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# Modulation of Glucocorticoid-Inducible Gene Expression by Metal Ions

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## SUMMARY

We examined the effects of various metal ions on the DNA-binding activity of the glucocorticoid receptor. Electrophoretic mobility shift assays demonstrated that the sequence-specific DNA binding activity of the receptor was decreased by metal ions in a dose-dependent fashion. The most potent inhibitor was Au(I). Cu(II), Cd(II), and Zn(II) were, in that order, less potent as inhibitors, whereas Fe(III), Al(III), and Mg(II) had no apparent effect. The inhibitory actions of metal ions were efficiently counteracted by the sulfhydryl reducing reagents 2-mercaptoethanol and *N*-acetyl-L-cysteine, indicating that metal ions interfere with the DNA binding activity of the glucocorticoid receptor through modification of sulfhydryl groups in the receptor molecule. Modification of sulfhydryls by metals seems to involve neither disulfide bond formation nor permanent destruction of the GR protein and is reversible. We also show that metal ions inhibit glucocorticoid-inducible gene transcription *in vivo*, presumably by interfering with the interaction between the glucocorticoid receptor and cognate DNA target sequences. In summary, these data demonstrate that metal ions are capable of modulating glucocorticoid receptor mediated intracellular signalling pathways.

Inorganic substances are primary components of several intracellular communication networks. Among them, transition metals contribute significantly to complex regulatory circuits within cells (1). For instance, a plethora of metals have been implicated in the regulation of expression of genes involved in respiration, metabolism, metal-specific homeostasis, and stress response systems (2). The notion that metals are likely to be involved in the regulation of expression of broad classes of genes has attracted much attention and provides new insights into biological, physiological, and pharmacological properties of metals. In fact, besides the traditional therapeutic uses of metal compounds, metal chemistry has a continuing role in the discovery of new drugs (3). For example, the lanthanide ions, which are suggested to have the ability to catalyze the hydrolysis of mRNA (3), may play a key role in developing novel antisense drugs when conjugated to oligonucleotides.

Glucocorticoids have been widely used in the treatment of conditions that involve inflammation and other immunological disorders (4). However, glucocorticoid therapy is frequently limited by severe side effects, which often leads to discontinuance of the therapy. It has not yet been possible to separate major therapeutic effects from undesirable side effects. Because glucocorticoids exert most of their effects through specific, ubiquitous intracellular receptors, differential regulation of GR function could contribute to reduce the incidence and severity of glucocorticoid-related side effects. The human GR is a member of the nuclear hormone receptor superfamily. After ligand binding, the members of this protein family are capable of regulating target genes after interacting with specific DNA sequences and thus function as ligand-inducible transcription factors (5–8). After cloning and functional analysis of the receptors, GR and the other members of the family have been shown to have a modular organization with at least three major functional domains: a carboxyl-terminal hormone binding domain, an amino-terminal domain involved in transactivation, and a central DBD. The DBD of the GR, which is sufficient for selective interaction with GREs, contains nine cysteine residues, eight of which are suggested to form two so-called zinc finger motifs with each finger coordinating one zinc atom. The zinc atoms in the DBD of the receptors have been shown to play a critical

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**ABBREVIATIONS:** GR, glucocorticoid receptor; GRE, glucocorticoid response element; CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary; DBD, DNA binding domain; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; MMTV, mouse mammary tumor virus; NAC, *N*-acetyl-L-cysteine; PMSF, phenylmethylsulfonyl fluoride; ppGR, partially purified glucocorticoid receptor; SDS, sodium dodecyl sulfate; 2ME, 2-mercaptoethanol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

role in specific DNA binding (9, 10) and correct folding of the DBD (11). Some metals were previously reported to interact with the GR (9, 12–14). We have described how gold can negatively modulate the DNA binding, transactivation, and transrepression function of the GR, and the target for the suppressive effects of gold is postulated to be cysteine residues within the GR protein (15, 16). Metal ions may thus be capable of posttranslational modification of GR function and may be possible regulators of glucocorticoid action.

To extend our understanding of the mechanism of metal action on GR-mediated signal transduction, we investigated the effects of various metal ions on GR function in conjunction with sulfhydryl modifying reagents. In this study, we demonstrated that some metal ions, including Au(I), Cu(II), and Cd(II), can negatively regulate the sequence-specific DNA binding activity of GR and that the negative effects of those metal ions were reversed by either 2ME or NAC. Moreover, glucocorticoid-inducible gene activation was inhibited by these metal ions, presumably through interference with productive GR-DNA interaction.

## Materials and Methods

**Cell culture.** The GR-expressing Chinese hamster ovary cell line CHO-pMTGR was originally developed and kindly provided by Dr. Stefan Nilsson, Karo Bio, Huddinge, Sweden (17). In brief, CHO-K1 cells were stably transfected with the full-length GR expression vector under the control of the metallothionein gene promoter. These cells express full-length GR in amounts of 300,000–500,000 molecules per cell (17). The cells were maintained in Ham's F-12 medium supplemented with antibiotics and 10% FCS in the presence of cadmium and zinc ions to a concentration of 40 $\mu$M. One day before each experiment, the medium was replaced with metal-free serum-containing Ham's F-12 medium (GIBCO Laboratories, Grand Island, NY). COS-1 cells were supplied by Japan Cancer Resource Bank and were maintained in Dulbecco's modified Eagle medium, supplemented with 10% heat-inactivated FCS and antibiotics. In all experiments, serum steroids were stripped with dextran-coated charcoal, and cells were cultured in a humidified atmosphere at 37° with 5% $CO_2$.

**Metal reagents.** We used these reagents as sources of metal ions: Au(I), sodium aurothiomalate (Aldrich, Milwaukee, WI); Cu(II), $CuCl_2$; Cd(II), $CdCl_2$; Zn(II), $ZnSO_4$; Fe(III), $FeCl_3$; Al(III), $Al_2(SO_4)_3$; Mg(II), $MgCl_2$ (Wako Pure Chemical, Tokyo, Japan). Note that neither sodium thiomalate nor malate influences GR DNA binding and GR-mediated gene expression (15, 16).

**Plasmids.** The reporter constructs MTV-CAT and ΔMTV-CAT (18), and the various expression plasmids coding human GR derivatives have been described elsewhere (18–20) and were kindly provided by Dr. R. M. Evans (Salk Institute, La Jolla, CA). GREtkCAT (21), which was originally named PRE-PBL7 and contains two repeats of a 23-base pair GRE/progesterone response element of the rat TAT gene, was obtained from Dr. B. O'Malley (Baylor College of Medicine, Houston, TX) through Dr. H. Oshima (National Institutes of Health, Bethesda, MD). mGREtkCAT, in which wild-type GREs in GREtkCAT were exchanged with mutant GREs, was also obtained from Dr. H. Oshima (22). The pCAT-Control vector was obtained from Promega (Madison, WI). The β-galactosidase expression plasmid pCH110 (Pharmacia LKB Biotechnology, Uppsala, Sweden) was used as an internal control for transfection efficiency.

**Preparation of whole cell extract and partial purification of GR.** CHO-pMTGR cells were homogenized in buffer containing 10 mM Tris·HCl, pH 7.8, 1 mM EDTA, 10% glycerol, and 0.4 M NaCl. The homogenates were centrifuged at 100,000 × $g$ for 1 hr at 4°, and the resulting supernatants were used as crude whole-cell extracts (23). ppGR was prepared from CHO-pMTGR whole-cell extract essentially as described by Cairns *et al.* (24). Briefly, whole-cell extract was prepared in the presence of molybdate and chromatographed through a phosphocellulose column. The flow-through material was then applied to a DEAE-sepharose column and the absorbed material was eluted with 200 mM NaCl. Salt and molybdate were removed from the pooled, eluted material by chromatography on Sephadex G-25. After transformation at 25° for 60 min, the receptor fraction was further purified by fast protein liquid anion-exchange Mono Q chromatography (Pharmacia LKB Biotechnology). Fractions containing receptor were identified by ligand binding and specific DNA binding assays. These fractions contained 10–20% pure receptor and were used for protein-DNA interaction experiments.

