Direct Evidence for Intra- and Intermolecular Disulfide Bond Formation in the Human Glucocorticoid Receptor

INHIBITION OF DNA BINDING AND IDENTIFICATION OF A NEW RECEPTOR-ASSOCIATED PROTEIN*

(Received for publication, September 26, 1988)

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We have investigated the potential for the steroid affinity-labeled human glucocorticoid receptor to form both intramolecular and intermolecular disulfide bonds. Glucocorticoid receptors labeled in intact HeLa S3 cells with the covalent affinity label [3H]dexamethasone mesylate ([3H]DLM) were analyzed on denaturing 5-12% polyacrylamide gels under both nonreducing and reducing conditions. Under nonreducing conditions the affinity-labeled receptor migrated as a heterogeneous species having an average molecular mass of ~96 kDa whereas, under reducing conditions, the receptor migrated as a more discrete form. These data suggest that a reducing environment can influence the structure of the glucocorticoid receptor monomer and further imply that sulfhydryl groups within the affinity-labeled receptor are available for modification. To pursue this observation in greater detail, we tested the effect of oxidizing conditions on the structure of the glucocorticoid receptor. The presence of low concentrations (0.125–0.5 mM) of three oxidizing reagents (sodium tetrathionate, disulfiram, and iodosobenzoate) altered the migration of the affinity-labeled receptor resulting in forms of apparent lower molecular mass (as low as 78 kDa). This altered migration, not seen with most other cytosolic proteins, is consistent with the formation of intramolecular disulfide bonds within the receptor which presumably cause it to assume a folded conformation and migrate faster through the gel. At higher concentrations of these reagents (up to 5.0 mM), we also detect a saturably labeled [3H]DLM band which has a higher molecular mass (~140 kDa), indicating the formation of intermolecular disulfide bonds between the [3H]DLM-labeled receptor and another closely associated protein(s) having a molecular mass of approximately 40 kDa. The effects which these oxidizing reagents have on glucocorticoid receptor structure are completely reversed upon the addition of dithiothreitol, indicating that the observed changes in migration do not reflect receptor proteolysis but rather a folding and unfolding within the receptor monomeric protein. We have also analyzed the effect of this oxidation/reduction on the function of the glucocorticoid receptor. Oxidation of the [3H]DLM-labeled receptor complex with 0.5 mM sodium tetrathionate inhibited activation of receptor to a form capable of binding to DNA-cellulose. This inhibition can be reversed with dithiothreitol at 25 °C but not at 0 °C, suggesting that these oxidizing reagents are inhibitory at the transformation and/or activation steps. Furthermore, by utilizing a nitrocellulose blotting procedure, we demonstrated that only the reduced form of the receptor can bind to DNA containing the glucocorticoid regulatory element. These studies provide the first direct evidence that disulfide bonds can form within the glucocorticoid receptor and that only the reduced forms of the receptor can bind to DNA.

Glucocorticoids exert a variety of effects on target tissues which are mediated through the glucocorticoid receptor. Upon interaction with steroid, the glucocorticoid-receptor complex becomes activated to a form which can associate with specific regions of DNA and thus regulate gene expression. Therefore, the structure of the receptor protein is key to the regulation of gene expression by glucocorticoids. Advances such as the development of the covalent affinity label, [3H]dexamethasone mesylate (1), and more recently the cloning of the cDNA for the human glucocorticoid receptor and determination of its amino acid sequence (2) have allowed a more rigorous analysis of glucocorticoid receptor structure. Several laboratories have focused on analyzing potential post-translational modification in receptor structure which may be prerequisites for steroid binding and ultimately activation of the steroid-receptor complex to its DNA binding form. These investigations have shown that both phosphate and sulfhydryl groups on the receptor are involved in its ability to bind steroid (1, 3-5). Dephosphorylation as well as sulfhydryl modification inactivates the receptor to a state which is unable to bind glucocorticoid (4–10). The importance of these post-translational modifications on transformation, activation, and binding of the glucocorticoid receptor complex to DNA is less clear. Recent evidence suggests that, even though the receptor is a phosphoprotein (11–13), a phosphorylation/dephosphorylation mechanism is not involved in activation of the glucocorticoid receptor to a DNA binding form (14–19). However, it has become evident that sulfhydryl groups are involved in the transformation and activation processes of the oligomeric glucocorticoid receptor complex (17). Initial studies showed that addition of sulfhydryl modifying reagents to crude receptor preparations inhibited binding of glucocorticoid-receptor complexes to DNA (18–20). Subsequently, other laboratories have supplied evidence for the importance of sulfhydryl interactions in the transformation and activation of the glucocorticoid-receptor complex to a DNA binding form as well as its ability to be stabilized against thermal inactivation (18-24). Sulfhydryl groups may also play a role in association of the receptor with the nuclear matrix (25). Although these studies

* This work was supported by National Institutes of Health Grants AM32459, AM32460, and AM32078. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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provide compelling evidence for the role of sulfhydryl groups in steroid receptor structure and function, there is no direct evidence that sulfhydryl modifications occur within the glucocorticoid receptor monomer itself. The abundance of cysteine residues within the DNA binding region of the glucocorticoid receptor (10 residues within a 60-amino acid stretch) is consistent with the role of these residues in DNA-receptor interaction (26). Additionally, both the steroid and immunological domains of the receptor contain 5 cysteine residues, each of which are potentially available for interaction. In this paper we provide evidence for the formation of both intramolecular disulfide bonds within the glucocorticoid receptor monomer as well as intermolecular disulfide bonds between the receptor monomer and another protein(s) of approximately 40 kDa. Furthermore, we demonstrate that there is a close association between the oxidation-reduction state of the glucocorticoid receptor monomer and its capacity to bind DNA.

**MATERIALS AND METHODS**

[1H]Dexamethasone mesylate (48.9 Ci/mmol) and EN[1H]ANCE were obtained from Du Pont-New England Nuclear. A solution of radioinert dexamethasone (Steroidal, Wilton, NH) was prepared in distilled water at 100 mg/ml. Chemicals used for polyacrylamide gels (SDS, 1-acrylamide, bisacrylamide, TEMED, and ammonium per sulfate) were ultrapure grade from Bethesda Research Laboratories. High molecular weight prestained protein standards used for gel electrophoresis were also obtained from Bethesda Research Laboratories. Centricon™ 30 microconcentrators were purchased from Amicon Corp. (Danvers, MA) and nitrocellulose from Schleicher and Schuell.

