p53 Mediates Repression of the BRCA2 Promoter and Down-regulation of BRCA2 mRNA and Protein Levels in Response to DNA Damage*

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Adriamycin and other DNA-damaging agents have been shown to reduce BRCA2 mRNA levels in breast cancer cell lines, but the mechanism by which this occurs is unknown. In this study, we show that adriamycin and mitomycin C, but not other DNA-damaging agents, repress BRCA2 promoter activity in a dose- and time-dependent manner. We demonstrate that the effect is dependent on wild type p53 and that adriamycin and p53 mediate repression of the BRCA2 promoter by inhibiting binding of an upstream stimulatory factor protein complex to the promoter. In addition, we present evidence indicating that adriamycin and other DNA-damaging agents reduce BRCA2 mRNA and protein levels by altering both BRCA2 mRNA stability and protein stability. Thus, BRCA2 levels in the cell are regulated by three independent mechanisms in a p53-dependent manner.

The BRCA2 gene was identified in 1996 as a breast and ovarian cancer susceptibility gene (1, 2). The BRCA2 gene encodes a 3,418-amino acid, cell cycle-regulated, nuclear phosphoprotein (3, 4) that has been implicated in the response to DNA damage. The evidence for a role in DNA repair came initially from the observation that BRCA2 binds directly with RAD51 through the exon 11-encoded BRCA repeats (5, 6) and through an additional C-terminal binding site in the mouse (7). This association with a protein involved in meiotic and mitotic recombination and DNA double-stranded break repair suggests a similar role for BRCA2. Further support for a role in DNA repair comes from the observation that cells expressing a wild type BRCA2 BRCA4 domain show hypersensitivity to γ-irradiation, an inability to form RAD51 radiation-induced foci, and a failure of radiation-induced G2/M, but not G1/S, checkpoint control (8). Moreover, cells expressing mutant BRCA2 are more sensitive to methyl methanesulfonate-induced DNA damage than cells expressing wild type BRCA2 (9). Animal models have been used to demonstrate an association between BRCA2 mutants (10) and BRCA2 and homologous recombination using CAPAN-1 BRCA2 mutant cell lines and homozgyous mutant brca2 embryonic stem cells (12, 13) and in transcription-coupled repair in response to 8-oxoguanine treatment (14). Most recently, the C terminus of BRCA2 has been shown to bind directly to single-stranded DNA and to promote strand transfer and RAD51 loading onto DNA during homologous recombination (15).

The finding that BRCA2 was involved in the response to DNA damage led to the hypothesis that DNA damage might result in the induction of BRCA2 expression. However, the opposite has proven true. Specifically, BRCA2 mRNA levels were significantly down-regulated in breast and ovarian cancer cell lines after exposure to various DNA-damaging agents including adriamycin (ADR)1 and camptothecin (16, 17). In an effort to characterize this response to DNA damage better we investigated whether ADR and other DNA-damaging agents affected BRCA2 mRNA and protein levels in a p53-dependent manner. Thus, BRCA2 levels in the cell are regulated by three independent mechanisms in a p53-dependent manner.

EXPERIMENTAL PROCEDURES

Plasmids—The preparation of a series of pGL3 luciferase reporter constructs (Promega) containing partial fragments of the BRCA2 promoter has been described previously (18). A pcDNA3.1 plasmid containing the wild type p53 cDNA (wtp53) was provided by Wilma Lingle. CMV-USF1 and USF2-VP16 expression constructs were provided by Michele Sawadogo and Howard Towle, respectively. An R273L dominant negative p53 mutant construct (dnp53) was generated by site-directed mutagenesis of the wtp53 construct using the QuikChange kit (Stratagene) according to the manufacturer’s instructions. PCR primers for the site-directed mutagenesis were 5′-GGAACAGCTTGAAGTCTTGTGTTTGTCCTGCCTGTCCTGG-3′ (forward) and 5′-CCAGGACAGGACAACAAACAGACCTCAAGAGCTGTTCCC-3′ (reverse). Plasmid DNA was isolated from colonies, and the presence of the mutation was confirmed by DNA sequencing.

