Poly(ADP-ribose) Polymerase-1 Is a Positive Regulator of the p53-mediated G₁ Arrest Response following Ionizing Radiation

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Poly(ADP-ribose) polymerase-1 (PARP-1) and the p53 tumor suppressor protein are both involved in the cellular response to genotoxic stress. Upon binding to the site of DNA strand breakage, PARP-1 is activated, leading to rapid and transient poly(ADP-ribose)ylation of nuclear proteins using NAD⁺ as substrate. To investigate the role of PARP-1 in the p53 response to ionizing radiation in human cells, PARP-1 function was disrupted in wild-type p53 expressing MCF-7 and BJ/TERT cells using two strategies: chemical inhibition with 1,5-dihydroxyisoquinoline, and trans-domain inhibition by overexpression of the PARP-1 DNA-binding domain. Although a number of proteins can catalyze poly(ADP-ribosyl)ation in addition to PARP-1, we show that PARP-1 is the only detectable active species in BJ/TERT and MCF-7 cells. 1,5-Dihydroxyisoquinoline treatment prior to ionization radiation delayed and attenuated the induction of two p53-responsive genes, p21 and mdm-2, and led to suppression of the p53-mediated G₁-arrest response in MCF-7 and BJ/TERT cells. Trans-domain inhibition of PARP-1 by overexpression of the PARP-1 DNA-binding domain in MCF-7 cells also led to a delay and attenuation in p21 induction and suppression of the p53-mediated G₁ arrest response to ionizing radiation. Hence, inhibition of endogenous PARP-1 function suppresses the transactivation function of p53 in response to ionizing radiation. This study establishes PARP-1 as a critical regulator of the p53 response to DNA damage.

The p53 tumor suppressor protein plays a critical role in the cellular response to DNA damage leading to cell cycle arrest or apoptosis depending on cell type, culture conditions, and the extent of DNA damage. Loss of the p53-dependent DNA damage response can lead to genomic instability and the survival of cells carrying mutations and carcinogenic lesions thereby contributing to malignancy (1). DNA strand breaks produced by ionizing radiation (IR)³ or by DNA repair intermediates following treatment with UV radiation or chemotherapeutic agents result in the accumulation of p53 protein and in the activation of its transcriptional activity (reviewed in Refs. 2 and 3). Elevated levels of p53 protein are believed to be important to initiate the events that lead to G₁ arrest or apoptosis after DNA damage. There is compelling evidence that post-translational modification of p53 is required for its stabilization, as well as for activation of its latent sequence-specific DNA-binding and transactivation functions. Once p53 becomes activated it binds as a tetramer to p53 responsive elements on double stranded DNA consisting of two half-sites (5'-PuPuPuCTA/TGPyPyPyPy-3') separated by a spacer consisting of 0–13 nucleotides (4). The site-specific DNA-binding activity of p53 leads to transcriptional activation of p53 target genes. Covalent modification of p53 has also been shown to regulate its subcellular localization, tetramerization, interaction with other proteins, and degradation. p53 protein is modified in vivo through phosphorylation, acetylation, poly(ADP-ribose)ylation, ubiquitination, and sumoylation reactions (reviewed in Refs. 2 and 5).

The events upstream of p53 activation are complex and not well understood. Several DNA damage sensory molecules are believed to relay the DNA damage signal to p53; each may be involved in the response to one or more types of DNA damage. For example, ataxia-telangiectasia-mutated kinase protein is involved in the activation of p53 in response to IR (6, 7), and ataxia telangiectasia-related kinase protein is involved in the activation of p53 in response to UV irradiation (8).

Poly(ADP-ribose) polymerase (PARP-1) is an abundant nuclear enzyme that binds to, and is activated by, DNA single and double strand breaks (reviewed in Refs. 9–11). Its activation represents one of the earliest responses to DNA damage in the cell. PARP-1 catalyzes the sequential transfer of ADP-ribose monomers onto nuclear protein acceptors using NAD⁺ as substrate. During the process of poly(ADP-ribose)ylation, NAD⁺ is hydrolyzed and released as free nicotinamide. More than 30 nuclear proteins have been identified as poly(ADP-ribose) (pADPr) acceptors, with PARP-1 itself being the major target, via its automodification domain. pADPr acceptor proteins may be modified through covalent as well as through non-covalent association with pADPr, either free or bound to PARP-1. Poly-(ADP-ribose) glycohydrolase is the major enzyme responsible for the hydrolysis of pADPr. There is general agreement, based on genetic and biochemical studies, that PARP-1 plays a critical role in the maintenance of genomic integrity.