**Cell Culture Techniques—HeLa S3 cells were grown in suspension culture at 37°C in Joklik's minimum essential medium (JMEM) containing 75 units/ml penicillin G and 50 μg/ml streptomycin sulfate (GIBCO) supplemented with 2 mM glutamine (Sigma) and a 3% mixture (1:1) of heat-inactivated fetal calf serum/calf serum (HyClone Laboratories, Logan, UT). Approximately 1–2 liters of cell suspension were centrifuged at 2900 x g in 250-ml polystyrene bottles. The cell pellets were combined and resuspended in unsupplemented JMEM. This cell suspension was then washed in 50 ml of unsupplemented media. The resulting pellet (~2–5 ml volume) was resuspended in the appropriate volume of unsupplemented JMEM to give a final cell concentration of 5–10 x 10⁶ cells/ml. Cell number was determined by counting an aliquot of the cell suspension in a Zet Counter Coulter (Coulter Electronics, Hialeah, FL).

**Whole Cell Labeling of Glucocorticoid Receptors and Cytosol Preparation—**Suspensions of HeLa S3 cells in unsupplemented media (5–10 x 10⁶ cells/ml) were incubated at 0°C with 2 x 10⁻⁸ M [1H]dexamethasone mesylate minus or plus a 1000-fold excess of unlabeled dexamethasone (27). The total cell suspension volume was between 1 and 4 ml depending on the experiment. After 2 h of incubation with hormone at 0°C, the cells were centrifuged at 1525 x g, the supernatants were discarded, and the cell pellets were homogenized with a Tekmar Ultra Turrax homogenizer (Tekmar, Cincinnati, OH) in the following buffers: (a) 10 mM Tris-HCl, pH 8.3, 1 mM EDTA (TE pH 8.3); or with the following additions: (b) 0.5–2.0 mM sodium tetrathionate, (c) 0.5–2.0 mM disulfiram, (d) 0.5–2.0 mM iodosobenzoate, (e) 10 mM iodosacetic acid, (/) 10 mM dithiothreitol. The homogenates were then centrifuged at 100,000 x g for 1 h at 0°C. Each high speed supernatant (cytosol) was treated with a pellet from an equal volume of dextran-coated charcoal (1% activated charcoal, 0.1% dextran in 1.5 mM MgCl₂) in order to remove free steroid. Alternatively, cytosols were prepared in TE pH 8.3, treated with dextran-coated charcoal, and then incubated for 30 min at 0°C with varying concentrations of the sulfhydryl reagents listed above. This allowed more control over the experimental conditions but had the same effect as homogenizing in the presence of the reagents. Cytosols were then concentrated 3-5-fold by volume using a Centricon™ 30 microconcentrator.

**DNA-Cellulose Binding Assay—**Cytosols were prepared as described above in TE, pH 8.3. Calf thymus DNA-cellulose was prepared in the same buffer at a concentration of 215 μg of DNA/ml. 100 μl of this DNA-cellulose suspension was aliquoted into Eppendorf tubes and maintained at 0°C. Cytosols were activated at 25°C for 30 min in buffer alone or in the presence of the following: 1) 10 mM iodosacetic acid; 2) 0.5 mM sodium tetrathionate; or 3) 10 mM diithiothreitol. The activated cytosols were used for affinity-labeling of the DNA-cellulose suspension and incubated at 0°C for 1 h to determine percent binding to DNA. In order to assess background binding, cytosol which had not been activated but had been kept at 0°C was also added to an aliquot of DNA-cellulose suspension. After the 1-h incubation at 0°C, the DNA-cells were pelleted and washed twice with 1-ml volumes of TE, pH 8.3, at 0°C. The ability of DTT to reverse the effect of these reagents on DNA binding was analyzed at both 0 and 25°C. For reversibility studies: 1) cytosols were incubated with DNA-cellulose at 0°C (as above) except in the presence of 10 mM DTT; 2) cytosols were reincubated at 25°C for 30 min in the presence of 10 mM DTT and then with 5 mM EDTA for 30 min.

**DISCUSSION**

The activity present in these pellets was analyzed by dissolving them in ScintiVerse E and quantitating tritium using a Beckman LS 3801 scintillation counter.

**SDS-Polyacrylamide Gel Electrophoresis—**Samples which had been concentrated to ~200 μl were divided into half. Half of each sample was mixed 1:1 with electrophoresis buffer containing 4% SDS (6.25% w/v SDS, 37.5% (w/v) glycerol, 4.6% w/v SDS, 0.125 M Tris, pH 6.8) and half with reducing 2 x sample buffer (+20 mM DTT). Samples were then heated at 100°C for 2 min and then frozen at ~70°C until electrophoresis. Samples were electrophoresed on 5–12% polyacrylamide gels (3 x 160 x 140 mm) according to the method of Laemmli (28) at 150–200 V until the bromphenol blue dye front had reached the bottom of the gel. Preincubated molecular weight markers were electrophoresed as standards and have the following apparent molecular mass (according to Bethesda Research Laboratories specifications): myosin, 205 kDa; phosphorylase b, 105 kDa; bovine serum albumin, 67 kDa; ovalbumin, 42 kDa. Gels were transferred to nitrocellulose (BA85) according to the method of Towbin et al. (29). Blots were sprayed with EN[1H] ANCE and fluorographed for 7–10 days. To analyze DNA binding, gels were prepared as described previously (30). Briefly, gels were soaked for 2 h in renaturation buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.0, 20 mM EDTA, 0.1 mM dithiothreitol, 4 mM urea) and then transferred to a vertically placed nitrocellulose filter. The blots were preincubated for 2 h in binding buffer (5% nonfat dry milk in 50 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) to block nonspecific binding and then incubated for 2 h at room temperature in the same buffer containing nick-translated [³²P]-labeled MTV long terminal repeat DNA which contains the glucocorticoid regulatory element (GRE) (Specific activity, ~7.5 x 10⁶ cpm/pg DNA). Blots were washed with binding buffer, air dried, and autoradiographed. This procedure allows visualization of the selective interaction between the glucocorticoid receptor and DNA containing the GRE (30). Little or no interaction occurs between the receptor and non-GRE-containing DNA (31).

