Glucocorticosteroid Receptor Dimerization Investigated by Analysis of Receptor Binding to Glucocorticosteroid Responsive Elements Using a Monomer−Dimer Equilibrium Model†

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ABSTRACT: The aim of this study was to analyze the role of regions of the glucocorticosteroid receptor (GR) outside the DNA binding domain (DBD) in GR binding and homodimerization efficiencies by using a model according to which GR monomers and dimers are in equilibrium and able to bind to each half-palindromic motif of a GRE. We studied wild-type human GR (hGR), an N-terminal domain deleted mutant (lacking amino acids 1−417), a C-terminal deleted mutant (lacking amino acids 550−777, the main part of the ligand binding domain), and two rat GR derivatives limited to the DNA binding domain and proximal sequences. Specific GR monomer and dimer complexes with ^{33}P-labeled palindromic or half-palindromic GREs were identified by gel-shift and methylation interference experiments. The different complexes were quantified, and the multiple equilibrium constants for their formation were determined. The affinity of the monomer for the GRE was not affected by the deletions of regions outside the DBD. However, the affinity of the dimer for the GRE was clearly increased by the presence of the N-terminal domain and, to a lesser extent, by that of the main part of the C-terminal domain. By using this model, we also obtained a GR dimerization constant in the absence of specific binding to GRE. Dimerization of the DBD was not increased by the presence of only one of the GR terminal domains, but an increase in dimerization efficiency was observed when both domains were present, suggesting a structural synergy between the N- and C-terminal domains in GR homodimerization.

Gene transcription requires a complex set of interactions on target gene promoters involving both ubiquitous and cell-specific transcription factors (Diamond, 1990; Truss & Beato, 1993). Among these factors are the steroid hormone receptors which recognize specific DNA regulatory sequences in the promoter of target genes. A consensus motif has been defined for the glucocorticosteroid receptor (GR)[1] and is shared by receptors belonging to the same nuclear receptor subclass (progestin, mineralocorticoid, and androgen receptors) (Tsai et al., 1988; Beato et al., 1989). In most positively regulated genes, the consensus sequence of the glucocorticosteroid responsive elements (GRE) (GGTACAnnnTGTYCY) is formed of an imperfect palindromic sequence separated by a 3 bp hinge. GR binds to this symmetric structure as a dimer, which is thought to be the active regulatory form (Chalepakis et al., 1990; Drouin et al., 1992). There is controversy over the molecular form of the GR that initially binds to GRE, either the dimer (Drouin et al., 1992) or the monomer followed by cooperative binding of the second monomer, as indicated in experiments with the GR DNA binding domain (DBD) (Tsai et al., 1988; Dahlman-Wright et al., 1990, 1991; Truss et al., 1991; Luisi et al., 1991). The main intermonomeric contact occurs within the DBD, as shown by the cooperative binding properties of GR DBD (Dahlman-Wright et al., 1990; Härd et al., 1990) and crystallographic studies (Luisi et al., 1991). Recent work with a perfect palindromic GRE has indicated the contribution of the LBD of the GR (Dahlman-Wright et al., 1992), in agreement with reports describing a dimerization region in the C-terminal extremity of the estrogen receptor (Sabbah et al., 1989; Fawell et al., 1990). In addition, the N-terminal part of the GR may both improve the recognition of GRE sequences by GR and reduce interactions with nonspecific DNA (Chalepakis et al., 1990; Eriksson & Wrange, 1990).

The possible involvement of the GR monomers in positive and negative regulations by glucocorticosteroids has recently been proposed (Glass, 1994). In positively regulated genes, stabilization of the monomer complexes, either by reciprocal interactions and/or additional interactions with (an)other factor(s), would be required for biological activity (Slater et al., 1993; Chan et al., 1991). However, the possible formation of dimer GR complexes on apparent half-palindromes must be carefully evaluated since unrelated half-sites may exist (Garlatti et al., 1994), and, further, recent

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; DBD, DNA binding domain; DMS, dimethyl sulfate; DNA, deoxyribonucleic acid; ds, double-stranded; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; GR, glucocorticosteroid receptor; GRE, glucocorticosteroid responsive element; LBD, ligand binding domain; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ss, singled-stranded; TA, triamcinolone acetonide; TAE, Tris−acetate−EDTA buffer; TBE, Tris−borate−EDTA buffer.

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work on the MMTV regulatory promotor has underlined the role of sequences adjacent to GRE in the selection of binding sites by the GR DBD fragment (La Baer et al., 1994). Half-palindromes may also be involved in repression, suggesting a biological role for GR monomers alone (Tverberg & Rosso, 1992; Heck et al., 1994), or in higher polymerization states of the GR (Drouin et al., 1993), so that the analysis of the binding of GR to GRE in the monomer and dimer forms and of the equilibrium between these two forms may contribute to improving our understanding of glucocorticosteroid-mediated regulations.

This work aims to specify the role of the domains of the GR outside the DBD with respect to the efficiency of GR dimer and monomer interactions with different GREs and with respect to GR dimerization in the absence of specific GRE binding. We chose to study the functional GRE II of the tyrosine-amino-transferase gene, which has an imperfect palindromic structure (Ip) (Jantzen et al., 1987), and two derived sequences containing either a consensus perfect palindrome (Pp) or only a consensus half-palindrome ($^1/_2$-pc). Wild-type and GR derivatives with the N-terminal domain or the main part of the LBD deleted were expressed in the baculovirus Sf9 system, and GR-enriched extracts were prepared. Binding of these GR derivatives to GREs was analyzed by gel-shift experiments and complexes characterized by methylation interference and quantified. Binding parameters were determined, taking the monomer−dimer equilibrium and the stepwise binding of GR to each half-palindromic motif of GRE into account. Comparison with the binding parameters of the GR DBD expressed in bacteria allowed us to further specify which steps involved in the GR−GRE interaction might be dependent on the regions outside the DBD. It was found that the affinity of the monomer for the GRE was not significantly modified upon the deletion of one or both of the N- and the main part of the C-terminal domains. The binding of the dimer to GRE was stabilized more efficiently by a receptor containing the N-terminus than that containing the LBD, as compared to a receptor limited to the DBD and proximal flanking sequences. Finally, without binding to GRE, the presence of both the N-terminal domain and the main part of the LBD for dimer formation is required for full dimerization efficacy.

