Estrogen response element binding induces alterations in estrogen receptor- α conformation as revealed by susceptibility to partial proteolysis

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ABSTRACT

Genes whose expression is highly induced by estradiol (E_2) contain multiple estrogen response elements (EREs) in their promoters. Previously we reported that estrogen receptor- α (ER α) binds cooperatively to and E_2 synergistically activates reporter gene expression from three or four tandem copies of a consensus ERE (ERec38). Here we evaluated how ER α binding to one, two, three or four tandem copies of ERec38 affects ER α conformation as detected by altered ER α trypsin digestion patterns in Western blots. E_2 - or 4-hydroxytamoxifen (4-OHT)-occupied ER α bound to the pS2 ERE or to a single copy of ERec38 showed enhanced susceptibility to trypsin digestion

compared to E_2 - or 4-OHT-ER α incubated with DNA lacking an ERE. ER α binding to multiple tandem copies of ERec38 further increased sensitivity to trypsin digestion. These results correlate with synergistic transcription and cooperativity of ER α binding to multiple tandem copies of ERec38. These observations suggest that ERec38 binding alters the overall conformation of ER α and that multiple tandem copies of ERec38 enhance these conformational changes. We hypothesize that ERE-induced alterations in ER α conformation modulate interaction with coregulatory proteins, resulting in synergistic transcriptional activation.

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INTRODUCTION

Estrogen receptor (ER) is a member of the steroid/thyroid superfamily of proteins that act as hormone-inducible transcription factors (Mangelsdorf *et al.* 1995). Two ER gene products, i.e. ER α and ER β , mediate the actions of estrogens in target tissues, thus regulating estrogenic effects on reproduction, bone homeostasis, and mammary gland structure and function (Katzenellenbogen & Korach 1997). Ligands, e.g. estradiol (E_2), bind in the ligand-binding domain (LBD) of ER α and induce conformational changes leading to dimerization and high-affinity ER α binding to estrogen response elements (EREs). Once bound to an ERE, interaction between specific motifs within the DNA-bound ER α and coactivator proteins, e.g. SRC-1 (Oate *et al.* 1995), TIF1 (Le Douarin *et al.* 1995), TIF2 (Voegel *et al.* 1996), and the TATA binding complex, e.g. TFIIB (Ing *et al.* 1992) and TATA-box binding protein (Sadovsky *et al.* 1995), results in increased gene transcription (reviewed in McKenna *et al.* 1999).

Crystal structure studies of the ER α LBD occupied by an antiestrogen, i.e. 4-hydroxytamoxifen (4-OHT) (Shiau *et al.* 1998), the select ER modulator raloxifene (RAL) (Brzozowski *et al.* 1997), E_2 (Brzozowski *et al.* 1997) or diethylstilbestrol (DES) (Shiau *et al.* 1998) show distinct changes in the positioning of helix 12, which is involved in AF-2 function and interaction with the LXXLL-motif of coactivators. Whereas the position of helix 12 in E_2 - or DES-liganded ER α facilitates formation of a hydrophobic groove allowing interaction with specific coactivators, displacement of helix 12 in 4-OHT- or RAL-liganded ER α allows no interaction with these proteins.

We reported that E_2 -ER α binds cooperatively to stereoaligned EREs that are surrounded by naturally occurring AT-rich flanking sequences (Klinge *et al.* 1992a,b, 1996a,b, Anolik *et al.* 1993, 1995, 1996). In contrast, when ER α is occupied by 4-OHT, ER α binds EREs non-cooperatively, regardless of spacing of the EREs on the DNA helix or the presence of AT-rich ERE-flanking

sequence (Klinge *et al.* 1992a, Anolik *et al.* 1993, 1995, 1996). Further, upon ERE binding, an allosteric change is induced in the 4-OHT-ER α protein and one molecule of 4-OHT dissociates from the 4-OHT-ER α dimer (Klinge *et al.* 1996a).

Response elements recognized by nuclear transcription factors, including members of the steroid/nuclear receptor superfamily, were proposed to contain information that is interpreted by bound regulator factors (Lefstin *et al.* 1998). In this model, DNA is envisioned to act as an allosteric ligand whose binding alters the transcription factor's affinity for other ligands, such as coactivators or corepressors. This model predicts that the DNA-binding domain (DBD) of the ER α harbors activities beyond DNA recognition. Indeed, the ER α DBD constitutes surfaces for both intramolecular and intermolecular protein-protein contacts. Thus, alterations in the ER α conformation induced by ER α -ERE binding are predicted to alter ER α interaction with other proteins, e.g. coactivators or corepressors (Klinge 2000).

