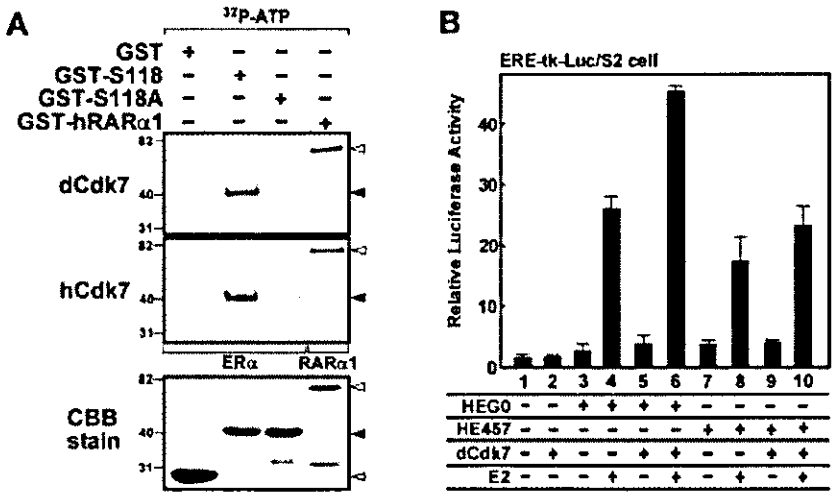

Figure 5 *In vitro* phosphorylation of hERα at Ser[118] by dCdk7. (A) Ten micrograms of GST-fused hERα (amino acids 56–180) and GST-fused hRARα1 were incubated with 9 μg dCdk7 or hCdk7. Phosphorylation and expression of GST-fused hERα amino acids (open arrow head), GST-fused hRARα1 (black arrow head), and GST (grey arrow head) were detected by autoradiography and CBB staining, respectively. (B) Schneider cells were co-transfected with 0.5 μg dCdk7 expression plasmid, 0.5 μg ERE-tk-luc reporter plasmid, 0.2 μg actin-GAL4 plasmid and hERα mutants and then incubated with or without 10^{-8} M E2. Luciferase activity data are shown as the average and standard deviation of three independent experiments.

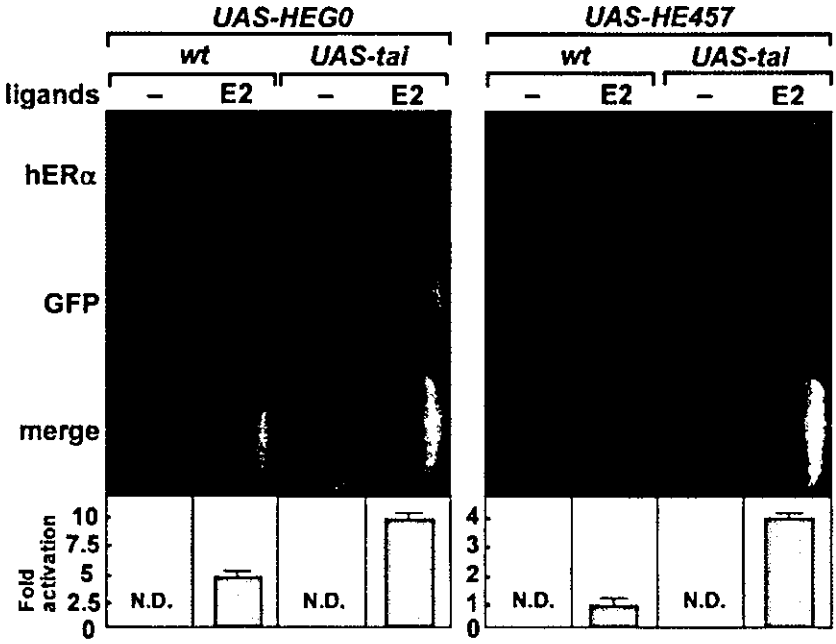

Figure 6 TAI enhancement of hERα transactivation is not dependent on Ser[118] phosphorylation status. hERα expression (red) and transactivation (green) in eye imaginal discs of either HEG0 or HE457 expression lines are shown. TAI is also expressed driven by *GMR-GAL4*.

addressed a question whether enhancement of hERα transactivation by the p160/CBP complex is dependent on the receptor Ser[118] phosphorylation status. Although the hERα S118A mutant was less effective in the ligand-induced transactivation, TAI significantly enhanced transcriptional activity of both the mutant and the wild-type receptor (Fig. 6). This suggests that modulation of the ligand-induced hERα transactivation by the p160/CBP co-activator complex does not depend on the receptor phosphorylation.

Discussion

hERα expressed in *Drosophila* is functional in ligand-induced transactivation

We have previously shown that the human androgen receptor (hAR) ectopically expressed in *Drosophila* tissues was transcriptionally functional and responsive to hAR ligands similar to that in mammalian cultured cells and intact tissues (Takeyama *et al.* 2002). In the present study, we utilized the same approach and demonstrated

that hERα expressed in *Drosophila* was able to activate the ERE reporter gene and respond to hERα agonists and antagonists in the same manner that had been observed in mammalian cells and tissues (McDonnell *et al.* 1995; Metzger *et al.* 1995; Watanabe *et al.* 2001). As hERα transgenic flies appear to be normal in terms of growth and reproduction, without any overt abnormalities, it seems that human steroid hormone receptors do not significantly interfere with endogenous signalling pathways. It can also be inferred that exogenous human steroid receptors do not compete with endogenous NRs at the fly NR-responsive elements in target gene promoters (Talbot *et al.* 1993; McKenna & O'Malley 2002). Therefore, our results provided evidence that transgenic *Drosophila* expressing hERα represent a potent and functionally relevant system in which to evaluate NR synthetic ligands and to genetically identify and characterize novel NR co-regulators.

Pivotal role of Ser^{118} in the hERα ligand-induced transactivation function *in vivo*

Both N-terminal AF-1 and C-terminal AF-2 domains contribute to the hERα ligand-induced transactivation function, with each AF-1 and AF-2 activity dependent on promoter-context and cell type (Kumar *et al.* 1987; Tora *et al.* 1989). The balance between hERα AF-1 and AF-2 is thought to be responsible, at least in part, for the tissue-specific action of selective oestrogen receptor modulators (SERMs) such as tamoxifen (Berry *et al.* 1990; McDonnell *et al.* 1995; Metzger *et al.* 1995; Brzozowski *et al.* 1997; Shiau *et al.* 1998). In particular, the activity of hERα AF-1 is believed to support the oestrogenic actions of SERMs (Endoh *et al.* 1999; Watanabe *et al.* 2001), leading to beneficial actions of SERMs in certain tissues such as the improved bone properties in oestrogen-related pathophysiological states (Shang & Brown 2002). Therefore, while the physiological and pharmacological significance of hERα AF-1 activity has been well addressed, the molecular basis underlying AF-1 function remains to be elucidated in terms of identifying the relevant specific co-regulators and co-regulator complexes (Endoh *et al.* 1999; Watanabe *et al.* 2001). The core activation region of hERα AF-1 has been mapped to the middle of the A/B domain (Kobayashi *et al.* 2000), and a number of *in vitro* studies have indicated that the Ser^{118} residue in this core region appears to play a crucial role and can be phosphorylated by several kinases in response to extracellular signals (Kato *et al.* 1995; Chen *et al.* 2000). Nevertheless, the impact of Ser^{118} phosphorylation *in vivo* remains obscure because of lack of studies involving intact animals. The present findings provide for the first time *in vivo* evidence for the significance of Ser^{118} phosphorylation in the transcriptional activity of the AF-1 domain alone and in the transactivation function of hERα as a whole receptor.

In vivo potentiation of hERα AF-1 through Cdk7-mediated phosphorylation of Ser^{118}

It has been shown that hERα Ser^{118} can be phosphorylated by several kinases (Ali *et al.* 1993; Le *et al.* 1994; Kato *et al.* 1995; Chen *et al.* 2000). Cdk7 has been chosen for the present study as mutant flies with inactive Cdk7 appear to suffer more general defects in gene regulation (Austin & Biggin 1996). We have shown that Cdk7 phosphorylates hERα Ser^{118} *in vivo* and that this phosphorylation enhanced hERα AF-1 activity in normal flies. It has been shown recently that, besides direct receptor phosphorylation, MAPKs are also able to potentiate function of some hERα co-activators, including AIB1, through phosphorylation of the cofactor protein (Font de Mora & Brown 2000). This suggests an additional mechanism for downstream cross-talk between different signalling pathways. Our transgenic *Drosophila* provides an experimental system in which to further study whether MAPKs activated by growth factors or stress-induced signalling pathways can also modulate hERα activity.

Ser^{118} phosphorylation-dependent and -independent co-activators for hERα

The S118A hERα mutant retained ligand responsiveness, albeit with reduced transactivation. Transactivation in the S118A hERα mutant has nevertheless been significantly enhanced by over-expression of TAI, *Drosophila* AIB1 homologue. Therefore, it appears that hERα activity is modulated *in vivo* by both phosphorylation-dependent and phosphorylation-independent co-activators. However, the timing of the recruitment of these co-activators, presumably within co-factor complexes associated with the AF-1 domain, remains unclear. p68/p72 have been identified as hERα AF-1-specific co-activators that physically associate with the hERα AF-1 domain (Endoh *et al.* 1999; Watanabe *et al.* 2001). Significantly, this interaction was clearly not dependent on Ser^{118} phosphorylation. It is not clear, however, whether recruitment of most of known hERα co-activators is dependent on phosphorylation status of the receptor. In this respect, the transgenic *Drosophila* lines that express hERα and its mutants represent a powerful tool for genetic screening of phosphorylation-dependent and -independent co-factors.