Cell Culture—HCT116/p53−/− and HCT116/p53+/− cells were provided by Junjie Chen. HCT116/p21−/− and HCT116/p21+/+ cells were provided by Wafik El-Deiry. MCF7/pCMV and MCF7/E6 cells were provided by Scott H. Kaufmann. All other cell lines were obtained from

Received for publication, November 5, 2002, and in revised form, February 13, 2003

1 The abbreviations used are: ADR, adriamycin; CBP, cAMP response element-binding protein; p53, p53; PAF, platelet-activating factor; CMV, cytomegalovirus; dnp53, dominant negative p53 mutant; EMSA(s), electrophoretic mobility shift assay(s); GADPH, glyceraldehyde-3-phosphate dehydrogenase; MeSO2, dimethyl sulfoxide; MMC, mitomycin C; TR, thymidine kinase; USF, upstream stimulatory factor; wtp53, wild type p53.

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This paper is available on line at http://www.jbc.org

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the American Type Culture Collection. Human breast adenocarcinoma MCF7 cells, human colon carcinoma SW480 cells, HCT116/p53−/− cells, HCT116/p53−/−, cells and human osteosarcoma U2OS cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (HyClone), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human osteosarcoma p53-null Saos2 cells were propagated in McCoy’s 5A medium supplemented with 15% bovine calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. T47D, MCF7, and MCF7/E6 breast adenocarcinoma cells were maintained in RPMI 1640 with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. MCF-10A cells were maintained in MEGM (Clonetics).

**Transient Transfection and Luciferase Reporter Assays**—Transient transfections were performed in six-well plates using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) with 0.5–2.0 μg of **BRCA2** promoter luciferase reporter construct and 0.1 μg of pRL-TK–Renilla luciferase control vector (Promega). For ADR (Sigma) treatment experiments, cells were transfected, grown for 24 h, and exposed to 0, 2.5, 5.0, or 10 μM ADR for 1 h in standard medium. The cells were washed with serum-free medium and incubated at 37 °C in fresh culture medium for another 24 h or the indicated time. Because less than 10% of any cell type exhibited a toxic response to 5 μM ADR, drug toxicity had no effect on the outcome of the study. In cotransfection experiments, cells were also transfected with 0.5 μg of wtP53, dnp53, USF1, USF2–VP16, or pcDNA3.1 control. Protein lysates were prepared from the cells, and luciferase activities were measured as described previously (18). Renilla luciferase activity from a cotransfected pRL-TK control vector was used for normalization. In some lower dose experiments, cells were exposed to 0.7 μM ADR for 1 h, or the indicated time, in standard medium and processed immediately.

**Electrophoretic Mobility Shift Assays (EMSAs)**—Double-stranded oligonucleotides containing bp −10 to −35 of the **BRCA2** promoter and either a wild type or mutated USF binding site (−20 to −13) were labeled with [γ-32P]ATP and used in EMSAs (18). Double-stranded DNA probes were purified from the reaction mixture using a Bio-Gel P-100 column (Bio-Rad), incubated with whole cell extract from MCF7 cells, and separated on 5% polyacrylamide gels as described previously (18). Supershift assays using anti-USF1 (Santa Cruz), and anti-USF2 (Santa Cruz) antibodies were also performed as described previously (18).

**Northern Blotting**—Cells were transiently transfected with pcDNA3.1, wtP53, or dnp53 expression constructs or exposed to 5 μM ADR for 1 h. After further incubation for 24 h, poly(A)+ RNA was isolated. RNA samples (1.5 μg/lane) were used for Northern blotting as described previously (18).

**Western Blotting**—Cells were collected, washed with cold phosphate-buffered saline twice, and then lysed at 4 °C for 1 h in EBC buffer (0.5% Nonidet P-40, 120 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), 1 mM β-mercaptoethanol, and Complete protease inhibitor mixture (Roche Molecular Biochemicals)). Equal aliquots of cell lysate (30–100 μg/lane) were electrophoresed through 7 or 12% SDS-polyacrylamide gels after incubating for 15 min at 65 °C in 1× loading buffer (100 mM Tris-HCl (pH 6.8), 10% glycerol, 0.01% bromphenol blue, 2% SDS, 100 mM dithiothreitol). Proteins were then transferred to nitrocellulose membranes (Schleicher & Schull) and blocked in TBST with 5% bovine albumin (fraction V) (ICN Biomedicals). Membranes were blotted with anti-BRCA2 (Ab2, Oncogene Research), anti-USF1 (Santa Cruz) or anti-p53 (Santa Cruz) antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). Signals were visualized using the SuperSignal chemiluminescent detection system (Pierce).

