Protein-Protein Interactions Facilitate DNA Binding by the Glucocorticoid Receptor DNA-binding Domain*

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We have studied the interaction of the DNA-binding domain of the glucocorticoid receptor with a glucocorticoid response element from the tyrosine aminotransferase gene. This response element consists of two binding sites (half-sites) for the glucocorticoid receptor DNA-binding domain. The sequences of these two half-sites are not identical, and we have previously shown that binding occurs preferentially to one of the half-sites (Tsai, S.-Y., Carlstedt-Duke, J., Weigel, N. L., Dahlman, K., Gustafsson, J.-Å., Tsai, M.-J., and O'Malley, B. W. (1988) Cell 55, 361–369). We show here that binding to the low affinity half-site is dependent on previous occupancy of the high affinity half-site. This facilitated binding is dependent on the distance between the two half-sites and their relative orientation but is not dependent on the integrity of the DNA backbone. This is consistent with a model where DNA binding is not only dependent on interactions between the protein and its DNA target sequence but is also influenced by interactions between the protein molecules bound.

The glucocorticoid receptor (GR)† belongs to a family of ligand-inducible nuclear transcription factors. In addition to the GR, this family also consists of the receptors for other steroid hormones, thyroid hormone, retinoic acid, and 1,25-dihydroxy vitamin D₃. Molecular cloning and sequence analysis of cDNAs encoding these receptors demonstrated that all members of this superfamily of receptor proteins contain a highly conserved domain that mediates receptor binding to specific DNA sequences termed hormone response elements. The DBD interacts with the hormone response element. Protein-protein interactions between two bound molecules could increase the stability of the complex; alternatively, binding of the first protein molecule could induce a change in the structure of the neighboring half-site along the DNA molecule, thereby increasing the affinity of the protein for its site. In this report, we conclude that protein-protein interactions are responsible for the facilitated binding of the isolated DNA-binding domain of the glucocorticoid receptor to DNA.

EXPERIMENTAL PROCEDURES

Expression of the GR DBD in E. coli and Purification of the Recombinant Protein The GR DBD was expressed as a fusion protein with protein A in E. coli as previously described (6), except that E. coli strain JM109 containing the plasmid pNF 2690 was used as the host strain for the expression plasmid pEHI13790. The plasmid pNF 2690 confers kanamycin resistance and encodes a λ repressor derivative which is temperature-sensitive due to the c857 mutation. The isolated DBD was purified as described previously (6) with the following modifications. 1 mM DTT was included in all the buffers, and Tween was excluded from buffer B. Buffer C was 20 mM sodium phosphate, pH 7.4, 300 mM NaCl, 1 mM DTT. Buffer D was 20 mM sodium phosphate, pH 7.4, 50 mM NaCl, 1 mM DTT. The homogenization of the cell lysate was omitted. The α-chymotrypsin concentration used for the cleavage of the fusion protein was 4 µg/ml. The cleaved protein from the IgG-Sepharose column was brought to 300 mM NaCl, incubated for 5 min, diluted with 2 volumes of 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and immediately loaded on the DNA cellulose column. The DNA cellulose column was washed with 10 column volumes of buffer B before eluting the protein.

The same protein preparation was used for all experiments in this report. It was stored at +4 °C in the elution buffer. The protein preparation was estimated to be at least 95% pure from Coomassie...
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staining of SDS-polyacrylamide gels. The protein preparation was checked repeatedly during the course of this study by SDS-polyacrylamide gel electrophoresis and DNA binding assays. No degradation or loss of DNA binding activity could be detected.

SDS-Polyacrylamide Gel Electrophoresis and Determination of Protein Concentration—SDS-polyacrylamide gel electrophoresis was performed as described (12). Protein concentrations were determined spectrophotometrically using an extinction coefficient \( \epsilon_{280} = 6,000 \text{ M}^{-1} \text{ cm}^{-1} \) (calculated from tyrosine absorption data).

Oligonucleotides—Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. The synthesized tyrosine aminotransferase GRE was 41 base pairs long. Oligonucleotides with inserted or deleted base pairs were correspondingly longer or shorter. DNA concentrations were determined spectrophotometrically using \( \epsilon_{280} = 13,200 \text{ M}^{-1} \text{ cm}^{-1} \).

