

Suppression of Estrogen Receptor-mediated Transcription and Cell Growth by Interaction with TR2 Orphan Receptor*

Received for publication, April 12, 2002, and in revised form, June 14, 2002
Published, JBC Papers in Press, July 1, 2002, DOI 10.1074/jbc.M203531200

Yueh-Chiang Hu, Chih-Rong Shyr, Wenyi Che, Xiao-Min Mu, Eungseok Kim,
and Chawnsang Chang‡

From the George Whipple Laboratory for Cancer Research, Departments of Pathology, Urology,
and the Cancer Center, University of Rochester Medical Center, Rochester, New York 14642

The transcriptional activity of the estrogen receptor (ER) is known to be highly modulated by the character and amount of coregulator proteins present in the cells. TR2 orphan receptor (TR2), a member of the nuclear receptor superfamily without identified ligands, is found to be expressed in the breast cancer cell lines and to function as a repressor to suppress ER-mediated transcriptional activity. Utilizing an interaction blocker, ER-6 (amino acids 312–340), responsible for TR2 interaction, the suppression of ER by TR2 could be reversed, suggesting that this suppression is conferred by the direct protein-protein interaction. Administration of antisense TR2, resulting in an enhancement of ER transcriptional activity in MCF7 cells, indicates that endogenous TR2 normally suppresses ER-mediated signaling. To gain insights into the molecular mechanism by which TR2 suppresses ER, we found that TR2 could interrupt ER DNA binding via formation of an ER-TR2 heterodimer that disrupted the ER homodimerization. The suppression of ER transcription by TR2 consequently caused the inhibition of estrogen-induced cell growth and G₁/S transition in estrogen-dependent breast cancer cells. Taken together in addition to the potential roles in spermatogenesis and neurogenesis, our data provide a novel biological function of TR2 that may exert an important repressor in regulating ER activity in mammary glands.

The human TR2 orphan receptor (TR2),¹ a member of the nuclear hormone receptor superfamily, was cloned from human testis and prostate cDNA libraries and has no identified ligand(s) (1, 2). TR2 is mapped to locate on chromosome 12q22 (3), which is known to be frequently deleted in various tumors, including testicular and ovarian germ cell tumors (4, 5). Four RNA isoforms, TR2-5, -7, -9, and -11, have been identified.

* This work was supported by National Institutes of Health Grant DK47258. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: University of Rochester Medical Center, 601 Elmwood Ave., Box 626, Rochester, NY 14642. Tel.: 585-275-9994; Fax: 585-756-4133; E-mail: chang@urmc.rochester.edu.

¹ The abbreviations used are: TR2, TR2 orphan receptor; LBD, ligand binding domain; DBD, DNA binding domain; aa, amino acids; CAT, chloramphenicol acetyltransferase; E2, 17 β -estradiol; ER, estrogen receptor; ERE, estrogen response element; GST, glutathione S-transferase; CNTF, ciliary neurotrophic factor; PR, progesterone receptor; AS, antisense; MMTV, mouse mammary tumor virus; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RXR, retinoic X receptor; AR, androgen receptor.

Although TR2-11 encodes the full-length receptor, TR2-5, -7, and -9 encode truncated receptors with distinct deletions in the ligand binding domains (LBD) (1). TR2 has high homology with TR4, which places them in a unique subfamily within the nuclear hormone receptor superfamily (6). TR2 is evolutionarily conserved among species from primitive creatures to mammals, including sea urchin, rainbow trout, axolotl, *Xenopus*, *Drosophila*, mouse, and human (1, 2, 7–11). The facts that TR2 is broadly expressed in many tissues throughout development starting at as early as mid-gestation stage (12–15) and that *Drosophila* with null mutations of DHR78 nuclear receptor, a homolog of human TR2, is lethal at the third-instar larval stage with severe defects in ecdysteroid-triggered metamorphosis (16), imply that the biological importance of TR2 is involved in the development process. With prominent expression throughout the active proliferating zones of the neural areas, the sensory nerve-targeted organs, and the testes during development, TR2 has been proposed to play an important role in the early development of the nervous system and the male reproductive system (12–15). Also, it has been shown that TR2 is primarily expressed in the mouse testis, particularly in the developing germ cells, indicating a role of TR2 in spermatogenesis (12, 17).

TR2 functions as a transcription factor that binds to its consensus response elements (AGGTCA) in a direct repeat orientation (AGGTCA_nAGGTCA, x = 1–6) (15). Many TR2 target genes have been discovered, such as cellular retinoid binding protein II (CRBP2), retinoic acid receptor β , SV40, erythropoietin, histamine H1 receptor, muscle-specific aldolase A, and ciliary neurotrophic factor (CNTF) receptor (13–15, 18–21), suggesting that TR2 has a broad range of biological functions. In terms of the regulation of TR2 expression, TR2 can be induced during neuronal differentiation in P19 embryonic carcinoma cells stimulated by CNTF. In return, TR2 activates its target gene, CNTF receptor, expression, which mediates CNTF signaling and is required for the motor neuron development (13, 22). These may provide a linkage between TR2 and neurogenesis. The tumor suppressor genes p53 and Rb which induce cell cycle arrest, can down-regulate TR2 expression in cells after ionizing radiation and in cells overexpressing p53 or Rb (23, 24). TR2 can then go through a feedback control mechanism to induce HPV-16 E6 and E7 target gene expression, which are known to enhance the p53 protein degradation and inactivate the Rb function, respectively (23, 25). TR2 is, therefore, thought to be involved in the cell cycle regulation.

Estrogen receptors (ERs), including ER α and ER β , belong to the nuclear hormone receptor superfamily and mediate estrogen actions in regulation of cell growth and differentiation, particularly in mammary glands and uterus in females (for reviews, see Refs. 26 and 27). The proliferation of mammary

glands is mainly dependent on estrogen stimulation; however, the proliferating epithelial cells detected in terminal end buds at the tip of elongating ducts in mammary glands are usually ER-negative (28–30). Despite the unclear role of ER in this process, in mice with a homozygous disruption of the ER gene, the mammary glands remain undeveloped, as demonstrated by the lack of terminal end buds and alveolar structures, even though the serum estrogen level is 10 times higher than those in wild-type mice (31, 32). This indicates an indispensable role of the ER in the growth of mammary glands. Also, the fact that more than two-thirds of breast cancers from patients are ER-positive and benefit from antiestrogen or ovariectomy therapies strengthens the importance of the ER in the stimulation of cell growth in mammary glands in response to estrogen (33). Therefore, understanding the mechanisms involving the suppression of ER-mediated gene expression and cell proliferation may eventually help us to develop better drugs to battle breast cancer.

In addition to functioning as a transcription regulator, TR2 can modulate other signaling via different mechanisms. For example, TR2 suppresses RXR and RXR/retinoic acid receptor-mediated transcription by binding to the same DNA response element with a higher binding affinity (15) and represses thyroid receptor α /RXR signaling by competing for limited amounts of DNA response elements (20). TR2 can also exert its suppressive effects via the recruitment of class I and class II histone deacetylases (34). Here, we demonstrate a new role of TR2 in the breast cancer cells where TR2 suppresses ER-mediated transcription and cell growth by direct protein-protein interaction, thus representing a novel signaling pathway within the nuclear hormone receptor superfamily.

MATERIALS AND METHODS

Antibodies—ER rabbit polyclonal (H-184), ER mouse monoclonal (C-314), progesterone receptor (PR) rabbit polyclonal (H-190), and actin goat polyclonal (C-11) were obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-TR2 IgM antibody (G204) was described previously (14). Monoclonal anti-FLAG antibody (M2) was purchased from Sigma. Alkaline phosphatase-conjugated secondary antibodies (goat anti-rabbit IgG, donkey anti-goat IgG, and goat anti-mouse IgM) were from Santa Cruz Biotechnology.