**Flow Cytometry**—MCF7 cells treated with ADR were washed with phosphate-buffered saline, collected by centrifugation, and fixed in ice-cold 95% ethanol at −20 °C for 12 h. The fixed cells were permeabilized and stained with 0.05% Triton X-100, 10 μg/ml RNase A, and 40 μg/ml propidium iodide at 37 °C for 30 min and analyzed by flow cytometry. The data were processed with VERITY ModFit software, version 5.2, for DNA distribution analysis.

**mRNA Stability**—MCF7 cells were incubated in the presence or absence of 10 μg/ml a-aminatin (Sigma) and 5 μM ADR for 1 h. Medium was removed, and cells were incubated further in the presence or absence of a-aminatin. Total RNA was extracted from the cells by standard methods after different periods of incubation. Total cDNA was prepared by oligo(dT) priming of 1 μg of RNA template from each time point using the cDNA preamplification kit (Invitrogen). The amount of **BRCA2** mRNA present in the cells at each time point was measured by semiquantitative reverse transcription PCR using oligonucleotide primer pairs from the 5′- and 3′-ends of the **BRCA2** cDNA. GAPDH was used as an internal control. The **BRCA2** forward primer was 5′-CAAGGGACTGAGGTTGTTGTAGC; **BRCA2** reverse, 5′-AGAACTAAGGGTTGTTGTAGGC; **BRCA2** forward, 5′-GCAGTAGGAAATGCACAGCA; **BRCA2** reverse, 5′-CAATACGGCAACTTCCACAGC; GAPDH forward, CAACATCATGGTTATAGTGG; GAPDH reverse, GCAATGGACTGAGGTTGTTGTAGC. PCR products were separated on 10% polyacrylamide gels, stained with Sybr Green, and scanned on a PhosphorImager.

**Protein Stability**—MCF7 cells were incubated with 20 μg/ml cycloheximide and 5 μM ADR for 1 h. Medium was removed, and cells were incubated further in the presence of cycloheximide. Total protein was extracted from the cells by standard methods after different periods of incubation, and Western blotting with the **BRCA2** Ab-2 antibody was performed. The intensities of Western blot signals were quantitated by densitometry using NIH Image software, version 1.62.

**RESULTS**

The **BRCA2 Promoter Is Repressed by ADR**—ADR has recently been shown to down-regulate **BRCA2** mRNA levels in human breast cancer cells such as MCF7 (16). To address whether the reduction in **BRCA2** mRNA levels by ADR is dependent on the **BRCA2** promoter regulation, MCF7 cells were transiently transfected with a **BRCA2** promoter reporter gene construct (pGL3Prom) (18) and exposed to various concentrations of ADR for 1 h. After removal of the drug and further propagation for 24 h, cell lysates were harvested for luciferase reporter assays. As shown in Fig. 1A, ADR treatment reduced **BRCA2** promoter activity in a dose-dependent manner, with 5 μM ADR down-regulating the promoter activity by 85%. Similar effects of ADR treatment on the **BRCA2** promoter were detected in both MCF10A normal breast epithelial cells (Fig. 1B) and U2OS osteosarcoma cells (data not shown). Continuous exposure of MCF7 cells to lower doses of ADR (0.7 μM) for 24 h
also reduced promoter activity by 60% (Fig. 1C). Furthermore, treatment of MCF7 cells with 5 μM ADR for 1 h resulted in a gradual reduction in promoter activity over time (Fig. 1D). Thus, ADR appears to repress the BRCA2 promoter in a dose- and time-dependent fashion in both tumor and normal cell lines. To confirm that the ADR-dependent repression was specific to the BRCA2 promoter and was not a result of a generalized effect on gene transcription, several other reporter constructs were evaluated using luciferase assays. MCF7 cells were transiently transfected with reporter constructs containing the Rous sarcoma virus, CMV, nuclear factor-κB, and β-galactosidase promoters and treated with 5 μM ADR. No repression of these promoters was observed (data not shown), suggesting that ADR specifically represses the BRCA2 promoter.