MATERIALS AND METHODS

Generation of Recombinant Baculoviruses. The full-length hGR cDNA, between the *Kpn*I and *Xho*I restriction sites of pRShGRα (Giguère et al., 1986), and the deleted cDNAs, between the *Kpn*I and *Xho*I restriction sites of plasmids that express I550* (Hollenberg et al., 1989) and Δ428−490 GR (Hollenberg et al., 1987) derivatives or between the *Kpn*I and *Bam*HI restriction sites of the Δ1−417 (ΔGG) expression plasmid (Thompson & Evans, 1989) (Figure 1), were inserted into the *Nhe*I site of the pBlueBac vector (Invitrogen), after filling in all protruding ends. Recombinant baculoviruses were purified and used for the production of wild-type hGR and derivatives as previously described (Summers & Smith, 1987; Vialard et al., 1990; Cadepond et al., 1994).

Sf9 Cell Culture, Infection, and Hormonal Treatment. Sf9 growth and infection were performed as previously described (Cadepond et al., 1994). For receptors with a ligand binding domain, 1 μM triamcinolone acetonide (TA) was introduced in the culture medium 1 h before harvesting cells. In all cases, expressed GR was analyzed for its molecular weight and for its ability to bind steroid. Steroid binding was measured by the hydroxylapatite method (Erdos et al., 1970). The steroid binding capacities of the different extracts are as follows: 137 pmol/mg protein for hGR, 155 pmol/mg for ΔGG, and 59 pmol/mg for Δ428−490. High-salt extracts were prepared as described by Elliston et al. (1992).

Sequence, Purification, and Labeling of Oligonucleotides. The GRE-containing oligonucleotides were derived from the functional imperfect palindromic GRE II of the tyrosine-amino-transferase (TAT) promoter. They consist of the 31 bp ds DNA (−2517 to −2487) of the TAT−GRE II (Jantzen et al., 1987), bordered by GATC sequences filled in for radioactive labeling, (GATC)ATCTCTGC**TGTACA**GGA**TGTTCT**AGCTACTT(GATC), the perfect palindromic equivalent sequence (Pp) (GATC)ATCTCTGC**AGAACA**GGA**TGTTCT**AGCTACTT(GATC), the consensus half-palindromic GREs ($^1/_2$pc) (GATC)ATCTCTGCTTGCTCGGA**TGTTCT**AGCTACTT(GATC), and the less conserved half-palindrome ($^1/_2$plc) (GATC)ATCTCTGC**TGTACA**GGACTCGTTTAGCTACTT(GATC). Receptor binding specificity of the different complexes was assessed by competition with a 10- or 25-fold molar excess of GRE, an estrogen responsive element (ERE) GTCCAAAGTC**AGGTCA**CAG**TGACCT**GATCAAAGTT, or an unrelated sequence (Random, Rd) CCTCCATGACTCCAGAACTAA in the presence of 2 μg of sonicated salmon sperm DNA. The ERE oligonucleotide used corresponds to the −342 to −308 sequence of the vitellogenin A2 gene promoter (Kumar et Chambon, 1988).

Single-stranded (ss) oligonucleotides (from Institut Pasteur, Unité de chimie Organique) were purified on a 50% urea−10% polyacrylamide gel. For the methylation interference studies, ss oligonucleotides were labeled with [γ-^{32}P]ATP using T4-polynucleotide kinase, and then complementary strands were annealed. For electrophoretic shift assays, double-stranded (ds) oligonucleotides with free 5′-ends were labeled by filling in the ends using the Klenow fragment of DNA polymerase in the presence of [α-^{32}P]- or [α-^{33}P]dATP. Double-stranded oligonucleotides were purified on a 12% polyacrylamide gel.

Electrophoretic Mobility Shift Assay (EMSA). Extracts were preincubated for 15 min at 0 °C with or without 2 μg of sonicated salmon sperm DNA in a 12 μL volume (final concentrations: 20 mM Tris Cl, 10% glycerol, 40−60 mM NaCl, 4 mM DTT, and 0.05% BSA). We tested various quantities and types of nonspecific DNA and found that 2 μg of sonicated salmon sperm DNA provided the best compromise between the detection of specific GR complexes and the extinction of nonspecific bands (Figure 3, lanes 13 and 14). ^{33}P-labeled oligonucleotide (0.2−20 ng), alone or with competitor DNA, was added in an 18 μL final volume, and the mixture was incubated for 1 h at 25 °C. Samples were layered on native polyacrylamide gels (5−10%) in recirculating 0.25× TBE (TBE: 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 7.8−8 at 4 °C).

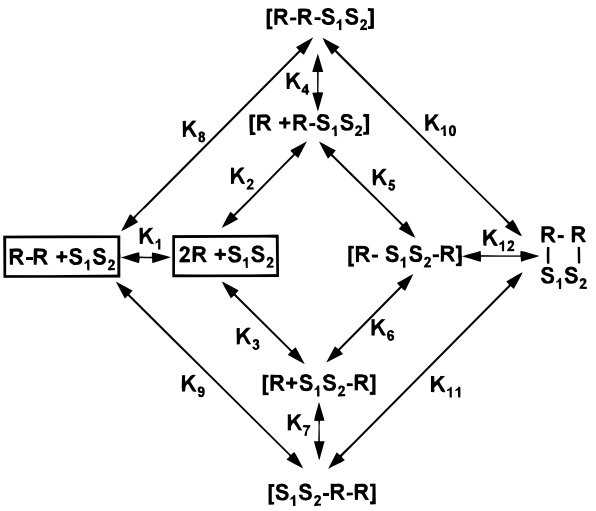
Anti-GR Rabbit Polyclonal Antibodies. Antibodies directed against the hGR molecule were used to supershift hGR-containing complexes: a polyclonal rabbit antibody raised against a peptide of the 149−168 hGR N-terminal region (A1) (Cadepond et al., 1993), another antibody

obtained from purified rat liver GR (A2; Ö. Wrange et al., Karolinska Institutet, Stockholm), and a polyclonal antibody raised against a bacterially expressed fragment of rat GR lacking the N-terminal region (αP1, A3) (Hoeck et al., 1989). One or two microliters of antibody solution were introduced into the incubation mixture for 20 min after the 1 h incubation period.