**RESULTS**

**Affinity Labeling of Glucocorticoid Receptor—**HeLa S3 cells were incubated with [1H]dexamethasone mesylate ([1H]DM) at 0°C for 2 h according to the whole cell labeling procedure we have described previously (27). Cytosols were prepared from these cells and electrophoresed on denaturing 5–12% polyacrylamide gels under nonreducing (+DTT) or reducing (+DTT) conditions (see "Materials and Methods"). Fig. 1 is a fluorograph from a representative experiment. Under nonreducing conditions, the affinity-labeled receptor appears as a heterogeneous protein having an apparent molecular mass ranging from ~97 to 85 kDa (Fig. 1, a). This labeling is competed for by an excess of unlabeled dexamethasone (+DEX) indicating a specific receptor-hormone interaction. A second protein having a molecular mass of ~76 kDa (Fig. 1, b) is also saturably labeled with [1H]DM. Both the 97-kDa
disulfiram, and iodosobenzoate, which are oxidizing reagents that induce the formation of disulfide bonds between conformationally available sulphydryl groups. Low concentrations (below \( \sim 2.0 \text{ mM} \)) of the three oxidizing reagents induce predominantly intramolecular disulfide bonds to form, whereas higher concentrations will cause not only intra- but also intermolecular disulfides to form (32). These reagents were chosen because they are relatively specific for modifying sulphydryl groups, and therefore the effects seen on the structure of the glucocorticoid receptor by these reagents is likely to represent a sulphydryl group modification.

**Effect of Alkylation or Reduction of Sulphydryls on Glucocorticoid Receptor Structure**—HeLa cells which had been incubated with \([^{3}H] \text{DM} \) were homogenized either in TE, pH 8.3, alone (CON) or in the presence of 10 mM iodoacetamide (IAA) or 10 mM dithiothreitol (DTT). Cytosols were prepared and samples were electrophoresed under either nonreducing (−DTT) or reducing (+20 mM DTT) conditions. The fluorograph from a representative gel is shown in Fig. 2. Neither reagent, iodoacetamide, nor dithiothreitol interferes with saturable affinity labeling of the receptor since receptor labeling in the CON (no added reagent) and the IAA and DTT lanes is the same. This indicates that the cysteine within the steroid binding domain to which dexamethasone mesylate specifically binds (33) does not interact with these sulphydryl modifying reagents after the \([^{3}H] \text{DM} \) has bound. These sulphydryl reagents do, however, compete effectively with the nonsaturable binding mentioned previously since the proteins migrating just above the 42-kDa marker (c or c') are labeled to a lesser extent in the samples treated with either IAA or DTT than in the control (CON). In all cases the alternate receptor form (b) is present and is affected in a fashion similar to the intact receptor. As seen in Fig. 1, the receptor migrates as a heterogeneous band which ranges over 6 kDa (a) in the absence of any added sulphydryl reagents (CON) and under nonreducing conditions. In contrast, addition of either an alkyllating (IAA) or reducing (DTT) reagent causes the \([^{3}H] \text{DM} \)-labeled receptor to migrate as a discrete band which ranges over only 3 kDa (a). This effect is similar to that seen in Fig. 1 for CON receptor which was electrophoresed under reducing conditions. Furthermore, when these same samples are electrophoresed on Western blots by an antibody to the glucocorticoid receptor. Thus, this \( \sim 76 \text{-kDa} \) protein is probably a form of the receptor which may result from proteolytic degradation or translation of an alternate receptor transcript and will be referred to as the alternate receptor form throughout the paper. Several other proteins of molecular mass \( \sim 55 \) to 50 kDa (Fig. 1, c) are labeled with \([^{3}H] \text{DM} \). The extent of labeling of these proteins varies with the experiment; however, the labeling is not competed for by excess unlabeled dexamethasone, indicating that these are nonreceptor proteins. When the same cytisos are electrophoresed in the presence of DTT (reducing conditions) the migration of the 97- and 76-kDa receptor forms is altered, and they appear as more discrete bands (Fig. 1, a and b, respectively). \([^{3}H] \text{DM} \)-labeled glucocorticoid receptor consistently displays this behavior in response to DTT suggesting that sulphydryl modification occurs and that the oxidation/reduction state of sulphydryls in the receptor influences its electrophoretic migration.

**Fig. 1.** Affinity labeling of human glucocorticoid receptor. HeLa cells were incubated with \( 2 \times 10^{-8} \text{ M} \) \([^{3}H] \text{DM} \) for 2 h at 0 °C, in the absence (−DEX) or presence of a 1000-fold excess of unlabeled dexamethasone (+DEX). Cells were homogenized in TE, pH 8.3, buffer; cytosols were prepared and processed as described under "Materials and Methods." Half of each sample was mixed 1:1 with sample buffer (NONREDUCING) and half with sample buffer plus 20 mM DTT (REDUCING). Samples were then heated to 100 °C, electrophoresed on 5–12% polyacrylamide gels, and fluorographed. Prestained molecular mass markers were electrophoresed in an adjacent lane: myosin, 205 kDa; phosphorylase b, 103 kDa; bovine serum albumin, 67 kDa; ovalbumin, 42 kDa. a, intact receptor; a', reduced intact receptor; b, alternate receptor form; b', reduced alternate receptor form; c and c', nonsaturably labeled proteins, untreated and reduced, respectively.

**Fig. 2.** Effect of alkylation and reduction on affinity-labeled receptor. HeLa cells were incubated for 2 h at 0 °C with \( 2 \times 10^{-8} \text{ M} \) \([^{3}H] \text{DM} \). Cells were then homogenized in: TE pH 8.3 alone (CON); plus 10 mM iodoacetamide (IAA); or plus 10 mM dithiothreitol (DTT). Cytosols were prepared as described under "Materials and Methods." Half of each sample was mixed 1:1 with sample buffer alone (NONREDUCING) and half with sample buffer plus 20 mM DTT (REDUCING); then heated to 100 °C, electrophoresed on a 5–12% polyacrylamide gel, and fluorographed. Molecular mass markers and labels are as in Fig. 1.

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*C. M. Silva and J. A. Cidlowski, unpublished results.*
resed under reducing conditions the receptor migrates as a tight discrete band which ranges over only 2-3 kDa in all cases (a.). Although these changes we see upon reduction (or alklylation) are subtle, we have consistently seen them in 10 separate experiments. These experiments demonstrate that when the sulfhydryl groups on the receptor are alkylated or reduced, either during homogenization and/or during electrophoresis, the receptor migrates as a single discrete form. Since reduction and alklylation were found to change the migration of the receptor relative to the untreated [3H]DM-labeled receptor, this implies that the sulfhydryls within the receptor are oxidized either in the cell and/or during the homogenization step. Therefore, we next evaluated the ability of sulfhydryls within the receptor to be oxidized by analyzing the effect of various oxidizing reagents on the structure of the affinity-labeled glucocorticoid receptor.