Experimental procedures

Transfection and luciferase activity

hERα mutants and dCdk7 expression vectors were constructed using the pCaSpeR vector for expression in Schneider cells. hERα mutants and dCdk7 expression plasmids (0.05 μg) were co-transfected with 0.2 μg actin-GAL4 plasmid and 0.5 μg ERE-tk-luc plasmid, along with 10 ng pRL-CMV-luc plasmid as an internal control. Three hours after transfection, the ligands 10^{-8} M 17β-oestradiol (Sigma, St Louis, MO), 10^{-8} M tamoxifen (Sigma) or 10^{-8} M ICI 182.780 (Tocris Cookson, Ballwin, MO) were added. After 20 h, dual luciferase assays were performed as previously described (Yanagisawa *et al.* 2002).

Generation of transgenic flies and *Drosophila* stocks

For germ-line transformation into *Drosophila*, cDNA encoding hERα mutants and GFP reporter under control of ERE-containing promoter were inserted into pCaSpeR. Transgenic constructs together with pπ25.7wc transposase were microinjected into w^{1118} embryos using a micromanipulator (Leica). Several independent transformant lines were established. To express hERα in *Drosophila* eyes, transgenic lines were crossed with a *GMR-GAL4* line that expressed GAL4 in the retina under the control of the glass multimer reporter. The *tai*^{k05809}, *UAS-tai*, *Df(1)J8254*-Pw^{+}]*snf*$^{+}$, *dhd*$^{+}$] and *cdk7*^{6} mutants were obtained from the Bloomington *Drosophila* Stock Center. The *nej*^{1} and *GMR-GAL4* line were the generous gifts of Drs S. Ishii and Y. Hiromi, respectively.

Histology

Eye imaginal discs from third instar larvae were dissected and fixed for 20 min in 4% formaldehyde at 25 °C. Eye discs were incubated with primary antibodies HC-20 (Santa Cruz Biotechnology, Santa Cruz, CA) or B10 that recognize the C- and N-terminal regions of hERα, respectively. Cy5-conjugated Affinity Pure donkey anti-rabbit or anti-mouse IgG (Jackson Immuno-Research, West Grove, PA) were used as secondary antibodies for immunofluorescence staining. hERα and GFP expression were detected using a Zeiss Confocal Laser Scanning System 510.

Western blotting

To confirm hERα and GFP expression in *Drosophila*, cell lysates from the heads of adult flies of third instar larvae were separated by 15% SDS–PAGE and detected with anti-ERα antibodies (HC-20 or B10) and anti-GFP antibody (Santa Cruz Biotechnology), and expression levels measured using Adobe Photoshop software facility. Fold-activation of hERα in *Drosophila* was shown as GFP expression signal intensity normalizing with hERα expression signal intensity.

In vitro phosphorylation

293T cells were transfected with FLAG tagged dCdk7 expression plasmid, lysed in lysis buffer, and immunoprecipitated with anti-FLAG affinity gel (Sigma). hCdk7 was obtained from 293T cells by immunoprecipitation with Cdk7 (N-19) antibody (Santa Cruz Biotechnology). dCdk7 or hCdk7 (9 μg) were incubated for 20 min at 30 °C with purified bacterially produced 10 μg of GST-fused hERα (amino acids 56–180 of hERα) and its mutants or GST-fused human retinoic acid receptor α1 (hRARα1) (Rochette-Egly *et al.* 1997), in 50 mM Tris-HCl, 0.5 mM EDTA, 25 mM $MgCl_2$, 1 mM DTT, 20 μM ATP, 0.01 μCi [γ-^{32}P]ATP and 10% glycerol. Phosphorylation of substrates was analysed by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Expression of GST-hERα mutants and GST-hRARα1 were detected by CBB staining.

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Wnt/β-Catenin and Estrogen Signaling Converge *in Vivo**

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Wnt and estrogen signaling represent important regulatory pathways, each controlling a wide range of biological processes. While an increasing number of observations suggest potential convergence between these pathways, no direct evidence of their functional interaction has been reported. Using human colon and breast cancer cells, we found that estrogen receptor (ER) α- and β-catenin precipitated within the same immunocomplexes, reciprocally enhanced the transactivation of cognate reporter genes, and were reciprocally recruited to cognate response elements in the promoters of endogenous target genes. Using transgenic *Drosophila* that ectopically expressed human ERα alone or together with metabolically stable β-catenin/Armadillo mutants, we demonstrated genetic interaction between these signal transducers *in vivo*. Thus, we present here the first direct evidence of cross-talk between Wnt and estrogen signaling pathways via functional interaction between β-catenin and ERα.

Estrogens regulate a plethora of physiological functions in the developing and adult organism and act predominantly via the activation of ERα[1] and ERβ. Liganded ER dimers bind to promoter estrogen response elements (EREs) and regulate the transcription of target genes. This ER-mediated regulation requires the recruitment of different co-factor complexes and is associated with rearrangement of chromatin structure at EREs within target gene promoters (1, 2). ER can also act as a co-factor at non-ERE sites via interaction with other DNA-bound transcriptional factor complexes, such as c-Jun/c-Fos on the AP-1 site (3) or c-Jun/NFκB on the tumor necrosis factor response element (4). The physiological significance of ERs is demonstrated by the severe abnormalities in development and function of major organs and tissues in mice with ablated ERα and/or ERβ (5). Also, both positive and negative impacts of estrogens in different types of cancer have been well documented (6).

Wnt signaling plays a critical role in numerous processes of development and in adult tissues and appears to be conserved across all animal taxa. β-Catenin is an intracellular transducer of canonical Wnt or Wnt/β-catenin signaling and, thus, has a dual function: as a transcriptional factor and, in a cadherin-bound form, as a regulator of cell adhesion and migration. Cytoplasmic or signaling β-catenin is unstable and rapidly targeted to phosphorylation-ubiquitination-coupled proteasomal degradation. Wnt signaling inhibits this degradation, resulting in the accumulation of β-catenin in the nucleus and its association with members of the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcriptional factors that leads to the activation of Wnt target genes. Mutations that increase the stability of cytoplasmic β-catenin have been implicated in numerous malignant transformations and represent a leading cause of colorectal tumorigenesis (7–9).

Consistent with the concept of morphogen gradients (10) β-catenin exerts different biological effects, such as induction of cell proliferation and apoptosis or stimulation and repression of the same target genes, in a threshold-dependent manner (11, 12). Thus, slight modulation of β-catenin signaling through cross-talk with other pathways may trigger serious physiological consequences. Potential cross-talk between Wnt/β-catenin and estrogen signaling *in vivo* has been implicated in physiological studies on tissues as different as brain (13) and uterus (14). Furthermore, although males and females develop colorectal cancer with approximately the same frequency, its incidence rate is significantly lower in women undergoing hormone replacement therapy (15, 16). While these and other observations suggested the possibility of functional interaction between ER and β-catenin, previous attempts failed to detect such an interaction (13, 17, 18), and no direct evidence of Wnt and estrogen signaling pathway convergence has been reported.

Compared with vertebrates, Wnt signaling has been far better characterized in *Drosophila*, in which it is not obscured by involvement of other, evolutionary more recent multiple pathways. Thus, *Drosophila* provides a powerful experimental system for analysis of functional interaction *in vivo* between Wnt signaling and other regulatory pathways, including those immerged at the later stages of evolution. Therefore, in addition to mammalian cells, to detect functional interaction between Wnt/β-catenin and estrogen signaling *in vivo* we used transgenic *Drosophila* that ectopically expressed human ERα coupled to an ERE-dependent green fluorescent protein (GFP) reporter gene alone or together with constitutively active mutants of Armadillo, a *Drosophila* homologue of β-catenin. Using different approaches, we obtained in this study the first evidence of physical association and transcriptional and genetic interaction *in vivo* between ERα and β-catenin.

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[1] The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; TCF, T cell factor; LEF, lymphoid enhancer factor; TBE, TCF/LEF binding element; CSFCS, charcoal-stripped fetal calf serum; ChIP, chromatin immunoprecipitation; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; LBD, ligand binding domain; wt, wild-type.

EXPERIMENTAL PROCEDURES

Immunoprecipitation and Immunoblotting—Cells grown in the presence of charcoal-stripped fetal calf serum (CSFCS) were transfected with FLAG-hERα expression vector and harvested 28–30 h post-transfection, after treatment for 3 h with vehicle (ethanol) or 10^{-8} M 17β-estradiol (Sigma), tamoxifen (Sigma), or ICI 182,780 (Tocris). Anti-β-catenin E-5 or H-102 antibodies (Santa Cruz Biotechnology) or preimmune rabbit serum IgG (as a negative control) were used for immunoprecipitation. Western blots were visualized with anti-FLAG M2 (Sigma) or anti-ERα HC-20 (Santa Cruz Biotechnology) antibodies.

Transfection and Reporter Assay—Cells grown in Opti-MEM, 5% CSFCS were transfected with 250 ng of reporter (ERE-tk-luc or tk-luc for MCF7 cells and TOPFLASH or FOPFLASH for colon cancer cells) and 1 ng of pRl (Promega) plasmid (control for transfection efficiency) together with 100 ng of empty (control) or cDNA (β-catenin S33Y for MCF7 cells and ERα for colon cancer cells) expression vector and treated for 16–20 h with vehicle or 10^{-8} M ligand, as indicated. To nullify nonspecific effects on basal promoters, TOPFLASH and ERE-tk-luc reporter activities were normalized against FOPFLASH and tk-Luc reporter activities, respectively, from parallel experiments.

Chromatin Immunoprecipitation (ChIP) Assay—Association of ERα and β-catenin with ERE in the pS2 gene promoter (19) and TCF/LEF binding element (TBE) in the Axin2 gene promoter (20) was analyzed using the Chromatin Immunoprecipitation Kit (Upstate Biotechnology) and HC-20 or E-5 antibody, respectively. As a control for nonspecific chromatin precipitation with these antibodies, a set of primers was used to amplify a pS2 gene DNA segment that does not have ERE or TBE sequences. In addition, IgG from normal preimmune rabbit serum was used as a negative control.

Histology and Immunostaining—All techniques were performed as described previously (21, 22). Expression of ERα and GFP in *Drosophila* eye discs were detected using Zeiss Confocal Laser Scanning System 510 and quantified by calculation of pixels of the corresponding signals using Adobe Photoshop 7 software facilities. TUNEL labeling was performed using the TACS2 TdT-Fluor *In Situ* Apoptosis Detection Kit (Trevigen).

