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BRCA1 Modulates Xenobiotic Stress-inducible Gene Expression by Interacting with ARNT in Human Breast Cancer Cells*

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Previously, we have reported that BRCA1 regulates the expression of various classes of genes, including genes involved in xenobiotic stress responses (Bae, I., Fan, S., Meng, Q., Rih, J. K., Kim, H. J., Kang, H. J., Xu, J., Goldberg, I. D., Jaiswal, A. K., and Rosen, E. M. (2004) *Cancer Res.* 64, 7893–7909). In the present study, we have investigated the effects of BRCA1 on xenobiotic stress-inducible gene expression. In response to aryl hydrocarbon receptor (AhR) ligands, cytoplasmic AhR becomes activated and then translocates to the nucleus where it forms a complex with the aryl hydrocarbon receptor nuclear translocator (ARNT). Subsequently, the AhR·ARNT complex binds to the enhancer or promoter of genes containing a xenobiotic stress-responsive element and regulates the expression of multiple target genes including cytochrome P450 subfamily polypeptide 1 (CYP1A1). In this study, we have found that endogenous and overexpressed exogenous wild-type BRCA1 affect xenobiotic stress-induced CYP1A1 gene expression. Using a standard chromatin immunoprecipitation assay, we have demonstrated that BRCA1 is recruited to the promoter regions of CYP1A1 and CYP1B1 along with ARNT and/or AhR following xenobiotic exposure. Our findings suggest that BRCA1 may be physiologically important for mounting a normal response to xenobiotic insults and that it may function as a coactivator for ARNT activity. Using immunoprecipitation, Western blotting, and glutathione *S*-transferase capture assays, a xenobiotic-independent interaction between BRCA1 and ARNT has been identified, although it is not yet known whether this is a direct or indirect interaction. We have also found that the inducibility of CYP1A1 and CYP1B1 transcripts following xenobiotic stress was significantly attenuated in BRCA1 knockdown cells. This reduced inducibility is associated with an altered stability of ARNT and was almost completely reversed in cells transfected with an ARNT expression vector. Finally, we have found that xenobiotic (TCDD) treatments of breast cancer cells containing reduced levels of BRCA1 cause the transcription factor ARNT to become unstable.

Inherited mutations in the breast cancer susceptibility gene BRCA1 confer increased risk of breast and ovarian cancer (1, 2). In addition, because BRCA1 expression is often decreased or even absent in sporadic breast and ovarian cancer, abnormal BRCA1 expression may also have a role(s) in nonhereditary tumors (3, 4). Although these observations indicate that BRCA1 may act as a tumor suppressor in breast cancer, the specific function(s) of BRCA1 that could have this effect is still not completely understood. However, numerous studies have shown that BRCA1 regulates various pivotal cellular processes, such as cell cycle progression, DNA repair, apoptosis, and transcription, and many of the mechanisms involved have been identified (see Ref. 5 for a review). Recent studies show that BRCA1 proteins are also required for maintaining chromosome stability by regulating centrosome duplication and mitotic spindle checkpoints (6–8).

Although BRCA1 regulates transcription, the mechanisms involved are unlike classical transcriptional factors that directly bind DNA sequences. Rather, BRCA1 regulates transcription via protein-protein interactions. The BRCA1 C-terminal activation domain is responsible for transactivation in yeast or mammalian cells (9). In addition to the terminal activation domain (aa[2] 1560–1863, now called AD2), a second activation domain of BRCA1 (designated AD1, aa 1293–1560) has been identified (10). These domains enable BRCA1 to interact with p53 and enhance its transcriptional activity (11, 12) or to interact with various co-repressors (*e.g.* RB1, RbAp46/48, HDAC-1/2, LMO4, and CtIP (which recruits the repressor CtBP)) (see review in Ref. 5). BRCA1 can also regulate transcription by interacting with components of the basal transcription machinery (*e.g.* RNA helicase A, p300) (13, 14), the SWI/SNF transcriptional complex (15), and a BRCA1-interacting protein (COBRA1) that mediates large scale chromatin folding (16). BRCA1 also interacts with STAT1 to stimulate interferon-γ-mediated transcription (17). BRCA1 interacts with estrogen receptor (ER)-α to inhibit ER-α transcription regulation activity (18–20). The absence of BRCA1 caused ligand-independent activation of ER-α (21). BRCA1 also interacts with androgen receptor and enhances androgen receptor-mediated transcription activation (22, 23). BRCA1 binds c-Myc and inhibits its

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[2] The abbreviations used are: aa, amino acid(s); 5F203, 2-(4-amino-3-methylphenyl)-5-fluoro-benzothiazole; DF203, 2-(4-amino-3-methylphenyl)benzothiazole; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; BRCA1, breast cancer susceptibility gene-1; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation; CHX, cycloheximide; CMV, cytomegalovirus; CYP1A1, cytochrome P450/subfamily 1/polypeptide 1; ER, estrogen receptor; GST, glutathione *S*-transferase; Luc, luciferase; NC, negative control; RT, reverse transcription; siRNA, small interfering RNA; TCDD, 2,3,7,8-tetracholrodibeco-*p*-dioxin; WB, Western blotting; XRE, xenobiotic response element; IVT, *in vitro* translated; GFP, green fluorescent protein.

transcriptional and transforming activities (24). We recently reported that BRCA1 stimulates antioxidant response element-driven transcriptional activity and enhances the activity of the antioxidant response transcription factor, nuclear factor, erythroid-derived 2-like 2 (also called NRF2 (NFE2L2)) (25).

The aryl hydrocarbon receptor nuclear translocator (ARNT) is the dimerization partner of a large family of transcriptional factors that act as environmental sensors and control the response of an organism to a wide array of environmental stimuli (26, 27). One of its heterodimeric partners, the aryl hydrocarbon receptor (AhR), becomes activated in response to xenobiotic stress (for example, TCDD). Prior to ligand binding, AhR is sequestered in a cytoplasmic complex containing heat shock protein 90 (28), co-chaperone p23 (29), and the immunophilin homolog XAP2 (30). Following ligand binding, the AhR-containing complex moves to the nucleus where AhR dissociates from the chaperone complex and forms a heterodimer with ARNT, the basic helix-loop-helix/Per-ARNT-Sim protein. These heterodimers can then bind to xenobiotic response elements (XREs) in the promoter and enhancer regions of target genes where they regulate transcription. The regulation of cytochrome P450A1 (CYP1A1) expression has been studied extensively and has become a model for studying AhR activation (31). The cytochrome P450s are essential enzymes involved in the metabolism of drugs, foreign chemicals, arachidonic acid, cholesterol, steroids, and other physiologically relevant lipids. Recently, several drugs targeting particular P450 enzyme activities have been developed. Two of these drugs, DF203 and 5F203, potently inhibit human cancer cell growth, in part by affecting the AhR signaling pathway (32).

In previous studies, we found that BRCA1 regulates multiple xenobiotic stress-inducible genes, including NQO1, UGT1A1, and gsta2 (25). The promoters or enhancer regions of each of these genes contain a XRE and respond to xenobiotic stressors (33–36). In this study, we used TCDD, DF203, or 5F203 as xenobiotic stressors to investigate the role of BRCA1 in regulating the expression of CYP1A1 and CYP1B1, two genes whose xenobiotic stress-inducible responses in human breast cancer cells are well documented.

EXPERIMENTAL PROCEDURES

Cell Lines, Culture Conditions, and Drug Treatments—Human breast cancer cell lines MCF-7, T47D, and ZR-75–1 were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. All cell culture reagents were purchased from BioWhittaker, Inc. (Walkersville, MD). TCDD, at >99% purity, was obtained from Cambridge Isotopes Laboratories (Andover, MA). TCDD was maintained as a stock solution (31 μM) in anhydrous tissue culture-grade dimethyl sulfoxide (Me_2SO). The final concentration of Me_2SO in all experiments was 0.1%. Both DF203 (National Service Center 674495) and its fluorinated analog 5F203 (National Service center 703786) were obtained from the drug repository of the Developmental Therapeutics Program of the National Cancer Institute (Rockville, MD) and were prepared in Me_2SO at a concentration of 100 mM, aliquoted, and stored frozen at −20 °C until used.

