitant increase in the 0.1 μM As peak until at 1×10^{-11} M Dex this was the only stimulatory peak remaining.

Although these experiments manipulated the levels of activated GR by different mechanisms, they showed similar trends. At high levels of activated GR, the stimulatory phase of the As response was minimal, and as the levels of activated GR were decreased, multiple stimulatory peaks appeared. Interestingly, at levels of GR where the number and magnitude of the stimulatory peaks were maximal, GR was more resistant to the inhibitory effects of the higher As concentrations. This was most pronounced in the hGR (although the trend exists for both mGR and rGR), whereas at the 2.7 μM As point in both the DNA titration experiment (Figure 6A, 0.5 μg of hGR DNA) and the Dex titration experiment (Figure 6D, 5×10^{-10} M Dex) there was still stimulation of Dex-activated GR transcription by As. The Dex titration experiments allowed us to more precisely manipulate the lower levels of activated GR. As the concentration of Dex was progressively lowered, stimulation by As appeared to be shifted to lower As concentrations (peak at 0.1 μM As in Figure 6D), and GR also became much more sensitive to the inhibitory effects of higher As concentrations. At 1×10^{-11} M Dex, there was greater inhibition at 0.67 μM As than at the 2.77 μM As point with the 5×10^{-8} M Dex treatment. It appears as if the entire As dose−response curve was left-shifted at these lower Dex concentrations. These results suggest that the qualitative and quantitative responses of GR-mediated gene regulation to As may be quite different in different tissues depending on their levels of GR expression and their hormone status (see Discussion).

Free Cysteines in the GR DBD Are Not Necessary for As Effect, but Their Mutations Alter the Response. Our experiments with the GR deletion mutants described above strongly implicated the central DBD of GR for being important in the mediation of the As response. As^{+3} is well-known to react with free sulfhydryl groups, particularly vicinal dithiols. The DBD contains two clusters of four cysteines that form the two zinc fingers. However, these have been shown to have very low affinity for As as compared to Zn^{2+} or Cd^{2+}, which cannot displace the normally bound zinc except at extremely high concentrations (*42*). However, the other two DBD cysteines external to the zinc fingers represent two free sulfhydryl groups that are potentially accessible and could provide one or more binding sites for As. To examine this, we mutated these two cysteines, using specific mutations in the rGR because of available information from structure−activity studies (described below).

The two free cysteines in the rat DBD are at residues 450 (C450) and 500 (C500). Previous X-ray crystallography studies indicated that these two cysteines are in quite different chemical environments (*43*−*45*). C450 appears to be near the surface and readily accessible to solvent whereas C500 is buried well within the hydrophobic core of the molecule. The results from mutating these residues were consistent with predictions based on these two locations. Mutation of C450 to alanine (450A) in both the mouse and the rat had minimal effects on either normal activity or their response to As (data not shown). However, in contrast to previous reports (*46*), mutation of C500 was more problematic, with many substitutions showing no or minimal activity. Mutation of C500 to serine resulted in good activity, and we subsequently used this mutant as the basis for a double mutant where C450 was altered to alanine and C500 to serine (450A-500S).

To prevent differences in the numbers of GRs/cell from confounding direct comparisons between WT and mutant GRs, we verified cellular GR content in all experiments. Although the number of GRs obtained per cell was directly proportional to the amount of DNA transfected (Figure 5A insert) for given individual DNA preparation, the slope of this response was different for each preparation. Consequently, we transfected cells with a range of concentrations of each GR and compared only those treatments containing an equivalent number of WT and mutant GRs. A saturating amount of Dex (50 nM) was used to treat the cells, ensuring that the majority of GRs transfected would become activated.

