Mapping of Glucocorticoid Receptor DNA Binding Domain Surfaces Contributing to Transrepression of NF-κB and Induction of Apoptosis*

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Yunxia Tao, Cheryl Williams-Skipp, and Robert I. Scheinman†
From the Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center, Denver, Colorado 80262

Glucocorticoids (GCs) function, in part, through the ability of the glucocorticoid receptor (GR) to activate gene expression and in part through the transrepression of AP-1 and NF-κB. Here we characterize the effect of GR DNA binding domain (DBD) mutations, previously analyzed for changes in the ability to activate gene expression or transrepress AP-1. We have identified a GR mutant capable of distinguishing between transrepression of NF-κB and AP-1. Using circular dichroism spectroscopy, we show that this mutation does not appreciably alter GR DBD conformation, suggesting that functional differences between the mutant and wild type protein are the result of an alteration of a specific interaction surface. These data suggest that transrepression of NF-κB and AP-1 occurs through distinct protein-protein interactions and argue against the hypothesis that transrepression occurs through competition for a single coactivator protein. Introduction of these mutations into GC-resistant CEM lymphoblastic T cells restored dexamethasone (DEX)-mediated apoptosis as did wild type GR regardless of whether these mutants were transrepression or activation defective. Thus, DEX-mediated apoptosis in transformed T cells is more complex than originally appreciated.

Glucocorticoids (GCs) have long been used as anti-inflammatory, immunosuppressive, and chemotherapeutic drugs. Initially, GCs were thought to mediate their therapeutic actions through the transcription of GC-responsive genes (1). This mechanism, however, did little to explain how GCs could inhibit the transcription of many cytokine genes, a critical part of its anti-inflammatory and immunosuppressive actions (2). Subsequently, it was discovered that GR could inhibit the activity of the transcription factor, AP-1, in the absence of a GC-responsive element (GRE) (3–6). This ability to inhibit directly a transcription factor activity in the absence of a GRE was termed transrepression and was shown to involve a direct physical interaction between GR and AP-1. GCs were also found to inhibit the activity of the transcription factor NF-κB (7–11). As NF-κB and AP-1 together regulate genes involved in inflammation and immunosuppression, these discoveries allowed the construction of a model for the therapeutic properties of GCs involving both activation and transrepression (12, 13).

Footprinting studies of the AP-1-driven collagenase promoter after GC treatment showed no change in protein occupancy, suggesting that the GR/AF-1 interaction functions to promote a restructuring of the complex of factors binding to the promoter rather than a simple inhibition of promoter occupancy (14). Domain mapping studies identified the zinc finger DNA binding domain (DBD) of GR as essential for transrepression of AP-1, suggesting that the GR DBD is responsible for both transrepression and activation (4–6). DNA binding and the subsequent activation of gene expression require the dimerization of GR and its binding to a palindromic GRE (15). Careful dissection of the GR DBD, through mutagenesis, demonstrated that disruption of the D loop within the second zinc finger could abolish the ability of GR to dimerize and, consequently, inhibit GC-mediated activation (16). Others (17, 18) have shown that mutations in this region, while inhibiting GR-mediated activation, did not affect transrepression of AP-1. Further mutations within the first zinc finger of the GR DBD were identified that disrupted transrepression of AP-1 but did not inhibit the ability of GR to transactivate through a GRE (18), suggesting that activation and transrepression are separable phenomena.

Evidence in support of GR-mediated inhibition of NF-κB as one mechanism for the clinical actions of GCs has been generated in various systems (19, 20). Transrepression of NF-κB by GCs has turned out to be mediated by a number of separate mechanisms, however. NF-κB comprises dimers of a protein family that shares homology with c-Rel, including relA (p65), RelB, c-Rel, p50, and p52. Classic NF-κB is a dimer of RelA and p50. Most forms of NF-κB are held in the cytoplasm by members of the IκB family. NF-κB can be activated through multiple signaling pathways through the activation of the IκB kinase (IKK) and the phosphorylation of IκB. Initial reports of GC-mediated transrepression of NF-κB included data demonstrating a physical interaction between NF-κB subunits and GR similar to that shown for AP-1 (7, 8, 10). Subsequently, we and others (9, 11) have demonstrated that GCs could induce transcription of the NF-κB inhibitor, IκBα. Most recently, it was demonstrated that both NF-κB and GR can compete for interactions with coactivators such as p300 and CREB-binding protein (CBP), which are present in limited amounts (21–23). Interestingly, however, a number of conflicting reports have emerged demonstrating systems in which GCs transrepress NF-κB in the absence of IκBα induction (24–26). In addition, others have reported that GC-mediated transrepression can occur also when the coactivators p300 and SRC-1 are over-expressed (27). Thus, the molecular basis for GC-mediated transrepression is controversial and may, to some extent, be tissue-specific.

