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Dimerizing the Estrogen Receptor DNA Binding Domain Enhances Binding to Estrogen Response Elements*

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In this work, we provide a rationale for the finding that the estrogen receptor (ER) binds to its DNA response element as a homodimer *in vivo*. Binding of the monomer estrogen receptor DNA binding domain (ER DBD) to a palindromic, consensus estrogen response element (ERE) is increased 5–6-fold when the ER DBD is dimerized either by a monoclonal antibody that recognizes an attached epitope tag or by expressing the ER DBD as a single molecule in which the two monomers are joined by a peptide linker. Most of the increase in binding is due to stabilization of the ER DBD·ERE complex. We observed only an approximately 2.5-fold reduction in binding when a consensus ERE was replaced with widely spaced ERE half-sites, suggesting that the interaction between ER DBDs on the ERE is relatively weak, and that in full-length ER the DBDs can move independently of each other. To test binding to an imperfect palindrome, typical of the imperfect EREs found in almost all natural estrogen receptor responsive genes, we used the pS2 ERE. Even at high concentrations of ER DBD, specific binding of the ER DBD to the imperfect pS2 ERE was undetectable. Both of the dimerized ER DBDs exhibited efficient binding to the imperfect pS2 ERE, with an affinity at least 25-fold greater than monomer ER DBD. These data support the view that steroid receptor dimerization provides an important mechanism facilitating the recognition of naturally occurring, imperfect hormone response elements.

The intracellular actions of estrogens are mediated by the estrogen receptor. The estrogen receptor (ER)[1] and other members of the steroid/nuclear receptor superfamily of transcription factors share a common domain structure with discrete regions of the receptors responsible for ligand binding, DNA binding, dimerization, nuclear localization, and transcription activation. Two estrogen receptor domains play a role in dimerization. A strong dimerization interface is located in the ligand binding domain of nuclear receptors (1). A weak dimerization interface is located in the DNA binding domain of steroid receptors (2, 3). However, this interface is insufficient to induce solution dimerization, inasmuch as even at high protein concentrations the isolated recombinant ER DNA binding domain is a monomer in solution (4, 5). When two ER DBD monomers bind to the half-sites of an estrogen response element (ERE), a dimerization interface stabilizes binding of the DBD monomers to the response element (2). We refer to this as ERE-dependent DBD dimerization. Although the ER DBD is a monomer in solution, in this work, we did not observe stable occupancy of only one of the two ERE half-sites by a single ER DBD. The ER DBD monomers on the ERE occupy both half-sites and are in the form of ERE-dimerized DBD. The DNA-dependent dimerization interface in the glucocorticoid receptor (GR)-DBD is sufficiently stable that it can alter binding of GR-DBD monomers to a glucocorticoid response element with an incorrect 4-base spacing (6).

It is widely, but not universally (7), accepted that steroid receptors bind to their DNA recognition sequences as dimers. However, ligand binding domain-mediated dimerization is not always required for binding of steroid receptors to their hormone response elements. For example, the ER DBD monomer expressed in *Escherichia coli* binds to the ERE with a reduced but still significant affinity (3). It has been widely assumed, but never actually demonstrated, that dimerization of steroid receptors increases their affinity for their DNA response elements.

In this work, we address the effect of dimerization on the interaction of a steroid receptor with a hormone response element, and provide a biological rationale for receptor dimerization. Although ER mutants that are impaired in ligand binding domain dimerization have been reported (1), it appears that these mutants still exhibit some ability to dimerize (8). We therefore elected to use the ER DBD, which has been shown clearly to be a monomer in solution (4, 5). We used two very different techniques to artificially dimerize the ER DBD in solution and compared binding to EREs by the dimerized DBD and by the ER DBD monomer. By measuring both equilibrium binding and the association and dissociation of the ER DBDs from a consensus ERE, we show that most of the increase in binding seen with the dimerized ER DBDs is to due to stabilization of the dimerized ER DBD complexes on the ERE. Virtually all EREs identified in ER-regulated genes contain imperfect ERE palindromes. The ER DBD was unable to recognize and bind to an imperfect, non-consensus ERE (from the human PS2 gene), whereas the artificially dimerized ER DBDs bound with high affinity. The striking dimerization-dependent enhancement of binding to an imperfect ERE provides a rationale for dimerization of the estrogen receptor and the other steroid receptors.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were obtained from Sigma unless otherwise indicated. Enzymes were obtained from either New England Bio-

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[1] The abbreviations used are: ER, estrogen receptor; ER DBD, estrogen receptor DNA binding domain; AbD-DBD, antibody-dimerized estrogen receptor DNA binding domain; LD-DBD, linker-dimerized estrogen receptor DNA binding domain; PAL3, palindrome with 3 nucleotides between ERE half-sites; EV15, everted ERE with 15 nucleotides between half-sites; DR15, direct repeat of ERE half-sites separated by 15 nucleotides; PCR, polymerase chain reaction; GR, glucocorticoid receptor; ERE, estrogen response element.

labs (Beverly, MA) or Life Technologies, Inc. Radioisotopes were obtained from ICN (Costa Mesa, CA).

Construction of pET-DBD and pET-DDb Expression Plasmids—The plasmid pET-DBD was constructed by insertion of a PCR generated product into the *Hin*dIII and *Nhe*I sites of the plasmid pET-21b(+) (Novagen, Madison, WI). The insert was amplified from the human ER coding sequence present in the CMV-hER plasmid (9) using the primers 5′-TCAGGATCCACCATGGCTAGCGACTACAAGGACGACGATGACAAGATGTACCCTAGGGGCAAGGAGACTCGCTACTGT-3′ and 5′-ATTGATAAGCTTGGATCCTTACTACCCTCCTCTTCGGTCTT-3′.