Figure 7 shows two representative experiments comparing the 500S and 450A-500S mutants to WT GR at different cellular GR levels. First, these results demonstrate that neither of the two free cysteines are necessary for the As response since the 450A-500S double mutant showed a pronounced response to As. Second, they suggest that structural alterations within the DBD qualitatively alter the As response, since that the double mutant had both a greater degree of stimulation at low As levels and inhibition at high As levels than did WT GR. The 450A-500S mutant was strongly inhibited for Dex activation by 1.6 μM As, with a greater degree of inhibition than was observed with WT GR at 2.7 μM As. This combination of increased stimulation and increased inhibition was counter to what we had observed for WT GR. As shown in Figure 6A−C, lowering the GR levels results in an increase in the magnitude of the stimulatory peaks at low levels of As that was invariably linked to a lesser degree of inhibition at the higher As levels. It appeared that both mutations were necessary for this phenotype. The 450A mutant was essentially indistinguishable from the WT GR (data not shown). The 500S mutant consistently showed greater stimulation at low As levels than WT GR. However, 500S was only slightly more sensitive than WT to the inhibitory effects of higher As concentrations. Thus, it was only with the double mutant that we observed the large increase over WT in the inhibition that occurred at the higher levels of As, suggesting a cooperativity of these two mutations and further indicating that this central DBD is important for the GR response to As.

DNA Conformational Mutants Have Altered Responses to As. When GR binds DNA, it induces a conformation change in the dimerization interface of the DBD (region between cysteine 476 and cysteine 482, also known as the dimerization loop or D-box; see Figure 4, lower section) (*47*). This DNA-bound conformation generates a new transcriptional activation surface that is thought to bind factors necessary for proper transcription (*43* and references therein). Two different point muta-

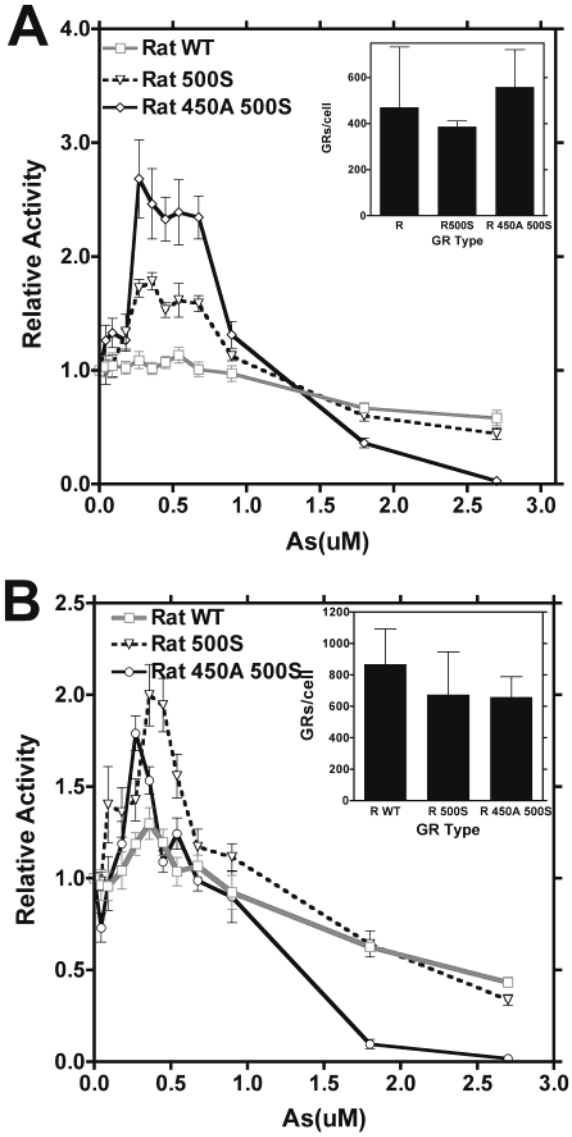

Figure 7. Free cysteines in DBD are not necessary for response to As, but the response is altered by their mutations. EDR3 cells were transfected with different levels of DNA encoding WT or mutant (500S or 450A-500S) rGR along with the G2T reporter gene. The cells were treated and processed, and the results were normalized as described in Figure 6. The cellular GR content was determined for each transfection (see Experimental Procedures) to ensure that WT and mutant GR levels matched. Two representative experiments are presented with the insert displaying the average GRs/cell for each GR type. No As control values for panel A; (B) WT, 54.8 (26.4); 500S, 21.0 (14.3); and 450A-500S, 7.7 (7.3) kRLU/mg.