ER α conformation also depends on bound ligand. Digestion of ER α -expressing MCF-7 cells with chymotrypsin or trypsin revealed different sized fragments of ER α when liganded by E₂ and tamoxifen (Hutchens *et al.* 1987). More recently, different trypsin digestion patterns of both ER α and ER β were detected when the receptors were occupied by agonist vs antagonist ligands (Kraichely *et al.* 2000). However, not all investigators detected ligand-specific alterations in ER α conformation by altered sensitivity to trypsin digestion (Fritsch *et al.* 1993). While specific amino acid mutations that activate ER α were shown to result in proteolytic digestion patterns similar to those of the wild-type ER α (Lazennec *et al.* 1997), two reports indicate conflicting results on the effect of DNA binding on ER α conformation as measured by alterations in trypsin digestion (Fritsch *et al.* 1992b, Wood *et al.* 1998). One report showed that rat uterine ER α conformation was unaffected by DNA binding (Fritsch *et al.* 1992b). The other report showed that yeast-expressed, recombinant human (rh) ER α was more sensitive to trypsin or chymotrypsin digestion when bound to the vitellogenin A2 ERE, containing a perfect palindromic ERE, than when ER α was incubated with the ERE from the human pS2 gene, containing a single nucleotide change in the 3' half-site (Wood *et al.* 1998). Recently we reported that a correlation exists between ER α binding affinity, transcriptional activation in transiently transfected cells, and sensitivity of ER α to α -chymotrypsin digestion for two synthetic palindromic EREs, a direct repeat 5 sequence, and the EREs from the estrogen responsive c-fos, progesterone

receptor, and pS2 genes (Klinge *et al.* 2001). No one has used protease sensitivity to examine if the cooperative ER α binding and synergistic activation of reporter gene expression from multiple EREs is reflected in altered ER α conformation when bound to these elements.

In the present study, we utilized trypsin digestion to compare ER α conformation when the receptor was occupied by E₂ or 4-OHT and when ER α was free in solution or bound to one or more tandem copies of a consensus ERE called EREc38. We report that ERE binding enhances the sensitivity of ER α to proteolysis by trypsin. In contrast, incubation of ER α with DNA sequences to which ER α does not bind does not alter ER α sensitivity to proteolysis. We report that there is a correlation between the sensitivity of ER α to trypsin digestion and cooperativity of ER α -EREc38 binding and synergistic transcriptional activation. These results are consistent with a role for the sequence of the ERE as an allosteric regulator of ER α activity.

MATERIALS AND METHODS

Preparation of ERE-containing oligomers

The sequence of the synthetic EREc38 used in these experiments is: 5'-CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG-3' (Peale *et al.* 1988). The sequence of the pS2 ERE is 5'-CTTCCCCTGCAAGGTCAGCGTGGCCACCCCGTGAGCC-ACT-3' that was cloned into the pGL3-Promoter plasmid (Promega, Madison, WI, USA) as described (Klinge *et al.* 1999). Single EREc38 or multiple, head-to-tail, tandem copies of double-stranded EREc38 oligomers, referred to as *n* EREc38 where *n*=number of tandem copies, were cloned into the pGEM-7Zf(+) or pGL3-Promoter plasmids as described (Klinge *et al.* 1992b, 1997b). EREc38 oligomers were prepared by restriction digestion of the pGEM-7Zf(+) plasmid with EcoRI and BamHI and electroelution (Klinge *et al.* 1996a). pS2 and NOTERE (containing the plasmid DNA flanking the insertion site of the EREs in pGL3-Promoter) were restricted from the pGL3-Promoter plasmid by XhoI/HindIII digestion and purified by PAGE and electroelution (Klinge 1999). The concentration of the EREc38, pS2 and NOTERE oligomers was measured using the PicoGreen fluorescence assay with λ DNA as the standard (Molecular Probes, Eugene, OR, USA).

Preparation of ER

Baculovirus-expressed rhER α was prepared as nuclear extracts from Sf-21 cells as previously

described (Klinge *et al.* 1998, 1999). ER α concentration was determined by 17β -[2,3,6,7- ^3H (N)]-estradiol ($[^3\text{H}]\text{E}_2$, 84.1 Ci/mmol; NEN, Boston, MA, USA) binding and adsorption to hydroxylapatite (Pavlik *et al.* 1976). All receptor concentrations refer to dimeric ER α (i.e. with two molecules of bound ligand).

Trypsin digestion

Liganded ER α (250 fmol per reaction) was pre-incubated with ERE (1.25–2.5 pmol per reaction), non-specific DNA (a fragment from the region of the pGEM-7Zf(+) plasmid into which EREc38 was cloned), or an equal volume of TE buffer for 30 min at room temperature (RT, 22 °C). Trypsin was added to give the concentration (0.1–1.5 $\mu\text{g}/\text{ml}$) indicated in the figure legends and the reaction (20 μl total reaction volume) was allowed to proceed for 10 min at RT. The reaction was terminated by addition of 6 μl Laemmli loading buffer (Klinge *et al.* 1997b). Samples were applied to a 10% polyacrylamide mini-SDS PAGE gel.