**Purification of a recombinant DNA DBD of the GR.** The GR DBD, spanning Lys-419 to Gln-501 of the human GR (25), was overexpressed using the plasmid pT7–7 in the *E. coli* strain BL21[DE3]/pLysS. Bacteria were grown at 37° in Luria broth medium containing 1% casamino acids, 1% glucose, 100 $\mu$g/ml ampicillin, and 30 $\mu$g/ml chloramphenicol to an $A600$ = 0.6. Expression of the recombinant protein was induced with 0.2 mM isopropyl β-D-thiogalactopyranoside for 2 hr. The cells were resuspended in lysis buffer (50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 $\mu$g/ml leupeptin, and 10 $\mu$g/ml trypsin inhibitor), and deoxycholic acid was added to a final concentration of 0.05%. The lysate was centrifuged at 39,000 × $g$ for 30 min. Polyethylenimine was added to the clarified lysate to a final concentration of 0.2%, and the nucleic acid precipitate was removed by centrifugation at 39,000 × $g$ for 30 min. The lysate was then treated with ammonium sulfate to 70% saturation. The precipitate was collected by centrifugation, and resuspended in 50 mM Tris·HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 5 mM DTT, 1 mM $ZnSO_4$, 1 mM PMSF, 1 $\mu$g/ml leupeptin, and 10 $\mu$g/ml trypsin inhibitor; it was then dialyzed against the same buffer. The dialyzed lysate was loaded onto a CM-sepharose column (Pharmacia LKB Biotechnology) and eluted with a linear NaCl gradient in 20 mM phosphate buffer, pH 7.6 with 150 mM to 1 M NaCl and 1 mM DTT. The eluted protein was loaded onto a Hiload Superdex 75 prep-grade fast protein liquid chromatography column (Pharmacia LKB Biotechnology) and eluted with 20 mM phosphate buffer at pH 7.6 with 150 mM NaCl and 1 mM DTT. The eluted protein was dialyzed against 50 mM Tris·HCl, pH 7.4, 50 mM NaCl, and 1 mM DTT for storage. The protein was characterized by Western blot analysis using the anti-human GR DBD antibody and electrophoretic mobility shift assays using a $^{32}$P-labeled oligonucleotide that encompassed a GRE.

**SDS-PAGE and Western immunoblot analysis.** 1.5 $\mu$g of protein was mixed 1:1 with nonreducing 2 × sample buffer (20% v/v glycerol, 4.6% w/v SDS, 0.125 M Tris·HCl, pH 6.8) or reducing 2 × sample buffer (+ 20 mM DTT). Samples were then heated to 100° for 2 min and subjected to electrophoresis through 15% polyacrylamide gels according to the method of Laemmli (26) at 150–200 V, followed by Coomassie blue staining. Prestained molecular weight markers (Bio-Rad laboratories, Hercules, CA) were used as standards. For immunoblotting, ppGR and GR DBD were separated by 8% and 15% SDS-polyacrylamide gels, respectively. They were then electrically transferred to a nitrocellulose filter and probed with anti-human GR antibody PA1–512, a polyclonal rabbit antiserum raised against a synthetic peptide spanning human GR amino acids 346–367 (Affinity Bioreagents, Neshanic Station, NJ), or anti-human GR DBD antibody, a monoclonal antibody raised against a peptide fragment encompassing amino acids 393–500 of the human GR (27) (kindly provided by Dr. Ann-Charlotte Wikström, Department of Medical Nutrition, Karolinska Institute, Sweden), respectively. Antigen-antibody complexes were detected by the Prot Blot system (Promega) according to the manufacturer's instructions.

**EMSA.** EMSA was carried out as previously described (15). Briefly, double-stranded oligonucleotide probes were end-labeled with [α-$^{32}$P]dCTP (Amersham, Buckinghamshire, UK) using the Klenow fragment of DNA polymerase I (TaKaRa, Kyoto, Japan), and unincorporated nucleotides were chromatographically removed by a

Nick column (Pharmacia LKB Biotechnology). ppGR (usually 10 ng of protein per reaction) was incubated with 0.2 ng of $^{32}$P-labeled GRE oligonucleotide probe (approximately 20,000 cpm) in a 20-μl reaction mixture containing 5 mM HEPES, pH 7.9, 60 mM KCl, 2.5 mM EDTA, 2.5 mM $MgCl_2$, 10 mM spermidine, 0.25 mM DTT, 10% glycerol, and 100 ng poly(dI-dC) (Pharmacia LKB Biotechnology) for 15 min on ice. Radioinert competitor DNA was included when indicated. The reaction mixture was loaded onto a 4% nondenaturing polyacrylamide gel containing 0.25 × Tris/borate/EDTA solution (1 × = 89 mM Tris-borate, 89 mM boric acid and 2 mM EDTA). The gels were run at 350 V for 2 hr and were then dried. Results were visualized by autoradiography. The sequence of the oligonucleotide encompassing GRE is: 5′-CGAGTAGCTAGAACAGACTGTTCTGAGG-3′. For quantitation, the radioactivity of appropriate bands was counted using a BAS2000 phosphorimage analyzer (Fuji Film, Minamiashigara, Japan).

**Transfection and CAT assays.** Transient transfections were performed as previously described (28). Briefly, cells were plated in plastic culture dishes (10-cm diameter; IWAKI Glass, Funabashi, Japan) to 30–50% confluency, washed with phosphate-buffered saline three times; the medium was then replaced with OPTI-MEM medium (GIBCO Laboratories). Plasmid cocktail was mixed with 30 μl of Lipofectin reagent (GIBCO Laboratories) and added to the culture. After 12 hr of incubation, the medium was replaced with fresh medium supplemented with 2% dextran-coated, charcoal-stripped FCS, and the cells were further cultured in the presence or absence of various ligands for 24 hr. After normalization of transfection efficiency from determination of β-galactosidase expression, CAT enzyme activity was determined essentially as previously described (28). For quantitation, the radioactivity of relevant spots was counted using a BAS2000 phosphorimage analyzer (Fuji Film).

## Results

**Metal ions inhibit the sequence-specific DNA binding activity of ppGR.** To investigate the effect of metal ions on GR DNA binding we prepared ppGR from CHO-pMTGR cells (see Materials and Methods). EMSA using this ppGR showed a single class of DNA-protein complex (Fig. 1). This complex was shown to be GRE-specific, because the formation of the complex was competed with by addition of a molar excess of radioinert GRE oligonucleotide but not by an oligonucleotide containing an unrelated DNA sequence motif (Fig. 1). In the experiments, to achieve maximum DNA binding activity of ppGR, 0.25 mM DTT was included in the binding buffer (data not shown). Preincubation of ppGR with various metal ions resulted in a concentration-dependent inhibition of GR-GRE complex formation (Fig. 2A). Quantitative analysis of the effects of these metal ions on GR-GRE complex formation demonstrates a possible correlation between the inhibitory effect and the affinity for thiols (29): Au(I) showed the most potent inhibitory effect, followed by Cu(II), Cd(II), Zn(II), and Fe(III) (Fig. 2B). Al(III) and Mg(II) had no apparent effects (Fig. 2B). The effect of metals on GR DNA binding was unaltered in the presence of Fenton reagents ($FeCl_2$, 10 μM; EDTA, 10 μM; $H_2O_2$, 1 mM) (30), which indicates that an oxidative or free radical reaction is not likely to be involved (data not shown). The kinetics of the inhibitory action of Au(I) is shown in Fig. 3. Simultaneous addition of both Au(I) and probe DNA to ppGR (preincubation time: 0 min) resulted in a significant reduction in the amounts of protein-DNA complexes; this inhibition effect nearly reached plateau at 15–30 min preincubation of GR protein (Fig. 3). Notably, the DNA binding activity of ppGR remained suppressed even after dialysis of metal-treated ppGR to remove metals before incubating with probe DNA (data not shown), which suggests that metals act directly on the GR protein rather than on the DNA. Western blot analysis of metal-treated ppGR demon-