Constructs—pCMV-TR2, pGEX-3x-TR2, and pCMX-VP16-TR2 were constructed by insertion of full-length TR2 cDNA (1, 2) into individual vectors. The doxycycline-inducible expression vector pBIG2i bearing hygromycin B resistance gene was a gift from Dr. Jay Reeder (University of Rochester, Rochester, NY) (35). pBIG2i and pBIG2i-FLAG-TR2 were used for generating MCF7-pBIG and MCF7-TR2 stable clones, respectively. The GAL4-ER (aa 282–595) and pCMV-mER β were gifts from Dr. Hinrich Gronemeyer (Strasbourg, France) and Vincent Giguère (McGill University, Québec, Canada), respectively. To construct GST-ER fragments, ER cDNA fragments were released from pSG5-ER (36) using adequate restriction enzymes and inserted into the pGEX vector series (Amersham Biosciences) to produce pGEX-3X-ER-1 (aa 1–165), pGEX-2T-ER-2 (aa 123–340), pGEX-2T-ER-3 (aa 312–595), pGEX-3X-ER-4 (aa 552–595), pGEX-2T-ER-5 (aa 123–312), and pGEX-2T-ER-6 (aa 312–340). The pGEX-KG-TR2-1, -2, and -3 plasmids were constructed by insertion of PCR-generated cDNA fragments corresponding to aa 1–112, 88–196, and 179–603, respectively, into pGEX-KG vector (a gift from Dr. Frank B. Furnari, University of California, San Diego, CA). pCDNA3-TR2-fl AS and pIRES-TR2-N AS were constructed by insertion of antisense orientation cDNAs, encoding full-length and N terminus (aa 1–112), into pCDNA3 (Invitrogen) and pIRES (CLONTECH), respectively.

Transient Transfections—Transfections in the chloramphenicol acetyltransferase (CAT) assay were performed using the calcium phosphate precipitation method, as described previously (37). CAT reporter plasmids containing one copy of estrogen response element (ERE-CAT) or mouse mammary tumor virus (MMTV-CAT) were used as indicated. Also, a β -galactosidase expression plasmid, pCMV- β -gal, was used for internal control. For the luciferase assay, luciferase reporters (ERE-luc and MMTV-luc) were transfected into cells using the calcium phosphate precipitation method or SuperFect Transfection Reagent (Qiagen) as indicated. pRL-TK vector (Promega) encoding *Renilla* luciferase was

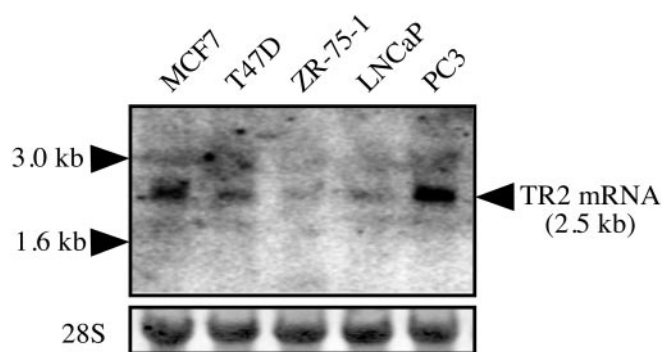


FIG. 1. Expression of TR2 mRNA in the breast cancer cell lines. 20 μ g of total RNA was extracted from cancer cells. A TR2 cDNA encoding LBD (aa 179–603) was random primed-labeled with [α - 32 P]dCTP and used as a probe. Northern hybridization was performed using Rapid-hyb buffer (Amersham Biosciences) according to manufacturer's instructions. 28 S ribosome RNA was stained with 0.04% methylene blue in sodium acetate, pH 5.0, for RNA integrity and quantity control. kb, kilobases.

used for internal control, and luciferase activity was analyzed using the dual-luciferase reporter assay system (Promega) following the manufacturer's instructions.

Co-immunoprecipitation—MCF7 cells plated on 100-mm dishes were solubilized in 1 ml of radioimmune precipitation assay buffer containing 0.5% Nonidet P-40 and protease inhibitors. Immunoprecipitation was performed using rabbit anti-ER antibody (1:100) (H-184) and then analyzed by Western blotting with anti-ER (1:1000) (H-184) or anti-TR2 (1:1000) (G204) antibodies, followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgM antibodies and visualized with alkaline phosphatase conjugate substrate kit (Bio-Rad).

GST Pull-down Assay—GST alone and GST fusion proteins were purified by glutathione-Sepharose 4B beads as instructed by manufacturer (Amersham Biosciences). The pull-down assay was performed with 5 μ l of *in vitro* translated 35 S-labeled proteins as described previously (37).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was carried out as described previously (38) with some modifications. Human complement C3 ERE (containing one imperfect palindromic inverted repeat: 5'-AGGTGGCCCTGACCC-3') end-labeled with [γ - 32 P]ATP was used as a probe. ER and TR2 were *in vitro* translated by the TNT system (Promega) as instructed by manufacturer. Reactions were performed in 20 μ l of EMSA binding buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 1 mM dithiothreitol, 0.5 mM EDTA, 2.5 mM MgCl $_2$, and 6% glycerol). For the antibody supershift analysis, 1 μ l of the monoclonal anti-ER α antibody (C-314) was used. The protein-DNA complexes were analyzed on a 5% polyacrylamide native gel containing 2.5% glycerol in 1 \times Tris borate EDTA.

RESULTS

TR2 mRNA Is Expressed in the Breast Cancer Cell Lines—The studies of TR2 tissue distribution demonstrate that TR2 is expressed in many tissues with higher expression in brain and male reproductive organs (1, 2, 13, 14). To explore whether TR2 is expressed in female tissues, the expression of TR2 mRNA was examined in several breast cancer cells using Northern blot analysis. As shown in Fig. 1, TR2 transcripts around 2.5 kilobases were detected in three ER-positive breast cancer cell lines (MCF7, T47D, and ZR-75-1) at different expression levels. Also, TR2 transcripts could be detected in LNCaP and PC-3 prostate cancer cells as a control (Fig. 1).

TR2 Selectively Suppresses ER-mediated Transcription—The ER is known to be highly involved in the breast cancer development because two-thirds of breast tumors contain a functional ER that mediates estrogen responsiveness to stimulate cell growth. Because the TR2 was detected in the breast cancer cells, we wondered if TR2 could affect ER function. Using the ERE-CAT reporter system, TR2 was found to consistently suppress the transcriptional activity of exogenous (in PC-3 and H1299 cells) and endogenous (in MCF7 and T47D