Gel Retardation Assays—Complementary DNA strands were annealed at a concentration of 100 \( \mu \text{g/ml} \) in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl by heating at 65 °C for 15 min and cooling to room temperature over a period of 2 h. To obtain the oligonucleotide containing a nick in one of the DNA strands, two contiguous oligonucleotides were annealed with a complementary oligonucleotide. 10 pmol of oligonucleotide was labeled with polynucleotide kinase (Boehringer Mannheim) and \( \gamma^32\text{P}\)ATP (Amersham Corp.) to a specific activity of \( 1 \times 10^{7} \text{ cpm/ug} \). Labeled oligonucleotides were separated from unincorporated nucleotides using an NAP 5 column (Pharmacia LKB Biotechnology Inc.) and stored at -70 °C. Binding reactions (volume 10 \( \mu \text{l} \)) were carried out at room temperature for 15 min. with reaction mixtures containing 10 mM HEPES, pH 7.5, 10% glycerol, 2.5 mM MgCl₂, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 200 ng of poly(di- dC)-poly(dG), and 0.27 ng of labeled oligonucleotide. Protein-DNA complexes were separated from protein-free DNA by nondenaturing electrophoresis (13) in 5% polyacrylamide (29:1, acrylamide/bisacrylamide) gels. Gels were run at 14 °C in 50 mM Tris borate acid, pH 8.0, 1 mM EDTA at a constant voltage of 200 V, dried, and exposed to Kodak XAR5 film. The binding was specific as determined by competition analysis and methylation interference assays (Fig. 3 and data not shown). Data was quantified by liquid scintillation counting of bands excised from gels. Experiments were repeated at least three times with similar results. In each case one representative experiment is shown.

Methylation Interference Assays—Single-stranded oligonucleotides were labeled with polynucleotide kinase and \( \gamma^32\text{P}\)ATP. Labeled oligonucleotides were separated from unincorporated nucleotides using an NAP 5 column. Labeled single-stranded oligonucleotides were annealed with unlabeled oligonucleotides as described above. 2.5 pmol of oligonucleotide was added on ice to 200 \( \mu \text{l} \) of 50 mM sodium cacodylate, pH 8.0, 1 mM EDTA, containing 200 ng of salmon sperm DNA, and mixed with 1 \( \mu \)l of dimethyl-sulfate. After 2.5 min at 25 °C, 5 mM sodium citrate was added, 1.5 M \( \beta \)-mercaptoethanol was added to stop the reaction, followed by ethanol precipitation in the presence of 0.5 \( \mu \text{g} \) of tRNA. Gel retardation assays were carried out as described above but on a 10-fold larger scale. Gels were exposed to Kodak XAR5 film to reveal retarded and unbound DNA. The bands were cut out, and DNA was eluted in 400 \( \mu \text{l} \) of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA at room temperature overnight. 4 \( \mu \text{g} \) of tRNA was added followed by ethanol precipitation. The dried pellet was resuspended in 100 \( \mu \text{l} \) of water, and 1 \( \mu \)l of 10 M piperidine was added. After 30 min at 90 °C, probes were lyophilized followed by five cycles of resolubilization in 20 \( \mu \text{l} \) of water and relyophilization. Approximately equal amounts of radioactivity from each sample were separated on 15% polyacrylamide, 7 M urea sequencing gels which were dried and exposed on Kodak XAR5.

RESULTS

Influence of Point Mutations on Binding of GR DBD to the Tyrosine Aminotransferase GRE—It has previously been shown that two symmetry point mutations of a synthetic palindromic GRE abolish glucocorticoid-mediated induction of gene transcription in transient expression assays (14). We recreated the same mutations in the context of the tyrosine aminotransferase GRE to study the effect of these mutations at the level of protein-DNA interaction. Gel retardation analysis showed that the affinity of GR DBD for the mutant binding site is at least 1 order of magnitude lower than for the wild type binding site (data not shown). More importantly, we introduced the corresponding single point mutations into the tyrosine aminotransferase GRE to study how mutation of one half-site influences binding to the other nonmutated half-site. Fig. 1A shows that mutation of the high affinity half site (TGTTC, mutant GREM2) decreases binding to both half-sites since at protein concentrations where binding to both half-sites is seen for the wild-type tyrosine aminotransferase GRE, no complexes are formed when the high affinity half-site is mutated (Fig. 1A). On the other hand, mutation of the low affinity half-site, mutant GREM3, does not significantly influence binding to the high affinity half-site (Fig. 1A). We conclude that the initial binding to the high affinity half-site facilitates subsequent binding to the low affinity half-site. Fig. 1B depicts two models which could account for this facilitated binding. In model 1, protein-protein interactions between bound protein molecules increase the stability of the protein-DNA complex. In model 2, binding of DBD to the high affinity half-site changes the conformation of the low affinity half-site such that the affinity of DBD for this site is increased. To distinguish between these two models, we studied the binding of DBD to several variants of the tyrosine aminotransferase GRE binding site. If protein-protein interactions were important for the facilitated binding, we predicted that they would be disrupted by changing the distance between the two half-sites or by changing their relative orientation. Alternatively, if binding of the first protein molecule changed the structure of the DNA, we expected that change to be dependent on the integrity of the intervening DNA sequence and that introducing a nick in one of the DNA strands between the half-sites would abolish the facilitated binding. The dramatic reduction in the affinity of DBD for the low affinity half-site when it does not cooperate with a molecule bound at the high affinity half-site gives rise to very clear changes in the DNA binding characteristics for the various mutant binding sites when analyzed by gel retardation assay.