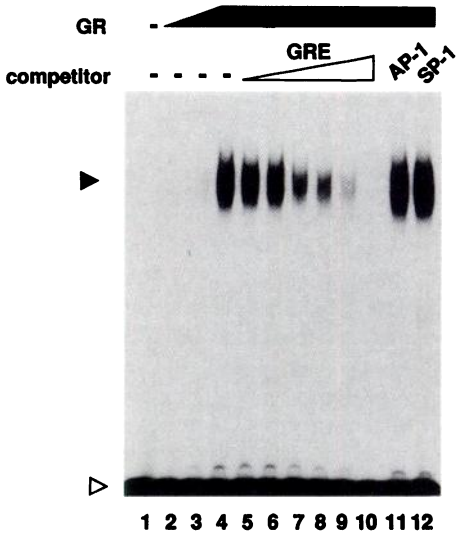


**Fig. 1.** Sequence-specific DNA binding activity of ppGR. Increasing amounts of ppGR (2.5, 5, 10 ng of protein per lane, respectively) were incubated with $^{32}$P-labeled GRE-oligonucleotide probe in the absence (*lanes 2–4*) or the presence (*lanes 5–9*) of a molar excess of radioinert competitor DNA (1-, 1.25-, 2.5-, 5-, 10-, and 20-fold molar excess, respectively), and specific protein-DNA complex formation was analyzed by EMSA. A 20-fold molar excess of nonspecific competitor DNA (AP-1 or Sp1) was included as indicated. AP-1 or Sp1 represent synthetic oligonucleotides encompassing the binding sites for the transcription factor AP-1 or Sp1, respectively (*lanes 11 and 12*). *Filled arrowhead*, position of specific protein-DNA complexes; *open arrowhead*, position of free probe.

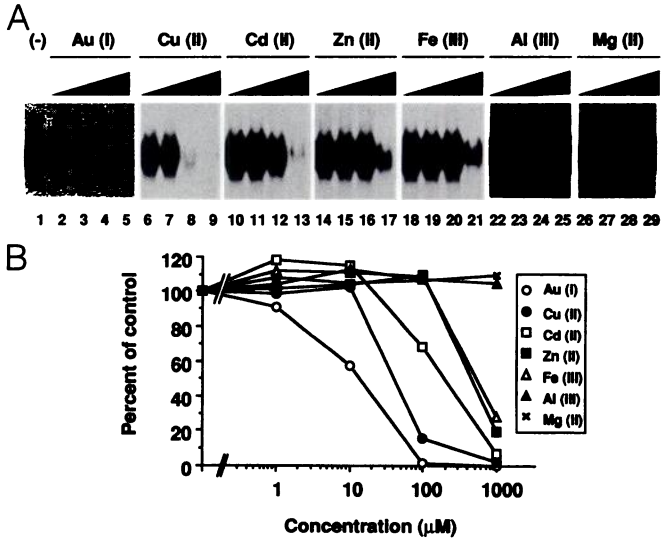


**Fig. 2.** Metal ions repress the sequence-specific DNA-binding activity of GR. A, 10 ng of ppGR were incubated with 1, 10, 100, and 1000 μM metal ions (indicated) for 30 min on ice and mixed with the $^{32}$P-labeled GRE oligonucleotide probe. Formation of protein-DNA complexes was monitored by EMSA and visualized by autoradiography. *Lane 1*, control radioactivity. Experiments were repeated three times with almost identical results; a representative experiment is shown. B, Quantitative analysis of the results from A. The radioactivity of the corresponding bands was quantified by a BAS 2000 phosphorimage analyzer. All results are shown as means of percentages of the control values.

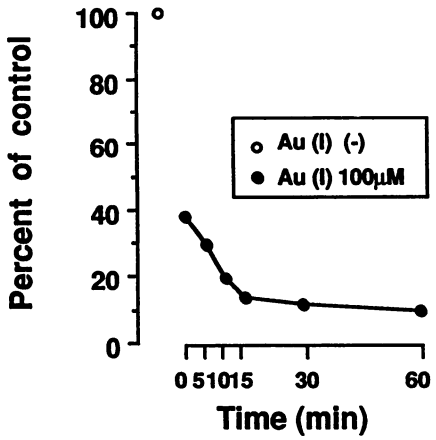


**Fig. 3.** Time-course of the inhibitory effect of Au(I) on GR-GRE complex formation. ppGR was incubated with 100 μM Au(I) on ice and aliquots were transferred to tubes containing $^{32}$P-labeled GRE probe at the indicated time. After an additional incubation for 15 min on ice, formation of protein-DNA complexes was analyzed by EMSA. Radioactivity was quantified by a BAS 2000 phosphorimage analyzer. The results are given relative to the radioactivity of the complex formed before addition of Au(I) and shown as percent of the control.

strated that these metal ions do not induce apparent alterations either in the molecular weight or in the amount of the GR protein (Fig. 4), which suggests that GR degradation is not responsible for the decreased DNA binding activity. Importantly, neither malate nor thiomalate significantly affected GR DNA binding activity (data not shown) (15, 16).

**Sulfhydryl modifying reagents reverse metal-mediated inhibition of GR DNA binding.** Because metal ions with thiol affinity inhibited the sequence-specific DNA binding activity of the GR, we next investigated how sulfhydryl-modifying reagents influence this inhibitory effect of metal ions. After preincubation of ppGR with relevant metal ions for 30 min, various concentrations of the sulfhydryl reducing reagent 2ME or the cysteine derivative NAC was added and the DNA binding activity of ppGR was assayed by EMSA. 2ME (Fig. 5A) and NAC (Fig. 5B) reversed the metal-mediated inhibition of DNA binding activity of the GR in a dose-dependent fashion. Neither 2ME alone nor NAC alone seemed to have an effect on GR-GRE complex formation (data not shown). In summary, the data indicate that metal ions exert their inhibitory action via a reversible modification of sulfhydryls in the GR protein.

**The GR DBD is a target for negative modulation by metal ions.** The DBD of the GR is sufficient for specific interaction with GREs (20, 31) and it has been shown to be one of the targets for repression of DNA binding activity by

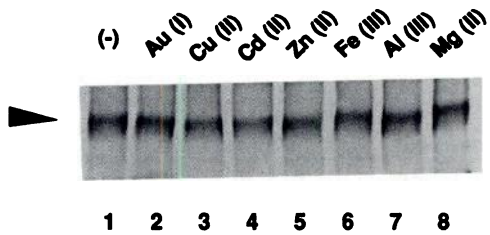


**Fig. 4.** ppGR protein levels are not affected by treatment with metal ions. The ppGR was incubated with 100 μM of the indicated metal ions for 60 min on ice, and ppGR immunoreactivities were determined by Western immunoblotting using anti-human GR antibodies. *Arrowhead*, position of GR.

sulfhydryl oxidizing reagents (32–34). To investigate whether metal-mediated inhibition of DNA binding involves the DBD, we performed EMSA using a minimal DBD of the receptor, GR DBD. GRE-specific DNA binding by GR DBD was, as in the case of ppGR, negatively effected by preincubation with metal ions (Fig. 6). Inhibition of the DNA binding activity of the GR DBD by metal ions was restored by 2ME and/or NAC (data not shown). These results indicate that negative modulation of GR function by metal ions, is mediated, at least in part, via reversible modification of DBD.