PARP-1 and p53 have been shown to interact in a number of

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1 The abbreviations used are: IR, ionizing radiation; PARP, poly(ADP-ribose) polymerase; pADPr, poly(ADP-ribose); IQR, 1,5-dihydroxyisoquinoline; DBD, DNA-binding domain; PBS, phosphate-buffered saline; MeSO₂, dimethyl sulfoxide; GFP, green fluorescent protein; PI, propidium iodide; Gy, gray; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Cells were irradiated in a 37°C Gammacell Exactor (Nordion International, Inc.) at a dose rate of ~1 Gy/min at room temperature. Initially, a dose of 6 Gy was employed for the MCF-7 Western blot analysis; however, we decided to use a dose of 2 Gy for future experiments with MCF-7 and BJ/TERT cells. Both these doses are sufficient to activate the p53-mediated DNA damage response; however, the extent of cell death is less following 2 Gy than 6 Gy in these cell lines (9, 34), thus allowing for a larger surviving fraction for subsequent analysis.

Transfection experiments were carried out by electroporation. Cells were washed in PBS, trypsinized, collected, and washed in α-minimal essential medium (without antibiotics or fetal bovine serum), and re-suspended in the same media in combination with the appropriate gene plasmids. Cells were electroporated using a Bio-Rad Gene Pulser, 250 V, 950 microfarads. For the MCF-7 Western blot analysis, MCF-7 cells were transiently transfected with 10 μg of pPARP6 plasmid that encodes the PARP-1 DBD (35) or the control plasmid pcDNA3 (Invitrogen) per 5 × 10⁶ cells. To determine the transfection efficiency, cells were transfected with 1 μg of EGFFP-NL, a green fluorescent protein (GFP) reporter plasmid. For the MCF-7 cell cycle analysis, 5 × 10⁶ cells were transiently co-transfected with 2 μg of pCMVDC20, a plasmid encoding the B-cell marker CD20, and 10 μg of pPARP6 or the control plasmid pcDNA3.

Preparation of Nuclear and Cytoplasmic Extracts—Cells were grown to 75% confluence and washed twice with PBS. Lysis Buffer A (300 mM sucrose, 0.1 M Tris-HCl, pH 8.0, 1 m M MgCl₂, 20 mM 2-mercaptoethanol, 0.03% Nonidet P-40 supplemented with Mini-Pill protease inhibitors (Roche Diagnostics)) was added and the cells were allowed to swell 7 min. Cells were centrifuged at 800 × g and the cytoplasmic fraction decanted. The nuclear pellet was washed twice with lysis Buffer A. The nuclear and cytoplasmic fractions were diluted in 1 × loading buffer (62.5 mM Tris–Cl, pH 6.8, 10% glycerol, 2% SDS, 6 M urea, 5% β-mercaptoethanol (added fresh) and 1% (w/v) bromphenol blue), sonicated (3 pulses, 15 s each), and heated at 65°C for 15 min. Activity-Western blot analysis—Protein extracts were resolved by gel electrophoresis on a 10% SDS-polyacrylamide gel. Gels were soaked at 37°C in 1× SDS-PAGE running buffer (125 mM Tris base, 960 mM glycine, 0.1% SDS) containing 0.1% SDS) containing 0.1% SDS (Sigma). The proteins were transferred onto a Hybond C nitrocellulose membrane (APBiotech). After re-folding of the membrane-bound proteins in renaturation buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol, 0.3% Tween), the membranes were incubated in the same buffer supplemented with 2 μg/ml DNase I-activated calf thymus DNA (Sigma), 2 mM MgCl₂, 20 μM ZnCl₂, and 2 μCi/ml [α-32P]-labeled dNTP. To remove non-covalently bound pADPr, the membranes were washed in renaturation buffer until no radioactivity was detected in the washes. Covalently bound pADPr was detected by autoradiography.

