The effects of estrogen and anti-estrogen are mediated through the estrogen receptors (ER) α and β, which function as ligand-induced transcriptional factors. Recently, one of the phthalate esters, 2,5-bis(3-oxo-3-phenylpropyl)benzyl phthalate (BBP), has been shown to induce estrogen receptor-mediated responses. By using the truncated types of ER mutants, we revealed that activation function-1 (AF-1) activity was necessary for the BBP-dependent transactivation function of ERα. AF-1 is also known to be responsible for the partial agonistic activity of tamoxifen. Whereas tamoxifen exhibits an anti-estrogenic effect on proliferation of the MCF-7 breast cancer cell line, BBP showed an estrogenic effect on MCF-7 to stimulate proliferation. In vivo and in vitro binding assays revealed that whereas 4-hydroxytamoxifen (OHT) induced binding of ERα to both an AF-1 coactivator complex (p68/p72 and p300) and corepressor complexes (N-CoR/SMRT), BBP selectively enhanced the binding to the AF-1 coactivators. We also showed that the transcriptional activity of OHT-bound ERα was modulated by the ratio between the AF-1 coactivator and corepressor complexes.

Expression of a dominant-negative type of N-CoR inhibited the interaction between OHT-bound ERα and N-CoR/SMRT and enhanced the transcriptional activity of OHT-bound ERα. Furthermore, the cell growth of MCF-7 stably expressing the dominant-negative type of N-CoR was enhanced by the addition of OHT. These results indicated that fully activated AF-1 induces the stimulation of breast cancer growth and that the ratio between AF-1 coactivators and corepressors plays a key role to prevent proliferation of tumor by tamoxifen.

The effects of estrogens are mediated primarily via estrogen receptor α and β (ERα and -β), which are members of the nuclear hormone receptor superfamily. Estrogen (E2) binding to its receptor induces the ligand binding domain to undergo a characteristic conformational change, whereupon the receptor dimerizes, binds to DNA, and subsequently stimulates gene expression (1–7). ERα is stimulated by two distinct activation regions, activation function-1 (AF-1) and AF-2 (2–4, 8). AF-1, which is located in the N-terminal A/B domain, is constitutively activated in a cell-specific and promoter-specific manner (9). AF-2 is located in the C-terminal ligand binding domain (LBD) and exerts ligand-dependent transcriptional activity (10). AF-1 and AF-2 activate transcription independently and synergistically and act in a promoter-specific and cell-specific manner (11).

The ligand-dependent activation of ERα requires ligand-dependent association of coactivator complexes (12–15). The coactivator complexes for AF-2 contain histone acetylases p300/CBP (16, 17), p300/CBP-associated factor (pCAF) (18), p160 protein family members steroid receptor coactivator-1 (SRC-1) (19, 20), transcription intermediary factor 2 (TIF2) (21), p300/CBP-interacting protein (p/CIP) (22–24), non-acetylase vitamin D receptor-interacting protein/TR-associated proteins (DRIP/TRAP) (25–29) or transformation/transcription domain-associated protein (TRRAP) (30–32), and general control of amino acid protein-5 (GCN5) (32, 33). AF-1 transcriptional activity is enhanced by p300 and DEAD box protein p68/p72, which form a protein complex with p160 family proteins and p300/CBP, and directly bind to the A/B domain to potentiate AF-1 activity (34, 35). The phosphorylation of the serine residue at position 118 in the A/B domain stabilizes the complex formation of ERα and the coactivator complex containing p68/p72 to potentiate the AF-1 activity (34–36).

Estrogen is known to stimulate hormone-dependent tumors such as endometrial cancer and breast cancer (37). Recently, it was suggested that some endocrine disrupters may also contribute to the development of hormone-dependent cancers (38, 39). Several studies have demonstrated that many endocrine disrupters, such as 2,5-bis(3-oxo-3-phenylpropyl)benzyl phthalate (BBP), a phthalate ester used as a plasticizer, are capable of interacting with estrogen receptors and induce estrogen receptor-mediated responses, suggesting that estrogenic or anti-estrogenic effects elicited by these substances may be receptor-mediated (38–41).
For the endocrine therapy of these cancers, the development of inhibitory ligands for ERs has yielded important therapeutic treatments, including the use of tamoxifen (37, 42, 43). Tamoxifen exhibits a wide range of estrogen-like and anti-estrogen actions according to the target tissue examined (44). 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 (45–49). 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 (50–53). Wolf et al. (54, 55) 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 351 (ERα(D351Y)), located within the LBD of ERα (54, 55).