Repression of the BRCA2 Promoter by ADR Is Dependent on p53—ADR is a potent DNA-damaging agent that induces p53 accumulation and p53-dependent cell death in wtp53-expressing cell lines (19). Given that ADR regulates the BRCA2 promoter and induces p53, we investigated whether ADR down-regulates the BRCA2 promoter in a p53-dependent manner. Initially, BRCA2 reporter assays were performed in p53-positive U2OS and p53-null Saos2 osteosarcoma cells in an effort to address the role of p53 in regulation of the promoter while minimizing tissue-specific differences. Treatment with either 5 μM ADR for 1 h or 0.7 μM ADR for 24 h did not affect BRCA2 promoter activity in Saos2 cells, whereas inhibition of the promoter was observed in U2OS cells (Fig. 2A). Similarly, ADR treatment of matched p53-null (HCT116/p53−/−) and p53 wild type (HCT116/p53+/+) HCT116 cells resulted in repression of the BRCA2 promoter in p53 wild type cells but not in p53-null or mutant cells (Fig. 2B). In addition, reporter assays were performed in MCF7 cells stably expressing either the human papilloma virus type 16 (HPV-16) E6 gene (MCF7/E6) or a CMV vector control (MCF7/CVM). The E6 protein stimulates degradation of p53 through a ubiquitin pathway (20, 21), resulting in very low levels of p53 expression in these cells. Reduced levels of p53 in MCF7/E6 cells was verified by Western blotting (data not shown). A decrease in BRCA2 promoter activity in response to ADR treatment was only detected in the MCF7/CVM cells (Fig. 2C). Taken together, these data suggest that repression of BRCA2 promoter activity by ADR is dependent on the presence of wtp53.

Given that ADR-associated repression of the BRCA2 promoter is dependent on p53, it seemed likely that p53 could regulate the BRCA2 promoter independently of ADR treatment. To address this hypothesis a series of BRCA2 promoter reporter assays was performed in MCF7 and Saos2 cells transiently transfected with wtp53, dnp53 (R273L), or control vector. F, MMC and actinomycin D (Act-D) inhibit BRCA2 promoter activity in a p53-dependent manner. Luciferase activity from pGL3Prom was measured after 24 h of treatment with 10 ng/ml actinomycin D or 30 μg/ml MMC in MCF7 (wtp53), Saos2 (p53-null), and T47D (p53 mutant) cells.
BRCA2 expression is down-regulated by p53 and Adriamycin

BRCA2 expression in vivo through inhibition of BRCA2 promoter activity.

In Vivo Reduction in BRCA2 Protein Levels by ADR and Wtp53—Although ADR appears to down-regulate BRCA2 promoter activity and BRCA2 expression levels, down-regulation of BRCA2 protein levels must also be evident to speculate that ADR-dependent repression of BRCA2 transcription can affect BRCA2 function. To evaluate the effect of ADR on in vivo BRCA2 protein levels, MCF7, U2OS, and MCF10A cell lines were treated with 5 \( \mu \text{M} \) ADR. As shown in Fig. 4A, BRCA2 levels were decreased significantly in ADR-treated cells. In addition, the amount of BRCA2 in MCF7 cells decreased in a dose-responsive manner after treatment with 0.5, 2.0, and 5.0 \( \mu \text{M} \) ADR (Fig. 4B). The expected dose-dependent increase in p53 levels was also observed. To evaluate whether ADR treatment had a time-dependent effect on BRCA2 protein expression, MCF7 cells were treated with 5 \( \mu \text{M} \) ADR for 1 h, and BRCA2 protein levels were measured over a 24-h period. A gradual decrease in BRCA2 levels over time was observed with the most significant changes occurring between 5 and 11 h post-treatment (Fig. 4C). Interestingly, the decrease in BRCA2 correlated inversely with changes in p53 levels. Similar results were obtained in response to continuous treatment with 0.7 \( \mu \text{M} \) ADR over a 24-h period, although the most significant changes in BRCA2 and p53 were delayed and occurred between 9 and 13 h after initiation of treatment (Fig. 4D).