Drosophila Lines and Stocks—The *UAS-ΔArm* and *UAS-ArmS10* mutants were obtained from the Bloomington *Drosophila* Stock Center. Generation and characterization of the used *UAS-ERα*, *ERE-GFP* transgenic *Drosophila* lines were described in Ref. 23. Briefly, cDNA encoding full-length human ERα, ligand binding domain (LBD) deletion mutant ERα-(1–302), or GFP reporter under control an ERE containing promoter were recloned into the pCaSpeR vector. Transgene constructs together with pπ25.7wc transposase were microinjected into w^{1118} embryos using a micromanipulator (Leica). Several independent transformant lines have been generated. To target ERα expression into the eye disc, transgenic *Drosophila* were crossed with flies of a *GMR-GAL4* line expressing GAL4 driver in the retina under control of the tissue-specific glass multimer gene promoter.

RESULTS

Physical Association of ERα and β-Catenin—Human colon cancer HCT116 cells express metabolically stable β-catenin due to mutation at its putative phosphorylation site. These cells, however, do not express detectable ER. HCT116 cells were transfected with a FLAG-tagged human ERα expression plasmid, and endogenous β-catenin was immunoprecipitated from cell lysates following 3-h preincubation with estrogen or vehicle. IgG from normal rabbit serum was used as a control for nonspecific immunoprecipitation. Obtained immunocomplexes were subjected to Western blotting and analyzed by immunostaining with antibodies against FLAG-tag and ERα.

ERα co-immunoprecipitated with β-catenin even in the absence of ligand; however, ERα-β-catenin association was markedly stimulated by estrogen (Fig. 1*A*). Similar results (data not shown) were obtained using SW480 human colon cancer cells, in which non-mutant β-catenin was stabilized by a loss-of-function mutation in the gene of tumor suppressor Adenomatous polyposis coli, an essential component of the β-catenin degradation machinery. Brief exposure to ligand did not affect FLAG-ERα expression in this (Fig. 1*A*) or further experiments.

As anti-β-catenin antibodies co-precipitated a C-terminally truncated FLAG-ERα-(1–396) (Fig. 1*B*), it appeared that an intact LBD was not essential for the ER interaction with β-catenin. Predictably, C-terminal truncation of ERα abolished the ligand sensitivity of the interaction.

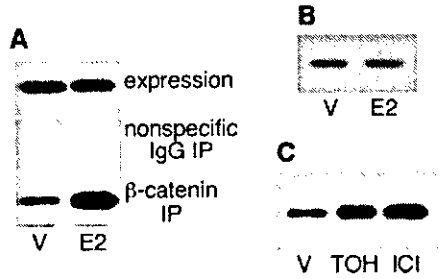

FIG. 1. **Association between ERα and β-catenin in mammalian cells.** *A–C*, anti-FLAG immunostaining of Western blots of immunocomplexes precipitated with antibodies against β-catenin or with nonspecific rabbit IgG (*A*) from HCT116 cells expressing FLAG-tagged full-length ERα (*A*, *C*) or C-terminally truncated ERα (*B*) pretreated with vehicle (*V*), estradiol (*E2*), tamoxifen (*TOH*), or ICI 182,780 (*ICI*), as indicated. *IP*, immunoprecipitation.

We then analyzed whether ligands that inhibited the transcriptional activity of ERα would also affect its interaction with β-catenin. Immunoprecipitation of ERα with antibodies against β-catenin was significantly stimulated by the ERα partial, tamoxifen, and complete, ICI 182,780, antagonists (Fig. 1*C*).

Transcriptional Interaction between ERα and β-Catenin—Next, we investigated whether the apparent physical association between ERα and β-catenin was consequential for transcriptional function of the proteins. Transactivation of an ERE-dependent reporter by endogenous ER was studied in human breast cancer MCF7 cells, in which the Wnt pathway is practically silent. Expression of stabilized β-catenin S33Y in these cells enhanced ligand-dependent expression of the reporter without affecting its basal activity in the absence of ligand (Fig. 2*A*). Expression of ERα in human colon cancer SW480 (Fig. 2B) and HCT116 (data not shown) cells enhanced the activation of the Wnt-responsive TOPFLASH reporter by endogenous β-catenin in the absence of ligand. Treatment with estrogen resulted in further moderate activation of reporter expression, while ER antagonists appeared not to affect reporter gene activity (Fig. 2*B*).

The reciprocal activation of cognate reporters in the transfection experiments suggested that ERα and β-catenin might reciprocally recruit each other to their corresponding response elements in endogenous target gene promoters. Indeed, antibody against β-catenin precipitated ERE of the pS2 gene promoter from chromatin of β-catenin S33Y expressing MCF7 cells in an estrogen-dependent manner (Fig. 2*C*). Conversely, anti-ERα antibody precipitated in a ligand-dependent manner Axin2 gene promoter putative TBE from chromatin of SW480 cells transfected with an ERα expression construct, while recruitment of β-catenin to the TBE was not sensitive to the presence of estrogen (Fig. 2*D*). The used antibodies did not display nonspecific chromatin precipitation (Fig. 2*E*).

Consistent with the results obtained using MCF7 cells, ERα transactivation was markedly enhanced *in vivo* by the stabilized Armadillo mutants ΔArm (24) (Fig. 2*F*) or ArmS10 (25) (data not shown) when ectopically co-expressed in the *Drosophila* eye disc.

Genetic Interaction between ERα and β-Catenin—Constitutive activation of Armadillo in the *Drosophila* eye disc has been shown to induce apoptosis and consequent degeneration in the adult eye (26, 27). Potentiation of β-catenin transcriptional activity by ERα in SW480 cells (Fig. 2*B*) and functional interaction between ERα and Armadillo (Fig. 2*F*) would predict activation of endogenous Armadillo by the ectopic ERα expression in

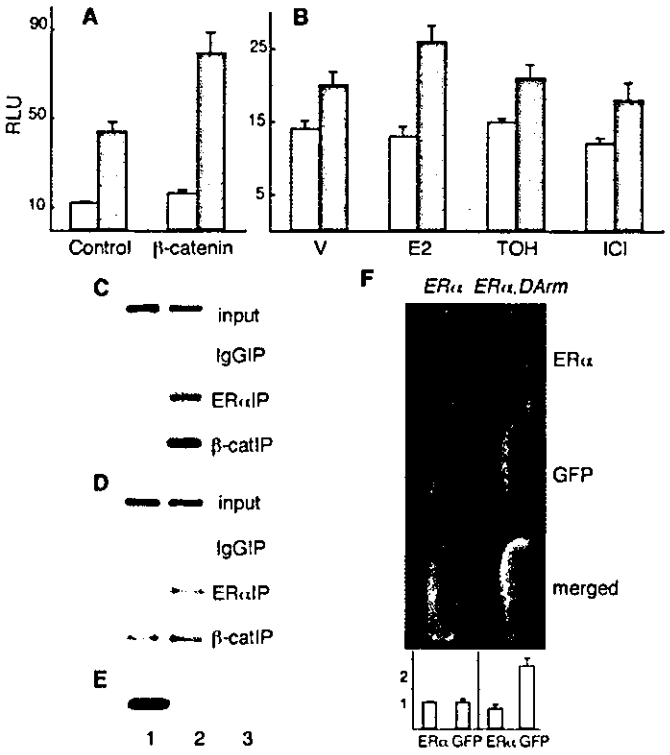

FIG. 2. **Transcriptional interaction between ERα and β-catenin.** *A*, MCF7 cells were transfected with ERE-tk-Luc reporter construct together with empty (*Control*) or β-catenin S33Y expression vector and treated with vehicle or estradiol (*open* and *filled bars*, respectively). *B*, SW480 cells were co-transfected with Wnt/β-catenin-responsive reporter and empty or ERα expression vector (*open* and *filled bars*, respectively) and treated with vehicle (*V*), estradiol (*E2*), tamoxifen (*TOH*), or ICI 182,780 (ICI), as indicated. The data represent the mean ± S.D. of three independent experiments. *C* and *D*, ChIP assay of the putative ERE of the pS2 gene promoter in MCF7 cells (*C*) and the putative TBE of the Axin2 gene promoter in SW480 cells (*D*) with anti-ERα (*ERαIP*) or β-catenin (*β-catIP*) antibodies or preimmune rabbit IgG (*IgGIP*) as a negative control. Cells were pretreated for 3 h with vehicle (*left column*) or estradiol (*right column*). *E*, control for a nonspecific chromatin immunoprecipitation: amplification of a pS2 gene DNA segment that does not contain ERE or TBE sequences from DNA samples used for PCR presented in C, *right column*: input (*lane 1*), ChIP with anti-ERα (*lane 2*), or anti-β-catenin (*lane 3*) antibodies. The data shown are representative of typical results of at least three independent ChIP experiments. *F*, estrogen-induced expression of an ERE-dependent GFP reporter (*green*) in *Drosophila* third instar larva eye discs ectopically expressing human ERα (*red*) alone or together with constitutively active Armadillo mutant ΔArm. Similar results were obtained with a different constitutively active Armadillo mutant, ArmS10.

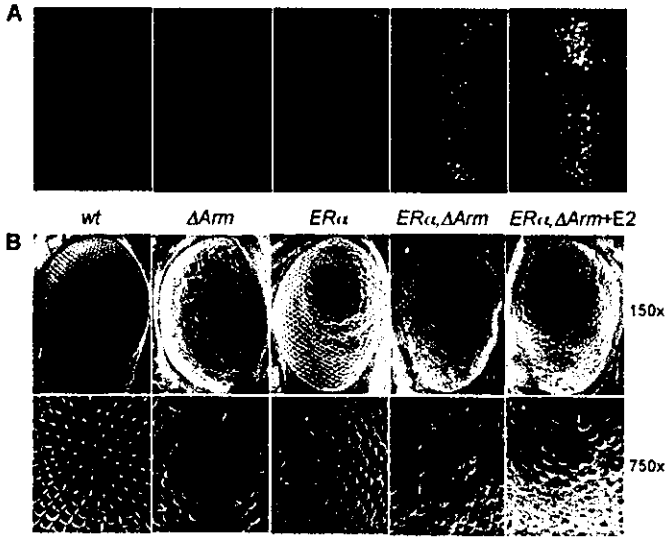

FIG. 3. **Genetic interaction between ERα and β-catenin/Armadillo in *Drosophila*.** *A*, apoptosis (*green*, fluorescein isothiocyanate TUNEL-labeled cells) in third instar larval eye discs; *B*, scanning electron microscope images of adult eye from *Drosophila* with ectopic expression of ERα, stabilized Armadillo mutant ΔArm, or both, as indicated. The same phenotypes were produced in experiments using other independently obtained *Drosophila* lines with different chromosomal localization of the ERα transgene.

the *Drosophila* eye disc leading to development of a phenotype characteristic of abnormal Wnt/β-catenin activation.