**Metal-mediated repression of GR DNA binding does not involve disulfide bond formation.** Disulfide bond formation in GR DBD by sulfhydryl-oxidizing reagents has been suggested to cause a significant decrease in the DNA binding activity (34). We therefore examined by SDS-PAGE whether disulfide bonds are formed in metal-treated GR DBD. As shown in Fig. 7, SDS-PAGE analysis of GR DBD followed by Coomassie staining revealed two bands. The slower migrating band was identified as GR DBD by immune-clearance experiments using anti-human GR DBD antibody-conjugated protein A sepharose beads (Fig. 7A). Nonspecific human IgG-conjugated beads precipitated neither the slower nor the faster migrating band (lane 2). In addition, the anti-GR DBD antibody exclusively recognized the slower migrating band with a molecular mass of 13 kDa (Fig 7A), thus the slower migrating band is GR DBD and the faster migrating band is a GR-unrelated product derived from *E. coli*. The difference in migration pattern of proteins on denaturing polyacrylamide gels under reducing (+DTT) and nonreducing (-DTT) conditions is considered to be diagnostic of the formation of disulfide bonds (35, 36). Treatment of GR DBD with 0.1–5 mM diamide, which is a potent inducer of disulfide bond formation, altered the migration pattern when resolved on nonreducing SDS-PAGE (Fig. 7B); the bands representing monomeric form of GR DBD became diffuse, and the formation of polymeric GR DBD became apparent with increasing concentrations of diamide. These changes in migration pattern were not seen in reduced SDS-PAGE analysis (Fig. 7B). In contrast, the faster migrating GR-unrelated bands were not affected by treatment with diamide. Thus, it is suggested that diamide did not induce the aggregation of proteins but intra- and/or intermolecular disulfide bond formation in GR DBD. In contrast with diamide, pretreatment with 100 μM Au(I), the most potent metal in inhibiting GR DNA binding, caused no remarkable change in the migration pattern of GR DBD under reducing or nonreducing conditions (Fig. 7B). This suggests that Au(I)- or metal-mediated inhibition of DNA binding by GR DBD does not involve disulfide bond formation, which affects protein migration pattern in the gel.

**Metal ions suppress glucocorticoid-inducible gene expression through the interference with GR-GRE interactions.** Possible effects of metal ions on glucocorticoid hormone action *in vivo* were investigated in transient transfection experiments. COS-1 cells were transfected with the wild type GR expression vector pRShGRα and various glucocorticoid-inducible reporter constructs, and incubated in the presence or absence of dexamethasone and metal ions as indicated (Table 1). The reporter construct MTV-CAT is a hybrid gene in which the well-known glucocorticoid inducible promoter from the MMTV is inserted in front of the CAT gene (18). Incubation with 100 nM dexamethasone produced a

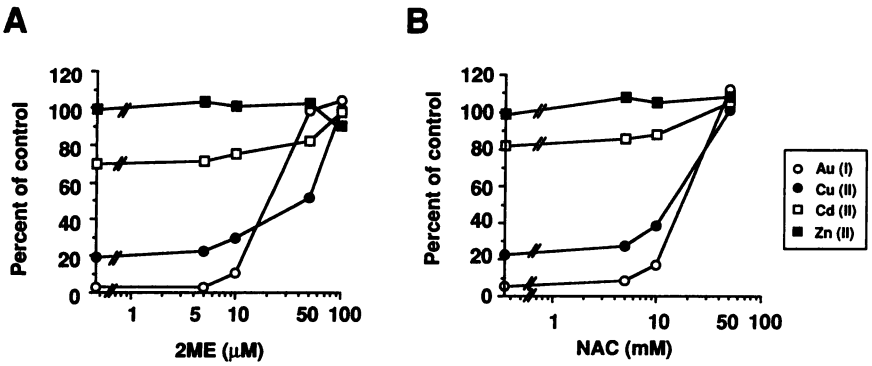


**Fig. 5.** 2ME and NAC counteract the repression of GR DNA binding activity by metal ions. 10 ng of ppGR were incubated with 100 μM metal ions (indicated) for 30 min on ice and was further incubated with increasing amounts of 2ME (A) or NAC (B) for 30 min, after which $^{32}$P-labeled GRE probe was added. After an additional incubation for 15 min on ice, formation of protein-DNA complexes was analyzed by EMSA. For quantitative analysis, the radioactivity of the bands was quantified by a phosphorimage analyzer. The bands that represent the formation of complex between untreated ppGR and GRE probe served as control; all results are shown as means of percentages of the control values.

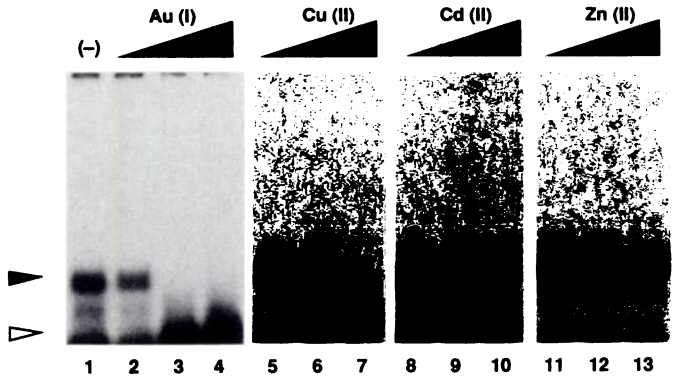


**Fig. 6.** Metal ions repress the sequence-specific DNA-binding activity of the recombinant DBD of GR. 50 ng of GR DBD was incubated with 1, 10, and 100 μM metal ions (indicated) for 30 min on ice, and mixed with $^{32}$P-labeled GRE probe. Formation of protein-DNA complexes was monitored by EMSA and visualized by autoradiography. Experiments were repeated three times with almost identical results; a representative result is shown. *Open arrowhead,* free probe; *filled arrowhead,* protein-DNA complex.

17.4-fold induction of cellular CAT activity, indicating that the expressed GR is functional. Au(I) (100 μM) significantly decreased the dexamethasone-induced activation of the MMTV promoter by 42% (17.4- to 7.3-fold). In parallel with the results of the DNA binding experiments, Cu(II) showed weaker reduction of hormone induction. Cd(II) seemed to cause cell toxicity and was excluded from the study. Zn(II) and other metal ions showed only marginal effects even at 100 μM.

To further investigate the mechanism of the negative effect of metal ions on hormone responsive gene transcription, we performed transfection assays using wild-type and mutant glucocorticoid-inducible reporter constructs and the most potent negative modulator Au(I) (Table 2). The expression of ΔMTV-CAT, a reporter plasmid lacking the hormone response unit of MTV-CAT (18), was not activated by dexamethasone and no repressive effect of Au(I) was observed. On the other hand, treatment with dexamethasone markedly induced expression of GREtkCAT, in which two tandem repeats of a palindromic GRE are linked to the thymidine kinase promoter driving the CAT gene (22) (26.0-fold), and moreover, Au(I) repressed hormone induction of CAT expression (26.0- to 9.1-fold). Mutations in both of the GREs in GREtkCAT to give the construct mGREtkCAT (22) completely abolished both the hormone induction response and the Au(I)-mediated repression. These results strongly suggest that Au(I) negatively modulates the productive interaction between GR and its cognate DNA sequences. Notably, Au(I) did not affect the expression of the pCAT-Control Vector, which constitutively expresses CAT under the control of the SV40 promoter and enhancer sequences (data not shown), indicating that Au(I) has no effect on the basal activity of this promoter. In summary, the antagonistic effect of Au(I) on glucocorticoid inducible gene expression was specific for GR-GRE interactions both *in vitro* and *in vivo*.

**Au(I)-mediated repression of glucocorticoid-inducible gene expression occurs independent of ligand-inducible activation of GR.** To investigate the effect of Au(I) on the ligand-inducible function of GR, we studied the influence of Au(I) on the transactivation function of wild type GR and a GR-deletion mutant lacking the hormone binding domain. When the expression vector encoding wild-type GR, pRShGRα, was cotransfected with GREtkCAT, dexamethasone dramatically activated CAT expression and Au(I) markedly repressed the hormone induction response (Fig. 8). As expected, cotransfection of the constitutively active GR mutant I550*, which has a deletion of the ligand binding domain of GR (18–20), showed hormone-independent activation of transcription of the reporter genes. Au(I) also inhibited this ligand-independent activation of gene expression, which indicates that negative regulation by Au(I) occurs independently of the presence of ligand.