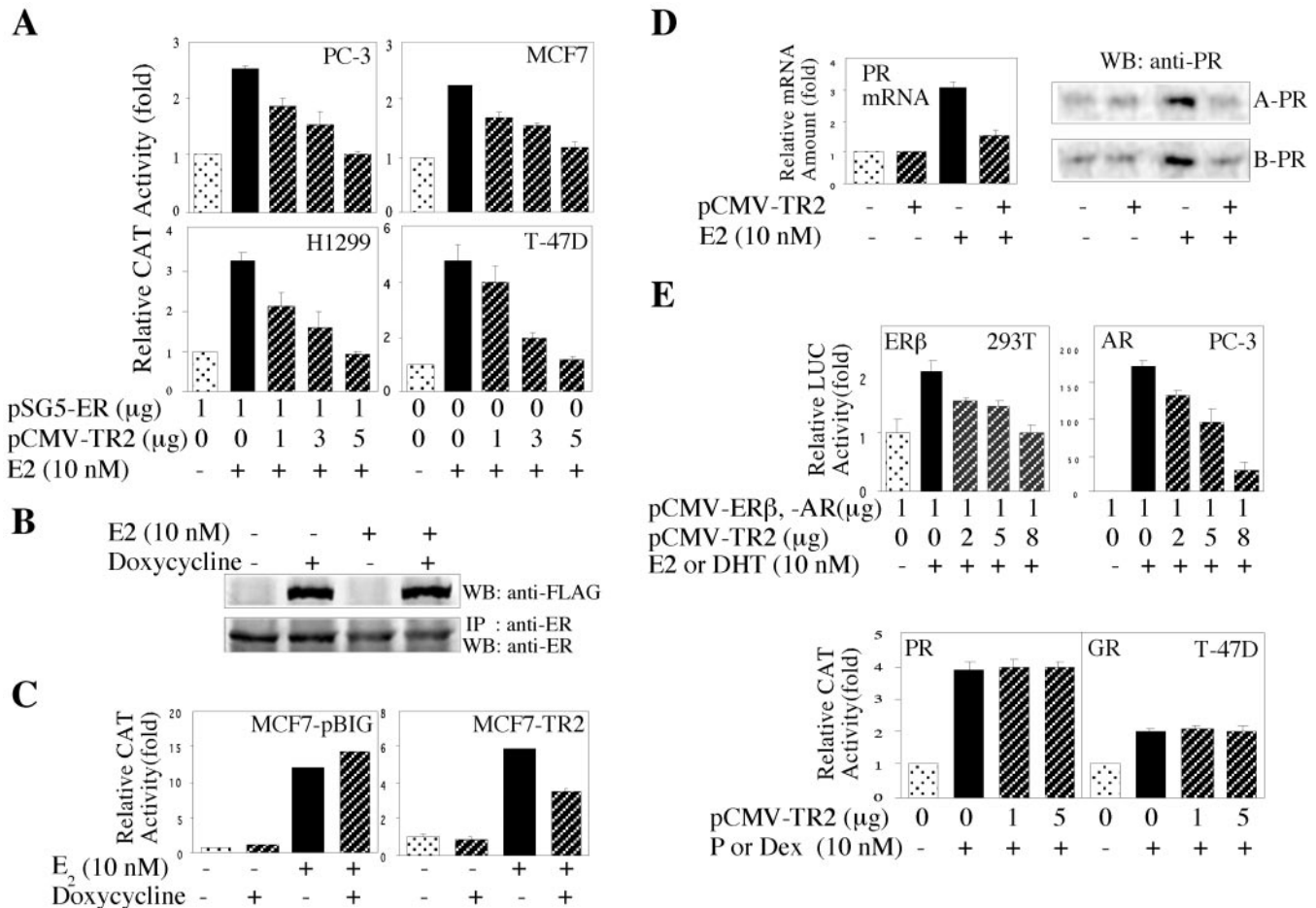


FIG. 2. The effects of TR2 on ER α -, ER β -, AR-, PR-, and glucocorticoid receptor-mediated transactivation. **A**, cells in 60-mm dishes were transfected with 2 μ g of ERE-CAT and expression plasmids, as indicated, by calcium phosphate precipitation methods. 1 μ g of β -galactosidase expression plasmid, pCMV- β -gal, was used as an internal control for transfection efficiency. Sixteen hours after transfection, cells were treated with ethanol or 10 nM E2 for another 16 h and then harvested for CAT assay. **B**, MCF7-TR2 cells were treated with 10 nM E2 and/or 2 μ g/ml doxycycline for 24 h. Cell lysates were subjected to Western blotting (WB) using anti-FLAG antibody (M2) to monitor the induction of FLAG-tagged TR2. The ER expression level was determined by co-immunoprecipitation (IP) followed by Western blotting with anti-ER antibody (H-184) using cell lysates containing 400 μ g of total proteins. **C**, MCF7-pBIG and MCF7-TR2 cells were transfected with 2 μ g of ERE-CAT and 1 μ g of pCMV- β -gal and, after 16 h, treated with or without 2 μ g/ml doxycycline. Cells were then harvested for CAT assay. **D**, T-47D cells seeded in 60-mm dishes were transfected with 10 μ g of pCMV or pCMV-TR2 for 16 h followed by treatment with 10 nM E2 for another 16 h. Cell extracts (80 μ g) and total RNA (15 μ g) were used for Western blotting with anti-PR antibody (H-190) and Northern blotting, respectively. Relative mRNA expression amounts were normalized by 28 S expression and quantitated by ImageQuant V.1.2 (Molecular Dynamics). **E**, methods used are the same as described in **A**. ERE- and MMTV-luciferase reporter genes were used for examination of ER β and AR transactivation, respectively. MMTV-CAT reporter was used for PR and glucocorticoid receptor transactivation. Luciferase activity was analyzed following the manufacturer's instructions (Promega). 10 nM of E2, 5 α -dihydrotestosterone (DHT), progesterone (P), and dexamethasone (Dex) were used as indicated. CAT and luciferase activity are presented relative to the response to ethanol, which is set as one. Values are the means \pm S.D. of three independent experiments.

cells) ER in a dose-dependent manner (Fig. 2A). To determine whether the expression level of the ER was affected by TR2, we used a stable clone of MCF7-TR2 cells, where the expression of FLAG-tagged TR2 driven by pBIG2i vector could be induced by doxycycline treatment (2 μ g/ml) and detected by Western blotting with anti-FLAG antibody (Fig. 2B). First, the expression of doxycycline-induced FLAG-TR2 was not influenced by 17 β -estradiol (E2) treatment. Secondly, the expression level of the ER was not suppressed by overexpressed FLAG-TR2 induced by doxycycline in MCF7-TR2 cells, indicating that the suppression of the ER by TR2 does not result from the down-regulation of ER expression. Also, we found that, in transient transfection assays, both endogenous and exogenous ER levels were not affected by transiently overexpressed TR2 in MCF7 and COS-1 cells (data not shown). Consistent with the phenomenon in Fig. 2A, ER was suppressed by doxycycline-induced TR2 in MCF7-TR2 cells (Fig. 2C). By contrast, the doxycycline treatment did not affect ER transcriptional activity in the MCF7-pBIG cells,

which were stably transfected with the pBIG2i parent vector (Fig. 2C). To rule out the artificial effects linked to foreign reporters, as demonstrated in Fig. 2, A and C, PR expression, an endogenous target gene of the ER, was examined. As shown in Fig. 2D, TR2 could repress E2-induced PR expression at mRNA and protein levels in T47D cells as well as in MCF7 cells (data not shown). Interestingly, TR2 could also suppress the basal level of ER transcription in the absence of E2 (data not shown). For examining the specificity, we also tested the effect of TR2 on other classical steroid receptors. As shown in Fig. 2E, whereas TR2 could also suppress ER β - and androgen receptor (AR)-mediated transcription in HEK293 (no detectable ER) and PC-3 cells, respectively, TR2 has little effect on the PR- or glucocorticoid receptor-mediated transcription in T47D cells. Taken together, results from Fig. 2 demonstrate that TR2 can suppress ER α -mediated transcription, and these suppressive effects are rather receptor-specific.

TR2 Physically Associates with the ER—To investigate

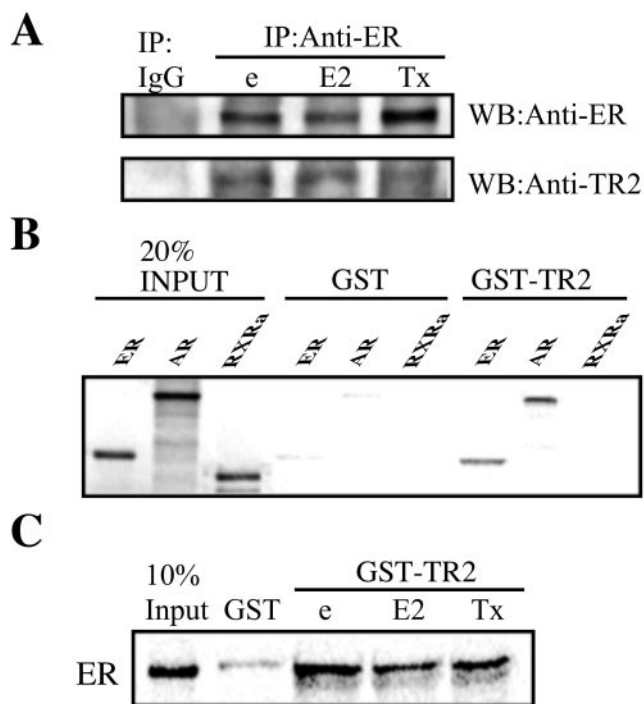


FIG. 3. The physical association analysis between the ER and TR2. A, 500 μ g of total proteins from MCF7 cells treated with ethanol (e), 10 nM E2, or 1 μ M tamoxifen (Tx) for 24 h were immunoprecipitated (IP) with normal rabbit IgG or rabbit anti-ER antibody (H-184) as indicated. The immunoprecipitates were subjected to Western blotting (WB) with anti-ER (1:1000, H-184) or anti-TR2 (1:1000, G204) antibodies. B, the GST and GST-TR2 fusion proteins were purified as instructed by the manufacturer (Amersham Biosciences). 5 μ l of *in vitro* translated 35 S-labeled AR, ER, and RXR α were incubated with the GST or GST-TR2 fusion proteins bound to glutathione-Sepharose beads in a pull-down assay. After extensive washing, bead-bound protein complexes were loaded onto 8% SDS-PAGE and analyzed by PhosphorImager (Molecular Dynamics). The input represents 20% of the 35 S-labeled proteins used in each pull-down assay. C, ligand effects on the interaction between the ER and TR2. Three kinds of treatments (ethanol, 10 nM E2, 1 μ M tamoxifen) were added individually in each GST pull-down reaction as indicated. The input represents 10% of the 35 S-labeled ER used in each pull-down assay.

whether TR2 and the ER are physically associated, co-immunoprecipitation and GST pull-down assays were carried out for examination of *in vivo* and *in vitro* interaction, respectively. Cell extracts from MCF7 cells treated with ethanol, E2, and tamoxifen were co-immunoprecipitated with anti-ER antibody (H-184). Immunocomplexes were then Western-blotted with anti-TR2 antibody (G204). As shown in Fig. 3A, TR2 was in ER immunocomplexes in the presence of ethanol, E2, or tamoxifen. Using GST pull-down assays, GST-TR2 fusion protein could directly interact with *in vitro* translated 35 S-labeled ER and AR but not RXR α (Fig. 3B). For testing ligand effects on the ER-TR2 binding, not much difference was found among different treatments (Fig. 3C). Collectively, these results suggest that ER and TR2 are directly associated with each other in a ligand-independent manner.