Recent studies (56–60) have suggested that tamoxifen promotes the binding of ERα to nuclear receptor corepressor (N-CoR) or related factors silencing mediator of retinoid and thyroid receptors (SMRT), which mediate repression by recruiting histone deacetylases (HDACs). We reported previously (61) that ERα (D351Y) exhibited reduced interaction with N-CoR and SMRT in the presence of 4-hydroxytamoxifen (OHT).

These observations raised the possibility that OHT-dependent and SMRT in the presence of 4-hydroxytamoxifen (OHT) may not bind to corepressors and enhanced the proliferation of the ERα/ER/estrogen receptor (ER) histone deacetylases (HDACs). We reported previously (61) that ERα (D351Y) exhibited reduced interaction with N-CoR and SMRT in the presence of 4-hydroxytamoxifen (OHT). These observations raised the possibility that OHT-dependent interaction between ERs and corepressor complexes may be essential for the anti-estrogenic effect of OHT and that abrogation of OHT-dependent binding of corepressors to ERα may convert OHT from antagonist to agonist to stimulate cancer growth.

In this paper, we identified BBP as an agonist for AF-1 of ERα. Although BBP exhibited the same properties as tamoxifen in a transient transfection assay, BBP–occupied ERα did not bind to corepressors and enhanced the proliferation of the breast cancer cell line MCF-7. The transcriptional activity of OHT-occupied ERα was modulated by the ratio of the expression levels between AF-1 coactivators and corepressors. Moreover, MCF-7 breast cancer cell lines expressing the dominant-negative type of N-CoR exhibited a growth phenotype in the presence of OHT. These results indicate that activation of AF-1 induces the stimulation of breast cancer growth and that the ratio between AF-1 coactivators and corepressors plays a key role to prevent proliferation of tumor by tamoxifen.

EXPERIMENTAL PROCEDURES

Materials—17β-Estradiol (E2) and OHT were purchased from Sigma. BBP was from Wako Chemicals Co., Japan. IC182,780 (ICI) was synthesized by Taiho Pharmaceutical Co.

Measuring IC₅₀ Values of E2 and BBP—For measurement of the binding constant value of BBP to ERα, IC₅₀ measuring kit was purchased from Wako Chemicals Co., and IC₅₀ was examined according to the manufacturer's protocol.

Plasmid Construction—The ERα β expression plasmids (HEGO/ERG0β) and their deletion mutants (HE19/HE19β) were described previously (3, 11, 61, 64). The p300, p68, p72, SRC-1, TRAP220, and TRRAP expression plasmids were also described previously (32, 33, 62–64). Human N-CoR cDNA was cloned into pEF1-V5-His A for V5-N-CoR. Reporter constructs (17m5-luc, MH100-tk-luc, and ERE3-tk-luc) have been described previously (35, 61, 64). The ligand binding domain of ERα was inserted into the pCM vector (Clontech) to generate GAL-DEF. VP-SRC-1, VP-TRAP220, and VP-p300 were described previously (11, 64). Nuclear receptor interaction region in TRRAP was previously (11, 64). The p300, p68, p72, SRC-1, TRAP220, and N-CoR plasmids were inserted into pVP16 vector to generate VP-TRRAP (32). C-terminal fragments of N-CoR and SMRT (including the NR interaction domains ID1 and ID2) were inserted into the pVP16 vector (Clontech) to generate VP-N-CoR, VP-SMRT, and pGEX-2T vector to GST-ID1-2 of N-CoR and SMRT and pcDNA3 vector (Invitrogen) for FLAG-N-CoR ID1-2. ERα mutation in amino acid replacement D351Y was introduced into full-length ERα and GAL-DEF plasmid by PCR-based point mutagenesis (Stratagene).