## Discussion

Glucocorticoid hormones are known to modulate gene expression after binding to their receptor, the GR, which therefore acts as a hormone-dependent transcription factor. Here we show that metal ions, possibly through a modification of sulfhydryls in the GR, reversibly inhibit the specific DNA binding activity of the GR, resulting in negative regulation of the hormone responsiveness of target genes.

In addition to genetic alterations of the GR protein (13, 37, 38), posttranslational modification of the GR protein may constitute an important mechanism for regulation of glucocorticoid responsiveness *in vivo*. The majority of previous reports focused on the effects of posttranslational modification on GR function with regard to ligand binding activity and/or nonspecific binding to DNA-cellulose, using nonphysiological inorganic chemicals as effectors (32–34, 39–42). The metals that we used in this study exist endogenously as trace elements, are frequently taken as drugs, and/or occur in the environment as pollutants. We showed that metal ions including Au(I), Cu(II), and Cd(II) inhibit the specific binding of the GR to GRE target sequences. In the first series of experiments, the GR was partially purified from CHO-pMTGR cell extracts. Because purified GR is known to be a

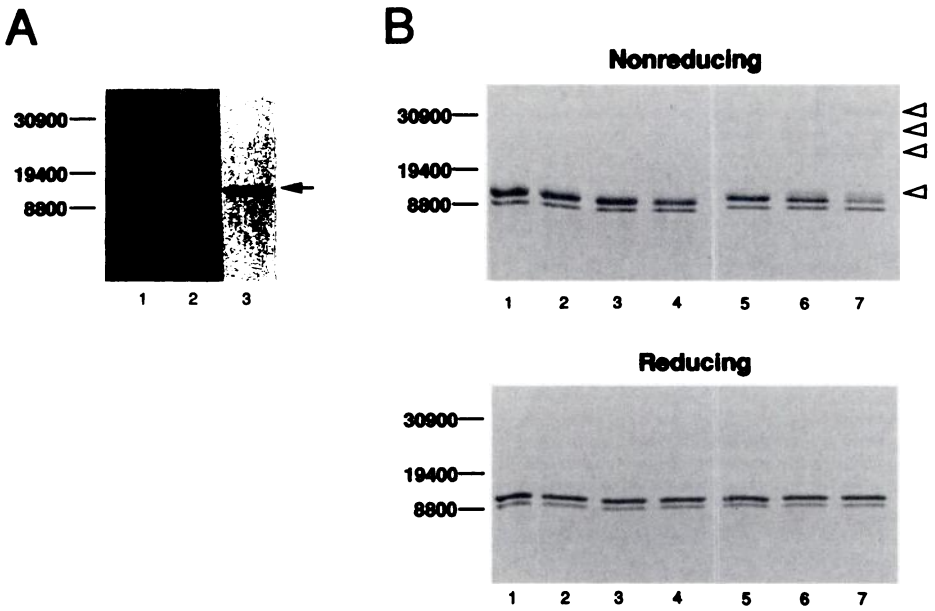


**Fig. 7.** Au(I) causes neither intra- nor intermolecular disulfide bond formation in the GR DBD (DBD). A, SDS-PAGE and Western blot analysis of GR DBD. *Lanes 1 and 2*, 1.5 μg of GR DBD was pretreated with anti-human GR DBD antibody- (*lane 1*) or nonspecific human IgG- (*lane 2*) conjugated protein A sepharose beads. After centrifugation of the mixture, the supernatant was analyzed by SDS-PAGE, followed by Coomassie blue staining. *Lane 3*, Western blot analysis of the GR DBD using the anti-human GR DBD antibody. *Arrow*, position of the GR DBD. The numbers refer to the sizes of the reference proteins in daltons. B, 3.0 μg of GR DBD was incubated without (*lane 1*) or with increasing amounts of Au(I) (*lane 2*, 1 μM; *lane 3*, 10 μM; and *lane 4*, 100 μM) or diamide (*lane 5*, 0.1 mM; *lane 6*, 1 mM; and *lane 7*, 5 mM) for 30 min on ice. Half of each sample was mixed 1:1 with nonreducing sample buffer and half with reducing sample buffer containing 200 mM DTT. The samples were analyzed by SDS-PAGE (15%) followed by Coomassie blue staining. *Numbers on the left*, sizes of reference proteins in daltons; *open arrowheads*, positions of polymeric protein forms.

TABLE 1

**Repression of hormone responsiveness of the MTV-CAT by metal ions**

MTV-CAT plasmid (5 μg) was cotransfected into COS-1 cells with 5 ng of the wild-type GR expression vector pRShGRα. The cells were cultured in the presence of dexamethasone (100 nM) and/or 100 μM of the indicated metal ions for 24 hr; cell extracts were then prepared for CAT enzyme assay. Transfection efficiency was monitored by the β-glaactosidase activity of the reference plasmid. For quantitation, radioactivity of each spot was quantitated using a phosphorimage analyzer; results expressed as percent are conversion of chloramphenicol into its acetylated forms. Experiments were repeated twice with almost identical results; mean values are presented.

| | Conversion to acetylated forms | | Induction |
|---|---|---|---|
| | −dexamethasone | +dexamethasone | |
| | % | | *fold* |
| Control | 2.2 | 38.3 | 17.4 |
| Au(I) | 2.1 | 15.5 | 7.3 |
| Cu(II) | 2.2 | 27.6 | 12.3 |
| Zn(II) | 2.1 | 38.8 | 16.9 |
| Fe(III) | 2.1 | 37.9 | 17.9 |
| Al(III) | 2.1 | 38.0 | 18.1 |
| Mg(II) | 1.8 | 35.2 | 18.5 |

TABLE 2

**Effect of Au(I) on glucocorticoid-inducible reportor gene expression**

The indicated reporter constructs (5 μg each) were cotransfected into COS-1 cells with 5 ng of wild-type GR expression plasmid pRShGR α, and the cells were cultured in the absence or presence of dexamethasone (100 nM) and 100 μM of Au(I) as indicated for 24 hr and cellular CAT activity was determined. For quantitation, radioactivity of each spot was counted using a phosphorimage analyzer; the results are expressed as percent conversion of chloramphenicol into its acetylated forms. Experiments were repeated twice with almost identical results; mean values are presented.

| | | Converison to acetylated forms | | Induction |
|---|---|---|---|---|
| | | −dexamethasone | +dexamethasone | |
| | Au(I) | % | | *fold* |
| MTV-CAT | − | 2.2 | 38.3 | 17.4 |
| | + | 2.1 | 15.5 | 7.3 |
| ΔMTV-CAT | − | 2.0 | 2.4 | 1.2 |
| | + | 1.9 | 2.5 | 1.3 |
| GREtkCAT | − | 1.9 | 49.4 | 26.0 |
| | + | 1.8 | 16.4 | 9.1 |
| mGREtkCAT | − | 2.0 | 2.2 | 1.1 |
| | + | 2.1 | 2.4 | 1.1 |

ligand-independent regulator (43), it is not likely that metal ions inhibited the process of activation or promoted reassociation with factors inhibiting activation, e.g., hsp90. The purified DNA binding form of the GR is recovered as a homodimer (43; data not shown); thus, inhibition of receptor dimerization is not likely to be involved in the mechanism of action of metal ions. In addition, dialysis of metal-treated ppGR to remove free metals did not affect the metal-mediated repression of DNA binding activity of the GR, which indicate that metals do not affect the DNA. Therefore, the most plausible model is that metal ions act directly on the GR protein to interfere with its interaction with the GRE. Moreover, the inhibitory effect of metal ions on the GR DNA binding activity did not appear to be due to destruction of the GR protein, as assessed by Western blot analysis or the reversibility of the inhibitory action. Thus, metal ions seemed to affect functional properties of the GR.

