Modulation of estrogen receptor α function and stability by tamoxifen and a critical amino acid (D538) in helix 12*

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Running title: helix 12 and 4-OHT modulate ER levels and activity

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**SUMMARY**

Estrogen receptor α (ER) is a ligand-activated transcription factor implicated in breast cancer growth. Selective estrogen receptor modulators (SERMs), such as tamoxifen (4-OHT), bind to the ER and affect the position of helix 12, thereby influencing coregulator binding and ER transcriptional activation. Previous studies have shown that a triple mutation in helix 12 (3m; D538A, E542A and D545A) caused a change in ER stability and obliterated 4-OHT action (Liu, et al. Cancer Res 61:3632-3639, 2001). Two approaches were taken to determine the role of individual mutants (D538A, L540Q, E542A and D545A) on the activity and stability of the 4-OHT:ER complex. Firstly, mutants were evaluated using transient transfection into ER negative T47D:C4:2 cells with an ERE3-luciferase reporter and secondly, transforming growth factor alpha (TGFα) mRNA was used as a gene target *in situ* for stable transfectants of MDA-MB-231 cells. Transcriptional activity occurred in the presence of estrogen in all of the mutants, although a decreased response was observed in the L540Q, 3m and D538A cells. The 3m and D538A mutants lacked any estrogenic responsiveness to 4-OHT, whereas the other mutations retained estrogen-like activity with 4-OHT. Unlike the other mutants, the ER was degraded in the D538A mutant with 4-OHT treatment. However, increasing the protein levels of the mutant with the proteasome inhibitor MG132 did not restore the ability of 4-OHT to induce TGFα mRNA. We suggest that D538 is a critical amino acid in helix 12 that not only reduces the estrogen-like actions of 4-OHT, but also facilitates the degradation of the 4-OHT:D538A complex. These data further illustrate the complex role of specific surface amino acids in the modulation of the concentration and the estrogenicity of the 4-OHT:ER complex.
INTRODUCTION

Estrogen receptor $\alpha$ (ER)$^1$ is a member of the steroid hormone superfamily of nuclear receptors, which are gene regulatory transcription factors. Similar structural domains, designated A-F, are shared between the nuclear receptors [for review, see (1,2)]. Two transcriptional activation functions, activation function 1 (AF1) and activation function 2 (AF2), are present in the ER (Fig. 1). AF1 is a constitutive activation function located in the A/B region and AF2 is a ligand-dependent activation function in the E region or ligand binding domain (LBD). The activity of AF1 and AF2 is largely mediated by the cell and promoter context (3,4) and can be independent or synergistic (5).

The ER is an important therapeutic target for the treatment and prevention of breast cancer. Selective estrogen receptor modulators (SERMs) are compounds that bind to the ER and exert tissue-specific effects. Tamoxifen was the first SERM approved clinically for the treatment and prevention of breast cancer. Tamoxifen acts as an antiestrogen in the breast, but has estrogenic properties in that it maintains bone density (6), lowers circulating cholesterol (7) and causes an increased risk of endometrial cancer in women over 50 (8). Raloxifene is a chemically related SERM that is used for the prevention of osteoporosis but also lowers cholesterol and reduces the risk of both breast cancer and endometrial cancer (9). ICI 182,780 is considered to be a pure antiestrogen in that it displays no agonist activity at the ER (10). This occurs because ICI 182,780 interferes with receptor dimerization (11) and increases ER protein turnover (12).

Analysis of the crystal structure of the ligand:ER complex has been instrumental in understanding ER conformation at the molecular level and highlights the importance of
helix 12 in modulating estrogenic and antiestrogenic actions. Helix 12 is located in the LBD of the ER, but the composition and orientation of helix 12 differs depending on the ligand bound to the ER (13). When the ER LBD is complexed with the ER agonists estrogen (E2) or diethylstilbestrol (DES), helix 12 is positioned over the ligand binding pocket (Fig. 2A)(13,14). This proper positioning generates AF2 and forms a surface for the recruitment of coactivators. However, when 4-hydroxytamoxifen (4-OHT, the active metabolite of tamoxifen) or raloxifene is bound to the ER LBD, the antiestrogenic side chain displaces helix 12 from its normal position, thereby preventing the formation of a functional AF2 (Fig. 2B)(13,14). Having excluded AF2, the reported partial agonist activity of 4-OHT can only be mediated by AF1 (15). In a previous study, a binding site responsible for the estrogen-like action of 4-OHT was defined that is referred to as AF2b (16). This site contains two critical components: D351 and a portion of helix 12 (D538, E542 and D545). AF2b is proposed to be a docking site for coactivators or corepressors that modulate the estrogenicity of the 4-OHT or raloxifene ER complex (17-19). Therefore, different ligands induce different receptor conformations, and the positioning of helix 12 is the key event that permits discrimination between ER agonists and antagonists by influencing the interaction of the ER with coregulators.

The estrogenic or antiestrogenic action of ligands at the ER depends on the subtle changes in ER shape that programs the ER to form an active or inactive transcription complex or to be degraded by the proteasome. The amount of available ER in the cell is controlled by a balance between synthesis and degradation. ER stability is influenced by the nature of the bound ligand such that ligand-induced conformational changes modulate the ability of the ER to interact with proteins involved in the degradation process (20).
The transcriptional activity of the resulting ER pool is also influenced by the ligands present. The ER is activated if the ligand is estrogenic, and the established estrogens can be classified as class I or class II (21). Class I estrogens, such as DES or E2, are planar compounds that use the AF2 site for optimal action. Class II estrogens, represented by angular triphenylethylene compounds such as 4-OHT and fixed ring 4-hydroxy triphenyl pentene (4OHTPP), utilize AF2b for activity. However, ligands such as SERMs or pure antiestrogens can block the activity of the ER by creating a ligand:ER complex that is inactive. Overall, the complex decision making network depends upon the protein recognition sequences exposed on the external surface of the relevant SERM-ER complex in response to ligand binding.