Transfection, Luciferase Assay, Mammalian Two-hybrid Assay, and Repression Assay—293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Two days before transfection, medium was changed to phenol red-free DMEM containing 5% charcoal-stripped FBS. Transfection was performed with Lipofectin reagent (Invitrogen) according to the manufacturer's protocol. For luciferase assays, 250 ng of ERE3-tk-luc plasmid was cotransfected with 25 ng of ER expression vector (HEGO/ERG0β) or mutants. For mammalian two-hybrid assays, 250 ng of ERE3-tk-luc or 17m5-luc vector was cotransfected with 250 ng of HEG0, GAL-DEF, or GAL-DEF(D351Y) in combination with 250 ng of indicated VP16-conjugated constructs and/or p300, p68, N-CoR, or N-CoR ID1-2 plasmids. For repression analysis, 1 µg of MH100-tk-luc vector was cotransfected with 250 ng of GAL-DEF. As a reference plasmid to normalize for transfection efficiency, either 5 ng of pRL-CMV vector (Promega) or 125 ng of pEG6Gal vector was transfected in all experiments. After transfection, culture medium was replaced with fresh medium containing 0.2% FBS. At this time, either E2 (10 nM), OHT (100 nM), ICI182,780 (100 nM), ethanol vehicle, or 5 ng/ml trichostatin A (TSA) was added, and cells were incubated for an additional 24 h. Preparation of cell extracts and luciferase assays were performed following the manufacturer's protocol (Promega). β-Galactosidase activity was measured to control the efficiency for each transfection. Individual transfections, each consisting of triplicate wells, were repeated at least three times. For establishing MCF-7 stable transfectant of N-CoR ID1-2, Lipofectin reagent was used for introduce pcDNA-ID1-2, and transfectants were selected by 500 µg/ml G418 (Sigma), and several clones were isolated.

GST Pull-down Assay—For GST pull-down assays, bacterially expressed GST fusion proteins or GST bound to glutathione-Sepharose 4B beads (Amersham Biosciences) were incubated on ice with [35S]methionine-labeled proteins expressed by in vitro translation using the TNT-coupled transcription-translation system (Promega). After 1 h of incubation, free proteins were removed by washing the beads 5 times with phosphate-buffered saline containing 10% glycogen and protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µM phenylmethylsulfonyl fluoride). Specifically bound proteins were eluted by boiling in SDS sample buffer and analyzed by 6% SDS-PAGE. After electrophoresis, radiolabeled proteins were visualized by autoradiography.

Cell Proliferation Assay—Two days before assay, MDA-MB-231 (ERα-negative) and MCF-7 (ERα-positive) cells were cultured in a 24-well plate in phenol red-free DMEM supplemented with 0.2% charcoal-stripped fetal bovine serum. As experimental medium, either E2 (10 nM), OHT (1 µM), BBP (1 µM), or ethanol vehicle was supplemented. Cells were harvested for the indicated times, and the number of viable cells was counted with hemocytometer.

RESULTS

BBP Binds Ligand-binding Pocket of ERα and Induces the Transcriptional Activity of AF-1—It has been reported that BBP binds to ERα and enhances the transcriptional activity of ERα. To confirm the binding of BBP to ERα, we performed an in vitro competitive ligand binding assay to investigate the abilities of BBP to compete with E2 for binding to ERα and β. E2
The transcriptional activity of ERα was measured by luciferase assay. Bars indicate fold change in luciferase activity relative to that of no ligand. Results represent the average of three independent experiments; error bars indicate S.D.

**FIG. 1.** BBP enhances the transactivation function of ERα. A, illustration of organic structures for BBP, OHT, and E2. B, BBP binds to the ligand pocket of ERs in vitro. Fluorescein-labeled estrogen bound to ERs was displaced competitively by increasing concentrations of E2 (squares) or BBP (triangles), and the ratio of displacement was measured using the luminescence meter. Displacement curves were obtained for E2 and BBP. The data were performed in triplicate and resulted from a representative experiment of the three repeated experiments. C, BBP enhances the transcriptional activity of ERα. 293T cells were transiently transfected with a plasmid expressing ERα (HEG0) or ERβ (ERG0) and a reporter plasmid bearing 3×-ER-responsive elements (ERE3-tk-luc) and incubated for 24 h with 10 nM to 100 μM of either E2, OHT, or BBP. Transcriptional activity of ERs was measured by luciferase assay. Bars indicate fold change in luciferase activity relative to that of no ligand. Results represent the average of three independent experiments; error bars indicate S.D.