Au(I), Cu(II) and Cd(II) have a high affinity for sulfhydryl groups (29). Based on their thiolate coordinating chemistry (44), it is suggested that these metals may interact with sulfhydryls in the GR as thiolate ligands. The observation that the repressive effects of these metal ions were efficiently antagonized by the sulfhydryl reducing reagents 2ME and

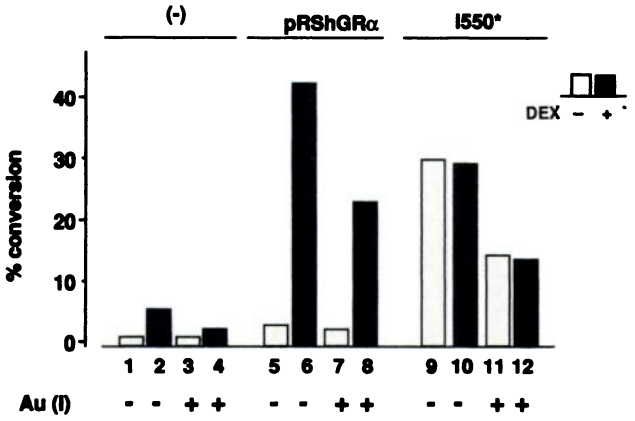


**Fig. 8.** The ligand-binding domain is not required for Au(I)-mediated repression of GR transactivation function. Glucocorticoid-inducible GREtkCAT reporter construct (5 μg) was transfected into COS-1 cells, without (*columns 1–4*) or with (*columns 5–8*) wild-type GR expression vector pRShGRα, or with the ligand binding domain-deleted GR expression vector I550* (*columns 9–12*). Cells were incubated with 100 nM dexamethasone (*DEX*) and 100 μM Au(I) as indicated for 24 hr; cellular CAT activities were determined. For quantification, the radioactivity of each spot was counted using a phosphorimage analyzer; results are expressed as percent conversion of chloramphenicol into its acetylated forms for each reporter. Experiments were repeated twice with similar results; mean values are presented.

NAC suggest a competition between 2ME or NAC and thiols in GR for binding to these metals. To determine whether these metals directly bind to the protein, in analogy with the case for inhibition of AP-1 DNA binding activity by Au(I) and selenium (30), requires biophysical studies, i.e., atomic absorption analysis, nuclear magnetic resonance spectroscopy, or X-ray crystallography, and biochemical identification of responsible amino acids by mutational analysis, which are now ongoing. Alternatively, metal ions may cause displacement or removal of zinc atoms in the DBD and thereby alter the function of this domain. However, zinc ions themselves abolish the DNA binding activity of the GR when present in excess, which shows that the mechanism for the inhibitory action of metal ions can not simply be explained by displacement or removal of zinc.

Sulfhydryl groups in the GR are included as part of cysteine residues. The human GR contains 20 cysteine residues, concentrated in the central region spanning the DBD, and in the carboxyl-terminal region spanning the hormone binding domain (45). Numerous *in vitro* studies employing sulfhydryl oxidizing reagents have shown that the conversion of free sulfhydryls in cysteine residues to disulfides results in critical alterations in the function of each domain of the GR (32–34, 41, 46, 47). In this study, disulfide bond formation is shown not to be involved in metal-mediated inhibition of GR DNA binding, and the modulation of sulfhydryls by metal ions is suggested to be distinct from that by sulfhydryl-oxidizing reagents. It has recently been reported that modification of critical cysteine residues in transcription factors, for example, AP-1 (48, 49), NF-κB (49, 50), Myb (51), v-Rel (52), BZLF1 (53), papilloma virus E2 protein (54), and p53 (55), results in alterations in their DNA binding activity *in vitro*. Notably, the DNA binding activity of NF-κB (56) and AP-1(30) are susceptible to metal ions with thiol affinity, as is the case with our results. The other members of the nuclear hormone receptor superfamily may be affected by metal ions in a similar fashion, because the DBD of those receptors is highly conserved (6). However, the inhibitory action of metal ions on transcription factors is not ubiquitous and there seem to be differences in the sensitivity to metal ions between transcription factors. In fact, DNA binding by AP-2, TFIID, and NF-1 is less sensitive than that of AP-1(30), and the difference in susceptibility is suggested to be caused by the electrostatic environment of cysteine residues (30, 57).

We have also demonstrated that metal ions repress GR-mediated gene activation in transient transfection assays using a variety of glucocorticoid responsive reporter genes. The rank order of the inhibitory action of metal ions *in vivo* was similar to their inhibitory effect on DNA binding *in vitro*, which indicates that the reduction in DNA binding activity leads to a decrease in transactivation activity *in vivo*. Additionally, metal ions may inhibit ligand-dependent activation of the GR, which is one of the key steps in signal transduction by glucocorticoids *in vivo*. We have previously shown that Au(I) partially decreases the ligand binding activity of the GR in a whole cell assay (15). However, in the present study, we have further demonstrated that Au(I) also interfered with the ligand-independent transactivation function of I550*, a constitutively active GR mutant. Thus, it seems most unlikely that Au(I) affects only ligand-GR interaction.

We only observed a rather modest effect of Au(I)-mediated repression on the MMTV promoter when compared with the inhibitory effect of Au(I) on the DNA binding activity. A possible explanation for this discrepancy may be a difference in the intracellular concentration of Au(I) versus the concentration to which the GR is exposed under *in vitro* conditions. Alternatively, as-yet-unidentified factors may be present in the cell or the culture medium and may attenuate Au(I) action. For example, thioredoxin has been suggested to be an endogenous GR-activating factor (58) and also to facilitate DNA binding by NF-κB (50); thus, still unidentified endogenous factors may be involved in the metal-mediated regulation of GR function *in vivo*.

The therapeutic concentrations of Au(I) in the sera of patients under crysotherapy are 10–50 μM (59), and the hepatic Cu(II) concentrations in patients with Wilson's disease (60) or the renal Cd(II) concentrations in intoxicated patients (61) often exceed 100 μM; thus, the concentrations of metals used in this study may be clinically and/or pathologically attainable, and metal-mediated repression of cellular glucocorticoid responsiveness may be physiologically relevant. Examination of the hypothalmus-pituitary-adrenal axis activity in patients under chrysotherapy or studies using GR extracted from the liver of metal-fed animals may be helpful to verify the physiological significance of our observation. Intravenous administration of NAC efficiently reversed the adverse hematologic reactions to gold compounds in patients with rheumatoid arthritis, suggesting that Au(I) is capable of modulating sulfhydryls *in vivo* (62).

In summary, we have shown that metal ions can reduce the DNA binding activity of the GR and inhibit receptor-mediated activation of gene expression. In addition to the direct effect on the interaction between GR and its target DNA sequences, metal ions may also effect protein-protein interactions between the receptor and other transcription factors (63–66) or other cellular components, such as immunophilins (67), calreticulin (68), or the RB protein (69). Moreover, it is possible that metals may interfere with other zinc finger

proteins and/or proteins in which the cysteine residues and metals play pivotal roles for protein functions. At this moment, we have indicated a novel aspect of the roles of metal ions in intracellular signalling pathways for glucocorticoids. To investigate medical roles for metal-containing compounds, it will be necessary to further investigate interactions between metal ions and cellular signal transduction cascades.

