Determinants of High-Affinity DNA Binding by the Glucocorticoid Receptor: Evaluation of Receptor Domains outside the DNA-Binding Domain†

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ABSTRACT: In this study, we have investigated the influence of regions outside the DNA-binding domain of the human glucocorticoid receptor on high-affinity DNA binding. We find that the DNA-binding domain shows a 10-fold lower affinity for a palindromic DNA-binding site than the intact receptor. The N-terminal part of the receptor protein does not influence its DNA-binding affinity, while the C-terminal steroid-binding domain increases the DNA-binding affinity of the receptor molecule. It has previously been shown that both the intact glucocorticoid receptor and the glucocorticoid receptor DNA-binding domain bind to a palindromic glucocorticoid response element on DNA as dimers. It is likely that differences in DNA-binding affinity observed result from protein–protein interactions outside the DNA-binding domain betwen receptor monomers, as has been shown for the estrogen receptor. We have previously identified a segment involved in protein–protein interactions between DNA-binding domains of glucocorticoid receptors. This, in combination with results presented in this study, suggests that there are at least two sites of contact between receptor monomers bound to DNA. We suggest that the interaction between the DNA-binding domains may act primarily to restrict DNA binding to binding sites with appropriate half-site spacing and that additional stability of the receptor dimer is provided by the interactions between the steroid-binding domains.

The glucocorticoid receptor (GR)[1] belongs to a family of nuclear receptors which activate transcription in response to binding of their respective ligands. Transcriptional activation is accomplished by DNA-bound receptors. DNA-binding sites for the GR are called glucocorticoid response elements (GREs) and are characterized by palindromic half-sites which are separated by a three amino acid spacer of variable sequence [for reviews, see Gustafsson et al. (1987), Evans (1988), Beato (1989), and O'Malley (1990)].

The palindromic nature of their response elements suggests interaction with receptor dimers, and this has been demonstrated for the GR (Tsai et al., 1988; Wrange et al., 1989; Chalepakis et al., 1990), the estrogen receptor (ER) (Kumar & Chambon, 1988; Fawell et al., 1990), and the thyroid hormone receptor (TR) (Brent et al., 1991; Selmi & Samuels, 1991; Lazar et al., 1991). The GR (Wrange et al., 1989), the ER (Kumar & Chambon, 1988; Fawell et al., 1990), and the progesterone receptor (PR) (Rodriguez et al., 1990; DeMarzo et al., 1991) also show some dimerization in the absence of DNA.

The ER is the best characterized steroid receptor with respect to the segments involved in receptor dimerization. Using ER expressed in yeast cells, it was shown that two segments of the ER are involved in dimerization: one in the steroid-binding domain and one in the DNA-binding domain (Kumar & Chambon, 1988). However, the major dimerization activity was shown to reside in the steroid-binding domain (Kumar & Chambon, 1988; Fawell et al., 1990). A more detailed characterization has revealed the importance of amino acids at the C-terminal end of the steroid-binding domain for ER dimerization (Fawell et al., 1990). The corresponding segment has also been implicated in heterodimerization between retinoic acid and thyroid hormone receptors (Glass et al., 1989). For the PR, mutagenesis studies have indicated a dimerization activity within the ligand-binding domain (Guiochon-Mantel et al., 1989). Finally, it has been suggested that the N-terminal domain of the GR mediates protein–protein interactions between molecules constituting receptor dimers (Eriksson & Wrange, 1990).

Two molecules of the GR DNA-binding domain (DBD) bind to a GRE cooperatively due to protein–protein interactions between bound DBDs (Dahlman-Wright et al., 1990). Mutation of a five amino acid segment close to the second zinc ion has been shown to abolish this activity (Dahlman-Wright et al., 1991). Transient transfection studies using mutated receptor derivatives have shown that this five amino acid segment functions to restrict the transactivation capacity of the glucocorticoid receptor to response elements where the distance between the half-sites is three base pairs (Umesono & Evans, 1989). Thus, protein–protein interactions between DBDs within GR dimers are involved in the specificity of binding site selection.

This study was initiated to determine whether the interactions between GR DBDs, in addition to their role in binding site selection, are the sole determinants for GR dimerization. For the ER, it has been shown that receptor dimerization is required for high-affinity DNA binding (Kumar & Chambon, 1988; Fawell et al., 1990). In this study, we have measured the DNA-binding affinity of various GR derivatives to evaluate the role of DBD in relation to other receptor domains in receptor dimerization.