BBP exhibited an IC_{50} of ~1.0 nM to both ERs (Fig. 1B), which is within the range of previously reported IC_{50} values (40, 65). The IC_{50} values for BBP to ERα and ERβ were ~50 μM, indicating that BBP weakly bound to both receptors (Fig. 1B). Therefore, we next evaluated the ability of BBP to induce a transcriptional response using a 293T cell line that were cotransfected with a luciferase reporter plasmid bearing estrogen response elements (ERE3-tk-luc) and either ERα or ERβ expression vector (HEG0) or ERβ expression vector (ERG0). The results in Fig. 1, C and D, show that BBP treatment of 293T cells transiently transfected with ERα caused a concentration-dependent increase in luciferase activity. However, the transcriptional activity of ERβ was not enhanced by BBP treatment (Fig. 1C). E2 treatment of either ERα- or ERβ-transfected cells significantly induced luciferase activity (Fig. 1C). Induction of luciferase activity by BBP in transiently transfected 293T cells was completely abolished if the ERα construct, HEG0, was not cotransfected. In addition, 100 nM of the “pure ER antagonist” ICI182,780 completely inhibited BBP-induced transcriptional activity of ERα (Fig. 1D, lanes 5 and 6). These results indicate that BBP acts as an ERα-selective agonist. To investigate the reason why BBP stimulates the transcriptional activity of ERα but not ERβ, BBP-dependent transcriptional activity of truncated types of ERs (Fig. 2A) was estimated by luciferase assay. E2 enhanced the transcriptional activity of full-length ERα and a truncated type of ERα, HE19 (Fig. 2B, lanes 2 and 6), which does not have an A/B region and exhibits no AF-1 activity. In contrast, BBP stimulated only the transcriptional activity of full-length ERα (Fig. 2B), suggesting that AF-1 is essential for the BBP-dependent transcriptional activity of ERα. It is known that the non-steroidal anti-estrogen tamoxifen, which is the most commonly used endocrine in the treatment of all stages of breast cancer in both pre- and postmenopausal women, also exhibits AF-1 agonistic activity (45–49). In this experiment, OHT induced full-length ERα-mediated transcriptional activity (Figs. 1C and 2B) but did not stimulate the transcriptional activity of HE19 (Fig. 2B), as expected. The transcriptional activity induced by BBP was about three times higher than that induced by OHT (Fig. 2B, compare lane 3 to 4). These results raise the possibility that BBP may possess the same biological properties as tamoxifen.
A Contrastive Effect between BBP and OHT on the Proliferation of Breast Cancer Cells—Thus, we next examined the effects of BBP on proliferation of two human breast cancer cell lines: MCF-7 and MDA-MB-231. The MCF-7 cell line expresses ERs, and its proliferation is estrogen-dependent. MDA-MB-231 cells, whose growth is not affected by estrogens, were used to control for false-positive responses. Whereas E2 induced MCF-7 cell proliferation at concentrations at 10 nM, OHT strongly inhibited the proliferation of MCF-7 cells (Fig. 2C, right panel) (41). In contrast with OHT, BBP exerted an increase in MCF-7 proliferation at 1 to 10 μM, and the maximum effect represented 70% of the presence of E2 (Fig. 2C, right panel). None of the three compounds we tested affected proliferation of MDA-MB-231, which does not express ERα (Fig. 2C, left panel).

To investigate whether the AF-1 activity is necessary for the BBP-dependent cell proliferation, newly synthesized DNA was evaluated by BrdUrd incorporation method. NIH3T3 fibroblasts were transiently transfected with expression plasmid encoding either ERα (HEG0) or ERα mutant (HE19). The transfected NIH3T3 fibroblasts were made quiescent and then stimulated for 24 h with either E2, BBP, or OHT (86). BrdUrd was added to the medium together with the ligands, and its incorporation into DNA was analyzed. BrdUrd-positive cells expressing either ERα or HE19 were counted. The NIH3T3 cells transfected with control vector did not respond to ligand treatments. Although E2 and BBP strongly stimulated progression of ERα-transfected NIH3T3 cells toward S-phase, BrdUrd incorporation into HE19-transfected cells was enhanced by E2 but not by BBP (Fig. 2D). These results indicate that the BBP-dependent S-phase entry requires the AF-1 activity of ERα.