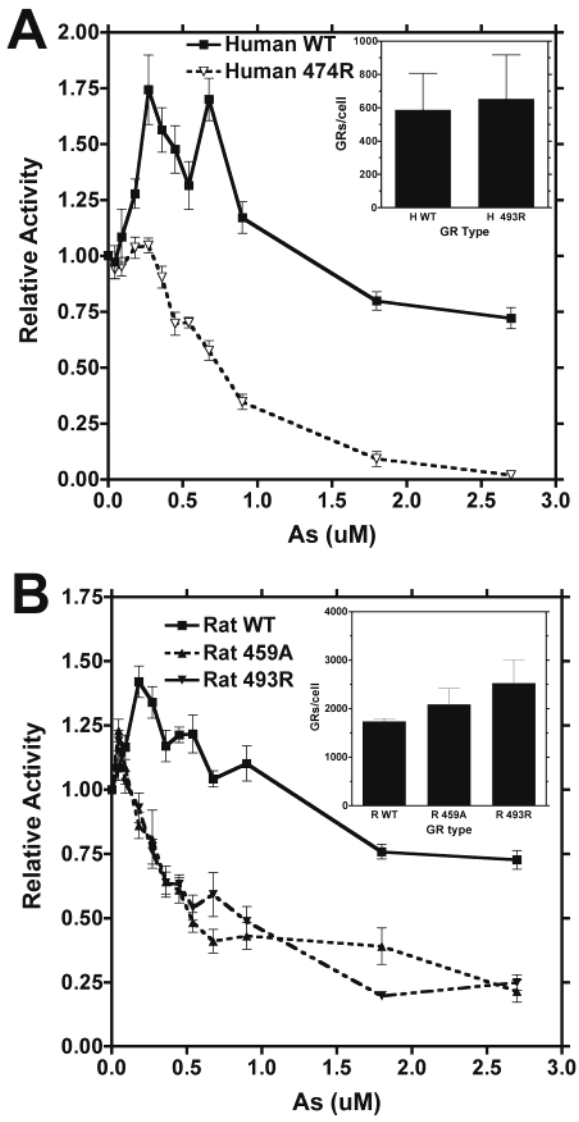

Figure 8. DNA bound conformational mutants alter the response to As. The experimental setup was the same as in Figure 7 except that the DNA bound conformation mutants 459A or 493R were compared to WT rGR. A homologous mutation (474R) in hGR was also compared to WT hGR. No As control values for (A) WT, 26.6; 474R, 7.3 kRLU/mg. (B) WT, 69.8; 459A, 3.8; and 493R, 10.4 kRLU/mg.

tions (Figure 4, lower section), serine 459 to alanine (459A) and proline 493 to arginine (493R), cause GR to spontaneously assume this conformation even in the absence of DNA binding. When these mutants are expressed at low levels, they activate gene transcription, but at higher levels, they suppress transcription, presumably by binding and sequestering one or more required factors in a dominant negative manner.

Representative experiments for each of these two rat mutants and one for the corresponding human equivalent of 493R (human 474R) are presented in Figure 8. In each of these experiments, we measured receptor number and only compared treatments containing equivalent numbers of expressed GR. These mutants had a similar response to As, which was substantially different from WT GR. In contrast to WT GR, the concentrations at which As stimulated Dex inducible expression from both 459A and 493R occurred over a much narrower range and at a substantially lower level (~0.05 μM) without evidence of multiple peaks of stimulation at higher As concentrations. Above 0.1 μM As, there was substantially greater inhibition than seen with WT rGR. The 474R mutant of the hGR showed a qualitatively similar profile to the rat 493R, but there was no discernible shift of stimulation to lower As levels and inhibition started at 0.3 μM. This was slightly higher than the rat mutant but still at much lower values than seen with WT hGR. Because there are no published structural studies of this hGR mutant, it could differ because this mutation does not produce the same conformational changes as in rGR. Nonetheless, these mutants demonstrate interesting qualitative differences with WT GR, further supporting a central role for the DBD in the response of GR to As.