To construct the linker-dimerized DBD plasmid pET-DDb, one copy of the DBD coding sequence was inserted into the *Nhe*I and *Bam*HI sites of the pET-21b(+) plasmid. This insert was prepared by PCR amplification from the human ER coding sequence using the primers 5′-TCAGGATCCACCATGGCTAGCGACTACAAGGACGACGATGACAAGATGTACCCTAGGGGCAAGGAGACTCGCTACTGT-3′ and 5′-ATAGGATCCACCTCCACCTGAGCCACCCCCTCCTCTTCGGTCTTT-3′. The second copy of the DBD was initially cloned into the *Eco*RI and *Hin*dIII sites of pTZ18u. The second DBD was prepared by PCR using the primers 5′-ACAAGAATTCAAGGAGACGCGTTACTGTGCAGTGT-3′ and 5′-ATTGATAAGCTTGGATCCTTACTACCCTCCTCTTCGGTCTT-3′. The *Bam*HI site was removed from this construct by filling in with Klenow. The second copy of the DBD was then subcloned into the *Eco*RI and *Hin*dIII sites of the pET-21b(+) construct, which contained the first copy of the DBD. The resulting construct was digested with *Sph*I and *Xho*I to prepare an insert containing both DBDs, which was subsequently ligated into the *Sph*I and *Sal*I sites of pTZ18u, which had its *Bam*HI site destroyed by filling in with Klenow. Linker oligonucleotides 5′-CTCTGGATCCGGTGGAGGTGGTTCTGGAGGAGGTGGTTCCGGAGGTGGAGGAAAGGAGACGCGTTACT-3′ and 5′-AGTAACGCGTCTCCTTTCCTCCACCTCCGGAACCACCTCCTCCAGAACCACCTCCACCGGATCCAGAG-3′ were annealed and inserted into the *Bam*HI and *Mlu*I sites of the latter construct. An insert was prepared by *Hin*dIII and *Nhe*I digestion of this construct and inserted into the *Hin*dIII and *Nhe*I sites of the pET-21b(+) to generate the pET-DDb plasmid. All constructs were confirmed by sequencing. This complex cloning procedure was performed to minimize the selection of mutants in the DNA binding domain with a reduced affinity for the ERE. These mutants are selected due to the toxicity of wild-type ER DBD in *E. coli*.[2] However, the pET-DDb plasmid does not appear to be highly toxic to the cells.

Protein Expression and Purification—The pET-DBD and pET-DDb plasmids were expressed in *E. coli* BL21(DE3)pLysS (Novagen, Madison, WI) to yield DBD and linker-dimerized DBD (LD-DBD), respectively. Crude bacterial lysates were prepared as described (4), and the ER DBDs were purified by affinity chromatography with the flag M2 monoclonal antibody (Eastman Kodak Co.) using synthetic flag peptide to elute the DBDs (10). Purified proteins were concentrated using Centricon-3 or Centriplus-3 protein concentrators (Amicon, Beverly, MA). Protein purity was assessed on 15% polyacrylamide gels (11) and visualized by silver staining (12). Protein concentration was determined using Coomassie Blue (Bio-Rad) with a bovine serum albumin standard.

Gel Mobility Shift Assays—Gel mobility shift assays were performed essentially as described (4, 13), with modifications. Binding reactions contained 80 mM KCl, 15 mM Tris-HCl (pH 7.9), 4 mM dithiothreitol, 0.2 mM EDTA, 10 μM $ZnCl_2$, 25 ng/μl poly(dI-dC), and 10% glycerol (in 20 μl, total volume). Added proteins were diluted in binding buffer without glycerol or poly(dI-dC). Equilibrium experiments were performed by incubating the samples for 15 min at room temperature and then loading the samples onto a gel running at 4 °C (for samples containing antibody, a 10-min preincubation at room temperature before the addition of probe was also performed). All kinetics experiments were performed by incubation of the binding reaction at 4 °C for 1 h before the addition of probe (on-rate) or a 10-fold excess ERE containing competitor (off-rate), and then loaded onto a gel running at 4 °C at the times indicated. Quantitation was performed by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Expression and Purification of DBD Constructs—We constructed a core human ER DBD containing a minimum sequence, which retains efficient binding to the ERE. This ER DBD consisted of amino acids 180–262, including the nuclear localization sequence near the C terminus and a flag epitope at the N terminus (Fig. 1*A*). A thrombin cleavage site was inserted between the flag epitope and the DBD. In the LD-DBD construct, two copies of the DBD (amino acids 180–262) were joined by a flexible peptide linker (Fig. 1*B*). To identify the length of the linker we would use, we worked from the crystal structure of the ER DBD bound to the ERE (2), and chose a linker of 80 Å extended length, which was approximately 133% of the estimated distance between the C terminus of the first copy of the DBD and the N terminus of the second copy. Because the nuclear localization sequence does not produce a region of sufficient order to be observed in the crystal structure, its length was estimated. To provide flexibility in the linker, we used five repeats of the sequence Gly-Gly-Gly-Gly-Ser (without a Ser on the last repeat).

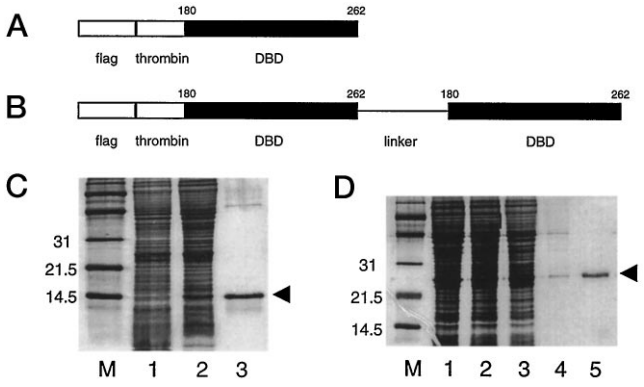

FIG. 1. **Purification of the ER DBDs.** Schematics (not drawn to scale) of the DBD and the LD-DBD are shown in *panels A* and *B*, respectively. The DBDs were purified and analyzed by polyacrylamide gel electrophoresis as described under "Experimental Procedures." For the purification of the DBD (*panel C*), the lanes on the gel are as follows: *M*, molecular weight markers; *1*, crude *E. coli* high speed supernatant; *2*, M2-agarose column flow-through; *3*, purified DBD eluted with the flag peptide. For the purification of the LD-DBD (*panel D*), the lanes are as follows: *M*, molecular weight markers; *1*, a high speed supernatant from uninduced *E. coli*; *2*, a high speed supernatant from induced cells; *3*, the material which flowed through the column, *4*, the proteins in the second of four 10-ml washes of the column; *5*, the purified flag peptide-eluted LD-DBD.