Western blot analysis—Cells were washed with PBS, lysed with either SDS loading buffer or STG buffer (5% SDS (w/v), 6% urea, 10% glycerol, 125 mM Tris–HCl, 125 mM Tris–HCl, pH 6.8, with protease inhibitors), collected, and sonicated for 20 s. For the BJ/TERT and BJ/TERT-DD protein analyses, DNA was removed by centrifugation in a Qiagen rebinder column (Qiagen). Protein content of the cell extracts was measured using a modified Lowry assay (Sigma). Following addition of diethiothreitol (100 mM) and bromphenol blue (1% w/v) the protein samples were denatured at 70°C for 5 min, resolved by gel electrophoresis on an SDS-polyacrylamide gel, and transferred by electroblotting onto polyvinylidene difluoride membranes. Primary antibodies against p53 (PAb1801, 1:10 for MCF-7; PAb421, 1:100 for BJ/TERT and BJ/TERT-DD), p21 (1:1000; BD Pharmingen), mdm-2 (2A10, 1:1000), β-actin (1:1000; Sigma), and PARP-1 (C2–9, 1:10,000) were used in conjunction with horseradish peroxidase-conjugated anti-mouse (1:2000; Sigma) or anti-sheep secondary antibodies (1:5000; Cedarlane) and the blot visualized using the Renaissance ECL detection system (PerkinElmer Life Sciences). For the BJ/TERT and BJ/TERT-DD blots, Coomassie Blue stain was used to determine equal loading of protein.

Cell mRNA Analysis—Total RNA was isolated from BJ/TERT and MCF-7 cells using a modified guanidinium isothiocyanate method (Trizol, Invitrogen) according to the manufacturer’s instructions. RNA content was measured by A260. Twelve μg of each RNA sample was separated on a 1% denaturing agarose gel and transferred to a Hybond N+ membrane (APBiotech). The blot was hybridized with 32P-radiolabeled cDNA probes to p21 and GADPH. Following standard washes, phosphorimage was acquired using a BAS-Radiodine System (Fuji). To quantify the data, the blots were analyzed using ImageQuaNT software (Amersham Biosciences).

Cell Cycle Analysis—To assess the G2 arrest response in MCF-7 and BJ/TERT cells, asynchronously dividing cells were plated on 150-mm tissue culture dishes (MCF-7 at 1 × 10⁶ cells, BJ/TERT at 5 × 10⁶ cells).

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The BJ/TERT, BJ/TERT-DD, MCF-7, and MCF-7-p53A135V cells were grown in α-minimal essential medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO₂ atmosphere.

To generate BJ/TERT-DD cells, a 700-bp fragment encoding the C-terminal dimerization domain of p53 was cloned into the EcoRI site of pcDNA3. The resulting plasmid p53Δbeta33ΔNneo was cotransfected with pC-AmpNeo into 293T cells and the resulting viral supernatants were used to infect BJ/TERT cells. The resulting BJ/TERT-DD cells were subsequently analyzed and shown to lack a p53-dependent G1 checkpoint in response to ionizing radiation. This data supports the hypothesis that PARP-1 is a critical regulator of p53 function in response to DNA damage.

For inhibition of PARP activity IQ (Sigma-RBI) was added to the cells 1 h prior to IR. The optimal IQ doses determined by immunofluorescence microscopy were 200 and 350 μM for MCF-7 and BJ/TERT cells, respectively (data not shown). Twenty minutes post-IR, cells were washed with phosphate-buffered saline (PBS) without calcium and magnesium and incubated with fresh media containing IQ. IQ was dissolved in dimethyl sulfoxide (Me₂SO) such that the final concentration of Me₂SO to which cells were exposed did not exceed 0.5% (v/v).