Methylation Interference. Double-stranded oligonucleotides labeled at one end (~75 ng in 10 μL) were treated with dimethyl sulfate (DMS in 50% ethanol) in 50 mM cacodylate buffer containing 10 mM $MgCl_2$ and 1 mM EDTA (pH 8), in the presence of 2 μg of salmon sperm DNA. We used salmon sperm DNA instead of poly(dAdT) (Chalepakis et al., 1990) to maintain the same conditions as in EMSA. Methylated probes were used for gel retardation as before. Bands corresponding to bound and free DNA were excised and eluted from the gel overnight at 37 °C with a buffer containing 0.5 M ammonium acetate, 10 mM Tris Cl, 0.1% SDS, and 1 mM EDTA (pH 8). The eluted DNAs were cleaved in 1 M piperidine for 90 min at 30 °C and analyzed on sequencing gels (50% urea−10% acrylamide), which were dried and autoradiographed. The radioactivity of each band corresponding to G cleavage was quantified (as percent of the total radioactivity of the slot) by using a Millipore densitometry scanner. For free DNA, direct absorbances are shown. To illustrate the modifications in the methylation pattern of the protein-bound DNA, the absorbance values of each band are given after subtraction of the free DNA value.

Determination of Binding Parameters. Bands corresponding to DNA-bound GR dimer and monomer and free DNA were quantified with a Berthold radioactivity analyzer. When the intensities of the bands were too weak or when they were very close to a stronger one, their relative intensities were quantified by a Millipore density scanner. Monomer and dimer GR forms were assumed to be in equilibrium, and polynomial regression analysis of the binding data was achieved by using Excel 5 (Microsoft) on a PC-compatible computer.

The model used for the GR monomer (R) dimerization (dimer: RR) and binding to the two sites of palindromic (S_1S_2) is illustrated in the following schema. Complex formation is broken up into 12 binding reactions with the corresponding dissociation constants (starting reagents are boxed):



The total receptor concentration, $[R_T]$, is

$$[R_T] = [R] + 2[RR] + [R - S_1S_2] + [S_1S_2 - R] + 2[RR - S_1S_2] + 2[R - S_1S_2 - R] + 2[S_1S_2 - RR] + 2\begin{bmatrix} R\text{-}R \\ | \quad | \\ S_1S_2 \end{bmatrix} \quad (1)$$

The three quantified concentrations are that of the free oligonucleotide ($[O] = [S_1S_2]$) and those of the oligonucleotide binding one [RO] and two [DO] receptor molecules, where

$$[RO] = [R{-}S_1S_2] + [S_1S_2{-}R] \quad (2)$$

$$[DO] = [RR{-}S_1S_2] + [R{-}S_1S_2{-}R] + [S_1S_2{-}RR] + \begin{bmatrix} R\text{-}R \\ | \quad | \\ S_1S_2 \end{bmatrix} \quad (3)$$

Thus,

$$[R_T] = [R] + 2[RR] + [RO] + 2[DO] \quad (4)$$

The unknowns in eq 4, [R] and [RR], can be obtained from the corresponding mass action equations:

$$K_2 = [R][S_1S_2]/[R{-}S_1S_2], \qquad K_3 = [R][S_1S_2]/[S_1S_2{-}R]$$

By substituting into eq 2 and rearranging,

$$[R] = [RO]/[S_1S_2]\left(\frac{1}{K_2} + \frac{1}{K_3}\right) \quad (5)$$

Similarly,

$$K_8 = [RR][S_1S_2]/[RR{-}S_1S_2], \qquad K_9 = [RR][S_1S_2]/[S_1S_2{-}RR]$$

$$K_{10} = [RR{-}S_1S_2] \Big/ \begin{bmatrix} R\text{-}R \\ | \quad | \\ S_1S_2 \end{bmatrix}, \qquad K_{12} = [R{-}S_1S_2{-}R] \Big/ \begin{bmatrix} R\text{-}R \\ | \quad | \\ S_1S_2 \end{bmatrix}$$

By substituting into eq 3 and rearranging,

$$[RR] = [DO]/[S_1S_2]\left(\frac{1}{K_9} + \frac{1}{K_8}\left(1 + \frac{1}{K_{10}}(1 + K_{12})\right)\right) \quad (6)$$

By substituting eqs 5 and 6 into eq 4 and rearranging (with $[O] = [S_1S_2]$),

$$[RO] + 2[DO] = [R_T] - \frac{[RO]}{[O]\left(\frac{1}{K_2} + \frac{1}{K_3}\right)} - \frac{2[DO]}{Z[O]} \quad (7)$$

where

$$Z = \left(\frac{1}{K_9} + \frac{1}{K_8}\left(1 + \frac{1}{K_{10}}(1 + K_{12})\right)\right)$$

While the unknown parameters in eq 7 (K_2, K_3, and Z) should be obtainable by nonlinear regression, the nonindependence between the variables DO and RO and practical problems of receptor instability (especially for the monomer) meant that a check had to be made on those sets of results that could be retained for the calculation. For these reasons, eq 7 was expressed as two equivalent equations. Given that $K_1 = [R]^2/[RR]$, and substituting eqs 5 and 6 into it,

$$K_1 = \frac{[RO]^2 Z}{[O][DO]\left(\frac{1}{K_2} + \frac{1}{K_3}\right)^2}$$

From eq 7,

$$[RO] + 2[DO] = [R_T] - \frac{[RO]}{[O]\left(\frac{1}{K_2} + \frac{1}{K_3}\right)} - \frac{2[RO]^2}{[O]^2 K_1 \left(\frac{1}{K_2} + \frac{1}{K_3}\right)^2} \quad (8)$$

$$[RO] + 2[DO] = [R_T] - \sqrt{\frac{K_1[DO]}{Z[O]}} - \frac{2[DO]}{Z[O]} \quad (9)$$

These equations are quadratics ($y = ax^2 + bx + c$), with x being [RO]/[O] or $\sqrt{[DO]/[O]}$; notice that $[RO]^2/([O][DO]) = [K_1(1/K_2 + 1/K_3)^2]/Z$ is a constant that can be determined experimentally. From both eqs 8 and 9, polynomial regression was used to obtain the unknown parameters and $[R_T]$. Sets of results for which the two equations did not give the same value for $[R_T]$ (±10%) were not taken into consideration.

In these equations, if the affinities of both sites of a palindromic oligonucleotide are the same, then $K_2 = K_3$. In the case of the imperfect palindromic sequence, $K_2 \neq K_3$, then the value of K_2 (and thence K_3) must be found by using results with the perfect palindrome. Further, for the perfect palindromic GRE, Z can be simplified given that $K_8 = K_9$ and $K_{10} = K_{11}$ and by assuming that $K_{10} \ll 1$ and $K_{12} \ll 1$. Thus, $1/Z \cong K_8K_{10}$ ($=K_9K_{11}$), which is the dissociation constant for the binding of dimerized receptor to both oligonucleotide binding sites.

The significances of the differences between equilibrium constant values were assessed by using the *t*-test.