The DBD and the LD-DBD were purified from crude bacterial lysates by affinity chromatography using immobilized monoclonal antibody to the flag epitope (10). Elution with synthetic flag peptide resulted in isolation of highly purified DBD and LD-DBD (Fig. 1, *C* and 1*D*). The proteins appeared to be resistant to denaturation or degradation, although gel electrophoresis showed that a small percentage of the LD-DBD was cleaved after the first copy of the DBD.

Binding of the DBD to the ERE—To determine whether the presence of immunoglubulins in the reactions, or of the flag epitope on the DBD, altered binding to the ERE, we carried out control gel mobility shift assays. Nonspecific IgG, even at higher concentrations than were used in our studies, did not alter binding of the DBD to the ERE (Fig. 2*A*, compare *lanes 1–4* with *8–10*). To evaluate the effect of the N-terminal flag epitope we compared binding of the flag-DBD to DBD from which the flag epitope was removed by digestion with thrombin. Complete removal of the flag epitope was evident by the presence of a single gel-shifted band, which migrated more rapidly than the flag-DBD·ERE complex (Fig. 2*A*, compare *lanes 2–4* with *5–7*). In gel mobility shift assays carried out at three DBD concentrations, the DBD showed similar binding to the ERE with and without the flag epitope (Fig. 2*A*, *lanes 5–7* and *8–10*).

[2] M. Kuntz, K. Glenn, S. Chusacultanachai, A. Rodriguez and D. Shapiro, unpublished observations.

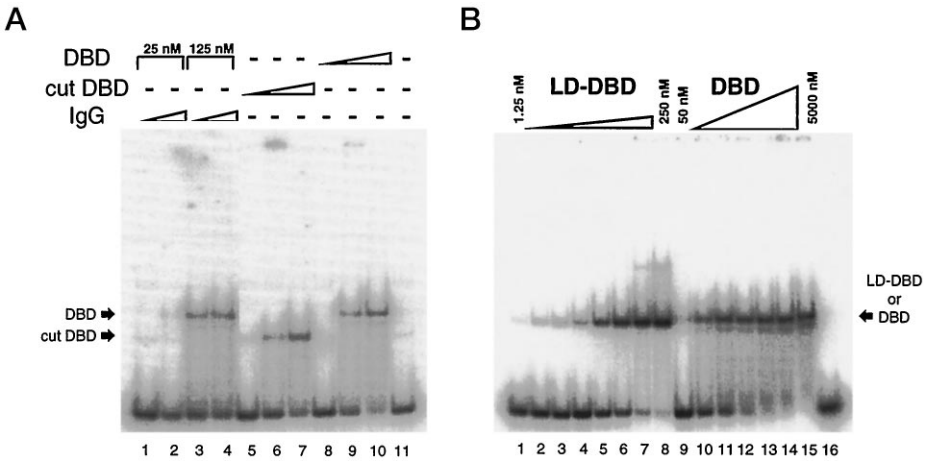

FIG. 2. **Binding of ER DBD and linker-dimerized ER DBD to a consensus ERE.** Gel mobility shift assays were carried out as described under "Experimental Procedures." In *panel A*, the concentrations of DBD and cut DBD used were 25 nM (*lanes 1*, *2*, *5*, and *8*), 125 nM (*lanes 3*, *4*, *6*, and *9*) and 500 nM (*lanes 7* and *10*). Cut DBD indicates ER DBD that has had the flag epitope removed by thrombin cleavage. The cut DBD has a single non-native glycine residue on the N terminus. In *panel B*, increasing amounts of DBD or of LD-DBD were incubated with the ERE and analyzed in gel mobility shift assays. The nanomolar concentrations of protein used for LD-DBD (*lanes 1–8*) were: 1.25, 2.5, 5, 12.5, 25, 50, 125, and 250 nM; and for ER DBD (*lanes 9–15*): 50, 125, 250, 500, 1250, 2500, and 5000 nM.

To determine the affinity of the monomer and dimer DBDs for the ERE, we carried out protein titration experiments. Increasing concentrations of purified monomer ER DBD (Fig. 2*B*, *lanes 9–15*) or of LD-DBD (Fig. 2*B*, *lanes 1–8*) were incubated with the labeled ERE, and binding was analyzed in gel mobility shift assays. For example, in *lanes 6* and *11*, in which approximately equal proportions of probe were gel-shifted, the concentration of LD-DBD was 50 nM and the concentration of DBD was 250 nM. These data indicated that the dimerized LD-DBD bound far more efficiently to the ERE than monomer ER DBD.

Antibody-dimerized DBD (AbD-DBD) Exhibits Strongly Enhanced Binding to the ERE—To carry out a protein titration with antibody-dimerized ER DBD, it was necessary to first determine whether the M2 monoclonal antibody could effectively dimerize the ER DBD. Because we can readily distinguish between the ER DBD·ERE complex and the M2-ER DBD·ERE complex in gel-shift assays, we used this method to define antibody-DBD ratios. A titration with increasing concentrations of antibody was carried out at 40 nM, 80 nM, and 160 nM ER DBD. The DBD was effectively dimerized across a broad range of ratios of DBD to antibody (Fig. 3*A*). Even at the highest concentrations of ER DBD (160 nM), intermediate and high concentrations of antibody effectively dimerized the ER DBD, as no gel-shifted band was observed at the position of the DBD·ERE complex (Fig. 3*A*, *lanes 3–5*). At the optimum ratio of DBD to antibody, there were approximately 2 molecules of DBD/molecule of antibody. In addition to illustrating that the M2 monoclonal antibody effectively dimerized the ER DBD, these data also indicated that antibody dimerization of the DBD greatly increased binding to the ERE. At 40 nM ER DBD, binding of the ERE by the DBD monomer was barely detectable (Fig. 3*A*, *lane 13*). In contrast, at the same DBD concentration, most of the ERE was complexed by AbD-DBD (Fig. 3*A*, *lanes 14–17*).