To dissect the TR2 interaction domain on the ER, six ER peptides fused with GST were tested in GST pull-down assays. As shown in Fig. 4A, GST-ER-2 (aa 123–340) and GST-ER-3 (aa 312–595), but not GST-ER-1 (aa 1–165) and GST-ER-4 (aa 552–595), can interact with TR2 in the presence or absence of E2. Furthermore, GST-ER-6 (aa 312–340), the overlapping region between GST-ER-2 and -3, but not GST-ER-5 (aa 123–312), showed positive interaction with TR2, indicating that ER-6 domain is responsible for this interaction. On the other hand, three GST-fused TR2 fragments, GST-TR2-1, -2, and -3, corresponding to N terminus (aa 1–112), DBD (aa 88–196), and

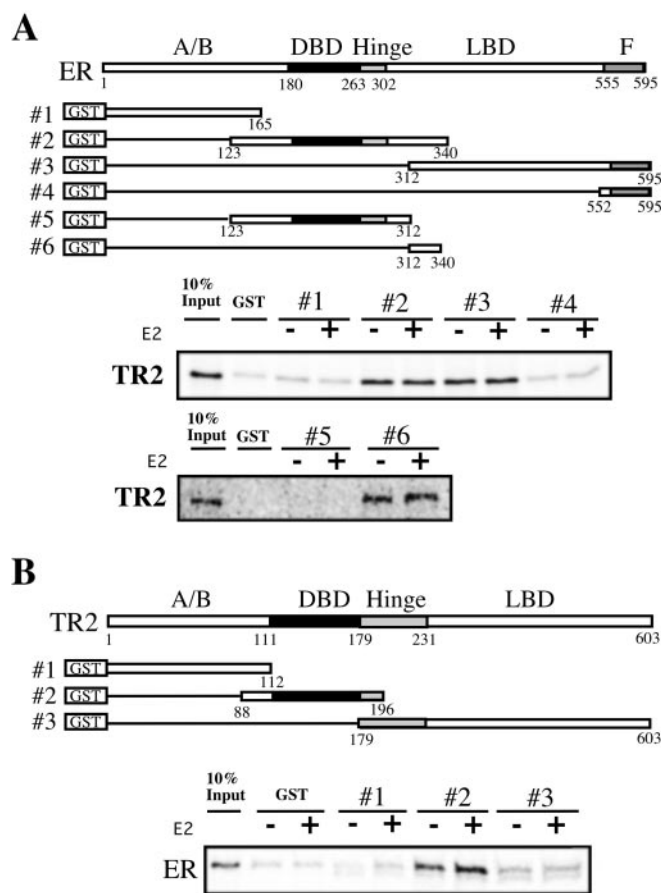


FIG. 4. Mapping the interaction domains between the ER and TR2. A, the construction of GST-ER fragments is illustrated schematically on the upper panel. GST alone and GST fusion proteins were purified as described by the manufacturer's instructions (Amersham Biosciences). 5 μ l of 35 S-labeled TR2 was incubated with GST or GST-ER fusion proteins bound to glutathione-Sepharose beads in the absence or presence of 1 μ M E2. After extensive washing, bead-bound protein complexes were loaded onto 8% SDS-PAGE and analyzed by PhosphorImager (Molecular Dynamics). B, schematic representation of GST-TR2 constructs is illustrated on the upper panel. GST or three GST-TR2 fusion proteins were purified and incubated with 5 μ l of 35 S-labeled ER in a pull-down assay. The input represents 10% of the 35 S-labeled proteins used in each pull-down assay.

LBD (aa 179–603), respectively, were also examined to locate the ER binding region. As shown in Fig. 4B, GST-TR2-2, but not GST-TR2-1 or -3, was responsible for binding to the ER.

Direct Association Is Required for TR2-mediated Suppression of the ER—Small proteins (<20–30 kDa) are presumably capable of quickly crossing nuclear pore complexes via passive diffusion (39). Ideally, introducing small peptides containing interaction sequences may mask the binding sites from binding to the interacting proteins in either cytoplasm or nucleus. The small peptide ER-6 was, therefore, tested to determine whether it can serve as an interaction blocker to interfere with ER-TR2 binding by using the GST pull-down assay and mammalian two-hybrid system where *in vitro* translated HA-tagged ER-6 and pCDNA3-HA-ER-6 plasmid were introduced, respectively. First, the interaction of GST-TR2 with 35 S-labeled ER was inhibited by increasing amounts of HA-ER-6 peptide (Fig. 5A). Secondly, GAL4-ER can interact with VP16-TR2 in the presence of E2, according to the induction of CAT activity, and this ER-TR2 interaction was suppressed when co-transfecting with pCDNA3-HA-ER-6 (Fig. 5B). Thus, based on these results, ER-6 is able to be an interaction blocker. Next, to determine whether direct association is required for TR2 to suppress the ER, pCDNA3-HA-ER-6 was applied in an ERE-CAT reporter

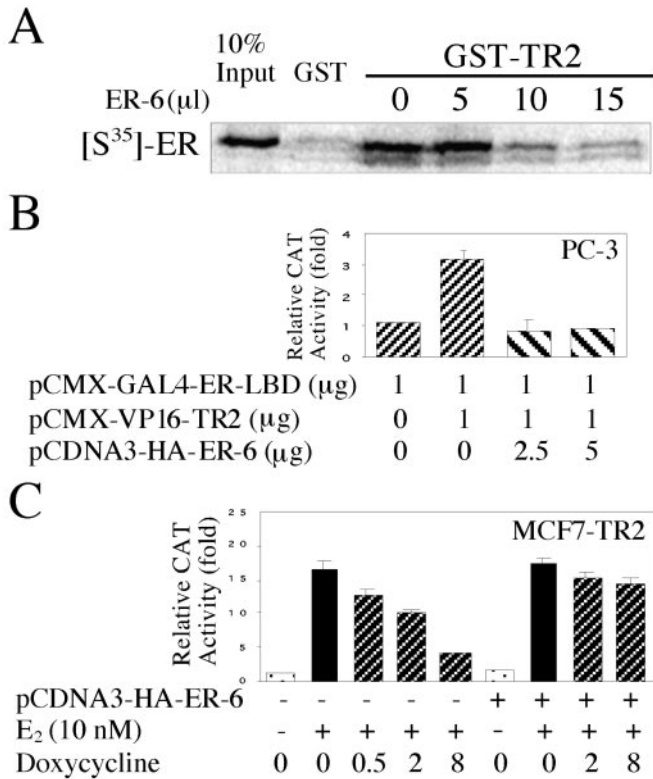


FIG. 5. ER-6 serves as the ER-TR2 interaction blocker capable of reversing the suppression of the ER by TR2. *A*, ER-6 blocks ER-TR2 interaction in a GST pull-down assay. GST and GST-TR2 fusion proteins were purified as described by the manufacturer (Amersham Biosciences). Glutathione-Sepharose bead-bound GST-TR2 was then incubated with 5 μ l of ³⁵S-labeled ER with increasing amounts of HA-ER-6, which was *in vitro* translated from a pCDNA3-HA-ER-6 plasmid for 2 h at 4 °C in the absence of E₂. After extensive washing, bead-bound protein complexes were loaded onto 8% SDS-PAGE and analyzed by PhosphorImager (Molecular Dynamics). The input represents 10% of the ³⁵S-labeled ER used in each pull-down assay. *B*, ER-6 inhibits the ER-TR2 interaction in the mammalian two-hybrid system. PC-3 cells plated on 60-mm dishes were co-transfected with 2 μ g of pG5-CAT reporter with expression plasmids as indicated. 1 μ g of pCMV- β -gal was also used as an internal control for transfection efficiency. CAT activity was analyzed in the presence of 10⁻⁸ M E₂. *C*, ER-6 reverses TR2-mediated suppression of ER transactivation. MCF7-TR2 cells were co-transfected with 2 μ g of ERE-CAT, 1 μ g of pCMV- β -gal, and 7 μ g of pCDNA3 or pCDNA3-HA-ER-6. Sixteen hours after transfection, cells were treated with ethanol, 10 nM E₂, and/or increasing amounts of doxycycline as indicated for another 16 h. Cells were then harvested for a CAT assay. CAT activity is presented relative to the response to ethanol, which is set as one. Values are the means \pm S.D. of three independent experiments.

gene assay. As shown in Fig. 5C, the E₂-induced ER transcription was significantly repressed by the doxycycline-induced TR2 in a dose-dependent fashion in MCF7-TR2 cells. The addition of ER-6 was then capable of reversing this suppression, suggesting that TR2 suppresses the ER through direct interaction.