We performed TUNEL staining of the third instar larval eye discs with ectopic expression of ERα alone or together with the constitutively active Armadillo mutant ΔArm. When expressed singly, ERα and ΔArm both induced a slight increase in apoptosis compared with wild-type (wt) eye discs from *Drosophila* of the parental line. Co-expression of ERα and ΔArm resulted in a marked increase in apoptotic cell number. Importantly, while estrogen had no discernible effect on apoptosis in wt eye discs and those expressing either ERα or ΔArm alone (data not shown), treatment with estradiol significantly increased apoptosis rates when ERα and ΔArm (*ERα,ΔArm*+E2) were co-expressed (Fig. 3*A*). Activated Armadillo has a mild apoptotic effect in the third instar larva eye disc, reportedly due to the protective counteraction at this stage by the EGFR/MAPK signaling (27). This allowed us to detect differences in apoptosis patterns in transgenic fly eye discs at this developmental stage that would otherwise be difficult to distinguish due to the onset of massive cell death at the later stages.

We compared adult eye phenotypes of flies from these transgenic lines and the wt (Fig. 3*B*). The normal *Drosophila* eye is composed by regularly spaced ommatidia with regularly oriented interommatidial bristles. Expression of ERα in the eye disc leads to development of phenotypes similar to those caused by expression of ΔArm: rough eye appearance and disorientation or loss of interommatidial bristles. Co-expression of ΔArm and ERα synergistically enhanced this abnormal eye development. Again, while estradiol appeared not to affect the separate ΔArm or ERα expression phenotypes (data not shown), treatment with estrogen, however, further aggravated the severity of eye abnormalities in the ERα and ΔArm co-expression mutants (*ERα,ΔArm*+E2). Consistent with β-catenin-ERα-(1–396) co-immunoprecipitation, ectopic expression of the LBD deletion mutant ERα-(1–302) in the eye disc produced a phenotype closely resembling that with the full-length ERα (data not shown).

DISCUSSION

We found that β-catenin associated with ERα even in the absence of ligand and that estrogens further enhanced this interaction. While it is possible that the ligand-independent association was due, at least in part, to the overexpression of one of the interacting proteins, the association between β-catenin and C-terminally truncated ERα suggested that the ligand binding was not essential but might rather induce a more favorable conformation for ERα to interact with β-catenin. This may be of functional significance at physiological concentrations of the interacting proteins. Interestingly, β-catenin recruitment to EREs and ERα recruitment to TBEs in the promoters of endogenous target genes were both highly ligand-dependent. The apparently equal stimulation of ERα-β-catenin interaction by ER agonists and antagonists may have important implications for the design of novel therapeutic strategies.

Our most significant finding was that ERα functionally interacted with β-catenin/Armadillo *in vivo* in transgenic *Drosophila*. The ligand-dependent transactivation function of ERα was significantly enhanced by the co-expression of stabilized Armadillo mutants. Abnormalities in the eye development in-

duced by targeted expression of activated Armadillo and ERα were of a similar nature. Co-expression of both proteins synergistically enhanced the abnormal phenotype that was further aggravated by treatment with estradiol. Importantly, in mammals, estradiol is shown to have a prominent neuroprotective activity thought to be mediated by ER (28).

Physical and transcriptional interaction between β-catenin and androgen receptor has been observed previously (17, 18). However, in experiments presented in these reports no interaction between β-catenin and other nuclear hormone receptors, including ER, has been detected.

Thus, we have shown that Wnt and estrogen signaling pathways cross-talk *in vivo* through functional interaction between ERα and β-catenin. This interaction may underlie mechanisms of estrogen effects in pathological conditions and processes in which abnormalities of Wnt/β-catenin signaling have been implicated, such as in colorectal cancer. In addition, we have established a novel experimental system in which to identify factors conserved between humans and *Drosophila* that may be involved in regulation of cross-talk between Wnt and estrogen signaling and for the screening of novel compounds able to interfere with this cross-talk.

Although other mechanisms may be involved (*e.g.* intranuclear sequestration), transcriptional modulation appears to be the major mechanism of functional ERα-β-catenin interaction. The genomic function of nuclear receptors is dependent on the recruitment of different coactivator and chromatin remodeling complexes (1, 2, 29, 30). β-Catenin has been shown to recruit coactivators, such as the p300/CBP complex (31), and components of the mammalian SWI/SNF and RSC chromatin remodeling complexes (32) that are also known to interact with ERα. Recruitment of additional co-activator and chromatin remodeling complexes may account for the transcriptional outcome of ERα-β-catenin interaction. The physiological consequences of this interaction may also depend on cell and tissue specificity in composition of the recruited regulatory complexes. Further experiments to identify all ERα-β-catenin complex components are required to determine whether the ERα-β-catenin interaction results only in quantitative changes in the composition of the recruited regulatory proteins or if factors specific to ERα-β-catenin protein complexes are involved.

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

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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, *n*-butylbenzyl 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),[1] 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 butylbenzyl 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).

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[1] The abbreviations used are: ER, estrogen receptor; AF, activation function; BBP, *n*-butylbenzyl phthalate; N-CoR, nuclear receptor corepressor; SMRT, silencing mediator for retinoid and thyroid hormone receptor; LBD, ligand binding domain; ID, interaction domain; OHT, 4-hydroxytamoxifen; ICI, ICI182,780; TSA, trichostatin A; GST, glutathione *S*-transferase; TK, thymidine kinase; ERE, estrogen-responsive element; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BrdUrd, 5-bromouridine 5′-triphosphate; E2, 17β-estradiol; SRC-1, steroid receptor coactivator-1; TRAP, TR-associated proteins; TRRAP, transformation/transcription domain-associated protein.

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 interaction between ERα 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. ICI182,780 (ICI) was synthesized by Taiho Pharmaceutical Co.

Measuring IC_{50} Values of E2 and BBP—For measurement of the binding constant value of BBP to ERα, IC_{50} measuring kit was purchased from Wako Chemicals Co., and IC_{50} was examined according to the manufacturer's protocol.

Plasmid Construction—The ERα/β expression plasmids (HEG0/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, 35, 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 pM vector (Clontech) to generate GAL-DEF. VP-SRC-1, VP-TRAP220, and VP-p300 were described previously (11, 61, 64). Nuclear receptor interaction region in TRRAP was 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 (HEG0/ERGβ0) 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 pRSVβGAL vector was cotransfected in all experiments. Six hours 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), ethanolic 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 [^{35}S]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% glycerol 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.

Coimmunoprecipitation and Western Blotting—293T cells were transfected with the indicated plasmids, lysed in TNE (10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 0.15 M NaCl, 1 mM EDTA, 1 μM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin) buffer, and immunoprecipitated with anti-FLAG M2 monoclonal antibody (Sigma) or anti-ERα (Chemicon). Interacting proteins were separated by 6% SDS-PAGE, transferred onto polyvinylidine difluoride membranes (Millipore), and detected with anti-ERα, anti-p300 (Santa Cruz Biotechnology), anti-FLAG M2, or anti-V5 tag (Invitrogen), and secondary antibodies were conjugated with horseradish peroxidase. For detecting the expression of ERα and N-CoR ID1-2, isolated clones were lysed in TNE, and each lysate was detected by immunoblotting using anti-ER or anti-FLAG and secondary antibodies.

Cell Proliferation Analysis—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.

S-phase Entry Analysis—For S-phase entry analysis, NIH3T3 cells were cultured in phenol red-free DMEM supplemented with 5% charcoal-stripped FBS and seeded onto glass coverslips at 60–70% confluence. Transfection with 3 μg of wild-type or mutant ERα expression plasmid was performed by using Perfectin Reagent (Gene Therapy Systems) according to the manufacturer's protocol. Incubation medium was changed after 24 h into phenol red-free DMEM supplemented with 0.2% charcoal-stripped FBS. Cells were left in this medium for 24 h and then cultured with 100 μM 5-bromouridine 5′-triphosphate (BrdUrd) in the presence of either E2 (10 nM), BBP (1 μM), or OHT (1 μM) for an additional 24 h. After incubation, the cells were fixed for immunostaining.

