The Tamoxifen-responsive Estrogen Receptor α Mutant D351Y Shows Reduced Tamoxifen-dependent Interaction with Corepressor Complexes*

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The effects of estrogen and anti-estrogen are mediated through the estrogen receptors ERα and β, which function as ligand-induced transcriptional factors. The non-steroidal anti-estrogen tamoxifen is the most commonly used endocrine in the treatment of all stages of breast cancer in both pre- and postmenopausal women. Several lines of evidence have indicated that tamoxifen promotes association between ERα and corepressors N-CoR or silencing mediator for retinoid and thyroid hormone receptor (SMRT). Our results indicate that N-CoR/SMRT recognize and interact with helices H3 and H5, and ERα ligand-binding domain in a 4-hydroxy tamoxifen-dependent manner. The mutant ERα(D351Y), derived from a tamoxifen-stimulated tumor and containing an amino acid substitution at position 351 within H3, showed reduced interaction with a tamoxifen-induced active mutant of ERα(D351Y) was ERα, the mutant estrogen activity in the presence of tamoxifen compared with wild-type ERα(D351Y) can be stimulated by 4-hydroxy tamoxifen (D351Y) can be stimulated by 4-hydroxy tamoxifen (D351Y) compared with wild-type ERα, indicating this fact.

The crystal structures of several nuclear receptor ligand-binding domains (LBDs) have now been determined and reveal structural conservation despite modest sequence homology (39–46). The LBD forms a structure described as a sandwich of 12 α-helices (H1-H12) with a central hydrophobic ligand binding pocket (47, 48). In the presence of ligand, the hinge region between H11 and H12 is moved closer to H3 and H5, and H12 is positioned over the ligand binding pocket formed by H3, H4, and H5. The repositioned H12 releases the corepressors from the LBD and forms a hydrophobic groove with H3 and H5 (39, 49, 50). This hydrophobic groove is known to be important for interaction with LXXLL motifs found in p160 family members as well as in other coactivator molecules (51–55).

The estrogen dependence of some breast cancers is well known, and endocrine therapy is used to control this disease (56). The development of inhibitory ligands for nuclear receptors has yielded important therapeutic treatments, including the use of tamoxifen for the endocrine therapy of breast cancer (57). Tamoxifen exhibits a wide range of estrogen-like and anti-estrogen actions according to the target tissue examined (58). While tamoxifen may exert anti-estrogenic activity by silencing the transcriptional activity of AF-2, agonist activity of tamoxifen can be mediated through AF-1 in a cell- or tissue-dependent manner (59–63). The tamoxifen-related compound raloxifene is used clinically for the prevention of osteoporosis...
and is being tested for the prevention of breast cancer in high-risk women (58). However, most patients undergoing long-term treatment of breast cancer with tamoxifen eventually experience recurrence of tumor growth. One of the reasons for this treatment failure is the acquisition by the tumor of the ability to respond to tamoxifen as a stimulatory rather than inhibitory ligand (64–67). Wolf et al. identified a mutant ERα from a tamoxifen-stimulated tumor that contained a point mutation that led to a tyrosine for aspartate substitution at amino acid D351 (ERα(D351Y)), located within the LBD of ERα (68, 69). However, the molecular mechanism of this substitution in tamoxifen-stimulated tumor that contained a point mutation led to a tyrosine for aspartate substitution at amino acid D351 (ERα(D351Y)), located within the LBD of ERα (68, 69). However, the molecular mechanism of this substitution in tamoxifen-stimulated tumor that contained a point mutation led to a tyrosine for aspartate substitution at amino acid D351 (ERα(D351Y)), located within the LBD of ERα (68, 69).