The Biological Significance of TR2 on ER Activity—Antisense TR2 expression plasmids, pCDNA3-TR2-fl AS and pIRES-TR2-N AS, were assessed in a ERE-luciferase assay to determine whether reducing endogenous TR2 expression might significantly enhance ER activity in MCF7 cells. First, using Western blotting with anti-TR2 antibody (G204), those two antisense constructs were proven to be able to reduce the expression of endogenous TR2 as well as overexpressed TR2 (Fig. 6A). This reduction of endogenous TR2 by antisense plasmids resulted in an increase in ER transcription in a dose-dependent manner (Fig. 6B), indicating that endogenous TR2 normally suppresses ER activity in MCF7 cells.

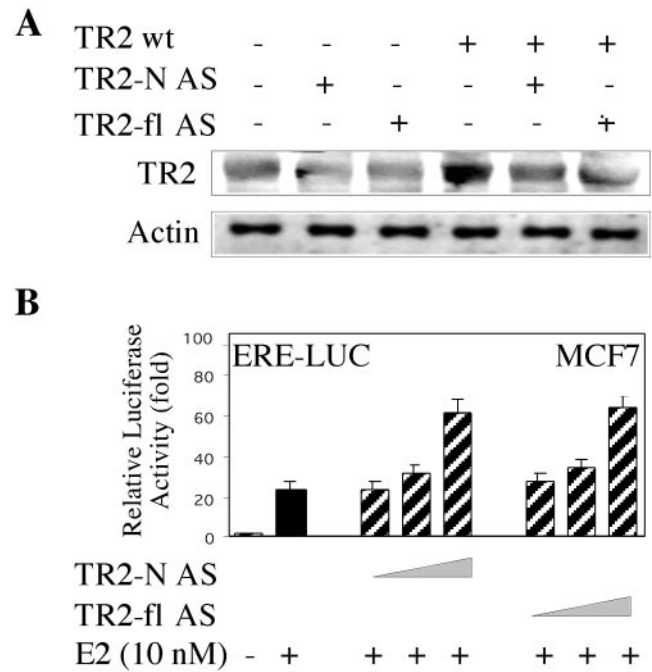
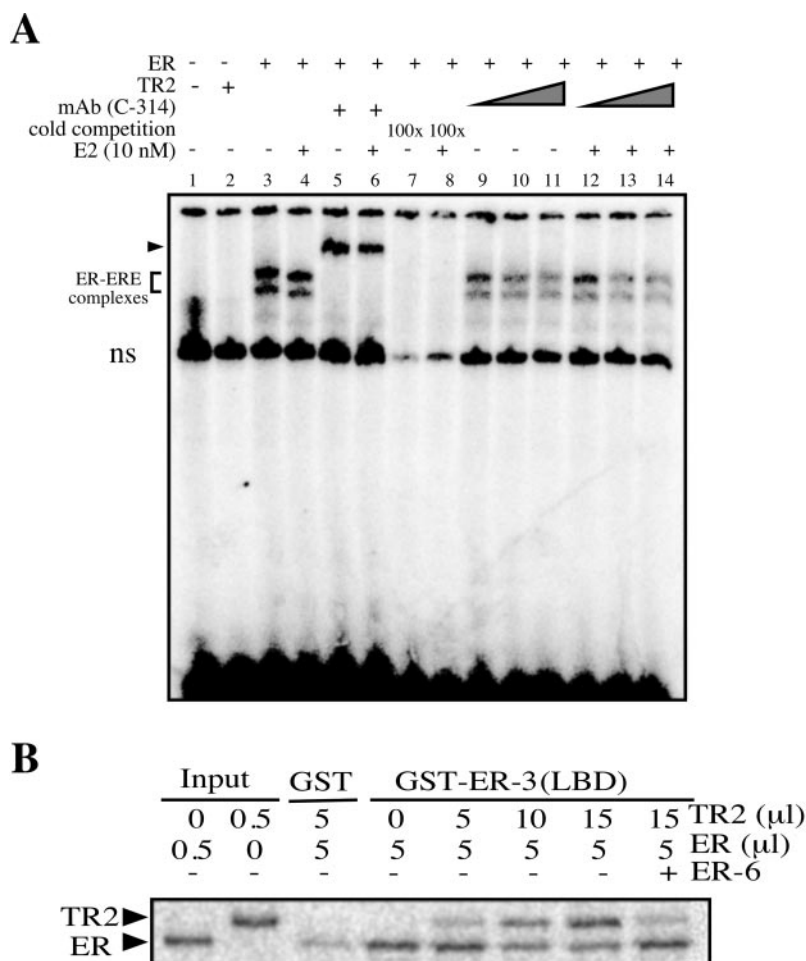


FIG. 6. Enhancement of ER transcriptional activity by administration of antisense TR2 in MCF7 cells. *A*, MCF7 cells cultured in 35-mm dishes were transfected with 1 μ g of pCDNA3-TR2-fl AS, pIRES-TR2-N AS, and pCMV-TR2 plasmids using SuperFect Transfection Reagent (Qiagen). The total amount of plasmids in each dish was made up to 2 μ g by adding the parent vectors. After 32 h, cells were harvested, and 80 μ g of cell lysates were subjected to Western blotting with anti-TR2 antibody (G204) and anti-actin antibody (C-11). *B*, MCF7 cells cultured in 35-mm dishes were transfected with 0.125 μ g of ERE-Luc and increasing amounts (0.5–1.875 μ g) of pCDNA3-TR2-fl AS and pIRES-TR2-N AS plasmids as indicated. 10 ng of pRL-TK (Promega) was used for the internal control. The total amount of plasmids in each dish was made up to 2 μ g by adding the pCDNA3 parent vector. Sixteen hours after transfection, cells were treated with or without 10 nM E₂ for another 16 h. Luciferase activity was analyzed according to manufacturer's instructions (Promega). Luciferase activity is presented relative to response to ethanol, which is set as 1. Values are the means \pm S.D. of three independent experiments.

ER DNA Binding and Homodimeric Formation Are Disrupted by Associating with TR2—To elucidate the molecular mechanisms by which the ER was suppressed by interacting with TR2, we have tested the effect of TR2 on ER expression, stability, nuclear translocation, DNA binding, and interaction with coregulators. We found that overexpression of TR2 did not influence ER expression (Fig. 2B), stability, or nuclear translocation (data not shown). Using GST pull-down assays and mammalian two-hybrid assays, TR2 did not affect the binding between the ER and some coregulators such as SRC-1, TIF-II, and ARA70 (data not shown). After excluding those possibilities, we found that TR2 may mainly influence the ER on DNA binding. Using the EMSA assay as shown in Fig. 7A, two specific ER-ERE bands could be detected (lanes 3 and 4) and were supershifted by ER antibody (C-314) (lanes 5 and 6, indicated as an arrowhead). Two ER-ERE bands are presumably composed of a monomer and a dimer of ER bound to ERE. Because the sequences of ERE used in this assay contain one imperfect palindromic inverted repeat, the ER was bound to the half side of ERE as monomer. However, the monomer of ER bound to ERE does not occur *in vivo* because of the instability (40). Then, the addition of a 100-fold molar excess of unlabeled ERE could effectively eliminate these bands (lanes 7 and 8). Interestingly, the intensity of these ER-ERE complexes was decreased upon the addition of increasing amounts of TR2 in either the absence (lanes 9–11) or the presence of 10 nM E₂ (lanes 12–14). Because no ERE-TR2-specific band (lane 2) and

FIG. 7. Interference with ER binding to ERE by ER-TR2 heterodimer formation.

