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Genetic variations within the ERE motif modulate plasticity and energetics of binding of DNA to the ERα nuclear receptor

Brian J. Deegan, Vikas Bhat, Kenneth L. Seldeen, Caleb B. McDonald, Amjad Farooq *

Department of Biochemistry and Molecular Biology and USylvester Braman Family Breast Cancer Institute, Leonard Miller School of Medicine, University of Miami, Miami, FL 33136, United States

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ABSTRACT

Upon binding to estrogens, the ERα nuclear receptor acts as a transcription factor and mediates a multitude of cellular functions central to health and disease. Herein, using isothermal titration calorimetry (ITC) and circular dichroism (CD) in conjunction with molecular modeling (MM), we analyze the effect of symmetric introduction of single nucleotide variations within each half-site of the estrogen response element (ERE) on the binding of ERα nuclear receptor. Our data reveal that ERα exudes remarkable tolerance and binds to all genetic variants in the physiologically relevant nanomolar–micromolar range with the consensus ERE motif affording the highest affinity. We provide rationale for how genetic variations within the ERE motif may reduce its affinity for ERα by orders of magnitude at atomic level. Our data also suggest that the introduction of genetic variations within the ERE motif allows it to sample a much greater conformational space. Surprisingly, ERα displays no preference for binding to ERE variants with higher AT content, implying that any advantage due to DNA plasticity may be largely compensated by unfavorable entropic factors. Collectively, our study bears important consequences for how genetic variations within DNA promoter elements may fine-tune the physiological action of ERα and other nuclear receptors.

Introduction

Nuclear receptors (NRs)[1] act as ligand-modulated transcription factors and orchestrate a plethora of cellular functions central to health and disease [1–4]. Some notable examples of ~50 members of the NR family are the androgen receptor (AR), estrogen receptor α (ERα), glucocorticoid receptor (GR) and progesterone receptor (PR). All members of the NR family are evolutionarily related and share a core modular architecture comprised of a central DNA-binding (DB) domain flanked between an N-terminal trans-activation (TA) domain and a C-terminal ligand-binding (LB) domain [5–7]. A typical scenario for the activation of nuclear receptors involves the secretion of lipophilic messengers such as hormones and vitamins by appropriate tissues. Upon their diffusion through the cell membrane, the binding of these ligands to the LB domain culminates in a series of events involving the translocation of nuclear receptors into the nucleus and subsequent modulation of expression of target genes [8–10]. While the DB domain recognizes specific promoter response elements within target genes, the LB domain additionally serves as a platform for the recruitment of a multitude of cellular proteins, such as transcription factors, co-activators and co-repressors, to the site of DNA transcription and thereby allowing nuclear receptors to exert their action at genomic level in a concerted fashion [11,12]. While the trans-activation function of the LB domain is ligand-dependent, the TA domain operates in an autonomous manner and it is believed to be responsive to growth factors acting through the MAPK signaling and thus it may further synergize the action of various co-activators and co-repressors recruited by the LB domain at the site of DNA transcription [13,14]. In this manner, nuclear receptors mediate a diverse array of cellular functions from embryonic development to metabolic homeostasis and their aberrant function has been widely implicated in disease [15–19,7].

ERα mediates the action of estrogens such as estradiol and its hyperactivation leads to the genesis of large fractions of breast cancer [20–26]. The DB domain of ERα binds as a homodimer with a twofold axis of symmetry to the ERE motif, containing the AGGTCAnnnTGACCT consensus sequence, located within the promoters of target genes [27]. DNA-binding is accomplished through a pair of tandem C4-type zinc fingers, with each finger containing a Zn^{2+} ion coordinated in a tetrahedral arrangement by four highly conserved cysteine residues [28,29]. It is important to note that

* Corresponding author. Fax: +1 305 243 3955.
E-mail address: amjad@farooqlab.net (A. Farooq).

[1] *Abbreviations used:* AR, androgen receptor; CD, circular dichroism; DB, DNA-binding; ERα, estrogen receptor α; ERE, estrogen response element; GR, glucocorticoid receptor; ITC, isothermal titration calorimetry; LB, ligand-binding; MAPK, mitogen-activated protein kinase; MM, molecular modeling; NMR, nuclear magnetic resonance; NR, nuclear receptor; PR, progesterone receptor; SEC, size-exclusion chromatography; TA, trans-activation; TB, terrific broth; Trx, thioredoxin; XRC, X-ray crystallography; ZF, zinc finger.

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the DB domain contains two zinc fingers. The first zinc finger (ZF-I) within each monomer of DB domain recognizes the hexanucleotide sequence 5′-AGGTCA-3′ within the major groove at each end of the ERE duplex, while the second zinc finger (ZF-II) is responsible for the homodimerization of DB domain upon DNA binding.

Although nuclear receptors recognize the target genes in a DNA-sequence-dependent manner, genetic variations within specific promoter response elements are extremely common within the eukaryotic genomes [27]. Given that the nucleotide sequence is a key determinant of the ability of DNA to behave as a flexible polymer and undergo physical phenomena such as bending, stretching, deformation and distortion coupled with its ability to exist in various structural conformations (such as the B-DNA, A-DNA and Z-DNA) [30–32], our knowledge of how genetic variations within the promoter elements influence the ability of nuclear receptors to bind and subsequently affect gene transcription remains largely elusive. Several lines of evidence indeed suggest that genetic variations within the cognate response elements play a key role in modulating the affinity and specificity of binding of AR, GR and PR nuclear receptors [33–36]. In an effort to build on these earlier studies, we set out here to investigate how single nucleotide variations within the estrogen response element (ERE) affect the plasticity and energetics of binding of DNA to the ERα nuclear receptor.