Next we evaluated whether the effect of ADR on BRCA2 protein levels was dependent on p53. Matched p53-null (HCT116/p53\(-/-\)) and p53 wild type (HCT116/p53\(+/+\)) HCT116 cells, matched MCF7/E6 and MCF7/CMV cells, SW480 p53 mutant cells, and Saos2 p53-null cells were treated with 5 \( \mu \text{M} \) ADR, and cell lysates were Western blotted for BRCA2 with the Ab2 antibody. As shown in Fig. 4E, an ADR-dependent reduction in BRCA2 protein levels was observed in cells expressing wt53 but not in p53-null or p53 mutant cells. Further analysis showed that ectopic expression of wt53 in Saos2 cells (Fig. 4F) and MCF7 cells (data not shown) also caused a significant reduction in BRCA2 levels. When combining all of these data, it is evident that there is a strong correlation between the effects of ADR and p53 on BRCA2 protein levels and on BRCA2 promoter activity.

The ADR- and p53-responsive Element Is Adjacent to the Transcription Initiation Site—Having determined that ADR and p53 repress BRCA2 promoter activity, we sought to understand the mechanism of repression. We began by identifying the ADR-responsive region within the BRCA2 promoter using a series of luciferase reporter constructs containing deleted forms of an 8-kb form of the BRCA2 promoter in reporter assays (18). MCF7 cells were transfected with the various constructs, and luciferase activities were measured before and after treatment with 5 \( \mu \text{M} \) ADR. All promoter constructs that contained the minimal promoter, located between nucleotides −58 and −1, were repressed equivalently by ADR treatment (data not shown), suggesting that the ADR-responsive cis-element is located in the minimal BRCA2 promoter. However, as deletion of the minimal promoter between −58 and −19 eliminated basal promoter activity (18), we could not directly evaluate the role of this region in the ADR response. To overcome this problem a series of promoter reporter constructs containing substitution mutations in the putative ATF and MLTF binding sites, in the known USF-binding element, and in the two repeats of the GCGTCACG tandem repeat sequence within the −58 to −19 region of the BRCA2 promoter was used (18). The reporter constructs containing the mutated −13 to −20 USF transcription factor binding site and the overlapping −17 to −24 repeat sequence were 3-fold less repressed in the pre-
ence of ADR than any other reporter construct (data not shown). Similar results were obtained after ectopic expression of wtp53. These data suggest that this USF binding site that has been implicated previously in regulation of basal activity of the BRCA2 promoter (18) also regulates the response to ADR and wt53.

Wtp53 Inhibits Binding of USF to the BRCA2 Promoter—To determine whether the USF transcription factor is directly involved in p53- and ADR-dependent regulation of BRCA2 promoter activity, the ability of USF to bind to the BRCA2 promoter was evaluated. Two 26-bp (~10 to ~35) oligonucleotide probes containing either a wild type or mutated USF binding site (18) were used in gel shift assays with lysates from MCF7 cells that had been treated with Me2SO or 5 μM ADR. As shown in Fig. 5A, a specific protein complex bound to the wild type oligonucleotide but not to the mutant oligonucleotide and the ability of this complex to bind DNA decreased over time when cells were treated with ADR (Fig. 5A).

To determine whether wt53 was needed for ADR-dependent inhibition of complex formation, we repeated the gel shift assays using lysates from matched Saos2 and U2OS cells, matched HCT116/p53−/− and HCT116/p53+/+ cells, and matched MCF7/CMV and MCF7/E6 cells (Fig. 5, B–D). A similar protein-oligonucleotide complex was evident in all cells, but the relative intensity of the complex was significantly greater in p53 mutant or p53-null cells than in matched cells with endogenous wt53. Moreover, exposure to ADR further reduced the intensity of the protein-DNA complex in wt53-expressing cells but had no effect in p53-null or mutant cells (Fig. 5, B–D).

In addition, complex formation was reduced substantially by ectopic expression of wt53, similarly to the ADR treatment, but was enhanced by expression of dnp53. This suggests that dnp53 inactivates endogenous or induced wt53 and prevents p53-dependent inhibition of complex formation (Fig. 5E).