Although these data indicated that the AbD-DBD exhibited a strongly enhanced binding to the ERE, they were unsuitable for quantitation because the concentrations of AbD-DBD used formed complexes with most of the ERE probe. To more precisely determine the affinity of the AbD-DBD for the ERE, we carried out a protein titration across a broad range of DBD concentrations (Fig. 3*B*). At each concentration of DBD, we titrated with three concentrations of antibody, with the middle concentration representing the approximately 2:1 DBD:Ab ratio, which produces optimum dimerization. The other samples of each set contain 10-fold more or less antibody to ensure that the maximum effect on binding affinity actually occurred at the expected concentration of antibody. Maximum binding did occur at the expected 2:1 DBD:Ab ratio, except for the samples at the lowest DBD concentrations, which formed complexes with a very small proportion of the ERE probe.

Quantitation of Binding of Monomer and Dimer DBDs to the ERE—For each DBD concentration (Fig. 3*B*), the amount of ERE probe up-shifted in the sample exhibiting maximum ERE binding was quantitated and used to plot a binding curve (Fig. 4). In a similar fashion, the protein titrations for the ER DBD monomer (Fig. 2*B*) and the LD-DBD (Fig. 2*B*) were quantitated. On these binding curves, the concentration of DBD required to elicit half-maximal binding represents the K_D. The K_D for binding of the monomer ER DBD to the ERE was 160 nM. For the AbD-DBD and the LB-DBD, the K_D values were 29 nM and 38 nM, respectively. Thus, dimerization of the DBD increased binding to the ERE by 5–6-fold. The difference in affinity between the two dimerized constructs may be due to steric hindrance by the linker in the double DBD (see below).

Analysis of the Association and Dissociation of DBD Monomer and DBD Dimers with the ERE—Our data indicated that dimerization of the DBD increased binding of the DBD to the consensus ERE palindrome. However, it was not clear whether binding was enhanced due to more rapid binding to the ERE (an increased on-rate) or to a reduced rate of dissociation from the ERE (a decreased off-rate). To determine the off-rate for the monomer DBD dimerized on the ERE half-sites and for the AbD-DBD and LD-DBD dimers, the DBDs were each incubated separately with the labeled ERE probe, and the reaction was quenched with an excess of unlabeled ERE. Aliquots of the samples were removed at the indicated times and loaded directly on to a native polyacrylamide gel. As shown in Fig. 5*A*, even at the earliest time point (30 s), the ERE-dimerized DBD had largely dissociated from the ERE. In contrast, at least 10 min was required for the LD-DBD to approach the same level of dissociation, and the AbD-DBD remained largely bound to the ERE through the entire 10-min time course of the experiment. These data indicate that externally dimerizing the DBD using a linker or an antibody greatly increases the time the DBD remains associated with a consensus ERE palindrome. To evaluate the association rate, each of the DBDs was separately incubated with the labeled ERE probe. At the indicated times, an aliquot of each reaction mixture was removed and loaded directly on to a native polyacrylamide gel running at 4 °C. Even at the earliest time point (30 s), the AbD-DBD was largely bound to the ERE. Binding by the monomer ER DBD and the LD-DBD was slower, and was largely complete within 6 min. Under the conditions of our experiments, an increase in the time the DBD remains associated with the ERE is the major factor in increased binding by the dimerized DBDs.

Binding to ERE Half-sites with Nonconsensus Spacing—The ER can bind to direct repeats of the ERE half-site separated by

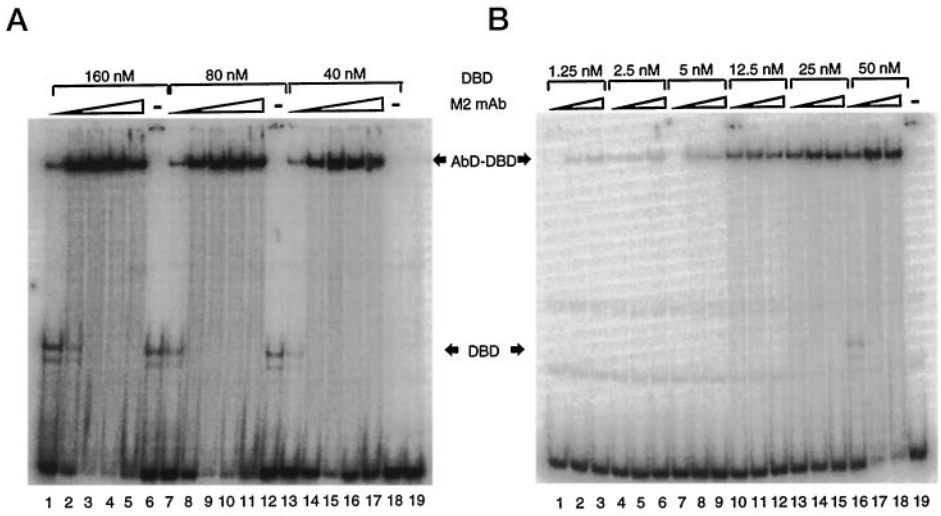

FIG. 3. **Dimerization with antibody increases ER DBD binding to a consensus ERE.** Incubations and gel-shift assays were carried out as described under "Experimental Procedures." In *panel A*, *lanes 6*, *12*, and *18* contain 160, 80, and 40 nM of DBD, respectively, and have no added antibody. For each set of DBD concentrations in *panel A* (160, 80, and 40 nM), the antibody concentration in each lane was progressively increased 10-fold. A titration to determine the binding affinity of antibody-dimerized ER DBD complex is shown in *panel B*. DBD concentrations are as indicated. The *center lane* of each concentration of DBD has the 2 molecules of flag antibody:molecule of DBD ratio determined to produce the maximum upshift. The *other two lanes* contain either 10-fold more or less antibody as indicated.