RESULTS

Expression of Wild-Type and Mutated hGRs. Wild-type and three GR derivatives used in this study (Figure 1A) were produced in the baculovirus Sf9 cell system (wild-type hGR, I550*, Δ1−417, and Δ428−490). The correct expression of hGR derivatives in SF9 cells was checked by Western blot analysis (Figure 1B). Each of the three GR deleted mutants studied has one of the main domains of the GR molecule deleted: the N-terminal region (Δ1−417 or ΔGG), most of the DBD (Δ428−490), or most of the LBD (I550*). The Δ428−490 mutant is an inactive GR molecule unable to bind DNA, and the I550* derivative is a constitutively active GR, while the ΔGG has reduced transcriptional activity (Hollenberg & Evans, 1988; Hollenberg et al., 1989; Thompson & Evans, 1989).

To compare the effects of deletions of N- and the main part of C-terminal regions of the hGR with the effects of the addition of the same regions to a receptor limited to the DBD, we studied two GR DBD fragments. Taking into account the unavailability of the corresponding recombinant baculovirus and the very high homology of the DBD sequences found between GRs from different species, we used, in our defined experimental conditions, two rat DBD fragments expressed in *Escherichia coli* (DBD fragments X556 and 440−525), generously provided by L. Freedman (Freedman et al., 1988; Alroy & Freedman, 1992). The 440−525 fragment, corresponding to the 421−506 sequence in hGR, has only one amino acid exchange at the last position (Ala instead of Thr), while X556, corresponding to the 487−536 sequence in hGR, displays other additive minor modifications [five amino acid exchanges and two point deletions (Hollenberg et al., 1985; Miesfield et al., 1986)].

Identification of Retarded Bands Containing Wild-Type and Mutated Receptor Complexes with Palindromic and half GREs. Complexes formed with wild-type hGR are shown in Figures 2 and 3. When Pp GRE was used (Figure 2, lanes 2−8), a major slow-migrating band was observed (lanes 2 and 6). This band disappeared in the presence of an excess of Pp GRE (lane 3), but not of ERE (lane 4) or of a random sequence (Rd, lane 5), and should correspond to a GR dimer. We also detected two weak faster migrating bands displaying a specific response by oligonucleotide competition (lane 2). These bands could be supershifted by two different antibodies (Al, lane 7; A2, lane 8; control, lane 6). With ½pc GRE (lanes 1, 9−14), a major band that migrates similarly to the faster small band previously observed with palindromic GRE was detected (lanes 1 and 9). This band, which was extinguished by the introduction of excess Pp GRE (lane 10) into the incubation medium but not by ERE (lane 11) or Rd (lane 12) and was supershifted by the anti-GR antibodies (A1, lane 13; A2, lane 14), should correspond to a monomeric GR complex.

Migration patterns of complexes obtained with the I550* mutant (Figure 3, lanes 5 and 6) are very similar to those

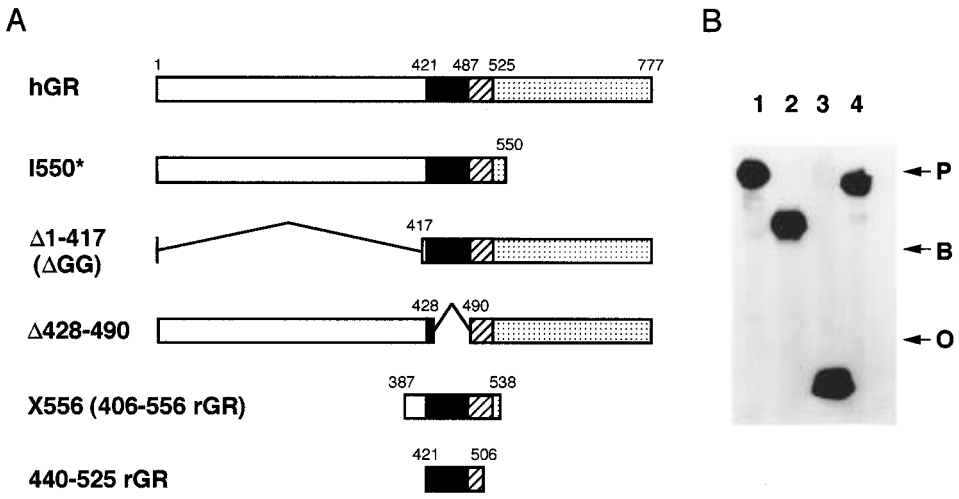


FIGURE 1: Overexpression of various GR derivatives. (A) Schematic representation of the wild-type and mutated hGRs: open area, N-terminal domain; closed area, DBD; striped area, hinge region; stippled area, LBD. For the rat DBD fragments, the numbers of the amino acids indicated correspond to the equivalent amino acids in the hGR. (B) Expression of wild-type hGR (lane 1), I550* (lane 2), ΔGG (lane 3), and Δ428−490 (lane 4) mutants in baculovirus-infected Sf9 cells. Extracts were prepared as described in Materials and Methods and analyzed after SDS−PAGE by Western blotting using the αP1 antibody (A3). P: phosphorylase B, 92.5 kDa. B: bovine serum albumin, 69 kDa. O: ovalbumin, 46 kDa.

