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BRIEF REPORT

Breast cancer-derived M543V mutation in helix 12 of estrogen receptor α inverts response to estrogen and SERMs

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Abstract We have isolated from human breast cancers several mutations in the Helix 12 component of activation function 2 (AF-2) in the estrogen receptor alpha (ERα). We used a novel approach to detect changes in the hormone-binding domain of ERα, based on the evidence that anti-estrogens, such as 4-hydroxytamoxifen (ZOHT) and ICI 182,780, block the function of ERα by binding and folding the AF-2 transcriptional domain in a way that inhibits its association with coactivator proteins. We have identified a Helix 12 mutation, M543V, which leads to greater ERα transcription with ZOHT and other antiestrogens (including 1,1-dichloro-2,2,3-triarylcyclopropanes, DTACs) than with 17-β estradiol (E2). We also found an independent mutation at the same position, M543I, which did not show this inverted ligand phenotype. In comparison to further Helix 12 mutations made in vitro, it appears that relative hydrophobicity of the amino acid side chains on the inner face of Helix 12 is key to maintaining the transcriptionally active, agonist conformation with bound E2. This active conformation can be induced, resulting in increased transcription, by adding excess p160 coactivator AIB1 in transcriptional assays with E2-bound receptors, while the ZOHT-bound receptors were not further activated by AIB1. Other experiments show that the cross talk between ERα and AP-1 protein from AP-1-binding sites is not dependent on Helix 12 integrity. We show that two alleles containing a proline substitution in Helix 12 that inactivate AF-2 function of ERα at EREs have little negative effect on function through AP-1 elements, supporting a prominent role for the N-terminal AF-1 of ERα in AP-1/ERα transcriptional cross talk.

Keywords Estrogen receptor alpha · Selective estrogen receptor modulator · Helix 12 mutation · p160 coactivator · Activating function 2

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Introduction

Tamoxifen has been one of the most widely used and effective drugs for treatment and prevention of breast cancer. However, there are a substantial number of patients with breast cancer (30%) who fail to respond to tamoxifen, even if they have estrogen receptor alpha (ERα+), or who become resistant to the effects of tamoxifen after initial response. Insight into the basis for such resistance to the potential benefits of tamoxifen would be expected to lead novel strategies or drugs to overcome the problem. Tamoxifen therapy requires a functional ER, yet the predominant clinical use of immunohistochemistry for ERα is an indirect determination of receptor status; the receptor protein may have mutations that inactivate or alter its function or steady state levels.

We have used a unique approach to detect functional changes in ERα from breast cancers and tissues. Among tumors analyzed, several have demonstrated mutations in ERα affecting the p160 coactivator-binding site within Helix 12 (H12). The p160 coactivators function to amplify transcription at estrogen responsive genes and are the prime targets of tamoxifen inhibition, by blocking the activation function, AF-2 domain [1, 2]. These coactivators, such as amplified in breast 1 (AIB1), have common nuclear receptor interaction motifs (LxxLL, NR box) and have been shown to bind over Helix 12, a key structural component of the hormone-stimulated AF-2, resulting in full transcription at estrogen target genes [3–5]. The X-ray structures of ER [5–9] show different final positions of Helix 12 when either hormone or antihormone is bound. The NR box interaction site for AF-2 function is only formed with bound agonists, e.g., estradiol (E2) or diethylstilbestrol, and not with SERMs, such as raloxifene, 4-hydroxytamoxifen (ZOHT; 4-OHT), lasofoxifene, or ICI 182,780 [5–10]. Altered Helix 12 positioning with SERMs for ER, such as 4-hydroxytamoxifen, leads to occlusion of the coactivator-binding cleft by the misaligned Helix 12 [5].

As the AF-2 region of the ER hormone-binding domain is the most important for ligand-regulated transcription and is the prime target of antiestrogen action, we have developed an assay in yeast to report on AF-2 folding and can detect mutations that alter that critical domain for ER activity. Such mutations will likely affect antiestrogen efficacy, coactivator protein interactions, and transcription. We have found several mutations in and around Helix 12 that alter the transcriptional properties of ERα and may explain one type of SERM resistance.