The ability of PARP-1 to interact with pADP-riboseylate p53 suggests a functional relationship between these two proteins. An intriguing model is that PARP-1 acts as a DNA damage sensor that regulates p53 function in response to DNA damage. A number of studies have investigated the role of PARP-1 in the p53 response to DNA damage. Several studies indicate that disruption of PARP function by chemical inhibition leads to suppression of p53-dependent transactivation in response to IR (12, 20, 21). Chemical inhibition of PARP leads to loss of the G1 checkpoint and accumulation of cells in G2/M following N-methyl-N-nitro-N-nitrosoguanidine treatment (22) or IR (20). Other studies employing dominant negative PARP-1 mutants (23), and PARP-1 knockout mice (24–27) have yielded confusing and apparently conflicting data. Studies using certain PARP-1-deficient mouse cells are confounded by the fact that these cells retain poly(ADP-ribose)lation activity (28, 29). Thus, although PARP-1 plays a clear role in the maintenance of genomic integrity, the functional relationship between PARP-1 and p53 remains unclear.

Here we have disrupted PARP-1 function in human cells using two experimental strategies: chemical inhibition with the inhibitor 1,5-dihydroxyisouquinoline (IQ), and trans-dominant inhibition by overexpression of the catalytically inactive DNA-binding domain (DBD) of PARP-1. We have investigated the consequences of PARP-1 disruption on p53-dependent DNA damage response pathways in MCF-7 human breast adenocarcinoma cells and in BJ/TERT cells derived from normal human BJ fibroblasts that have been immortalized by ectopic expression of the telomerase enzyme (30). These experiments reveal that inhibition of PARP-1 activity with IQ or with PARP-1 DBD overexpression suppresses the transactivation function of p53 and the ability of p53 to mediate G1 arrest in response to ionizing radiation. This data supports the hypothesis that PARP-1 is a critical regulator of p53 function in response to DNA damage.

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Following overnight incubation at 37 °C, cells were treated with IQ (MCF-7 at 200 μM, BJ/TERT at 350 μM) and 1 h later irradiated at 2 Gy. Cell cycle distribution was examined by flow cytometry using the propidium iodide (PI) DNA staining method.

BJ/TERT and BJ/TERT-DD cells were washed once with PBS and incubated on ice in 0.1% sodium citrate, 0.2% Nonidet P-40, and 50 μg/ml PI to release nuclei. Cells were collected and nuclei were analyzed for DNA content by flow cytometry. For the time course analysis, BJ/TERT cells were washed once with PBS and incubated with PBS containing 0.5% EDTA for 10 min. Cells were scraped into a minimal medium and collected by centrifugation. Following one wash in cold PBS, cells were fixed with 75% ethanol overnight at 4 °C. Cells were washed in PBS containing 0.5% bovine serum albumin, and resuspended in the same buffer containing 50 μg/ml PI (form 5 mg/ml stock in 38 mM sodium citrate, pH 7.4), and 200 μg/ml RNase A. After incubation for 30 min at 37 °C, cells were analyzed for DNA content by flow cytometry. MCF-7 and MCF-7/p53A135V cells were prepared for flow cytometry as described for the BJ/TERT time course.

For analysis of transient overexpression of the PARP-1 DBD, MCF-7 cells were transfected as described previously. Transfected cells were plated on 150-mm tissue culture dishes and incubated at 37 °C for 24 h. The mock-transfected cells were treated with IQ (MCF-7 at 200 μM, BJ/TERT at 350 μM) and 1 h later irradiated at 2 Gy. Cell cycle distribution was determined with a FACScalibur flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences). The relative proportion of cells in each phase of the cell cycle was determined using ModFit LT 2.0 software (Verity Software House Inc.).

RESULTS

Human MCF-7 and BJ/TERT Cells Express a Single Species with NAD- and DNA-dependent PARP Activity Corresponding to PARP-1—The recent demonstration that mouse cells express at least two proteins with DNA-dependent PARP activity prompted us to examine PARP activity in human MCF-7 and BJ/TERT cells using an in situ based activity assay. Activity Western blots were performed with nuclear and cytoplasmic extracts equivalent to 100,000 BJ/TERT and MCF-7 cells. Purified bovine PARP-1 was included as a positive control. It has been previously shown that normal mouse embryo fibroblasts have two active poly(ADP-ribose)ation species; a major active band corresponding to the 113-kDa PARP-1 and a second active band with an approximate molecular mass of 60 kDa corresponding to sPARP-1 (29). In contrast, the human cell lines BJ/TERT and MCF-7 express only one protein species capable of NAD+-dependent self-poly(ADP-ribose)ation in the presence of DNA strand breaks, provided by the DNase I-activated calf thymus DNA (Fig. 1A). This protein is present predominantly in the nuclear fraction and corresponds to PARP-1. NAD+-dependent self-poly(ADP-ribose)ation of PARP-1 is also observed in the activity blot performed without DNase I-activated calf thymus DNA (Fig. 1B), but to a significantly lesser extent, confirming that PARP-1 poly(ADP-ribose)ation activity is activated by DNA strand breaks.