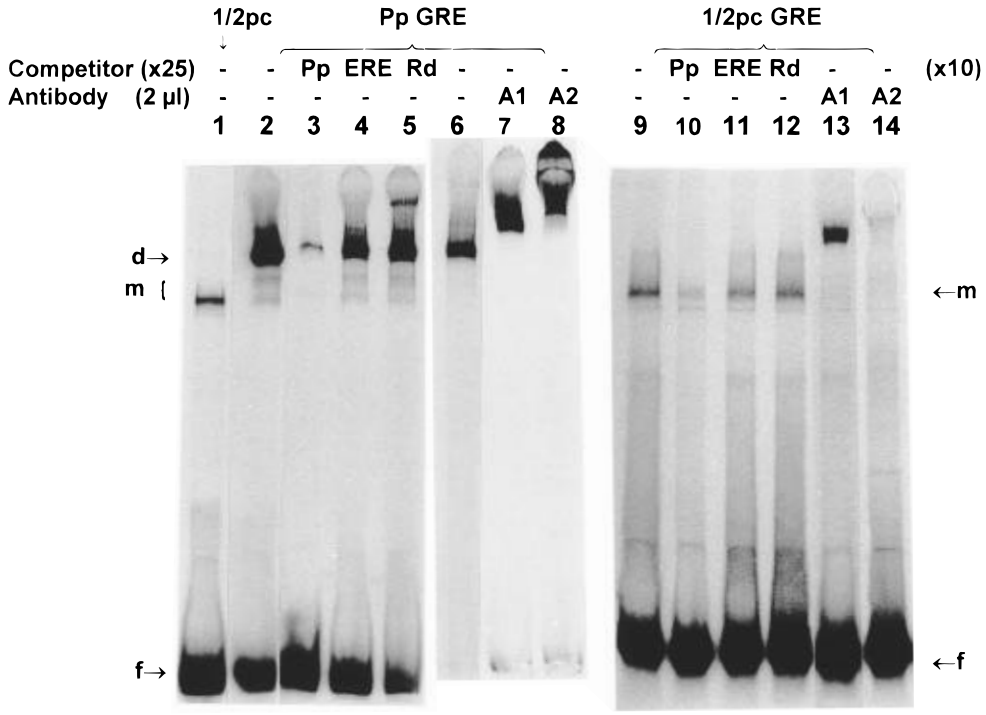


FIGURE 2: Specific binding of wild-type hGR to perfect palindromic (Pp) and half-palindromic consensus ($^1/_2$pc) GREs. Whole cell extracts containing hGR (3.5 μg) were incubated with [^{33}P]GRE (3 ng) (Pp GRE in lanes 2−8 and $^1/_2$pc GRE in lanes 1, 9−14) in either the absence (lanes 1, 2, 6, and 9) or presence of 25- or 10-fold excess of unlabeled perfect palindromic GRE (Pp) (lanes 3 and 10), ERE (lanes 4 and 11), or random sequence (Rd) (lanes 5 and 12). For supershift experiments, 2 μL of antibody solution A1 (lanes 7 and 13) or A2 (lanes 8 and 14) was introduced into the reaction medium; d, dimer; m, monomer; f, free DNA.

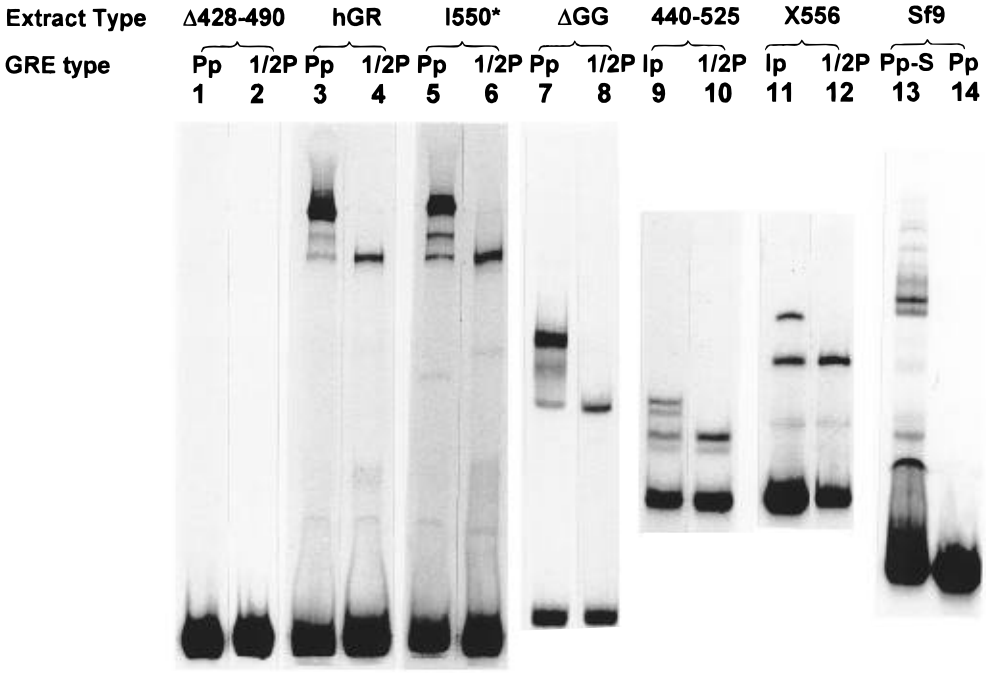


FIGURE 3: Specific binding of various hGR derivatives to palindromic (Pp or Ip) and consensus half-palindromic ($^1/_2$pc) GREs. Whole cell extracts containing the Δ428−490 mutant (9 μg, lanes 1 and 2), the wild-type hGR (3.5 μg, lanes 3 and 4), the I550* mutant (4.8 μg, lanes 5 and 6); ΔGG (8.2 μg, lanes 7 and 8), rat DBD fragments 440−525 (50 ng, lanes 9 and 10) and X556 (75 ng, lanes 11 and 12), and uninfected Sf9 cell extracts (11.2 μg, lanes 13 and 14) were incubated with [^{33}P]GRE (3 ng) (Pp GRE in lanes 1, 3, 5, 7, 13, and 14, Ip GRE in lanes 9 and 11, and $^1/_2$pc GRE in lanes 2, 4, 6, 8, 10, and 12). Lane 13 shows Sf9 extracts incubated with Pp GRE in the absence of nonspecific DNA (2 μg of salmon sperm DNA).

described for hGR−GRE complexes (lanes 3 and 4). However, with palindromic GRE, the two bands migrating faster than the main band are more intense than those observed with hGR.

For the ΔGG mutant interacting with Pp GRE (Figure 3, lanes 7 and 8), two major bands migrating faster than the above-mentioned major band found with hGR or I550* are detected as well as, between these, a diffuse third band displaying variable relative intensity (lane 7). All of these bands disappeared in the presence of an excess of GRE, but not of ERE or Rd (not shown). The A3 antibody, but not the others, shifted the three ΔGG bands (not shown). This