Materials and methods

Cells

Estrogen receptor negative CV-1 kidney cells were maintained in DMEM with 4.5 g/l glucose (Mediatech, Cellgro, VA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT) and 100 units/ml penicillin–streptomycin (GIBCO, Invitrogen, Carlsbad, CA) at 37°C in a humidified 5% CO_2 atmosphere. The human estrogen–dependent breast cancer cell line MCF-7 was purchased from ATCC and maintained in RPMI 1640 supplemented with 10% FBS, L-Glutamine, and Pen/Strep.

Chemicals

17-β-E2 and 4-hydroxytamoxifen (ZOHT) were purchased from Sigma (St. Louis, MO), and ICI 182,780 was from Tocris (Ellisville, MO). The ER ligands were prepared as 10 mM stocks in 100% ethanol and stored at −20°C. Z-1,1-dichloro-2,3-diphenylcyclopropane (a.k.a. Analog II) and the DTACs (dichloro-triarylcyclopropanes) were synthesized as described previously [11, and references within], prepared as 1 mM stocks in DMSO, and stored at −20°C. Prior to use in an experiment, each of the ligands was further diluted into the requisite amounts of DMSO and medium so as to keep vehicle concentrations constant.

Constructs

CMV-ERα (±mutations) contains the full-length coding sequence of human ERα, 595 amino acids. ERE-tk-luciferase contains a single estrogen response element (ERE) cloned upstream of the thymidine kinase promoter and the luciferase gene. AP-1-tk-luciferase contains a single AP-1 response element upstream of the thymidine kinase promoter and the luciferase gene and was purchased from BD Biosciences Clontech (Palo Alto, CA). CMV-SPORT-β-galactosidase was purchased from Invitrogen.

Transfections

For transient transfection assays, cells were plated in 6-well dishes at a density of 2×10^5 cells per well in phenol red-free DMEM (HyClone) containing 10% charcoal-dextran–stripped FBS (HyClone). CV-1 cells were transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Transfections contained 0.5 μg of reporter plasmid and 0.2 μg of ER expression vector. After 16 h, the medium was replaced, and the cells were treated with vehicle or ligand for an additional 24 h. Cells were harvested and assayed for luciferase and β-galactosidase. Transfections were performed in duplicate, and each experiment was repeated at least three times.

Luciferase assay

Cell monolayers were washed twice with ice-cold PBS and incubated for 15 min in 250 μl of 1× cell culture lysis reagent (Promega, Madison, WI). Cell extracts were transferred to an eppendorf tube and centrifuged for 2 min at 13,000 rpm at room temperature. The supernatants were transferred to a fresh tube and assayed using the Luciferase Assay System (Promega). For each assay, 10 μl of extract was diluted with 90 μl of 1× cell culture lysis reagent. Luminescence was read using an AutoLumat LB953 luminometer (Berthold, Pforzheim, Germany).

Preparation of whole cell extracts

CV-1 cells were transfected with the ERα allele and treated as described for transfection assays. Twenty-four hours

after treatment, the cells were collected and lysed in TX100/SDS buffer (1% TX-100, 0.1% SDS, 50 mM Tris–HCl pH 7.8, and 150 mM NaCl) supplemented with protease (BD Biosciences PharMingen) and phosphatase inhibitor (Calbiochem) cocktails. Then the extracts were centrifuged (10 min, 13,000*g*) at 4°C. Proteins were quantified using the BioRad protein assay as per the manufacturer's instructions.

Immunoblotting

Proteins (30 μg) were resolved by electrophoresis through precast 10% polyacrylamide Tris–HCl gels (BioRad) and then transferred to PVDF membrane. Membranes were probed using anti-ERα antibody (sc-543, Santa Cruz Biotechnology, Santa Cruz, CA). Complexes were detected using stabilized horseradish peroxidase-conjugated anti-rabbit secondary antibody and the Western Lightening chemiluminescence system (Perkin Elmer). Actin was detected using the Ab-1 kit (Oncogene Research Products).

