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Original Research Communication

BRCA1-Mediated Ubiquitination Inhibits Topoisomerase IIα Activity in Response to Oxidative Stress

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ABSTRACT

Topoisomerase IIα is known to be critically involved in both cell proliferation and cell death. The mechanisms responsible for stress-dependent topoisomerase IIα alterations, however, remain unclear. This study focused on the behavior of topoisomerase IIα in response to oxidative stress induced by hydrogen peroxide (H_2O_2). The catalytic activity of topoisomerase IIα in MOLT-4 cells treated with H_2O_2 decreased in parallel with the alteration of topoisomerase IIα expression. The ubiquitination of topoisomerase IIα was dependent on oxidative stress. BRCA1, a tumor-suppressor gene, appeared to be involved in these alterations in topoisomerase IIα. Furthermore, the retinoblastoma protein (pRb) was required for the ubiquitination of topoisomerase IIα by BRCA1. We conclude that the functions of topoisomerase IIα are regulated by ubiquitination on exposure to oxidative stress. *Antioxid. Redox Signal.* 10, 939–949.

INTRODUCTION

Oxidative stress has been implicated in the initiation and development of many diseases (5, 31) and causes various types of cellular injury, including DNA and protein damage, lipid peroxidation, and damage to other biomolecules (3). Oxidative stress also occurs during apoptotic cell death (21, 41). H_2O_2, a reactive oxygen species (ROS) generated in response to oxidative stress, induces apoptosis (23). One feature of apoptotic cell death is fragmentation of nuclear DNA into nucleosomal DNA ladders (25, 35), a process thought to involve a number of nucleases (20, 39). Interestingly, the formation of DNA fragments in apoptotic cell death is associated with topoisomerase IIα–mediated DNA fragmentation in cells (24, 38). DNA fragmentation induced by topoisomerase IIα inhibition is similar to that occurring in apoptotic cell death (7, 45). Available evidence thus suggests a relation between topoisomerase IIα and oxidative stress. However, the effect of oxidative stress on the functions of topoisomerase IIα remains largely unknown.

Topoisomerase IIα is a nuclear enzyme essential for chromosomal segregation during mitosis (44). Topoisomerase IIα is reported to have other biologic functions and is associated with G_2 cell-cycle checkpoints and tumor differentiation (9, 10). The level of topoisomerase IIα is cell-cycle regulated, peaking in G_2/M phase and declining to a minimal level at the end of M phase (59). Decreased topoisomerase IIα levels are frequently accompanied by a reduction in mRNA levels, suggesting alterations in the transcriptional regulation of proteins (30). In addition, posttranslational modifications, such as phosphorylation, ubiquitination, and sumoylation, can alter protein stability or localization (8, 27, 29, 36). Our previous studies demonstrated that protein kinase Cδ is activated in response to oxidative stress (47) and is, at least in part, involved in regulation of topoisomerase IIα expression (49, 53, 59). Cellular levels of topoisomerase IIα may also be regulated by certain types of stress. Activation of the mammalian unfolded-protein response induces resistance of cells to topoisomerase II–targeting agents (15) by dramatically reducing topoisomerase IIα levels (37). Moreover, the expression of topoisomerase IIα is downregulated in cancer cells exposed to glucose-regulated stress (18). However, the mechanisms underlying stress-dependent alterations in topoisomerase IIα otherwise remain poorly understood. To gain insight into the mechanisms involved, we studied the response of topoisomerase IIα to oxidative stress. Our results suggest that the activity of topoisomerase IIα is downregulated by BRCA1-induced ubiquitination in response to oxidative stress.

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MATERIALS AND METHODS

Cell cultures and treatments

Human leukemia MOLT-4 and HL-60 cells, human osteosarcoma U2OS cells, and human breast cancer HCC1937 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 m*M* L-glutamine. African green monkey kidney fibroblast Cos-7 cells and human cervical carcinoma HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 m*M* L-glutamine, and 4.5 g/L glucose. Cells were treated with H_2O_2 (Santoku Chemical), tumor necrosis factor-α (TNF-α) (Peprotech), *n*-propyl gallate (Nacalai Tesque), etoposide (Sigma-Aldrich), or MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; Nacalai Tesque).

Preparation of nuclear extracts

To prepare nuclear extracts for topoisomerase II catalytic activity assays and immunoblot analysis, 1×10^7 to 3×10^7 cells were washed with phosphate-buffered saline followed by 1 ml of buffer A (10 m*M* HEPES, pH 7.6, 15 m*M* KCl, 2 m*M* $MgCl_2$, 0.1 m*M* EDTA, 1 m*M* DTT, 0.5 m*M* PMSF, and 10 μg/ml leupeptin) and were then resuspended in buffer A containing 0.2% NP-40. The cells were centrifuged and resuspended in 1 ml of buffer A containing 0.25 *M* sucrose. Subsequently, samples were collected by centrifugation and resuspended in buffer D (50 m*M* HEPES, pH 7.9, 400 m*M* KCl, 0.1 m*M* EDTA, 10% glycerol, 1 m*M* DTT, 0.5 m*M* PMSF, and 10 μg/ml leupeptin). The samples were mixed and gently rocked for 30 min at 4°C and were then centrifuged for 5 min at 15,000 rpm. Supernatants were used as nuclear extracts.

Topoisomerase IIα catalytic activity assays

Topoisomerase II catalytic activity was assayed by the decatenation of kinetoplast DNA (kDNA) (59). The decatenation assays were performed by incubating 0.2 μg kDNA (Nippon Gene) with nuclear extracts in assay buffer (50 m*M* Tris-HCl, pH 8.0, 120 m*M* KCl, 10 m*M* $MgCl_2$, 0.5 m*M* ATP, 0.5 m*M* DTT, and 30 μg/ml bovine serum albumin). After incubation for 10 min at 37°C, reactions were stopped by the addition of stop buffer (5% sarkosyl, 0.0025% bromophenol blue, and 25% glycerol). The reaction products were analyzed on 1% agarose gels containing 0.5 μg/ml ethidium bromide.