Immunoprecipitation (IP) and Western Blotting (WB)—All IP procedures were carried out at 4 °C. Cells grown on 100-mm dishes were washed twice with phosphate-buffered saline and then lysed in a buffer (50 mM Tris (pH, 8.0), 150 mM NaCl, 1% Nonidet P-40) and immunoprecipitated with either anti-ARNT rabbit antibody (H-172, Santa Cruz Biotechnology) or a combination of anti-human BRCA1 mouse monoclonal antibodies (Abs) against N- and C-terminal epitopes on BRCA1 (Ab-1 + Ab-2 + Ab-3, Oncogene Research Products) as previously described (19). Immunoprecipitated proteins were detected by WB using either an anti-ARNT rabbit antibody (H-172, Santa Cruz Biotechnology) or an anti-BRCA1 rabbit antibody (C-20, Santa Cruz Biotechnology).

Glutathione S-transferase (GST) Capture Assays—Briefly, purified GST fusion proteins or GST alone, produced in *Escherichia coli* BL21 (DE3) pLys cells, were incubated with glutathione-Sepharose-4B beads (Amersham Biosciences) to concentrate and immobilize them. Thereafter, the GST proteins immobilized on beads were mixed with a probe (*in vitro* translated [^{35}S]-labeled ARNT) in a reaction buffer (40 units of RNasin Rnase inhibitor, 40 mM HEPES-KOH (pH 7.6), 6 mM ATP, 20% (v/v) glycerol, 8 mM $MgCl_2$, 0.1% (v/v) Nonidet P-40, 6 mM creatine phosphate). The beads were centrifuged and then washed three times with a washing buffer (0.5 mM dithiothreitol, 0.1 mM EDTA, 4 mM $MgCl_2$, 0.05% (v/v) Nonidet P-40, 20 mM HEPES-KOH (pH 7.6), 300 mM KCl, 10% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride). The captured proteins were eluted in sample buffer and fractionated by SDS-PAGE. The gels were dried under vacuum at 80 °C and autoradiographed.

Quantitative Real-time PCR—Quantitative real-time PCR was performed in duplicate using the 1× TaqMan Universal PCR Master Mix (Roche Applied Science), on an Applied Biosystems-Prism Sequence Detector System 7700 and analyzed with SDS software. Real-time PCR conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15-s denaturation at 95 °C, 60-s annealing/extension at 60 °C. SYBR Green was used to monitor the amounts of amplified double-stranded DNA fragments. Cycle threshold values were obtained to determine the fold change. The mRNA level of β-actin was also determined for each RNA sample and was used for normalization. The following primer sequences were used: CYP1A1 forward and reverse primers 5′-ctt gga cct ctt tgg agc tg-3′ and 5′-cga agg aag agt gtc gga ag-3′; CYP1B1 forward and reverse primers 5′-cac caa ggc tga gac agt ga-3′ and 5′-gat gac gac tgg gcc tac at-3′; β-actin forward and reverse primers 5′-tag cgg ggt tca ccc aca ctg tgc ccc atc ta-3′ and 5′-cta gaa gca ttt gcg gtg gac cga tgg agg g-3′, respectively.

Semiquantitative RT-PCR—Well controlled semiquantitative reverse-transcription-PCR assays were performed as described previously (25). GAPDH, whose expression is unaffected by TCDD or BRCA1-siRNA, was used as the loading control. The PCR products were analyzed on 1% agarose gels, stained with ethidium bromide (0.1 mg/ml) and photographed under UV illumination. The CYP1A1 and CYP1B1 primers described above were also used for the semiquantitative RT-PCR. Forward and reverse primers for BRCA1 were 5′-ttg cgg gag gaa aat ggg tag tta-3′ and 5′-tgt gcc aag ggt gaa tga tga aag-3′, respectively. Forward and reverse primers for GAPDH were 5′-atg ttc gtc atg ggt gtg aa-3′ and (5′-ttc agc tca ggg atg acc tt-3′, respectively.

Luciferase Reporter Assays—Cells (~60,000 cells/well) grown in 24-well plates were transfected with Lipofectamine Plus (Invitrogen) transfection reagents. Total amounts of transfecting DNA/well were adjusted to a constant amount with empty vector (pcDNA3, for example). The transfected cells were incubated for an additional 24 h before being treated with chemicals, harvested, and assayed for luciferase activity as previously described (25). For normalization, a constant amount of pCMV-β-galactosidase was co-transfected and β-galactosidase activity was monitored as previously described (37). Two promoter constructs, p(XRE-1A1)-Luc (38) or p(Cyp1a1)-Luc (39), were used for this study.

Chromatin Immunoprecipitation (ChIP) Assays—Exponentially proliferating MCF-7 and T47D cells were treated with 10 nM TCDD. ChIP assays were performed as suggested in the manufacturer's instruction

manual (Upstate Biotechnology). In brief, cross-linking was achieved by adding formaldehyde to 1% and incubating at 37 °C for 10 min. The cells were then washed twice with ice-cold phosphate-buffered saline and then collected in 200 μl of SDS lysis buffer and incubated on ice for 10 min. The resuspended, lysed cells were sonicated, yielding DNA fragments raging in size from 200 to 900 bp. The samples were then centrifuged for 10 min at 4 °C (13,000 revolutions/min) and the supernatants collected and diluted 10-fold in ChIP dilution buffer. The diluted samples were precleared with 75 μl of salmon sperm DNA/protein A-agarose 50% slurry for 30 min at 4 °C with agitation. Supernatants were then immunoprecipitated with specific antibodies (anti-ARNT, -AhR, or -BRCA1) or control IgG as described above. Immunoprecipitated complexes were eluted from protein A beads with an elution buffer (1% SDS, 0.1 M $NaHCO_3$). The immunoprecipitated DNA was separated from the protein complexes and used as a template for PCR reactions as described above. The following genomic sequences were used: CYP1A1 forward and reverse primers, 5′-taa gag ccc cgc ccc gac ttc ct-3′ and 5′-tag ctt gcg tgc gcc ggc gac at-3′), respectively, and CYP1B1 forward and reverse primers, 5′-gtt ccc tta taa agg gag-3′ and 5′-ctg cga tgg aag ccg ttg-3′), respectively, as previously reported (40).

Transfection with siRNA—Exponentially proliferating cells were transfected with chemically synthesized siRNA (control-siRNA or BRCA1-siRNA) and Lipofectamine 2000 for 72 h as previously described (25). Alternatively, we used pKD-negative control (NC) empty vector or the pKD-BRCA1 siRNA expression vector (Upstate Biotechnology, Inc.) (see Fig. 8*A*). For ARNT knockdown, we used pooled siRNA sequences obtained from Dharmacon Inc.

Statistical Methods—Statistical comparisons were made using the two-tailed Student's *t* test where appropriate.