SDS-PAGE, Western blotting and densitometric quantitation

Proteins were analyzed on 10% SDS-polyacrylamide mini-gels as described (Klinge *et al.* 1997a). For Western blotting, proteins were electroblotted onto PVDF (NEN). The transfer was monitored by transfer of the prestained protein markers (SeeBlue Standard from Invitrogen, Carlsbad, CA, USA). Following the transfer, the blots were processed as described (Klinge *et al.* 1997b). Multiple exposures of each blot (from 10 s to 4 min) were taken to ensure that the film's range was not exceeded. The resulting films of the immunoblots were scanned into Adobe Photoshop v. 4.0 using a Microtek ScanMaker III scanner (Redondo, Beach, CA, USA). Un-Scan-It v. 5.1 for Windows (Silk Scientific, Orem, UT, USA) was used to digitalize and quantitate the amount of ER α in the immunoblot bands and to determine the molecular mass (MM) of each ER α band, based on migration of the prestained protein standards. The densitometric value from each band was entered into Microsoft Excel 97 and relative values determined. Regression analysis across multiple experiments determined EC $_{50}$ values for digestion of the intact receptor. Computer analysis of the potential trypsin sites and anticipated peptide mass of the resulting tryptic fragments of the human ER α sequence was performed using Expert Protein Analysis System proteomics server (ExPASy) of the

Swiss Institute of BioInformatics; web site <http://expasy.cbr.nrc.ca/cgi-bin/peptide-mass.pl>.

Transient transfection experiments

Chinese hamster ovary cells (CHO-K1) were purchased from ATCC (Manassas, VA, USA) and maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum. All other cell culture reagents were purchased from Gibco BRL. Transient transfection was performed using Transfast (Promega) according to directions supplied by the manufacturer and as described previously (Klinge *et al.* 1999, 2000, 2001). Cells were treated with 10 nM E $_2$ (Sigma Chemical Co., St Louis, MO, USA), 100 nM 4-OHT (Research Biochemicals International, Natick, MA, USA), or 10 nM E $_2$ + 100 nM 4-OHT, diluted in IMDM without phenol red. Cells were harvested 30 h later and luciferase and β -galactosidase (β -gal) activities assayed (Klinge *et al.* 1997b). All data for transient transfections were normalized by β -gal to account for transfection efficiency. Statistical analyses were performed using Student's *t*-test in Microsoft Excel 97.

Other statistical treatments

The concentration of trypsin required to decrease the amount of intact ER α by 50% (EC $_{50}$) was calculated from densitometric data using logistic regression (GraphPad Prism, v.3, GraphPad Software, San Diego, CA, USA). Apparent equilibrium dissociation constants (K_d) were calculated from electrophoretic mobility shift assay (EMSA) data using a one-site competition model (GraphPad Prism, v.3) (Tyulmenkov *et al.* 2000). These values and the mean luciferase activities from transfection experiments were dichotomized using the criteria described in the text and organized in 2×2 contingency tables to employ chi-square independence tests in Microsoft Excel 97. We investigated possible correlations between EC $_{50}$, K_d , and fold-induction using parametric and non-parametric regression tools in GraphPad Prism, v.3.

RESULTS

EREc38 binding increases ER α susceptibility to trypsin digestion when occupied by E $_2$ or 4-OHT

To examine the effect of ERE binding on the conformational state of baculovirus-expressed, rhER α

FIGURE 1. ERE binding to ERα enhances trypsin proteolysis. Western blots of PVDF membranes probed with Ab-10 to ERα. ERα (12.5 nM final) was preincubated with E₂ (A–C) or 4-OHT (D and E) followed by incubation with TE, NOTERE (B and D) and EREc38 (C and E) as detailed in Materials and Methods. The liganded ERα-DNA mix was then incubated with increasing concentrations of trypsin (0–1.5 μg/ml, as indicated) for 10 min at RT. The reaction was terminated and the samples were applied to 10% polyacrylamide SDS-PAGE gels. Conditions for electrophoresis and Western blotting with ERα antibody Ab-10 are described in Materials and Methods. Sizes of protein markers are indicated in kDa. The sizes of the ERα bands are: T1=57 kDa, T2=45 kDa, T3=36 and 34 kDa bands combined.

TABLE 1. Summary of ER α interaction with select ERE sequences. Comparison of the EC₅₀ value for trypsin digestion of the intact ER α band vs K_d for E₂-ER α -ERE binding and fold-induction of luciferase activity in CHO-K1 cells transiently transfected with ER α and treated with 10 nM E₂. Details of the experiments from which these data are derived are described in Materials and Methods. Values are means $\pm$ S.E.M. for 3–11 experiments

	K_d for E ₂ -ER α -ERE binding (nM) ¹	Fold-induction of luciferase activity	EC ₅₀ for E ₂ -ER α (trypsin μ g/ml)	EC ₅₀ for 4-OHT-ER α (trypsin μ g/ml)
DNA				
NOTERE	N/A ²	N/A ²	1.21	1.57
pS2	1.069 $\pm$ 0.03	2.41 $\pm$ 0.21	0.89	0.91
1 EREc38	0.089 $\pm$ 0.0088	3.71 $\pm$ 0.31	0.81	0.84
2 EREc38	0.038 $\pm$ 0.0086	6.34 $\pm$ 0.81	0.70	0.71
3 EREc38	0.0024 $\pm$ 0.0001	24.86 $\pm$ 3.93	0.61	0.51
4 EREc38	0.018 $\pm$ 0.0016	29.96 $\pm$ 5.60	0.35	0.22

¹Tyulmenkov & Klinge (2001).