Changing the Distance between the Two Half-Sites Abolishes Facilitated Binding of DBD to the Tyrosine Aminotransferase GRE—In the consensus GRE, the two GR binding half-sites are separated by 3 base pairs of nonconserved sequence (3, 15, 16). To test if this spacing is critical for the facilitated binding, we changed the distance between the two half-sites to 2, 4, and 13 base pairs, respectively. The interaction between DBD and the corresponding oligonucleotides (GREM4, GREM5, and GREM6) was studied using gel mobility shift assays (Fig. 2). The different complexes were quantified and plotted as a function of the protein concentration. The small differences in total binding observed between experiments reflect experimental variation and are not significant compared with the scale of the differences between the binding sites. The methylation interference assay (Fig. 3) confirms our previous data (4). At complexes I and II correspond to one and two molecules of DBD bound, respectively, and that complex I represents binding only to the high affinity half-site (TGTTC). Fig. 2 shows that the formation of complex II is drastically reduced when the distance between the two binding sites is changed. Increasing or decreasing the distance between the two half-sites by 1 base pair rotates the binding sites on the DNA helix in relation to each other. The failure to form complex II probably reflects loss of protein contacts which would be disrupted by a corresponding rotation of bound protein molecules.

We suggest that increasing the distance between the half-sites by 13 base pairs, which increases the distance between the binding sites by one helical turn while making only a small change to their relative positions on the face of the
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A

TAT GRE <TGTACA>GGA <TGTTCT>

32 16 8 4 2 1 0.5 0.25 -

II I I I II -

F -

QREM (~TTZTJGGAITGATC~)

32 16 8 4 2 1 0.5 0.25 -

QREHJ -QQAm

FIG. 1. Band shift assays of GR DBD binding to wild type and mutant tyrosine aminotransferase (TAT) GREs. A, band DNA helix, disrupts protein-protein contacts because the distance between the binding sites is too large.

Changing the Relative Orientation of the Two Half-sites Abolishes Facilitated Binding of DBD to the Tyrosine Aminotransferase GRE—The sequence of the high affinity half-site was inverted to give the oligonucleotide GREM12. Inversion of the low affinity half-site was not done since it recreates the original sequence (TGTACA). Fig. 4 shows that inversion of the high affinity half-site leads to a dramatic reduction in the amount of complex II formed, similar to that seen in Fig. 2. We suggest that when the high affinity half-site is inverted, DBD will bind in the reverse orientation and therefore the proposed protein-protein contact surfaces would not be able to interact.

Facilitated Binding of DBD to the Tyrosine Aminotransferase GRE Is Not Affected by a Nick in the DNA Molecule—Introduction of a nick in one of the DNA strands (NICK) does not influence the DNA binding characteristics of GR DBD (Fig. 5). On the other hand, introduction of a single nucleotide gap in one of the strands leads to a reduction in the amount of complex II formed (data not shown). Since cutting of one of the DNA strands between the binding sites would be expected to disrupt transmission of any structural change in the second site which results from binding to the first site, we conclude that such a mechanism is unlikely to account for the cooperativity of binding. Conversely, the nick is unlikely to alter the relative position of the binding sites significantly, since all base pair contacts are maintained, and therefore facilitated binding mediated by protein-protein interactions would not be impeded. In contrast, a nucleotide gap would have a marked effect on the gross structure of the DNA molecule and completely disrupt the normal spatial relationship between the two half-sites.

In summary, we find that facilitated binding is dependent on the distance between the two half-sites and their relative orientation but that it is not influenced by the integrity of the DNA backbone. We therefore conclude that protein-protein interactions are responsible for the cooperative DNA binding observed for DBD, as schematically shown in Fig. 6 where the postulated protein-protein contact surface within GR DBD is shown in black.

DISCUSSION

We have described the importance of protein-protein interactions for optimal binding of GR DBD to the tyrosine aminotransferase GRE. This interaction could occur as part of two distinct mechanisms for DNA binding. First, dimerized DBD might represent a component of the free DBD in solution which has a higher affinity for the tyrosine aminotransferase GRE than monomeric DBD. Alternatively, dimers might be formed in association with DNA as a result of prior binding of DBD to the high affinity half-site. Also it is possible that both mechanisms occur simultaneously. However, un-
Fig. 2. Binding of GR DBD to tyrosine aminotransferase (TAT) GREs with varying spacing between the half-sites. Binding of GR DBD to wild type tyrosine aminotransferase GRE or variants containing 2 (GREM4), 4 (GREM5), or 13 (GREM6) intervening base pairs was assayed by band shift analysis. Annotations are the same as in Fig. 1.