Results

Screening for altered ERα was performed on RNA prepared from samples of human breast cancers, as described [12]. After RT-PCR recovery from human tumors of the 3′-half of ERα (EagI-SacI fragment, amino acids 312–595), it was subcloned into a FLP-ER plasmid [13] for expression in *Saccharomyces cerevisiae*. We developed this assay as a rapid screen for mutations that was not solely reliant on DNA sequence analysis [12]. Numerous isolates from each original tissue sample have been transformed into the yeast assay strain, and plate assays were performed with a panel of ligands, causing a white to red color change (due to an adenine auxotrophy) [12]. A wild-type FLP-ER control was included in each experiment to allow direct comparison of redcircle size, indicative of ligand-binding and ER protein conformation. Many of the samples exhibited the very reproducible pattern consistent with the wild-type ERα sequence. Others that were obviously different from that wild-type pattern, due to a mutation in the ERα domain exhibiting either altered ligand activation, weak or no activation, or unregulated constitutive activity, were sequenced to determine the causative amino acid change.

Of the breast cancer samples that we screened as previously described [12], we found a subset of mutations in the hormone-binding domain that affect Helix 12, a key component of the hormone-responsive activation function, AF-2. Figure 1 shows the X-ray crystal structure of the outside and inside surfaces of the amphipathic alpha Helix 12 of ERα [6] and the sites of amino acid changes that we found from breast cancer samples. The M543A/L544A

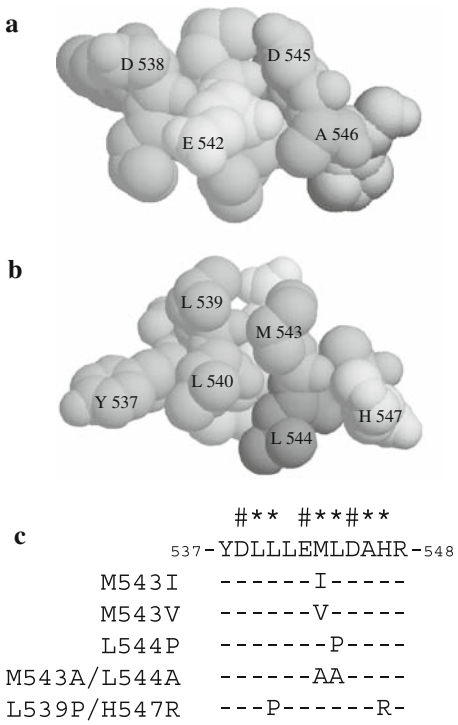

Fig. 1 Positions of the mutations found in Helix 12 of ERα from breast cancers. The X-ray structure data are shown for Helix 12 of ERα [6] from a *top view*, exposed to the outside (**a**) or a *bottom view*, to the inside of the protein (**b**). Key amino acids are labeled for the acidic top (D538, E542, and D545; marked by #) or the hydrophobic underside (L539, L540, M543, and L544; marked by *) of Helix 12. The mutations recovered from breast cancers are shown (**c**). The double mutation, M543A/L544A, was generated in vitro for comparison

double mutation was constructed using PCR in vitro [14], for comparison with subsequent transcriptional experiments. We cloned all of the mutations independently into a full-length expression construct of ERα to test their effects on transcription from an ERE–luciferase reporter. Most of the mutations diminished the levels of transcription in the presence of E2, as might be expected for changes in a key component of AF-2 (Fig. 2). Most interesting was a mutation, M543V, which showed much greater activity with 4-hydroxytamoxifen (ZOHT) than with E2, while another mutation at the same amino acid, M543I, showed diminished, yet wild-type character with respect to E2 and ZOHT activation. The double mutation, M543A/L544A, like M543V, also showed much greater relative activity with ZOHT than with E2. The other two Helix 12-mutated alleles of ERα that were recovered, L544P and a double mutation, L539P/H547R, showed almost no transcriptional activity with either E2 or SERMs.