Recent studies have suggested that tamoxifen promotes the binding of ERα to N-CoR/SMRT and that the relative expression levels of coactivators and corepressors may modulate the ability of tamoxifen to regulate ERα transcriptional activity (77–81). Here, we report that N-CoR/SMRT recognized and associated with ERα helices H3 and H5 in the presence of 4-hydroxy tamoxifen (OHT) or raloxifene (RAL). ERα(D351Y), derived from a tamoxifen-stimulated tumor, contains an amino acid substitution in H3 and exhibited reduced interaction with N-CoR and SMRT in the presence of OHT or RAL. In contrast, the 17β-estradiol (E2)-dependent coactivator binding affinity of the D351Y mutant was comparable with that of wild-type ERα. Our observations may help explain the observation that the transactivation function of ERα(D351Y) is enhanced by both tamoxifen and estrogen.

**EXPERIMENTAL PROCEDURES**

**Materials—**17β-estradiol (E2) and OHT were purchased from Sigma. RAL and ICI182,780 (ICI) were synthesized by Taiho Pharmaceutical Co.

**Plasmid Construction—**The LBD regions of ERα or RXRα were inserted into the pM vector (CLONTECH) to generate GAL-ERα DEF (GAL-DEF) and GAL-RXRα DEF (GAL-RXRα DEF), respectively. C-terminal fragments of N-CoR and SMRT (including the nuclear receptor interaction domains ID1 and ID2) were inserted into the pGEX-4T-1 vector (Amersham Pharmacia Biotech) to generate VP-N-CoR, VP-SMRT, Myc-N-CoR, Myc-SMRT, GST-ID1(SMRT), GST-ID2(SMRT), GST-ID1(N-CoR), and GST-ID2(N-CoR), respectively. The ERα expression vector constructs (17 m8-luc, MH100-tk-luc, ERE-tk-luc) have been described previously (3, 33, 82–84).

**Transfection, Luciferase Assay, Mammalian Two-hybrid Assay, and Immunoprecipitation—**Nuclear extracts of transfected 293T cells were prepared as described (85). Whole cell lysates were added, and cells incubated for additional 12 h. Preparation of cell lysates was performed as described (84).

**GST-pull down assays** were performed using GST-fused ID1 and ID2 proteins produced by *Escherichia coli* and [35S]-methionine-labeled ERα translated in vitro in the presence of OHT (1 µM), 17β-estradiol (E2) (10 nM), and 0.2% fetal bovine serum. For transfection, media were replaced with fresh medium containing 0.2% fetal bovine serum. Media were added, and cells were incubated for additional 24 h. Preparation of cell extracts and GST-pull down assays were performed following the manufacturer's instructions (Promega). After transfection, media were replaced with fresh medium for an additional 12 h.
GST Pull Down Assay—For GST-pull down assays, bacterially expressed GST fusion proteins or GST bound to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) were incubated at 30 °C with [35S]methionine-labeled proteins expressed by in vitro translation using the TNT coupled transcription-translation system (Promega). After 2 h of incubation, free proteins were removed by washing the beads with NET-N buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Specifically bound proteins were eluted by boiling in SDS sample buffer and analyzed by 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, radiolabeled proteins were visualized using an image analyzer (BAS1500, Fuji Film, Tokyo, Japan).

RESULTS
The ERα Binding Surface for N-CoR/SMRT Is Located in the Helix H3 and H5—Several reports have indicated that tamoxifen promotes interaction between ERα and the N-CoR or SMRT corepressors (77–81). Therefore, we first confirmed the tamoxifen- or raloxifene-dependent interaction between ERα and N-CoR/SMRT by coimmunoprecipitation. Using an anti-FLAG antibody, FLAG-ERα fusion proteins were immunoprecipitated from nuclear extracts of 293T cells that were cotransfected with FLAG-tagged ERα and either Myc-tagged N-CoR or Myc-tagged SMRT. Strong anti-Myc antibody binding was observed on immunoblots of anti-FLAG immunoprecipitates from both Myc-N-CoR or Myc-SMRT cotransfectants treated with OHT or RAL (Fig. 1, lanes 3, 4, 8, and 9), whereas no coimmunoprecipitates were observed in cotransfectants treated with E2 or in the absence of ligand (Fig. 1, lanes 1, 2, 6, and 7). Weak interactions were observed in cotransfectants treated with pure anti-estrogen, ICI (Fig. 1, lanes 5 and 10).