Analysis of the helix 12 region of AF2b using the 3m mutation (D538A, E542A and D545A) yielded important insight into mechanism of 4-OHT agonism. The transforming growth factor alpha (TGFα) gene is recognized as a target of estrogen action and is involved in cell growth stimulation by estrogen (22,23) so the biological activity of the 4-OHT:ER complex can be assessed using Northern blotting for TGFα mRNA. Expression of TGFα mRNA is normally induced by E2 and 4-OHT treatment in MDA-MB-231 human breast cancer cells stably transfected with the wild type ER (S30 cells) (24). The 3m mutation resulted in a decreased induction of TGFα in response to E2 and no response to 4-OHT (18). Therefore, the 3m mutation abolished the agonist activity of 4-OHT and decreased the agonist activity of E2. In addition, a slight degradation of the ER was observed when the 3m mutant stable cell line was treated with E2, 4-OHT and ICI (18). This is in contrast to stable cell lines containing the wild type ER, which displayed a large downregulation of the ER in the presence of E2 and ICI, but an increase
in ER protein with 4-OHT treatment. Although the effect of E$_2$ on the 3m mutation and the three individual amino acids comprising the 3m mutation has been studied using ERE-luciferase assays (4,25-27), the majority of the studies were not performed in breast cancer cell lines and in a comprehensive manner. In addition, the precise interaction between 4-OHT and the individual mutations is not known.

Amino acid L540 is a nearby amino acid of interest on the underside of helix 12 when it is sealing estrogen in the hydrophilic pocket of the LBD. The L540Q mutation was initially generated by random chemical mutagenesis and is a dominant negative ER mutant (28-31). Previous studies in MDA-MB-231 breast cancer cells have shown that an ERE-CAT reporter is activated by 4-OHT and ICI 164,384, but not by E$_2$, in the presence of the L540Q mutant (27). Therefore, the L540Q mutation reverses the pharmacology of E$_2$ and ICI 182,780 that is normally observed at the wild type ER in MDA-MB-231 cells.

We have stably transfected individual mutant ER cDNAs into MDA-MB-231 human breast cancer cells to create an in vitro model to address the contribution of specific amino acids in helix 12 (D538A, E542A, D545A, and L540Q) to the agonist activity of 4-OHT at the AF2b site. We have found that D538 is the critical amino acid in helix 12 that not only reduces the estrogen-like actions of 4-OHT, but also enhances the degradation of the ER upon 4-OHT treatment.
EXPERIMENTAL PROCEDURES

Cell culture and reagents---Stable cell lines were maintained in phenol red-free MEM media supplemented with 5% calf serum treated 3x with dextran-coated charcoal, 0.5 mg/ml G418 (Geneticin, Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin and 6 μg/ml insulin. This media is referred to as stripped media, indicating that it is free of E2.

S30 cells are MDA-MB-231 human breast cancer cells stably transfected with wild type ERα (24) and are referred to as wild type cells. These cells are grown in stripped media. T47D:C4:2 cells are ERα negative human breast cancer cells (32) that were propagated in phenol red free RPMI media containing 10% fetal serum calf serum treated 3x with dextran-coated charcoal, as well as the concentrations of amino acids, penicillin, streptomycin, and insulin described above. ER- represents a G418-resistant clone that is ER-negative. The 3m stable cell line (18) containing the triple mutation (D538A, E542A, D545A) was also grown in stripped media.

4-OHT and E2 were purchased from Sigma (St. Louis, MO). ICI 182,780 was obtained from AstraZeneca (Macclesfield, England). Raloxifene was a generous gift from Eli Lilly and Company (Indianapolis, IN). All drugs were dissolved in ethanol and stored at –20°C. MG132 was dissolved in DMSO and obtained from Calbiochem (San Diego, CA).

Mutagenesis---Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit according to the manufacturer’s instructions (Stratagene, LaJolla, CA). The ERαPSG5 plasmid (HEGO, kindly provided by P. Chambon) was
used as a template for PCR. Primers used were as follows: D538A (5’GCA TCT CCA GCA GCA GGG CAT AGA GGG GCA CCA CG3’ and 5’CGT GGT GCC CCT CTA TGC CCT GCT GCT GGA GAT GC3’), L540Q (5’GGC GTC CAG CAT CTC CAG CTG CAG GTC ATA GAG GGG3’and 5’CCC CTC TAT GAC CTG CAG CTG GAG ATG CTG GAC GCC3’), E542A (5’GGG CGT CCA GCA TCG CCA GCA GCA GGT C3’and 5’GAC CTG CTG CTG GCG ATG CTG GAC GCC C3’) and D545A (5’GTA GGC GGT GGG CGG CCA GCA TCT CCA GC3’ and 5’GCT GGA GAT GCT GGC CGC CCA CCC CCT AC3’). Miniprep DNA was isolated from the resulting bacterial colonies using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The miniprep DNA was sequenced for the presence of the mutation. A larger scale DNA preparation was made from a chosen mutant using the Qiagen Maxiprep Kit and the entire ER DNA was sequenced. Each mutant was then cloned into the pIRESneo2 plasmid (Clontech, Palo Alto, CA) using the EcoRI site flanking the ER cDNA.