Materials and methods

Protein preparation

The DB domain (residues 176–250) of human ERα (Expasy# P03372) was cloned into pET101 bacterial expression vector with a C-terminal polyhistidine (His)-tag, to aid in protein purification through Ni–NTA affinity chromatography, using Invitrogen TOPO technology. The protein was subsequently expressed in *Escherichia coli* BL21*(DE3) bacterial strain (Invitrogen) and purified on a Ni–NTA affinity column using standard procedures. Briefly, bacterial cells were grown at 20 °C in TB media supplemented with 50 μM $ZnCl_2$ to an optical density of 0.5 at 600 nm prior to induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The bacterial culture was further grown overnight at 20 °C and the cells were subsequently harvested and disrupted using a BeadBeater (Biospec). After separation of cell debris at high-speed centrifugation, the cell lysate was loaded onto a Ni–NTA column and washed extensively with 20 mM imidazole to remove non-specific binding of bacterial proteins to the column. The recombinant protein was subsequently eluted with 200 mM imidazole and dialyzed against an appropriate buffer to remove excess imidazole. Further treatment on a Hiload Superdex 200 size-exclusion chromatography (SEC) column coupled in-line with GE Akta FPLC system led to purification of recombinant DB domain to apparent homogeneity as judged by SDS–PAGE analysis. The identity of recombinant protein was confirmed by MALDI-TOF mass spectrometry. Final yield was typically between 5 and 10 mg protein of apparent homogeneity per liter of bacterial culture. Protein concentration was determined by the fluorescence-based Quant-It assay (Invitrogen) and spectrophotometrically using an extinction coefficient of 14,940 M^{-1} cm^{-1} calculated for the recombinant DB domain using the online software ProtParam at ExPasy Server [37]. Results from both methods were in an excellent agreement.

DNA synthesis

21-mer DNA oligos containing the consensus ERE motif (AGGTCAnnnTGACCT) and all possible symmetric single nucleotide variants were commercially obtained from Sigma Genosys. The design of such oligos and their numbering relative to the central 3-bp spacer is illustrated in Fig. 1. Oligo concentrations were determined spectrophotometrically on the basis of their extinction coefficients derived from their nucleotide sequences using the online software OligoAnalyzer 3.0 (Integrated DNA Technologies) based on the nearest-neighbor model [38]. Double-stranded DNA (dsDNA) oligos were generated as described earlier [39].

ITC measurements

Isothermal titration calorimetry (ITC) experiments were performed on a Microcal VP-ITC instrument and data were acquired and processed using fully automized features in Microcal ORIGIN software. All measurements were repeated at least three times. Briefly, protein and DNA samples were prepared in 50 mM sodium phosphate containing 5 mM β-mercaptoethanol at pH 7.0 and degassed using the ThermoVac accessory for 5 min. The experiments were initiated by injecting 25 × 10 μl aliquots of 50–200 μM of a dsDNA oligo containing the ERE motif, or a variant thereof, from the syringe into the calorimetric cell containing 1.8 ml of 5–10 μM of DB domain of ERα at 25 °C. The change in thermal power as a function of each injection was automatically recorded using Microcal ORIGIN software and the raw data were further processed to yield binding isotherms of heat release per injection as a function of molar ratio of dsDNA oligo to dimer-equivalent DB domain. The heats of mixing and dilution were subtracted from the heat of binding per injection by carrying out a control experiment in which the same buffer in the calorimetric cell was titrated against the dsDNA oligo in an identical manner. Control experiments with scrambled dsDNA oligos generated similar thermal power to that obtained for the buffer alone, implying that there was no non-specific binding of DB domain to non-cognate DNA. To extract various thermodynamic parameters, the binding isotherms were iteratively fit to a built-in one-site model by non-linear least squares regression analysis using the ORIGIN software as described previously [40,39].

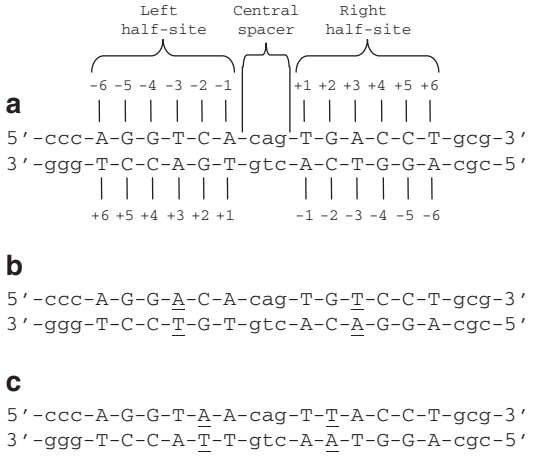

Fig. 1. Nucleotide sequence of dsDNA oligos. (a) Consensus ERE motif. The AGGTCA and TGACCT half-sites are clearly marked. The consensus nucleotides within each half-site are capitalized while the flanking nucleotides and the intervening nucleotides within the central spacer are shown in small letters. The numbering of various nucleotides within each half-site relative to the CAG central spacer in the sense (upper) and antisense (lower) strands are indicated. (b) Am3Tp3 motif, wherein adenine and thymine are, respectively, substituted at −3 and +3 positions within the sense strand in a symmetric manner within each half-site. The variant nucleotides relative to the consensus ERE motif in both strands are underlined. (c) Am2Tp2 motif, wherein adenine and thymine are, respectively, substituted at −2 and +2 positions within the sense strand in a symmetric manner within each half-site. The variant nucleotides relative to the consensus ERE motif in both strands are underlined.