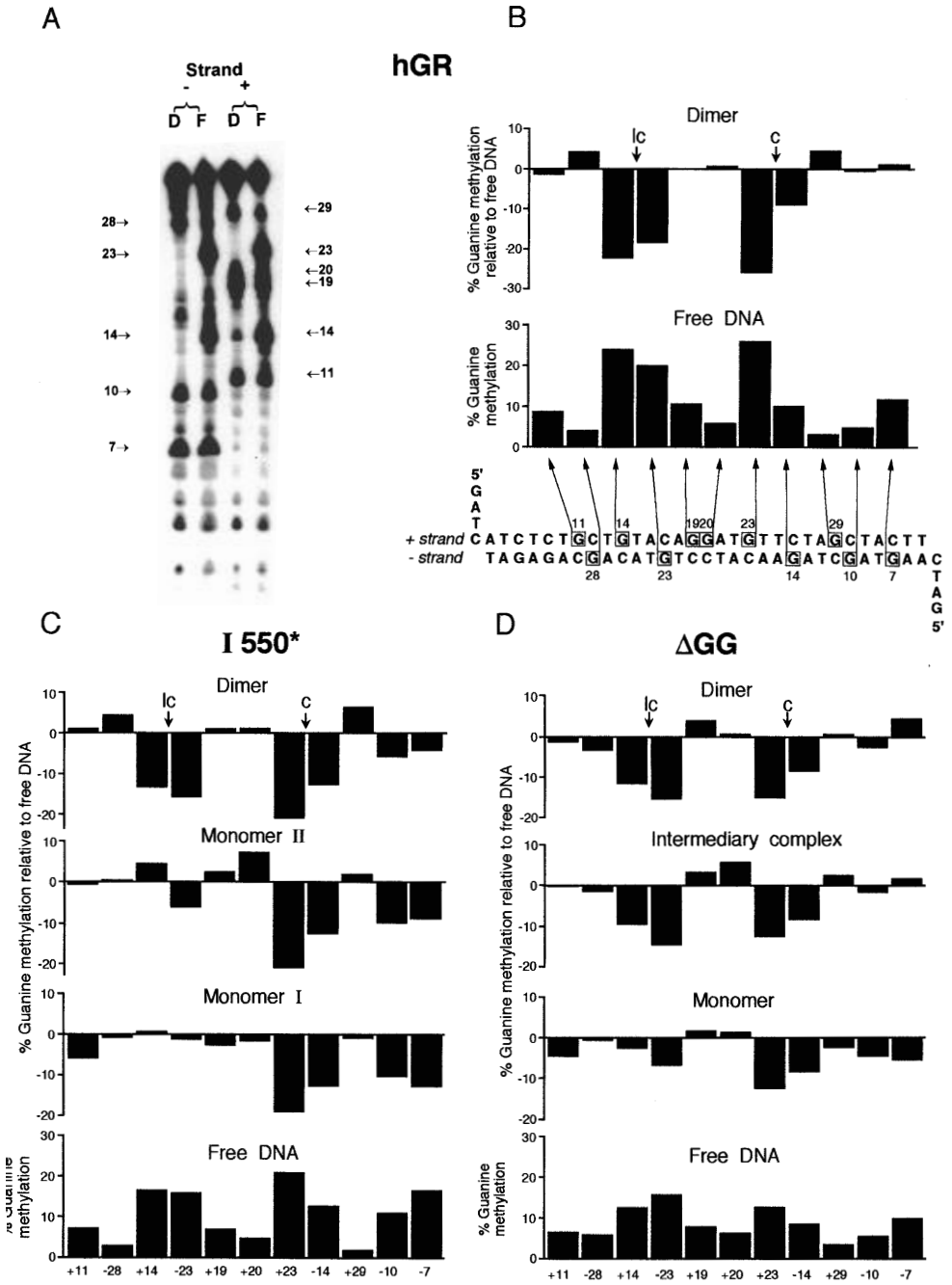


FIGURE 4: Methylation interference analysis of the complexes formed with wild-type hGR and derivatives: (A) autoradiogram showing the methylated guanines in the hGR dimeric complex (D) and in free DNA (F); (B) quantification of the band intensities (corresponding to guanine cleavage) in the dimeric Ip GRE−hGR complex; (C) quantification of the band intensities in the Ip GRE−I550* complexes; (D) quantification of the band intensities in the Ip GRE−ΔGG complexes. Band absorbance values are given after subtraction of the free DNA values. For the free DNA, direct absorbances are shown. The consensus half-GRE site (c) and the less conserved half-GRE site (lc) are indicated.

would be expected since the A3 antibody, but not the A1 and A2 antibodies, recognizes epitopes outside the N-terminal region of GR, which is lacking in ΔGG. The major bands were taken to represent the dimeric and monomeric forms of ΔGG bound to GRE. With $^1/_2$pc GRE (lane 8), the major band migrated like the faster one observed in the presence of palindromic GRE, and tests with competing DNAs and A3 antibody showed the specificity of the binding.

Under our defined experimental conditions, the rat GR DBDs and the X556 (lanes 11 and 12) and 440−525 fragments (lanes 9 and 10) gave dimeric and monomeric complexes with Ip GRE (or Pp GRE, not shown). Only monomeric complexes were observed with $^1/_2$pc GRE. The duplicated bands observed with the 440−525 rat GR were due to a particular sensitivity of this derivative to limited proteolysis.

As expected, given the absence of the main part of the DBD, the Δ428−490 mutant did not yield any specific bands with GREs (Figure 3, lanes 1 and 2), as observed with control Sf9 cells (lane 14).

Analysis of Various Complexes by Methylation Interference. Methylation interference experiments were undertaken to map the protein−DNA contacts for each specific complex and to determine whether one or two half-GRE sites are involved in complexes formed with receptor. The major complex formed between Ip GRE and hGR (Figure 4A,B) had the characteristic pattern of undermethylated guanines (essentially nos. 14 and 23 of the + and − strands), which

Table 1: Binding Parameters for the Interaction of Wild-Type and Mutant GRs with Perfect (Pp) and Imperfect (Ip) Palindromic GREs[a]

		equilibrium dissociation constants (nM)				
		K_1	K_2	$1/(1/K_2 + 1/K_3)$	K_3	$1/Z = K_8K_{10}$
hGR	Pp	3.9 ± 1.9 ($n = 5$)	62 ± 18 ($n = 5$)			1.21 ± 0.58 ($n = 5$)
	Ip	3.1 ± 1.7 ($n = 5$)		45 ± 12 ($n = 5$)	158	2.56 ± 0.64 ($n = 5$)
I550*	Pp	15 ± 7.5 ($n = 5$)	61 ± 18 ($n = 5$)			1.39 ± 0.88 ($n = 5$)
	Ip	21 ± 4.0 ($n = 4$)		33 ± 9.0 ($n = 4$)	72	3.25 ± 1.13 ($n = 4$)
ΔGG	Pp	34 ± 17 ($n = 5$)	70 ± 30 ($n = 5$)			3.08 ± 1.75 ($n = 5$)
	Ip	36 ± 7 ($n = 4$)		45 ± 5.0 ($n = 5$)	122	5.9 ± 1.96 ($n = 4$)
X556 (406−556)rGR	Pp	13 ± 2.2 ($n = 6$)	62 ± 10 ($n = 6$)			7.14 ± 3.35 ($n = 6$)
	Ip	15 ± 4 ($n = 4$)		34 ± 7.0 ($n = 5$)	72	16 ± 3.6 ($n = 4$)
440−525 rGR	Pp	21 ± 6.7 ($n = 4$)	92 ± 22 ($n = 4$)			16.8 ± 10 ($n = 4$)
	Ip	23 ± 9.8 ($n = 4$)		50 ± 18 ($n = 5$)	110	25.7 ± 13.7 ($n = 4$)