RESULTS

BRCA1 Overexpression Enhances Xenobiotic Stress-induced Gene and Protein Expression—TCDD is the prototype for one class of xenobiotics, halogenated aromatic hydrocarbons, which induce stress in human cells. To determine whether BRCA1 protein levels affect the expression of genes known to be induced by this class of xenobiotics, control and BRCA1-transfected cells were incubated for 24 h and then treated with 10 nM TCDD for an additional 24 h. Thereafter, changes in CYP1A1 and CYP1B1 mRNA levels were measured by quantitative real-time PCR. We found that overexpressed BRCA1 enhanced TCDD-induced expression of both CYP1A1 (Fig. 1*A*) and CYP1B1 at the mRNA level (Fig. 1*B*) ($p > 0.005$ for comparisons of cells transfected with pCDNA3 *versus* BRCA1 in the presence of TCDD). To determine whether this enhanced response could reflect XRE-dependent transcriptional regulation, we transfected cells (MCF-7, T47D, and ZR-75–1) with pcDNA3, BRCA1, and p(XRE-1A1)-Luc (which contains three tandem XREs from human CYP1A1), and 24 h later, we added TCDD (10 nM). Only the BRCA1-expressing plasmid enhanced TCDD-stimulated XRE promoter reporter activity in a dose-dependent manner in these three ER(+) breast cancer cell lines (Fig. 2, *A–C*) ($p < 0.005$ or < 0.05 for comparisons of cells transfected with pCDNA3 *versus* BRCA1). Similar results were obtained when p(Cyp1a1)-Luc, a reporter plasmid containing 1195 bp of the rat Cyp1a1 promoter cloned into pGL3, was used (Fig. 2*D*). This BRCA1 effect is not limited to TCDD-induced stress, because stress-induced transcription caused by two other xenobiotic drugs of the same class, DF203 and 5F203, was also enhanced by the BRCA1 expression plasmid (Fig. 3) ($p < 0.005$ or < 0.05).

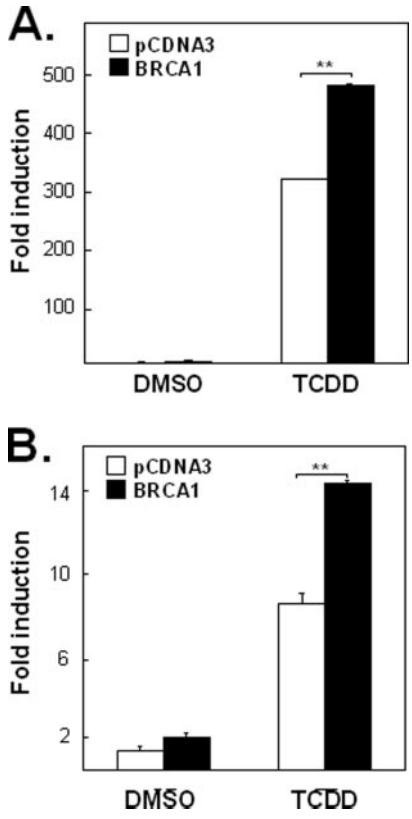

FIGURE 1. **The effect of exogenous BRCA1 on TCDD-induced gene expression.** Subconfluent MCF-7 cells transfected with a BRCA1 expression vector or pcDNA3 (empty vector) were incubated for 24 h and then treated with 10 nM TCDD for an additional 24 h. Total cellular RNA was then extracted to measure the relative amounts of CYP1A1 (*A*) and CYP1B1 (*B*) by quantitative reverse transcription-PCR. The mRNA values were normalized to the levels of a control gene (β-actin). The values (*Fold induction*) represent means ± S.E. of five wells ($n = 3$) of three independent experiments. BRCA1-transfected cells have a higher fold induction of CYP1A1 and CYP1B1 than empty vector-transfected control cells ($p < 0.005$, two-tailed Student's *t* tests). *DMSO*, dimethyl sulfoxide (Me_2SO). **, $p < 0.005$.

BRCA1 Is Recruited to Endogenous CYP1A1 Promoters in Response to TCDD—To determine whether BRCA1 is recruited to endogenous promoters containing XREs, we used a ChIP assay. Recruitment of AhR, ARNT, and BRCA1 to the human genomic CYP1A1 and CYP1B1 proximal enhancer regions was monitored for 2 h following treatment of cell cultures with TCDD (10 nM). Genomic DNA, bound to proteins immunoprecipitated by antibodies specific for BRCA1, ARNT, or AhR, was used as a template for PCR to determine whether the association between these proteins and the CYP1A1 or CYP1B1 promoters was dependent on TCDD treatment (Fig. 4). We found that both BRCA1 and ARNT were detectably associated with the genomic CYP1A1 promoter in untreated MCF-7 and T47D cells and that TCDD treatment enhanced this association in both cell lines (Fig. 4*A*). In contrast, antibodies to BRCA1 and ARNT failed to immunoprecipitate detectable amounts of CYP1B1 promoter DNA from either cell line in the absence of TCDD treatment. Nevertheless, TCDD treatment enhanced BRCA1 and ARNT binding to CYP1B1 promoter DNA in both MCF-7 and T47D cells (Fig. 4*B*). The association of AhR with either promoter in either cell line was only weakly or not at all detectable under our conditions, possibly because of the relatively shorter half-life of activated AhR. We determined the effect of TCDD treatment on the total cellular levels of BRCA1 protein by WB analysis and found that they are unchanged after treatment with TCDD (Fig. 4*C*). To determine whether promoter occupancy by BRCA1 required ARNT, we performed ARNT knockdown (Fig. 4*D*) and then treated

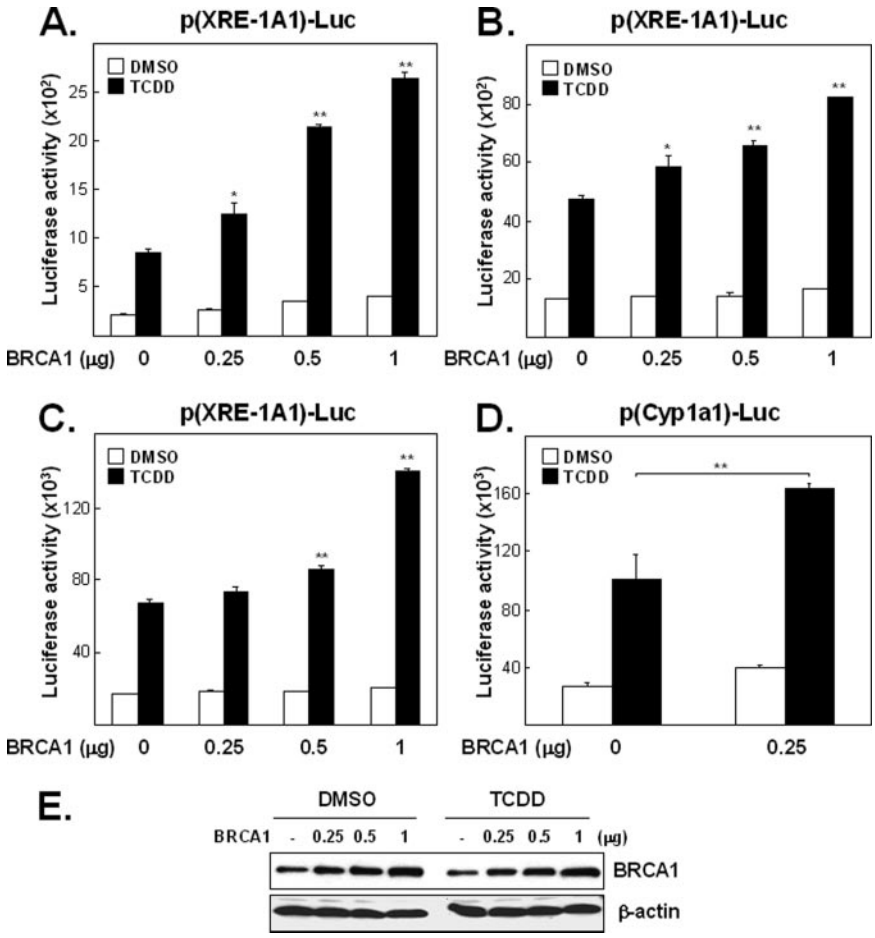

FIGURE 2. **The effect of exogenous BRCA1 on a XRE-containing promoter in TCDD treated cells.** Exponentially proliferating cells (MCF-7 (*A*), T47D (*B*), and ZR-75–1 (*C*)) were transiently transfected with empty vector or the BRCA1 expression vector and a p(XRE-1A1)-Luc reporter plasmid, treated with 10 nM TCDD, and assayed for luciferase activity as described under "Experimental Procedures." *D*, MCF-7 cells were transfected with BRCA1 and the p(Cyp1a1)-Luc reporter (34) and treated with 10 nM TCDD, and the promoter activity was monitored. The data are presented as the means ± S.E. of four independent experiments, each performed in triplicate. For normalization of the luciferase assay data, triplicate transfections with pCMV-β-galactosidase and pcDNA3 vectors were done in parallel (25). The values represent means ± S.E. of quadruplicate wells ($n = 4$) of three independent experiments. BRCA1-transfected cells showed a higher fold induction of CYP1A1 than control cells in the presence of TCDD ($p < 0.005$–0.05). *E*, the expression level of BRCA1 was determined by WB analysis. β-actin was used as the loading and transfer control. *, $p < 0.05$; **, $p < 0.005$.