Immunoblot analysis

Nuclear extracts were analyzed by immunoblotting as previously described (40, 50, 51, 57). In brief, nuclear extracts or immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were then incubated with anti-topoisomerase IIα (MBL), anti-ubiquitin (Santa Cruz Biotechnology), anti-BRCA1 (Ab-4; Calbiochem), anti-retinoblastoma (BD Pharmingen), anti-PCNA (Santa Cruz Biotechnology), or anti-Lamin B1 (Santa Cruz Biotechnology). The antigen–antibody complexes were visualized by chemiluminescence (Perkin-Elmer).

RT-PCR analysis for topoisomerase IIα gene expression

Total cellular RNA was extracted from cells with the use of ISOGEN-LS (Nippon Gene). First-strand cDNA synthesis and the following PCR were performed with 300 ng of total RNA, by using a SuperScript one-step reverse transcriptase PCR (RT-PCR) system (Invitrogen) according to the manufacturer's protocol. For topoisomerase IIα gene expression, the nucleotide sequence of 5′-GCCCTCCTGCTACACATTTC-3′ was used as the sense primer, and 5′-AACACTTGGGCTTTACTTCACTT-3′, as the antisense primer. For β-actin gene expression, the nucleotide sequence of 5′-CAGGGCGTGATGGTGGGCA-3′ was used as the sense primer, and 5′-CAAACATCATCTGGGTCATCTTCTC-3′, as the antisense primer. The amplified DNA samples were resolved on 2% agarose gels.

Immunoprecipitation

The immunoprecipitation was performed as described elsewhere (52, 55, 56, 58). In brief, nuclear extracts from cells were incubated with anti-topoisomerase IIα (Bethyl), anti-BRCA1 (Ab-3; Calbiochem), anti-retinoblastoma (BD Pharmingen), or anti-HA (Roche) antibodies for 2 h at 4°C, followed by 1 h of incubation with protein A/G-Sepharose beads (Amersham Biosciences). The immune complexes were washed 3 times with 0.1% NP-40 lysis buffer (0.1% NP-40, 50 m*M* Tris-Cl, pH 7.6, 150 m*M* NaCl, 10 μg/ml aprotinin, 1 m*M* DTT, 10 m*M* NaF, 1 m*M* PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 m*M* Na_3VO_4) and were then eluted by boiling for 5 min in 50 m*M* Tris-Cl, pH 6.8, containing 2% SDS, 6% 2-mercaptoethanol, 0.01% bromophenol blue, and 10% glycerol. The eluted samples were subjected to immunoblot analysis.

Plasmid construct and transfection

Full-length BRCA1 was cloned into the pME18S-FL3 vector *via* the Not I site. HA-ubiquitin was described elsewhere (46). Transfection of plasmids was performed by using FuGENE 6 (Roche) according to the manufacturer's instructions.

Small Interfering RNA duplex oligoribonucleotides (siRNAs) and transfection

Synthetic siRNAs for pRb were purchased from Invitrogen (Stealth RNAi). The pRb-specific Stealth RNAi used in this study has the following sequences: Rb siRNA1, 5′-UCAAGAUUCUGAGAUGUACUUCUGC-3′ (sense) and 5′-GCAGAAGUACAUCUCAGAAUCUUGA-3′ (antisense); and Rb siRNA2, 5′-AUAAAGGUGAAUCUGAGAGCCAUGC-3′ (sense) and 5′-GCAUGGCUCUCAGAUUCACCUUUAU-3′ (antisense); and Rb siRNA3, 5′-UUCAGUCUCUGCAUGAAGACCGAGU-3′ (sense) and 5′-ACUCGGUCUUCAUGCAGAGACUGAA-3′ (antisense). The scramble siRNA was purchased from QIAGEN. Transfection of siRNAs was performed

by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

RESULTS

Catalytic activity and expression of topoisomerase IIα in response to oxidative stress

To induce oxidative stress response, MOLT-4 and HL-60 cells were treated with H_2O_2. We assayed the catalytic activity of topoisomerase IIα by the decatenation of kinetoplast DNA. The decatenation activity of topoisomerase IIα in MOLT-4 cells was found to decrease exponentially in response to oxidative stress (Fig. 1A). In contrast, the decatenation activity of topoisomerase IIα in HL-60 cells did not appreciably decrease under the same conditions (Fig. 1B). To examine whether the decreased catalytic activity of topoisomerase IIα depended on alteration of topoisomerase IIα expression in MOLT-4 cells, we performed an immunoblot analysis. On exposure to oxidative stress, the expression of topoisomerase IIα decreased in MOLT-4 cells, but not in HL-60 cells (see Fig. 1A and B). In this context, the change in topoisomerase IIα activity in response to oxidative stress was relatively consistent with the change in topoisomerase IIα expression. To determine whether alterations in topoisomerase IIα activity and expression depended on immediate oxidative stress in cells, we used TNF-α, because this cytokine indirectly induces oxidative stress (28, 60). When MOLT-4 cells were treated with TNF-α, the catalytic activity of topoisomerase IIα did not decrease (data not shown). Furthermore, topoisomerase IIα expression was also not decreased by TNF-α (data not shown). When HL-60 cells were treated with TNF-α, the catalytic activity and expression of topoiso-