Effect of Oxidation of Sulfhydryl Groups on the Structure of the Glucocorticoid Receptor—HeLa cells which had been incubated with [3H]DM as described previously were homogenized either in TE, pH 8.3, buffer alone (CON) or in the presence of a low (0.5-1.0 mM) concentration of one of the three oxidizing reagents mentioned above. Cytosols were then electrophoresed under either nonreducing or reducing conditions (see “Materials and Methods”). A representative fluorograph showing the effect of oxidizing conditions on the receptor is seen in Fig. 3. Again, in samples to which no sulfhydryl reagents were added (CON), the receptor (a) migrates as a heterogeneous species of protein under nonreducing conditions and as a discrete moiety under reducing conditions (a.). However, the addition of low concentrations of sodium tetrathionate (NaTT), iodosobenzonate (ISBA), or disulfiram (DIS) to the samples significantly influences the migration of the [3H]DM-labeled receptor when compared to receptor prepared in the absence of any of these reagents (CON). In the presence of these oxidizing reagents and under nonreducing electrophoretic conditions, the receptor migrates faster through the gel resulting in a broad species of receptor which spans ~13 kDa and has molecular masses which range from 91 to 78 kDa (a.). For example, in the sample treated with NaTT, the receptor migrates as three discernible forms which all have a lower apparent molecular weight than that of the untreated sample (CON) which spans only 6 kDa at a molecular mass range of 93-87 kDa. In the presence of DIS or ISBA, the receptor also migrates as a heterogeneous population with some forms having a lower molecular weight than untreated receptor (CON), although the discrete species are not as apparent as in the NaTT-treated sample. These forms (a,) range over 11 kDa and migrate as small as 75 kDa. The migration of the ~76-kDa alternate receptor form is similarly affected. As in Fig. 2, the effect of the sulfhydryl-containing reagents on the nonsaturably labeled proteins (c) is evident. These proteins bind [3H]DM to a much lesser extent in the presence of the sulfhydryl reagents (NaTT, ISBA, and DIS) than in untreated samples (CON). We propose that these oxidizing reagents, NaTT, DIS, and ISBA, at low concentrations (0.5 or 1.0 mM) induce the formation of intramolecular disulfide bonds between available sulfhydryl groups within the receptor monomer. This presumably causes the receptor to assume a folded conformation and thus migrate faster through the gel than untreated receptor (CON). In contrast, when these same samples are electrophoresed under reducing conditions (by adding 20 mM DTT to the sample buffer) the receptor migrates as a single discrete band spanning only 4 kDa just below the 103-kDa marker (a.). The reversibility of this effect upon the addition of DTT indicates that the altered migration of the receptor is due to a mechanism involving oxidation-reduction of disulfide bonds. Upon addition of DTT to the samples, the disulfide bonds that were induced to form are reduced, thus causing unfolding of the receptor monomer. This change is reflected in a homogeneous population of receptor which migrates slower through the gel as a single discrete band. We have used the three oxidizing reagents under various times and concentrations in a total of 17 separate experiments and have routinely seen this same effect on the migration of the receptor which is then reversed by the addition of DTT to the sample. Although the calculated molecular weight for the receptor may differ between experiments, the magnitude of change upon oxidation-reduction is consistent (13 kDa ± 2). Together, these results indicate that the sulfhydryl groups within the receptor can be induced to form disulfide bonds and thus influence the conformation (folding) of the receptor. This effect occurs despite the fact that one sulfhydryl group is covalently linked to the affinity label [3H]DM. Furthermore, the extent of folding induced by the addition of these oxidizing reagents compared to the untreated sample indicates that additional sulfhydryls within the receptor have the potential to form disulfide bonds.

Effect of Sulfhydryl Reagents on Cytosolic Proteins—We next sought to determine whether the effect on the migration of the receptor upon addition of these sulfhydryl reagents was specific for receptor or a general effect on all cytosolic proteins. Fig. 4 is a Coomassie Blue staining pattern of cytosols which were prepared in the presence of these sulfhydryl-modifying reagents. Each lane is labeled with the reagent used. Both low (0.5 mM) and high (2.0 mM) concentrations of NaTT were tested. Samples were electrophoresed under both nonreducing and reducing conditions. Treatment with NaTT seems to cause aggregation of a few proteins at the top of the gel as well as minor changes in the staining pattern in particular areas (indicated by the arrows). However, the overall pattern of migration is the same for the NaTT-treated samples, and the proteins in these lanes migrate as discrete homogeneous bands. These data indicate that the altered migration of the [3H]DM-labeled receptor upon oxidation with NaTT is a property of the receptor protein itself and not an effect of these reagents on the general migration of cytosolic proteins under these conditions.
**Effect of Low and High Concentrations of Oxidizing Reagents on Glucocorticoid Receptor Structure**—To characterize in greater detail the effect of the oxidizing reagents on the structural conformation of the receptor, samples were treated with a wide concentration range of these reagents. In these experiments, reagents were added to the cytosol rather than to the homogenization buffer in order to control more precisely for reagent concentration as well as time of treatment. Cytosols were prepared as described under “Materials and Methods” and then treated for 30 min at 0°C with various concentrations of NaTT ranging from 0.125 to 5.0 mM. Time course experiments revealed that 30 min at 0°C was sufficient for the sulfhydryl reaction to occur (data not shown). Fig. 5 is the fluorograph showing the [3H]DM labeling pattern of samples treated with these concentrations of NaTT and electrophoresed under either nonreducing or reducing conditions. With no added reagent (0 mM, nonreducing), the receptor migrates as a heterogeneous protein species (a) at 101-93 kDa in this experiment. The alternate receptor form (b) as well as the nonspecific [3H]DM binding proteins (c) are also seen under these conditions. As NaTT is added to the cytosol, receptor forms which migrate faster through the gel than untreated receptor (0 mM) are evident (a1). Concentrations of NaTT as low as 0.125 mM alter the migration of affinity-labeled receptor and two forms of receptor, one having a molecular mass of 96-91 kDa and the other having a molecular mass of 85 kDa appear (a2). As the concentration of NaTT is increased to 0.25 mM, three different forms of receptor which are comparably labeled and have molecular mass of 93, 90, and 85 kDa are induced. At 0.375 and 0.5 mM concentrations of NaTT, those forms which have the fastest mobility (at 89 and 83 kDa) become most prominent. These studies indicate that NaTT can induce the formation of multiple intramolecular disulfide bonds within the affinity-labeled human glucocorticoid receptor. The relative amounts of each species vary depending on the concentration of NaTT used. Under these oxidizing conditions (0.125–0.50 mM NaTT) the alternate receptor form (b) and the nonspecific [3H]DM binding proteins (c) are not seen, indicating an effect of NaTT on labeling. When these same samples (0.125–0.50 mM NaTT) are electrophoresed under reducing conditions, the receptor migrates as a single discrete form (a3) at 98–93 kDa, indicative of an unfolded homogeneous population. Again, the fact that the effect on migration can be reversed upon addition of DTT suggests that the altered mobility is due to oxidation-reduction of disulfide bonds and not the result of proteolysis.