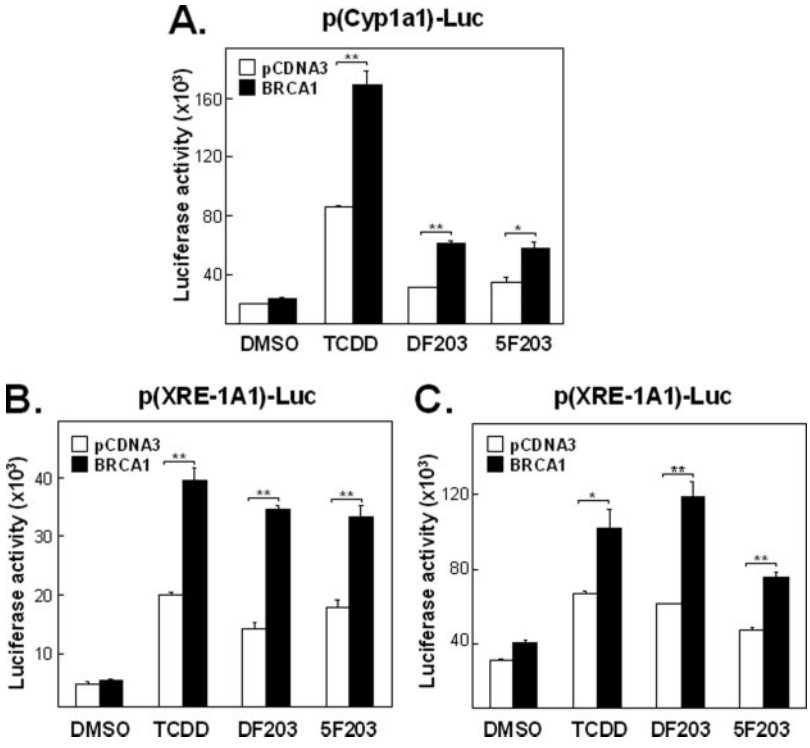

FIGURE 3. **The effect of other AhR ligands on the activity of a XRE-containing promoter in the presence of exogenous BRCA1.** *A*, exponentially proliferating MCF-7 cells were transiently transfected with BRCA1 and p(Cyp1a1)-Luc reporter, incubated with AhR ligands, 10 nM TCDD, 1 μM DF203, or 1 μM 5F203, harvested, and assayed for luciferase activity as described in the legend to Fig. 2. Cells (MCF-7 (*B*) and T-47D (*C*)) were transfected with BRCA1 or p(XRE-1A1)-Luc reporter plasmid and treated with 10 nM TCDD, 1 μM DF203, or 1 μM 5F203 for only 8 h before luciferase activities were assayed. These data are presented as the means ± S.E. of four independent experiments, each performed in triplicate. Normalization of the luciferase assay data were done as described in the legend for Fig. 2. BRCA1-transfected cells showed higher fold induction of CYP1A1 than control cells in the presence of DF203 or 5F203 ($p < 0.005$ or 0.05). *, $p < 0.05$; **, $p < 0.005$.

cells with TCDD and performed ChIP assays. We found that reduced amounts of ARNT resulted in reduced binding of BRCA1 to the CYP1A1 promoter (Fig. 4*E*).

BRCA1 Interacts with ARNT—Because BRCA1 regulates transcription via interactions with transcription factors, we investigated whether BRCA1 also interacts, directly or indirectly, with the transcription fac-

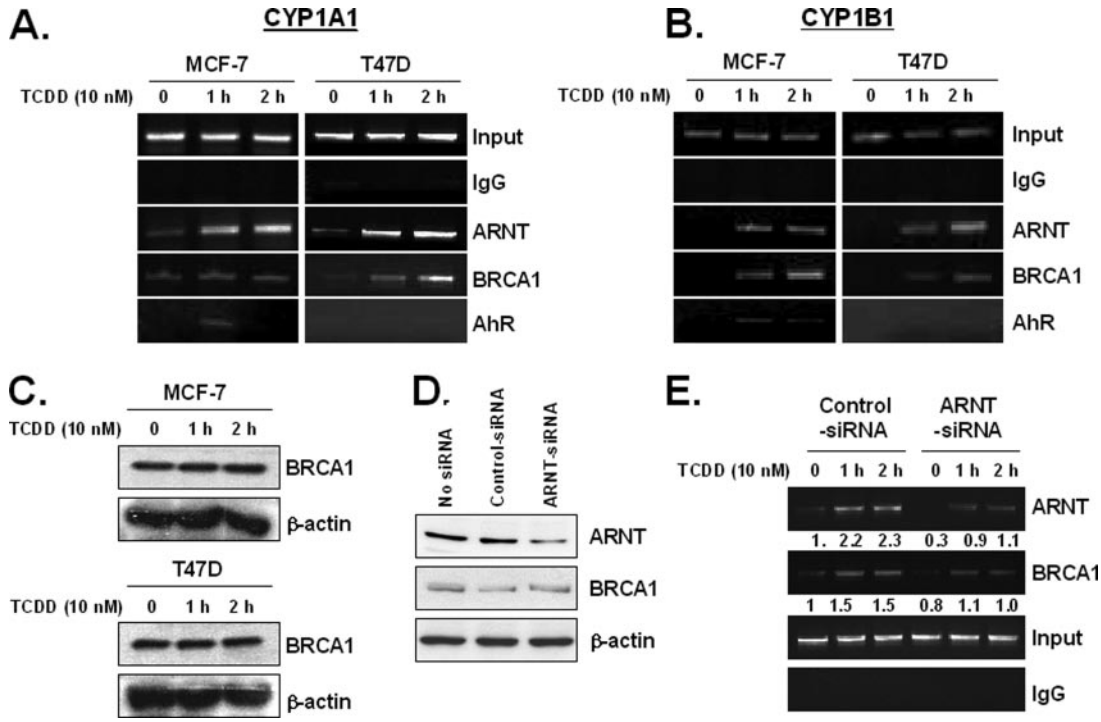

FIGURE 4. The effect of TCDD on recruiting endogenous BRCA1 to the promoter regions of endogenous CYP1A1 (*A*) and CYP1B1 (*B*). Cells treated with 10 nM TCDD for 0, 1, or 2 h were used for ChIP assays. Endogenous promoter regions associated with BRCA1, ARNT, or AhR in MCF-7 and T47D cells were immunoprecipitated with anti-BRCA1, anti-ARNT, or anti-AhR antibody, respectively. The relative amounts of promoter-specific DNA in the immunoprecipitated complexes were then determined by PCR as described under "Experimental Procedures." *C*, the expression level of BRCA1 following TCDD exposure was monitored by WB analysis using the anti-BRCA1 antibody. *D*, ARNT was knocked down by a specific siRNA, which was confirmed by WB analysis using anti-ARNT antibody. *E*, cells (*MCF-7*) transfected with ARNT-specific siRNA for 48 h were treated with TCDD and immunoprecipitated with anti-BRCA1 for ChIP assay analysis. The presence of BRCA1 on the CYP1A1 promoter region DNA was determined by PCR, PCR band intensity was quantitated by densitometry, and values are indicated.