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[1] Abbreviations: GR, glucocorticoid receptor; ER, estrogen receptor; TR, thyroid hormone receptor; PR, progesterone receptor; DBD, DNA-binding domain; GRE, glucocorticoid response element; ERE, estrogen response element; DTT, dithiothreitol; TAT, tyrosine aminotransferase.

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EXPERIMENTAL PROCEDURES

Plasmid Constructions. The yeast expression vector, pKV50, used for expression of GR derivatives has been described (Wright et al., 1990). Using this vector, proteins are expressed from the PGK promoter, the upstream activation sequences of which have been replaced with those from the *GAL1,10* promoter. In this expression system, expression is repressed during growth on glucose-containing media and induced on galactose-containing media. The plasmids pKVNX, pKVXE, pKVAX, and pKVNXΔτ1 have been described previously (Wright & Gustafsson, 1991) and express the following receptor derivatives, respectively: GR (intact receptor, residues 1–777); GRDBD (residues 370–500); GRNtermΔ (residues 415–777); and GRτ1Δ (residues 77–262 deleted). The plasmid pKVNE encodes the GRCtermΔ protein (residues 1–500) followed by Asp-His-Gly at the C-terminus. Plasmids were transformed into the *Saccharomyces cerevisiae* strain W303-1A (*MATa, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1*) according to Beggs (1978).

Growth of Yeast Strains and Preparation of Extracts. Yeast strains were grown to late log phase in SD medium (Sherman et al., 1986) without leucine at 30 °C, harvested by centrifugation, and washed in the same medium without added carbon source. The cells were harvested and resuspended in SGE medium (0.67% yeast nitrogen base without amino acids, 3% glycerol, and 1% ethanol) without leucine, at a density of $A_{600} = 0.5$. After 24-h growth at 30 °C, during which glucose repression was relieved, the cells were diluted to a density of $A_{600} = 0.5$ in SGE medium without leucine, and galactose was added to a final concentration of 2% to induce expression of the recombinant proteins. Cells were harvested after 8-h growth at 30 °C. Yeast extracts were prepared essentially as described (Wright et al., 1990) except that the extraction buffer was 200 mM Tris-HCl, pH 8.0, 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 2 mM DTT, and 1 mM PMSF. Extracts were dialyzed against 10 mM Hepes, pH 8.0, 10% glycerol, 2.5 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, and 1 mM DTT.

Gel Mobility Shift Assays. For DNA-binding assays, 5 μL of extract was incubated in 10 mM Hepes, pH 8.0, 10% glycerol, 2.5 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 μg of poly(dI-dC)·poly(dI-dC), and 100 ng of a 41 base pair palindromic estrogen response element (ERE1) described previously (Zilliacus et al., 1991) with various labeled DNA fragments in a total volume of 10 μL. DNA concentrations were determined spectrophotometrically, assuming $A_{260} = 1$ corresponds to 20 μg/mL oligonucleotide. The GRE in Figure 1 is derived from the tyrosine aminotransferase (TAT) gene (Jantzen et al., 1987) and is 41 base pairs in length. Oligonucleotides with inserted or deleted base pairs were correspondingly longer and shorter (Dahlman-Wright et al., 1990). The palindromic GRE studied in Figures 2–5 is a 41 base pair palindromic GRE (GRE1) described previously (Zilliacus et al., 1991). In Figures 1 and 2B, 0.2 ng of labeled oligonucleotide was added to each incubation. In Figures 3–5, the amount of oligonucleotide added is indicated in the figures. High concentrations of DNA were achieved by diluting the labeled oligonucleotide with nonlabeled oligonucleotide. Protein–DNA complexes were separated from protein-free DNA by nondenaturing electrophoresis (Fried & Crothers, 1981) in 5% polyacrylamide gels. The gels were run at 4 °C in 50 mM Tris–boric acid, pH 8.0, 1 mM EDTA, and 0.1% Triton X-100 at a constant voltage of 200 V. Following electrophoresis, gels were dried and autoradiographed. Data were quantified by scintillation counting of bands excised from gels. In some cases, the bands were also quantified by densitometry of autoradiographs which gave very similar results. The amount of DNA bound was calculated from the specific activity of the probe. Scatchard analysis was used to calculate the dissociation constants for the different proteins. The values measured were reproducible, with individual values varying from the mean of two or more experiments by about 5%, except for the GRNtermΔ protein where the variation was 18% from the mean of two experiments. The *R* values show the reliability of the regression lines used in the Scatchard plots. The Scatchard plots for the GRDBD and GRCtermΔ proteins are nonlinear at low DNA concentrations, due to cooperative DNA binding, and, consequently, these points were excluded from the analysis.