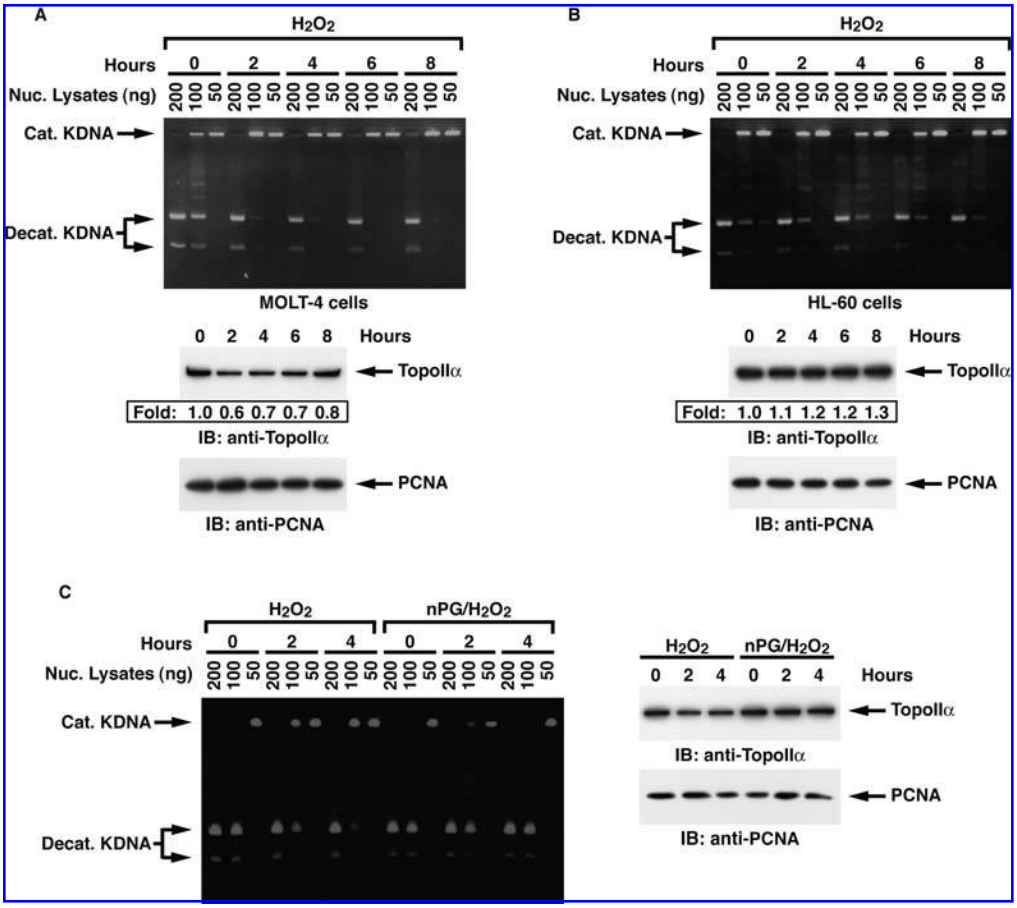

FIG. 1. The catalytic activity and expression of topoisomerase IIα. MOLT-4 cells (**A**) or HL-60 cells (**B**) were treated with 500 μ*M* hydrogen peroxide (H_2O_2) for the indicated times. Topoisomerase IIα (TopoIIα) activity was analyzed by decatenation assays (*top*). These assays used reaction mixtures containing nuclear lysates and kinetoplast DNA (kDNA). Reaction products were analyzed on 1% agarose gels containing 0.5 μg/ml ethidium bromide. Nuclear lysates were also analyzed by immunoblotting (IB) with anti-TopoIIα (*middle*) or anti-PCNA (*bottom*). A ratio of topoisomerase IIα expression was determined by the densitometric analysis with the Image J program. The amount of nuclear topoisomerase IIα in control cells was defined as 1.0. (**C**) MOLT-4 cells were treated with 500 μ*M* H_2O_2 for the indicated times in the presence or absence of 500 μ*M* *n*-propyl gallate (nPG). TopoIIα activity was analyzed by decatenation assays (*top*). Nuclear lysates were also analyzed by immunoblotting with anti-TopoIIα (*middle*) or anti-PCNA (*bottom*).

merase IIα did not change appreciably (data not shown). We next checked the effect of antioxidant on topoisomerase IIα. In the presence of the antioxidant *n*-propyl gallate (nPG) (34), the catalytic activity of topoisomerase IIα was not decreased by H_2O_2 (Fig. 1C). Under the same conditions, the expression of topoisomerase IIα also did not decrease (Fig. 1C).

Effect of proteasome inhibitor on topoisomerase IIα expression in MOLT-4 cells exposed to oxidative stress

We next performed a quantitative RT-PCR analysis to evaluate topoisomerase IIα gene expression directly and thereby clarify whether such expression is regulated at the transcription level in response to oxidative stress. Expression of the topoisomerase IIα mRNA in MOLT-4 cells was not altered by oxidative stress on RT-PCR assay (Fig. 2A). Because topoisomerase IIα is a key target enzyme of many anticancer drugs, we next examined expression of the topoisomerase IIα mRNA in the presence of etoposide, a topoisomerase II inhibitor, by RT-PCR assays. Etoposide was found to decrease expression of the topoisomerase IIα mRNA (Fig. 2B). To determine whether the oxidative stress–induced decrease in topoisomerase IIα expression was dependent on posttranslational regulation, MOLT-4 cells were treated with H_2O_2, in the presence or absence of MG-132, a proteasome inhibitor. The decrease in topoisomerase IIα expression at the protein level was attenuated in the presence of MG-132 (Fig. 2C). These results indicated that the oxidative stress–induced decrease in topoisomerase IIα expression did not depend on transcriptional regulation in MOLT-4 cells.

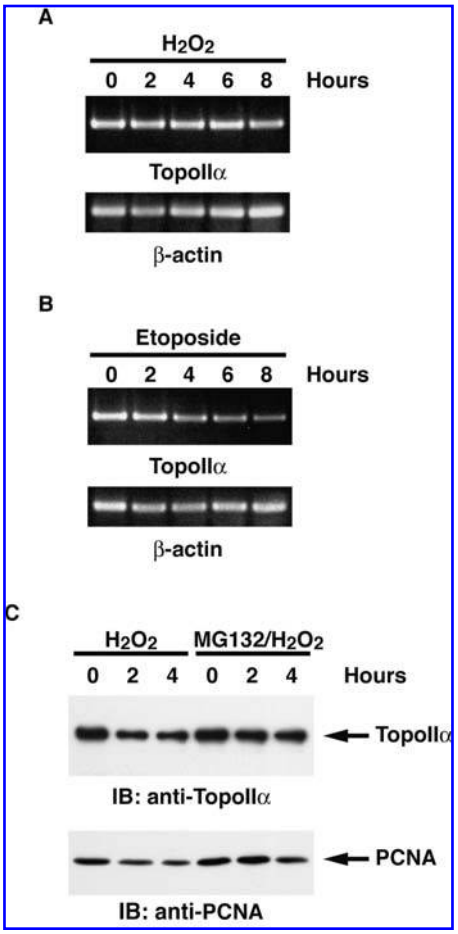

FIG. 2. Alteration of topoisomerase IIα expression by translational or posttranslational regulation in MOLT-4 cells. MOLT-4 cells were treated with 500 μM H_2O_2 (**A**) or 10 μM etoposide (**B**) for the indicated times. Total cellular RNA was extracted from MOLT-4 cells, and then RT-PCR assays were carried out by using primer sets for TopoIIα or β-actin. (**C**) MOLT-4 cells were treated with 500 μM H_2O_2 for the indicated times in the presence or absence of 2.5 μM MG-132. Nuclear lysates were analyzed by immunoblotting with anti-TopoIIα or anti-PCNA.