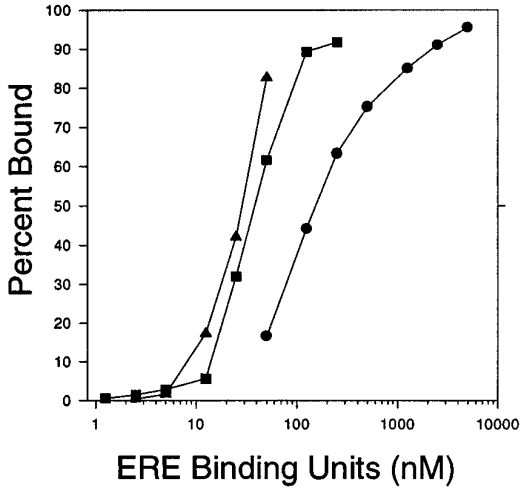

FIG. 4. **Binding of monomer and dimer DBDs to the consensus ERE as a function of protein concentration.** Data for the binding of ER DBD (*filled circles*), LD-DBD (*filled squares*), and AbD-DBD (*filled triangles*) was generated by PhosphorImager quantitation of the gel mobility shift assays, which appear in Figs. 2 and 3. For all constructs, ERE binding units is the concentration of DBD dimers. This is equal to the nanomolar concentration of the dimerized constructs (which each contain 2 DBD monomers) and is equal to one-half the concentration of the added ER DBD monomer.

substantial distances on the DNA (14, 15). Although it has never been demonstrated that ER can bind to widely spaced, everted ERE half-sites (Fig. 6*A*), this might be expected to occur. We tested binding of the LD-DBD to direct and everted EREs with 15 nucleotides between their half-sites (Fig. 6*A*), a spacing previously determined to be optimal for ER binding to direct repeats of the ERE half-site (15). The affinity of the LD-DBD for everted widely spaced ERE half-sites was approximately 2.5-fold lower than the affinity for the consensus ERE palindrome (Fig. 6*B*). Binding to the ERE direct repeat with a 15-base spacing was considerably less effective than binding to the everted EREs with the same spacing (Fig. 6). Inefficient binding of the LD-DBD to the direct repeat ERE half-sites with a 15-base spacing may be due to steric constraints imposed by the length of the linker. The linker is required to bridge the distance between the half-sites for the direct repeat, whereas the linker attachment sites are brought closer together in the everted repeat.

Binding of the ER DBDs to a Naturally Occurring ERE Which Is an Imperfect Palindrome—All but one known ERE contains at least one nonconsensus base pair in its sequence. To analyze the role of dimerization in binding to the imperfect EREs, which are found in almost all natural estrogen-regulated genes, we investigated binding of the DBDs to the imperfect ERE found in the pS2 gene, an estrogen-inducible gene identified in studies of human breast cancer cells (16). The pS2 promoter contains a single ERE centered at −437 with the imperfect palindrome 5′-GGTCANNNTGGCC-3′ (17). Although the ER has been shown to bind with higher affinity to the perfect ERE palindrome 5′-GGTCACAGTGACC-3′ than to the imperfect pS2 ERE (3), the influence of dimerization on binding to the pS2 ERE has not been examined.

Dimerizing the ER DBD had a much greater effect on binding to the imperfect pS2 ERE palindrome than on binding to the perfect ERE palindrome (Fig. 7). The K_D values for binding of the LD-DBD and of the AbD-DBD complex to the pS2 ERE were 110 nM and approximately 200 nM, respectively (Fig. 7). In contrast, the DBD monomer did not produce a discrete gel-shifted band, even at 10,000 nM DBD. There was some smearing near the free probe, especially at the highest concentration of added DBD (10,000 nM). This smearing may have been the result of DBD binding in the initial reactions with complete dissociation during electrophoresis. The failure of the DBD to exhibit detectable binding to the pS2 ERE makes it difficult to determine the -fold increase in binding induced on DBD dimerization. However, it seems clear that the LB-DBD has >25-fold higher affinity for the imperfect pS2 ERE than the DBD monomer.

DISCUSSION

The DNA recognition sequences of the estrogen receptor and the other steroid hormone receptors are usually palindromic half-sites. In principle, the steroid receptors could interact with their half-sites by binding to them independently as monomers, by binding as monomers and forming interacting dimers on the DNA, or by forming dimers in solution and binding to their recognition sequences as dimeric units. Although several studies have demonstrated that the isolated ER and GR DNA

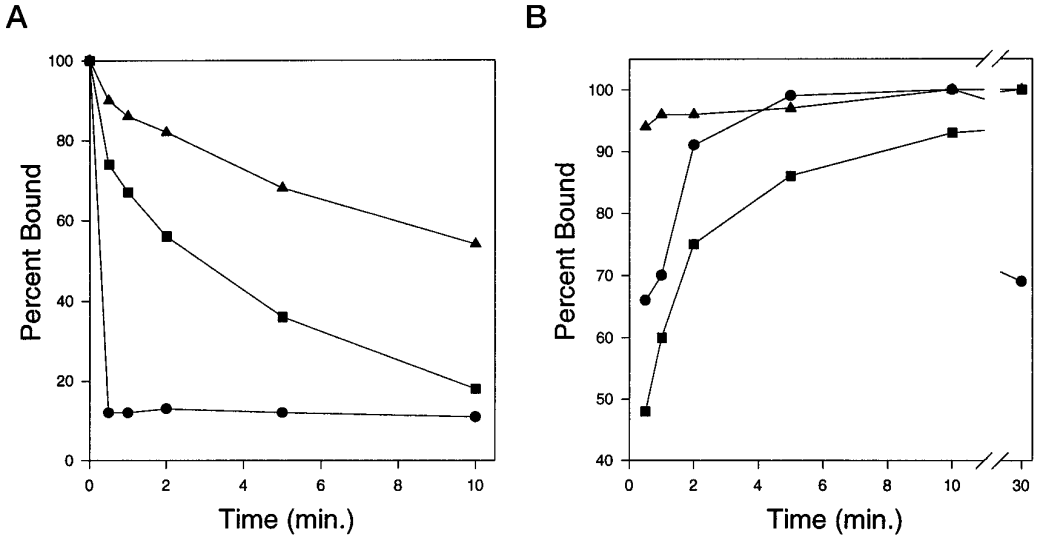

FIG. 5. **Determination of the kinetics of association and dissociation of the ER DBDs from the ERE.** Dissociation (*panel A*) and association (*panel B*) studies for the ER DBD (*filled circles*), LD-DBD (*filled squares*), and AbD-DBD (*filled triangles*) were carried out as described under "Experimental Procedures." Percent bound was normalized to 100% binding for the maximum upshift in each sample set.