A, interruption of ER binding to ERE by TR2 in EMSA. ^{32}P -End-labeled ERE probe (4×10^8 dpm/ μg) was incubated with *in vitro* translated TR2 and ER proteins (ratios from 1:1 to 1:4) in EMSA binding buffer and analyzed on a 5% acrylamide native gel containing 2.5% glycerol. $1 \mu\text{l}$ of anti-ER α monoclonal antibody (mAb) (C-314) was added for antibody supershifts (lane 5 and 6). A 100-fold molar excess of unlabeled ERE probe was added as a cold competitor (lane 7 and 8). Ethanol or 10 nM E2 was added as indicated. The migration positions of the supershifted band formed by Ab-ER-ERE are indicated as an arrowhead. ns, non-specific binding. **B**, ER homodimeric formation is disrupted by TR2 but rescued by ER-6. GST-ER-3 (LBD) and GST proteins were purified as described by the manufacturer (Amersham Biosciences). *In vitro* translated ^{35}S -labeled ER with increasing amounts of ^{35}S -labeled TR2 were co-incubated with GST-ER-3 or GST alone that were bound to glutathione-Sepharose beads. ER-6 peptide was obtained using the thrombin protease cleavage method (Roche Molecular Biochemicals) to release ER-6 peptide from bead-bound GST-ER-6. Equal amounts of GST-ER-3 and GST-ER-6 were used as determined by a Coomassie stained gel. After extensive washing, bead-bound protein complexes were loaded onto 8% SDS-PAGE and analyzed by PhosphorImager (Molecular Dynamics). The input represents $0.5 \mu\text{l}$ of ^{35}S -labeled ER and TR2 used in each reaction.



no extra supershifted band formed as TR2-ER-ERE complexes (lanes 3–4 versus 9–14) were found, we may conclude that TR2 interacts with the ER, resulting in the ER dissociating from binding to DNA. Accordingly, the competition assay (Fig. 7B) showed that the ER homodimer formation, as illustrated by the interaction between GST-ER-LBD and ^{35}S -labeled ER, was decreased by the presence of TR2, and conversely, the heterodimeric formation of ER-TR2 was increased along with the increasing amounts of TR2. It indicates that TR2 forms a TR2-ER heterodimer, but not a TR2-ER-ER complex, to interfere with ER homodimerization. Furthermore, the reduction of ER homodimerization by TR2 could be reversed when the ER-6 peptide, which prevents TR2 from binding to ER, was added (Fig. 7B). Taken together, Fig. 7 suggests that TR2 may suppress ER-mediated transactivation via the formation of a TR2-ER heterodimer that reduces ER homodimerization and causes ER dissociation from ERE.

TR2 Suppresses E2/ER-induced Cell Growth and G₁/S Transition—E2, through the ER, is known to enhance G₁/S transition and stimulate cell proliferation in estrogen-dependent breast cancer cells (41–45). To investigate whether the suppression of the ER by TR2 can affect breast cancer cell growth in response to estrogen, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were carried out to examine the cell viability. The data from MTT assays (Fig. 8A) showed that the addition of E2 for 5 days apparently stimulated cell growth in both MCF7-pBIG (MTT_{A570} 0.82 ± 0.008) and MCF7-TR2 cells (MTT_{A570} 0.74 ± 0.019) as compared with both cells treated with ethanol for 5 days (MTT_{A570} 0.58 ± 0.007 and 0.48 ± 0.032 , respectively). Although doxycycline had mild toxic effect on cell growth, as demonstrated by

causing the slight growth inhibition in MCF7-pBIG cells, the presence of doxycycline obviously arrested cell growth of the MCF7-TR2 cells (MTT_{A570} 0.30 ± 0.014 in ethanol treatment and 0.36 ± 0.054 in the presence of E2), indicating that TR2 expression abrogated the E2-induced cell proliferation in breast cancer cells. To determine whether TR2 can interrupt E2/ER-induced G₁/S transition, the cell cycle profile was obtained from flow cytometric analysis using MCF7-TR2 cells, which were treated with ethanol, E2, and doxycycline for 12 h. As shown in Fig. 8B, the addition of E2 to MCF7-TR2 cells cultured in RPMI medium with 10% of charcoal-dextran-treated fetal bovine serum can induce the G₁/S transition (G₁, from 42.6 to 32.9%; S, from 27.5 to 37.1%). In contrast, TR2 expression inhibited the E2-induced G₁/S transition, leading to the G₁ arrest (G₁, from 32.9 to 55.3%; S, from 37.1 to 21.4%). In the absence of E2, doxycycline treatment also resulted in an accumulation of G₁ cells (G₁, from 42.6 to 54.6%), which is consistent with the data that overexpression of TR2 could also suppress the basal level of ER transactivation in the absence of E2 in a ERE-luciferase reporter assay (data not shown). Meanwhile, we also observed that the cell size of MCF7-TR2, but not MCF7-pBIG cells, became larger after 3 days of doxycycline induction. Whether the changes of the cell size might be due to cell cycle arrest, as occurred in many other cases (46, 47), remains to be further investigated.

DISCUSSION

The discovery that TR2 is expressed in breast cancer cells (Fig. 1) and suppresses ER-mediated signaling (Fig. 2) may demonstrate a novel biological function of TR2 in the estrogen-responsive organs in addition to its potential physiological roles

A

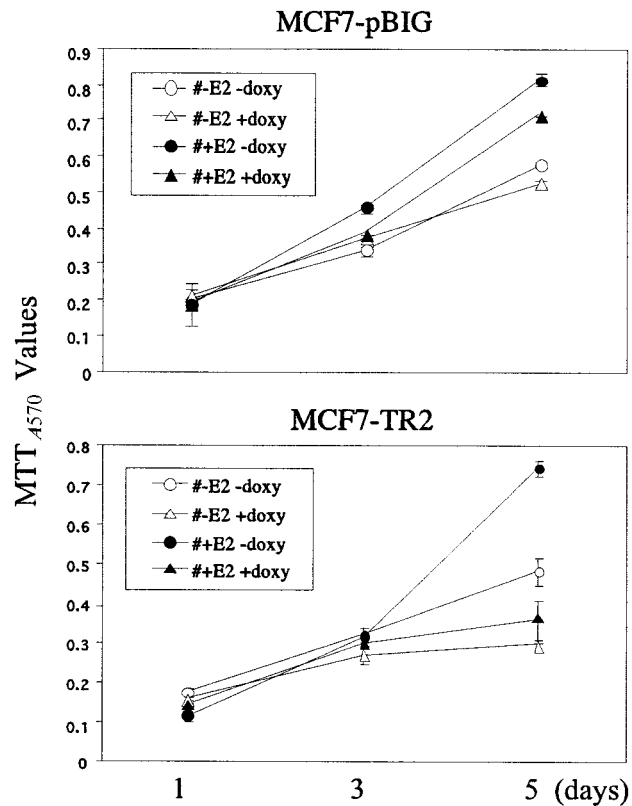
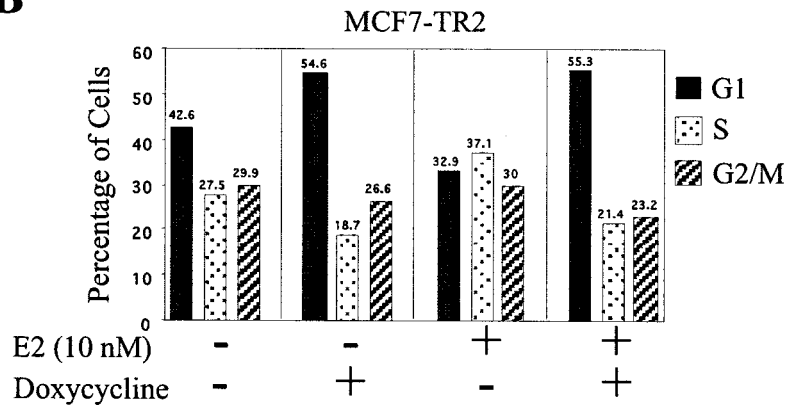


FIG. 8. The TR2 suppresses E2-induced breast cancer cell growth and G₁/S transition. A, growth assays were performed by the MTT method as instructed by the manufacturer (Sigma). 5×10^3 MCF7-pBIG and MCF7-TR2 cells were seeded in 24-well plates and incubated in RPMI with 10% charcoal-dextran-treated fetal bovine serum for 48 h. Cells were then treated with ethanol, 10 nM E2, and/or 2 μ g/ml doxycycline as indicated. After 1, 3, and 5 days of treatments, cells were harvested for an MTT assay. Values are the means \pm S.D. of A_{570} from three independent wells of cells. B, the inhibition of E2-induced G₁/S transition by TR2 in MCF7-TR2 cells. Cells were incubated in RPMI with 10% charcoal-dextran-treated fetal bovine serum for 48 h and then treated with ethanol, 10 nM E2, and 2 μ g/ml doxycycline as indicated for 12 h. Cells were then trypsinized and fixed overnight in 70% ethanol. After cells were incubated with 1 μ g/ml RNase A (Sigma) and propidium iodide (Roche Molecular Biochemicals), the DNA contents of cells were measured by flow cytometry.