Recently, it was shown that the RXR and TR LBDs associate with N-CoR or SMRT via the N-CoR/SMRT domains ID1 and ID2 (26, 29, 30, 37, 38) and that substitution of an isoleucine to arginine in H3 or a valine to arginine in H5 of TR or RXR, respectively, reduced N-CoR or SMRT binding (38). Therefore, to map the regions responsible for the interaction between ERα and N-CoR/SMRT, we first assessed whether GST-ID1 or -ID2 fusion proteins could associate with in vitro translated ERα. A schematic representation of ERα showing amino acid substitutions in helices H3, H4, and H5. The binding domain of ERα consists of helices H3 and H5.

To confirm the tamoxifen- or raloxifene-dependent interaction between ERα and N-CoR or SMRT by coimmunoprecipitation. Nuclear extracts were prepared from 293T cells coexpressing either FLAG-tagged ERα, ERα(I358R), ERα(G366T) or ERα(D376R) and either Myc-tagged N-CoR or Myc-SMRT. FLAG-ERα or ERα mutants were immunoprecipitated by an anti-FLAG antibody, and Myc-N-CoR or Myc-SMRT detected by immunoblotting with anti-Myc antibodies.

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GST-pull down assay showed that both GST-ID1 and -ID2 directly bound ERα in the presence of OHT (Fig. 2B) or RAL (data not shown). We then studied whether N-CoR or SMRT physically interacted with the ERo-LBD in the presence of OHT or RAL using a mammalian two-hybrid assay. Plasmid constructs consisting of a GAL4 DNA-binding domain fused with the DEF region (i.e., the LBD) of RXR (GAL-RXR) or ERα (GAL-DEF) were cotransfected with either VP16 transactivation domain-fused N-CoR (VP-N-CoR), SMRT (VP-SMRT) or coactivator TIF2 (VP-TIF2) constructs into 293T cells. In the presence of OHT or RAL, a strong interaction between the DEF region and N-CoR or SMRT was observed (Fig. 2C, lanes 22, 23, 26 and 27). As reported previously, unliganded-RXR was observed to bind N-CoR/SMRT. In the absence of ligand, a weak interaction between the DEF region and N-CoR or SMRT was detected (Fig. 2C, lanes 21 and 25), whereas no interaction was observed in the presence of E2 orICI (Fig. 2C, lanes 36, 37, 24 and 28). E2-dependent interaction between ERα and TIF2 was also detected by this assay (Fig. 2C, lane 38). These results indicated that the ID1 and ID2 in N-CoR/SMRT and the LBD in ERα were responsible for the interaction between corepressors and ERα.

We next evaluated corepressor binding to a series of ERα ligand-binding domain mutants within helices H3, H4, and H5. We introduced substitutions of isoleucine to arginine at residue 358 in H3 (ERα(D358R)), valine to arginine at 376 in H5 (ERα(V376R)), and glycine to threonine at 366 in H4 (ERα(G366T)) of ERα (Fig. 3A). In the case of TR and RXR, amino acid substitutions at equivalent positions 358, 376, and 366 in H3, H4, and H5, respectively, of ERα are known to reduce N-CoR/SMRT binding (21, 22, 23). These results showed reduced interaction of tamoxifen-bound ERα obtained from mamalian two-hybrid assays with N-CoR and SMRT appear to be components of HDACs that repress transcriptional activity (32–36). To investigate whether SMRT complexes recruited to ERα repress transcriptional activity of a thymidine kinase (TK) promoter located downstream from GAL4 binding elements (17m × 4). Consistent with previous studies, unliganded RXR repressed transactivation (Fig. 4, lane 25). In the presence of either OHT or RAL, basal transcriptional TK promoter activity was repressed ~40% when cotransfected with the GAL4-DEF constructs on the basal transcriptional activity of TR and RXR (38).