²NA=not applicable, since ER α did not bind NOTERE (data not shown).

occupied by either E₂ or 4-OHT, ER α was incubated with EREc38 and subjected to limited trypsin proteolysis in the presence of ERE-containing oligomers or a DNA sequence containing no EREs, called 'NOTERE' for simplicity. Figure 1 shows Western blots of five representative trypsin digestion experiments in which ER α , occupied by either E₂ or 4-OHT, was incubated with TE (Fig. 1A), NOTERE (Fig. 1B and D) and EREc38 (Fig. 1C and E). Without trypsin treatment, Western blotting of E₂-ER α with a C-terminal specific monoclonal antibody to ER α , Ab-10, revealed the most prominent band to be 66 kDa (Fig. 1A–E). This corresponds in size to intact ER α . Thus, there was no degradation of ER α with incubation with TE buffer or with DNA oligomers at RT prior to trypsin digestion. Cleavage of ER α by trypsin creates a concentration-dependent disappearance of the 66 kDa intact band concurrent with the appearance of increasingly smaller digestion products, i.e. 60, 55, 45 and 36–34 kDa.

Incubation of ER α with trypsin and either E₂ or 4-OHT in the presence of a non-cognate DNA sequence (NOTERE) or TE buffer alone resulted in digestion patterns comparable to patterns observed without added DNA (Fig. 1A–C). These results agree with a recent report which detailed ER α conformational changes induced by ligand binding (Kraichely *et al.* 2000). When ER α was incubated with EREc38 instead of NOTERE, however, the results diverged from both our NOTERE results and other published findings (Kraichely *et al.* 2000) (Fig. 1C and E). ER α is more sensitive to trypsin digestion when bound to EREc38. This is readily seen in the densitometric quantitation of the ER α

band intensities for repeated trypsin digestion experiments of E₂- and 4-OHT-occupied ER α incubated with NOTERE or EREc38 shown in Fig. 2. The EC₅₀ value for disappearance of the intact ER α band decreased from 1.2 and 1.5 μ g/ml trypsin for E₂- and 4-OHT-occupied ER α receptor respectively, in the presence of NOTERE, to 0.81 and 0.84 μ g/ml trypsin with the addition of EREc38 (Fig. 2, Table 1). This increased sensitivity of ER α to trypsin digestion in the presence of EREc38 suggests that conformational changes in addition to those induced by ligand binding occur upon interaction with EREc38, reflecting the allosteric effect of DNA on ER α conformation (Lefstin & Yamamoto 1998).

Comparison of ER α digestion when incubated with EREc38 vs pS2

We reported a correlation between the apparent binding affinity (K_d) of ER α binding to specific ERE sequences, as determined by EMSA, and E₂-induced transactivation potential in transient transfection experiments (Klinge *et al.* 1992b, 1996a, 2001, Tyulmenkov *et al.* 2000). As anticipated, a trypsin concentration-dependent loss of intact ER α was detected for both E₂- and 4-OHT-occupied ER α bound to pS2 (Fig. 3A and B). Densitometric quantitation of the ER α band intensities for repeated trypsin digestions of ER α incubated with pS2 gave an identical EC₅₀ value of 0.89 μ g/ml for E₂- and 4-OHT-ER α respectively (Fig. 3C and D). There is no statistical difference between the EC₅₀ values for trypsin digestion of ER α bound to pS2 vs EREc38.

A. Trypsin digestion of E₂-ER α -NOTERE **B. Trypsin digestion of 4-OHT-ER α -NOTERE**

FIGURE 2. (A and B, legend opposite).

Effect of multiple tandem copies of EREc38 on sensitivity of ER α to trypsin digestion

Multiple copies of EREc38 cloned head-to-tail as a tandem array demonstrated cooperative E₂-ER α binding and synergistic transactivation potential (Tyulmenkov *et al.* 2000). This result, combined with the data showing that binding to 1 EREc38 enhanced trypsin digestion of ER α (Fig. 1D and E and Fig. 2C and D), indicates that further ER α conformation changes may occur upon ER α binding to multiple tandem copies of EREc38. To test this

hypothesis, we examined the effect of ER α binding to 2, 3 and 4 tandem copies of EREc38 on ER α trypsin sensitivity. These experiments utilized the same ER α proteolysis assay described in Fig. 1 and Materials and Methods. As expected from the K_d and fold-induction values for these constructs (Tyulmenkov *et al.* 2000), ER α incubated with 2, 3 or 4 EREc38 displayed increasing susceptibility to proteolysis by trypsin. Binding of ER α to 2 EREc38 resulted in an EC₅₀ of 0.70 and 0.71 μg/ml trypsin for E₂- and 4-OHT-ER α respectively (Fig. 4A and B and Fig. 5A and B), whereas ER α

C. Trypsin digestion of E₂-ER α -ERec38

D. Trypsin digestion of 4-OHT-ER α -ERec38

FIGURE 2. (C and D).