Discussion

The mechanism(s) by which As is able to affect its various pathophysiological processes remains unclear. We and others have shown in previous work that low dose As treatment of cultured cells and intact animals has profound but highly selective effects on gene expression (*11*, *12*). In particular, As appears to target highly inducible genes whose regulation is controlled by environmental, hormonal, or other exogenous signals. For example, in one series of studies, the treatment of cells or animals with a single nonovertly toxic dose of As was shown to have little or no effect on expression of most genes, including all of the constitutively expressed genes that we examined (*11*). Conversely, As had profound effects on both the basal and the inducible expression of several inducible genes, including several different cytochrome P450s, stress- and heat shock-associated genes, and genes associated with specific metabolic pathways including heme biosynthesis and glucose homeostasis (*11*, *12*). These affected genes are all highly inducible, and many are regulated by glucocorticoids and other steroid hormones. Interestingly, gene array experiments have shown that the pattern of gene alterations at low (nontoxic) doses is almost completely nonoverlapping with the pattern seen in the same cells at a higher dose producing overt cytotoxicity (*48*). At the low dose (5 μM, 4 h), the number of genes that were increased or decreased was approximately equal, whereas at the higher dose (50 μM, 4 h) virtually all of the altered genes were increased in expression. Most striking was the observation that there were only a very small number of genes common to the two doses, suggesting a fundamental difference in biological response to low and high doses of As. This may have important implications for understanding the various health effects seen in people exposed to As chronically in drinking water. These observations reinforce the idea that if one is to understand the role of As in chronic human diseases, it is vitally important to investigate cellular effects of As at doses below those causing overt cytotoxicity, e.g., as measured by a decrease in survival in a colony-forming assay. In most culture systems, this is equivalent to concentrations at or below 3−5 μM arsenite. We have observed significant effects on gene expression as low as 50 nM As in cell culture, which is 100-fold below the highest noncytotoxic dose, suggesting that such changes are likely to be relevant to human exposures of concern.

In investigating the selective effects of As on gene expression, we previously discovered that As is a potent endocrine disruptor, altering glucocorticoid hormone signaling and gene regulation at very low, environmentally relevant doses (*11*, *12*). These studies suggested that the GR itself was the principal mediator for these effects. GR is a ligand-dependent transcription factor and a member of the steroid receptor superfamily (*49*, *50*). In the absence of hormone, GR is found in a multimeric complex with other proteins and is located predominantly in the cytoplasm of the cell (*49*). The composition of this complex and the regulatory cycle needed to prepare GR to bind steroid are both complex (*51*). Under physiological conditions, hormone binding to GR causes dissociation of the complex (*52*, *53*). GR then becomes activated and translocates to the nucleus where it binds to DNA. Activated GRs associate as homodimers with specific gene regulatory sequences known as GREs (*30*, *54*), and in association with various coactivators and basic transcription factors, GR can modulate expression of hormone responsive genes such as phosphoenolpyruvate carboxykinase (PEPCK) and TAT.

PEPCK is the rate-limiting step in gluconeogenesis (*55*, *56*) and is primarily regulated by changes in transcription. We showed that treatment of rat H4IIE hepatoma cells in culture with As(III) (0.3−3.3 μM) significantly suppressed the glucocorticoid inducible expression of the endogenous rat PEPCK gene as well as transfected luciferase reporter genes regulated by either the GRE-containing glucocorticoid response region from the PEPCK promoter or the two tandem GREs from the TAT gene (*11*). As treatment of H4IIE cells did not change cellular levels of GR nor did it appear to alter hormone binding to GR or nuclear translocation of hormone-activated GR since both Western blotting following cell fractionation and confocal microscopy of green fluorescent protein-tagged GR in intact cells (*12*) showed no effect of As on the Dex-induced cytosolic to nuclear translocation of GR associated with hormone activation. Thus, As appeared to be exerting its effects after these well-defined steps in the mechanism of glucocorticoid action, suggesting that it was altering the function of GR as a nuclear transcription factor.