BBP and OHT Induce the Binding of ERα to AF-1 Coactivators but Not to AF-2 Coactivators—Our results indicate that both BBP and OHT are selective agonists for ERα AF-1 and show an antagonistic effect on AF-2, whereas BBP exhibits an estrogenic effect and OHT shows an anti-estrogenic effect on the cell proliferation. In order to reveal a reason for this discrepancy, we tested the interaction between ERα and coactivators or corepressors in the presence of either BBP or tamoxifen. By using a mammalian two-hybrid assay, we first evaluated the BBP- or OHT-dependent binding of AF-1 coactivators to ERα. HEG0 was cotransfected with either VP16 transactivating domain-fused p72 (VP-p72), p68 (VP16-p68), or p300 (VP-p300) constructs into 293T cells. All of the three ligands we tested induced the interaction of ERα with either p68, p72, or p300 (Fig. 3A). The BBP-dependent interaction between ERα and AF-1 coactivators was confirmed using a coimmunoprecipitation method. By using an anti-ERα antibody, ERα was immunoprecipitated from a nuclear extract of 293T cells that were cotransfected with ERα and either FLAG-tagged p72, FLAG-tagged p68, or p300. Strong anti-FLAG or anti-p300 antibody binding was observed on immunoblots of anti-ERα immunoprecipitates from cotransfectants treated with either E2, BBP, or OHT (Fig. 3B).
We then examined the binding of the DEF region of ERα with AF-2 coactivators. A GST pull-down assay showed that whereas E2 induced the direct binding of ERα(GAL-DEF) to AF-2 coactivators SRC-1, TRAP220, or TRRAP, BBP and OHT abrogated the interaction between GAL-DEF and AF-2 coactivators (Fig. 3C). In a mammalian two-hybrid experiment, E2-bound GAL-DEF exhibited significant binding to either SRC-1, TRAP220, or TRRAP (Fig. 3D, left panel). Conversely, neither BBP nor OHT induced the interaction between GAL-DEF and AF-2 coactivators (Fig. 3D, middle and right panels). These results were in good agreement with the observation that BBP and OHT act as antagonists for AF-2.

**OHT but Not BBP Induces the Interaction between ERα and Corepressor Complexes**—In a previous paper, we showed that OHT induces the binding between GAL-DEF and corepressor complexes to repress the basal transcriptional activity of TK promoter located downstream from GAL4-binding elements (17 × 5 m) (61). This OHT-induced binding of ERα to corepressor complexes is reduced by the amino acid substitution at position 351 in ERα (ERα(D351Y)), which is derived from tamoxifen-induced tumors (Fig. 4A) (54, 55). Consistent with previous studies, OHT-bound GAL-DEF repressed the transcriptional activity of the TK promoter (Fig. 4B, lane 2). This repressive activity was inhibited by the addition of TSA, a specific histone deacetylase inhibitor (Fig. 4B, lane 5). The D351Y mutation, which exhibited decreased corepressor association, impaired the tamoxifen-dependent repression by GAL-DEF (Fig. 4B, lane 5). In the presence of BBP, the repressive activity of GAL-DEF was not observed (Fig. 4B, lane 3), indicating that BBP-bound ERα would not interact with corepressor complexes.

To test if the interaction between ERα and corepressors, a mammalian two-hybrid assay was performed. Either GAL-DEF or GAL-DEF(D351Y) was cotransfected into 293T cells with either VP, VP-SMRT, or VP-N-CoR. Whereas the interaction between GAL-DEF and corepressors was observed in the presence of OHT (Fig. 4C, middle panel), BBP-bound GAL-DEF exhibited no binding to corepressors as expected (Fig. 4C, right panel). Consistent with previous studies, the D351Y mutation reduced the OHT-induced interaction with corepressors (Fig. 4C, lanes 10–12) (61). The corepressor binding was further investigated by a coimmunoprecipitation method. By using an anti-ERα antibody, ERα was immunoprecipitated from nuclear extracts of 293T cells that were cotransfected with ERα and V5-tagged N-CoR. In the presence of OHT, anti-V5 antibody binding was observed on immunoblots of anti-ERα immunoprecipitates from cotransfectants (Fig. 4D, lane 5). In contrast, N-CoR was not coprecipitated with E2- or BBP-bound ERα (Fig. 4D, lanes 4 and 6).