To explore further the transcriptional properties of the M543V ERα compared to wild-type ERα, we tested a greater set of compounds for their effects on transcription from an ERE reporter. We included a set of new

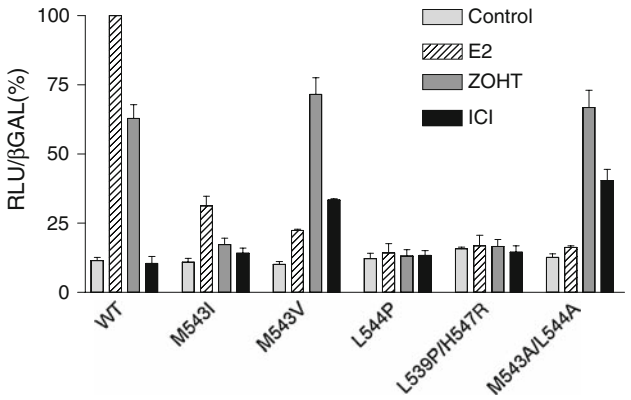

Fig. 2 Helix 12 mutations differentially alter ERα transcription. CV1 cells were cotransfected with CMV-ERα (wild-type or indicated mutants), CMV-*β*-galactosidase, and ERE-tk-luciferase. After 16 h, cells were treated with DMSO alone (control), 10 nM E2, ZOHT, or ICI 182,780. After an additional 24 h, the cells were harvested and luciferase and *β*-galactosidase activity measured. To control for transfection efficiency, luciferase activity was normalized to *β*-galactosidase activity. Data are expressed relative to the activity of E2 on the wild-type ERα, which was set at 100%. *Bars* represent the mean ± SD of at least three independent experiments. Relative ligand-induced expression data are representative of more than ten independent experiments

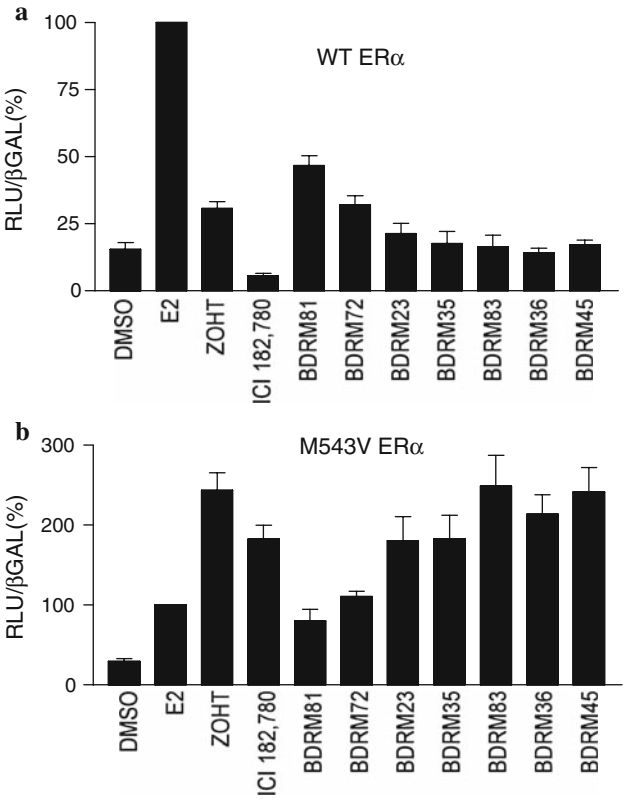

Fig. 3 M543V ERα exhibits an inverted phenotype in that antiestrogenic compounds have more activity than estrogenic compounds in transcription from an ERE reporter. CV-1 cells were co-transfected with CMV-ERα (WT or M543V) and ERE-tk-luciferase. After 16 h, cells were treated with DMSO alone (control), 10 nM E2, ZOHT, or ICI 182,780, or 1.0 μM DTAC compounds (BDRM#) [11]. After an additional 24 h, the cells were harvested and luciferase activity measured. Data are expressed relative to the activity of E2 alone, which was set at 100%. *Bars* represent the mean ± SD of at least three independent experiments