To verify that the protein complex binding to the wild type oligonucleotide contained USF, it was demonstrated that anti-USF1 and anti-USF2 antibodies demonstrated that the levels of these proteins were not altered in response to ADR or p53 expression (data not shown). Together these data suggest that the repressive effect of ADR and p53 on the BRCA2 promoter does not involve regulation of USF1 or USF2 levels but is dependent on altered binding of USF2 and/or USF1 to the promoter.

p53 Inhibits USF-dependent Induction of the BRCA2 Promoter—To establish further the relevance of USF to p53- and ADR-dependent repression of the BRCA2 promoter, we tested whether p53 could block induction of the BRCA2 promoter by USF. Initially, a USF2-VP16 fusion protein that binds to the USF site was used to transactivate the BRCA2 promoter in MCF7 cells (18), and then the effect of wt53 and ADR on the activated promoter was evaluated. As shown in Fig. 6A, wt53 partially inhibited USF2-VP16-associated activation of the BRCA2 promoter, whereas dnp53 further activated the promoter. Subsequently, the ability of wt53 to inhibit activation of the promoter by USF1 alone was evaluated. Ectopic expression of USF1 in SW480 (p53 mutant) cells up-regulated the BRCA2 promoter 4.5-fold. However, ectopic expression of wt53 completely blocked this effect (Fig. 6B). Similar effects were observed in the Saos2 p53-null and T47D p53 mutant cell lines (data not shown). Thus, p53 inhibits USF-dependent promoter activation.

ADR Enhances BRCA2 mRNA and Protein Degradation—The data described above show that ADR, MMC, and actinomycin D repress the BRCA2 promoter in a p53-dependent manner. However, although the effect is significant it does not entirely account for the rapid decrease in BRCA2 mRNA and protein levels. In fact, a comparison among promoter activity, mRNA levels, and protein levels at various time points after ADR exposure (Figs. 1D and 4C) indicates that BRCA2 mRNA and protein levels decreased more rapidly than BRCA2 promoter activity. This suggests that ADR can influence BRCA2 mRNA and protein levels through additional mechanisms and provides support for earlier observations that DNA-damaging agents can decrease BRCA2 mRNA levels (16, 17) and that
certain damaging agents such as UV irradiation can enhance BRCA2 protein degradation (22).

To determine whether ADR altered the stability of BRCA2 mRNA, MCF7 cells were treated with \(10^{-9}\)M ADR and Me\(_2\)SO to block transcription, and the rate of decrease in BRCA2 mRNA in cells treated with \(5 \mu M\) ADR and Me\(_2\)SO was compared by semiquantitative reverse transcription PCR. As shown in Fig. 7, A and B, ADR substantially enhanced the rate of BRCA2 mRNA degradation. Quantitation by densitometry showed that the half-life of BRCA2 mRNA in MCF7 cells is normally 9 h, whereas in the presence of ADR it is reduced to \(\sim 3\) h. In addition, by comparing mRNA levels from MCF7 cells treated with ADR alone or with ADR and \(\alpha\)-amanitin at various time points we determined that promoter inhibition accounted for 50% of the reduction in BRCA2 mRNA levels.

We also attempted to measure the rate of BRCA2 protein degradation in the presence and absence of ADR. MCF7 cells were treated with cycloheximide, and BRCA2 protein levels were measured by Western blotting and densitometry. The half-life of the BRCA2 protein was estimated to be 5 h (data not shown).
Interestingly, a finding that BRCA2 protein levels are reduced by 50% and \( \alpha \)-amanitin was performed. Oligonucleotide pairs for PCR were located at the 3'-end (A) and 5'-end (B) of the BRCA2 coding region.

**Repression of the BRCA2 Promoter by ADR and p53 Is Independent of Cell Cycle Arrest**—It is not known whether p53-dependent cell cycle arrest is required for repression of the BRCA2 promoter or whether these events are independent of each other and are simply concurrent responses to p53 induction by ADR. To address this question, cell cycle profiles of cells were measured at various time points after treatment with ADR, and the results were compared with time course studies of BRCA2 promoter activity and BRCA2 mRNA and protein levels from cells that had been treated similarly. It was noted that ADR-dependent repression of the BRCA2 promoter preceded ADR-associated induction of an S and G2/M cell cycle checkpoint arrest (Fig. 8, A and B). This was noted after exposure to a short pulse of 5 \( \mu \)M ADR and in response to continuous exposure to 0.7 \( \mu \)M ADR. Down-regulation of BRCA2 mRNA and protein also appeared to precede the onset of cell cycle arrest.