The **Ratio of AF-1 Coactivator/Corepressor Complexes in Cells Is an Essential Determinant for the Transcriptional Activity of OHT-occupied ERα**—Our results indicated that al-
through OHT induced the binding of ERα to both AF-1 coactivators and corepressors, BBP-bound ERα selectively associated with AF-1 coactivators. Thus we next examined the effect of AF-1 coactivators and corepressors on transcriptional activity of ERα induced by OHT or BBP. The expression of p300 and p68 enhanced the transcriptional activity of ERα in the presence of E2, OHT, or BBP (Fig. 5A). On the contrary, N-CoR reduced OHT-dependent transactivation function of ERα (Fig. 5B, middle panel) but not E2- and BBP-dependent activation (Fig. 5B, left and right panels). The stimulation of OHT-dependent transcriptional activity of ERα(D351Y) by the expression of AF-1 coactivators was much higher than that of wild-type ERα (Fig. 5A, compare left to middle panel), suggesting that the association of endogenous corepressor complexes with OHT-bound ERα reduces AF-1 activity. The maximum OHT-dependent transcriptional activity induced by coactivator expression was comparable with the BBP-dependent activity (Fig. 5A, compare lanes 14–16), raising the possibility that the overexpression of coactivators may convert OHT from antagonist into agonist to stimulate cancer growth. In addition, as shown in Fig. 5D, enhancement of transcriptional activity by the coactivator expression was reduced by the expression of corepressor, N-CoR. These findings indicate that the ratio of coactivator/corepressor in cells determines the transcriptional activity of OHT-bound ERα and that the inhibition of corepressor binding to ERα may convert OHT from partial agonist to full agonist for AF-1 to stimulate cell growth of MCF-7.

Inhibition of the Interaction between OHT-bound ERα and Corepressors Stimulates Proliferation of Breast Cancer Cells in the Presence of OHT.—To investigate this hypothesis, we first determined the regions in N-CoR/SMRT, which are responsible for the interaction between N-CoR/SMRT and OHT-occupied ERα. Recently, it was shown that retinoid X receptor and TR LBDs associate with N-CoR/SMRT via the N-CoR/SMRT domains ID1 and ID2 (66–69). Therefore, we assessed whether GST-ID1-2 fusion proteins could associate with in vitro translated ERα. A GST pull-down assay showed both GST-ID1-2 derived from N-CoR and SMRT directly bound to ERα (Fig. 6B). In this assay, the binding of N-CoR/SMRT to OHT-bound ERα was inhibited by the expression of the ID1-2 region derived from N-CoR (Fig. 6C). Coexpression of the ID1-2 region with ERα enhanced the OHT-dependent transactivation function of ERα (Fig. 6D, left panel) but not that of ERα(D351Y).
Fig. 6A. A competitive effect between AF-1 coactivator and corepressor complexes on the OHT-induced transcriptional activity of ERα. Bars indicate fold change in luciferase activity relative to ERα in the absence of ligand. Results represent the average of at least three independent experiments; error bars indicate S.D.

**DISCUSSION**

BBP is a phthallic ester that is present in papers and paperboards used as packaging materials for aqueous, fatty, and dry foods (38, 40, 41). BBP has been shown to possess estrogenic properties in *vitro* and in *vivo* (40, 41). We showed that whereas BBP bound to ERα and ERβ, it stimulated the transcriptional activity of ERα but not that of ERβ. The results obtained from the experiments using the truncated type of ERα, HE19, revealed that the A/B region of ERα was responsible for the transactivation induced by BBP. This property of BBP resembles the property of tamoxifen which engenders a conformational change in the ligand binding domain distinct from that induced by E2 and inhibits the activity of the hormone-dependent AF-2 but not AF-1 (70, 71). Whereas both BBP and tamoxifen acted as an AF-1-selective agonist, BBP and tamoxifen exhibited a contrastive effect on proliferation of the breast cancer cell line MCF-7. OHT showed an anti-estrogenic effect on MCF-7 cells, and BBP had an estrogenic effect to stimulate proliferation of MCF-7.

The Structure of LBD Induced by BBP Is Different from That Induced by OHT—Accumulating evidence suggests that the
differential ability of partial antagonists to modify gene expression cannot be accounted for by alterations in the ligand-receptor complex alone but also must take into consideration coregulator (coactivator and corepressor) proteins that regulate ER interaction with the general transcriptional machinery and chromatin (15, 32, 72–75). Therefore, coactivators and corepressors of the ERα were tested to determine whether these coregulators interact with ERα in the presence of these compounds. All of the three compounds we tested induced the interaction between ERα and AF-1 coactivators p68/p72 and p300 as expected. E2 induced the binding of LBD to AF-2 coactivators but not to corepressors (32, 59, 74, 75, 78). Consistent with previous reports (70, 71), OHT induced the interaction with corepressors instead of AF-2 coactivators. In contrast, BBP-occupied LBD bound to neither AF-2 coactivators nor corepressors.