B



in neurogenesis and spermatogenesis (12–15). TR2 was originally identified as a transcriptional factor that can modulate target gene expression via binding to its response elements (1, 2, 15) and can influence the activity of other transcription factors such as RXR, retinoic acid receptor, and thyroid receptor α through competing with the same DNA response elements in the cells (15, 20). In this study, we found that TR2 is also capable of interacting and regulating ER-mediated transcription in breast cancer cells (Figs. 2–4). Furthermore, the interaction blocker, ER-6, an ER fragment (aa 312–340) responsible for TR2 binding (Fig. 4A) was able to rescue the ER from suppression by TR2 (Fig. 5), and administration of antisense TR2 could enhance ER transcription in MCF7 cells (Fig. 6). Thus, these data provide a new molecular function of TR2 in modulating other nuclear receptor activity via the mechanism of direct protein-protein interactions, implying that TR2 may function as one of the repressors of ER-mediated signaling.

To further understand the molecular mechanisms by which TR2 suppresses the ER, TR2 may possibly influence the ER on expression, protein stability, nuclear translocation, DNA bind-

ing, interacting with coregulators, and/or post-translational modifications such as phosphorylation and acetylation. From our preliminary studies, we found that TR2 neither affects ER expression levels (Fig. 2B) and the nuclear translocation nor the interaction with some coactivators, such as SRC-1a, TIF-II, and ARA70 (data not shown). Despite not ruling out other possible mechanisms, such as post-translational modifications, data from EMSA (Fig. 7A) clearly demonstrate that the addition of TR2 may interrupt ER binding to DNA, and this dissociation may be due to the disruption of ER homodimerization as a result of the formation of non-functional TR2-ER heterodimers (Fig. 7B). It may consequently result in the suppression of ER transcription. Similarly, Resnick *et al.* (48) also demonstrate that the disruption of ER homodimerization by interaction with truncated estrogen receptor product-1 causes an interruption of ER-ERE binding, resulting in the suppression of ER-mediated transcription. By contrast, a tumor suppressor, p53, suppresses the ER via interfering with the DNA binding without affecting the dimerization (49). However, the mechanism by which p53 suppresses the ER remains unclear.

Although it is still unknown whether the region spanning aa 341 to 551 on the ER provides the binding sites for TR2, ER-6 (aa 312–340) is sufficient to bind TR2 and functions as an interaction blocker (Figs. 4 and 5). ER-6 covers the region spanning helix 1 to part of helix 3 within the N terminus of the ER LBD, which is located outside of the ligand-binding pocket and has no critical amino acids responsible for hormone binding (50). This binding region for TR2 is different from the region known as the AF-2 domain for most other ER coactivators, such as SRC-1 and the related p160 family, which contain the signature motif of the NR box (LXXLL) responsible for interacting with the ER in the presence of ligands (51). The AF-2 interaction surface is comprised of the specific amino acids in helix 3, 4, 5, and 12 and, upon ligand binding, forms a hydrophobic cleft where helix 12 is positioned over the ligand binding pocket, providing a surface for those coregulators binding (52, 53). The different binding sites for TR2 and ligand-dependent coactivators on the ER may provide an explanation for our results showing that ER-TR2 interaction was ligand-independent (Figs. 3 and 4) and that TR2 did not interfere with the ER interacting with those coactivators (data not shown). Consistent with this phenomena, an antiestrogen, tamoxifen, did not affect their interaction, as shown in Fig. 3, A and C. It is also consistent with the finding that a signature motif, LXXLL, located on the TR2 LBD (aa 547–551), is not required for ER interaction since the ER binding region is located on the TR2 DBD (Fig. 4B). The similar phenomenon has also been demonstrated by Delage-Mourroux *et al.* (54). They demonstrated that REA, a repressor of estrogen receptor activity, interacts with the ER through a ligand-independent fashion, where the LXXLL motif of REA and the helix 12 of the ER are not involved in the binding. However, they showed that the integrity of the LXXLL motif is still important for REA to perform its suppressive effect on the ER, although it is not required for interaction. Therefore, it will be interesting to determine whether the LXXLL motif within TR2 is also necessary for suppression of the ER.

It has long been known that the beneficial effects of antiestrogens on ER-positive breast tumors is probably due to blockage of E2/ER-mediated cell growth (33). Therefore, to identify the repressors of the ER and understanding their molecular mechanisms may provide information toward the development of therapeutic drugs to battle the E2/ER-dependent tumors. However, few ER suppressors have been identified and characterized (55), and the detailed suppression mechanisms also remains largely unknown. Early reports suggest several possible mechanisms including 1) the interference of the binding capacity of ER homodimers to ERE, such as p53 (49) and truncated estrogen receptor product-1 (48), 2) competition with coactivators for binding to the ER, such as short heterodimer partner (SHP) (56), DAX-1 (57), truncated estrogen receptor product-1 (48), and REA (54), and 3) recruitment of histone deacetylases to the ER, such as metastatic-associated protein 1 (58) and SMRT (59). Here our data provide another repressor, TR2, functioning through the formation of nonfunctional ER-TR2 heterodimers that result in the ER dissociating from ERE.

The ER is also known to function as a modulator to regulate the function of other nuclear receptors, such as TR, retinoic acid receptor, and RXR, through protein-protein interaction (60). The ER also interacts with and suppresses the transcriptional activity of proapoptotic forkhead transcription factor in the presence of estrogen (61). Unexpectedly, we also found that the ER could suppress TR2-mediated transcription in a ligand-independent manner (data not shown). This suppression was not mediated via interruption of TR2 DNA binding, although

the ER interaction site is located on the TR2 DBD (data not shown). The mechanism by which the ER suppresses TR2 remains unclear at this moment. Nevertheless, these findings represent a mutual regulation between the ER and TR2 within the nuclear hormone receptor superfamily.

Fig. 8 demonstrates that TR2 can suppress E2/ER-induced G₁/S transition and cause cell growth inhibition in MCF7-TR2 cells, where TR2 could be induced by treatment with doxycycline. This growth suppression is suggested to mainly go through suppression of the ER signal since TR2 lost this suppressive effect on cell growth in the presence of tamoxifen (data not shown). However, we cannot rule out the possibility that TR2 mediates growth inhibition through the pathways independent of the ER. Earlier studies show that TR2 induction is involved in neuronal differentiation in mouse P19 stem cells stimulated by either retinoic acid or CNTF (13, 62), suggesting that TR2 may have a role in cell differentiation and negative regulation of cell proliferation. Because TR2 is located in chromosome 12q22, a known region frequently deleted in various tumors including testicular and ovarian germ cell tumors (4, 5), it will be interesting to link TR2 as one of the tumor suppressor candidates that can negatively regulate cell growth.

Taken together, TR2 may function not only as a transcription factor but also an important repressor in regulating ER-mediated transcription in mammary glands. Therefore, our future study may expand to determine the physiological roles of TR2 in TR2 knockout mice, especially in the development of mammary glands as well as brain, nervous, and reproductive systems, where the ER is known to exert an essential role.

Acknowledgments—We thank Drs. Hinrich Gronemeyer (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France), Jay Reeder (University of Rochester, Rochester, NY), Frank B. Furnari (University California, San Diego, CA), and Vincent Giguère (McGill University, Québec, Canada) for the generous gifts of plasmids. We also thank Karen Wolf for grammar and vocabulary corrections.