The goal of the current study was to determine the structural basis for effects of As on GR transcriptional regulation. Our results demonstrate the importance of the central DBD in the response of GR to the effects of low, environmentally relevant concentrations of As on GR-mediated gene transcription. These results further indicate that the cellular level of activated GR has a substantial qualitative and quantitative effect on the relative stimulatory and inhibitory alterations of GR function by As that are observed. Our previous studies, using a GR reporter gene similar to G2T in H4IIE cells, which express GR, showed only inhibitory effects of As over the range of 0.3−3.3 μM As. When those experiments were performed, we were not aware of the important influence of cellular GR and hormone levels on the response. It seems likely, based on the current results, that a combination of the high number of GRs per H4IIE cell (∼10 000−15 000) and the high level of hormone treatment (50 nM Dex, approximately 10-fold over the Km for the rGR) produced only inhibition at 0.3 μM As. Alternatively, it is possible that H4IIE cells are fundamentally different than EDR3 cells in their response to As; however, we think that this is unlikely, since we have observed the dual stimulatory/inhibitory response to different As concentrations in cells as diverse as rat C6 glioma and COS-7 monkey kidney cells (data not shown) and with different reporter genes as well as the endogenous TAT gene.

Simons and co-workers, working with cytosolic semipurified GR preparations in vitro, demonstrated that hormone and DNA binding of GR could be decreased by the addition of arsenite (*40*−*44*). The inhibitory effect of As on steroid binding was half-maximal at 7 μM in their in vitro preparation and appeared to result from As interactions with one or more vicinal dithiols on GR located in the LBD. We previously observed no apparent effect of As on hormone activation or nuclear translocation of GR in H4IIE cells treated with As in the culture medium at physiologically relevant concentrations, i.e., at or below 5 μM (extracellular) As (*12*). However, it is unlikely one could achieve an intracellular concentration

of 7 μM As at noncytotoxic concentrations. Similarly, Sanchez and co-workers had previously shown that treatment of cells with 100−200 μM As led to a hormone-independent activation of GR, its subsequent translocation to the nucleus, and increases in GR-regulated gene expression (*57*, *58*). However, As is known to be heat shock mimetic at very high, cytotoxic doses, and in fact, GR can be activated in a similar hormone-independent manner by heat shock itself (*57*, *58*). However, this does not occur at noncytotoxic doses of As, and we did not observe any effect of As alone on activation or nuclear translocation of GR in our previous experiments (*12*), indicating that these are strictly high dose effects associated with a stress response and/or apoptosis. Moreover, our current results demonstrating that complete removal of the LBD from GR has minimal effects on its response to As further suggest that As binding to or interfering with the LBD is not critical to the effects on GR transcription that we have observed.

The effect of As on DNA binding by GR was also evaluated by the Simons group and was reported to be much less sensitive to As than the effect on steroid binding (*4*). The addition of 100 μM As produced about a 10% decrease in DNA binding. However, this dose is 10-fold higher than a completely cytotoxic dose in our cells and suggests that the doses that we used, which are 2−4 orders of magnitude lower, are likely to have minimal effects on the GR−DNA interaction.