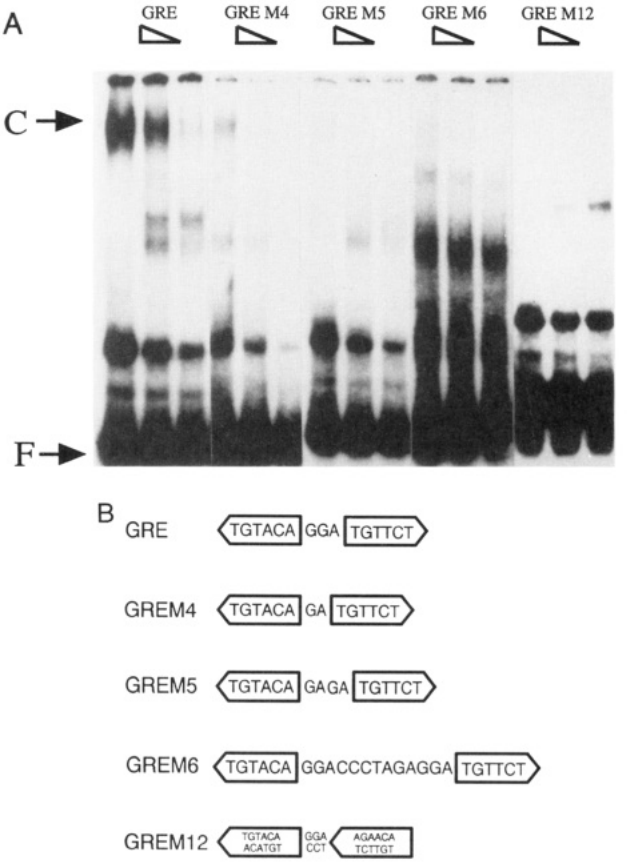

FIGURE 1: GR dimerization correlates with high-affinity DNA binding. (A) Binding of intact GR to the TAT GRE or variants of this GRE was assayed by band shift analysis. The variant GREs contain 2 (GREM4), 4 (GREM5), or 13 (GREM6) base pairs between the half-sites or have an altered relative orientation of the half-sites (GREM12). F and C show the positions of the free probe and the GR–DNA complex, respectively. Extracts are diluted 2-fold serially from left to right. (B) Sequence of the variant GREs studied in (A).

RESULTS

GR Dimerization Is Required for High-Affinity DNA Binding. To evaluate the role of GR dimerization for high-affinity DNA binding, binding of intact GR expressed in yeast cells to a GRE from the TAT gene and variants of this GRE, where the distance between the two half-sites or their relative orientation is changed, was studied using a gel retardation assay (Figure 1A). The sequences of the TAT GRE and the variant GREs are depicted in Figure 1B. Binding of the GR, expressed in yeast cells, resulted in the complex marked C in Figure 1A which is specific to the GR since extracts expressing