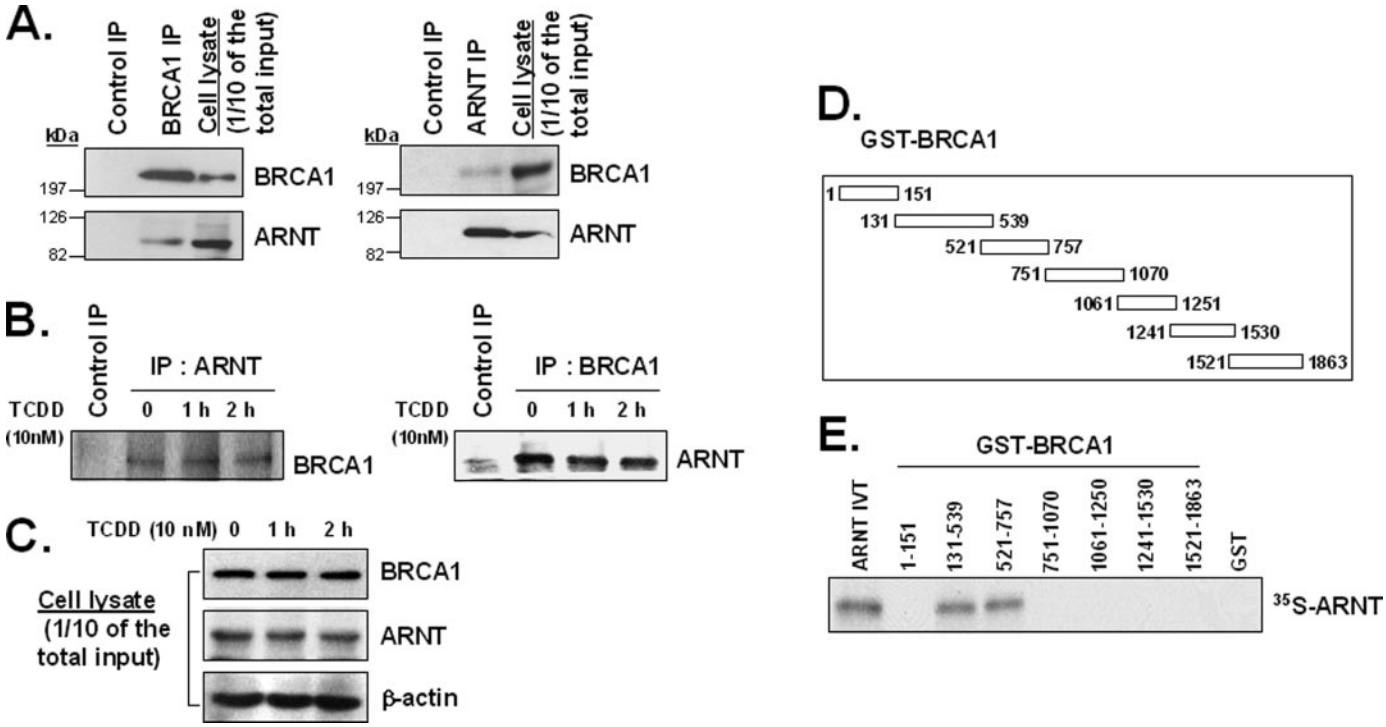

FIGURE 5. **Interactions between BRCA1 and ARNT.** *A*, the association of endogenous BRCA1 with endogenous ARNT in total cell extracts from MCF-7 cells was detected by immunoprecipitation with either anti-BRCA1 and anti-ARNT followed by Western blotting and detection by anti-ARNT and anti-BRCA1, respectively. The third column of each blot represents 10% of the total cell lysate input. *B*, the interaction of BRCA1 and ARNT in lysates from TCDD-treated cells was monitored using a bidirectional IP-WB assay as in *A*. *C*, the expression levels of BRCA1 and ARNT following TCDD exposure in the cell lysates used for IP experiments (*A* and *B*) as determined by WB analysis. *D*, this panel shows the GST-BRCA1 protein fragments used to capture *in vitro* translated (IVT) ARNT. *E*, the GST capture assay results obtained with the GST-BRCA1 fragments shown in *D*.

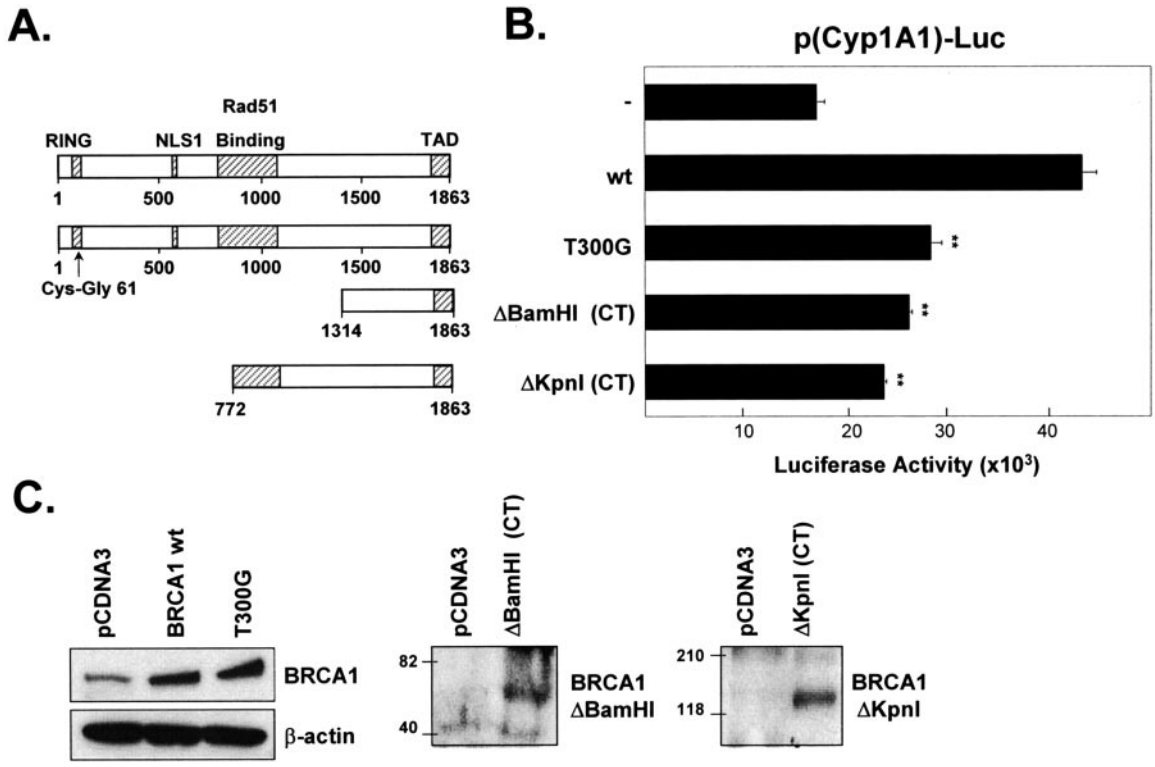

FIGURE 6. **Effect of BRCA1 mutants on TCDD-induced CYP1A1 promoter activity.** *A* and *B*, MCF-7 cells were co-transfected with the p(Cyp1a1)-luciferase reporter and either wild-type BRCA1 or mutant BRCA1 constructs (T300G, ΔBamHI and ΔKpnI) as indicated. After 24 h, the cells were treated with 10 nM TCDD for ~24 h and then luciferase activity was assayed. pCMV-β-galactosidase and pcDNA3 vectors were included for data normalization as described in the legend to Fig. 2. The data are presented as the means ± S.E. of four wells ($n = 4$) in three independent experiments. *C*, the levels of exogenous wild-type BRCA1, T300G, ΔBamHI (CT), and ΔKpnI (CT) were determined with an anti-BRCA1 antibody (C-20, Santa Cruz Biotechnology). **, $p < 0.005$.