REFERENCES

- Chang, C., Kokontis, J., Acakpo-Satchivi, L., Liao, S., Takeda, H., and Chang, Y. (1989) *Biochem. Biophys. Res. Commun.* **165**, 735–741
- Chang, C., and Kokontis, J. (1988) *Biochem. Biophys. Res. Commun.* **155**, 971–977
- Lin, D. L., Wu, S. Q., and Chang, C. (1998) *Endocrine* **8**, 123–134
- Faulkner, S. W., and Friedlander, M. L. (2000) *Gynecol. Oncol.* **77**, 283–288
- Murty, V. V., Renault, B., Falk, C. T., Bosl, G. J., Kucherlapati, R., and Chaganti, R. S. (1996) *Genomics* **35**, 562–570
- Chang, C., Da Silva, S. L., Ideta, R., Lee, Y., Yeh, S., and Burbach, J. P. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 6040–6044
- Kontogianni-Konstantopoulos, A., Vlahou, A., Vu, D., and Flytzanis, C. N. (1996) *Dev. Biol.* **177**, 371–382
- Zelhof, A. C., Yao, T. P., Evans, R. M., and McKeown, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 10477–10481
- Wirtanen, L., Huard, V., and Seguin, C. (1997) *Differentiation* **62**, 159–170
- Huard, V., and Seguin, C. (1998) *DNA Sequencing* **9**, 113–120
- Le Jossic, C., and Michel, D. (1998) *Biochem. Biophys. Res. Commun.* **245**, 64–69
- Lee, C. H., Chang, L., and Wei, L. N. (1996) *Mol. Reprod. Dev.* **44**, 305–314
- Young, W. J., Lee, Y. F., Smith, S. M., and Chang, C. (1998) *J. Biol. Chem.* **273**, 20877–20885
- Lee, H. J., Young, W. J., Shih, C. Y., and Chang, C. (1996) *J. Biol. Chem.* **271**, 10405–10412
- Lin, T. M., Young, W. J., and Chang, C. (1995) *J. Biol. Chem.* **270**, 30121–30128
- Fisk, G. J., and Thummel, C. S. (1998) *Cell* **93**, 543–555
- Lee, C. H., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Wei, L. N. (1995) *Genomics* **30**, 46–52
- Lee, H. J., and Chang, C. (1995) *J. Biol. Chem.* **270**, 5434–5440
- Lee, H. J., Lee, Y., Burbach, J. P., and Chang, C. (1995) *J. Biol. Chem.* **270**, 30129–30133
- Chang, C., and Pan, H. J. (1998) *Mol. Cell Biochem.* **189**, 195–200
- Lee, H. J., Lee, Y. F., and Chang, C. (1999) *Mol. Cell. Biochem.* **194**, 199–207
- DeChiara, T. M., Vejsada, R., Poueymirou, W. T., Acheson, A., Suri, C., Conover, J. C., Friedman, B., McClain, J., Pan, L., and Stahl, N. (1995) *Cell* **83**, 313–322
- Mu, X., Liu, Y., Collins, L. L., Kim, E., and Chang, C. (2000) *J. Biol. Chem.* **275**, 23877–23883
- Lin, D. L., and Chang, C. (1996) *J. Biol. Chem.* **271**, 14649–14652
- Collins, L. L., Lin, D. L., Mu, X. M., and Chang, C. (2001) *J. Biol. Chem.* **276**, 27316–27321
- Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G.,

- Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J. A. (2001) *Physiol. Rev.* **81**, 1535–1565
27. Couse, J. F., and Korach, K. S. (1999) *Endocr. Rev.* **20**, 358–417
28. Zeps, N., Bentel, J. M., Papadimitriou, J. M., D'Antuono, M. F., and Dawkins, H. J. (1998) *Differentiation* **62**, 221–226
29. Jensen, E. V., Cheng, G., Palmieri, C., Saji, S., Makela, S., Van Noorden, S., Wahlstrom, T., Warner, M., Coombes, R. C., and Gustafsson, J. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 15197–15202
30. Sapino, A., Macri, L., Gugliotta, P., and Bussolati, G. (1990) *J. Histochem. Cytochem.* **38**, 1541–1547
31. Couse, J. F., Curtis, S. W., Washburn, T. F., Eddy, E. M., Schomberg, D. W., and Korach, K. S. (1995) *Biochem. Soc. Trans.* **23**, 929–935
32. Bocchinfuso, W. P., Hively, W. P., Couse, J. F., Varmus, H. E., and Korach, K. S. (1999) *Cancer Res.* **59**, 1869–1876
33. Early Breast Cancer Trialists' Collaborative Group. (1998) *Lancet* **351**, 1451–1467
34. Franco, P. J., Farooqui, M., Seto, E., and Wei, L. N. (2001) *Mol. Endocrinol.* **15**, 1318–1328
35. Strathdee, C. A., McLeod, M. R., and Hall, J. R. (1999) *Gene* **229**, 21–29
36. Green, S., Issemann, I., and Sheer, E. (1988) *Nucleic Acids Res.* **16**, 369
37. Yeh, S., Hu, Y. C., Rahman, M., Lin, H. K., Hsu, C. L., Ting, H. J., Kang, H. Y., and Chang, C. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 11256–11261
38. Lee, Y. F., Shyr, C. R., Thin, T. H., Lin, W. J., and Chang, C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 14724–14729
39. Gorlich, D., and Kutay, U. (1999) *Annu. Rev. Cell Dev. Biol.* **15**, 607–660
40. Klinge, C. M. (2001) *Nucleic Acids Res.* **29**, 2905–2919
41. Dickson, R. B., and Lippman, M. E. (1987) *Endocr. Rev.* **8**, 29–43
42. Katzenellenbogen, B. S., Montano, M. M., Ekena, K., Herman, M. E., and McNerney, E. M. (1997) *Breast Cancer Res. Treat.* **44**, 23–38
43. Donovan, J. C., Milic, A., and Slingerland, J. M. (2001) *J. Biol. Chem.* **276**, 40888–40895
44. Lazennec, G., Alcorn, J. L., and Katzenellenbogen, B. S. (1999) *Mol. Endocrinol.* **13**, 969–980
45. Planas-Silva, M. D., and Weinberg, R. A. (1997) *Mol. Cell. Biol.* **17**, 4059–4069
46. Rogatsky, I., Trowbridge, J. M., and Garabedian, M. J. (1997) *Mol. Cell. Biol.* **17**, 3181–3193
47. Kerkhoff, E., and Ziff, E. B. (1995) *EMBO J.* **14**, 1892–1903
48. Resnick, E. M., Schreihof, D. A., Periasamy, A., and Shupnik, M. A. (2000) *J. Biol. Chem.* **275**, 7158–7166
49. Liu, G., Schwartz, J. A., and Brooks, S. C. (1999) *Biochem. Biophys. Res. Commun.* **264**, 359–364
50. Tanenbaum, D. M., Wang, Y., Williams, S. P., and Sigler, P. B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 5998–6003
51. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) *Nature* **387**, 733–736
52. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) *Nature* **389**, 753–758
53. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) *Cell* **95**, 927–937
54. Delage-Mourroux, R., Martini, P. G., Choi, I., Kraichely, D. M., Hoeksema, J., and Katzenellenbogen, B. S. (2000) *J. Biol. Chem.* **275**, 35848–35856
55. Montano, M. M., Ekena, K., Delage-Mourroux, R., Chang, W., Martini, P., and Katzenellenbogen, B. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 6947–6952
56. Johansson, L., Thomsen, J. S., Damdimopoulos, A. E., Spyrou, G., Gustafsson, J. A., and Treuter, E. (1999) *J. Biol. Chem.* **274**, 345–353
57. Zhang, H., Thomsen, J. S., Johansson, L., Gustafsson, J. A., and Treuter, E. (2000) *J. Biol. Chem.* **275**, 39855–39859
58. Mazumdar, A., Wang, R. A., Mishra, S. K., Adam, L., Bagheri-Yarmand, R., Mandal, M., Vadlamudi, R. K., and Kumar, R. (2001) *Nat. Cell Biol.* **3**, 30–37
59. Smith, C. L., Nawaz, Z., and O'Malley, B. W. (1997) *Mol. Endocrinol.* **11**, 657–666
60. Lee, S. K., Choi, H. S., Song, M. R., Lee, M. O., and Lee, J. W. (1998) *Mol. Endocrinol.* **12**, 1184–1192
61. Schuur, E. R., Loktev, A. V., Sharma, M., Sun, Z., Roth, R. A., and Weigel, R. J. (2001) *J. Biol. Chem.* **276**, 33554–33560
62. Lee, C. H., and Wei, L. N. (2000) *Biochem. Pharmacol.* **60**, 127–136