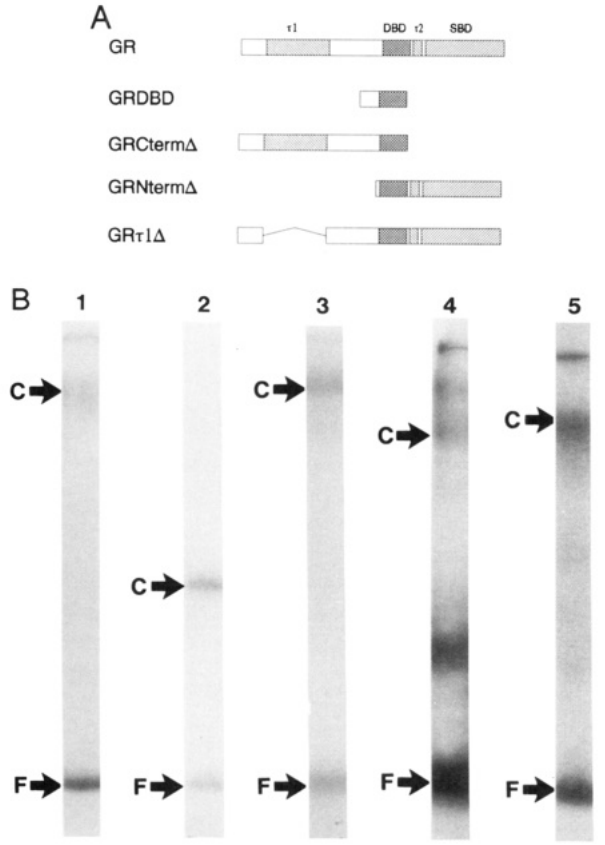

FIGURE 2: Sequence-specific DNA binding by GR derivatives expressed in yeast. (A) GR derivatives expressed in the yeast extracts utilized in this study. The steroid-binding domain (SBD), the DNA-binding domain (DBD), and the transactivation domains (τ1 and τ2) are indicated. (B) Complexes formed by GR derivatives expressed in yeast using a palindromic GRE (AGAACATGATGTTCT) in a gel mobility shift assay. Extracts contained the expressed proteins as follows: GR (lane 1), GRDBD (lane 2), GRCtermΔ (lane 3), GRNtermΔ (lane 4), and GRτ1Δ (lane 5). F and C show the positions of the free probe and the protein–DNA complexes, respectively. Note that the lanes shown should not be compared quantitatively since they are taken from DNA titration experiments and contain different amounts of protein and/or DNA.

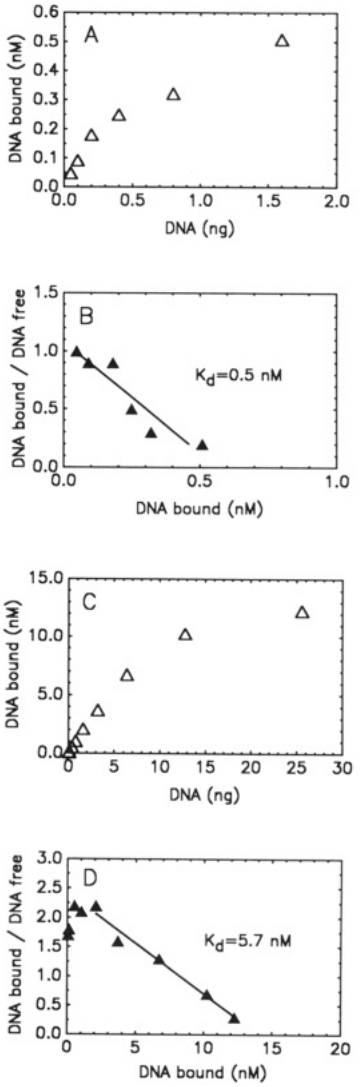

FIGURE 3: DNA-binding affinity of the intact GR and the GR DBD. Scatchard analysis of gel shift assays with yeast extracts containing the GR (B) and the GRDBD (D) proteins. The DNA fragment was a palindromic GRE with sequence AGAACATGATGTTCT. Shown are the saturation curves for GR and GRDBD (A and C, respectively) and the Scatchard plots for GR and GRDBD (B and D, respectively). The *R* values for the regression analysis of the Scatchard data were 0.94 and 0.98, respectively.

smaller GR derivatives do not give this complex (Wright & Gustafsson, 1991). Changing the distance between the half-sites constituting a GRE, or changing their relative orientation, changes the relative position of receptor molecules on the DNA, potentially interfering with interactions between receptor monomers. Figure 1A shows that the affinity of GR for DNA-binding sites where the distance between the half-sites or their relative orientation has been changed is much reduced. This is consistent with the binding behavior previously shown for the GR DBD (Dahlman-Wright et al., 1990) and supports the view that a dimerized receptor structure, that is compatible with specific interaction with both half-sites of the GRE, is required for high-affinity DNA binding of the GR contained in crude yeast extracts. A similar correlation between half-site spacing and DNA-binding affinity has previously been demonstrated for GR purified from rat liver (Chalepakis et al., 1990).

DNA-Binding Affinities of the Intact GR and the GR DBD. To evaluate the role of the protein–protein contact within the DBD for receptor dimerization and high-affinity DNA binding, the DNA-binding affinities of the intact GR and the GR DBD were compared. The GR and GRDBD proteins expressed in yeast are depicted in Figure 2A. Western blot analysis showed that these proteins, as well as the other GR derivatives used in the study, appeared to be intact in the extracts (data not shown). The DNA-binding activity of the expressed proteins was analyzed using a gel retardation assay. As can be seen in Figure 2B, incubation of extracts expressing GR (lane 1) or GRDBD (lane 2) with a palindromic GRE results in formation of a protein–DNA complex. The migration of the complexes varies according to the size of the receptor derivative in the extracts, and therefore they reflect specific interactions with the GRE. The palindromic GRE used for the affinity determinations was chosen because it shows a lower level of nonspecific binding compared to the TAT GRE (compare Figure 2B and Figure 1A).