tor ARNT and whether this interaction increases after xenobiotic stress. To answer this question, lysates from MCF-7 cells were prepared and immunoprecipitated with anti-BRCA1 antibody. The presence of ARNT in the IP complex was assayed with anti-ARNT antibody. Vice versa, the presence of BRCA1 in IP complexes generated with an anti-ARNT antibody was assayed with an anti-BRCA1 antibody. Both types of IP complexes contained both BRCA1 and ARNT (Fig. 5*A*). Next, cells lysated from MCF-7 cells treated or not treated with 10 nM TCDD were prepared and bidirectional IP-WB was performed, as in Fig. 5*A*. The interaction between BRCA1 and ARNT was not affected by TCDD treatment, and TCDD did not change the levels of BRCA1 and ARNT expression in MCF-7 cells (Fig. 5*B*). Next, the regions of BRCA1 required for detecting an interaction with ARNT in our GST capture assays were investigated using *in vitro* translated ARNT (ARNT-IVT) as previously described (37, 41, 42). After incubating ARNT-IVT with a panel of GST-BRCA1 protein fragments, we found that ARNT-IVT was captured by GST-BRCA1 (aa 131–539) and GST-BRCA1 (aa 521–757). These data suggest that the N-terminal region of BRCA1 (aa 131–757) may be both necessary and sufficient for interacting with ARNT. The GST-alone control and other GST-BRCA1 regions did not detectably capture ARNT-IVT in this assay (Fig. 5*D*).

N-terminal BRCA1 Mutants Have Reduced Capacity to Enhance Expression from the TCDD-induced CYP1A1 Promoter—Because the N-terminal region of BRCA1 interacts with ARNT, we tested whether N-terminal BRCA1 mutants (see Fig. 6*A*) impact the ability of BRCA1 to regulate TCDD-induced CYP1A1 gene expression. T300G, a point mutant (C61G) in the N-terminal ring domain, was able to stimulate TCDD-induced CYP1A1 luciferase reporter activity only about half as much as wild-type BRCA1 (Fig. 6*B*) ($p < 0.005$ for comparisons of cells transfected with wtBRCA1 *versus* mutant BRCA1 expression vectors). These promoter activity studies were performed in four wells in three independent experiments. The luciferase values are the means ± S.E. This difference cannot be explained as being the result of differential expression of T300G and wild-type BRCA1, because no significant difference was found when exogenously expressed protein levels were measured on WB analysis with an anti-BRCA1 antibody (Fig. 6*C*). Next, we measured the effects of two BRCA1 N-terminal deletion mutants (ΔBamHI and ΔKpnI) on TCDD-induced CYP1A1 stimulation. Both deletion mutants had less capacity to stimulate TCDD-induced CYP1A1 transcription than wild-type BRCA1 (Fig. 6*B*). Equal amounts of the two mutant proteins were detected by WB analysis (Fig. 6*C*).

BRCA1 Knockdown Reduced TCDD-mediated CYP1A1 Gene Inducibility—The results presented thus far suggest that endogenous BRCA1 levels might also affect TCDD-induced CYP1A1 promoter activity. To test this possibility, we reduced endogenous BRCA1 levels by transfecting MCF-7 and T47D cells with BRCA1-siRNA or control-siRNA (scrambled) and incubating for 72 h to assure a maximal effect (25). The normal endogenous BRCA1 level was reduced by >80% in cells transfected with the siRNA plasmid vector construct as previously reported (25). Subsequently, these cells were transfected with p(XRE-1A1)-Luc or p(Cyp1a1)-Luc reporters treated with 10 nM TCDD for 24 h and harvested for luciferase activity assays. We found reduced TCDD-stimulated luciferase activity in both cell types transfected with BRCA1-siRNA (Fig. 7, *A–C*) ($p < 0.05$ for comparisons of cells transfected with control-siRNA *versus* BRCA1-siRNA in the presence of TCDD). This effect appears to occur at least partially at the mRNA level, because we found less TCDD-mediated induction of CYP1A1 and CYP1B1 mRNA in T47D cells transfected with BRCA1-siRNA than in the control-transfected cells when assayed by semiquantitative RT-PCR (Fig. 7*D*). This gel was scanned using densitometry, allowing a quanti-