compounds (DTACs) with antiestrogenic character, as described previously by us [11]. The wild-type ERα was most transcriptionally active with E2 and partially activated by ZOHT and the smallest DTAC compounds, BDRM81 and BDRM72 (Fig. 3a), consistent with previous experiments [11]. The larger DTAC compounds (BDRM23, -35, -83, -36, -45) failed to activate the ERα at an ERE reporter, consistent with their more complete antiestrogenic character [11]. However, the mutated M543V ERα showed the opposite results when tested with this series of compounds (Fig. 3b). The most estrogenic agonists on wild-type ERα, namely E2, BDRM81, and BDRM72, were the *least* activating compounds on M543V ERα. On the other hand, almost all of the antiestrogenic compounds that had little activity on wild-type ERα gave significant transcriptional activity (>twofold increase over E2) from the M543V ERα, on an ERE–luciferase reporter.

Ligands are known to alter the protein stability of ERα [15–18], which can greatly affect the resulting transcriptional readout. One study shows that inhibition of the 26S proteasome by specific reagents increases the steady state levels of ERα and the consequent estrogen-regulated gene products [19]. We compared the effects of several ligands on the steady state levels of transfected ERα in CV-1 cells, which have no endogenous estrogen receptors. We performed similar experiments using transfected M543V ERα or M543I ERα in CV-1 cells. With the wild-type ERα, E2 results in diminished protein levels, but not nearly as reduced as with the pure antagonist, ICI 182,780 (Fig. 4)

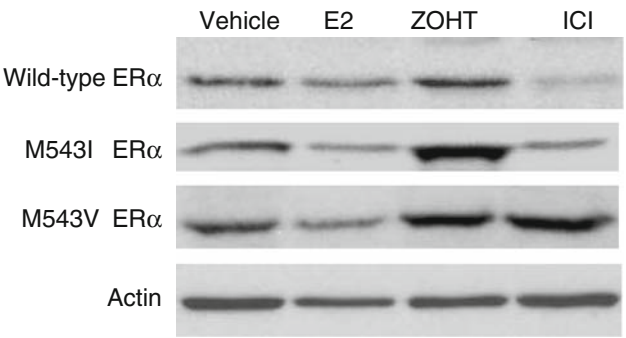

Fig. 4 Steady-state levels of ERα, M543I ERα, or M543V ERα, treated with various ligands. CV-1 cells were transfected with CMV-ERα, CMV-M543I ERα, or CMV-M543V ERα. The following day, cells were treated as indicated. E2, ZOHT, and ICI 182,780 were used at a final concentration of 10 nM. Cells were harvested and ERα protein measured by immunoblot after 24 h of treatment

[17]. ZOHT actually increases the steady state levels of wild-type ERα. The M543I ERα showed the same steady-state protein levels as the wild-type ERα in response to various ligand treatments (Fig. 4). With the M543V ERα, E2 again results in slightly diminished protein levels, but surprisingly, ICI 182,780 results in a significantly increased level of steady-state M543V ERα, similar to ZOHT. The pure antagonist ICI 182,780 does not lead to increased degradation of the M543V receptor as it does for the wild-type ERα.

Because the mutations affect H12 of AF-2, a known binding site of p160 coactivators, we tested each of the mutated ERα forms in transcriptional experiments that included a cotransfected p160 coactivator AIB1 and an ERE–luciferase reporter (Fig. 5). The wild-type ERα showed increased transcription in the presence of E2 when the AIB1 coactivator was included, as expected. The coactivator did not increase transcription from the wild-type receptor when cells were treated with ZOHT or ICI 182,780. For the most interesting allele, M543V, ZOHT was the most active ligand in the absence of added p160 coactivator. Adding exogenous coactivator AIB1 increased transcription for M543V only in the presence of E2, and not for antagonists ZOHT or ICI 182,780. This was similarly true for the double mutant, M543A/L544A. The M543I allele showed activity similar to wild type, in that E2 was the ligand most active, and increased by p160 AIB1 addition.