The DNA-binding affinities of the GR and GRDBD proteins were compared by Scatchard analysis of gel retardation experiments using the palindromic GRE (Figure 3). The DNA-binding affinity of the intact GR is about 10-fold higher than that of the isolated DBD, K_d = 0.5 nM versus K_d = 5.7 nM, respectively. This shows that sequences outside the DBD are required for high-affinity DNA binding by the GR. These sequences probably mediate protein–protein contacts between receptor monomers that stabilize the protein–DNA complex.

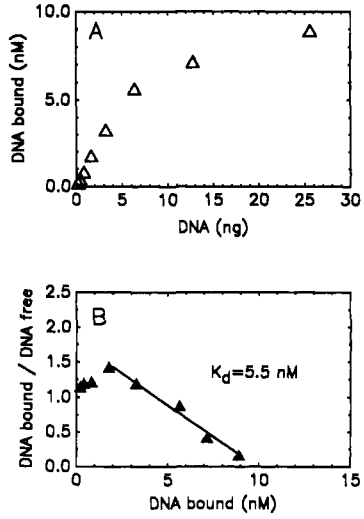

FIGURE 4: DNA-binding affinity of the C-terminally deleted GR. Scatchard analysis of gel shift assays with a yeast extract containing GRCtermΔ (B). The DNA fragment was a palindromic GRE with sequence AGAACATGATGTTCT. Shown are the saturation curve (A) and the Scatchard plot (B). The R value for the regression analysis of the Scatchard data was 0.99.

Table I: DNA-Binding Affinity of Intact and Truncated GR Proteins

GR derivative	K_d (nM)	GR derivative	K_d (nM)
GR	0.5	GRNtermΔ	0.95
GRDBD	5.7	GRτ1Δ	1.4
GRCtermΔ	5.5		

Having established the importance of sequences outside the DBD for high-affinity DNA binding, we investigated whether the N-terminal and/or the C-terminal part of the receptor protein contributed to the difference in affinity between the intact GR and the GR DBD. For the ER, the C-terminal steroid-binding part of the receptor protein has been shown to contribute to receptor dimerization and high-affinity DNA binding (Kumar & Chambon, 1988; Fawell et al., 1990). However, for the GR, it has been suggested that protein–protein interactions involving the N-terminal part of the receptor play a role in the interaction of the dimeric receptor with DNA (Eriksson & Wrange, 1990).

Role of the GR N-Terminus in High-Affinity DNA Binding. The N-terminal part of the GR (GRCtermΔ, Figure 2A) was expressed in yeast. Incubation of the extracts with a labeled GRE fragment led to the formation of a protein–DNA complex (Figure 2B, lane 3). This complex represents a specific DNA–protein complex since it was not seen in extracts expressing other GR derivatives (Figure 2B). Quantification of gel retardation experiments followed by Scatchard analysis shows that the DNA-binding affinity of the GRCtermΔ protein is very similar to that of the GRDBD protein, K_d = 5.5 nM versus K_d = 5.7 nM, respectively (Figures 3 and 4). This shows that sequences N-terminal of the GR DBD do not contain an independent activity that increases the DNA-binding affinity of the receptor protein.

Role of the GR C-Terminus in High-Affinity DNA Binding. The lack of dimerization activity in the N-terminal domain suggests that the C-terminal ligand-binding domain of the GR contributes to receptor dimerization, as has been shown for the ER (Kumar & Chambon, 1988; Fawell et al., 1990). To test this directly, receptor derivatives from which the whole N-terminus (GRNtermΔ) or part of the N-terminus (GRτ1Δ) were deleted, shown in Figure 2A, were expressed and the