**OHT Recruits Histone Deacetylase Complexes to ERα**

We then investigated whether N-CoR or SMRT recruited to ERα repressed transcriptional activity of a thymidine kinase (TK) promoter located downstream from GAL4 binding elements (17m × 4). Consistent with previous studies, unliganded RXR repressed transactivation (Fig. 4, lane 25). In the presence of either OHT or RAL, basal transcriptional TK promoter activity was repressed ~40% when cotransfected with the GAL4-DEF constructs on the basal transcriptional activity of TR and RXR (38).

**Fig. 4. Tamoxifen-induced repressional activity of ERα via HDAC was reduced by amino acid substitutions in H3 or H5.** Tamoxifen-induced repressional activity of GAL-DEF on basal transcriptional activity of TK promoter with the GAL4 control (Fig. 4, lanes 3–8). These results suggested that the spectrum of corepressor interaction of tamoxifen-bound ERα was reduced, whereas no interaction was observed in the presence of E2 or ICI (Fig. 2C, lanes 36, 37, 24 and 28). E2-dependent interaction between ERα and TIF2 was also detected by this assay (Fig. 2C, lane 38). These results indicated that the ID1 and ID2 in N-CoR/SMRT and the LBD in ERα were responsible for the interaction between corepressors and ERα.

**Fig. 5. Reporter activity were evaluated. The I358R and V376R mutations, of which bind corepressor complexes in a OHT-dependent manner, were observed in tamoxifen-stimulated tumors. Therefore, we examined the binding of ERα(D351Y) to N-CoR or SMRT complexes using a mammalian two-hybrid assay. In this assay, only weak ERα(D351Y) would be unable to bind corepressor complexes. Moreover, the aspartate at position 351 is located within H3, which has been identified as part of the binding surface for corepressor complexes in the presence of tamoxifen (Fig. 5A). Therefore, we examined the binding of ERα(D351Y) to N-CoR or SMRT complexes using a mammalian two-hybrid assay. In this assay, only weak ERα(D351Y)-corepressor interactions were observed in the presence of OHT or RAL (Fig. 5B, lanes 13–18). The results of coimmunoprecipitation experiments confirmed the weak OHT- or RAL-dependent interactions between ERα(D351Y) and the coactivators (Fig. 5C, lanes 3, 4, 8, and 9). Furthermore, GAL-DEF(D351Y) also showed reduced repressational activity against basal TK transcription (Fig. 5D, lanes 8 and 9).

**Transcription Function of the ERα(D351Y) Mutant Is Enhanced by Both E2 and OHT**—Although the binding of corepressor complexes to both ERα(D358R) and ERα(G366T) was as weak as that for ERα, only the ERα(D351Y) mutation was observed in tamoxifen-stimulated tumors. Therefore, we next examined for functional differences between ERα(D351Y), ERα(D358R), and ERα(G366T). It is well known that tamoxifen blocks ERα AF-2 but induces AF-1 activity (59–63). In the presence of OHT or RAL, wild-type ERα and ERα(G366T), both of which bind corepressor complexes in an OHT-dependent manner, showed only weak AF-1 activity (Fig. 6A, lanes 6, 8, 11, and 15).
Amino acid substitution D351Y in ER\textsubscript{H9251} reduces tamoxifen- and raloxifene-dependent binding to corepressors, such as ER\textsubscript{H9251}/N-CoR, ER\textsubscript{H9251}/SMRT, or VP. Indicated are representative results of at least three independent experiments; error bars indicate standard deviation.

**DISCUSSION**

Cumulative data from several recent studies have indicated that ERα also binds N-CoR/SMRT in the presence of tamoxifen or raloxifene and that this interaction may be essential for the antagonistic effect of tamoxifen and raloxifene on ERα transcriptional activity (77–81). In this paper, we showed that the binding between tamoxifen or raloxifene-bound ERα and N-CoR/SMRT was mediated by the ID1 and ID2 domains of N-CoR/SMRT and the H3/H5 region of ERα-LBD. We also found that amino acid residues 358 and 376 in the H3/H5 region of ERα-LBD were important for the binding of both coactivator and corepressor complexes. These results were consistent with previous studies (58, 70–75) and our result that the D351Y mutant was activated by both ligands (Fig. 6A, lane 25).
The transcriptional activity of the ERα(D351Y) mutant is enhanced by both tamoxifen and estrogen. A, transcriptional activity of ERα or ERα mutants in the presence of E2. Bars indicate fold-change in luciferase activity relative to GAL-DEF and VP vector in the presence of E2.