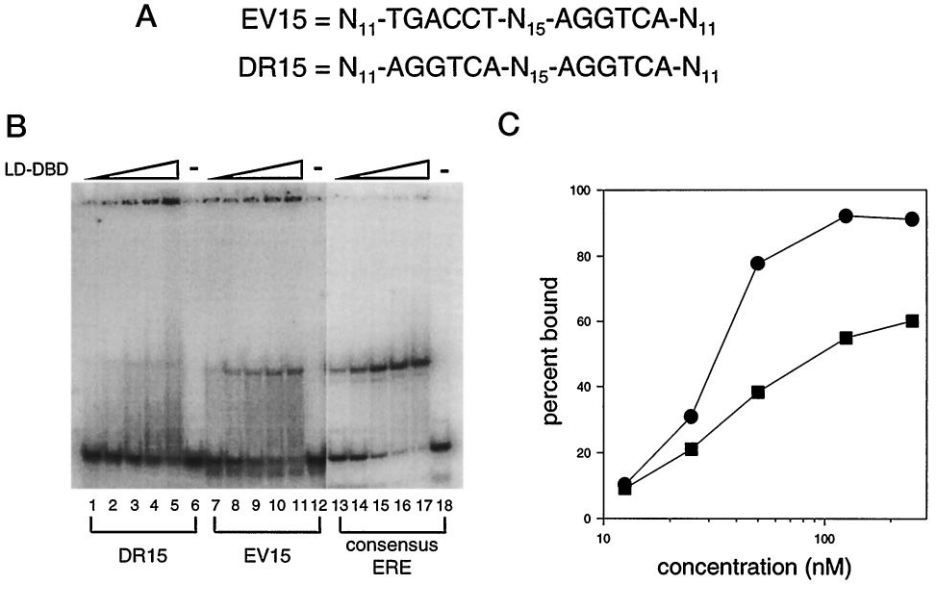

FIG. 6. **Binding of linker-dimerized ER DBD to widely spaced ERE half-sites.** *Panel A* contains a schematic of the half-sites used. EV15 has half-sites everted with respect to the consensus ERE separated by 15 base pairs. DR15 has half-sites directly repeated and separated by 15 base pairs. *Panel B* contains gel mobility shift assays showing a titration of the LD-DBD binding to each of the ERE probes. The concentrations of LD-DBD were: 12.5 nM (*lanes 7* and *13*), 25 nM (*lanes 1*, *8*, and *14*), 50 nM (*lanes 2*, *9*, and *15*), 125 nM (*lanes 3*, *10*, and *16*), 250 nM (*lanes 4*, *11*, and *17*), and 500 nM (*lane 5*). To make the shifted bands more visible, different segments of the gel represent two different scans of the same gel with different exposure times. In *panel C*, binding of the ERE probes is plotted as a function of LD DBD concentration quantitated from the gel mobility shift assay appearing in *panel B*. The *filled circles* represent the consensus ERE, and the *squares* are EV15.

binding domains are monomers in solution and undergo DNA dependent-dimerization after binding to their recognition sequences (4, 18), most evidence supports the view that the full-length liganded ER, GR, and progesterone receptor dimerize in solution and bind to their response elements as dimers (reviewed in Ref. 19). The recent observation that widely separated ERE half-sites can bind the ER and play a role in transcription activation (14, 15) underscores the question of whether dimerization actually confers a significant advantage to the receptor in forming a complex with its hormone response element. In the absence of any quantitative determination of the extent to which receptor dimerization results in enhanced binding to the hormone response element, there was no explanation of the advantage conferred on the receptor by dimerizing.

One approach to evaluating the effect of dimerization on interaction of the ER with the ERE would be to compare ERE binding by wild-type ER and by an ER mutant unable to dimerize. Although several ER mutants that show impaired dimerization on translation in a rabbit reticulocyte lysate have been reported (20), these mutants likely still retain some ability to dimerize *in vivo*. For example, one well characterized mutant in this group, L507R, retains significant ability to activate transcription from EREs (8), and is therefore likely to exhibit significant dimerization *in vivo*. We therefore elected to investigate binding of the ER DNA binding domain to the ERE. The ER DBD has been shown unambiguously to be a monomer in solution (4, 5), its interaction with the ERE has been described at high resolution (2), and it is available in pure form.