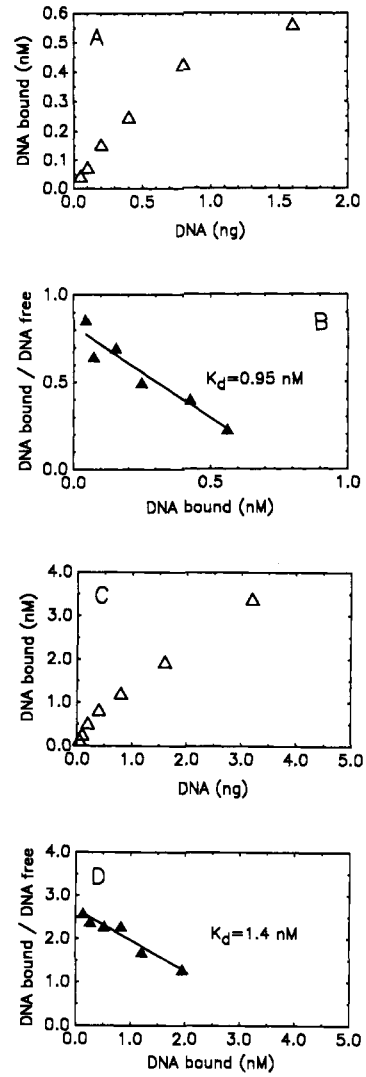

FIGURE 5: DNA-binding affinity of N-terminally deleted GR derivatives. Scatchard analysis of gel shift assays with yeast extracts containing the proteins GRNtermΔ (B) and GRτ1Δ (D). The DNA fragment was a palindromic GRE with sequence AGAACATGATGTTCT. Shown are the saturation curves for GRNtermΔ and GRτ1Δ (A and C, respectively) and the Scatchard plots for GRNtermΔ and GRτ1Δ (B and D, respectively). The R values for the regression analysis of the Scatchard data were 0.96 and 0.97, respectively.

extracts used to produce the specific complexes shown in Figure 2B (lanes 4 and 5). These proteins bound to the GRE with dissociation constants of 0.95 and 1.4 nM, respectively, indicating that the C-terminus does contribute to high-affinity DNA binding by the GR. The two proteins gave very similar results in spite of the low level of active protein in the GRNtermΔ extracts. This probably results from aggregation of the GRNtermΔ protein when it is overexpressed (Wright et al., 1990) and causes an increase in nonspecific binding to the GRE (Figure 2B), thus making quantitation more difficult.

DISCUSSION

In Figure 1A, we show that high-affinity DNA binding by the GR is practically abolished when the distance between, or the relative orientation of, the two half-sites constituting the GRE is altered. This probably reflects an influence on the ability of the receptor to form DNA-bound dimers as has previously been suggested (Chalepakis et al., 1990).

We have used a gel retardation assay, followed by Scatchard analysis, to evaluate the role of different parts of the GR

for maximal DNA-binding affinity, as summarized in Table I. The results show that regions outside of the DBD are required for maximal DNA-binding affinity by the GR (Figure 3). It is likely that this increased DNA-binding affinity is the result of protein–protein interactions between receptor monomers bound to the GRE. This has previously been demonstrated for the ER (Kumar & Chambon, 1988; Fawell et al., 1990). However, we cannot exclude that regions outside the DBD mediate direct contacts with DNA or that they indirectly influence DNA-binding affinity by, for example, causing structural changes within the DBD.

The N-terminal region of the GR has previously been implicated in receptor dimerization (Eriksson & Wrange, 1990). However, this domain does not appear to increase the DNA-binding affinity of the receptor and thus would not be expected to mediate contacts between receptor monomers (Figure 4), at least not in the absence of the steroid-binding domain. Addition of the C-terminal steroid-binding domain increases the DNA-binding affinity of the DBD (Figure 5). This suggests that this part of the receptor molecule contributes protein–protein interactions as has previously been shown for the ER (Kumar & Chambon, 1988; Fawell et al., 1990). The DNA-binding affinity of the GRNtermΔ and GRτ1Δ proteins is not quite as high as that of the intact GR. Thus, the N-terminus seems to be required to achieve maximal DNA-binding affinity. This could reflect an auxiliary role of the N-terminus that enhances the activity in the C-terminus and would be consistent with previous observations that the N-terminus does play a role in receptor dimerization (Eriksson & Wrange, 1990). Alternatively, since the contribution of the N-terminus is very small, it might be due to a general artifact associated with the use of abnormal truncated proteins. At present, we cannot distinguish between these possibilities. However, we can conclude that the C-terminus of the GR makes a major contribution to the receptor's DNA-binding affinity and that the N-terminus cannot contribute independently, although it might perform an auxiliary function in the intact GR.

In vitro mutagenesis has revealed the importance of amino acids at the C-terminal end of the steroid-binding domain for ER dimerization. A striking feature of this sequence is a heptad repeat of hydrophobic residues with additional hydrophobic residues at intermediate positions (Fawell et al., 1990). The segment including these amino acids, particularly the repeating pattern of hydrophobic residues, is conserved within the nuclear receptor family, and it will be interesting to see if the same amino acids are involved in high-affinity DNA binding by the GR.

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