Given that induction of cell cycle arrest by p53 is associated with induction of p21\(^{Waf1/Cip1}\), we also investigated whether p21\(^{Waf1/Cip1}\) was required for the ADR and p53 effect on the BRCA2 promoter. The BRCA2 luciferase reporter construct was transiently transfected into HCT116/p21\(^{−/−}\) and matched p21\(^{+/+}\) cells, and promoter activity was measured after treatment with 5 \( \mu \)M ADR and ectopic expression of wt p53, BRCA2 promoter activity was repressed equivalently in the matched cell lines by ADR (Fig. 8C) and p53 (Fig. 8D), indicating that the ADR and p53 effect is independent of p21\(^{Waf1/Cip1}\) and suggesting that the BRCA2 promoter is repressed independently of the induction of G1/S checkpoint arrest.

**DISCUSSION**

The effect of cellular stress and DNA-damaging agents on BRCA2 has been a topic of much interest. Initially, BRCA2 mRNA levels were shown to be down-regulated in cells in response to treatment with DNA-damaging agents such as ADR, MMC, and UV irradiation (16). In this study, we show that as part of the cellular response to ADR-associated DNA damage the BRCA2 promoter is significantly repressed in a p53-dependent manner. We have established that in response to ADR, MMC, and actinomycin D, p53 inhibits binding of the USF transcription factor to the BRCA2 minimal promoter, resulting in repression of basal promoter activity and substantial decreases in BRCA2 mRNA and protein levels. The inability of ADR treatment to repress the promoter and decrease...
mRNA and protein levels in p53 mutant or null cells or in cells expressing the dominant negative R273Lp53 indicates that this is a p53-dependent process. However, it is important to note that only ADR, MMC, and actinomycin D repressed the promoter by inhibiting USF binding in a p53-dependent manner, whereas other DNA-damaging agents such as UV irradiation, γ-irradiation, and camptothecin, which are also associated with induction of p53, had no effect. This raises the possibility that only certain forms of DNA damage can induce other factors, in addition to p53, which are required for inhibition of USF binding to the promoter.

Although UV irradiation, γ-irradiation, and camptothecin fail to inhibit USF binding, these agents all reduce BRCA2 mRNA and protein levels. This suggests that these agents either regulate transcription of the BRCA2 gene through cis-elements and enhancer/repressor binding sites that are not present in our 8-kb promoter construct or that these agents influence the stability of BRCA2 mRNA and protein. In this study we determined that ADR and other DNA-damaging agents enhance BRCA2 mRNA and protein turnover, resulting in decreased BRCA2 mRNA and protein levels. Given that BRCA2 mRNA and protein are only reduced by ADR in cells expressing wtp53, the suggestion is that p53 mediates BRCA2 promoter activity and BRCA2 mRNA and protein turnover in response to ADR, MMC, and actinomycin D but only regulates mRNA and protein turnover in response to other DNA-damaging agents. Thus, BRCA2 protein levels may be regulated by various DNA-damaging agents at the level of the promoter, mRNA stability, and protein stability.

In this work we determined that ADR and p53 repress the BRCA2 promoter by inhibiting the ability of USF to bind to the promoter. To our knowledge, this is the first report showing that p53-mediated transcriptional repression is functionally associated with USF binding. We have shown previously that binding of USF to the BRCA2 minimal promoter is required for basal transcription of BRCA2 and for induction of the promoter and increased expression of BRCA2 during the S and G2 phases of the cell cycle (18). Thus, by inhibiting binding of USF to the promoter it appears that ADR treatment and p53 actually prevent activation of the BRCA2 promoter during S and G2 phases of the cell cycle. Although p53 appears to regulate USF binding in response to ADR treatment, p53 does not seem to bind directly to USF, as evidenced by an inability of anti-p53 antibodies to supershift the USF complex and by failure of p53 to coimmunoprecipitate with USF1 or USF2 (data not shown). Because we have also shown that p53 does not regulate USF expression levels, it seems likely that ADR or p53 may regulate and p53 may interact with other protein(s) that in turn modulate the ability of USF to bind to the promoter. However, it is also possible that p53 may be a part of the USF protein complex, but its presence in the complex may not be detected easily by certain antibodies. USF1 has recently been identified as a phosphoprotein (23, 24) whose DNA binding activity is dependent on cyclin-dependent phosphorylation and can be inhibited by the p53-inducible cyclin-dependent kinase inhibitor, p21Waf1/Cip1, which blocks phosphorylation of USF1 (24). This suggests that ADR and p53 regulate BRCA2 expression through p21Waf1/Cip1. However, we found that p53 and ADR repressed BRCA2 promoter activity in cells lacking p21Waf1/Cip1, suggesting that p21Waf1/Cip1 plays no role in USF-dependent regulation of the BRCA2 promoter.