To artificially dimerize the ER DBD, we used a peptide

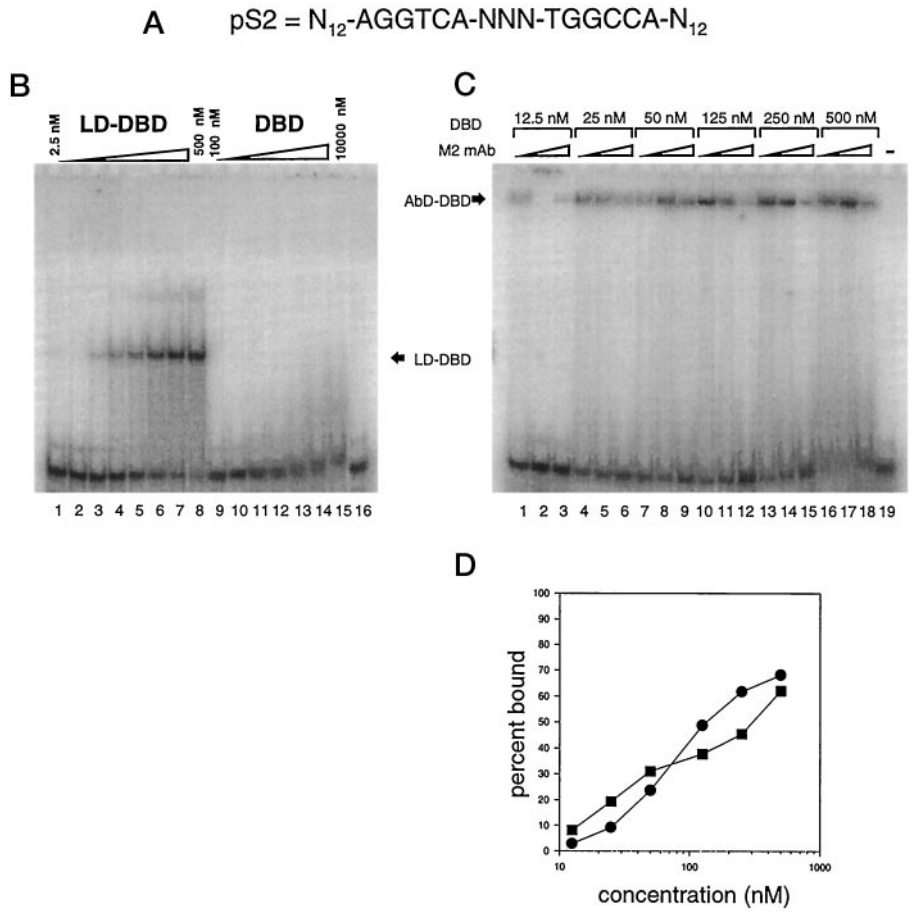

FIG. 7. **Dimerization greatly increases binding of the ER DBD to the non-consensus PS2 ERE.** *Panel A* shows the sequence of the pS2 ERE half-sites. *Panel B* is a gel mobility shift assay in which binding of increasing concentrations of monomer DBD (*DBD*) or LD-DBD to the pS2 ERE was assayed. The nanomolar concentrations of LD-DBD in *lanes 1–8* were: 2.5, 5, 12.5, 25, 50, 125, 250, and 500. The nanomolar concentrations of ER DBD in *lanes 9–15* were: 100, 250, 500, 1000, 2,500, 5,000 and 10,000. In *panel C*, binding of the pS2 ERE to the AbD-DBD is shown. The concentrations of AbD-DBD are indicated above the lanes. The *center lane* of each concentration of AbD-DBD was incubated with the amount of antibody calculated to produce the maximum upshift. The *flanking lanes* contain either 10-fold more or less antibody as indicated. *Panel D* is a graphical representation of binding of the LD-DBD and AbD-DBD to the pS2 ERE using the data of *panels B* and *C*. The *filled circles* are LD-DBD, and the *filled squares* are AbD-DBD. No plot could be made for monomer DBD, which did not show detectable binding to the pS2 ERE (*panel B*).

linker related to those used in the production of single chain antibodies. Similar linkers have recently been used in a few studies of DNA-binding proteins (21–24). In one such study, the possibility that a peptide linker very similar to the one we used enhanced DNA binding by altering the structure of the protein was explicitly examined (24). Fluorescence emission, circular dichroism, and DNase I and copper phenanthroline footprinting all indicated that the free *arc* repressor, and the *arc* repressor dimerized with a peptide linker had similar structures and interactions with DNA. Because none of these techniques could prove definitively that enhanced binding of the LD-DBD to the ERE was caused by dimerization and not by a linker-mediated structural change in the ER DBD, we also used a second entirely different antibody-based method to dimerize the ER DBD.

Although it has been reported previously that antibodies to ER can enhance dimerization (20, 25), quantitative studies of the interaction of an antibody-dimerized protein with its recognition sequence have not been reported. To dimerize the ER DBD, we used a monoclonal antibody to a short epitope added to the N terminus of the DBD. The short N-terminal flag epitope did not alter DNA binding by the ER DBD as the flag-ER DBD and DBD from which the flag epitope was removed by thrombin digestion showed similar binding to the ERE in gel-shift assays. The position of the epitope recognized by the antibody does not appear to be critical for antibody-mediated dimerization to enhance binding to the ERE. In qualitative studies, we also observed a substantial enhancement of ERE binding using the *Xenopus* ER DBD and the monoclonal antibody p1A3, which appears to recognize an epitope in the hinge region of the *Xenopus* ER DBD (data not shown). Inasmuch as this region is poorly conserved, this antibody does not bind to the human ER DBD, necessitating use of the *Xenopus* ER DBD for these studies.

In contrast to the LD-DBD, which will be a dimer independent of concentration, the antibody will only dimerize the DBD at the appropriate ratio of antibody and flag-DBD antigen. In practice, we found that the DBD bound to the ERE as an antibody-induced dimer across a wide range of antibody:DBD ratios (Fig. 3). One possible explanation for this is that when the antibody has dimerized the DBD, antibody-DBD complex binds rapidly to the consensus ERE (Fig. 5*B*), and once bound the ERE may be stabilizing the antibody complex with two bound DBDs. Support for this idea comes from the data for the AbD-DBD bound to the non-consensus pS2 ERE. Qualitative assessment of the amount of probe up-shifted at the three antibody concentrations indicates more variation than was seen for the consensus ERE.

Most available data indicate that the liganded full-length ER is a dimer in solution. However, the state of the DBDs in the solution dimer of ER is unknown. It is not clear whether the DBDs in the solution dimer of ER are linked to the rest of the protein through a conformationally flexible region (as has been proposed for nuclear receptors; Ref. 26) and are therefore free to move independently, or are associated through the dimerization interface seen in the x-ray structure of two ER DBDs bound to the ERE. The observation that an ERE with a direct repeat of the ERE half-sites with 15 nucleotides between the half-sites bound the ER and activated transcription approximately one-third as well as the consensus ERE palindrome (15) raised the possibility that the DBDs in the intact protein are not in a dimer interface but are free to rotate. To evaluate this question, we determined the effect of dimerization on binding of ER DBD to the consensus ERE palindrome with its 3 nucleotide spacer (PAL3) and to direct repeats (DR15) and everted repeats (EV15) with a 15-nucleotide spacer. Binding of LD-

TABLE I
Dissociation constants for DBD construct and ERE probe combinations

ERE probe	DBD construct	Dissociation constant[a]
		nM
Consensus ERE	DBD	160
	LD-DBD	38
	AbD-DBD	29
EV15	LD-DBD	~100
pS2 nonconsensus ERE	DBD	Undetectable
	LD-DBD	110
	AbD-DBD	~200

[a] The K_D values were calculated from the data presented in Figs. 2–4, 6, and 7.