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 ERs, 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

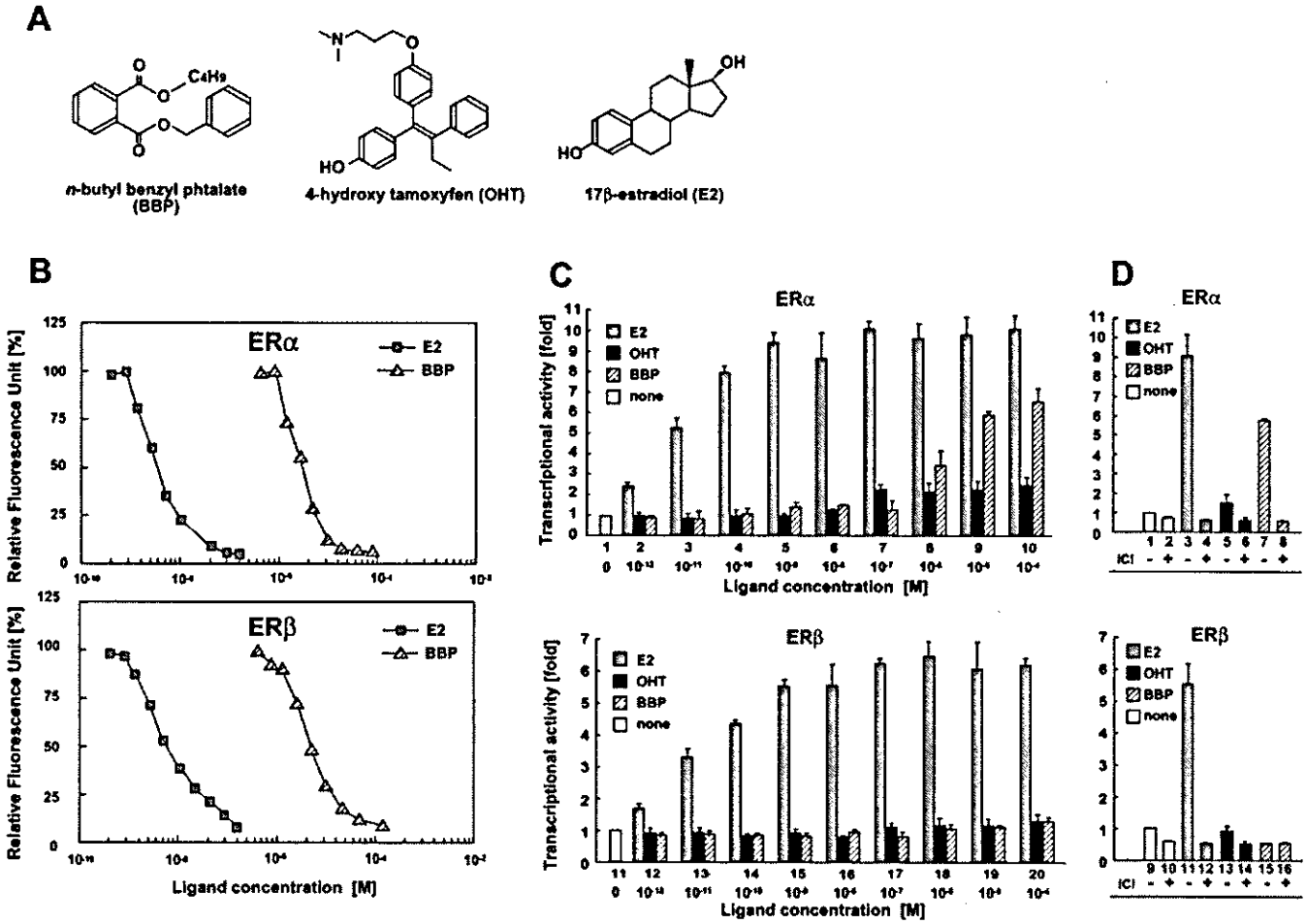

FIG. 1. **BBP enhances the transactivation function of ERα.** *A*, illustration of organic structures for BBP, OHT, and E2. *B*, BBP binds to the ligand pocket of ERs *in vitro*. Fluorescein-labeled estrogen bound to ERs was displaced competitively by increasing concentrations of E2 (*squares*) or BBP (*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 result 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 (ERG0β) and a reporter plasmid bearing 3× ER-responsive elements (ERE3-tk-luc) and incubated for 24 h with 10 fM 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. *D*, BBP-dependent transactivation of ERα is inhibited by ICI treatment. 293T cells were transfected with a plasmid expressing ERα (HEG0) or ERβ (ERG0β) and ERE3-tk-luc and incubated for 24 h in the presence of either E2 (10 nM), OHT (100 nM), or BBP (1 μM) with or without ICI (100 nM), and transcriptional activity 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.

exhibited an IC_{50} of ~1.0 nM to both ERs (Fig. 1*B*), 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. 1*B*). Therefore, we next evaluated the ability of BBP to induce an ER-mediated response by measuring luciferase activity using 293T cells that were cotransfected with a luciferase reporter plasmid bearing estrogen response elements (EREs) and either ERα expression vector (HEG0) or ERβ expression vector (ERG0β). 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. 1*C*). E2 treatment of either ERα- or ERβ-transfected cells significantly induced luciferase activity (Fig. 1*C*). 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. 1*D*, *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. 2*A*) was estimated by luciferase assay. E2 enhanced the transcriptional activity of full-length ERα and a truncated type of ERα, HE19 (Fig. 2*B*, *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. 2*B*), 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. 1*C* and 2*B*) but did not stimulate the transcriptional activity of HE19 (Fig. 2*B*), as expected. The transcriptional activity induced by BBP was about three times higher than that induced by OHT (Fig. 2*B*, compare *lane 3* to *4*). These results raise the possibility that BBP may possess the same biological properties as tamoxifen.

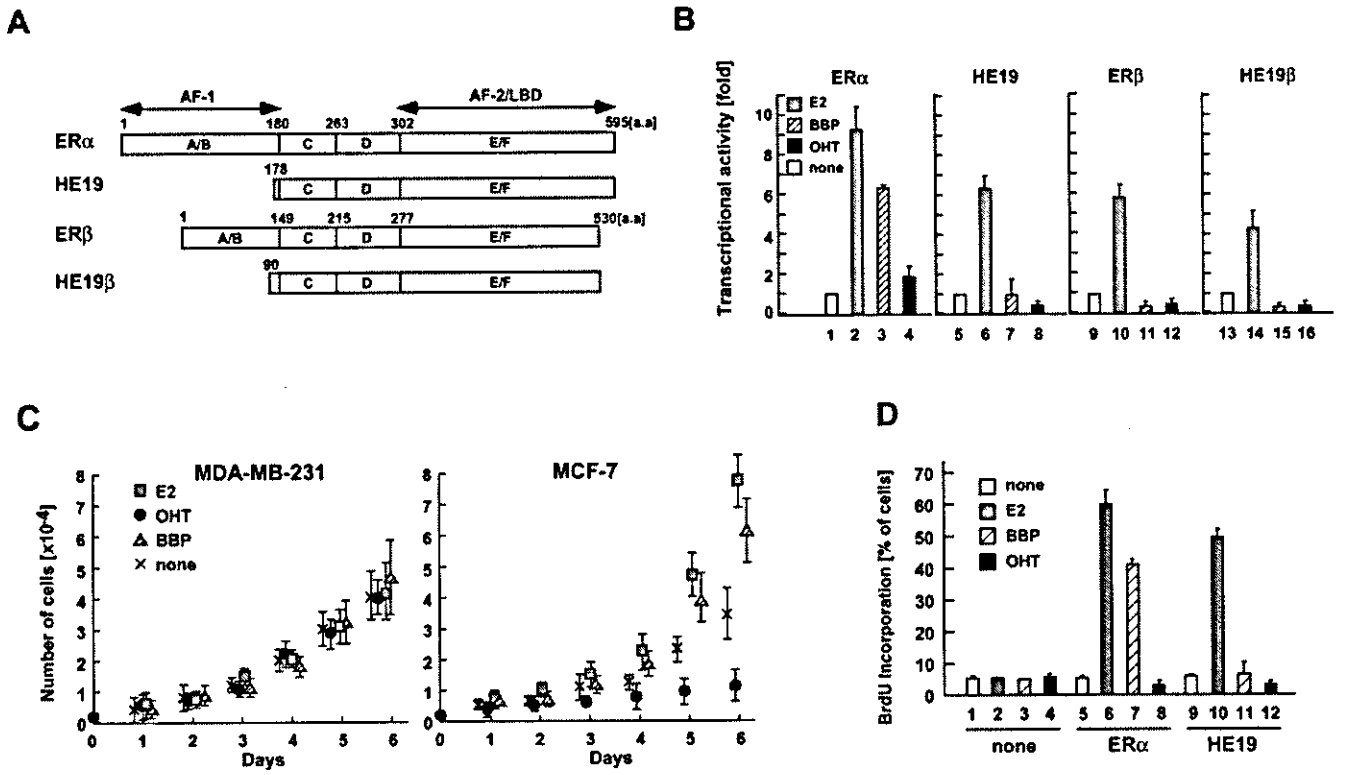

FIG. 2. **A contrastive effect between BBP and OHT on proliferation of breast cancer cells.** *A*, schematic representations of ERα expressed from HEG0 and HE19 and ERβ from ERG0β and HE19β. HE19 encodes truncated ERα defecting the N-terminal 1–177 amino acids and HE19β encodes truncated ERβ defecting 1–89 amino acids concerned with AF-1 activity. *B*, BBP- and OHT-dependent transactivation of ERα requires the ERα AF-1 activity. Expression plasmids of ERs (HEG0/ERG0β) or truncated ERs (HE19/HE19β) were transfected into 293T cells with ERE3-tk-luc in the absence (none) or presence of either E2 (10 nM), OHT (100 nM), or BBP (1 μM). *C*, BBP but not OHT stimulates proliferation of the breast cancer cell line MCF-7. MCF-7 and MDA-MB-231 cells were cultured for the indicated times in the absence (none) (*crosses*) or presence of either E2 (10 nM) (*squares*), OHT (1 μM) (*circles*), or BBP (1 μM) (*triangles*), and the number of viable cells was counted. Data are shown as means ± S.D. of triplicate cultures. *D*, BBP-dependent S-phase entry requires ERα AF-1 activity. NIH3T3 fibroblasts transfected with either HEG0 or HE19 were made quiescent and then left unstimulated (none) or stimulated with either E2 (10 nM), OHT (1 μM), or BBP (1 μM). BrdUrd was incubated in the cell medium, and its incorporation into DNA was assessed by immunofluorescent staining. Several coverslips were analyzed, and data from at least 200 scored cells were represented according to the formula: percentage of BrdUrd-positive cells = (number of BrdUrd-positive cells/number of transfected cells) × 100. In each case, the BrdUrd incorporation of non-transfected fibroblasts was evaluated and subtracted.

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. 2*C*, *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. 2*C*, *right panel*). None of the three compounds we tested affected proliferation of MDA-MB-231, which does not express ERα (Fig. 2*C*, *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. 2*D*). 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 transactivation 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. 3*A*). 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. 3*B*).