Ubiquitination of topoisomerase IIα in MOLT-4 cells exposed to oxidative stress

We next investigated the ubiquitination of topoisomerase IIα, because the oxidative stress–induced decrease in topoisomerase IIα was almost completely inhibited in the presence of MG-132. Topoisomerase IIα was immunoprecipitated from MOLT-4 cells or HL-60 cells treated with H_2O_2 in the presence or absence of MG-132. When topoisomerase IIα was immunoprecipitated from MOLT-4 cells, smears were detected on application of antibodies to ubiquitin (Fig. 3A and C). No smear was detected when topoisomerase IIα was immunoprecipitated from HL-60 cells (Fig. 3B and C). These results demonstrated that ubiquitination of topoisomerase IIα occurred specifically in MOLT-4 cells. The present data show that ubiquitin reactivity of anti-topoisomerase IIα-immunoprecipitates is detected in the smear that migrates not only slower but also faster than topoisomerase IIα (Fig. 3A and C). The possible explanation is that the immunoprecipitates co-migrate with numerous interacting proteins that are polyubiquitinated after oxidative stress. To address this issue, U2OS cells transfected with HA-ubiquitin were left untreated or treated with H_2O_2. Anti-topoisomerase IIα immunoblot analysis of anti-HA immunoprecipitates showed that, on exposure to H_2O_2, the smear was detected only slower migration from the band corresponding to topoisomerase IIα (Fig. 3D). This result suggested the specificity of polyubiquitinated topoisomerase IIα in response to oxidative stress. Further to determine whether ubiquitination of topoisomerase IIα occurred specifically in response to oxidative stress, topoisomerase IIα was immunoprecipitated from MOLT-4 cells treated with etoposide. In contrast to H_2O_2 treatment, no ubiquitination of topoisomerase IIα was detected after etoposide exposure (Fig. 3E). In this regard, the ubiquitination of topoisomerase IIα apparently affected the expression of topoisomerase IIα in MOLT-4 cells exposed to oxidative stress.

BRCA1 regulates the ubiquitination of topoisomerase IIα in response to oxidative stress

We next considered the possibility that BRCA1 regulates the catalytic activity of topoisomerase IIα *via* the ubiquitination of topoisomerase IIα in MOLT-4 cells exposed to oxidative stress. Tumor-suppressor gene BRCA1 is associated with transcriptional regulation, cell-cycle checkpoint control, and DNA damage repair (12, 54). The BRCT domains of BRCA1 bind topoi-