**Generation of stable transfectants**---MDA-MB-231 (clone 10A) cells (24) were grown in stripped media for 3-4 days prior to transfection. The cells were transfected with 10μg of the ER mutant in the pIRESneo2 plasmid. 5x10^6 cells were electroporated in a 0.4cm cuvette (Bio-Rad, Hercules, CA) at a voltage of 0.250 kV and a high capacitance of 0.95 μF in phenol red-free MEM media with no additives. The cells were transferred to a 10cm plate and incubated overnight in 10 ml of stripped media without G418, and the media was changed the next day. The following day, media containing 0.5 mg/ml of G418 was added and the cells were subsequently maintained in this media.
Individual colonies appeared approximately 1 month after transfection, and these were isolated and screened for stable expression of the ER by Western blotting.

**Transient transfections and luciferase assays**—The cells were transfected with 1 μg of the ERE3-luciferase plasmid (33) and 1 μg of the mutant or wild type ERαPSG5 plasmid. To normalize for transfection efficiency, 0.2 μg of the PCMVβ plasmid (Clontech, Palo Alto, CA) were also transfected. 5x10^6 cells were electroporated in a 0.4 cm cuvette (Bio-Rad, Hercules, CA) at a voltage of 0.320 kV and a high capacitance of 0.95 μF in serum-free media. The cells were transferred to 12 well plates and incubated overnight. The next day, the cells were treated with the appropriate compound for 24 hours.

The cells were washed once with cold 1x PBS and 100 μl of extraction buffer (0.1 M potassium phosphate pH 7.5, 1% TritonX-100, 100 μg/ml BSA, 2.5 mM PMSF, and 1 mM DTT) were added to each well. The cells were incubated on ice for 2 min, dislodged from the plates, and transferred to an eppendorf tube. The lysate was centrifuged for 2 min at top speed in a microfuge and the supernatant was used for the assay. 50 μl of the lysate was mixed with 350 μl of reaction buffer (160 mM MgCl₂, 75 mM glycylglycine pH 7.8, 0.5 mg/ml BSA, 19 mg/ml ATP, and 15 mM Tris-HCl pH 7.5) and 100 μl of luciferin (0.4 mg/ml). Luminescence was measured in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) for 10 sec. β-galactosidase activity was measured using 10 μl of each sample and the Galacto-Light Plus detection system (Applied Biosystems, Bedford, MA). Data is reported as relative light units (RLU), which is the luciferase reading divided by the β-gal reading.
Northern blots---Stable transfectants were treated with compounds for 24 hours. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. 20 μg of RNA were loaded per lane in a 1% agarose/0.66M formaldehyde gel. The RNA was transferred to a MagnaGraph nylon transfer membrane (Osmonics, Minnetonka, MN) overnight in 10x SSPE buffer (20x SSPE is 3.6 M NaCl/0.2 M NaH₂PO₄/0.02 M EDTA, pH 7.4). The RNA was fixed to the membrane by UV-crosslinking. The membrane was prehybridized in hybridization solution (0.5 M sodium phosphate, 10 mM EDTA, 1% BSA, 7% SDS, pH 7.2) for a minimum of 2 hours at 60°C. The TGFα probe (a gift from Dr. R. Derynck, Genentech, South San Francisco, CA) or the ERα probe (the EcoRI fragment from the ERαPSG5 plasmid) was labeled with ³²P dCTP using the Megaprime DNA labeling system (Amersham Biosciences, Piscataway, NJ) and the labeled probe was separated from free ³²P using Microspin columns (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions. The probe was heated at 95°C for 5 min, added to the hybridization buffer and incubated at 60°C overnight. The next day, the membrane was washed for 30 min at 60°C with 1x SSPE/0.1% SDS, 30 min with 0.5x SSPE/0.05% SDS, and 2x15 min with 0.1x SSPE/0.1% SDS. To visualize TGFα, the membrane was exposed to film overnight. Equal loading of samples was verified by stripping the membrane and reprobing with β-actin.

Protein isolation and western blots---Cells were treated for 24 hours with compound. To harvest protein, cells were washed once with PBS, scraped using a cell
scatter into 10 ml of PBS, and transferred to a 15 ml conical tube. The cells were pelleted and the supernatant was aspirated. The cell pellet was resuspended in 100μl of extraction buffer [50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.5% NP-40, 10 mM β-glycerophosphate, pH 8 containing a 1:100 dilution of a freshly added protease inhibitor cocktail (Sigma P8340, St. Louis, MO)], passed through a 22G1½ needle, and incubated on ice for 30 min. The cell lysate was centrifuged at 10,000xg for 10 min at 4°C and the supernatant was transferred to a new tube. Samples were quantitated using the Bio-Rad protein assay kit.

20 μg of cell lysate were separated on a 7.5% SDS-PAGE gel and transferred to nitrocellulose. The blot was blocked in blotto (2.5% dry milk/0.05% tween/0.5x PBS) for 1 hour to overnight. Blots were probed with polyclonal ERα antibodies at 1:200 (G20, Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal β-actin antibodies at 1:20,000 (Sigma A5441, St. Louis, MO) for 1 hour at room temperature. The membrane was then washed 3x5 min with wash buffer (0.5x PBS/0.05% tween). The blot was incubated in a 1:3000 dilution of HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. The membrane was washed 3x5 min with wash buffer and bands were visualized using chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ).