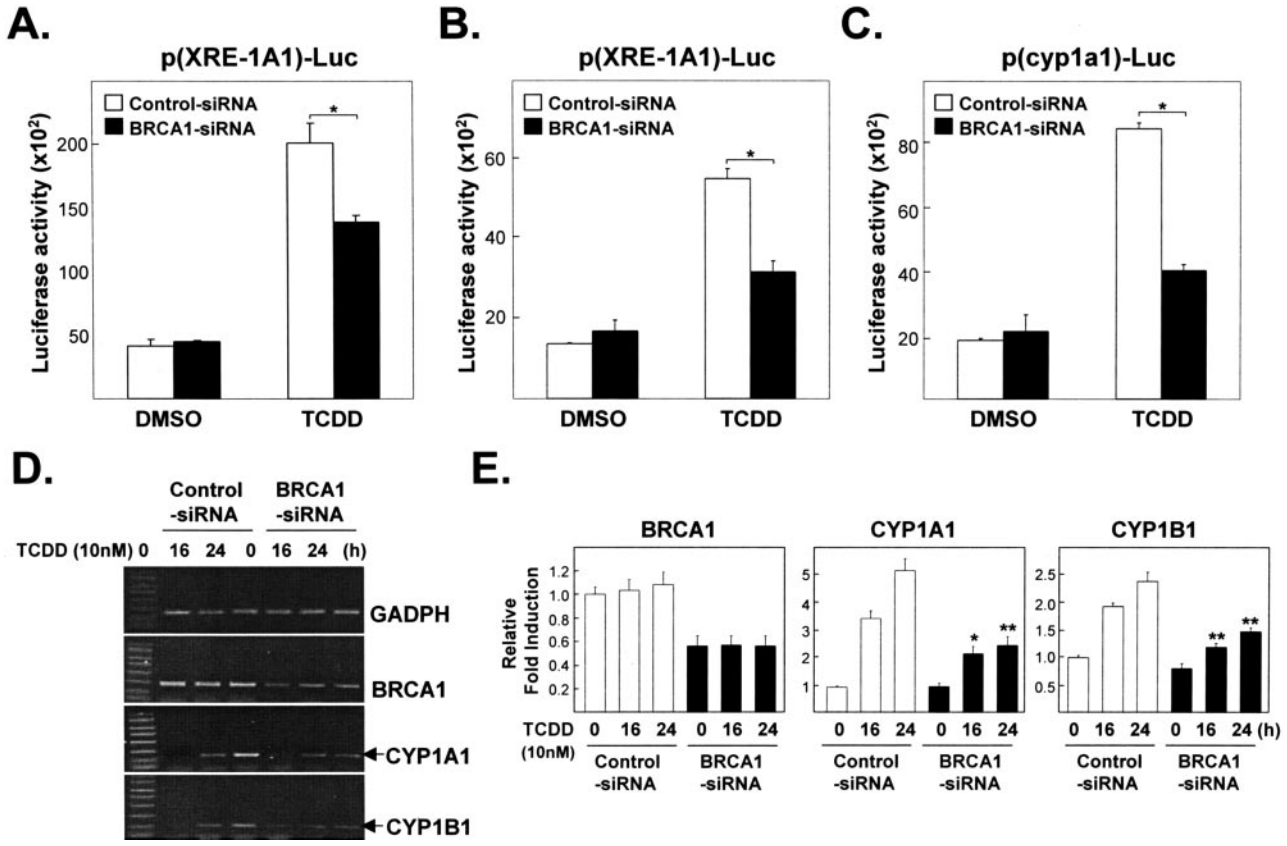

FIGURE 7. **The effect of BRCA1-siRNA on TCDD-induced XRE-dependent gene expression and XRE-luciferase activity.** Cells (MCF-7 (*A* and *C*) and T47D (*B*)) were preincubated with control-siRNA or BRCA1-siRNA (50 nM for 72 h). These cells were then transfected with p(XRE-1A1)-Luc and 24 h later treated with 10 nM TCDD for an additional 24 h and assayed for luciferase activity. Transfections with pCMV-β-galactosidase and pcDNA3 vectors were used for data normalization. *C*, a similar experiment done with the p(Cyp1a1)-Luc reporter and T47D cells. The data are presented as the means ± S.E. of four independent experiments, each performed in triplicate. Significantly decreased p(XRE-1A1)-Luc or p(cyp1a1)-Luc activity was found in cells transfected with BRCA1-siRNA compared with cells transfected with control-siRNA ($p < 0.05$) (*D*). Total RNA extracted from T47D cells transfected with BRCA1-siRNA (or control-siRNA), ±10 nM TCDD for the indicated times (in hours), was used in semiquantitative RT-PCR reactions. The amounts of GAPDH mRNA detected using appropriate primers were used as the loading control. *E*, RT-PCR results from *D* were quantitated by densitometry and presented as *bar graphs* for the relative expression levels of BRCA1, CYP1A1, and CYP1B1 after calculating means ± S.E. from three independent experiments of *D* ($p < 0.005$ or < 0.05 for comparisons of cells transfected with control-siRNA *versus* BRCA1-siRNA). *, $p < 0.05$; **, $p < 0.005$.

tative analysis of the data (Fig. 7*E*). Clearly, decreased induction of both CYP1A1 and CYP1B1 mRNA following TCDD occurs in BRCA1-depleted cells ($p < 0.005$ or < 0.05 for comparisons of cells transfected with control-siRNA *versus* BRCA1-siRNA in the presence of TCDD).

Altered Stability of ARNT in BRCA1-depleted Cells—To identify possible molecular mechanisms by which CYP1A1 and CYP1B1 induction by TCDD is reduced when BRCA1 levels are reduced, we performed a BRCA1 knockdown experiment and then treated cells with TCDD and prepared cell lysates for WB analysis to determine the ARNT expression level. No significant change in the levels in ARNT expression was found between pKD-NC- *versus* pKD-BRCA1-transfected and Me_2SO-treated cells (Fig. 8, *A* and *B*). However, ~30% less ARNT protein was found in cells transfected with pKD-BRCA1 and treated with TCDD (10 nM) ($p < 0.005$ comparisons of cells transfected with pKD-NC *versus* pKD-BRCA1). This decrease was not due to decreased ARNT mRNA (data not shown). It could be that TCDD differentially regulates the stability of ARNT in the presence and absence of BRCA1. To test this hypothesis, cells transfected with pKD-NC or pKD-BRCA1 were treated with TCDD (10 nM) for 3 h and then with cycloheximide (CHX, 100 μg/ml) for 0, 2, or 4 h before cell lysates were prepared for WB analysis. The results suggest that BRCA1 stabilizes ARNT when cells are under TCDD stress (Fig. 8*C*). Relatively unstable ARNT was detected in cells transfected with pKD-BRCA1 (Fig. 8*D*) ($p < 0.005$ for comparisons of cells transfected with pKD-NC *versus* pKD-BRCA1).

Overexpressed ARNT Reversed the BRCA1 Knockdown Effect on CYP1A1 Expression—The data in Fig. 8 suggests that the decreased ability of TCDD to induce CYP1A1 gene expression following BRCA1 knockdown is associated with reduced ARNT protein levels. If reduced ARNT levels are indeed important for this reduced gene expression, overexpression of ARNT in BRCA1 knockdown cells exposed to TCDD may restore CYP1A1 gene expression to a normal level. To test this possibility, we transfected an ARNT expression vector (GFP-ARNT) and p(XRE-1A1)-Luc into cells previously transfected with BRCA1-siRNA or control-siRNA. As predicted, overexpression of ARNT restored TCDD-stimulated CYP1A1 reporter activity to essentially normal levels in the BRCA1-siRNA-transfected cells (Fig. 9) ($p < 0.005$ for comparisons of cells transfected with pCDNA3 *versus* ARNT in BRCA1 depleted cells). Similar results were obtained with a CYP1B1 reporter (data not shown). Overexpressed ARNT had a small, but probably insignificant, effect in the control-siRNA-transfected cells, suggesting that endogenous nuclear levels of ARNT do not limit the ability of the cells to respond to TCDD (10 nM).

DISCUSSION

The goal of this study was to determine the role of BRCA1 in regulating the expression of xenobiotic stress-inducible genes, such as CYP1A1 and CYP1B1 in human breast cancer cells. We found that overexpressed BRCA1 significantly enhanced TCDD-stimulated

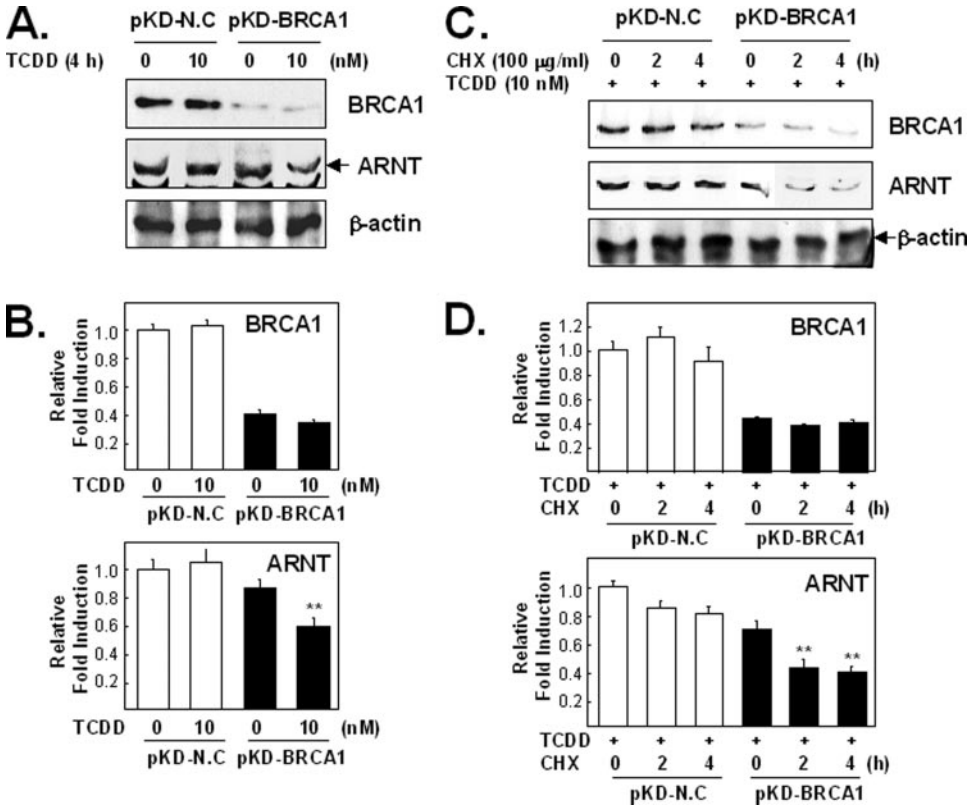

FIGURE 8. **The stability of ARNT protein in cells depleted of BRCA1 by transfecting with DNA-based siRNAs.** *A*, MCF-7 cells transiently transfected with pKD-NC or pKD-BRCA1 for three days were incubated with TCDD (0 or 10 nM) for 4 h. Total cell lysates were then prepared and analyzed by WB analysis for BRCA1 and ARNT as described under "Experimental Procedures." *B*, the relative amounts of BRCA1 and ARNT were quantified by densitometry and plotted as *bar graphs* normalized to the amounts present just before treatment with TCDD in pKD-NC-transfected cells. Values (means ± S.E.) obtained from three independent experiments are normalized to the β-actin inputs and to the value of a control sample (pKD-NC-transfected and treated with Me_2SO). Decreased ARNT expression after TCDD treatment was found only in the BRCA1 knockdown cells ($p < 0.005$ for comparisons of cells transfected with pKD-NC *versus* pKD-BRCA1). *C*, MCF-7 cells transfected with pKD-NC or pKD-BRCA1 were treated with TCDD (10 nM) for 3 h and then with CHX (100 μg/ml) for the times indicated. Cell lysates were subjected to WB analysis and the results plotted as a *bar graph* (*D*). Values (means ± S.E. in three independent experiments) are normalized to the β-actin inputs and to the value of a control sample (pKD-NC-transfected treated with TCDD but without CHX treatments). Decreased ARNT stability after TCDD treatment was found only in the BRCA1 knockdown cells treated with CHX ($p < 0.005$ for comparisons of cells transfected with pKD-NC *versus* pKD-BRCA1 in the presence of CHX). **, $p < 0.005$.