[a] Dissociation constants (±standard deviations) were determined as described in Materials and Methods, and n is the number of separate experiments. K_1 corresponds to the dissociation constant for receptor dimerization in the absence of GRE binding, K_2 corresponds to the binding of monomer GR to one site of palindromic GRE, and K_3 corresponds to the binding of GR monomer to the lower affinity site of imperfect palindromic GRE. K_3 is derived from the value of $1/(1/K_2 + 1/K_3)$ and the corresponding value of K_2. The complex constant K_8K_{10} corresponds to the binding of GR dimer to both oligonucleotide sites of a GRE.

would be expected if a dimer GR reacts with the two half-palindrome motifs of a complete GRE. For the I550* mutant, the major complex displayed the same characteristics as that described for hGR. The two minor, faster migrating bands obtained with the I550* mutant were analyzed and corresponded to a monomeric type interaction with the consensus half-GRE (Figure 4C). The slower migration of the monomer II band may result either from a monomer complex having a different conformation or from it including another protein component. Variations in the methylation of guanines in the less conserved half-palindrome and flanking nucleotides agree with such assumptions. In both cases, formation of this complex should depend on the palindromic context sequence, since it was not observed with the half-palindrome (Figure 3).

For the ΔGG mutant (Figure 4D), the slower and intermediate bands were characteristic of dimeric type complexes. The faster band did not exactly correspond to a monomeric type interaction: the consensus half-GRE ($^1/_2$-pc) contained the expected undermethylated guanines, but guanine 23 of the − strand of the less conserved half-GRE (TGTACA) was still partially undermethylated. Analysis of the gel shifts found after incubation of an oligonucleotide having only the less conserved half-GRE ($^1/_2$plc) with ΔGG demonstrated that this guanine undermethylation could be due to a nonspecific binding component in this region. With the consensus half-GRE, the major bands displayed characteristics of the monomeric type interaction. Identical methylation interference patterns were recorded for hGR, I550*, and ΔGG (not shown).

The identification of the complexes containing monomer and dimer GRs allowed the determination of the binding parameters of the GR−GRE interaction, following quantification of these complexes. Monomer complex concentrations of hGR and I550* were estimated from the sum of the intensities of the two weak bands. The dimer ΔGG complex concentrations included the contribution of the intermediary band.

Determination of the Binding Parameters of the Monomer and Dimer GR−GRE Complexes. The binding results were treated according to a monomer−dimer equilibrium model, as described under Materials and Methods. Polynomial regression analysis permitted the determination of the apparent equilibrium dissociation constants of various reactions possibly involved in the GR−GRE interaction.

The R_T values that we determined for each extract type represent an estimate of the number of receptor molecules able to bind to the GREs. Binding capacities of 63 pmol/mg protein for the hGR extract, 67 pmol/mg protein for the I550* extract, and 125 pmol/mg protein for the ΔGG extract were calculated. Thus, 46% of liganded hGR and 80% of liganded ΔGG bind to specific DNA.

Table 2: Effect of the Addition of N- and C-Terminal Regions of the GR on the Dimerization Efficiency (K_1) and GR Binding to GRE as Monomer (K_2) and Dimer (K_8K_{10})[a]

addition of N- or C-terminal or DBD flanking regions		K_{1a}/K_{1b},	K_{2a}/K_{2b},	$[K_8K_{10}]_a/[K_8K_{10}]_b$	
a⇒b		Pp	Pp	Pp	Ip
X556⇒I550*	+N	∼1	∼1	5.13	4.9
ΔGG⇒hGR	+N (C present)	8.5	∼1	2.5	2.3
X556⇒ΔGG	+C	0.4	∼1	2.3	2.7
I550*⇒hGR	+C (N present)	3.8	∼1	∼1	1.3
440−525⇒X556	+DBD flanking regions	1.6	1.5	2.4	1.6

[a] Values correspond to the quotient of the equilibrium dissociation constants of mutants lacking (a) or having (b) the considered region and represent the affinity gain resulting from the addition of the considered domain.

The binding and dimerization parameters calculated for each type of receptor interacting with Pp and Ip GREs are summarized in Tables 1 and 2. From the interaction of each extract type with Pp, the equilibrium dissociation constants of monomeric GR with the consensus half-site (K_2 in Table 1) could be determined. There was no decrease in the affinity of the GR monomer for GRE upon deletion of most of the LBD, the entire N-terminal domain, or both of these GR domains. A significant decrease (t-test, $p \leq 0.05$) was observed only for the 440−525 mutant with respect to hGR and I550*. With Ip GRE, the $1/(1/K_1 + 1/K_2)$ coefficient was always slightly higher than with Pp ($=K_2/2$), reflecting a decreased affinity of the GR monomer for the less conserved half-palindrome (K_3); however, K_3 values, of the same order of magnitude as K_2 values, could not be determined precisely.

The complex constant $1/Z$ value can be simplified with the assumptions described under Materials and Methods, so that $1/Z = K_8K_{10}$ corresponds to the successive binding of dimeric GR to the first and second GRE half-sites (Table 1) and, thus, to the affinity of the dimer for palindromic GRE. We observed no change in the affinity of the GR dimer upon the deletion of most of the LBD, while a significant decrease (t-test, $p \leq 0.05$) was observed upon deletion of the entire N-terminal domain (2.54-fold for Pp and 2.3-fold for Ip, respectively). An additional decrease in affinity was ob-

served when both of these DNA flanking regions were removed, as shown by the higher $1/Z$ values obtained with the X556 and 440−525 fragments (6.3- and 13.9-fold for Pp and 6.25- and 10-fold for Ip, respectively). With all extracts, the affinity of the dimer GR for Ip was lower than that for Pp GRE.

K_1 values for the different GR derivatives are dissociation constants for GR dimerization without binding to specific DNA. A significant decrease in dimer formation was observed for all of the deleted mutants when compared with hGR.

Some titrations were also performed in the absence of nonspecific salmon sperm DNA. An increase in the quantities of specific complexes was observed under these conditions. However, with more than 1−2 ng of GRE, additional bands were observed. The identical patterns observed with hGR, I550*, and ΔGG indicate that the bands correspond to the interaction of GRE sequences with nuclear factors present in infected SF9 cells (Figure 3, lane 13). The affinities estimated in the absence of nonspecific DNA were approximately 1 order of magnitude higher than those in its presence.