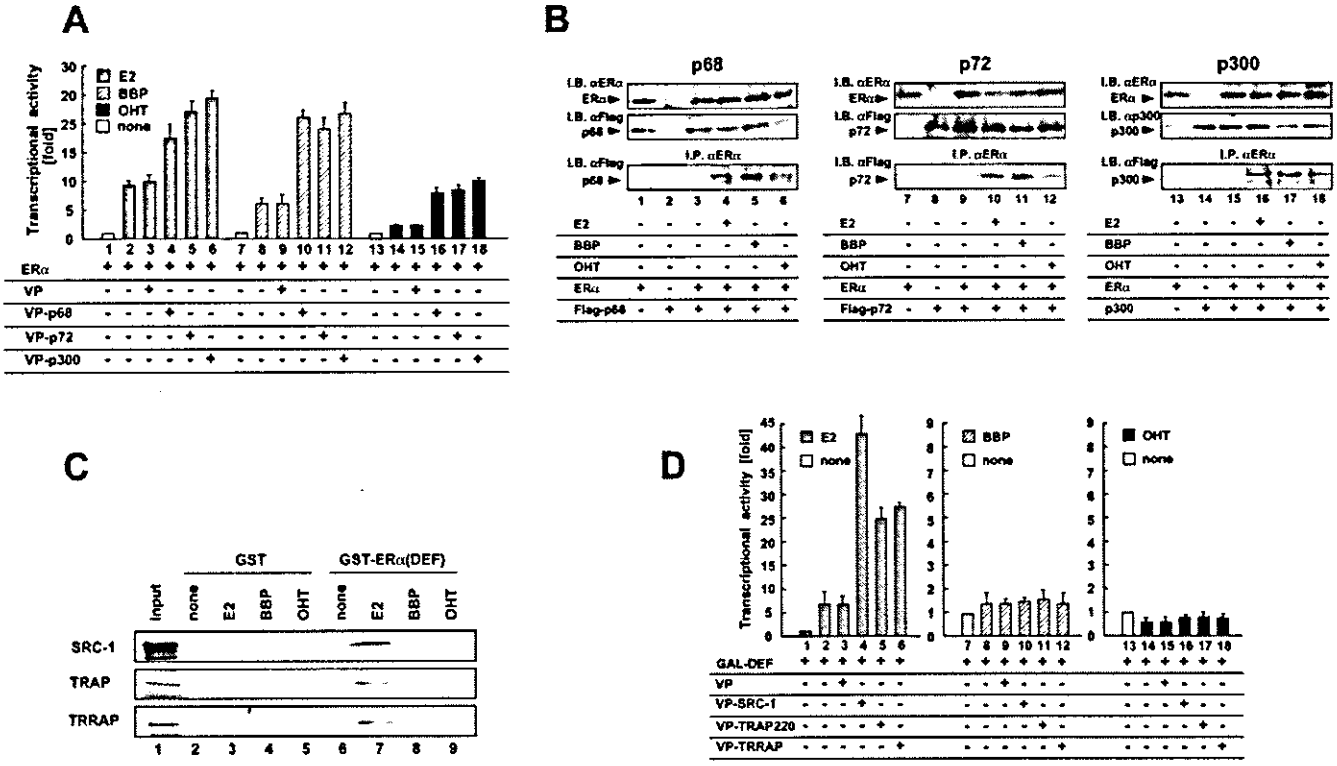

FIG. 3. **OHT and BBP induce the binding of AF-1-specific coactivators to ERα.** *A*, recruitment of AF-1 coactivators to BBP- or OHT-occupied ERα. Mammalian two-hybrid assays using ERα, along with either VP-p68, VP-p72, VP-p300, or VP, were performed. Indicated plasmids were transfected into 293T cells with HEG0 and ERE3-tk-luc in the absence (none) or presence of either E2 (10 nM), OHT (100 nM), or BBP (1 μM). *Bars* show fold change in luciferase activity relative to HEG0 and VP vectors in the absence of ligands. Results represent the average of three independent experiments; *error bars* indicate S.D. *B*, confirmation of OHT- and BBP-induced binding between ERα and p68/p72/p300 by coimmunoprecipitation. Nuclear extracts were prepared from 293T cells coexpressing ERα and FLAG-tagged p68, FLAG-tagged p72, or p300. ERα was then immunoprecipitated by anti-ERα antibody, and immunoprecipitates were detected by immunoblotting with anti-FLAG or anti-p300 antibody. *C*, BBP or OHT does not induce the binding of ERα to AF-2 coactivators *in vitro*. GST pull-down assays were performed using GST or GST-fused ERα DEF region produced by *Escherichia coli* with either [^{35}S]methionine-labeled SRC-1, TRAP220, or TRRAP translated *in vitro* in the presence of either E2 (10 nM), OHT (100 nM), or BBP (1 μM). *D*, BBP or OHT does not induce the binding of ERα to AF-2 coactivators *in vivo*. Mammalian two-hybrid assays using ERα (GAL-DEF), along with either SRC-1 (VP-SRC-1), TRAP220 (VP-TRAP220), or TRRAP (VP-TRRAP) were performed. Indicated plasmids were transfected into 293T cells with ERE3-tk-luc in the absence (none) or presence of either E2 (10 nM), OHT (100 nM), or BBP (1 μM). *Bars* show fold change in luciferase activity relative to GAL-DEF and VP vectors in the absence of ligands. Results represent the average of three independent experiments; *error bars* indicate S.D.

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. 3*C*). In a mammalian two-hybrid experiment, E2-bound GAL-DEF exhibited significant binding to either SRC-1, TRAP220, or TRRAP (Fig. 3*D*, *left panel*). Conversely, neither BBP nor OHT induced the interaction between GAL-DEF and AF-2 coactivators (Fig. 3*D*, *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 tumor (Fig. 4*A*) (54, 55). Consistent with previous studies, OHT-bound GAL-DEF repressed the transcriptional activity of the TK promoter (Fig. 4*B*, *lane 2*). This repressive activity was inhibited by the addition of TSA, a specific histone deacetylase inhibitor (Fig. 4*B*, *lane 5*). The D351Y mutation, which exhibited decreased corepressor association, impaired the tamoxifen-dependent repression by GAL-DEF (Fig. 4*B*, *lane 8*). In the presence of BBP, the repressive activity of GAL-DEF was not observed (Fig. 4*B*, *lane 3*), indicating that BBP-bound ERα would not interact with corepressor complexes.

To test 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. 4*C*, *middle panel*), BBP-bound GAL-DEF exhibited no binding to corepressors as expected (Fig. 4*C*, *right panel*). Consistent with previous studies, the D351Y mutation reduced the OHT-induced interaction with corepressors (Fig. 4*C*, *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. 4*D*, *lane 5*). In contrast, N-CoR was not coprecipitated with E2- or BBP-bound ERα (Fig. 4*D*, *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-