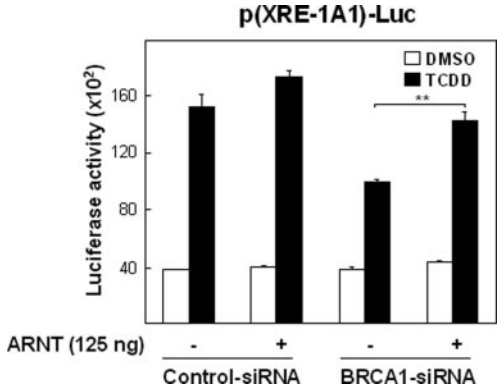

FIGURE 9. **Overexpressed ARNT rescues the reduced ability of TCDD to induce CYP1A1 expression in BRCA1 knockdown cells.** After transfection with siRNA (control or BRCA1) for 2 days, cells were co-transfected overnight with the GFP-ARNT expression vector and p(XRE-1A1)-Luc, treated with 10 nM TCDD for 24 h, and assayed for luciferase activity. The data are presented as means ± S.E. of four independent experiments, each performed in triplicate. Normalization of the luciferase assay data were performed as described in the legend to Fig. 2. Overexpression of ARNT rescued the reduced TCDD-mediated induction of CYP1A1 in BRCA1 knock-out cells ($p < 0.005$ for comparisons of cells transfected with empty vector *versus* the ARNT expression vector in the presence of TCDD in BRCA1 knockdown cells). **, $p < 0.005$.

p(XRE-1A1)-Luc reporter activity in all three ER(+) human breast cancer cell lines examined (MCF-7, T47D, and ZR-75–1). We studied only ER(+) cell lines, because TCDD-mediated CYP1A1 induction requires a functionally active estrogen receptor in human breast cancer cells (43). Consequently and as expected, neither TCDD treatment nor BRCA1 overexpression or the combination stimulated p(XRE-1A1)-Luc reporter activity in two ER(−) breast cancer cell lines, MDA-MB-231 and Hs578T (data not shown). (This finding may be important, for mutation (or down-regulation) of BRCA1 is associated with a higher incidence of breast and/or ovarian cancer, both of which are estrogen-responsive tissues). These results do not depend on the particular XRE-Luc reporter, for similar results were obtained when p(Cyp1a1)-Luc containing wild type CYP1A1 promoter sequences was used (Fig. 2*D*). The results are also not dependent on the particular AhR ligand used to stimulate transcription from the XRE-Luc reporter, as BRCA1 also enhanced the reporter plasmid response to two drugs that activate the AhR receptor, DF203 and 5F203 (Fig. 3).

BRCA1 interacts with multiple transcription factors and affects their ability to regulate transcription. Our IP-WB study demonstrated that BRCA1 interacts with ARNT in human breast cancer cells. However, we have not yet determined whether this is a direct or indirect interaction. Although ARNT immunoprecipitated from our IVT system is likely to be relatively purer than ARNT immunoprecipitated from total cell lysates, we cannot exclude the possibility that the IVT system contains species that promote ARNT-BRCA1 interactions. In any case, this BRCA1-ARNT interaction was detectable only with either of two BRCA1 fragments containing N-terminal amino acids, aa 131–757. This region is known to associate with other transcription factors, such as c-Myc (aa 175–511) (24), p53 (aa 224–500) (12), ER-α (aa 1–302) (19), Rad50 (aa 341–748) (44), hMSH2, hMSH6 (aa 1–625) (45), and Brg1 (aa 260–553) (15). BRCA1 is normally a nuclear protein, targeted to the nucleus via its two nuclear localization signal sequences (NLS1 (501 KLKRKRR) and NLS2 (607 KKNRLRRK)), one of which (NLS1) is thought to be more important for nuclear targeting (47–49). Because ARNT binds equally to GST-BRCA1 fusions containing either one of these signals (aa 131–539 and aa 521–757), it will be interesting to determine whether these ARNT-BRCA1 interactions, which we assume occur in the nucleus of intact cells, affects nuclear retention (or translocalization) of BRCA1.

Previous studies have demonstrated that ARNT·AhR-mediated transcriptional activation requires co-activators. The ARNT·AhR co-activators currently known are the p160 family of coactivators (50), estrogen receptor-associated protein (ERAP140) (51), CBP/p300 (52), and components of SWI/SNF (46) and TRIP230 (40). Our experiments, in particular

the transient transfection of a BRCA1 expression plasmid and reporter plasmids responsive to various AhR agonists, indicate that BRCA1 also functions as a co-activator for the ARNT-dependent transcription that occurs in response to TCDD, DF203, or 5F203. Although we do not yet know if this ARNT-dependent transcription is ARNT·AhR-dependent transcription, our ChIP assay data is consistent with such a hypothesis. That is, our ChIP assay showed that BRCA1 is recruited to sites of activated transcription at the CYP1A1 promoter *in vivo* in a TCDD-dependent fashion. On the other hand, reduced BRCA1 binding to the CYP1A1 promoter following TCDD was found in ARNT knocked down cells. This data suggests that BRCA1 is a physiologically important co-activator for ARNT/ARNT·AhR-dependent transcription in breast cancer cells.

Taken together, the BRCA1 mutant and siRNA studies show that the presence of functional BRCA1 is important for a normal response to TCDD exposure that includes stimulation of XRE-dependent signals. Although the N-terminal end of BRCA1 appears to be essential for detecting ARNT-BRCA1 interactions under our conditions (Fig. 5), it contributes to but does not appear essential for xenobiotically induced transcription (Fig. 6*B*). This apparent discrepancy could have several explanations. The conditions used to detect the BRCA1-ARNT interaction may not detect all of the functional interactions, the C-terminal regions of BRCA1 used in Fig. 6 are larger than any of the C-terminal fragments used in the Fig. 5 experiment and thus could detectably interact with IVT ARNT, or these larger C-terminal fragments (either alone or by interacting with the endogenous wild type BRCA1 present in these cells) may contribute to xenobiotic stress-induced transcription via some other route.

Our study found a correlation between reduced endogenous BRCA1 levels and an attenuated ability to achieve normal CYP1A1 and CYP1B1 expression in response to a classical xenobiotic stressor, TCDD. CYP1A1 is one of the cytochrome P450 enzymes that metabolically activates and detoxifies various xenobiotic stressors such as dioxins. Abnormal induction or expression of CYP1A1 may alter its activity in metabolizing xenotoxic chemicals and may have effects on detoxification processes. In any case, our findings suggest that breast cancer cells with mutations or reduced levels of BRCA1 may respond differently than normal breast cells to various endogenous or exogenous xenobiotic stresses. Whether the effects observed at the gene expression level (for CYP1A1 and CYP1B1) in BRCA1-depleted cultured cells indicates that similar effects occur at the enzymatic activity level (metabolic activation or detoxification) *in vivo* and whether such postulated effects influence tumor formation or progression needs to be investigated.

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BRCA1 Modulates Xenobiotic Stress-inducible Gene Expression by Interacting with ARNT in Human Breast Cancer Cells

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