FIGURE 2 (opposite and above). Plot of densitometric scans of NOTERE- and ERec38-bound ER digested by trypsin. The relative level of ER α in all bands was determined by densitometric scans of Western blots. The resulting numerical values for image intensity correlate to the amount of ER α protein recognized by Ab-10. The relative ER α amounts in the trypsin digestion bands are expressed relative to the intact, non-trypsinized ER α 66 kDa band that was assigned a value of 100%. (A and B) Data from E₂- and 4-OHT-occupied ER α incubated with the NOTERE DNA sequence respectively. (C and D) The same analysis applied to E₂- and 4-OHT-occupied ER α bound to ERec38 respectively. Data are means $\pm$ S.E.M. of $n=3-5$ experiments. The sizes of the ER α bands are: T1=57 kDa, T2=45 kDa, T3=36 and 34 kDa bands combined.

binding to 3 ERec38 showed 0.61 and 0.51 μ g/ml trypsin for E₂- and 4-OHT-ER α respectively (Fig. 4C and D and Fig. 5C and D). Finally, ER α binding

to 4 ERec38 yielded EC₅₀ values of 0.35 and 0.22 μ g/ml for E₂- and 4-OHT-occupied ER α respectively (Fig. 4E and F and Fig. 5E and F).

FIGURE 3. (A–C, legend opposite).

Incubation of ERα with 2–4 tandem copies of EREc38 also generated stabilized ERα tryptic digestion bands characteristic of each ligand, i.e. 28 kDa for E₂ and 25 kDa for 4-OHT (Kraichely *et al.* 2000). Comparison of the T4 bands of E₂- and 4-OHT-liganded 4 EREc38 in Fig. 6 clearly demonstrates this difference in the lowest visible MM band. These bands did not appear, at least at the trypsin concentrations used in this study, when ERα was incubated without DNA, with NOTERE, with pS2, or with 1 copy of EREc38 (Figs 1 and 3). Therefore, the appearance of the smallest ERα fragments, at the trypsin concentrations used here,

is dependent upon ERα interaction with multiple tandem copies of EREc38. Also, Fig. 6 highlights differences in sensitivity of ERα bound with E₂ vs 4-OHT. Importantly, increased numbers of tandem copies of EREc38 increased the sensitivity of ERα to trypsin digestion, resulting in the appearance of the low MM stabilized LBD fragments at lower trypsin concentration than those in the presence of non-cognate DNA, the pS2 natural, NOTERE, or 1 EREc38 (Figs 1 and 3). For example, this can be seen by comparing lane 6 in Fig. 1C (E₂-ERα-1 EREc38) with lane 6 in Fig. 4A, C and E (E₂-ERα-2–4 EREc38). Furthermore, the

D. Trypsin digestion of 4-OHT-ER α -pS2

FIGURE 3. (D).

FIGURE 3 (opposite and above). ER α binding to the pS2 ERE induces increased sensitivity to trypsin digestion. Shown are Western blots of PVDF membranes probed with Ab-10 to ER α . ER α (12.5 nM final), liganded by E₂ (A) or 4-OHT (B), was incubated with pS2, following partial digestion with trypsin at the indicated concentrations. Conditions for electrophoresis and Western blotting are described in Materials and Methods and Fig. 1. The sizes of the ER α bands are: T1=57 kDa band, T2=45 kDa, T3=36 and 34 kDa bands combined. (C and D) Quantitation of the relative amounts of ER α in all bands resulting from trypsin digestion of E₂-ER α (C) or 4-OHT-ER α (D) bound to pS2 ($n=3$ experiments each). Quantitation was performed as described in Materials and Methods and Fig. 2. Values are means $\pm$ S.E.M of three experiments.

ER α -trypsin digestion patterns generated by the multiple EREc38 repeats resemble those produced at significantly higher trypsin concentrations and in the absence of ERE binding as reported recently (Kraichely *et al.* 2000). These results indicate that ER α interaction with multiple tandem copies of EREc38 alters ER α conformation in a way that increases ER α sensitivity to trypsin digestion.

Correlation of EC₅₀ of trypsin digestion of intact ER α , K_d of ER α for ERE sequences, and fold-induction of ERE-driven luciferase reporters in response to E₂

We investigated whether there was a correlation between the EC₅₀ values for trypsin digestion determined here with the values for ER α -EREc38 binding affinity (K_d) and fold-induction of reporter gene activity of the same ER α -EREc38 combinations, determined previously (Tyulmenkov *et al.* 2000). The r^2 -value for the correlation between EC₅₀ trypsin concentration and K_d for E₂-ER α

interaction with 1, 2, 3 and 4 EREc38 is 0.5 (Fig. 7A). For the EC₅₀-E₂-induced reporter gene expression correlation, the r^2 -value is 0.82 (Fig. 7B). Therefore, there appears to be a stronger correlation between sensitivity to trypsin digestion and fold-induction of reporter gene expression for E₂-ER α interaction with 1, 2, 3 and 4 EREc38 than with K_d values.

The EC₅₀ value for 4-OHT-liganded ER α incubated with NOTERE indicates reduced susceptibility to trypsin digestion as compared to E₂-ER α incubated with NOTERE. The EC₅₀ values for trypsin digestion for 4-OHT-ER α incubated with 1, 2, 3 and 4 EREc38, however, were unexpectedly similar to those of E₂-ER α bound to the same EREs. As the number of tandem repeats of EREc38 increased, the EC₅₀ value decreased (Fig. 7C). This result was not expected given the lack of cooperativity of binding of 4-OHT-ER α to three or more tandem repeats of EREc38 (Klinge *et al.* 1992b, 1997b) and with 4-OHT's antagonist activity with ER α in MCF-7 and CHO-K1 cells (Klinge

