Cooperative interaction of oestrogen receptor ‘zinc finger’ domain polypeptides on DNA binding

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The consensus oestrogen response element (ERE) contains two inverted copies of an AGGTCA consensus hexameric half-site, spaced by three base pairs. It differs from many other hormone response elements, such as consensus thyroid (TREp) and retinoic acid (DR-5 RARE) response elements, only in the relative spacing and orientation of these sequences. In the present study we report values for cooperativity (w) of an oestrogen receptor DNA-binding domain polypeptide upon binding to these sequences. The polypeptide binds with negative cooperativity, or without cooperativity to retinoic acid and thyroid response elements respectively, but with high cooperativity to the ERE. We have also examined cooperativity upon binding of the polypeptide to an ERE variant. Since naturally occurring EREs commonly contain one hexamer which is considerably more degenerate than the other, we designed a hybrid response element in which one hexamer is a consensus ERE, while specific mutations were introduced into the other. We chose to mutate the second half-site to a glucocorticoid response element (GRE) half-site sequence (AGAACA), since normally no binding of the DNA-binding domain polypeptide to a GRE hexamer alone can be detected. In the hybrid response element, however, the GRE half-site is recognized with relatively high affinity, although binding to this sequence is dependent on the previous binding of a polypeptide to the ERE hexamer. Thus, cooperative interactions are capable of mediating the recognition of ERE sequence degeneracy. The ability of protein–protein interactions to mediate recognition of DNA sequence degeneracy may also have implications for transcription factors in general.

INTRODUCTION

The involvement of protein–protein interactions in DNA binding is now well documented. Dimerization interactions are seen in a number of classes of eukaryotic DNA-binding proteins such as leucine zipper (bZIP) [1], helix–loop–helix [2] and nuclear hormone receptors [3–5]. In dimeric DNA-binding proteins such as these, in which the DNA-binding sites typically consist of two half-sites, DNA recognition is often achieved by ‘reading’ two distinct types of information. While one type of information is the sequence of the half-sites themselves, the other type of information consists of the spacing and orientation of these half-sites relative to one another. Protein–protein interactions between DNA-binding domains are often responsible for mediating the recognition of the spacing and orientation of the half-sites, while recognition of the specific half-site sequences is mediated by direct protein–DNA contacts of the individual domains. While these two types of information are distinct, the mechanisms for reading these are generally interrelated. bZIP DNA-binding domain monomers, for instance, fail to bind DNA, although dimers are capable of specifically interacting with DNA. As well as being a prerequisite to DNA binding, dimerization specifies recognition of half-site spacing and orientation by physically restricting movement of the DNA-binding domains. The isolated DNA-binding domains of some nuclear hormone receptors such as the zinc finger regions of the glucocorticoid and oestrogen receptors, on the other hand, are capable of recognizing individual response element half-sites without prior dimer formation [4,6,7]. In these cases, dimerization appears to occur upon response element interaction and is manifested as cooperative binding to the two half-site motifs [6].

Characterization of a number of nuclear hormone receptor response elements (HREs) has revealed that most of these sequences are composed of two half-site motifs. While the half-site sequences in many HREs are virtually identical, they differ in the relative spacing and orientation of these motifs. An idealized thyroid response element (TREp), for instance, consists of a palindromic pair of hexamers, but there is no spacing between the palindromes [8]. The consensus DR-5 retinoic acid response element (DR-5 RARE), however, consists of a direct repeat of two AGGTCA hexamers with a 5 bp spacing [9,10]. On the other hand, the consensus oestrogen response element (ERE) consists of a hexameric sequence (AGGTCA) followed by a 3 bp ‘spacer’ and the palindrome of the hexamer [11]. Presumably, response element-binding specificity is mediated by recognition of the different spacing and orientations of the hexamers within the response elements.

We have developed a simple method to analyse and quantify the cooperative interactions of proteins binding to DNA. In this study we have utilized this approach to analyse and quantify the cooperative interactions of an oestrogen receptor DNA-binding-domain polypeptide upon binding ERE, TREp and DR-5 RARE, using idealized AGGTCA half-site sequences. Since sequence degeneracy is often observed in one of the half-sites of naturally occurring EREs, we also used an ERE with specific mutations in only one half-site to study the role of cooperative interactions in recognition of such sequence degeneracy, converting it into a glucocorticoid-response-element (GRE) half-site.

EXPERIMENTAL

Materials

The human oestrogen receptor DNA-binding-domain bacterial expression vector, pET31 HE81, was a gift from P. Chambon.