Disruption of PARP by Chemical Inhibition Suppresses Transcription of p21 mRNA following Ionizing Radiation—The p53-dependent elevation of p21 protein levels in response to IR is controlled at the transcriptional level. We wished to investigate whether PARP inhibition with IQ interfered with the transactivation function of p53. BJ/TERT and MCF-7 cells were treated with 350 or 200 μM IQ and γ-irradiated with 2 or 6 Gy, respectively. Total RNA was prepared at the indicated times and Northern blot analysis was performed using radiolabelled p21 and GAPDH cDNA probes (Fig. 3, top panel). Following normalization to GAPDH, RNA levels were quantitated and plotted (Fig. 3, bottom panel). In non-treated MCF-7 cells, p21 mRNA levels were induced by 2 h following IR, and remained elevated at subsequent time points. In MCF-7 cells treated with IQ, the induction of p21 mRNA following IR was delayed by 1.5 to 2 h compared to non-treated cells, an effect similar to that observed at the protein level (Fig. 2A). BJ/TERT cells showed a more dramatic effect: prior treatment with IQ led to almost complete suppression of p21 mRNA induction at all time points. These results indicate that IQ suppresses induction of p21 expression in response to IR by interfering with the transactivation function of p53.

Fig. 1. PARP-1 is the only detectable species capable of self-poly(ADP-ribose)ation in BJ/TERT and MCF-7 cells. Cytoplasmic and nuclear extracts equivalent to 100,000 BJ/TERT and MCF-7 cells were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Following incubation in renaturation buffer to refold proteins, membranes were incubated in buffer containing 32P-labeled NAD+, with (A) or without (B) DNase I-activated calf thymus DNA for 1 h. The membranes were subsequently washed in renaturation buffer to remove noncovalently bound pADPr and exposed to autoradiography film to detect poly(ADP-ribose)ated proteins. Purified recombinant PARP-1 (100 milliunits) was included as a positive control.
Disruption of PARP by Chemical Inhibition Suppresses the p53-medi­ated G1 Arrest Response following Ionizing Radiation—p21 is a critical effector of the p53-medi­ated G1 arrest response following DNA damage. Both MCF-7 cells and BJ/TERT cells undergo G1 arrest in response to low doses of ionizing radiation (33, 34). Our result showing that PARP inhibition suppresses p53-medi­ated transactivation of p21 indicates that PARP inhibition should lead to disruption of the p53-dependent G1 checkpoint. To test this possibility, cell cycle analysis using PI to assess DNA content was performed.

BJ/TERT and BJ/TERT-DD cells were treated with 350 μM IQ, γ-irradiated at 2 Gy, and 24 h later cell cycle analyses were performed by flow cytometry. The proportion of cells in each phase of the cell cycle was determined in three (BJ/TERT-DD) or six (BJ/TERT) independent experiments and the mean values summarized (Fig. 4A, top panel). Also shown are the G1/S ratios (Fig. 4A, bottom panel). In addition to G1 arrest, ionizing radiation can lead to arrest in G2, and this block can result in a decrease in the proportion of cells in the G1 and S phases of the cell cycle. For this reason, it is important to compare the G1/S ratios; an increase in the G1/S ratio following DNA damage has been used as an indicator of G1 arrest. BJ/TERT cells showed a significant increase (6.7-fold) in the G1/S ratio following IR, indicating that these cells were undergoing G1 arrest. In contrast, BJ/TERT-DD cells showed no increase in the G1/S ratio after IR confirming that G1 arrest is dependent on p53. Importantly, BJ/TERT cells treated with IQ showed a marked decrease in p53-mediated G1 arrest. We confirmed this observation by performing a time course experiment. BJ/TERT cells were treated with 350 μM IQ or Me2SO and γ-irradiated at 2 Gy. Flow cytometric analysis of cells was performed to determine the cell cycle profiles at various time points for both non-treated cells (Fig. 5A) and cells treated with IQ (Fig. 5B). The percentage of cells in each cell cycle phase and G1/S ratios were determined. The mean ± S.E. are shown for three independent experiments (Table I). BJ/TERT cells showed a strong G1 arrest response by 13 h post-IR and cells remained in a G1-arrested state at later time points. In contrast, IQ treatment of BJ/TERT cells delayed and attenuated this G1 arrest response.