B, estrogen-dependent recruitment of ERα and corepressors (Fig. 7). Indicated vectors were transfected into 293T cells along with a reporter plasmid bearing GAL4 binding elements (17 m8-luc). Bars indicate fold-change in luciferase activity relative to ERα/H9251 (100 nM), E2 (10 nM), or both OHT (100 nM) and E2 (10 nM). 293T cells were transfected with 0.25 g of luciferase reporter plasmid.

Several studies have indicated that the tamoxifen-dependent transcriptional activity of tamoxifen and raloxifene both possess a bulky side chain extension near the base of H12 that protrudes through the LBD surface near the base of H12 and folds back into the remainder of the hydrophobic cleft (41, 55, 85, 86). This extension displaces H12, which rotates the tertiary amine group in the tamoxifen and raloxifene extended side chains into a transcriptionally permissive state (16). However, while the D351Y mutant exhibited selective corepressor interaction, as expected, amino acid substitutions at Asp-351 might change the position of H12 that Asp-351 is thought to be key to the antagonistic character of these analogs (58, 70, 87). Thus, amino acid substitutions at Asp-351 might change the position of H12 induced by tamoxifen or raloxifene and prevent the formation of the interaction surface for corepressors.

While tamoxifen may exert its anti-estrogenic activity by silencing AF-2 transcriptional activity through the repositioning of H12 to block coactivator binding and promote corepressor recruitment, the agonist activity of tamoxifen is believed to be mediated through AF-1 in a cell- or tissue-dependent manner. Several studies have indicated that the tamoxifen-dependent AF-1 activity of D351Y mutant was increased compared with wild-type ERα (58, 70–75). However, previous characterization of the D351Y mutant found that the relative affinity for estradiol or tamoxifen was unaffected. In our study we found that the additional mutants I358R and V376R exhibited higher fold-change in luciferase activity in the presence of E2.

The crystal structures of tamoxifen and raloxifene-bound ERs revealed that the position of H12 is not required for corepressor interaction and in fact hinders corepressor interaction (38). We also found that deletion of H12 resulted in the potentiation of OHT-bound ERα to bind corepressors and repress transcription (data not shown). Therefore, it is possible that the tamoxifen-or raloxifene-induced positioning of H12 with respect to H3 and H5 may be important for not only blocking coactivator binding but also forming a interaction surface with the corepressor/nuclear receptor motifs found in N-CoR and SMRT. Indeed, E2-bound LBD bind coactivators, rather than corepressors, indicating that alternate H12 configurations create different recognition surfaces for coactivator or corepressor complexes.

The Asp-351 position of ERs was found to be mutated to a tyrosine in the MCF-7 breast tumor cell line that showed stimulated growth rather than inhibition by tamoxifen (68, 69). This D351Y mutant also exhibited increased tamoxifen- and raloxifene-induced activity of ERE-responsive genes. Since
tivity (33–36), suggesting that AF-1 coactivator and corepressor complexes may act and/or bind competitively to tamoxifen- or raloxifene-bound ERs. According to this hypothesis, the intercellular balance between corepressor and coactivator recognition and binding might be necessary for the acquisition of tamoxifen-stimulated tumor cell lines (68, 90). This raised the possibility that while tamoxifen-dependent interaction between ER and coactivator complexes may be the key event for the inhibition of ERα transactivation by tamoxifen, the disruption of this interaction may not be sufficient for the acquisition of a tamoxifen-stimulated growth phenotype. As ERα(D351Y) was the only mutant showing reduced N-CoR/SMRT binding that retained comparable estrogen response and binding ability to TIF2, it is possible that both the disruption of coactivator binding and retention of the overall spectrum of ERα/coactivator recognition and binding might be necessary for the acquisition of the tamoxifen-stimulated growth phenotype.

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