**Quantitation and Statistics**---Western and Northern blots were quantitated using the gel plot feature in Scion Image version 4.0.2. The results were statistically analyzed using SPSS 9.0.
RESULTS

Previous results in our laboratory showed that a triple mutation in helix 12 of the ER (3m; D538A, E542A and D545A) caused a change in ER stability and eliminated 4-OHT agonist activity (18). To analyze the role of individual amino acids in helix 12, multiple mutations were generated. These mutations include single point mutations of the 3m mutation (D538A, E542A, D545A) and the L540Q mutation (Fig. 1).

The transcriptional activity of the ER mutants was first tested by transient transfection of ER negative T47D:C4:2 cells with the mutant ER cDNA and an ERE3-luciferase reporter (33). Transfection with the empty vector PSG5 showed no induction of luciferase activity with any of the treatments used (Fig. 3). In addition, none of the mutants exhibited any response to treatment with the vehicle control ethanol (EtOH). E2 treatment resulted in a statistically significant induction of luciferase activity with the wild type ER and all of the mutants when compared to the EtOH control. The greatest induction was observed with the E542A mutant. The wild type, D538A, D545A and 3m mutants displayed an intermediate level and the L540Q mutant displayed the smallest induction. The L540Q mutant was the only mutant that showed a slight induction of luciferase activity during ICI 182,780 treatment, but this was not statistically significant. In addition, the wild type, E542A and D545A ERs displayed an induction of luciferase activity upon 4-OHT treatment, whereas the D538A, L540Q and 3m mutants did not. When wild type, 3m and D538A cells were treated with E2 plus 4-OHT, the response of the cells was the same as that observed with 4-OHT alone, indicating that 4-OHT acts as a complete antiestrogen in these cells (data not shown). Therefore, of the three mutations...
present in the 3m mutant, the D538A mutation is responsible for the elimination of the agonist activity of 4-OHT at an ERE in T47D:C4:2 cells.

To further evaluate these mutants in a reproducible manner, stable transfectants were generated in ER negative MDA-MB-231 cells. At least 5 clones were obtained representing each mutation and the clones were screened for the presence of the ER using Western blotting. Two clones harboring each mutation were initially screened using Northern blotting for TGFα mRNA levels. Both of the clones studied showed similar TGFα levels in response to various treatments, so a single representative clone was chosen for further analysis. Each of the stable clones was screened to ensure that the proper mutation was present using RT-PCR and sequencing.

ER protein levels were compared between each of the stable cell lines (Fig. 4). All of the cell lines contained similar levels of ER protein, so the characteristics observed in each cell line were not a result of varying ER levels. A clone that was stably transfected but ER negative by Western blot was used as a control and designated ER-.

The transcriptional activity of the ER mutants was also analyzed using Northern blot analysis of TGFα mRNA. The advantage of this assay is that the TGFα gene is an endogenous gene in MDA-MB-231 cells, and induction of TGFα mRNA levels reflects a process that is inherent to these cells. Cells from the ER- clone were treated with EtOH, E2, 4-OHT and E2 plus 4-OHT and no induction of TGFα mRNA was observed (data not shown). Wild type cells showed an induction of TGFα mRNA in response to E2 and 4-OHT, but 4-OHT did not act as an antiestrogen in these cells because it was not able to significantly block the E2 response (Fig. 5). A similar pattern of mRNA expression was observed in the E542A and D545A mutants. Although the 3m and D538A mutants
showed an increase in TGFα mRNA in response to E2 treatment, the level of induction was less than that observed for the other mutants. In addition, no induction occurred with 4-OHT treatment. This is in agreement with ERE-luciferase assay results and suggests that D538 is the single amino acid within the 3m mutation required for the agonist activity of 4-OHT at the ER. The L540Q mutant produced an induction of TGFα mRNA with ICI 182,780 and 4-OHT treatment, but not with E2 treatment. In addition, raloxifene (Ral) treatment had no effect on TGFα mRNA levels with any of the transfected stable cell lines (Fig. 5).

Since the expression of E2-induced TGFα mRNA was lower in the D538A and 3m stable transfectants compared with all other transfectants, E2 concentration response curves were completed to ensure that an E2 response was present (Fig. 6). Wild type cells displayed an induction of TGFα mRNA at the lowest concentration of E2 (10^{-10} \text{ M}) and the amount of TGFα mRNA continued to increase up to the highest concentration of E2 (10^{-6} \text{ M}). An EC_{50} value of 7 \times 10^{-10} \text{ M} was calculated for the wild type cells. A similar profile was observed in the D545A mutant, in that a 10^{-10} \text{ M} concentration of E2 induced TGFα mRNA, and the increase continued up to 10^{-6} \text{ M} E2. This is consistent with an EC_{50} of 2 \times 10^{-10} \text{ M}. The 3m and D538A mutants behaved similarly in that they first displayed an increase in TGFα mRNA at a 10^{-9} \text{ M} concentration of E2, and the induction continued with increasing concentrations of E2. Therefore, the 3m and D538A mutants required higher concentrations of E2 before TGFα mRNA was transcribed, when compared to the wild type and D545A cells. The EC_{50} of the 3m and D538A cells were 2 \times 10^{-9} \text{ M} and 4 \times 10^{-9} \text{ M}, respectively, which are approximately 10 times higher than the
wild type and D538A cells. In addition, TGFα mRNA levels in the D538A mutant were lower at each concentration of E2 tested in comparison to the other mutants.