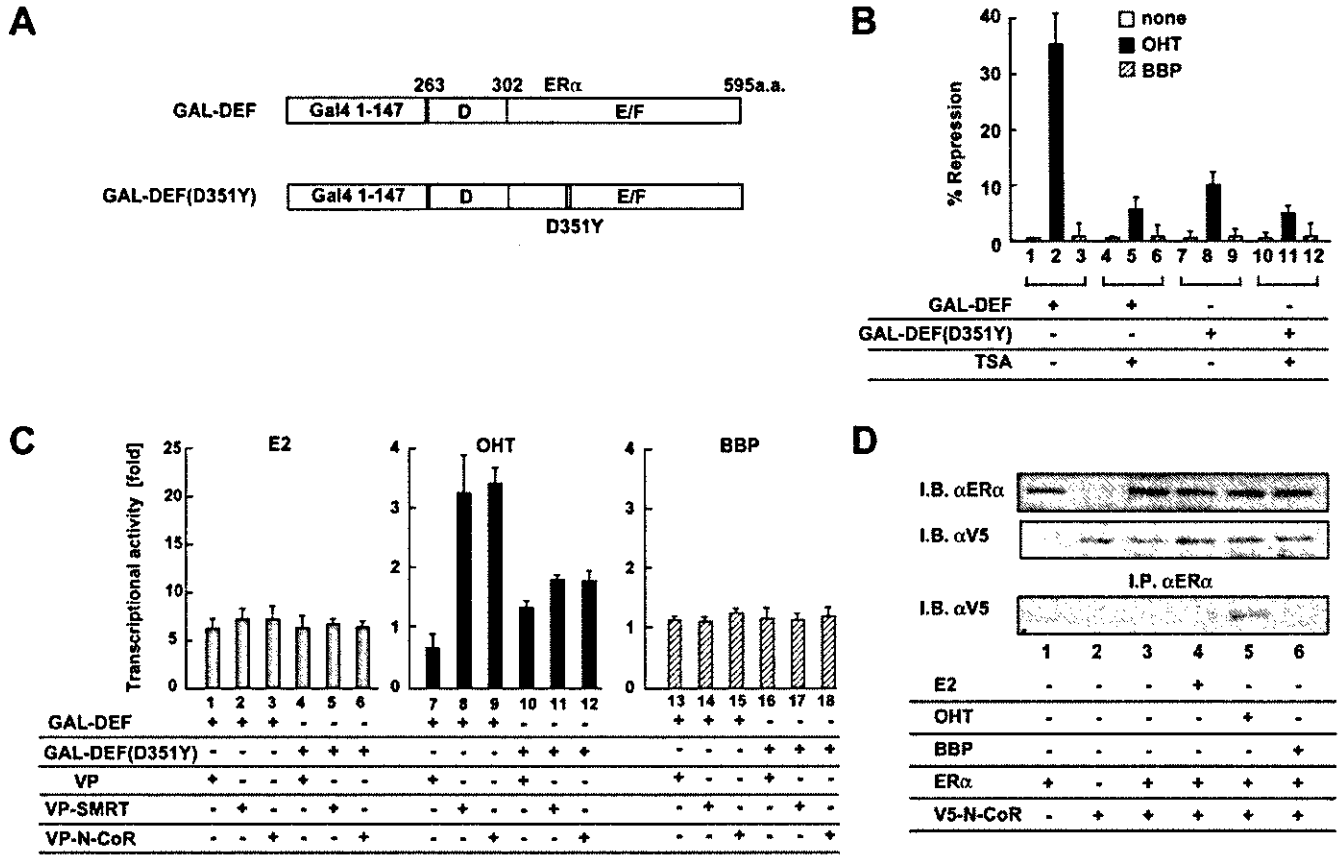

FIG. 4. **OHT but not BBP induces the binding of corepressor complexes to ERα.** *A*, schematic representation of GAL-DEF and GAL-DEF(D351Y). Ligand binding domain of wild-type ERα (263–595 amino acids (*a.a.*)) and ERα amino acid substitution D351Y were fused with GAL4 DNA binding domain. *B*, repressive activity of ERα AF-2 is induced by OHT but not by BBP. Indicated plasmids were transfected into 293T cells along with a reporter plasmid bearing GAL4-binding elements and the TK promoter (MH100-tk-luc) in the absence (none) or presence of OHT (100 nM), BBP (1 μM), or TSA (5 ng/ml). *Bars* indicate the repression rate of luciferase activity relative to GAL-DEF in the absence of ligands. Results represent the average of three independent experiments; *error bars* indicate S.D. *C*, BBP does not induce the binding of ERα to corepressors *in vivo*. Mammalian two-hybrid assays were performed using either GAL-DEF or GAL-DEF(D351Y), along with either VP-N-CoR, VP-SMRT, or VP. Indicated plasmids were transfected into 293T cells with a reporter plasmid bearing GAL4-binding elements (17m5-luc) in the absence (none) or presence of either E2 (10 nM), OHT (100 nM), or BBP (1 μM). *Bars* indicate fold change in luciferase activity relative to the GAL-DEF and VP vectors. Results represent the average of three independent experiments; *error bars* indicate S.D. *D*, the confirmation of OHT- and BBP-induced binding between ERα and N-CoR by coimmunoprecipitation. Nuclear extracts were prepared from 293T cells coexpressing ERα and V5-tagged N-CoR. ERα was immunoprecipitated by anti-ERα antibody, and V5-N-CoR in the anti-ERα immunoprecipitates was detected by immunoblotting with anti-V5 antibody.

though 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. 5*A*). On the contrary, N-CoR reduced OHT-dependent transactivation function of ERα (Fig. 5*B*, *middle panel*) but not E2- and BBP-dependent activation (Fig. 5*B*, *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. 5*A*, 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. 5*A*, 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. 5*D*, 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 in the presence of OHT (Fig. 6*B*). We then studied whether the expression of the ID1-2 region abrogates the interaction between OHT-occupied ERα and N-CoR/SMRT using a mammalian two-hybrid assay. 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. 6*C*). Coexpression of the ID1-2 region with ERα enhanced the OHT-dependent transactivation function of ERα (Fig. 6*D*, *left panel*) but not that of ERα(D351Y)

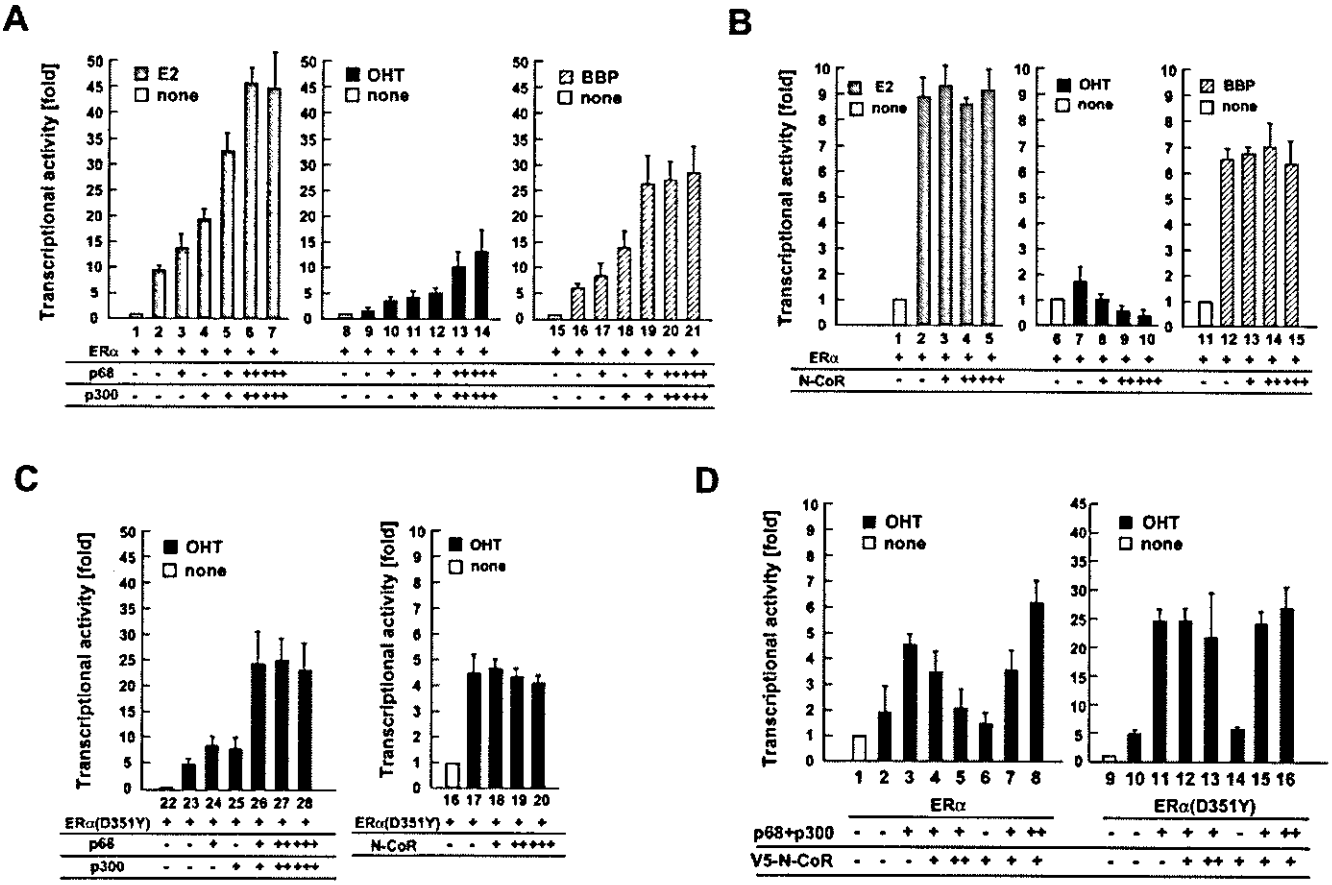

FIG. 5. **A competitive effect between AF-1 coactivator and corepressor complexes on the OHT-induced transcriptional activity of ERα.** *A*, E2-, OHT-, or BBP-dependent transcriptional activity is enhanced by expression of AF-1 coactivators. Indicated plasmids were transfected into 293T cells with HEG0 and ERE3-tk-luc in the presence of E2 (10 nM), OHT (100 nM), or BBP (1 μM). The amount of transfected plasmids for p68/p300 was 0.25 (+), 0.5 (++), and 1.0 μg (+++), respectively. *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. *B*, OHT-induced transcriptional activity is reduced by expression of N-CoR. Indicated plasmids were transfected into 293T cells with HEG0 and ERE3-tk-luc in the presence of either E2 (10 nM), OHT (100 nM), or BBP (1 μM). The amount of transfected plasmids for N-CoR was 0.25 (+), 0.5 (++), and 1.0 μg (+++), respectively. *Bars* indicate fold change in luciferase activity relative to ERα in the absence of ligand. *C*, effects of AF-1 coactivators and corepressors on OHT-induced transcriptional activity of ERα(D351Y). Indicated plasmids were transfected into 293T cells with ERE3-tk-luc in the presence of OHT (10 nM). The amount of transfected plasmids for p68/p300 and N-CoR was 0.25 (+), 0.5 (++), and 1.0 μg (+++), respectively. *Bars* indicate fold change in luciferase activity relative to ERα in the absence of ligands. *D*, the ratio between AF-1 coactivators and corepressors regulates the OHT-dependent transcriptional activity. Indicated plasmids were transfected into 293T cells with ERE3-tk-luc in the presence of OHT (100 nM). The amount of transfected plasmids for p68/p300 and N-CoR was 0.25 (+) and 0.5 μg (++), respectively. *Bars* indicate fold change in luciferase activity relative to ERα in the absence of ligand.

(Fig. 6*D*, *right panel*), indicating that the inhibition of corepressor binding enhances the AF-1 activity of ERα.

Next, we examined the effect of the expression of the ID1-2 region on the E2-dependent growth phenotype of the MCF-7 breast cancer cell line. The expression vector containing FLAG-tagged ID1-2 region was transfected into MCF-7 cells, and several stable cell lines constitutively expressing the ID1-2 region in MCF-7 cells (MCF-7(ID1-2)) were established. We picked up four independent clones, clones 1–4, and we examined the expression of ERα and ID1-2 by Western blot using specific antibodies against ERα and FLAG epitope. In all four clones we tested, the expression of FLAG-ID1-2 was observed (Fig. 7*A*). The expression level of ERα in these clones was unchanged when compared with the control MCF-7 cells (Fig. 7*A*). In the absence of E2, ID1-2 stable clones exhibited normal cell growth similar to the control MCF-7 (Fig. 7*B*). However, either E2 or BBP treatment enhanced the growth rate of the control and clones. OHT inhibited the growth of control MCF-7 cells but stimulated the growth of ID1-2 stable transformants (Fig. 7*B*), indicating that the inhibition of corepressors binding to ERα converts OHT from anti-estrogenic to an estrogenic effect on the proliferation of breast cancer cells.