As the concentration of NaTT is increased to 2.0 and 5.0 mM and samples are electrophoresed under nonreducing conditions we also observe the appearance of a higher molecular weight band which is saturably labeled with [3H]DM (a4). The overall amount of [3H]DM labeling in these lanes is greater than that in the previous lanes (0.125–0.50 mM NaTT) because the samples are from a different experiment. The molecular mass of this [3H]DM-labeled band ranges from 141 to 124 kDa, and we interpret this to represent interaction of the receptor monomer with another protein(s) (of ~45–35 kDa) through the formation of intermolecular disulfide bonds. We have consistently seen (in 10 separate experiments) the formation of this complex with each of the three oxidizing reagents (NaTT, DIS, and ISBA). Furthermore, disulfide bond formation between the affinity-labeled receptor and any additional proteins is not apparent. This band (a4) is not labeled with [3H]DM in the presence of excess unlabeled dexamethasone (data not shown) indicating that this intermolecular interaction involves saturably labeled receptor (a and b) and not the nonspecifically [3H]DM-labeled proteins (c). Our data suggest that the protein(s) which is interacting with the receptor is relatively closely associated with the receptor in the cytosol. No interaction between the receptor and the 90-kDa heat shock protein (hsp) is evident. This was a surprising observation since it has been suggested that the 90-kDa hsp is a component of the 8 S untransformed glucocorticoid receptor complex (34–36). In addition, other laboratories have reported that oxidation by hydrogen peroxide (22) or cupric o-phenanthroline (37) results in cross-linking of the 90-kDa hsp to the 8 S glucocorticoid receptor-containing complex, thus suggesting an intermolecular disulfide interaction between the glucocorticoid binding moiety and the 90-kDa hsp. In our studies, the utilization of NaTT, which is a different type of cross-linking reagent than those mentioned above (and works through a sulfinyl intermediate and not atmospheric oxygen or radical formation (32)), has enabled the identification of another receptor-associated protein(s) which has an apparent molecular mass of ~45–35 kDa. Preliminary data3 indicate that this ~45-kDa protein(s) is also a component of the [3H]DM-labeled 8 S glucocorticoid receptor complex previously described (27).

**Effect of Sulfhydryl-modifying Reagents on the Ability of the Glucocorticoid Receptor to Activate to a DNA-binding Protein**—The results presented thus far indicate that sulfhydryls within the glucocorticoid receptor monomer can be induced to form intramolecular and intermolecular disulfide bonds and that the oxidation-reduction state of these disulfide bonds can effect conformation and thus migration of the receptor during denaturing polycrylamide gel electrophoresis. We sought to determine whether the effect of this oxidation-reduction on the structure of the receptor had coordinate effects on the ability of receptor to activate to a DNA-binding protein. To evaluate this question, we used the standard DNA-cellulose binding assay as described previously (31, 38). Cy-

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3 C. M. Silva and J. A. Cidlowski, unpublished observations.
TABLE I
Influence of sulfhydryl reagents on glucocorticoid receptor activation

| Treatment*            | DNA-cellulose binding†  |
|-----------------------|-------------------------|
|                       | −DTT  | +DTT (0 °C) | +DTT (25 °C) |
| No additions          | 16.7 ± 4.3 | 13.5 ± 0.1 | 224 ± 2.9 |
| 0.5 mM NaTT           | 4.1 ± 4.9 | 5.6 ± 2.2 | 21.7 ± 6.8 |
| 10 mM IAA             | 1.9 ± 0.6 | 3.6 ± 0.2 | 2.0 ± 0.6 |
| 10 mM DTT             | 15.7 ± 7.3 | 16.9 ± 4.3 | 26.0 ± 1.3 |

* These treatments were during the 30-min activation step at 25 °C.

† Treatment with DTT was at 0 °C during the 1-h DNA-cellulose binding step.

†† Treatment with DTT was at 25 °C for 30 min after the initial activation step.

process, the ability of the [3H]DM receptor complex to activate to a DNA binding form is reduced to 4%. Similarly, if 10 mM IAA is present during the activation step, DNA binding is reduced to only 2%. The inhibitory effect of IAA on DNA binding has been seen previously (20), and the evidence suggests that this is an effect directly on DNA binding and not on transformation or activation (18, 22). However, 0.5 mM NaTT, a reagent which we have shown induces intramolecular disulfide bonds within the receptor (Figs. 3 and 5) also inhibits activation of the [3H]DM-labeled receptor to a form capable of binding to DNA (only 4% binding to DNA-cellulose). This result suggests that the folded conformation of receptor is inhibitory to transformation, activation, and/or DNA binding of the receptor. In contrast, if receptors are in a reducing environment during activation (+DTT) the ability to bind to DNA is similar to that of control (~16%). These results indicate that transformation and/or activation (as measured by DNA binding ability) is influenced by the oxidation-reduction state of sulfhydryls.

Reversing the Inhibition of DNA-Cellulose Binding by Reduction of Sulfhydryls—We next attempted to reverse the effect of NaTT on activation by adding DTT to the same sample which had been treated with NaTT, thereby reducing intra- or intermolecular bonds which had been induced to form. To provide some insight into the mechanism of inhibition by NaTT, DTT was added either at 0 °C after the receptor had been activated or at 25 °C during the activation step. As seen in Table I, addition of DTT at 0 °C after the activation step does not reverse the inhibition by NaTT. This result suggests that the effect which NaTT has on inhibiting DNA binding occurs during the 30-min receptor activation step at 25 °C. We reincubated NaTT-treated samples at 25 °C in the presence of DTT and then analyzed for binding to DNA-cellulose. Under these conditions the inhibition by NaTT is totally reversed by DTT, and the amount of binding...
to DNA-cellulose is comparable to the control sample (~22% in both). These results indicate that NaTT is inhibiting activation at 25 °C by a mechanism which is reversible by reduction of sulfhydryls at the activation step. Since we know from results presented earlier (Fig. 3) that 0.5 mM NaTT affects the conformation of the receptor, we postulate that this conformation prevents activation of the glucocorticoid receptor complex to a DNA binding form. In contrast, since alkylation by IAA is an irreversible process, subsequent incubation with DTT (either at 0 or 25 °C) does not reverse the inhibition by IAA. This sample serves, therefore, as an internal control. It is also important to note that reagents which modify proteins in a way similar to IAA but are reversible (i.e. methyl methanethiosulfonate) inhibit DNA binding of the receptor but in a manner that is reversible by DTT at 0 °C (18, 22). Since their effect is reversible by reduction at 0 °C, this indicates that these alkylation reagents have a direct effect on DNA binding and not on activation and/or transformation. Furthermore, in contrast to the effects seen with IAA or NaTT, the addition of DTT either during activation or during the DNA-cellulose binding step results in levels of DNA binding which are comparable to untreated samples (22–26%). These data indicate that reduction of sulfhydryls permits activation of the [3H]DM-labeled receptor to a DNA binding form but does not increase the extent of activation above control levels.