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**References**

1. Karlin, D. K. Metalloenzymes, Structural motifs, and inorganic models. *Science (Washington, D. C.)* **267:**701–708 (1993).
2. O'Halloran, T. V. Transition metals in control of gene expression. *Science (Washington, D. C.)* **261:**715–725 (1993).
3. Abrams, M. J., and B. A. Murrer. Metal compounds in therapy and diagnosis. *Science (Washington, D. C.)* **261:**725–730 (1993).
4. Vallee, B. L., and K. H. Falchuk. The biochemical basis of zinc physiology. *Physiol. Rev.* **73:**79–118 (1993).
5. Gustafsson, J. -Å., J. Carlstedt-Duke, L. Poellinger, S. Okret, A.-C. Wikström, M. Brönnegård, M. Gillner, Y. Dong, K. Fuxe, A. Cintra, A. Härfstrand, and L. Agnati. Biochemistry, molecular biology and physiology of the glucocorticoid receptor. *Endocrine Rev.* **8:**185–234 (1987).
6. Evans, R. M. The steroid and thyroid hormone receptor superfamily. *Science (Washington, D. C.)* **240:**889–895 (1988).
7. Beato, M. Gene regulation by steroid hormones. *Cell* **56:**335–344 (1989).
8. O'Malley, B. W. The steroid receptor superfamily: more excitement predicted for the future. *Mol. Endocrinol.* **4:**363–369 (1990).
9. Freedman, L. P., B. F. Luisi, Z. R. Korszun, R. Basavappa, P. B. Sigler, and K. R. Yamamoto. The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature (Lond.)* **334:**543–546 (1988).
10. J. Carlstedt-Duke, P.-E. Strömstedt, K. Dahlman, C. Rae, A. Berkenstam, J. Hapgood, H. Jörnvall, and J.-Å Gustafsson. Structural analysis of the glucocorticoid receptor protein, in *The Steroid/Thyroid Hormone Receptor Family and Gene Regulation* (J. Carlstedt-Duke, H. Eriksson, J.-Å. Gustafsson, eds.). Birkhäuser Verlag, Basel, 93–108 (1989).
11. Pan, T., L. P. Freedman, and J. E. Coleman. Cadmium 113 NMR studies of the DNA binding domain of the mammalian glucocorticoid receptor. *Biochemistry* **29:**9218–9225 (1990).
12. Luisi, B. F., W. X. Xu, Z. Otwinowski, L. P. Freedman, K. R. Yamamoto, and P. B. Sigler. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature (Lond.)* **352:**497–505 (1991).
13. Schena, M., Freedman, L. P., and Yamamoto, K. R. Mutations in the glucocorticoid receptor zinc finger region that distinguish interdigitated DNA binding and transcriptional enhancement activities. *Genes Dev.* **3:**1590–1601 (1989).
14. Dundersk, J., J. Stanosev, B. Ristic, D. Trajkovic, and G. Matic. *In vivo* effects of cadmium on rat liver glucocorticoid receptor functional properties. *Int. J. Biochem.* **24:**1065–1072 (1992).
15. Makino, Y., H. Tanaka, F. Hirano, E. Fukawa, and I. Makino. Repression of glucocorticoid receptor function by the anti-rheumatic gold compound aurothiomalate. *Biochem. Biophys. Res. Commun.* **197:**1146–1153 (1993).
16. Makino, Y., H. Tanaka, and I. Makino. Paradoxical derepression of the collagenase gene expression by the anti-rheumatic gold compound aurothiomalate. *Mol. Pharmacol.* **46:**1084–1089 (1994).
17. Alksnis, M., T. Barkhem, P.-E. Strömstedt, H. Ahola, E. Kutoh, J.-Å. Gustafsson, L. Poellinger, and S. Nilson. High level expression of functional full length and truncated glucocorticoid receptor in chinese hamster ovary cells. *J. Biol. Chem.* **266:**10078–10085 (1991).
18. Rangarajan, P. N., K. Umezono, and R. M. Evans. Modulation of glucocorticoid receptor function by protein kinase A. *Mol. Endocrinol.* **6:**1451–1457 (1992).
19. Hollenberg, S. M., V. Giguere, P. Segul, and R. M. Evans. Colocalization of DNA-binding and transcriptional activation function in the human glucocorticoid receptor cDNA. *Nature (Lond.)* **318:**635–641 (1987).
20. Hollenberg, S. M., and R. M. Evans. Multiple and cooperative transactivation domains of the human glucocorticoid receptor. *Cell* **55:**899–906 (1988).
21. Tsai, S. Y., M.-J. Tsai, and B. M. O'Malley. Cooperative binding of steroid hormone receptors contributes to transcriptional synergism at target enhancer elements. *Cell* **57:**361–369 (1989).
22. Oshima, H., and S. S. Simons, Jr. Sequence-selective interactions of transcription factor elements with tandem glucocorticoid-responsive elements at physiological steroid concentrations. *J. Biol. Chem.* **268:**26858–26865 (1993).
23. Dong, Y., L. Poellinger, J.-Å. Gustafsson, and S. Okret. Regulation of glucocorticoid receptor expression: evidence for transcriptional and post-translational mechanisms. *Mol. Endocrinol.* **2:**1256–1264 (1988).
24. Cairns, W., C. Cairns, I. Pongratz, L. Poellinger, and S. Okret. Assembly of a glucocorticoid receptor complex prior to DNA binding enhances its specific interaction with a glucocorticoid response element. *J. Biol. Chem.* **266:**11221–11226 (1991)
25. Lundbäck, T., J. Zilliacus, J.-Å. Gustafsson, J. Carlstedt-Duke, and T. Härd. Thermodynamics of sequence-specific glucocorticoid receptor-DNA interactions. *Biochemistry* **33:**5955–5965 (1994).
26. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227:**680–685 (1970).
27. Zilliacus, J., K. Dahlman-Wright, J. Carlstedt-Duke, and J.-Å Gustafsson. Zinc coordination scheme for the C-terminal zinc binding site of nuclear hormone receptors. *J. Steroid. Biochem. Molec. Biol.* **42:**131–139 (1992).
28. Tanaka, H., and I. Makino. Ursodeoxycholic acid-dependent activation of the glucocorticoid receptor. *Biochem. Biophys. Res. Commun.* **188:**942–948 (1992).
29. Handel, M. L., A. deFazio, C. K. W. Watts, R. O. Day, and R. L. Sutherland. Inhibition of DNA binding and transcriptional activity of a nuclear receptor transcription factor by aurothiomalate and other metal ions. *Mol. Pharmacol.* **40:**613–618 (1991).
30. Handel, M. L., C. K. W. Watts, A. deFazio, R. O. Day, and R. L. Sutherland. Inhibition of AP-1 binding and transcription by gold and selenium involving conserved cysteine residues in Jun and Fos. *Proc. Natl. Acad. Sci. USA* **92:**4497–4501 (1995).
31. Zilliacus, J., A. P. H. Wright, J. Carlstedt-Duke, and J.-Å Gustafsson. Structural determinants of DNA-binding specificity by steroid receptors. *Mol. Endocrinol.* **9:**389–400 (1995).
32. Tienrungro, W., S. Meshinchi, E. R. Sanchez, S. E. Pratt, J. F. Grippo, A. Holmgren, and W. B. Pratt. The role of sulfhydryl groups in permitting transformation and DNA binding of the glucocorticoid receptor. *J. Biol. Chem.* **262:**6992–7000 (1987).
33. Bodwell, J. E., N. J. Holbrook, and A. Munck. Sulfhydryl-modifying reagents reversibly inhibit binding of glucocorticoid-receptor complexes to DNA-cellulose. *Biochemistry* **23:**1392–1398 (1984).
34. Hutchinson, K. A., G. Matic, S. Meshinchi, E. H. Bresnick, and W. B. Pratt. Redox manipulation of DNA binding activity and BuGR epitope reactivity of the glucocorticoid receptor. *J. Biol. Chem.* **266:**10505–10509 (1991).
35. Matsumura, M., and B. W. Matthews. Control of enzyme activity by an engineered disulfide bond. *Science (Washington, D. C.)* **243:**792–794 (1989).
36. Opoku, J., and S. S. Simons, Jr. Absence of intramolecular disulfides in the structure and function of native rat glucocorticoid receptors. *J. Biol. Chem.* **269:**503–510 (1994).
37. Byravan, S., J. Milhon, S. K. Rabindran, B. Olinger, M. J. Garabedian, M. Danielson, and M. R. Stallcup. Two point mutations in the hormone-binding domain of the mouse glucocorticoid receptor that dramatically reduce its function. *Mol. Endocrinol.* **5:**752–758 (1991).
38. Chrousos, G. P., S. D. Detera-Wadleigh, and M. Karl. Syndromes of glucocorticoid resistance. *Ann. Intrn. Med.* **119:**1113–1124 (1993).
39. Miller, N. R., and S. S. Simons, Jr. Steroid binding to hepatoma tissue culture cell glucocorticoid receptors involves at least two sulfhydryl groups. *J. Biol. Chem.* **263:**15217–15225 (1988).
40. Meshinchi, S., G. Matic, K. A. Hutchinson, and W. B. Pratt. Selective molybdate-directed covalent modification of sulfhydryl groups in the steroid-binding *versus* the DNA-binding domain of the glucocorticoid receptor. *J. Biol. Chem.* **265:**11643–11649 (1990).
41. Chakraborti, P. K., W. Hoeck, B. Groner, and S. S. Simons, Jr. Localization of the vicinal dithiols involved in steroid binding to the rat glucocorticoid receptor. *Endocrinol.* **127:**2530–2539 (1990).
42. Simons, S., S., Jr, P. K. Chakraborti, and A. H. Cavanaugh. Arsenite and cadmium (II) as probes of glucocorticoid receptor structure and function. *J. Biol. Chem.* **265:**1938–1945 (1990).
43. Wrange, Ö., P. Eriksson, and T. Perlmann. The purified activated glucocorticoid receptor is a homodimer. *J. Biol. Chem.* **264:**5253–5259 (1989).
44. Zucconi, T. D., G. E. Janauer, S. Donahe, and C. Lewkowicz. Acid dissociation and metal complex formation constants of penicillamine, cysteine, and antiarthritic gold complexes at stimulated biological conditions. *J. Pharm. Sci.* **68:**426–432 (1979).
45. Hollenberg, S. M., C. Weinberger, E. S. Ong, G. Cerelli, A. Oro, R. Lebo, E. B. Tompson, M. G. Rosenfeld, and R. M. Evans. Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature (Lond.)* **318:**635–641 (1985).
46. Chakraborti, P. K., M. J. Garabedian, K. R. Yamamoto, and S. S. Simons, Jr. Role of cysteines 640, 656, and 661 in steroid binding to rat glucocorticoid receptors. *J. Biol. Chem.* **267:**11366–11373 (1992).
47. Bresnick, E. H., E. R. Sanchez, R. W. Harrison, and W. B. Pratt. Hydrogen peroxide stabilizes the steroid-binding state of rat liver glucocorticoid receptors by promoting disulfide bond formation. *Biochemistry* **27:**2866–2872 (1988).