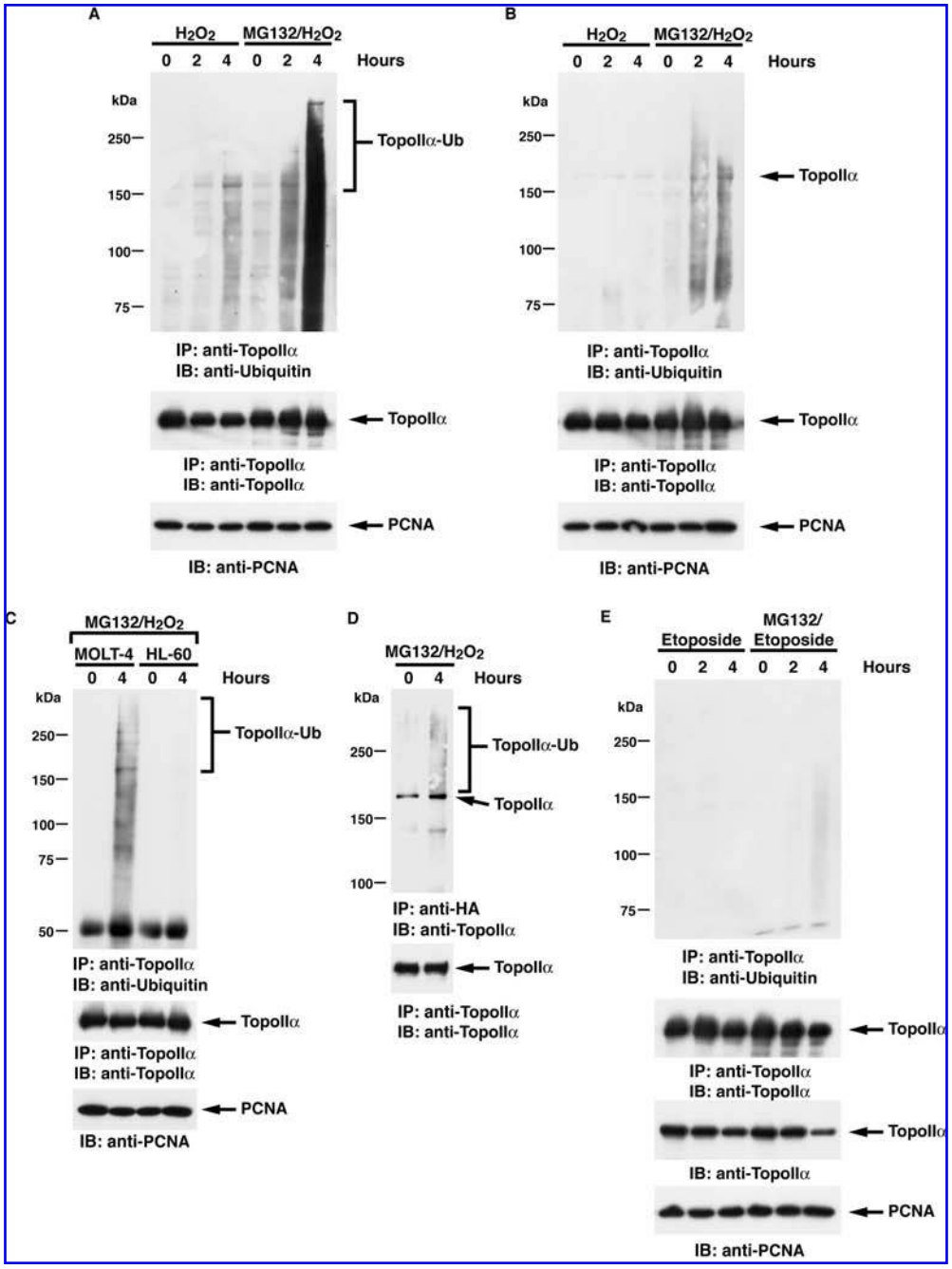

FIG. 3. The ubiquitination of topoisomerase IIα under oxidative stress in MOLT-4 cells. MOLT-4 cells (**A, C**) or HL-60 cells (**B, C**) were treated with 500 μM H_2O_2 for the indicated times in the presence or absence of 5 μM MG-132. Nuclear lysates were subjected to immunoprecipitation (IP) with anti-TopoIIα followed by immunoblot analysis with anti-ubiquitin or anti-TopoIIα. Nuclear lysates were also analyzed by immunoblotting with anti-PCNA. (**D**) U2OS cells transfected with the HA-Ub were treated with 1 mM H_2O_2 in the presence of 5 μM MG-132. Nuclear lysates were subjected to immunoprecipitation with anti-HA followed by immunoblot analysis with anti-TopoIIα. Nuclear lysates were also analyzed by immunoblotting with anti-TopoIIα. (**E**) MOLT-4 cells were treated with 10 μM etoposide for the indicated times in the presence or absence of 5 μM MG-132. Nuclear lysates were subjected to immunoprecipitation with anti-TopoIIα followed by immunoblot analysis with anti-ubiquitin or anti-TopoIIα. Nuclear lysates were also analyzed by immunoblotting with anti-TopoIIα or anti-PCNA.

somerase IIα in a phosphorylation-dependent manner (26). In addition, BRCA1 has a RING finger domain (12, 26, 54). BRCA1 may thus modulate the ubiquitination of topoisomerase IIα by acting as an E3 ubiquitin ligase in the ubiquitin–proteasome pathway. To confirm that BRCA1 is required for topoisomerase IIα ubiquitination, we used FLAG-tagged BRCA1 to overexpress BRCA1. When topoisomerase IIα was immunoprecipitated from Cos-7 cells transfected with FLAG-BRCA1