DBD to DR15 was inefficient, presumably because the short length of the linker required the introduction of an energetically unfavorable bend or kink in the DNA. In contrast, LD-DBD bound to EV15 with only about a 2.5-fold lower affinity than to PAL3 (Table I). Because the two DBD monomers are separated by an extra turn of the DNA helix and are in opposite orientation when bound to EV15, they are unlikely to form the DNA-dependent dimerization interface. The 2.5-fold difference in binding of LD-DBD to PAL3 and EV15 corresponds to a DBD dimerization energy of about 0.5 kcal/mol, which is approximately 4% of the 12 kcal/mol total energy of interaction of the full-length ER with the consensus ERE (which has been determined to have a dissociation constant of 2.0 ± 0.3 nM; Ref. 27). This relatively small contribution of DBD dimerization to the total binding energy implies that the DBDs in dimerized ER may be free to rotate and bind to functional EREs, which are not palindromes and do not have the familiar three-base pair spacing. Furthermore, our observation raises the possibility that widely spaced everted ERE half-sites may similarly function *in vivo*, although none have yet been reported.

Our kinetics studies examine two possible mechanisms by which the ER may bind to the ERE. Binding of the ER to the ERE may occur by a sequential mechanism in which one DBD binds followed by binding of the second DBD. Because binding of a single DBD to an ERE half-site is strongly disfavored (28), the second DBD must bind to one ERE half-site before the first DBD has dissociated from the other half-site. Alternatively, the two DBDs may transiently dimerize in solution and then rapidly bind to the ERE as a unit. For either of these mechanisms, we would expect an increase in the on-rate in response to externally induced dimerization of the DBD, such as that brought about by sequences in the ligand binding domain. In the case of the sequential mechanism, this would be due to the dimerization-induced high local concentration of the second DBD once the first DBD was bound. In the transient dimerization model, only a small proportion of the DBD is a dimer at any one time and dimerizing all of the DBD via sequences in the ligand binding domain would increase the proportion of DBD in the ER-binding dimer form.

We find that for the AbD-DBD binding is extremely rapid, even at 4 °C. However, the LD-DBD actually has a slower on-rate than the monomer DBD. This may be due to the linker interfering with binding or to the presence of the linker (which is attached near the outsides of the protein complex when bound to a consensus ERE) favoring a solution form of the linked DBD monomers in which the two DBDs are as far apart as possible and therefore have an orientation opposite to the orientation needed for binding.

It is the rate of dissociation from the ERE (the off-rate) that is most strongly affected by dimerizing the DBD. If the ER DBD monomers bind and dissociate sequentially from the DBD, the DBD·ERE complex is stabilized by the relatively weak dimerization interface between the two monomers on the ERE, and the interaction of each monomer with a half-site. Because binding of the ER or the ER DBD to a single half-site is not favored (28), dissociation of one DBD monomer results in an unstable complex of one DBD and an ERE-half-site. In contrast, when the DBDs are dimerized, one DBD would maintain transient contact with an ERE half-site after the other monomer had dissociated. Dimerizing the DBD would greatly increase the local ER DBD concentration and the probability that the monomer that had dissociated would rebind to the free ERE half-site. This view is consistent with the data showing that dimerization produces a dramatic increase in binding to an imperfect ERE. When one half-site contains an imperfect ERE, as is found in the pS2 ERE, the energetic contribution due to binding of the single ERE monomer is insufficient for forming a stable complex and no binding is observed. The inability of a single ER DBD monomer to bind to a half-site under these conditions is illustrated by the smear (presumably due to a rapid dissociation of half-site complexes) observed when the pS2 ERE is incubated with extremely high levels of ER DBD. The absence of a complex of ER DBD on both half-sites of the pS2 ERE is consistent with our view that the dimerization interface between the ER DBDs bound to the ERE is relatively weak and cannot stabilize binding of the DBD to the imperfect half-site.

Dimerizing the DBD produced a dramatic increase in binding to the non-consensus pS2 ERE, which differs from the ERE consensus sequence by only one nucleotide in one half-site. It was not possible to accurately quantitate the effect of dimerization on binding to the pS2 ERE, inasmuch as even at a concentration of 10,000 nM, the DBD monomer did not produce a discrete upshifted band in the gel mobility shift assay with the pS2 probe. At the highest concentrations used, there was some smearing near the probe alone band. Even if this smearing is considered to represent binding of the ER DBD to the ERE, the affinity of the dimerized DBDs is at least 25-fold higher than the affinity of the DBD monomer. This is a substantially larger effect than the 5–6-fold difference in binding seen with the consensus ERE palindrome. This suggests that dimerization stabilizes the interaction of the DBD with the imperfect ERE half-site. The imperfect pS2 ERE binds ER with only a 2–5-fold lower affinity than the consensus ERE (3, 29). The striking effect of dimerization on binding to the pS2 ERE suggests that for both the pS2 ERE and for the many other EREs that exhibit larger deviations from the consensus sequence, ER dimerization is a prerequisite for efficient ER binding.

In this work, we used two independent methods to quantitate the effect of dimerization on binding of the ER DBD to the ERE. Surprisingly, the major effect of dimerization was to stabilize the ER DBD·ERE complex rather than to increase the association rate. Our data support the view that, in the intact ER dimer, the DBDs can behave independently of each other. Most important, dimerization was required for binding to an imperfect naturally occurring ERE. These data support a model in which the ER must be dimerized to occupy both ERE half-sites in the imperfect EREs found in almost all ER-regulated genes.

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