48. Abate, C., L. Patel, F. J. Rauscher III, T. Curran. Redox regulation of *fos* and *jun* DNA-binding activation *in vitro*. *Science (Washington, D. C.)* **249**:1157–1161 (1990).
49. Meyer, M., R. Schreck, and P. A. Baeuerle. $H_2O_2$, and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J.* **12**:2005–2015 (1993).
50. Hayashi, T., Y. Ueno, and T. Okamoto. Oxidoreductive regulation of Nuclear Factor κB *J. Biol. Chem.* **268**:11380–11388 (1993).
51. Myrset, A. H., A. Bostad, N. Jamin, P.-N. Lirsac, F. Toma, and O. S. Gabrielsen. DNA and redox state induced conformational changes in the DNA-binding domain of the Myb protein. *EMBO J.* **12**:4625–4633 (1993).
52. Kumar, S., A. B. Rabson, and C. Gèlinas. The RxxRxRxxC motif conserved in all Rel/kappa B proteins is essential for the DNA-binding activity and redox regulation of the v-Rel onco protein. *Mol. Cell. Biol.* **12**:3094–3106 (1992).
53. Bannister, A. J., A. Cook, and T. Kouzarides. *In vitro* DNA binding activity of Fos/Jun and BZLF1 but not C/EBP is affected by redox changes. *Oncogene* **6**:1243–1250 (1991).
54. McBride, A. A., R. D. Klausner, and P. M. Howley. Conserved cysteine residue in the DNA-binding domain of the bovine papillomavirus type 1 E2 protein confers redox regulation of the DNA-binding activity *in vitro*. *Proc. Natl. Acad. Sci. USA* **89**:7531–7535 (1992).
55. Rainwater, R., D. Parks, M. E. Anderson, P. Tegtmeyer, and K. Mann. Role of cysteine residues in regulation of p53 function. *Mol. Cell. Biol.* **15**:3892–3903 (1995).
56. Yang, J.-P., J. P. Merin, T. Nakano, T. Kato, Y. Kitade, T. Okamoto. Inhibition of the DNA-binding activity of NF-κB by gold compounds *in vitro*. *FEBS Lett.* **361**:89–96 (1995).
57. Handel, M. L., S. Sivertsen, C. K. Watts, R. O. Day, and R. L. Sutherland. Comparative effects of gold on the interactions of transcription factors with DNA. *Agents Actions Suppl.* **44**:219–223 (1993).
58. Grippo, J. F., A. Holmgren, and W. B. Pratt. Proof that the endogenous, heat-stable glucocorticoid receptor-activating factor is thioredoxin. *J. Biol. Chem.* **260**:93–97 (1985).
59. Lorber, A., T. M. Simon, J. Leeb, and P. E. Carroll, Jr. Chrysotherapy: pharmacological and clinical correlates. *J. Rheumatol.* **2**:401–410 (1975).
60. Sternlieb, I., and I. H. Scheinberg. Prevention of Wilson's disease in asymptomatic patients. *N. Engl. J. Med.* **278**:352–359 (1968).
61. Kjellstrom, T., C. G. Elinder, and L. Friberg. Conceptual problems in establishing the critical concentrations of cadmium in human kidney cortex. *Environ. Res.* **33**:284–295 (1984).
63. Cippitelli, M., A. Sica, V. Viggiano, J. Ye, P. Ghosh, M. J. Birrer, and H. A. Young. Negative transcriptional regulation of the interferon-γ promoter by glucocorticoids and dominant negative mutants of c-Jun. *J. Biol. Chem.* **270**:12548–12556 (1995).
64. Liu, W., A. G. Hillmann, and J. M. Harmon. Hormone-independent repression of AP-1-inducible collagenase promoter activity by glucocorticoid receptors. *Mol. Cell. Biol.* **15**:1005–1013 (1995).
65. Caldenhoven, E., J. Liden, S. Wissink, A. Van de Stolpe, J. Raaijmakers, L. Koenderman, S. Okret, J.-Å. Gustafsson, and P. T. Van der Saag. Negative cross-talk between RelA and glucocorticoid receptor: a possible mechanism for the antiinflammatory action of glucocorticoids. *Mol. Endocrinol.* **9**:401–412 (1995).
66. Scheinman, R. I., A. Gualberto, C. M. Jewell, J. A. Cidlowski, and A. S. Baldwin, Jr. Characterization of mechanisms involved in transrepression of NF-κB by activated glucocorticoid receptors. *Mol. Cell. Biol.* **15**:943–953 (1995).
67. Renoir, J.-M., C. Mercier-Bodard, K. Hoffmann, S. L. Bihan, Y.-M. Ning, E. R. Sanchez, R. E. Handschumacher, and E.-E. Baulieu. Cyclosporin A potentiates the dexamethasone-induced mouse mammary tumor virus-chloramphenicol acetyltransferase activity in LMCAT cells: a possible role for different heat shock protein-binding immunophilins in glucocorticosteroid receptor-mediated gene expression. *Proc. Natl. Acad. Sci. USA* **92**:4977–4981 (1995).
68. Burns, K., B. Duggan, E. A. Atkinson, K. S. Famulski, M. Nemer, R. C. Bleackley, and M. Michalak. Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. *Nature (Lond.)* **367**:476–480 (1994).
69. Singh, P., J. Coe, and W. Hong. A role for retinoblastoma protein in potentiating transcriptional activation by the glucocorticoid receptor. *Nature (Lond.)* **374**:562–565 (1995).

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