FIGURE 4. ERα binding to multiple tandem copies of EREc38 further enhances trypsin proteolysis. Shown are Western blots of PVDF membranes probed with Ab-10 to ERα. ERα (12.5 nM final), liganded by E₂ (A, C and E) or 4-OHT (B, D and F), and incubated with two (A and B), three (C and D) or four (E and F) head-to-tail, tandem copies of EREc38, followed by partial digestion with the indicated trypsin concentrations. Western blotting and electrophoresis were performed as detailed in Materials and Methods and Fig. 1. Sizes of protein markers are indicated in kDa. The sizes of the ERα bands are: T1=57 kDa, T2=45 kDa, T3=36 and 34 kDa bands combined, and T4=28 kDa (A, C and E) or 25 kDa (B, D and F).

et al. 1997b, 1999, 2000, Tyulmenkov *et al.* 2000). While 4-OHT-ERα obviously exists in a different conformational state compared to E₂-ERα (Brzozowski *et al.* 1997, Shiau *et al.* 1998) and when bound to different EREs, at present, the exact

mechanism accounting for the similarity in trypsin digestion patterns is uncertain. We postulate that *in vivo*, or in transfected cells, ERα interaction with coactivators and other coregulatory proteins induces further alterations in ERα conformation.

DISCUSSION

Lefstin & Yamamoto (1998) proposed that response elements recognized by nuclear transcription factors, including members of the steroid/nuclear receptor superfamily, contain information that is interpreted by bound regulator factors. Based on Lefstin and Yamamoto's model, we postulated that DNA acts as an allosteric ligand whose binding alters ER's affinity for other ligands (Klinge *et al.* 1992b, 1996a, 1998, 2001, Sathya *et al.* 1997, Tyulmenkov *et al.* 2000). Other investigators have also proposed that DNA is an allosteric modulator of ER α activity (Wood *et al.* 1998). The present study is the first to address the hypothesis that the synergism of transcriptional activation and the cooperativity of ER α binding to multiple tandem EREs result, at least in part, from allosteric, ERE-mediated changes in ER α conformation. Previous studies with glucocorticoid receptor (GR) showed that transcriptional synergism requires an intact DBD and LBD (Ingiguez-Lluhi *et al.* 1999), and our results with ER α showing that E₂, but not 4-OHT, synergistically activates reporter gene expression also implicate the ER α LBD in transcriptional synergism (Tyulmenkov *et al.* 2000). Our data showing that ER α binding cooperativity correlates with transcriptional synergism implicate the DBD in these events as well (Tyulmenkov *et al.* 2000).

Here we showed that ERE binding makes ER α more sensitive to trypsin digestion and that increasing the number of tandem copies of EREc38 resulted in decreased EC₅₀ values for trypsin digestion, indicating that binding of ER α to multiple, tandem copies of EREc38 increases access of trypsin to digestion sites within EREc38-bound ER α . We conclude that ERE-bound ER α is in a different conformation than ER α in solution or when interacting with a non-ERE containing section of the genome with low affinity. Further, we demonstrated that there is a correlation between the sensitivity of ER α to trypsin digestion and cooperativity of ER α -EREc38 binding and transcriptional activation. These results also support our recent report on the increased sensitivity of ERE-bound ER α to α -chymotrypsin digestion. In that set of experiments, we found both E₂- and 4-OHT-specific and ERE sequence-specific differences in ER α sensitivity to chymotrypsin digestion in the presence of EREc38 and natural EREs from estrogen target genes: pS2, progesterone receptor, and c-fos (Klinge *et al.* 2001). The ERE-mediated increase in ER α sensitivity to chymotrypsin digestion correlated with E₂-stimulated transcriptional activity from the same

EREs in transiently transfected CHO-K1 cells (Klinge *et al.* 2001). Transcriptional activity also correlates with the affinity of ER α -ERE binding *in vitro* (Klinge *et al.* 2001).

Trypsin cleaves peptides C-terminal to lysine and arginine residues (Voet & Voet 1990). Computer analysis of the ER α sequence revealed 57 possible trypsin digestion sites (Fig. 8). Because the recognition region for ER α monoclonal antibody is amino acids (aa) 302–553, we compared the size of possible tryptic ER α fragments containing the epitope for ER α antibody Ab-10 to those obtained experimentally (Figs 1, 3 and 4). The band of 57 kDa detected corresponds to ER α aa 32–555 (MM=57.5 kDa); the band of 45 kDa detected corresponds to ER α aa 142–555 (MM=45.3 kDa); the band of 34–36 kDa corresponds to ER α aa 245–555 (MM=34.1 kDa) and aa 231–555 (MM=35.6 kDa); the band of 28 kDa detected corresponds to ER α aa 303–555 (MM=27.7 kDa); and the band of 25 kDa detected corresponds to ER α aa 303–531 (MM=25.1 kDa). These data correlate with the stability of the LBD and the relative proteolytic sensitivity of the N-terminus of ER α (Seielstad *et al.* 1995).

Although the domains of steroid and nuclear receptors function independently (Webster *et al.* 1988), there are few quantitative analyses describing how interactions between domains within an intact receptor modulate receptor activity, i.e. for ER (Fritsch *et al.* 1992b, Kraus *et al.* 1995); for androgen receptor (Zhou *et al.* 1995, Karvonen *et al.* 1997); for retinoid X receptor (RXR) (Kurokawa *et al.* 1994); for GR (Segard-Maurel *et al.* 1996). Such experiments are important to define the role of ligand when a nuclear receptor is bound to DNA *in vivo*.