DISCUSSION

BBP is a phthalic 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

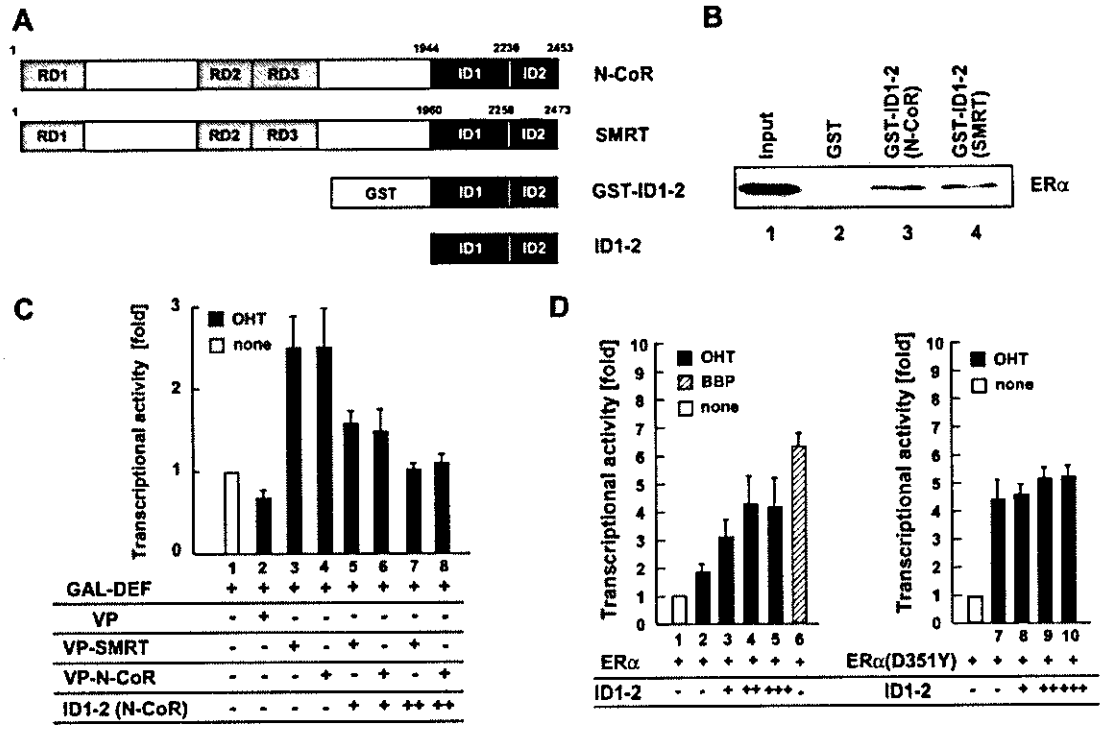

FIG. 6. **Overexpression of the N-CoR ID1-2 region abrogates the binding of corepressor complexes to OHT-occupied ERα.** *A*, schematic representation of corepressors N-CoR/SMRT. *B*, OHT induces the binding of the ID1-2 region of N-CoR/SMRT to ERα *in vitro*. GST pull-down assays were performed using GST or GST-fused ID1-2 region of N-CoR or SMRT produced by *E. coli* and [^{35}S]methionine-labeled ERα translated *in vitro* in the presence of OHT (100 nM). *C*, expression of ID1-2 region abrogates the binding between OHT-occupied ERα and corepressors. Mammalian two-hybrid assays were performed using GAL-DEF along with either VP-N-CoR, VP-SMRT, or VP. Indicated plasmids were transfected into 293T cells with 17m5-luc in the presence of OHT (100 nM). OHT-induced interactions between ERα and N-CoR/SMRT were inhibited by cotransfection of 0.25 (+) or 0.5 μg (++) of an ID1-2 expressing plasmid. *Bars* indicate fold change in luciferase activity relative to the GAL-DEF and VP vectors in the absence of ligand. Results represent the average of at least three independent experiments; *error bars* indicate S.D. *D*, expression of ID1-2 region stimulates OHT-dependent transcriptional activity. Indicated plasmids were transfected into 293T cells with ERE3-tk-luc in the presence of OHT (100 nM). OHT-induced transcriptional activity was enhanced by cotransfection of 0.25 (+), 0.5 (++), or 1.0 μg of an ID1-2 expressing plasmid. *Bars* indicate fold change in luciferase activity relative to ERα plasmids in the absence of ligand. Results represent the average of at least three independent experiments; *error bars* indicate S.D.

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, 59, 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

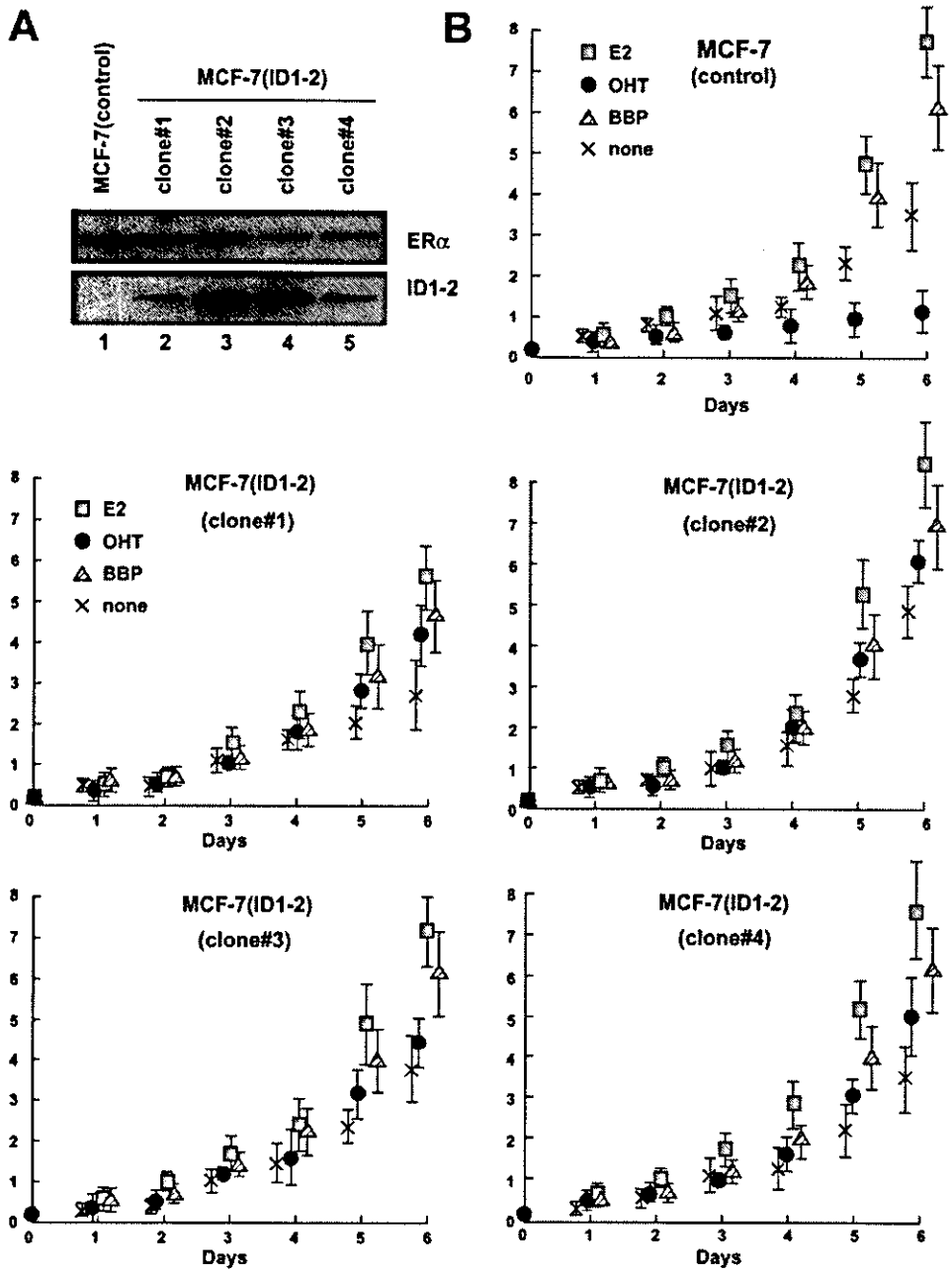

FIG. 7. **The breast cancer cell lines expressing the ID1-2 region exhibit OHT-stimulated growth phenotype.** *A*, expression levels of the ID1-2 region of N-CoR in ID1-2 stable transfectants (*clone 1–4*) measured by immunoblotting. Equal protein loading were ensured by ERα levels. *B*, MCF-7 stably expressing ID1-2 region exhibits the OHT-stimulated growth phenotype. Control MCF-7 cells and MCF-7 cell lines stably expressing the ID1-2 region (*clones 1–4*) were cultured for the indicated times in the absence (none) (*crosses*) or presence of either E2 (10 nM) (*squares*), OHT (1 μM) (*circles*), or BBP (1 μM) (*triangles*), and the number of viable cells was counted. Data are shown as means ± S.D. of triplicate cultures.

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. Hayashi and co-workers (85) reported that the relative transcriptional activity of AF-1 of ERα compared with that of AF-2 was 4-fold higher in Ishikawa cells than MCF-7 cells. They also mentioned that mitogen-activated protein kinase, which phosphorylates the serine residue at position 118 in ERα A/B domain, was constitutively activated in Ishikawa cells but not in MCF-7 cells. We reported previously (34–36) that the phosphorylation of serine 118 by mitogen-activated protein kinase stabilized the complex formation between ERα and p68/p72 DEAD box proteins, which are components of the p300/SRC-1 coactivator complex, to stimulate AF-1 activity of ERα. Together with these observations, our results suggest that the ratio between ERα AF-1-coactivator complex and ERα-corepressor complex in cells is an important determinant of AF-1 activity of OHT-occupied ERα and that the proliferation of cancer cells is regulated by AF-1 activity of OHT-bound ERα. From these observations, it is suggested that the tissue and cell type specificity of tamoxifen action is due to the difference of AF-1 activity of tamoxifen-bound ERα in various tissues and cells. Our results shed light on the molecular mechanism underlying tamoxifen-dependent inhibition of cancer growth and ring an alarm against endocrine disrupters.

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