In general, a SERM can negatively affect ER activity using two different mechanisms. First, the SERM could cause the degradation of the ER by creating a SERM:ER complex that is targeted for destruction. Second, a SERM:ER complex may not be degraded, but be present and have no intrinsic activity. To distinguish between these possibilities, wild type and mutant ER protein levels were analyzed in the presence of ICI 182,780, E2 and 4-OHT (Fig. 7A). ICI 182,780 degraded the wild type ER at all concentrations tested, and the D545A and D538A mutants were also degraded by ICI 182,780 (Fig. 7B). In contrast, the L540Q mutant exhibited no changes in ER protein levels at any ICI 182,780 concentration. The 3m and E542A mutants displayed intermediate ER levels after ICI 182,780 treatment.

When wild type cells were treated with E2, less ER protein was observed by Western blot (Fig. 7C). E2 treatment also resulted in a downregulation of ER levels in the D538A and D545A mutants, whereas ER levels were stable in the 3m, L540Q and E542A mutants. In the presence of 4-OHT, ER levels increased in the wild type cells and remained stable in the L540Q, E542A and D545A mutants. Importantly, 4-OHT treatment reduced ER protein levels in the D538A and 3m stables. The stable cell lines that showed an induction of ER transcriptional activity upon 4-OHT treatment (wild type, E542A and D545A) all contained ER protein levels that were maintained or increased when treated with 4-OHT. In contrast, the D538A and 3m mutants exhibited no transcriptional activity in response to 4-OHT, and they were the only mutants that showed a decrease in ER levels with 4-OHT treatment. This observation prompted us to
explore the possibility that the decreased transcriptional activity of the D538A mutant was a result of decreased ER protein levels in response to 4-OHT.

In the D538A mutant, the degradation of ER protein by 4-OHT could be a result of 4-OHT-induced transcriptional downregulation of the ER message, or 4-OHT-induced post-translational degradation. To distinguish between these possibilities, Northern blot analyses for ERα were performed in wild type and D538A cells that were treated with EtOH or 4-OHT (Fig. 7D). A comparison of ER mRNA and protein levels showed that 4-OHT upregulated both ER mRNA and protein in the wild type cells. In contrast, 4-OHT treatment did not change ER mRNA levels and decreased ER protein levels in the D538A cells by 70%. This indicated that the 4-OHT-mediated decrease in ER protein levels in the D538A mutant were not a result of decreased ER mRNA stability.

ER protein levels were measured in all of the stably transfected cell lines to establish whether the proteasome is involved in the degradation of the ER. The cells were treated with the proteasome inhibitor MG132 before the addition of ligand, and a Western blot was performed to analyze ER levels (Fig. 8). Pre-incubation with MG132 elevated the amount of ER protein present, indicating that the ligand-induced degradation of the ER could be mediated by the proteasome. However, subtle differences were observed, depending on the mutation and the ligand evaluated, especially in the case of ICI 182,780. MG132 was able to prevent the ICI 182,780-mediated degradation in D538A and E542A cells, but smaller increases were detected in wild type and D545A cells. It is possible that MG132 was not able to restore ER levels in the ICI 182,780 treated cells to control levels because a relatively high concentration of ICI 182,780 (10⁻⁶ M) was used. Experiments using lower concentrations of ICI 182,780 (10⁻⁶-10⁻⁸ M) in combination
with MG132 were performed in the wild type cells (data not shown), but MG132 treatment did not restore ER levels in the ICI 182,780 treated cells to control levels. In addition, MCF-7 cells were treated under the same conditions as described in Fig. 8, and the results were essentially the same (data not shown) in that MG132 treatment could not fully abrogate ICI 182,780 treatment. Therefore, MG132 was unable to restore ER protein to control levels in wild type, D545A and MCF-7 cells treated with ICI 182,780. This is in contrast to MG132 treatment in E2 treated cells, where the ER levels are greater than control cells. L540Q cells were not included in this experiment because the ER levels in these cells remained the same after treatment with E2, ICI 182,780 and 4-OHT (Fig. 7). These data indicate that the degradation of the ER in the stably transfected cell lines occurs through the proteasome.

When D538A cells were treated with MG132 and 4-OHT, ER protein levels were increased 2.3 fold, compared to the decreased levels of the receptor normally observed with 4-OHT treatment alone (Fig. 8). The D538A stable cells were treated with a range of MG132 concentrations and 4-OHT or E2 to determine whether preventing the degradation of the 4-OHT:D538A complex could restore 4-OHT agonist activity (Fig. 9). TGFα mRNA was induced with E2 treatment but no induction of TGFα mRNA levels was observed with MG132 treatment alone or MG132 in combination with 4-OHT. Therefore, increasing the amount of D538A ER that would normally be available in the presence of 4-OHT did not restore the agonist activity of 4-OHT. Therefore, adjusting the levels of the 4-OHT:D538A complex did not have an effect because the complex has no intrinsic activity.
DISCUSSION

The finding that D538 is an essential amino acid that modulates estrogen action with 4-OHT supports and extends the idea that helix 12 plays a vital role in the mechanics of estrogen action (13,14). D538 appears to be a central control mechanism for both the intrinsic activity of the 4-OHT:ER complex and the processing and degradation of the complex by the proteasome. These observations introduce a new dimension for consideration with SERMs as modulators of estrogen responsive genes.

Proteolysis is involved in the regulation of a variety of cellular functions such as cell cycle progression, oncogenesis, transcription, development, tissue growth, elimination of abnormal proteins, and antigen processing (34). Degradation of proteins can occur through three major pathways, which include mechanisms mediated through lysosomes, calpains (calcium-dependent cysteine proteases), and the proteasome. It has been shown previously that the ER is a ubiquitinated protein and that ubiquitination targets the protein to the proteasome, which causes ER degradation (35-37). Our data indicate that the proteasome is responsible for the degradation of ER protein observed in all of the stably transfected cell lines (Fig. 8).