**The Effect of Sulfhydryl-modifying Reagents on the Ability of Receptor to Bind to Specific DNA**—We have used the Southwestern blot procedure described previously in our laboratory (30) to analyze the ability of oxidized and reduced forms of the [3H]DM-labeled receptor to bind in a selective manner to DNA containing the glucocorticoid regulatory element (GRE). Cytosols were treated with different concentrations of oxidizing reagents, NaTT (or ISBA), as described previously (Fig. 5 and "Materials and Methods"). These samples were electrophoresed under either nonreducing or reducing conditions and then analyzed for both [3H]DM binding, or [32P]MMTV GRE binding forms of the intact receptor which has been treated with 0.5 mM NaTT migrates as a heterogeneous species of forms under nonreducing conditions ranging from 103 to 85 kDa (a). However, only a 103-kDa form of the receptor (a) is able to bind to the [32P]-labeled GRE DNA as seen in lane D. This [32P]-labeled band comigrates with the receptor in the CON sample (both lanes S and D) indicating that the oxidized (0.5 mM NaTT-treated) receptors (a) cannot bind DNA. The amount of DNA binding, as indicated by the intensity of the [32P]-labeled band, is also considerably less in the 0.5 mM NaTT sample than in the control. Furthermore, comparison of the [3H]DM labeling (S) with the [32P]DNA binding (D) suggests that the DNA is interacting with forms of the receptor which do not bind [3H]DM. This result is not without precedent, and there are a number of explanations for why it may be occurring. First, Willmann and Beato (40) have shown that receptor from crude rat liver cytosol which has been denatured in order to dissociate receptor-associated proteins can be activated and binds selectively to MMTV GRE without being bound to steroid. The Southwestern procedure which we use here to determine selective interaction with DNA also denatures cytosol, dissociating receptor-associated proteins as well as most probably effecting a conformational change (i.e. activation) during the electrophoresis and/or renaturation process. It is not surprising, therefore, that nonsteroid binding forms of the receptor will bind selectively to the [32P]MMTV GRE in our system. Second, the Southwestern technique which involves binding of a [32P]-labeled DNA fragment is more sensitive than

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**Fig. 6. Steroid binding and DNA binding patterns of oxidized affinity-labeled glucocorticoid receptor.** HeLa cells were incubated with [3H]DM, homogenized in TE, pH 8.3, buffer, and cytosols prepared (see "Materials and Methods"). Samples were then incubated at 0 °C for 30 min with TE, pH 8.3 (CON), 0.5 mM NaTT, or 5.0 mM NaTT buffer. Half of each sample was mixed 1:1 with sample buffer alone (NONREDUCING) or plus 20 mM DTT (REDUCING). Each of these was then electrophoresed on two separate 5–12% polyacrylamide gels. One gel was prepared for fluorography in order to analyze [3H]DM binding (S); and one gel was renatured and probed with a [32P]-labeled piece of DNA containing the GRE (D) as described under "Materials and Methods." Adjacent lanes, therefore, represent the same sample, electrophoresed under the same conditions, and then analyzed for either [3H]DM binding (S) or [32P]GRE binding (D). Prestained markers are as in previous figures. Labels indicate untreated, oxidized (,) or reduced (,) [3H]DM binding, or [32P]MMTV GRE binding forms of the intact receptor (a) or the alternate receptor form (b). Nonsaturably labeled nonreceptor proteins are denoted by c.
fluorography of \([\text{H}]\text{DM}\) binding. Finally, we have seen in other unrelated studies that nonsteroid binding antibody-reactive forms of the receptor do bind to \([\text{P}]\text{MMTV GRE}\) as analyzed by the Southwestern blot procedure. Analysis of the 5.0 mM NaTT-treated samples for DNA binding shows similar results. Interestingly, the 141-124-kDa band (seen with 5.0 mM NaTT) which represents intermolecular disulfide bonds between the receptor and another protein (a) cannot bind to \([\text{P}]\text{MMTV GRE}\) DNA. Only that form of receptor which has a molecular mass of 103 kDa is able to interact with \([\text{P}]\text{MMTV GRE}\) DNA. The amount of DNA binding is even less in this 5.0 mM NaTT sample (as compared to CON) presumably since more of the receptors are present in disulfide-bonded forms (either intramolecularly and/or intermolecularly) and are therefore unavailable for interaction with the DNA. For comparison, the same samples described above were also electrophoresed under reducing conditions. In all cases, the \([\text{H}]\text{DM}\)-labeled receptor migrates as a single discrete band just below the 103-kDa marker (a) and can bind to DNA as seen by a \([\text{P}]\text{MMTV GRE}\) band in the adjacent lanes (a). Furthermore, the extent of DNA binding is substantially increased over that seen in the nonreducing samples. Under reducing conditions, it appears that there is some binding of the lower molecular weight form of the receptor to \([\text{P}]\text{MMTV GRE}\) (b). These results clearly illustrate that only the reduced form (probably unfolded) of the receptor can bind to DNA. Forms of receptor which contain intramolecular disulfide bonds or are intermolecularly disulfide-bonded to another protein do not appear to bind to DNA. The results seen with NaTT on binding to a specific DNA fragment were confirmed using 0.2 and 0.5 mM concentrations of another oxidizing reagent, ISBA (data not shown). Therefore, the results of Fig. 6 are consistent with DNA-cellulose binding results in Table I and indicate that the receptor must be fully reduced in order to bind effectively to DNA.

**DISCUSSION**

Recent data from a number of laboratories suggest that sulfhydryl interactions play an important role not only in steroid binding but also in transformation and activation of the glucocorticoid-receptor complex (1, 4, 18–20). However, the direct role of those sulfhydryls which are part of the glucocorticoid binding monomer itself has not been established. In this paper we report that sulfhydryl groups within the human glucocorticoid receptor can form intramolecular disulfide bonds as well as intermolecular disulfide bonds with another protein(s). We have found that these interactions occur despite the fact that one sulfhydryl group within the steroid binding domain of the receptor is unavailable for interaction since it has been covalently modified with the affinity label, \([\text{H}]\text{dexamethasone mesylate}\). These observations indicate that the remaining sulfhydryls within the receptor are susceptible to oxidation-reduction and potentially can form disulfide bonds which influence receptor structure and function.