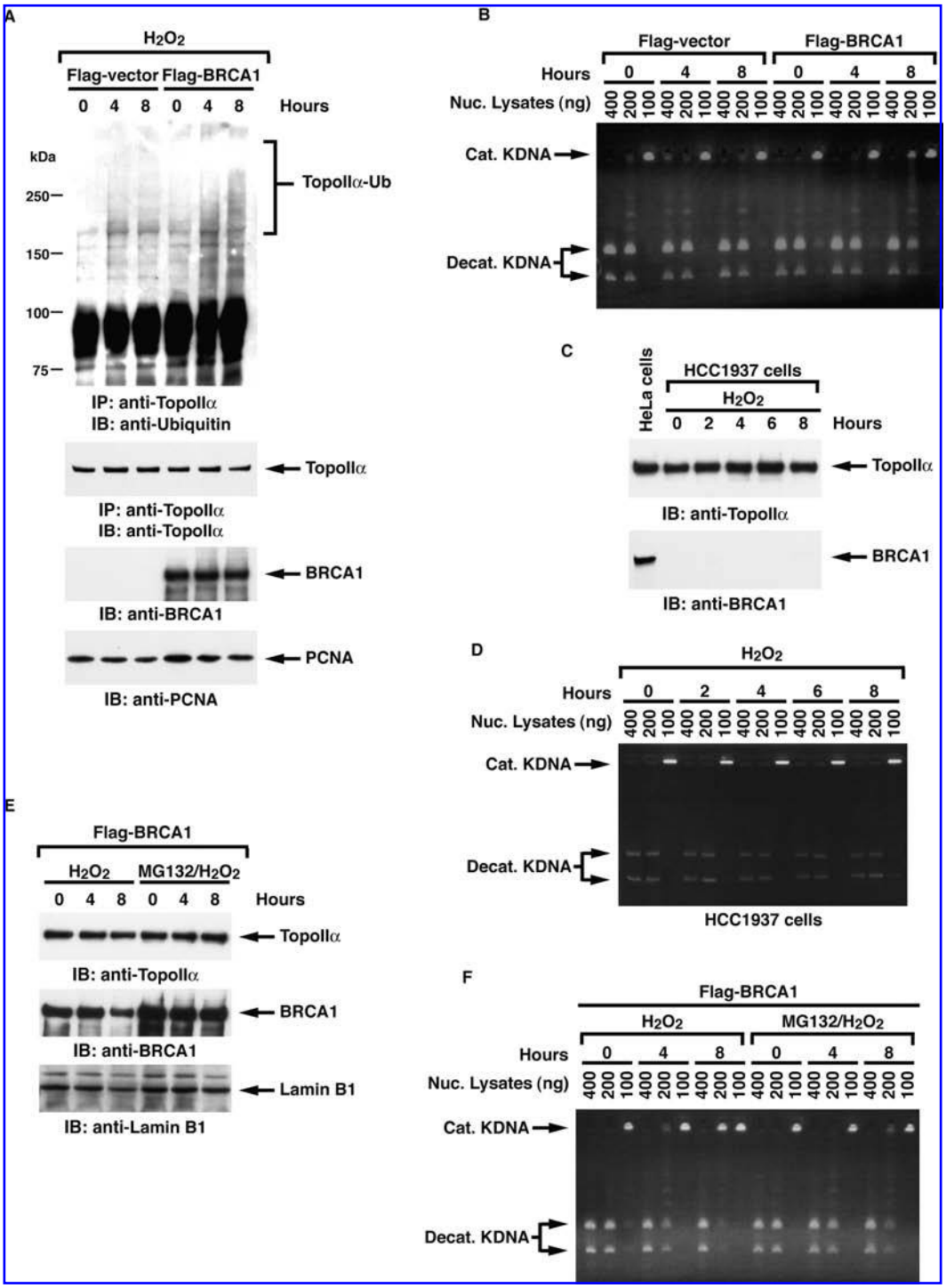

FIG. 4. Topoisomerase IIα is ubiquitinated in a BRCA1-dependent manner. (**A**) Cos-7 cells transfected with the FLAG vector or FLAG-BRCA1 were treated with 1 m*M* H_2O_2. Nuclear lysates were subjected to immunoprecipitation with anti-TopoIIα followed by immunoblot analysis with anti-ubiquitin or anti-TopoIIα. Nuclear lysates were also analyzed by immunoblotting with anti-BRCA1 or anti-PCNA. (**B**) The nuclear lysates of transfected Cos-7 cells mentioned earlier were analyzed with decatenation assays. (**C**) HCC1937 cells were treated with 1 m*M* H_2O_2. Nuclear lysates were analyzed by immunoblotting with anti-TopoIIα or anti-BRCA1. Nuclear lysates of HeLa cells were also analyzed by immunoblotting with anti-TopoIIα or anti-BRCA1. (**D**) The nuclear lysates of HCC1937 cells mentioned earlier were analyzed with decatenation assays. (**E**) Cos-7 cells transfected with the FLAG-BRCA1 were treated with 1 m*M* H_2O_2 in the presence or absence of 5 μ*M* MG-132. Nuclear lysates were analyzed by immunoblotting with anti-TopoIIα, anti-BRCA1, or anti-Lamin B1. (**F**) The nuclear lysates of transfected Cos-7 cells mentioned earlier were analyzed with decatenation assays.

and exposed to oxidative stress, smears were detected on application of antibodies to ubiquitin (Fig. 4A). In contrast, after transfection with FLAG vector, no smears were detected after treatment with antibodies to ubiquitin (see Fig. 4A). The catalytic activity of topoisomerase IIα in Cos-7 cells transfected with FLAG-BRCA1 and exposed to oxidative stress decreased slightly, as compared with control (Fig. 4B). To determine whether BRCA1 is required for downregulation of topoisomerase IIα expression and activity, HCC1937 cells, which are BRCA1 deficient, were left untreated or treated with H_2O_2. No significant decrease in expression and catalytic activity of topoisomerase IIα was found, demonstrating a critical role of BRCA1 in topoisomerase IIα regulation (Fig. 4C and D). Further to define whether BRCA1-mediated downregulation of topoisomerase IIα is associated with degradation by the ubiquitin–proteasome system, Cos-7 cells transfected with Flag-BRCA1 were treated with H_2O_2 in the presence or absence of MG-132. The decrease in topoisomerase IIα expression and catalytic activity was attenuated in the presence of MG-132 (Fig. 4E and F). Taken together, these results suggest that BRCA1 at least partly regulates the catalytic activity of topoisomerase IIα via ubiquitination.

pRb couples BRCA1 with topoisomerase IIα on exposure to oxidative stress

Because previous studies have demonstrated that the retinoblastoma protein (pRb) interacts with both BRCA1 and topoisomerase IIα (1, 4), we examined the possibility that pRb functions as a scaffold protein for interaction of BRCA1 with topoisomerase IIα. MOLT-4 cells were left untreated or were treated with H_2O_2, in the presence or absence of MG-132. Nuclear lysates were immunoprecipitated with anti-topoisomerase IIα followed by immunoblotting with anti-Rb or anti-BRCA1. The results demonstrated that topoisomerase IIα is associated with pRb or BRCA1 in response to oxidative stress (Fig. 5). Finally, the data revealed that topoisomerase IIα and BRCA1 form complexes with pRb after H_2O_2 exposure (see Fig. 5). These results suggest that pRb couples BRCA1 with topoisomerase IIα in MOLT-4 cells exposed to oxidative stress.

pRb is required for interaction between topoisomerase IIα and BRCA1 in cells exposed to oxidative stress

To determine whether pRb is required for interaction between topoisomerase IIα and BRCA1, pRb was knocked down by transfection of HeLa cells with Rb siRNAs. Of note, as shown for MOLT-4 cells, activity of topoisomerase IIα was attenuated after H_2O_2 exposure in HeLa cells (data not shown). The results demonstrated that knocking down pRb abrogated the interaction of topoisomerase IIα with BRCA1 as well as ubiquitin after exposure to oxidative stress (Fig. 6A). Moreover, downregulation of topoisomerase IIα activity was substantially attenuated in HeLa cells transfected with the Rb siRNA in response to oxidative stress (Fig. 6B). These results indicate that pRb is necessary for the interaction of topoisomerase IIα with BRCA1, thus suggesting that the complexes of topoisomerase IIα and BRCA1 coupled with pRb would affect the catalytic activity of topoisomerase IIα.