An important finding of this study was that although GR-mediated gene activation was highly sensitive to As, GR-mediated inhibition of AP1- and NF-κB-mediated transcription was refractory to As. Because this well-known inhibitory effect of GR on AP1 and NF-κB is thought to occur via a DNA-independent interaction with GR monomer, this raises the intriguing possibility that As might be affecting either GR dimerization or the interaction of DNA-bound GR with other effector molecules. It is interesting that the 459A and 493R mutants, which alter the conformation of the GR dimerization interface, have a very different response to As than WT GR. These mutants demonstrated enhanced inhibition by As, typically resulting in complete suppression of hormone induction down to basal levels, whereas the WT GR was only suppressed by approximately 25%. Although these two mutants apparently alter GR conformation through different mechanisms (*43*, *44*), they both cause reorganization of the hydrophobic core such that the D loop presents a DNA-bound conformation even in the absence of DNA binding. In WT GR in the unbound conformation, R496 hydrogen bonds to S459. Upon binding to a DNA GRE, the side chain of R496 is reoriented, breaking the H-bond, and the dimerization loop adopts the DNA-bound conformation. The R496-to-S459 H-bond is important for maintaining the unbound conformation. When S459 is mutated to alanine, this bond is broken and allows the DBD to assume the DNA-bound conformation. Normally, the aliphatic side chain of R496 contacts the pyrrolidine ring of P493. Likewise, the R493 mutation alters this interaction allowing the R496-S459 H-bond to be broken and the DBD to assume the DNA-bound conformation.

Stabilization of the DBD hydrophobic core has also been attributed to C500, and it is believed that this important role is the reason for its conservation in all known nuclear hormone receptors (*45*). We made a number of mutations to C500, including conversion to alanine and serine. Structural studies have been done with the DBD in which C500 has been mutated to alanine (C500A, *45*). This mutation produced changes in the NMR spectrum of the GR DBD indicating shifts in the position of five amino acid residues—one of which was R496. In the context of the intact GR molecule, C500A produced very little transcriptional activity, but we did find that the serine mutant (C500S) was quite active. Unfortunately, there are no NMR or X-ray structural studies on the C500S mutant, but the strong activity of this mutant suggests that conformational changes are probably more subtle than with C500A. It is possible that combining C500S with C450A to make the double mutant may have altered the position of R493 resulting in the much greater inhibitory response of the double mutant than the WT GR to the higher As levels.

The C450A-C500S mutant is interesting because it displays the enhanced inhibitory response to As at the higher doses as seen by the DNA-bound conformational mutants, yet also shows an enhanced stimulatory phase to low levels of As. This region of the dose−response is quite striking, revealing multiple peaks of activity, which suggest that the interactions of As in this dose−response range are very complex. One obvious possibility is that this involves titration of As into multiple sites of interaction on the GR and/or its partners that have different affinities. It has been postulated that when the DBD changes into the DNA bound conformation, transcription factor(s) interact with this newly formed surface and facilitate transcription. We are actively exploring the possibly that As is mediating some of its effects through alterations in these protein−protein interactions.

These studies further support the previous observation that As is a potent endocrine disruptor, altering hormone-mediated gene transcription at concentrations as low as 50 nM arsenite. Moreover, the lowest concentrations of As substantially increased hormone-mediated gene activation by GR, whereas higher but still noncytotoxic concentrations of As strongly suppressed hormone regulation. This suggests that one might see qualitatively different cellular effects of As on hormone regulation at different doses, which in turn implies that low doses of As might produce different patterns of disease in exposed populations than do higher doses. Our previous results measuring the patterns of changes in gene expression in DNA microarray experiments using different doses of As also support the hypothesis that there may be fundamentally different biological responses to low vs high As, which may be significant in assessing its health effects. One other intriguing possibility suggested by this study is that the qualitative differences in As effects on gene expression that we observed in cell culture with different levels of activated GR are indicative of physiological conditions in the body. In particular, different organs and tissues within the body, or the same organs or tissues under different developmental or physiological conditions, are known to have different cellular levels of GR, and effective concentrations of glucocorticoid hormone may also differ. Thus, these tissues may have quantitatively and qualitatively different responses to As depending on their cellular levels of GR and/or their effective concentrations of hormone. As is considered the number one environmental agent of concern in the United States and worldwide, with perhaps 20 million people being exposed to excess drinking water As in the United States alone, so accurately assessing its mechanism of action is of

paramount importance to understanding and predicting its overall health impact.

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