Abbreviations used: HRE, hormone receptor response element; TREp, thyroid response element; DR-5 RARE, retinoic acid response element; ERE, oestrogen response element; w, cooperativity parameter; bZIP, leucine zipper; IPTG, isopropyl-1-thio-β-D-galactopyranoside; E/G-RE, hybrid response element; GRE, glucocorticoid response element; DTT, dithiothreitol.

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The oestrogen receptor DNA-binding-domain polypeptide was purified essentially as described by us previously [7]. Briefly, the polypeptide was encoded in a T7 expression vector and expression was induced in mid logarithmic phase by 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) in the appropriate Escherichia coli host (BL21DE3 pLysS). After extraction of lipids and DNA, the polypeptide in the crude cell extract was enriched by DNA-cellulose chromatography, ammonium sulphate precipitation and further purification on a CM BioGel A column. Specific binding of the polypeptide to [32P] end-labelled synthetic DNA fragments was measured essentially as described [7]. Briefly, 5% polyacrylamide gels (40:1, acrylamide: bisacrylamide) were pre-run at 100 V in TAE buffer (6.7 mM Tris, 3.3 mM sodium acetate, 1 mM EDTA), with increasing concentrations of DNA-binding-domain polypeptide, ranging from 1 ng to 1 ng final concentration, were incubated with 5 nM poly-dIdC non-specific DNA and ~20 000 c.p.m. (~2 nM final concentration) of [32P]-labelled ERE-containing oligonucleotide: 5'-CTCGCAAGCTTCAGGTTAGTTCTGACAGTGACTGAACTGTTACAAGCTGTC-5'; GRE hexamer: 5'-CTGAGATGGTCTGCAGCTG-3', 3'-GACTCTACAAGCGTCAAGA-5'; ERE hexamer: 5'-CGCAAGCTTCAGGTTAGTTCTGACAGTGACTGAACTGTTACAAGCTGTC-5'; 3'-GACTCTACAAGCGTCAAGA-5'; DR-5 RARE: 5'-AGCTGATACAGGGCTGCT-3', 3'-TGGTCCAGTTAGTGGTTCAGGTTACGACG-5'; TREp: 5'-AGCTGATACAGGGCTGCT-3', 3'-TGGTCCAGTTAGTGGTTCAGGTTACGACG-5'; or DR-5 RARE: 5'-AGCTGATACAGGGCTGCT-3', 3'-TGGTCCAGTTAGTGGTTCAGGTTACGACG-5' (hexamers in bold). Samples were incubated in MSA buffer [12 mM Hepes, 4 mM Tris, 60 mM KCl, 12% glycerol, 1 mM dithiothreitol [(DTT), pH 7.9] for 30 min at room temperature. Subsequently electrophoresis was carried out at 180 V at room temperature for 1 and 1.5 h. These mobility-shift assays were used to quantify levels of bound and free DNA for determination of the cooperativity parameter ω, described later. The lack of smearing of the ‘bound’ bands in the mobility-shift experiments is consistent with a lack of dissociation of protein–DNA complexes during the electrophoretic separation, which is essential for accurate quantification. To quantify bound and free DNA, X-ray films exposed to the gel were densitometrically scanned (LKB Ultrascan XL) using a variety of exposure times to ensure a linear response. Internal standards, containing known amounts of radio-labelled DNA (ERE, TREp, DR-5 RARE or E/G-RE, as appropriate), were also run on each gel.

RESULTS

ERE binding

The ability to quantify cooperative dimerization of the polypeptide–ERE interaction on a mobility-shift assay is dependent on the formation of stable monomeric and dimeric complexes of the DNA-binding-domain polypeptide with DNA. Under the conditions employed here, we are able to observe both monomeric and dimeric complexes (present study and [7]), although it should be noted that under other conditions the monomeric complex appears too unstable to detect [12].

Various concentrations of the polypeptide were incubated with an end-labelled ERE-containing oligonucleotide (Figure 1). With increasing concentrations of the DNA-binding-domain polypeptide, differing amounts of two distinct complexes, labelled a and b, are observed. Methylation interference assays with the ERE reveal that in both complex a and b, guanine residues within both of the hexamers are involved in complex formation (Figure 2). In the slower-migrating complex b, there is an obvious decrease in intensity of these bands. These results are consistent with complex a representing a single polypeptide bound to the oligonucleotide, occupying only one of the two
hexameric sequences. Complex b consists of a complex with two polypeptides, each one bound to one hexamer. Since both hexameric half-site sequences in the ERE are identical, the polypeptide should have virtually identical affinities for either of the individual half-site motifs. Consistent with this expectation, we find comparable levels of methylation interference for guanines in each half-site of complex a.