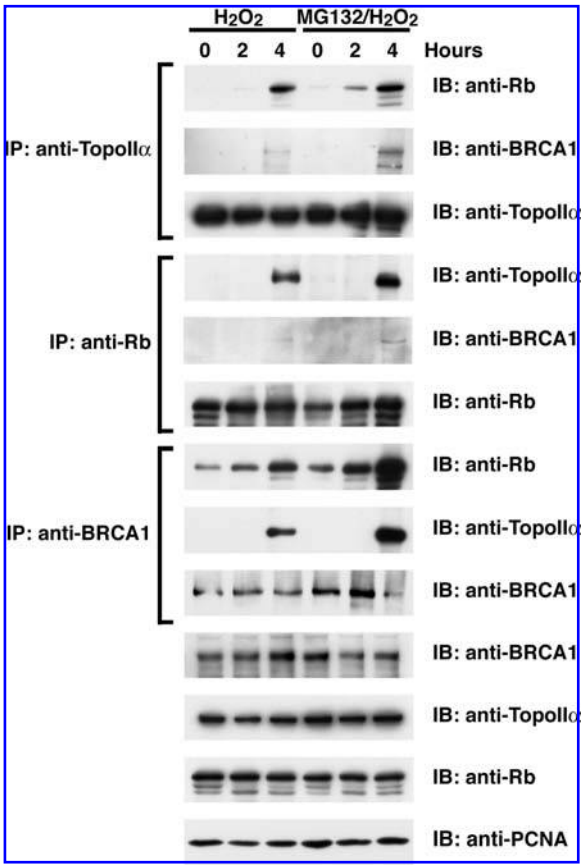

FIG. 5. Interaction among pRb, topoisomeraes IIα and BRCA1 exposed to oxidative stress. MOLT-4 cells were treated with 500 μM H_2O_2 for the indicated times in the presence or absence of 2.5 μM MG-132. Nuclear lysates were subjected to immunoprecipitation with anti-TopoIIα followed by immunoblot analysis with anti-retinoblastoma (anti-Rb), anti-BRCA1, or anti-TopoIIα. Similarly, nuclear lysates were subjected to immunoprecipitation with anti-Rb followed by immunoblot analysis with anti-TopoIIα, anti-BRCA1, or anti-Rb. Nuclear lysates were also subjected to immunoprecipitation with anti-BRCA1 followed by immunoblot analysis with anti-Rb, anti-TopoIIα or anti-BRCA1. The nuclear lysates were analyzed by immunoblotting with anti-BRCA1, anti-TopoIIα, anti-Rb, or anti-PCNA.

DISCUSSION

Although oxidative stress is ubiquitous in living organisms, few studies have examined the effects of oxidative stress on the functions of topoisomerase IIα. Our study showed that the catalytic activity of topoisomerase IIα decreased sharply in MOLT-4 cells exposed to H_2O_2. The change in topoisomerase IIα activity depended on the decrease in topoisomerase IIα expression (see Fig. 1A). By contrast, TNF-α–induced oxidative stress has few if any effects on topoisomerase IIα expression and activity (data not shown). Whereas treatment of cells with H_2O_2 rapidly generates hydroxyl radicals as ROS (11), TNF-α treatment induces elevated superoxide anions in mitochondria (17). Importantly, hydroxyl radicals induce cellular injury much more than do superoxide anions. In this context, these results

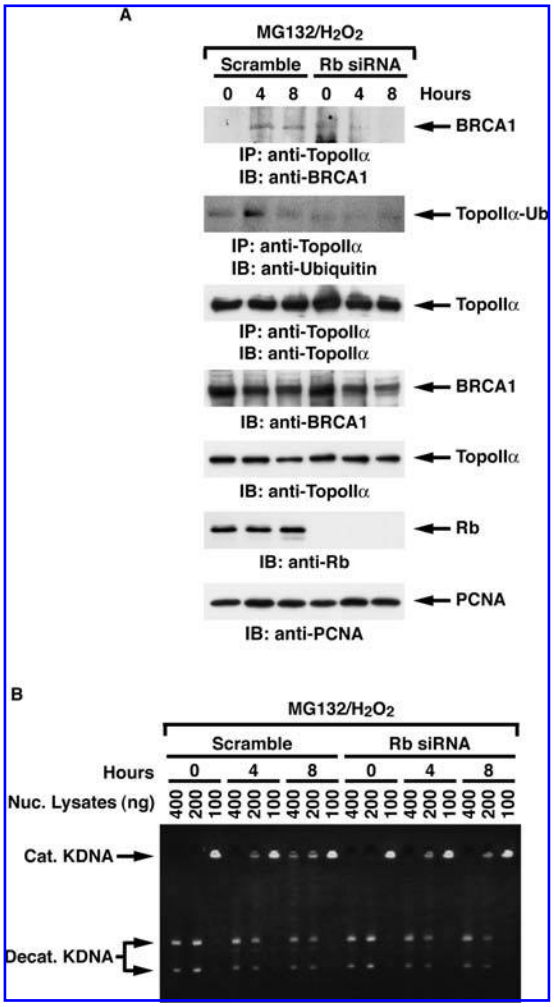

FIG. 6. pRb is required for interaction of topoisomerase IIα and BRCA1 in response to oxidative stress. (**A**) HeLa cells transfected with scramble siRNA or the Rb siRNA were treated with 1 m*M* H_2O_2 for the indicated times in the presence of 2.5 μM MG-132. Nuclear lysates were subjected to immunoprecipitation with anti-TopoIIα followed by immunoblot analysis with anti-BRCA1, anti-ubiquitin, or anti-TopoIIα. Nuclear lysates were also analyzed by immunoblotting with anti-BRCA1, anti-TopoIIα, anti-Rb, or anti-PCNA. (**B**) The nuclear lysates mentioned earlier were analyzed with decatenation assays.

demonstrating the different response of topoisomerase IIα between H_2O_2 and TNF-α could be mainly due to the difference of generated ROS. In addition, topoisomerase IIα plays a critical role in chromosome condensation and segregation during mitosis (44). Rapid decreases in topoisomerase IIα activity may thus affect cell division and then influence the cell cycle. Moreover, oxidative stress and fragmentation are common events during early apoptosis (33). Reactive oxygen species, such as superoxide, generated at ubiquinone sites of the mitochondrial respiratory chain, act as early major mediators in ceramide-induced apoptosis (32). Therefore, alterations in topoisomerase IIα induced by oxidative stress may also have important implications for apoptotic cell death.

We expected that oxidative stress–induced alterations in topoisomerase IIα would depend on posttranslational regulation, because expression of the topoisomerase IIα gene is not decreased by exposure to oxidative stress (see Fig. 2A). The ubiquitination of topoisomerase IIα has already been reported. Nakajima *et al.* (29) demonstrated that ubiquitin–topoisomerase IIα conjugates in MA1 epidermoid carcinoma cells and that topoisomerase IIα degradation during adenovirus E1A–induced apoptosis is meditated by the ubiquitin proteolysis system. Furthermore, Kim *et al*. (18) showed that stressful conditions stimulate proteasome accumulation in the nucleus, and such accumulation might contribute to the efficient degradation of topoisomerase IIα in stressed cells. Therefore, topoisomerase IIα is probably polyubiquitinated on exposure to oxidative stress, thereby affecting the expression of topoisomerase IIα in MOLT-4 cells. In addition, posttranslational modifications, such as phosphorylation and sumoylation, can alter protein stability or localization (8, 27). Such ubiquitination of topoisomerase IIα by oxidative stress might thus represent an adaptive change, thereby permitting cells to survive.