DISCUSSION

We studied the binding of wild-type hGR and mutants deleted from the C- and/or N-terminal parts of the molecule to the natural GRE II sequence of the tyrosine-aminotransferase gene, to the equivalent perfect palindromic sequence, or to the consensus half-palindromic sequence. By gel-shift experiments and methylation interference, we have provided evidence for the formation of both monomer and dimer GR complexes with palindromic GRE and of only monomeric complexes with the consensus half-GRE. Titration experiments, using the gel-shift technique, were performed to determine the dimerization and DNA binding parameters for wild-type and mutant GRs, according to a monomer−dimer equilibrium model for the stepwise binding of GR to each half-site motif of a GRE.

The equations provide a means for determining the dissociation constants for receptor dimerization without specific binding to GRE (K_1), for the binding of one receptor molecule to the consensus half-GRE site (K_2), and for the binding of dimer to the DNA (K_8K_{10}). They do not, however, provide information on the binding constants (K_5 and K_6) representing DNA binding of a second receptor monomer without receptor dimerization. Cooperativity of this second receptor monomer binding has been reported (Dahlman-Wright et al., 1990, 1992; Härd et al., 1990), and determination of K_5 and K_6 would permit the discrimination between true cooperativity ($K_5 < K_2$, $K_6 < K_3$) and a pseudo-cooperativity in which the measured increase in affinity for binding a second receptor monomer is provided by the binding energy of receptor dimerization ($K_5K_{12} < K_2$, $K_6K_{12} < K_3$).

N- or C-Terminal Regions of GR May Contribute to the Affinity of the Dimer for GRE but Not That of the Monomer. The affinities of the GR monomers of the various GR derivatives for GRE (K_2) were very similar (Tables 1 and 2), independent of GR derivatives, sources, and species. This demonstrated the absence of additional contacts between the DNA and the regions outside the DBD and underlined the crucial role of the DBD.

The affinity values that we obtained for the various GR dimers vary according to the GR derivative type. The respective contributions of the N-terminal region and the main part of LBD (amino acids 550−777) to the affinity of the dimer for GRE are illustrated in Table 2. Values indicate the affinity gain upon the addition of the considered region, and they correspond to the ratio of the K_8K_{10} constants of the two GR derivatives to be compared. The presence of the N-terminal region fused to the DBD (with immediate flanking regions) is sufficient to restore full binding affinity, while the addition of the main part of the LBD partially restored this affinity. Comparison of the values obtained for the X556 and 440−525 fragments underlines the stabilizing effect of the proximal rat DBD flanking regions.

The affinity of the dimer of the C-terminal truncated mutant I550* for GRE was not significantly lower than that of the wild-type hGR. A similar result has recently been reported for the androgen receptor (Palvimo et al., 1993; Kallio et al., 1994)). In apparent contrast with these data, Dahlman-Wright et al. (1992), using a GR C-terminal mutant truncated at amino acid 500, found a predominant role for the LBD in binding of the dimer to GRE. Nevertheless, the segment 500−550, which contains the GR LBD's transactivation regions (τ2), is retained in the C-terminal deleted mutant used here, and this may be the LBD region stabilizing dimer binding. This result is compatible with the high transcriptional efficiency of the I550* constitutive mutant (40% that of the wild-type GR), which would not be consistent with an important decrease in its efficiency of binding to GRE (Hollenberg et al., 1989).

For the N-terminal deleted mutant (ΔGG), we observed a decrease (2.3−2.5-fold) in the efficiency of binding of the dimer to GRE, in agreement with other reports (Dahlman-Wright et al., 1992). For DBD fragments, we obtained a 10-fold decrease as compared with intact GR as already reported (Dahlman-Wright et al., 1992), although these authors did not use the same DBD length and sequence.

Role of the N- and C-Terminal Domains in the Formation of GR Dimers. The similarly higher values of K_1 for all deleted GR derivatives compared with that for wild-type GR indicate that the presence of both terminal regions is necessary for optimum dimer formation. Addition of only one terminus to the DBD (Table 2) was inefficient, if not deleterious (ΔGG). The necessary requirement of both the N- and C-terminal regions for obtaining an increase in dimerization efficiency, as compared to the individual contribution of either of these regions for increasing the dimer−GRE binding, suggests that binding to GRE may induce modifications in interdimeric contacts in regions of the receptor outside the DBD.

The K_1 values obtained from our model indicate significant formation of GR dimers in the absence of specific binding to GRE. This was unexpected given the reported instability of dimers of intact hGR in solution without DNA (Eriksson & Wrange, 1990) and given the inability to detect DBD dimers by physicochemical methods even at high DBD concentrations (Freedman et al., 1988; Dahlman-Wright et al., 1990).

Influence of Nonspecific DNA on the Apparent Affinity of GR for GRE. Several reports have underlined the apparent increase in nonspecific binding upon the removal of the N-terminal region, probably related to its acidic amino acid content (Danielsen et al., 1987). Consistent with this, Eriksson and Wrange (1990) reported a ∼1.5 increase in ethylation of the N-truncated receptor compared to the wild-type GR in ethylation interference experiments. Our experiments did not provide evidence for any significant variation

in the influence of the nonspecific binding on specific affinity measurements for the different GR derivatives used in this study. Firstly, no modification in the binding affinity of the monomer was observed upon addition/deletion of the N-terminal region. Secondly, dimer affinities (at low oligonucleotide concentrations) in the absence of salmon sperm DNA were increased approximately 10-fold whatever the derivative used. Our affinity value obtained for hGR in the absence of nonspecific DNA is compatible with data reported for the purified wild-type GR (Drouin et al., 1992).

In conclusion, we have shown a role for the N- and C-terminal regions in the dimerization events and in the binding to GRE of the dimer, but no role for these regions in the binding of the monomer to the GRE. The proportions of monomeric complexes with respect to dimer complexes observed with the different GR derivatives are well-correlated with the variations observed in dimerization and dimer GRE binding affinities. The occurrence of more intense monomeric bands for I550* than for wild-type GR could be related to an increased monomer concentration for the same total receptor concentration. With ΔGG, the proportion of monomer is still more important, resulting from both decreases in dimerization and dimer binding to GRE. For the DBD fragments, a further decrease in dimer binding efficiency is consistent with the higher monomer/dimer ratios observed. Moreover, the relative affinities of the binding of monomer (K_2) and dimer GRs to GRE (K_8K_{10}) and of GR dimerization (K_1) show that, at least for the wild-type GR, GR dimerization followed by GRE binding may be the major path followed.

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