The stability of the ER complex has been shown to be influenced by the bound ligand. E2 and ICI 182,780 decrease ER levels and 4-OHT increases the accumulation of the ER in MCF-7 human breast cancer cells (38) and pituitary lactotrope PR1 cells (20). A similar situation occurs when MDA-MB-231 cells are stably transfected with the wild type ER (Fig. 7). However, the typical pattern of ER stability is changed by mutation of residues in helix 12. For example, treatment of cells with E2 does not downregulate the ER in the 3m, L540Q and E542A mutants, whereas downregulation is observed in the
D538A and D545A mutants. ICI 182,780 degrades the ER in all of the stable cell lines except for the L540Q cells, and ICI 182,780 has a reduced affect in the 3m and E542A cells. 4-OHT either increases or does not affect ER levels in L540Q, E542A and D545A cells, but has a dramatic effect on the degradation of the ER in 3m and D538A cells. This suggests that the presence of an aspartic acid at 538 prevents degradation of the ER when liganded by 4-OHT.

Alterations in other amino acids in helix 12 are reported to result in changes in the stability of the ER. The protein levels of the L539A/L540A double mutant remains constant upon E2 treatment, whereas 4-OHT induces degradation of the ER (38). The mouse ER mutants L543A/L544A (L539A/L540A in human) and M547A/L548A (M543A/L544A in human) (26) as well as the human ER mutants L540Q, E542A/D545A and L540Q/E542A/D545A (27) are not degraded by ICI 182,780. This indicates that helix 12 is important for maintaining the proper regulation of the ER protein in response to ligands, especially ICI 182,780.

The signal that ultimately targets the ER for ubiquitination and subsequent degradation has not been definitively established. Several possibilities have been proposed that modify the shape of a protein so that it is recognized by the E3 ubiquitin protein ligase, such as ER phosphorylation, binding of ancillary proteins to the ER, or binding of ligand. Modulation of the ubiquitination machinery or masking a degradation signal are also possibilities [for review, see (39-42)]. It is likely that a combination of these mechanisms could contribute to the ER degradation observed in the stable cell lines.
In addition to degradation, another consequence of changing the shape or charge distribution of the external surface of the ligand:ER complex is the modulation of ER transcriptional activity. Transcriptional activity was measured initially using transient transfection of the ER mutant and an ERE3-luciferase reporter into T47D:C4:2 cells (Fig. 3), but a further study of TGFα mRNA in the stably transfected MDA-MB-231 cells extended our observations (Figs. 5-6). The results of both of these assay approaches were consistent. In wild type cells, E2 and 4-OHT induced transcriptional activity, but ICI 182,780 did not. The 3m stable cells exhibited no response to ICI 182,780 and 4-OHT, and a decreased response to E2 compared to the wild type cells. The E542A and D545A cells showed essentially the same transcriptional activity as wild type cells. Little transcription occurred with E2 treatment in the L540Q mutant, but activation was observed upon ICI 182,780 and 4-OHT treatment. Remarkably, ICI 182,780 can produce a biological effect when the receptor is stable. The crystal structure of a pure antiestrogen and ERβ, but not ERα, is available. Although the ERβ:ICI 164,384 (a pure antiestrogen related to ICI 182,780) crystal structure has been solved, helix 12 is invisible in the experimental electron density maps (43) so structure/function speculations are not yet possible.

The D538A mutant exhibits decreased transcription in the presence of E2, and no transcription in the presence of ICI 182,780 and 4-OHT. This indicates that the D538A mutation is the single mutation responsible for the decreased activity of the 3m triple mutation. These data are important as it is now possible to redefine the components of AF2b as D351 and D538, which must interact with AF1, because only these regions are required for the estrogen-like activity of 4-OHT. By analogy with our approach to
defining the precise amino acids on helix 12, we are currently addressing the question of the critical amino acid(s) in AF-1 that may be required for SERM activity.

The external surface of the ER affects protein stability and transcriptional activity, but the amount of the ligand:ER complex does not necessarily correlate with activity. Because 4-OHT treatment results in rapid degradation of the D538A protein with an associated lack of transcriptional activity, we increased the level of the 4-OHT:D538A ER complex using an inhibitor of the proteasome. However, activity of the D538A mutant was not restored in the TGFα assay (Fig. 9), indicating that the 4-OHT:D538A complex has no intrinsic activity. An interesting point is that the amount of TGFα mRNA was reduced when the proteasome inhibitor was combined with E2 in the D538A cells. This observation is in agreement with a study by Lonard, et al. (44), which suggests that proteasomal degradation is required for E2-mediated ER transcription and that coactivator binding is required for ligand-mediated degradation of the ER.

Our studies and the known crystal structures of the ligand:ER complex emphasize the idea that the amino acids present in helix 12 are located in unique positions to influence the interaction of coregulators with the ER. In the E2:ER complex, helix 12 is positioned over the ligand binding pocket and the charged residues D538, E542 and D545 are on the outside of the complex (Fig. 2A) (14). L540 is positioned more toward the inside of the ligand binding pocket. E542 is uniquely positioned in the DES:ER crystal structure as an N-terminal capping amino acid that stabilizes the conformation of the coactivator GRIP1 peptide when this peptide is bound to the ER (13,45). Contacts are also made to the GRIP1 peptide in the DES:ER structure by D538 (13). In the 4-OHT:ER structure, the side chain of 4-OHT repositions helix 12 so that it binds to and blocks the
GRIP1 coactivator binding surface (Fig. 2B) (13). In fact, the side chains of L540, M543 and L544 on the inner hydrophobic surface of helix 12 mimic the interactions made by the coactivator’s nuclear receptor binding motif LxxLL (45). None of the helix 12 mutations utilized in our study are residues that contact the ligand or D351, which also modulates the estrogenicity of 4-OHT (16, 18, 46).