Repression of transcription of several genes by p53 is thought to be the consequence of p53-dependent inhibition of other transcriptional activators (25–27) or components of the basal transcription machinery (28–30). One mechanism of p53-associated repression utilizes histone deacetylases, mediated by interaction with Sin3a, to regulate target genes such as map4 and stathmin negatively (19) and to repress the CHK1 gene through the p21Waf1/Cip1 protein (31). Another mechanism of p53-dependent repression involves binding of p53 to p300/CBP and subsequent interference in coactivation of p300/CBP-dependent factors, such as AP-1 (26), hypoxia-inducible factor 1 (32), and nuclear factor-κB (33, 34). Interestingly, it has been reported that p300 interacts functionally with USF to potentiate the activation of USF target genes (35). Whether the USF-dependent regulation of the BRCA2 promoter by p53 is actually mediated by p300/CBP or histone deacetylases or as yet unidentified factors remains to be determined.

There is substantial evidence that BRCA2 plays a role in DNA repair. Therefore, it is surprising that BRCA2 is down-regulated by p53 in response to ADR-associated DNA damage. One possible explanation is that p53 must down-regulate BRCA2 to induce cell cycle arrest and DNA damage repair because BRCA2 appears to interact with p53 in a RAD51-p53-BRCA2 complex and may partially repress p53-dependent transactivation of target promoters such as p21Waf1/Cip1 (36). Alternatively, BRCA2 may interfere with induction of p53-dependent apoptosis in response to DNA damage in a similar manner. However, because BRCA2 is itself involved in DNA repair this seems unlikely. It is also possible that down-regulation of BRCA2 by p53 allows cells to initiate apoptosis in response to DNA damage more efficiently, while the presence of BRCA2 provides sufficient DNA repair to pro-
long cell viability. An alternative possibility is that p53 may down-regulate BRCA2 after DNA repair is complete to inactivate the DNA repair machinery and to release the cell from checkpoint arrest. This could also be a mechanism by which p53 regulates the extent and timing of DNA damage repair. The observation that p53 only significantly affects the checkpoint arrest. This could also be a mechanism by which p53 activates the DNA repair machinery and to release the cell from long cell viability. An alternative possibility is that p53 may down-regulate BRCA2 mRNA and protein levels in G1 and significantly elevated levels in S and G2 phase of the cell cycle (3, 4). In the work described above we have shown that repression of the BRCA2 promoter and associated down-regulation of BRCA2 mRNA and protein levels preceded p53-dependent S and G2/M phase cell cycle arrest in response to ADR treatment. This indicates that BRCA2 down-regulation is dependent on p53 but is independent of the effect of p53 induction on the cell cycle. Future experiments will determine whether BRCA2 down-regulation is required or contributes to p53-dependent cell cycle arrest in response to DNA damage.

In conclusion, we have demonstrated that the BRCA2 promoter is down-regulated by ADR in a p53-dependent manner and that repression of the promoter is mediated by altered binding of USF to the minimal promoter. We have also shown that inhibition of promoter activity results in decreased BRCA2 mRNA and protein levels over time, suggesting a direct effect on BRCA2 function. Furthermore, we found that ADR and other DNA-damaging agents increase the rate of BRCA2 mRNA and protein turnover in a p53-dependent manner. Given that BRCA2 appears to regulate the transactivation activity of p53 and that p53 inhibits BRCA2 expression, it appears that BRCA2 and p53 share a complex regulatory loop where p53 induction on the cell cycle. Future experiments will determine whether BRCA2 down-regulation is required or contributes to p53-dependent cell cycle arrest in response to DNA damage.

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J. Biol. Chem. 2003, 278:15652-15660.
doi: 10.1074/jbc.M211297200 originally published online February 18, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211297200

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