We also investigated the effect of IQ on the p53-mediated G1 checkpoint in MCF-7 cells and in MCF-7 cells expressing a dominant negative p53 allele (p53A135V). Cells were treated with 200 μM IQ, γ-irradiated at 2 Gy, and 16 h later the cell cycle profiles were analyzed by flow cytometry (Fig. 4B, top panel). The 16-h time point was chosen based on our previous observation that G1 arrest can be observed in asynchronous MCF-7 cell cultures 12 to 20 h following IR at 2 Gy (data not shown). Also shown are the G1/S ratios (Fig. 4B, bottom panel). MCF-7 cells showed a 5.2-fold increase in the G1/S ratio after
IR indicative of G1 arrest. Arrest in G1 was strongly suppressed in H9253-irradiated cells cultured in the presence of IQ. MCF-7/p53A135V cells showed only a 1.3-fold increase in the G1/S ratio after IR indicating that G1 arrest in these cells is primarily determined by p53. Similar effects were observed with a H9253-radiation dose of 6 Gy (data not shown). Together these data indicate that inhibition of PARP activity with IQ suppresses the transactivation function of p53 and the ability of p53 to mediate G1 arrest in response to IR.

An important consideration when using chemical inhibitors is the potential lack of specificity. IQ may target other NAD-dependent enzymes in addition to PARP-1. To address this important point, we wished to measure p53 activity in human cells where PARP-1 activity could be inhibited through non-chemical means. The expression vector pPARP6 encodes the DNA-binding domain of PARP-1, and this truncated molecule has been shown to function as a trans-dominant repressor of full-length PARP-1 through its ability to compete for binding to DNA strand breaks (35). Initially we established 3 stable BJ/TERT clones expressing PARP-1 DBD. In all of these clones,
poly(ADP-ribosyl)ation of nuclear proteins was observed even though PARP-1 DBD was abundantly expressed (data not shown). Retention of PARP activity is not the result of compensatory PARP enzymes such as sPARP-1, observed in PARP-1 −/− mouse embryo fibroblasts (29), because PARP-1 is the only NAD-dependent poly(ADP-ribosyl)ating enzyme detected in BJ/TERT cells. Rather, it may be the result of insufficient DBD expression in the clones. It is also possible that PARP-1 activity is required for long-term survival of the BJ/TERT cells. As a result, we employed transient expression assays. The plasmid pPARP-6 has been used effectively in transient expression analyses to inhibit PARP function (35, 36).

The transfection efficiency of BJ/TERT is extremely low and we were unable to achieve sufficient transient expression of PARP-1 DBD in these cells. We turned to MCF-7 cells that have higher transfection efficiency. Cells were transfected at an efficiency of ~31%. Forty-eight hours post-transfection with pPARP6, cells were γ-irradiated at 6 Gy, as with the MCF-7 Western blot time course previously described, and protein extracts were prepared from the entire cell population (transfected and non-transfected) at the indicated times and analyzed by Western blotting (Fig. 6). The PARP-1 DBD was expressed only in those cells transfected with pPARRP6, and not in cells transfected with the control vector pcDNA3. Notably, p21 basal levels were increased, and the induction of p21 protein following IR was delayed and attenuated in cells expressing the PARP-1 DBD.