By labeling with the covalent affinity label, \([\text{H}]\text{dexamethasone mesylate}\), and analyzing the receptor on denaturing polyacrylamide gels, we have shown that the apparent molecular weight of the receptor is altered during oxidation-reduction. We report an approximately 13-kDa difference in apparent molecular weight of the oxidized versus reduced form of the receptor (Fig. 3). The magnitude of this effect suggests that extensive conformational changes can occur within the receptor’s secondary structure upon oxidation-reduction. This apparent difference in molecular weight of a protein under oxidizing versus reducing electrophoretic conditions has been observed with a number of membrane-bound proteins including the \(\beta\)-adrenergic receptor (41), the interleukin-3 receptor (42), and the human chorionic gonadotropin receptor (43). Interestingly, the difference between the apparent molecular mass of these proteins under nonreducing versus reducing conditions is 13, 11, and 15 kDa, respectively. This difference in apparent molecular weight can be explained by the ability of sulfhydryls within a protein to form intramolecular disulfide bonds under oxidizing and/or nonreducing conditions. Formation of these intramolecular disulfide bonds within a protein can affect its conformation causing it to exist in a more folded globular state. A protein which has assumed this folded conformation would migrate more rapidly through a polyacrylamide gel than its unfolded form (41). Reduction of the intramolecular disulfide bonds results in unfolding of the protein causing it to assume a more rodlike conformation and to migrate more slowly through the gel. The molecular weight difference which we have observed with the affinity-labeled glucocorticoid receptor resembles the effects described for the \(\beta\)-adrenergic, interleukin-3, and human chorionic gonadotropin receptors which all form intramolecular disulfide bonds. Furthermore, this oxidation-reduction of intramolecular disulfide bonds has recently been proposed as a mechanism of activation for these membrane-bound receptors (44). Despite the fact that the redox environment is different for a membrane-bound protein versus a cytoplasmic protein such as the glucocorticoid receptor, it is not unreasonable to propose that a similar mechanism could be involved in the activation of the glucocorticoid receptor complex to a DNA binding form. Although the intracellular environment is generally a reducing one, various factors including microenvironments, protein-protein interactions, as well as physiological states of the cell could affect the redox potential of a cytoplasmic protein (45–47). Therefore, it is possible that the oxidation-reduction of disulfide bonds could play a role in the mechanism of glucocorticoid receptor activation. Our observation that the oxidation-reduction state of sulfhydryls within the receptor affects its ability to activate to a DNA binding form supports this hypothesis. We have found, using both a DNA-cellulose binding assay (Table I) as well as the Southwestern blot technique which directly analyzes protein-DNA interactions after gel electrophoresis (Fig. 6), that the same reagents which induce disulfide bond formation within the glucocorticoid receptor also inhibit binding of the glucocorticoid-receptor complex to DNA. Not only does 0.5 mM NaTT alter the mobility of the receptor during polyacrylamide gel electrophoresis (indicative of a conformational change), but it also inhibits activation of the receptor to a form capable of binding to DNA-cellulose (see Figs. 3 and Table I). Furthermore, both the effect on gel migration and the inhibition of DNA-cellulose binding is reversed by reduction with DTT. These results directly correlate the conformation of the receptor as influenced by sulfhydryl groups with the ability of the receptor to activate to a DNA binding form. The DNA-cellulose data are further supported by the Southwestern blot technique which directly analyzes the ability of the glucocorticoid receptor to bind to a specific DNA fragment. This technique allows analysis of \([\text{H}]\text{DM}\) binding and DNA binding simultaneously. Our data from both DNA-cellulose binding and Southwestern blot analysis suggest that covalent interaction of \([\text{H}]\text{DM}\) with cysteine 638 (33) is not essential for DNA binding. This, however, does not rule out the possibility that this sulfhydryl may be important for DNA-receptor interaction in vivo. Furthermore, the faster migrating (i.e. oxidized) forms of the receptor are unable to bind selectively to DNA containing the GRE. Only the reduced form of the glucocorticoid
receptor can bind to this DNA fragment. Based on our observations, we propose that activation of the receptor may involve reduction of intramolecular disulfide bond(s) which result in exposure of the DNA binding domain. Although this model is speculative, it is consistent not only with our results but with those from other laboratories (18, 19, 21, 22). Taken together, these data lend support to the hypothesis that oxidation-reduction of disulfide bonds within the glucocorticoid receptor protein plays a role in its activation to a DNA binding form.

In addition to the evidence we have presented for the possible role of intramolecular disulfide bonds, we also have evidence for the interaction of the glucocorticoid receptor with another protein(s) through intermolecular disulfide bonds. We have found that at increased concentrations (up to 5.0 mM) of the oxidizing reagents (NaTT, ISBA, DISS) the formation of a higher molecular mass form (ranging from 140 to 125 kDa) is induced (Figs. 5 and 6). The appearance of this form, which is saturably labeled with [3H]DM, is reversed by DTT. These observations indicate the formation (through intermolecular disulfide bonds) of a complex between the receptor and another protein(s) which has a molecular mass of approximately 45-35 kDa. Because this is the only higher molecular weight form seen with such high concentrations of oxidizing reagents, we suggest that there is a unique association between the receptor and this 45-35 kDa protein(s) in the cytosol. Furthermore, we found, using the Westblott technique, that the receptor was not able to bind selectively to DNA while associated with this protein(s) (Fig. 6). There is evidence from other laboratories that sulfhydryls are involved in the interaction of steroid receptors with other proteins. In both the glucocorticoid (17, 22, 23, 37) and the androgen (48) receptor systems, there is evidence that reduction of sulfhydryls plays a role in conversion of the 8 S oligomeric complex to the 4 S steroid binding monomer. Furthermore, it has been shown that this 8 to 4 S transformation, which requires reduction of sulfhydryls, involves dissociation of the 90-kDa heat shock protein (17, 22, 37). It is interesting to note, therefore, that with our conditions and reagents, we do not see interaction of the receptor with the 90-kDa heat shock protein through disulfide bonds, whereas the 45-35 kDa receptor-associated protein(s) appears to be part of the 8 S receptor complex. Based on the use of specific sulfhydryl group cross-linking reagents, our data suggest that this 45-35 kDa protein(s) is closely associated with the glucocorticoid receptor. Further analysis of the properties of this 45-35 kDa protein(s) could lead to insight into its function and possible role in glucocorticoid receptor action.