Here, we have begun to address this problem by introducing mutations into the GR DNA binding domain and analyzing the...
effect of these mutations on protein function and conformation on trans-repression of NF-{kappa}B. Using this structural information, we are able to propose that trans-repression of NF-{kappa}B and AP-1 is mediated through different interaction surfaces within the GR DBD. Our data argue against the hypothesis that trans-repression of both AP-1 and NF-{kappa}B is mediated by GR with a common factor.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Mutations in the GR DBD (S425G, L436V, and N454D/A458T) were constructed from our human GR expression construct, pYCGR (10), by in vitro site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) with the following oligonucleotides: GR S425G, 5'-CAACACTGTCCTAGAAGGTTGTCAGTGC-3'; GR L436V, 5'-CAGGATCTCATATGTTGAATCCT-3'; GR N454D/A458T, 5'-GTGGAAGACGACGATCATCAATGACTGAGAAGAATTGATTG-3'; and 5'-CATTTAATGCTTCCAAGATCCTCCTCATTAGCATCGT-3'; GR expression plasmid. DEX was assayed immunologically using the acridine orange/ethidium bromide double dye method (29).

**Northern Blot Analysis**—Total RNA was isolated from cells with TRIzol Regent (Life Technologies, Inc.) per the manufacturer's instructions. RNA integrity and loading consistency were assessed by visual inspection of ribosomal RNA bands after agarose gel electrophoresis and etidium bromide staining. 20 ng of RNA was separated on a 1% formaldehyde-containing agarose gel, transferred to Zeta-probe membranes (Bio-Rad) by standard capillary blotting, and cross-linked by UV irradiation. The cDNA probe for I{ beta} was labeled with [alpha-32P]dCTP using a random primer DNA labeling kit (Bio-Rad). The filter was washed twice in 40 mM NaPO4, 1 mM EDTA, 1% SDS, and data visualized by PhosphorImager.

**RESULTS AND DISCUSSION**

**Mutations in the GR DBD Do Not Cause Major Changes in Conformation**—Previously, we showed that GC-mediated trans-repression of NF-{kappa}B requires the DBD but not the t1 activation domain (10). To further dissect this requirement, we tested the DBD mutants S425G and L436V, shown to block trans-repression of AP-1 (18), and N454D/A458T, a D loop mutation that blocks GR dimerization and activation of transcription. We began by analyzing the effect of these mutations on GR conformation to determine whether the mutations affected one specific region of the GR DBD or whether the effects of these mutations were spread throughout the domain. This control experiment was critical for the appropriate structural interpretation of the subsequent functional data; however, to our knowledge, it had never been performed in the context of studying GR-mediated trans-repression. To this end, we expressed wild type and mutant GR DBD domains as GST fusion proteins in *Escherichia coli*. The GR DBD proteins were purified, GST domains cleaved, and contaminating DNA removed as described below “Experimental Procedures.” The extinction coefficient (epsilon) of 5470 M^-1 cm^-1 was determined by the method of Gill and von Hippel (30). SDS-PAGE analysis of the mutants generated an apparent molecular mass of 10 kDa, consistent with the theoretical molecular weights of our GR DBD proteins (data not shown).

**Structures of wild type and mutant GR DBDs were studied by CD spectroscopy.** Fig. 1 shows representative far-UV CD spectra of the DBDs. The strong negative maxima at 222 nm indicate that the DBDs possess an amount of alpha-helical structure consistent with earlier studies of the helix domain done by others using NMR spectroscopy and x-ray diffraction (31, 32). By visual inspection, the spectra for the S425G mutant appears to deviate slightly in intensity at the wavelengths representing alpha-helix, 208 and 222 nm. However,
measurements of the percentage of α-helical structure from three independent preparations, as measured by deconvolution analysis of the spectra, showed that these deviations were not statistically significant (Fig. 1, inset). These data indicate that within the error of the measurement, there are no obvious secondary structural differences between the wild type DBD and any of the mutants. To further test the structural similarity of these DBDs, thermal melting scans were collected on the wild type and each of the mutants. Although the transition was not thermodynamically reversible, the apparent melting temperatures ($T_m$) for all of the mutants were equivalent within error, indicating that the relative stability for the wild type DBD and the mutants are indistinguishable (data not shown). In conclusion, CD spectroscopy indicates that no global conformational changes exist in any of the mutants, and any functional changes that result from these mutations must be caused by either specific changes in the nature of the amino acids in an interaction interface region or by subtle local conformational changes of involving only several residues.