Partially purified, yeast-expressed ER α showed differential recognition by select ER α antibodies when bound to a perfect ERE from the *Xenopus laevis* vitellogenin A1 gene vs an imperfect ERE from the human pS2 gene, indicating that receptor epitopes were differentially exposed in response to these different ERE sequences (Wood *et al.* 1998). The authors interpreted these data to indicate that interaction of the ER α DBD with the vitellogenin A2 and pS2 EREs brings about global changes in ER α conformation. Together, this work, reports on thyroid receptor (TR) (Olson *et al.* 1997, 1998), and our recent analysis of ER α interaction with single copies of natural and synthetic EREs (Klinge *et al.* 2001) indicate that DNA architecture is important in determining receptor binding and ligand-activated transcription.

The effect of DNA binding on the protease sensitivity of other members of the steroid/nuclear

FIGURE 5. (A, B and C, legend opposite).

FIGURE 5. (D, E and F).

FIGURE 5 (opposite and above). Plot of densitometric scans of ER α bound to two to four tandem copies of EREc38 following trypsin digestion. (A–F) Quantitation of the data from the Western blots shown in panels A–F of Fig. 4. The relative amounts of ER α in all bands resulting from trypsin digestion of E₂-ER α (A, C and E) or 4-OHT-ER α (B, D and F) were analyzed by densitometric scanning as described in Materials and Methods and Fig. 2. The bars display the means $\pm$ s.e.m. of several experiments ($n=3-5$). T1=57 kDa band, T2=45 kDa, T3=36 and 34 kDa bands combined, and T4=28 kDa (A, C and E) or 25 kDa (B, D and F) bands.

4-ERec38 with E₂ or 4-OHT

FIGURE 6. Comparison of the trypsin digestion products of E₂- vs 4-OHT-occupied ERα incubated with 4 EREc38. Shown is a Western blot of a PVDF membrane probed with Ab-10 to ERα. ERα (12.5 nM final), liganded with E₂ (lanes 1–3) or 4-OHT (lanes 4–6), and incubated with four head-to-tail, tandem copies of EREc38, followed by partial digestion with the indicated trypsin concentrations. Western blotting and electrophoresis were performed as detailed in Materials and Methods and Fig. 1. Sizes of protein markers are indicated in kDa. The sizes of the ERα bands are: T3=36 and 34 kDa bands combined, and T4=28 kDa (lanes 2–3) or 25 kDa (lanes 5–6). This experiment was repeated twice with similar results.

receptor superfamily has been reported. One study reported that binding of the triiodothyronine (T₃)-activated TR/RXR heterodimer to transcriptionally active direct repeats of the ERE half-site, i.e. 5'-AGGTCA-3' with four nucleotides separating the half-sites, i.e. DR4, induced protease-resistance of TR, but not RXR (Ikeda *et al.* 1996). This result corresponds with the observation that although the T₃-TR/RXR heterodimer bound with similar affinity to a consensus or mutated DR4 oligomers in EMSA, transcription was not stimulated by T₃ from mutant DR4s. In contrast, the vitamin D receptor was more sensitive to trypsin digestion when bound to a DR3 vs a non-consensus vitamin D response element (Towers & Freedman 1998). We hypothesize that if these results are consistent for other members of the steroid/nuclear receptor superfamily, the specific nucleotide sequence of a receptor response element may modify ligand-induced transcription by altering receptor conformation.

Limited trypsin digestion of nuclei isolated from MCF-7 human breast cancer cells treated with 4-OHT or E₂ revealed [³H]4-OHT-ERα as a 67 kDa and [³H]E₂-ERα as a 60 kDa protein respectively (Hutchens *et al.* 1987). This report indicated that 4-OHT better protected ERα from

FIGURE 7. Relationship of EC₅₀ for trypsin digestion with the affinity of ER-ERE interaction, E₂-induced transcription and number of tandem EREs. (A) Correlation between K_d for E₂-ERα binding to one to four tandem copies of EREc38 and the EC₅₀ for trypsin digestion of intact E₂-ERα bound to the same EREs. (B) Correlation between the fold-induction of reporter gene expression from one to four tandem copies of EREc38 in transiently transfected CHO-K1 cells treated with 10 nM E₂ and the EC₅₀ for trypsin digestion of intact E₂-ERα bound to the same EREs. Linear regression equations are given within panels. (C) Relationship of EC₅₀ for trypsin digestion of intact E₂-ERα or 4-OHT-ERα (□, solid line and ●, dotted line respectively) bound to one to four tandem copies of EREc38 and the number of EREs. Lines were plotted in Microsoft Excel 97.