A general model for the response element recognition is illustrated in Figure 3. The polypeptide binds to either of the two hexameric half-sites with identical affinity, ‘K’. Binding of the second polypeptide occurs with an affinity equal to ωK, where K is the polypeptide association constant for a half-site and ω is the cooperativity parameter. The binding equation can be derived from the equilibria:

\[ P + f_A f_n \rightleftharpoons b_A f_n \]  \hspace{1cm} (1)

\[ P + f_A f_n \rightleftharpoons f_A b_n \]  \hspace{1cm} (2)

\[ P + b_A f_n \rightleftharpoons b_A b_n \]  \hspace{1cm} (3)

\[ P + b_A b_n \rightleftharpoons b_A b_n \]  \hspace{1cm} (4)

from the model depicted in Figure 3, where \( P \) = free protein, \( f_A f_n \) = free DNA, \( b_A f_n \) = protein bound to site A only, \( f_A f_n \) = protein bound to both sites A and B. Application of the law of mass action to these equations yields

\[ [f_A f_n] = K[P][f_A f_n][f_A b_n] \]  \hspace{1cm} (5)

\[ [b_A b_n] = K_{0}[P][b_A f_n] \]  \hspace{1cm} (6)

Substituting eqn. (5) into eqn. (6) yields

\[ [b_A b_n] = K_{0}^{\omega}P[f_A f_n] \]  \hspace{1cm} (7)

The fraction of DNA bound (x), then can be expressed as:

\[ x = \frac{(2K[P] + K^2\omega[P]^2)}{(2K[P] + K^2\omega[P]^2 + 1)} \]  \hspace{1cm} (8)

and when rearranged gives

\[ 0 = \omega[K[P]^2] + 2K[P] - x/(1-x) \]  \hspace{1cm} (9)

which can be solved as a quadratic equation. Note that the solution of this equation is independent of [P] but varies with the product of \( K[P] \). Thus, the cooperativity parameter can be determined without knowing the exact concentration of the protein. At any given fraction bound a value for \( K[P] \) can be determined from eqn. (9) and this, in turn, can be used to solve for the fraction monomer

Fraction monomer = \( \frac{(f_A b_n + b_A f_n)}{(f_A b_n + b_A f_n + b_A b_n + f_A f_n)} \)  \hspace{1cm} (10)

substituting eqn. (5) and eqn. (7) gives

\[ \frac{2K[P]}{(2K[P] + \omega[K[P]^2 + 1)} \]  \hspace{1cm} (11)

Similarly, Fraction dimer = \( \frac{\omega[K[P]^2]}{(2K[P] + \omega[K[P]^2 + 1)} \)  \hspace{1cm} (12)
Experimental values of $\omega$ were determined by least-squares fitting to eqns. 10 and 12 in the form of a species distribution plot. (Least-squares fitting was determined by a computer program designed by the authors. The amount of radioactivity in the monomeric and dimeric bound bands, as well as the free bands, were used to calculate $\omega$. Briefly, eqns. 11 and 12 were solved for a range of $\omega$s bracketing the final correct value. The final value for $\omega$ is that value which results in the curve which best approximates the experimental data, based on minimal least-squares fitting. The program is operated on a Macintosh computer using Hypercard and is available from the authors upon request.) We believe fitting to the equations is inherently more accurate than previously reported methods which rely on fitting only to one point, the maximal fraction of monomer [13].

A cooperativity parameter of $>1$ indicates positive cooperativity, while a value of 1 indicates no cooperativity and a value $<1$ indicates negative cooperativity. The species distribution as a function of fraction bound is unique for different cooperativity values and a best-fit curve to the ERE binding data is shown in Figure 4(a). In this case a value of 109 was obtained for $\omega$.

**Orientation and spacing discrimination**

The ability of the oestrogen receptor DNA-binding-domain polypeptide to discriminate alternative orientations and spacing between two AGGTCA hexamers was studied using a mobility-shift assay with different HREs. Various concentrations of the polypeptide were incubated with $^{32}$P end-labelled ERE-, TREp- and DR-5 RARE-containing oligonucleotides (for sequences, see Table 1). In each case two distinct complexes, labelled a (monomer) and b (dimer), are observed with increasing concentrations of polypeptide (results not shown), as seen with the ERE. However, while two bound complexes are seen for the interaction with each response element, the relative amounts of these complexes at given protein and DNA concentrations vary significantly. This reflects the highly cooperative nature of polypeptide binding to the ERE compared with TREp and DR-5 RARE DNAs. Least-squares fitting to the binding equation yielded cooperativity values for the polypeptide with TREp and DR-5 RARE of 1.8 and 0.14 respectively (see Figures 4b and 4c and Table 2).