**Analysis of Transrepression**—The mutations S425G, L436V, and N454D/A458T were introduced into our GR expression vector, pTcGR, as described under “Experimental Procedures.” We then tested their ability to transrepress NF-κB and AP-1 by transient transfection followed by treatment with DEX. The ability of these GR mutants to transrepress NF-κB and AP-1 reporter constructs is shown in Fig. 2. We found that the mutations L436V and N454D/A458T had no effect on the ability of GR to transrepress NF-κB, whereas the S425G mutation greatly reduced the ability of GR to transrepress NF-κB. N454D/A458T-mediated transrepression of AP-1 is consistent with the observation that GR can transrepress AP-1 as a monomer. Here we show that this property of GR can be extended to transrepression of NF-κB. In our hands, S425G and L436V both transrepress AP-1. These results are different from that reported by Heck et al. (18), which may be because of differences in the cell lines used by our respective laboratories. Additionally, these differences may be due to amino acid differences caused by the introduction of restriction sites flanking the DBD in their human GR expression plasmid (18). Given that our experiments were performed in one cell line with the same GR construct, we can state that the requirements for transrepression of AP-1 and NF-κB are not identical in that the S425G mutation can discriminate between these two functions. The serine to glycine substitution at position 425 removes an hydroxyl group, possibly altering hydrogen bonding patterns between the zinc finger domain and other proteins, suggesting that this hydrogen bonding pattern is important for the transrepression of NF-κB but not AP-1. Serine 425 is not thought to hydrogen bond either with DNA or within the zinc finger. X-ray crystallography data indicate that this amino acid is solvent-accessible (32), consistent with the possibility of its contribution to an intermolecular interaction interface. These data indicate that multiple elements within the GR DBD contribute to transrepression and argue against the hypothesis that transrepression primarily involves competition for a common factor, as this would predict that transrepression of NF-κB and AP-1 would not be separable phenomena.

**DEX-induced Apoptosis Is Restored Both by GR Mutants Deficient in Dimerization and by GR Mutants Deficient in Transrepression**—GCs induce apoptosis in both thymocytes and transformed T cells. To analyze the properties of these GR mutants in the context of T cell apoptosis, we used previously described sister human acute lymphoblastic leukaemic T cell lines, GC-sensitive CEM C7–14 and GC-resistant CEM C1–15 (33). We cotransfected CEM C1–15 cultures with pCI-neo along with an expression plasmid encoding either wild type GR or GR, containing one of the various GR mutants described above, and selected stable transfecteds by culturing in G418. Although endogenous (nonfunctional) GR migrated identically to transfected GR on SDS-PAGE, we found that GR immunoreactivity was significantly higher in transfected lines, indicating that our transgenes were expressed (Fig. 3A, inset). To investigate whether any of our GR mutants could restore GC sensitivity to apoptosis, our transfected CEM cell lines were treated with DEX for 48 h, and apoptosis was determined morphologically as described under “Experimental Procedures.” As expected, DEX induced apoptosis in ~25% of the C14 line after 48 h, with little apoptosis in the C15 line (Fig. 3A). Wild type GR restored DEX sensitivity to the C15 line as did the dimerization mutant, N454D/A458T, a result consistent with the results of Helmbert et al. (17). Interestingly, both the S425G and L436V mutants also restored DEX sensitivity to the C15 line. Statistical analysis of apoptosis levels indicated no significant differences in apoptosis between wild type GR transfected C15 cells and C15 cells transfected with S425G, L436V, or N454D/A458T GR mutants.

These data can be interpreted in several ways. As S425G is unable to transrepress NF-κB, and yet restores apoptosis, it would suggest that transrepression of NF-κB is not required for this process. Conversely, as N454D/A458T is unable to interact...
To our knowledge, we are the first group to analyze the structure of our GR mutants and systematically compare NF-xB and AP-1 in the study of GR-mediated transrepression. Through this work, we have identified a region of the first zinc finger, which is differentially involved in transrepression of NF-xB and with AP-1. Furthermore, we have shown that DEX-mediated apoptosis is dependent solely neither on transrepression nor on activation. Rather, our data suggest that GC-mediated apoptosis in CEM cells is mediated potentially through several independent pathways.

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Fig. 3. Stable transfection of GR DBD mutants into GR-deficient CEM T cells. CEM C1–15 (GR-resistant) cells were stably transfected with wild type GR or mutants. CEM C7–14 is a GC-sensitive cell line. A, apoptosis was determined morphologically using the ethidium bromide/acridine orange technique. Open columns, untreated; filled columns, DEX-treated; wt, wild type GR; SG, S425G; LV, L436V; dbI, N454D/A458T. Inset, Western blot of GR immunoreactivity in untransfected and transfected C1–15 cells. Nonfunctional (endogenous) and N454D/A458T. L436V mutant transgenes were capable of restoring the ability of gr to induce IκBα mRNA, indicating that the IκBα gene is not part of the subset of GC-responsive genes activated by this mutant.
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