Published data lead us to favor the second model since (i) we have failed to detect dimers in solution either by co-immunoprecipitation or by sucrose gradient sedimentation analysis, and (ii) the apparent affinity of DBD for the low affinity half-site is not dependent on the protein concentration as would be predicted if the former model was correct. Furthermore, quantification of the cooperativity factor using a palindromic GRE with two high affinity half-sites (TGTTCT) resulted in a cooperativity factor of about 100 for DBD binding. This corresponds to a $K_a$ of about $10^8$ M$^{-1}$ which is too low to allow the formation of a significant amount of dimers in solution.

Although we have studied only an isolated domain of GR, we believe that our findings are relevant also in the context of the native GR. It has been suggested that the native GR can occur as a dimer in solution (10) and that the NH$_2$-terminal region of the receptor influences dimerization (17). Therefore, DBD probably lacks at least one domain that is important for dimerization of the native GR. We believe that the native GR may have several contact surfaces that are involved in dimerization and that the dimerization surface in DBD, although not strong enough for dimer formation in solution, still contributes significantly to the stability of the dimer formation within the context of the native GR. More importantly, it is likely to be of importance for the correct spatial arrangement of the DNA binding region of the dimer and thereby for the correct positioning of the two molecules on the DNA binding sites. In natural GREs, the spacing between the half-sites is always three nucleotides. Furthermore, it has been shown by transient expression studies that it is the spacing and not the actual intervening sequence that is important (2, 15, 16). We suggest that the quaternary structure of the DBD dimer is responsible for this spacing requirement. Preliminary experiments with the progestin receptor DBD show that it binds to the same GRE sequence

$K_a$ = [GR]$_2$ [GRE]$_2$ / [GR] [GRE]

$K_a$ values for DBD binding to wild type tyrosine aminotransferase GRE or variants containing 2 (GREM4), 4 (GREM5), or 13 (GREM6) intervening base pairs were determined by band shift analysis. Annotations are the same as in Fig. 1.

Fig. 3. Methylation interference assay of GR DBD binding to wild type (right) and mutant (GREM4; left) tyrosine aminotransferase GRE. Methylation interference analysis was performed as described under "Experimental Procedures." Binding to the bottom strand is shown. F, I, and II refer to the bands recovered after band shift analysis (Fig. 2). With GREM4, only complex I is formed (Fig. 2). The G residues interfering with the protein binding in the respective complexes are marked I and II in the flanking sequences.

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FIG. 4. Binding of GR DBD to wild type and inverted tyrosine aminotransferase (TAT) GREs. The high affinity half-site has been inverted in the mutant GRE (GREM12). Annotations are the same as in Fig. 1.

FIG. 5. Binding of GR DBD to wild type and nicked tyrosine aminotransferase (TAT) GREs. A nick was introduced in one of the DNA strands within the intervening sequence (NICK). Annotations are the same as in Fig. 1.

FIG. 6. Summary of GR DBD binding to wild type and variant tyrosine aminotransferase GREs. Summary of the experimental data shown in Figs. 2–5. The direction of the arrows shows the orientation of the half-sites. The putative protein-protein contact surface within DBD is marked in black. Binding of the second DBD molecule occurs only if the normal protein-protein contacts are made in a cooperative manner (data not shown). Thus, this type of protein-protein interaction is not restricted to GR.

The nature of the protein-protein interactions involved in dimer formation of GR remains to be elucidated. However, a potential segment, near the second zinc binding site, which is involved in the interaction between two DBD molecules has been identified. It has been shown that GR DBD requires zinc for optimal folding and DNA binding (5, 7). The two zinc atoms are each tetrahedrally coordinated to four cysteine residues (5). Zinc and cadmium can contribute to dimer formation of other transactivating proteins (18), and since cadmium can replace zinc in DBD without affecting the structure or the cooperative DNA binding characteristics of the protein (data not shown), it cannot be excluded that the metal is of importance for dimerization of GR DBD. However, at present there is no evidence to support this, and we believe that the metal is primarily involved in a structural role within each individual DBD monomer.

It is becoming increasingly clear that protein-DNA interactions are not only influenced by specific amino acid-nucleotide base interactions but are also affected by subunit interactions. An ever increasing number of DNA binding proteins are shown to bind as dimers or even larger oligomeric structures. The formation of oligomers is of importance both for the specificity and for the affinity of the interaction with DNA. In many cases, specialized structures termed "leucine zippers" mediate these interactions (Refs. 19–22 and references therein). There is no structure reminiscent of the leucine zipper motif within the GR DBD. Furthermore, the interaction described here for DBD is much weaker than that seen in proteins containing the leucine zipper motif where the interaction occurs readily in solution (20, 21). It will be interesting to study the molecular basis for the protein-protein interactions described in this study.

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