ERα has been shown to have transcriptional activity at non-ERE binding sites, usually by interacting with other transcriptional factors that tether ERα to the sites [20–23]. Thus far, we have presented data for responses of ERα and mutated alleles from ERE reporter constructs. At EREs, we have seen that the M543V allele shows higher activity with ZOHT than with E2. We next examined the effects of the recovered mutations from an AP-1 luciferase reporter [23]. Unlike for the ERE reporter, the ligand activities from M543V ERα were not altered markedly when compared to those for the wild-type ERα (Fig. 6a). We examined the ligand effects at AP-1 reporters for each of the other alleles that we recovered from breast cancers. Almost all of the mutations in and around H12 of the ERα AF-2 showed the same relative activity responses to ligands as those for the wild-type ERα at an AP-1 reporter (Fig. 6b). The mutated L544P ERα, which showed almost no activity from an ERE (Fig. 2), exhibited almost normal activity from an AP-1 site, albeit with some loss of response to E2. A similar result was seen for the double mutant, L539P/H547R allele at AP-1 elements. Accordingly, the H12 component of AF-2 has minimal bearing on the transcriptional properties of ERα when tethered to AP-1 transcriptional sites.

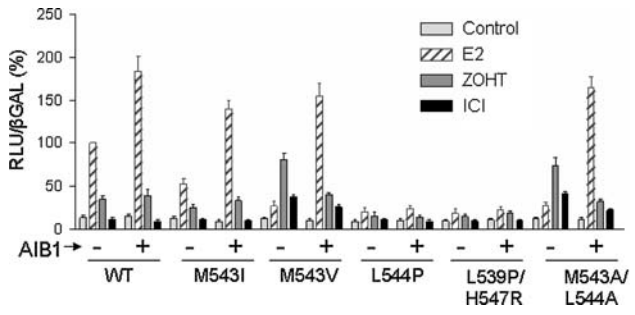

Fig. 5 p160 coactivator AIB1 stimulates transcription by ERα or mutated ERα, only if estradiol is bound. CV-1 cells were co-transfected with CMV-ERα, ERE-tk-luciferase, and minus or plus CMV-p160 coactivator (AIB1). After 16 h, vehicle control, E2, ZOHT, or ICI 182,780 compounds were added at 10 nM each. After an additional 24 h, the cells were harvested and luciferase and *β*-galactosidase activity measured. To control for transfection efficiency, luciferase activity was normalized to *β*-galactosidase activity. Data are expressed relative to the activity of E2 alone, which was set at 100%. *Bars* represent the mean ± SD of at least three independent experiments