Disruption of PARP-1 by Trans-dominant Inhibition Suppresses the p53-mediated G1 Arrest Response to Ionizing Radiation—We wished to determine whether PARP-1 DBD over-expression abrogates the p53-dependent G1 checkpoint in MCF-7 cells. pPARP6 or empty vector (pcDNA3) were co-transfected with the pCMVCD20 expression vector encoding the B cell surface marker CD20. Cells were irradiated at 2 Gy 38 h after transfection. Sixteen hours after IR, transfected cells were identified by CD20 staining, and the cell cycle profiles of the CD20-positive and CD20-negative cell populations were determined by flow cytometry (Fig. 7, A and B, top panel). The latter represent cells that were not successfully transfected and hence, serve as additional controls in these experiments. The CD20-negative populations from both the pcDNA3 co-transfection and the pPARP6 co-transfection exhibited G1 arrest following IR reflected by the increases in the G1/S ratio (9.5-fold, and 8-fold, respectively) (Fig. 7A, bottom panel). The CD20+ cells transfected with pcDNA3 showed a 7-fold increase in the G1/S ratio following IR (Fig. 7B, bottom panel). This is comparable with the CD20-negative cells and is indicative of arrest in G1. In contrast, CD20-positive cells co-transfected with the dominant-negative pPARP6, exhibited only a 2.2-fold increase in the G1/S ratio indicating a significant suppression of the G1 checkpoint.

DISCUSSION

Following DNA damage induced by ionizing radiation and alkylating agents, the nuclear enzyme PARP-1 is activated leading to rapid and transient poly(ADP-ribosyl)ation of nuclear proteins (37). Studies of PARP-1 knockout mice indicate a critical role for PARP-1 in the maintenance of genomic integrity (reviewed in Refs. 10 and 11); however, the molecular

![Fig. 6. Transient expression of the DBD of PARP-1 in MCF-7 cells.](image)

![Fig. 7. Trans-dominant inhibition of PARP-1 suppresses the p53-mediated G1 arrest response to ionizing radiation in MCF-7 cells.](image)
mechanisms through which PARP-1 functions in the cellular response to genotoxic stress and in DNA repair are not fully understood. Several studies suggest an indirect role for PARP-1 in base excision repair. In response to DNA damage, PARP-1 is recruited to strand breaks where it may act as a “nick protector” at sites of DNA damage to facilitate repair (38, 39). PARP-1-interacting proteins may also be recruited to DNA strand breaks to participate in the repair process. Poly(ADP-ribosyl)ation of histones may serve to open up chromatin following DNA damage, to allow access to DNA repair enzymes (40).

The p53 tumor suppressor also plays a critical role in the cellular response to DNA damage. In cells that have been exposed to genotoxic stresses such as ionizing radiation, UV irradiation, or alkylating agents, p53 activates transcription of target genes that are involved in either cell cycle arrest or apoptosis. These responses ensure that permanent mutations are not created, and that genetic integrity is maintained (1, 41).

We have previously shown that p53 protein can bind to PARP-1 in the human leukemia-derived OCI/AML-3 cell line. In the same study, we also demonstrated that analogs of NAD⁺ that selectively inhibit endogenous poly(ADP-ribosyl)ation are able to overcome p53-dependent senescence in early passage human fibroblasts (12). Other studies have shown that p53 can be poly(ADP-ribosyl)ated in vivo (17) and in vitro (15, 16, 18).

In the present work, we have investigated the functional relationship between PARP-1 and p53. Using two experimental strategies to inhibit PARP-1 function, we have observed an attenuation of the activity of the p53 protein as a transcription factor at the p21 promoter in two human cell types. These cell types exhibit a p53-dependent arrest in the G₁ phase of the cell cycle following ionizing radiation. We observed that inhibition of PARP-1 activity caused a marked disruption of p53-mediated G₁ arrest following ionizing radiation. These results demonstrate a critical role for PARP-1 in the p53-dependent G₁ checkpoint. Although inhibition of PARP-1 by IQ treatment led to comparable effects on p53 transactivation function in BJ/TERT and MCF-7 cells, it had different effects on p53 induction and accumulation. The induction of p53 protein in irradiated BJ/TERT cells was unaffected by IQ. In contrast, MCF-7 cells treated with IQ did not show p53 induction beyond the basal level following irradiation.