An active transcription complex contains coactivators that enhance the transcriptional activity of the ER. An inactive complex contains corepressor or is in a conformation that is unable to bind coactivators (47). Much of the transcriptional activity of the 4-OHT:D538A complex could be explained by the differential binding of coregulators. For example, the D538A mutation results in decreased transcriptional activity in the presence of E2. Because D538 contacts a coactivator, the decreased agonist activity of E2 in the D538A mutant (Figs. 3, 5, 6) could be a result of a decreased ability to recruit a coactivator. The agonist activity of 4-OHT in the wild type cells is mediated by constitutive AF1 activity, because the AF2 coactivator binding site has been disrupted by the side chain of 4-OHT. The D538A mutant eliminates the agonist activity of 4-OHT, suggesting that the D538A mutation in AF2 has allosterically affected AF1. The charge alterations produced by this mutation could favor the recruitment of a corepressor, because it has been demonstrated that 4-OHT can induce the formation of a ER-corepressor complex on the promoter (48, 49).

The dominant negative activity of the L540Q mutant occurs as a result of competition for ERE binding, formation of inactive heterodimers with the wild type receptor and transcriptional silencing (31). In addition, the L540Q mutant recruits a coregulator protein called repressor of estrogen receptor activity (REA) (50). These
mechanisms contribute to the activity of the L540Q mutant \textit{in vitro}, but activity is also observed \textit{in vivo}. The L540Q mutant was introduced into T47D human breast cancer cells using adenoviral infection, and when these cells were injected into athymic mice, tumor formation was inhibited (51). Injection of adenoviruses encoding the L540Q mutation into pre-existing T47D tumors resulted in tumor regression.

In summary, ER action is a complex and tightly regulated system involving interactions between the ligand, the receptor, and effectors that are all coordinated to modulate the appropriate action (52). Helix 12, more specifically D538, is central to these diverse interactions. The model we propose illustrates an unusually dramatic regulation of ER degradation and efficacy of the SERM:ER complex. The SERM 4-OHT normally causes an accumulation of the ER complex that is promiscuous and can induce estrogen-like action at the TGF\(\alpha\) target gene. 4-OHT degrades the ER complex if a specific amino acid (D538) is mutated, but if the 4-OHT:D538A complex is prevented from being destroyed by the proteasome, the complex is not estrogen-like. We believe that the modulation of the estrogenic and antiestrogenic properties of the SERM:ER complex occur through the multiple dimensions of ER destruction and the interaction of the ER with other coregulatory proteins. The finding that D538 in helix 12 can control both ER stability and the intrinsic activity of the 4-OHT:ER complex may not only provide new opportunities in drug design but also provide a new insight into the regulation of ER protein concentrations within the cell.
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FOOTNOTES

1 The abbreviations used are: ER, estrogen receptor α; AF, activation function; LBD, ligand binding domain; SERM, selective estrogen receptor modulator; E₂, estrogen; DES, diethylstilbestrol; 4-OHT, 4-hydroxytamoxifen; 4OHTPP, fixed ring 4-hydroxy triphenylpentene; TGFα, transforming growth factor α; Ral, raloxifene.
FIGURE LEGENDS

FIG. 1. Wild type and mutant ER helix 12 constructs. The human wild type ER is a 595 amino acid protein consisting of domains A-F. The location of activation function 1 (AF1) and activation function 2 (AF2) are indicated. The 3m mutation is a triple mutation composed of the D538A, E542A and D545A mutations. The individual mutations D538A, E542A and D545A were also constructed. The L540Q mutant is another mutation located within helix 12. The location of each mutation within the ER is denoted with an asterisk. Stable transfectants of each mutant were generated in MDA-MB-231 cells.

FIG. 2. Crystal structures of ER/ligand complexes show the position of helix 12 mutations. The estradiol:ER complex (A)(1ERE)(14) and the 4-OHT:ER (B)(3ERT)(13) complex are depicted. Helix 12 (amino acids 536-547 for E2 and 536-551 for 4-OHT) is shaded in yellow, the ligand in blue, D351 in green and D538, L540, E542 and D545 in red. L540 is not visible in the 4-OHT:ER structure because it is in the interior of the complex. In the diethylstilbestrol:ER structure, helix 12 comprises residues 538-546, whereas in the 4-OHT structure, it comprises residues 536-544 (13). For clarity, modified amino acids in helix 12 are shaded. The published crystal structures were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank and colored using Insight II.

FIG. 3. ER mutants display differential activation of an ERE-luciferase reporter. ER negative T47D:C4:2 cells were transiently transfected with 1 µg of an ERE3-luciferase
plasmid and 1 μg of the ER plasmid. The cells were also transfected with 0.2 μg of the PCMVβ plasmid to normalize for transfection efficiency. The next day, the cells were treated with EtOH, E₂ (10⁻⁹ M), ICI 182,780 (10⁻⁶ M) or 4-OHT (10⁻⁶ M) for 24 hours before performing the luciferase and β-gal assays. The data is presented as RLU, which is the luciferase reading divided by the β-gal reading and the resulting value was multiplied by 1000. The graph represents values from 3-4 independent experiments and the standard deviation is shown. (*p<0.05)

FIG. 4. **Protein expression levels are similar in wild type and mutant ER stable transfectants.** 20 μg of protein lysate from each stable clone were run on a 7.5% SDS-PAGE gel and a Western blot was performed using ERα and β-actin antibodies. ER- is a clone that was stably transfected but is ER negative and G418 resistant.