In contrast to oxidative stress, DNA damage prevents expression of topoisomerase IIα at a transcriptional level (see Fig. 2B). However, to the best of our knowledge, the precise mechanism for downregulation of topoisomerase IIα mRNA in the cellular response to etoposide remains obscure. The available evidence is that increased Sp-3 transcription factor acts directly to downregulate topoisomerase IIα promoter activity (22). This finding indicates that regulation of the level of transcription can be a critical factor in drug-induced downregulation of topoisomerase IIα mRNA. Another study demonstrated that expression of topoisomerase IIα mRNA is reduced by wild-type p53, which directly modulates topoisomerase IIα promoter activity (43). In this context, the possible explanation is that p53 is activated by etoposide, thereby inhibiting topoisomerase IIα promoter activity. Obviously, further studies are needed to clarify this issue.

We focused on BRCA1 as a regulator of the ubiquitination of topoisomerase IIα. This tumor-suppressor gene is associated with transcriptional regulation, cell-cycle checkpoint control, and DNA damage repair (12, 54). Moreover, BRCA1 has a

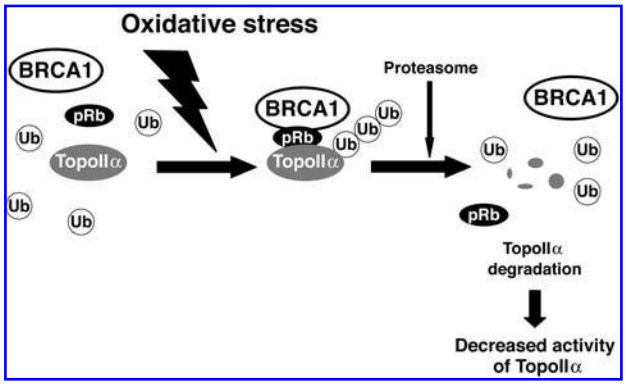

FIG. 7. BRCA1-mediated ubiquitination leads to topoisomerase IIα degradation and alteration of topoisomerase IIα activity in response to oxidative stress. pRb could function as a scaffold protein to BRCA1-induced ubiquitination of topoisomerase IIα in response to oxidative stress.

RING finger domain, and such domains have been documented to have E3 ubiquitin ligase activity (12, 26, 54). Gowen *et al.* (14) reported that BRCA1 participates, either directly or indirectly, in transcription-coupled repair of damage caused by oxidative stress. In this context, BRCA1 might modulate the ubiquitination of topoisomerase IIα in MOLT-4 cells exposed to oxidative stress. BRCA1 might also play a part in DNA damage repair by such modulation of topoisomerase IIα. Indeed, BRCA1 regulates key effectors that control G_2/M checkpoints and is therefore involved in regulating the onset of mitosis (48). In this regard, BRCA1 may specifically affect G_2/M checkpoints on exposure to oxidative stress and thereby regulate the ubiquitination of topoisomerase IIα.

The pRb tumor-suppressor protein is required for efficient activation of cell-cycle checkpoints in response to a variety of DNA lesions (13, 16). pRb can also affect the DNA damage response by regulating the expression of genes involved in DNA repair (6). In general, pRb is targeted to particular genes by interaction with sequence-specific DNA-binding transcription factors like the E2F family (4). In addition, topoisomerase IIα associates physically with pRb in interactions that appear to have functional significance (4). Moreover, BRCA1 forms complexes with the hypophosphorylated form of pRb (1). In this context, the present study revealed that pRb interacts with both topoisomerase IIα and BRCA1 in MOLT-4 cells after exposure to oxidative stress. Similarly, interaction among topoisomerase IIα, BRCA1, and pRb were detected when pRb or BRCA1 was respectively immunoprecipitated from cells (see Fig. 5), indicating that pRb couples BRCA1 with topoisomerase IIα exposed to oxidative stress. Kleiman *et al.* (19) demonstrated that E3 components containing BRCA1/BARD1 function for degradation of RNA polymerase II after DNA damage. In this regard, it is plausible that BRCA1 could ubiquitinate topoisomerase IIα for its degradation, possibly depending on complex formation including BRCA1, topoisomerase IIα, and pRb. We further demonstrated that pRb is required for degradation and inactivation of topoisomerase IIα on exposure to oxidative stress, possibly by regulating its ubiquitination.

The results demonstrated that, in addition to MOLT-4 cells, the catalytic activity of topoisomerase IIα is downregulated in HeLa, U2OS, or HCT116 cells in response to H_2O_2 (data not shown). By contrast, topoisomerase IIα activity remained unchanged in HL-60 or Cos-7 cells (Fig. 1B, and data not shown), Molecular mechanisms for cell type–specific regulation of topoisomerase IIα activity are currently under investigation. Nevertheless, we found that topoisomerase IIα activity remained constant in p53-deprived HCT116 (HCT116/p53$^{-/-}$) cells, suggesting the possibility that the p53 tumor suppressor might be involved in determination of topoisomerase IIα activity. p53 plays a central role in the cellular response to DNA damage. On exposure to genotoxic stress, p53 induces expression of numerous genes that lead to cell-cycle arrest, DNA repair, or apoptosis (42). Importantly, BRCA1 expression levels are controlled by p53, and the cellular response to stress involves an intracellular p53/BRCA1 pathway (2).

In conclusion, our results suggest that the activity of topoisomerase IIα is downregulated by BRCA1-induced ubiquitination in response to oxidative stress. A likely pathway involved is shown in Fig. 7. Oxidative stress–induced topoisomerase IIα alterations are most likely crucial to cell survival. Further studies are necessary to determine whether topoisomerase IIα alterations have an important role in cellular processes on exposure to oxidative stress. Understanding the effects of oxidative stress on topoisomerase IIα might help to elucidate the pathogenesis of many diseases associated with oxidative stress.

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ABBREVIATIONS

H_2O_2, hydrogen peroxide; kDNA, kinetoplast DNA; nPG, *n*-propyl gallate; pRb, retinoblastoma protein; ROS, reactive oxygen species; RT-PCR, reverse transcriptase PCR; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-alpha; TopoIIα, topoisomerase IIα.

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