Recent studies indicate the existence of a family of PARP enzymes that catalyze NAD-dependent poly(ADP-ribosyl)ation. These include tankyrase-1 (42), tankyrase 2 (43), and VAPAR (44), enzymes that are not activated by DNA strand breaks. Another family member, PARP-2, has been shown to bind to and be activated by DNA strand breaks, although this protein lacks the classical zinc finger DNA-binding domain of PARP-1 (45). Finally, sPARP-1, although lacking a DNA-binding domain, can be activated by DNA damage (29). In this study, we have only been able to detect one NAD-dependent ADP-ribosyltransferase species capable of self-poly(ADP-ribosyl)ation in BJ/TERT and MCF-7 cells, namely PARP-1. This finding lessens the possibility that another PARP family member is acting upstream of p53 in response to DNA damage.

Transdominant inhibition of PARP-1 led to a G₂ delay in both non-irradiated and γ-irradiated MCF-7 cells, suggesting that PARP-1 may be involved in G₂/M progression. A similar effect was observed in MCF-7 and BJ/TERT cells treated with IQ, in agreement with previous studies using the chemical inhibitor 3-aminobenzamide (20, 46); however, the G₂ delay was more pronounced in these cells as compared with those overexpressing the PARP-1 DBD. These results suggest that chemical inhibition by IQ may lead to PARP-1-independent effects on the G₂/M transition.

The effects of IQ treatment on the p53 response to IR observed in this study are consistent with previous inhibitor studies (12, 20, 21, 46); however, the use of NAD analogs to inhibit PARP activity can be criticized because of the possibility of nonspecific effects on other NAD-dependent processes, for example, those that require mono(ADP-ribosyl) transferases (47). In this study, trans-dominant inhibition of PARP-1 by PARP-1 DBD overexpression led to disruption of p21 induction and suppression of p53-mediated G₁ arrest in MCF-7 cells. These results confirm that chemical inhibition of PARP by IQ disrupts the p53-mediated G₁ checkpoint in MCF-7 cells by interference with PARP-1 activity.

Recent studies indicate an involvement of PARP-1 in transcriptional regulation. Various reports indicate that PARP-1 can either promote or repress transcription. Interestingly, this activity of PARP-1 requires the DNA binding but not the catalytic function of PARP-1 (reviewed in Ref. 48). Hence, PARP-1 DBD expression may affect gene expression in addition to inhibiting the enzymatic activity of PARP-1. This caveat emphasizes the importance of using different but complementary strategies to inhibit PARP-1 function. During the course of these experiments, we observed an elevated basal level of p53 and p21 protein in IQ-treated BJ/TERT and MCF-7 cells, and in DBD-expressing MCF-7 cells. p21 mRNA levels are not elevated in IQ-treated cells suggesting that this change in basal expression is controlled post-transcriptionally. We are currently investigating this further. In the context of DNA damage a possible novel regulatory function of the DBD would presumably not affect the transcription of DNA damage response genes because DNA damage would lead to preferential binding of the DBD to DNA strand breaks, relieving any ability to modulate transcription.

The precise mechanism through which PARP-1 positively regulates p53 activity remains to be determined. One possibility is covalent poly(ADP-ribosyl)ation of p53. Poly(ADP-ribosyl)ation of p53 has been observed in vivo in osteosarcoma cells undergoing spontaneous apoptosis (17, 49). To date there has been no report of poly(ADP-ribosyl)ation of p53 following DNA damage. Another possibility is that p53 interacts non-covalently with pADPr, either free or bound to nuclear protein acceptors such as PARP-1. Three regions within the tetramerization and DNA-binding domains have been found to interact with pADPr in vitro (14); these regions contain putative pADPr-binding sites as determined by sequence alignment analysis (13). It remains to be determined whether these regions interact non-covalently with pADPr in vivo. Covalent or non-covalent modification might serve to modulate the site-specific DNA-binding activity of p53 or its transcriptional activity (18, 19). Finally, PARP-1 activity may regulate other post-translational modifications of p53 such as phosphorylation or acetylation, in response to DNA damage.

In summary, using two different strategies to disrupt PARP-1 function, we have established that PARP-1 is a positive regulator of p53 transactivation function in response to ionizing radiation in both the transformed cell line, MCF-7, and the non-transformed cell line, BJ/TERT. The precise mechanism through which PARP-1 positively regulates the p53-mediated DNA damage response remains to be determined.

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PARP-1 Is a Positive Regulator of p53

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