**Hybrid response element binding**

The hybrid response element (E/G-RE) contains one hexameric half-site which is mutated to the GRE half-site sequence, AGAACA, and thus differs from the ERE half-site in two out of six positions. Not surprisingly, the oestrogen receptor DNA-binding-domain polypeptide does not bind to an isolated GRE half-site (results not shown), although it does bind an isolated ERE hexamer [7]. However, the polypeptide does bind the GRE half-site in the hybrid element, but only after prior binding of a polypeptide to the ERE half-site. On a mobility-shift assay, two bound complexes are observed. As illustrated in Figure 5, complex a predominates at lower polypeptide concentrations while the slower migrating complex b predominates at higher concentrations. Methylation interference assays (Figure 6) reveal that complex a consists of a monomeric complex of polypeptide bound to the ERE hexamer while complex b consists of a dimer interacting specifically with both the ERE and GRE hexamers. Essentially, a monomer forms as a polypeptide bound to the ERE hexamer which in turn 'creates' a site for binding of a second polypeptide molecule. This site consists of both the GRE hexamer and the protein contact surface from the polypeptide already bound to the DNA. Quantification of the cooperative interaction is possible in a manner similar to that described earlier. However, since a different binding model must be used, the mathematics also changes. In the case of E/G-RE binding though, the $\omega$ value ($\omega'$) is not really a cooperativity parameter, but is an indication of the affinity of the polypeptide for the GRE hexamer of complex a relative to its affinity for the ERE hexamer alone. We find, in fact, that this affinity is roughly equivalent to the affinity of the polypeptide for the ERE hexamer alone ($\sim$ 1.5 times higher).
Table 1  DNA sequences of different binding sites of interest

Upper-case letters indicate hexameric sequences and bold letters indicate nucleotides in the GRE hexamer different from those in the corresponding ERE hexamer. As no GRE sequence was specifically employed in the study, the three non-specific 'spacer' nucleotides between the hexamers are indicated as 'nnn' instead of any particular sequence.

<table>
<thead>
<tr>
<th>DNA name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERE</td>
<td>AGGTCAgacTVAGCTTT</td>
</tr>
<tr>
<td>GRE</td>
<td>AGAACAnnnTGGGCTCT</td>
</tr>
<tr>
<td>ERE hexamer</td>
<td>AGGTCAgacTGGAGCTA</td>
</tr>
<tr>
<td>GRE hexamer</td>
<td>taccctgagatTTGCTCT</td>
</tr>
<tr>
<td>E/G-RE</td>
<td>AGGTCAgacTGGGCTCT</td>
</tr>
<tr>
<td>TREp</td>
<td>AGGTCAgacTGGAGCTA</td>
</tr>
<tr>
<td>DR-5 RARE</td>
<td>AGGTCAgacTVAGCTTT</td>
</tr>
</tbody>
</table>

Table 2  Cooperativity values under various conditions

<table>
<thead>
<tr>
<th>DNA</th>
<th>(\omega)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERE</td>
<td>109</td>
</tr>
<tr>
<td>TREp</td>
<td>1.8</td>
</tr>
<tr>
<td>DR-5 RARE</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Figure 5  Typical mobility-shift assays with oestrogen receptor DNA-binding domain and E/G-RE

Increasing concentrations (from left to right) of oestrogen receptor DNA-binding domain were incubated with \(^{32}P\)-end-labelled E/G-RE-containing DNA. Monomeric (labelled 'a') and dimeric (labelled 'b') complexes are indicated. Methods are as described in the Experimental section.

DISCUSSION

Cooperative protein–protein interactions are a common aspect of many DNA-binding proteins. These interactions play a variety of biological roles, from modulating the formation of transcription-initiation complexes to directing DNA recognition. Thus, understanding these interactions is essential to our understanding of the transcriptional process.