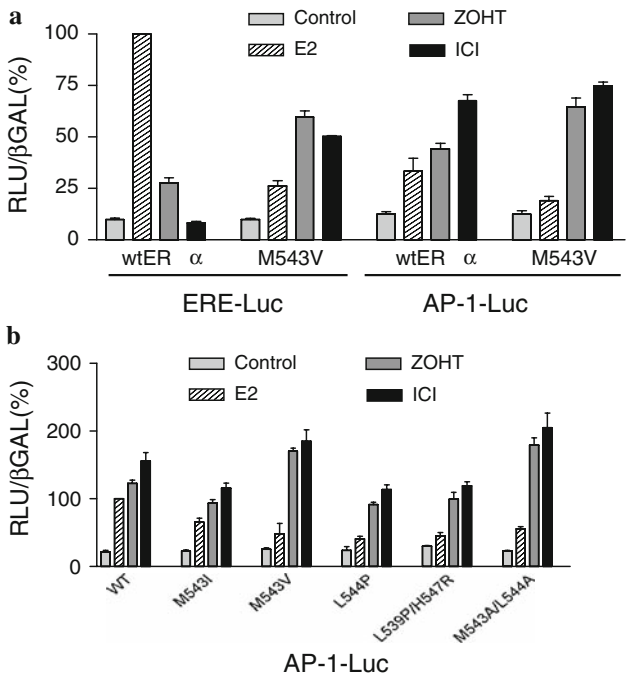

Fig. 6 Mutations in Helix 12 of ERα do not affect its ability to crosstalk with AP-1 protein at an AP-1 transcription reporter. CV1 cells were cotransfected with CMV-ERα or mutant noted and ERE-tk-luciferase or AP-1-tk-luciferase as noted. After 16 h, control, or E2, ZOHT, or ICI 182,780 compounds (10 nM final) were added. After an additional 24 h, the cells were harvested and luciferase and *β*-galactosidase activity measured. Comparison of wild-type ERα and M543V ERα on ERE or AP-1 reporters is shown in **a**. The complete set of results with the Helix 12 mutant ERα alleles from an AP-1 reporter is shown in **b**. To control for transfection efficiency, luciferase activity was normalized to *β*-galactosidase activity. *Bars* represent the mean ± SD of at least three independent experiments. Data are expressed relative to the transcription for E2 on wild-type ERα, which was set at 100%

Discussion

We have examined human breast cancer samples as outlined previously [12] to find altered ERα alleles that may change in vivo responses to ligands. We have isolated a number of ERα mutations, including several at the Helix 12 region of the hormone inducible AF-2. Mutations of ERα (M543V) have been found which cause 4-hydroxytamoxifen to be more active than E2 in transcription from an ERE. This is opposite to the character of the wild-type ER and is consistent with the clinical observation that some tumors seem to grow in the presence of tamoxifen and will regress if treatment is stopped [24, 25]. Though we do not find this mutation in a high proportion of tumors examined, it is direct evidence for the possibility that tamoxifen resistance could arise by such a mechanism. Tamoxifen inhibits growth signaling through ERα by binding directly to its hormone-binding pocket, so mutations in ERα should logically be one mechanism of resistance. Breast epithelial cells with an ERα variant that responds better to tamoxifen may form a population of cells that escape from growth repression by tamoxifen. In turn, their production of paracrine growth factors (TGFα, IGF) could lead to outgrowth of cells that form the eventual tumor. Such ERα mutations may also explain the sometimes observed growth stimulation by SERM therapy, or at least a loss of ER transcriptional repression, depending on to what degree coactivators or AF-1 transactivational domain can form active ERα transcriptional complexes. This report describes a naturally occurring mutation of ER from a breast cancer, whereby tamoxifen may stimulate rather than block ERα function and growth.

Interestingly, the M543V mutation showed greater transcription activation by ZOHT than E2, while the M543I mutation maintained the normal E2 greater than ZOHT response. While these may seem to be subtle amino acid substitutions at the equivalent position, our results point to a greater degree of hydrophobicity (isoleucine > valine) and length of the amino acid side chains in the interior of Helix 12 as a critical component for transcriptional activation by E2 and agonists over SERMs. We tested the double mutation M543A/L544A in similar transcriptional experiments and found that consistent with our premise, the reduction in hydrophobicity and side chain length for alanine correlated with SERM activation and E2 inactivity. We propose that the degree of hydrophobicity of the underside of Helix 12 is a key to maintaining its ligand-activated position over the E2-bound pocket, much like a "Velcro" attachment. With a reduction in the length of the aliphatic side chains such as occurs with M543V, the underside of Helix 12 will not associate as tightly over the ligand-bound pocket and may lead to the "antiestrogen" or another conformation of Helix 12, perhaps even unassociated with the hormone-binding domain surface. By overexpressing the p160 coactivator AIB1 with the M543V ERα, we recover relatively strong transcriptional activity with E2, consistent with increased p160 binding to further stabilize the "agonist" position of Helix 12. This model predicts a general feature of all nuclear hormone receptors, namely that mutations to reduce the hydrophobicity of specific amino acids on the undersides of H12 (amino acid alignment equivalents of ERα 539/540 and 543/544) of any of the nuclear receptors should "invert" the transcriptional activity by agonists and antagonists for that receptor. In the human androgen receptor, T877A, a missense mutation has been identified in the LNCaP cell line originally isolated from a patient with hormone-refractory prostate cancer. However, this mutation, T877A, is located in helix 11 and confers promiscuity to AR allowing activation by progesterone, estrogen, adrenal androgens, and hydroxyflutamide in addition to androgens [26].