The crystal structures of the LBDs of several nuclear receptors have been determined and described as a sandwich of 12 α-helices (H1–H12) with a central hydrophobic ligand-binding pocket (70, 71, 76, 77). In the presence of ligands, 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 (70, 71). This hydrophobic groove is known to be important for interaction with LXXLL motifs found in p160 family members (SRC-1, transcription intermediary factor 2, and p300/CBP-interacting protein) as well as in other coactivator molecules (49, 71, 79, 80). The structures of tamoxifen-bound ERα revealed that the position of H12 differed compared with that of H12 in E2-bound ERα and did not form a coactivator interaction surface but a recognition surface for corepressor complexes (70, 71). We observed that BBP induced the binding of LBD to neither AF-2 coactivators nor corepressors, indicating that the position of H12 in BBP-bound ERα is different from that of H12 in E2- or OHT-bound ERα. These results also suggest that the structural differences induced by compound binding would affect the binding affinity of ERα to the coregulators.

The Ratio between AF-1 Coactivator and Corepressor Complexes in Cells Is a Major Determinant of the Transactivation of OHT-occupied ERα—Our results indicated that OHT induced the binding of ERα to both AF-1 coactivators and corepressors. The enhancement of transcriptional activity induced by coactivator expression was reduced by the corepressor expression, indicating that the ratio of coactivator/corepressor in cells determines the transcriptional activity of OHT-bound ERα. It is well known that p300 possesses histone acetyltransferase activity that modifies local chromatin structure into a transcriptionally permissive state (16, 17). N-CoR/SMRT complexes contain histone deacetylase activity (62, 81–83), suggesting that AF-1 coactivator and corepressor complexes may act and/or bind competitively to tamoxifen-bound ERα. If the OHT-occupied ERα simultaneously binds coactivators and corepressors under these conditions, the repressor domain of corepressors may inhibit ERα transcriptional activity by blocking the activation function of coactivators. Alternatively, OHT may induce competitive binding between AF-1 coactivators and corepressors to abrogate full AF-1 activity. OHT may induce an LBD
conformation that enables the receptor to retain its ability to interact with corepressor to decrease the accessibility of AF-1 coactivators to the A/B region such that AF-1 activity is not efficiently activated.

Enhancement of the AF-1 Activity Stimulates Cancer Cell Growth—In a previous paper, we showed that the ERα/D351Y mutant, which is derived from an MCF-7 breast tumor cell line that showed stimulated growth rather than inhibition by tamoxifen, exhibited reduced OHT-dependent interaction with corepressors to increase OHT-induced AF-1 activity compared with wild-type ERα (61). The AF-1 activity induced by BBP was comparable with that of OHT-bound ERα(D351Y), since BBP induced the binding of ERα to AF-1 coactivators but not to corepressors. These results raise the possibility that fully activated AF-1 induces the proliferation of breast cancer cells and that the binding of corepressors to ERα is essential for the antagonistic effect of OHT to inhibit proliferation of breast cancer cells. Therefore, we generated cell lines stably expressing ID1-2 region of N-CoR to abrogate OHT-dependent binding of ERα to corepressors. The stable transformants expressing the ID1-2 region exhibited the OHT-stimulated growth phenotype, indicating that the binding between OHT-occupied ERα and corepressors plays a key role in inhibition of tumor growth by tamoxifen. In addition, these results also indicate that fully activated AF-1 stimulates cancer growth.

Tamoxifen is an effective treatment for all stages of hormone-responsive breast cancer and can prevent breast cancer in high-risk women (44, 84). However, tamoxifen has a partial...
estrogenic activity in the uterus and is associated with an increased incidence of endometrial hyperplasia and cancer. Recently, Brown and co-workers (75) showed that the expression level of SRC-1, a coactivator for ERα, is higher in an endometrial cancer cell line, Ishikawa, than in MCF-7. SRC-1 silencing by small interfering RNA in Ishikawa cells resulted in inhibition of tamoxifen-stimulated cell cycle progression. Hashimoto, H. G. (1998) Cell 94, 343–352.

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Full Activation of Estrogen Receptor α Activation Function-1 Induces Proliferation of Breast Cancer Cells

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