By using an oestrogen receptor DNA-binding-domain polypeptide we have been able to investigate the role of interactions between DNA-binding domains upon response element recognition. A value of 109 was obtained for the cooperativity parameter upon ERE binding (Figure 4), indicating that the second polypeptide binds with an association constant \(\sim 100\) times greater than that of the first polypeptide. Based on a recently solved crystal structure of the oestrogen receptor DNA-binding domain–ERE complex, the cooperativity is a manifestation of the protein–protein interactions between the DNA-bound polypeptides. This cooperativity is both qualitatively and quantitatively similar to the interaction of a glucocorticoid receptor DNA-binding domain with its cognate GRE [14]. It is not surprising, then, that both glucocorticoid and oestrogen receptor DNA-binding domains exhibit marked structural similarity both before and after binding DNA [15–18]. As well, the response elements for these two receptors are highly related, both consisting of similar half-site motifs, arranged as inverted repeats and spaced by 3 bp [11].

It seems a reasonable hypothesis that protein–protein interactions mediating cooperative binding of the polypeptide to an ERE are involved in the recognition of half-site spacing and orientation. If this is the case, then, one would not expect to observe cooperativity upon binding to elements with altered half-site positions. Our cooperativity analysis has enabled us to examine this assumption in further detail. While a high degree of cooperativity is seen for the interaction of the oestrogen receptor...
DNA-binding-domain polypeptide with the ERE (\(\omega = 109\)), no significant cooperativity is observed with the TREP element (\(\omega = 1.8\)), and a slight negative cooperativity is seen upon binding to the DR-5 RARE sequence (\(\omega = 0.14\)). This negative cooperativity is possibly the result of steric or other hindrance upon DNA binding of the polypeptides. The highly cooperative binding of the oestrogen receptor DNA-binding-domain polypeptide to the ERE but not TREP or DR-5 RARE demonstrates that the protein–protein interactions are specifically optimized for recognition of the particular orientation and spacing of hexamers observed in an ERE. It is likely that these interactions play a role in stabilizing oestrogen receptor–ERE interactions in vitro. The high cooperativity of binding observed only with the ERE suggests that this interaction dictates recognition of half-site orientation and spacing. Thus, while the oestrogen receptor may in principle be capable of interacting with a number of spacings and orientations of the hexamers, only the specific spacing and orientation observed with an ERE results in favourable interactions between the DNA-binding domains. This is manifested in vitro as cooperative interactions between the DNA-binding-domain polypeptides. However, in vitro, with the already dimerized oestrogen receptor, the cooperativity may provide additional binding energy, resulting in high-affinity recognition of the cognate response element. (Alternatively, interactions may occur between the two DNA-binding domains of the receptor dimer prior to response element binding, thus restricting the orientation and spacing of half-sites which can be simultaneously bound by the two domains.)

Although we used a consensus ERE to characterize the cooperative interactions of the oestrogen receptor DNA-binding-domain polypeptides, naturally occurring EREs frequently vary from the consensus. Interestingly, this sequence degeneracy occurs primarily in only one of the half-sites. Similar observations have been noted for GREs \(^{19}\). To test whether cooperative interactions could be detected with this type of sequence degeneracy, we synthesized a hybrid response element (termed an E/G-ERE) in which one half-site is that of a consensus ERE hexamer while the other is degenerate to that of a consensus GRE (AGAACA) hexamer.

An important aspect of receptor interaction with non-cognate sequences emerges from our analysis. Normally, a single GRE half-site is not recognized by the oestrogen receptor DNA-binding-domain polypeptide. However, binding to the GRE half-site in the hybrid element does occur, although it is dependent on prior binding of a polypeptide to the ERE half-site. In the mobility-shift assay, both monomeric (a) and dimeric (b) protein–DNA complexes are observed (Figure 5). Fitting of a binding curve to the data reveals that the association constant of the oestrogen receptor DNA-binding domain for the GRE hexamer of complex a is roughly identical with that of the oestrogen receptor DNA-binding domain for the ERE hexamer itself. Thus, cooperative interactions have mediated the recognition of a sequence that the protein would not normally bind.

We expect that this effect of cooperative interactions may also be of relevance to transcription factors in general.

In conclusion, we have developed a simple method to analyse and quantify the cooperative interactions of proteins binding to DNA. We have utilized this method to quantitatively analyse cooperativity of the oestrogen receptor DNA-binding domain upon binding to a variety of hormone responsive elements. Consistent with the in vitro role of the receptor, the polypeptide binds with high cooperativity to the ERE but with negative cooperativity or without cooperativity to retinoic acid and thyroid response elements, respectively. Finally, we have demonstrated that cooperative interactions are also capable of mediating the recognition of ERE sequence degeneracy.

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REFERENCES