We tested a wide set of compounds on the wild-type and M543V ERα to see how general SERM activation was for the mutated receptor. The results correlated well in that the more estrogenic the compound was on the wild-type ERα, the less it would activate M543V ERα at an ERE reporter. Alternatively, the more antiestrogenic a compound is, the more that M543V ERα was activated at an ERE reporter. Clearly, a common property, most likely Helix 12 position, was responsible for the resulting activities, which we refer to as "inverted" ligand phenotype.

Two of the mutant ERα alleles were not active from ERE-reporter constructs. Both of these alleles, L544P and the double mutant, L539P/H547R, involve proline substitutions in Helix 12. Proline is known to cause disruption of α-helices because the proline side chain is covalently bound to its peptide bond. Therefore, proline cannot form a hydrogen bond to maintain stable α-helical shape, instead bending the secondary structure, and in this case abrogating the role of Helix 12 in AF-2 function of ERα. This is consistent with the observation of virtually no transcriptional activation in the ERE-reporter transcription assays with agonists or antagonists (Fig. 2). However, these two ERE-inactive alleles maintained almost full activity from an AP-1 reporter (Fig. 6). While the transcriptional activity induced by E2 at the AP-1 reporter did drop with H12 mutations, the activities with ZOHT and ICI 182,780 were relatively unaffected, perhaps due in part to the increased receptor stability. This implies that the integrity of Helix 12 is not a major determinant of the cross talk between ERα and AP-1 proteins, and that this cross talk is dependent primarily on the N-terminal AF-1 function in ERα.

The steady state levels of ERα are differentially affected by the presence of ligands [15–18]. E2 leads to a reduction in receptor levels, consistent with the idea of a feedback inhibition of E2 action, through receptor turnover mediated

by ubiquitin and the 26S proteasome [15, 16]. Ligand-mediated turnover in the receptor appears to correlate with coactivator interaction [16] and transcriptional activity [27]. Blockade of coactivator binding by ZOHT is consistent with its ability to stabilize the receptor [27–29]. Indeed compounds have been described that direct Helix 12 to block the coactivator-binding site but also increase its surface hydrophobic exposure leading to faster receptor turnover [9, 30]. It has been shown that L539A/L540A ERα has increased stability and decreased transcriptional activity. However, attachment of a VP16-transactivating domain to L539A/L540A ERα increased its transcriptional activity and its degradation [29]. They further showed that degradation of ERα was proportional to the level of ubiquitination, namely that it was lowest with ZOHT and was highest with ICI 182,780, targeting ER degradation at the 26S proteasome. Our data suggest that ICI 182,780 may lead to ubiquitination/degradation of the M543I ERα but much less so for the M543V ERα. Thus, ICI 182,780 may not be as effective as an antiestrogen, leading to degradation, of the M543V protein.

Inhibition of the 26S proteasome has been shown to not only prevent ERα degradation but increase transcription of estrogen-stimulated genes [19]. On the wild-type ERα, ICI 182,780 is a pure antiestrogen that greatly reduces the level of receptor via nuclear immobilization and degradation [17, 30]. On the M543V ERα, ICI 182,780 did not cause rapid turnover and instead increased steady-state levels of the receptor. This may be an additional component of potential SERM resistance, if the receptor protein maintains higher levels than normal in the presence of SERMs.

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