FIG. 5. **Northern blot analysis of TGFα in wild type and mutant ER stable cell lines.** Stable cell lines were treated with EtOH, E₂ (10⁻⁹ M), ICI 182,780 (10⁻⁶ M), raloxifene (Ral, 10⁻⁶ M), 4-OHT (10⁻⁷ M), or combinations as indicated. RNA was harvested 24 hours after treatment and 20 μg of each sample was run on an agarose gel. The membrane was probed with TGFα and β-actin. The Northern were repeated at least 3 times, and a representative blot is shown. The quantitation is a combination of at least 3 independent experiments, with the standard error shown.

FIG. 6. **Concentration-dependent induction of TGFα mRNA expression by E₂ in wild type and mutant ER stable cell lines.** Stable cell lines were treated with EtOH or
varying concentrations of E₂ (10⁻¹⁰-10⁻⁶ M) for 24 hours and processed as described in Fig. 5. The top blot represents TGFα and the bottom represents β-actin. The EC₅₀ for each dose response curve was calculated by first subtracting the EtOH value and then setting the 10⁻⁶ M concentration to 100. The EC₅₀ is the concentration at which the E₂-induced TGFα mRNA increase is half maximal.

FIG. 7. Western blot analysis of ER levels in wild type and mutant stable cell lines.
A. Stable cell lines were treated with EtOH, E₂ (10⁻⁹ M), 4-OHT (10⁻⁶ M), or ICI 182,780 (10⁻⁸-10⁻⁶ M) for 24 hours. 20 μg of each sample were loaded onto a SDS-PAGE gel. The membrane was probed with ERα antibodies and β-actin antibodies were used to ensure even loading. The Westerns were repeated at least 3 times and a representative blot is shown. B and C. The quantitation is a combination of 3 independent experiments, with the standard error shown (*p<0.05). D. Wild type and D538A stable cell lines were treated with EtOH or 4-OHT (10⁻⁷ M) for 24 hours and RNA was processed for a Northern blot as described in Fig. 5. ER mRNA and protein levels (from part C) were compared. The quantitation is a combination of 3 independent experiments and the standard error is shown.

FIG. 8. Ligand-mediated degradation of the ER occurs through the proteasome.
Stable cell lines were treated with vehicle (Et/DM), 10μM of the proteasome inhibitor MG132 (MG), or media for 1 hour. E₂ (10⁻⁹ M), ICI 182,780 (10⁻⁶ M) or 4-OHT (10⁻⁶ M) were added for 4 hours before harvesting protein. 20 μg of protein lysate were run on a SDS-PAGE gel and the membrane was probed with ERα and β-actin antibodies.
FIG. 9. **Blocking 4-OHT mediated ER degradation in the D538A mutant does not restore the estrogenticity of 4-OHT.** D538A stable cells were treated with vehicle (Et/DM), MG132 (50μM, 10 μM or 1 μM) or media for 1 hour. 4-OHT (10⁻⁶ M) or E₂ (10⁻⁹ M) was added for 4 hours prior to harvesting RNA. The Northern blot was probed with TGFα and β-actin.
|            |     |   |   |   |   |   |
|------------|-----|---|---|---|---|---|
| **Wild type** | 1   | 180 | 263 | 302 | 553 | 595 |
| ERα        |     | A/B | C  | D  | E  | F  |
|            |     | AF1 |    |    |    |    |
| **3m**     |     | A/B | C  | D  | E  | ***F|
| **D538A**  |     | A/B | C  | D  | E  | * F |
| **E542A**  |     | A/B | C  | D  | E  | * F |
| **D545A**  |     | A/B | C  | D  | E  | * F |
| **L540Q**  |     | A/B | C  | D  | E  | * F |
EtOH E

ICI 4-OHT

RLU (luc/β-gal x1000)

PSG5 wild type D538A L540Q E542A D545A 3m

EtOH E2 ICI 4-OHT

* * * *

* * * *
D545A  
D538A  
3m  

wild type

EtOH  
E2 $10^{-10}$  
E2 $10^{-9}$  
E2 $10^{-8}$  
E2 $10^{-7}$  
E2 $10^{-6}$

EC_{50} = 7 \times 10^{-10} M  
EC_{50} = 2 \times 10^{-9} M  
EC_{50} = 4 \times 10^{-9} M  
EC_{50} = 8 \times 10^{-9} M

% of maximum

by guest on March 23, 2020http://www.jbc.org/Downloaded from
A

wild type

3m

D538A

L540Q

E542A

D545A

ERα

β-actin

B

% of control

control ICI 10^-6 ICI 10^-7 ICI 10^-8

wild type 3m L540Q D538A E542A D545A

C

% of control

wild type 3m D538A L540Q E542A D545A

D

% of control

EtOH E2 4-OHT


**Wild type**

**3m**

**D538A**

**E542A**

**D545A**
Modulation of estrogen receptor α function and stability by tamoxifen and a critical amino acid (D538) in helix 12
Sandra Timm Pearce, Hong Liu and V. Craig Jordan

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