FIGURE 8. Potential trypsin cleavage sites of ER α . Shown is the linear-domain structure of ER α . The potential trypsin digestion sites, indicated by the lines and aa number at which digestion may occur, and the anticipated peptide mass of the fragments of the human ER α sequence recognized by Ab-10 (double line below the LBD) were analyzed using the ExPASy proteomics server as described in Materials and Methods. Anticipated trypsin digestion products recognized by Ab-10 are indicated with the corresponding anticipated MM in kDa. Where no definitive data exist regarding the exact cleavage sites utilized, the aa numbers represent estimated cleavage points based on size of digestion products.

trypsin digestion than E₂. E₂ vs antiestrogen-specific differences in the sizes of proteolytic fragments after chymotrypsin digestion of baculovirus-expressed rhER α were also reported (Beekman *et al.* 1993). Here we report that when ER α was bound to multiple tandem copies of EREc38, 4-OHT increased the sensitivity of ER α to trypsin. On the other hand, when ER α was incubated with non-specific DNA, 4-OHT displayed the 'classical' protective effect (Hutchens *et al.* 1987). Lastly, when ER α was incubated with 1 EREc38 or pS2, 4-OHT effects were neutral relative to E₂, providing further evidence of the allosteric effect DNA sequence on ER conformation. Other investigators (Brown & Sharp 1990, Emmas *et al.* 1992, Anderson *et al.* 1998) and we have reported that E₂-ER α and antiestrogen-liganded ER α show different mobilities when bound to an ERE in EMSA (Klinge *et al.* 1996a), indicating ligand-specific differences in ER α con-

formation when bound to an ERE. However, not all studies detected conformational differences in E₂-ER α vs antiestrogen-liganded ER α . For example, both E₂ and 4-OHT induced similar decreases in surface hydrophobicity of ER α as detected by PEG-palmitate affinity partitioning (Fritsch *et al.* 1992a).

The collected observations indicating ligand-specific changes in receptor conformation within the LBD are substantiated by the elucidation of the LBD crystal structure of ER α (Brzozowski *et al.* 1997, Shiau *et al.* 1998, Pike *et al.* 1999). These studies indicate that, when liganded by E₂, the LBD is folded into a compact structure in which the ligand is completely buried within a hydrophobic pocket. In contrast, when liganded by the antiestrogen RAL, the extended side chain of RAL protrudes from the pocket between helices 3 and 11, altering the conformation of the LBD (Brzozowski *et al.* 1997). It is generally agreed that the compact

structure of ligand-bound LBD makes it more resistant to protease digestion. Ligand-specific conformational changes are thought to facilitate the dissociation of the protein components associated with non-activated ER, e.g. hsp90, hsp70, p56 and other proteins (Klinge *et al.* 1997a), and to unmask the DBD. Recent guanidine hydrochloride denaturation studies using ER α LBD show that the LBD undergoes a two-phase unfolding process and that occupation of the ER α LBD by E₂ protects against denaturation (Gee *et al.* 2001). These data suggest a role for E₂ in stabilizing the overall conformation of the ER α LBD (Gee *et al.* 2001). We suggest that, subsequent to ERE binding, further conformational changes critical for interaction with coactivators and with components of the transcriptional activation complex (Klinge 2000) occur within the ER α LBD in a ligand-specific manner. Alterations in ER α conformation may also be propagated from the LBD to the ER α N-terminus (Kraus *et al.* 1995).

In summary, the observations reported here indicate that DNA allosterically modulates the conformation of the LBD of ER α , resulting in enhanced susceptibility to proteolysis by trypsin. Binding of ER α to the imperfect ERE palindrome from the pS2 gene or to one or more tandem copies of EREc38 altered ER α conformation as detected by a decrease in the EC₅₀ for trypsin digestion. In contrast to our expectation that binding of E₂-ER α to three or four copies of EREc38 would result in a dramatic increase sensitivity to trypsin, a hypothesis based on our earlier observations of (i) cooperative E₂-ER α binding to three or four tandem copies of an ERE (Klinge *et al.* 1992, Tyulmenkov *et al.* 2000) and (ii) synergistic E₂-dependent activation of reporter expression from three and four, but not one or two, tandem copies of EREc38, as evidenced by a decrease of EC₅₀ (Sathya *et al.* 1997, Tyulmenkov *et al.* 2000), we detected a linear decrease in EC₅₀ with one to three tandem copies of EREc38. Interestingly, the EC₅₀ value for four tandem copies of EREc38 strayed from linearity (Fig. 6C). Thus, while the trypsin EC₅₀ values did not demonstrate the sharp demarcation between two and three copies of EREc38 that the binding affinity and fold-induction of reporter activity in transient transfections did (Tyulmenkov *et al.* 2000), increasing copy number of EREc38 substantially increased susceptibility to trypsin digestion. Despite this lack of sharp change in EC₅₀ between two and three tandem copies of EREc38, when we examined the fold-induction, EC₅₀ and K_d values for each of the ERE-ER α pairs statistically, we detected a strong correlation between EC₅₀ and fold-induction ($r=0.82$, $P<0.05$). Additionally, comparing the

EC₅₀ values of E₂-occupied ER α for 1 EREc38 or 4 EREc38 (0.81 vs 0.35) establishes the presence of a conformational difference in the EREc38-bound ER α . Together these observations suggest that specific alterations in ER α conformation result in cooperative binding of E₂-ER α to multiple EREs and transcriptional synergy. We also suggest that recruitment of coactivators also plays a role in transcriptional synergy. Our results support the hypothesis that the ERE sequence acts as an allosteric effector, altering ER conformation.

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