Certainly, the role of sulfhydryls in the mechanism of glucocorticoid receptor action is a complex one. The data point toward their role in steroid binding, 8-4 S transformation, activation, and DNA binding. Our results demonstrate that sulfhydryls within the glucocorticoid receptor monomer are available for interaction. These groups can play a direct role in altering receptor conformation (both tertiary and quaternary) and ultimately in its ability to bind to DNA. With the availability of the cDNA for the glucocorticoid receptor, there now exists the potential to probe the role which each cysteine within the receptor has in these various interactions.

Acknowledgments—We wish to thank Kerry Burnstein, Debbie Bellingham, and Victoria Allgood for their critical evaluation of this manuscript.

REFERENCES

1. Simons, S. S., Jr., and Thompson, E. B. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3541–3545
2. Hollenberg, S. M., Weinerberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G., and Evans, R. M. (1985) Nature 318, 635–641
3. Muncy, A., and Brinck-Johnsen, T. (1968) J. Biol. Chem. 213, 5556–5565
4. Reiss, A. M., and Bell, P. A. (1975) Biochim. Biophys. Acta 411, 121–132
5. Grippio, J. F., Holmgren, A., and Pratt, W. B. (1985) J. Biol. Chem. 260, 93–97
6. Nielsen, C. J., Sando, J. J., and Pratt, W. B. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 1338–1402
7. Nielsen, C. J., Sando, J. J., Vogel, W. M., and Pratt, W. B. (1977) J. Biol. Chem. 252, 7568–7578
8. Sando, J. J., LaForest, A. C., and Pratt, W. B. (1979) J. Biol. Chem. 254, 4772–4778
9. Housley, P. R., Dahmer, M. K., and Pratt, W. B. (1982) J. Biol. Chem. 257, 8615–8618
10. Kobelinski, M., Beata, M., Kalimi, M., and Feigelson, P. (1972) J. Biol. Chem. 247, 7897–7904
11. Housley, P. R., and Pratt, W. B. (1983) J. Biol. Chem. 258, 4630–4635
12. Singh, V. B., and Moudgil, V. K. (1986) J. Biol. Chem. 260, 3694–3699
13. Gzardis, P., Miller, A., Schmidt, T. J., and Litwack, G. (1984) Biochem. Biophys. Res. Commun. 120, 59–65
14. Smith, A. C., Elssasser, M. S., and Harmon, J. M. (1986) J. Biol. Chem. 261, 13285–13292
15. Mendel, D. B., Bodwell, J. E., and Munck, A. (1987) J. Biol. Chem. 262, 5644–5649
16. Tienrungroj, W., Sanchez, E. R., Housley, P. R., Harrison, R. W., and Pratt, W. B. (1987) J. Biol. Chem. 262, 17342–17349
17. Pratt, W. B. (1987) J. Cell. Biochem. 35, 51–68
18. Bodwell, J. E., Holbrook, N. J., and Munck, A. (1984) Biochemistry 23, 1392–1398
19. Bodwell, J. E., Holbrook, N. J., and Munck, A. (1984) Biochemistry 23, 4237–4242
20. Young, H. A., Parks, W. P., and Scolnick, E. M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3060–3064
21. Harrison, R. W., Woodward, C., and Thompson, E. (1983) Biochim. Biophys. Acta 759, 1–6
22. Tienrungroj, W., Meshanchi, S., Sanchez, E. R., Pratt, S. E., Grippio, J. F., Holmgren, A., and Pratt, W. B. (1987) J. Biol. Chem. 262, 6992–7000
23. Vedeckis, W. V. (1983) Biochemistry 22, 1983–1989
24. Bresnick, E. H., Sanchez, E. R., Harrison, R. W., and Pratt, W. B. (1988) Biochemistry 27, 2866–2872
25. Kaufmann, S. H., Okret, S., Wijkstrom, A-C., Gustafsson, J-A., and Shaper, J. H. Endocrinology 110, 987–1000
26. Weinberger, C., Hollenberg, S. M., Rosenfeld, M. G., and Evans, R. M. (1985) Nature 318, 670–672
27. Ciclowski, J. A., and Richon, V. (1984) Endocrinology 115, 1588–1597
28. Laemml, U. K. (1970) Nature 227, 680–685
29. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
30. Silva, C. M., Tully, D. B., Petch, L. A., Jewell, C. M., and Ciclowski, J. A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1744–1748
31. Tubb, D. B., and Ciclowski, J. A. (1987) Biochem. Biophys. Res. Commun. 144, 1–10
32. Webb, J. L. (1966) in Enzymes and Metabolic Inhibitors (Webb, J. L., ed) Vol. II, pp. 635–670, Academic Press, New York
33. Simons, S. S., Jr., Pumphrey, J. G., Rudikoff, S., and Eisen, H. J. (1987) J. Biol. Chem. 262, 9676–9680
34. Mendel, D. B., Bodwell, J. E., Gametchu, B., Harrison, R. W., and Munck, A. (1986) J. Biol. Chem. 261, 3758–3763
35. Sanchez, E. R., Toft, D. O., Schlesinger, M. J., and Pratt, W. B. (1986) J. Biol. Chem. 260, 12398–12401
36. Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M-G., Binart, N., Mester, J., and Baulieu, E-E. (1984) Nature 308, 852–853
37. Rexin, M., Busch, W., and Gehringer, U. (1988) Biochemistry 27, 5593–5601
38. Alberts, B., and Herrick, G. (1971) Methods Enzymol. 21, 198–217
39. Scheible, P. P., DeLorenzo, T. M., and Cidlowski, J. A. (1987) J. Steroid Biochem. 26, 181–187
40. Willmann, T., and Beato, M. (1986) Nature 324, 688–691
41. Moxham, C. D., and Malbon, C. C. (1985) Biochemistry 24, 6072–6077
42. Sorensen, P., Farber, N. M., and Krystal, G. (1986) J. Biol. Chem. 261, 3994–3997
43. Wimalasena, J., Abel, J. A., Wiebe, J. P., and Chen, T. T. (1986) J. Biol. Chem. 261, 9416–9420
44. Malbon, C. C., George, S. T., and Moxham, C. P. (1987) Trends Biochem. Sci. 12, 172–175
45. Gilbert, H. P. (1984) Methods Enzymol. 107, 330–351
46. Ziegler, D. M. (1985) Annu. Rev. Biochem. 54, 305–329
47. Mannervik, B., and Axelsson, K. (1980) Biochem. J. 190, 125–130
48. Wilson, E. M., Wright, B. T., and Yarborough, W. G. (1986) J. Biol. Chem. 261, 6501–6508