Mouse fibroblasts, deficient in DNA polymerase $\beta$, are hypersensitive to monofunctional DNA methylating agents such as methyl methanesulfonate (MMS). Both wild-type and, in particular, repair-deficient DNA polymerase $\beta$ null cells are highly sensitized to the cytotoxic effects of MMS by 4-amino-1,8-naphthalimide (4-AN), an inhibitor of poly(ADP-ribose) polymerase (PARP) activity. Experiments with synchronized cells suggest that exposure during S-phase of the cell cycle is required for the 4-AN effect. 4-AN elicits a similar extreme sensitization to the thymidylate analog, 5-hydroxymethyl-2′-deoxyuridine, implicating the requirement for an intermediate of DNA repair. In PARP-1-expressing fibroblasts treated with a combination of MMS and 4-AN, a complete inhibition of DNA synthesis is apparent after 4 h, and by 24 h, all cells are arrested in S-phase of the cell cycle. Continuous incubation with 4-AN is required to maintain the cell cycle arrest. Caffeine, an inhibitor of the upstream checkpoint kinases ATM (ataxia talangiectasia-mutated) and ATR (ATM and Rad3-related), has no effect on the early inhibition of DNA synthesis, but cells are no longer able to maintain the block after 8 h. Instead, the addition of caffeine leads to arrest of cells in G2/M rather than S-phase after 24 h. Analysis of signaling pathways in cell extracts reveals an activation of Chk1 after treatment with MMS and 4-AN, which can be suppressed by caffeine. Our results suggest that inhibition of PARP activity results in sensitization to MMS through maintenance of an ATR and Chk1-dependent S-phase checkpoint.

A particle of unnatural bases or single base lesions from DNA is predominantly by a glycosylase-initiated base excision repair (BER) pathway. Methylation bases are excised by N-methylpurine-DNA glycosylase, a monofunctional glycosylase. In the preferred “single-nucleotide” BER pathway, this is followed by strand cleavage on the 5′ side of the sugar by apurinic/apyrimidinic endonuclease, gap filling and cleavage on the 3′ side by the DNA synthesis and deoxyribosyl phosphate (dRP) lyase activities, respectively, of DNA polymerase $\beta$ (β-pol), and finally sealing of the nick by a DNA ligase (1). The essential role of β-pol in this pathway has been established using extracts from wild-type and β-pol null mouse fibroblasts (2). The requirement for β-pol, more specifically the dRP lyase activity of β-pol, in protection of cells against the cytotoxicity of methyl DNA lesions, has been demonstrated by the hypersensitivity of β-pol null cells to the monofunctional methylating agent methyl methanesulfonate (MMS) (2, 3). It is thought that the MMS hypersensitivity phenotype of β-pol null cells reflects accumulation of cytotoxic repair intermediates, such as the 5′ dRP group after removal of methylated bases from DNA (4).

Repair of oxidative DNA damage occurs by an alternate sub-pathway of single-nucleotide BER utilizing bifunctional glycosylases that have an associated lyase activity that nicks the DNA strand 3′ to the abasic site after base removal. Single-nucleotide BER is still preferred (5), but the 5′ dRP group is not an intermediate of repair, and there is no longer a requirement for the dRP lyase activity of β-pol. Less efficient repair of oxidized bases has been demonstrated in β-pol deficient cell extracts (6–8), and β-pol null cells are hypersensitive to oxidative damage (8, 9). Presumably it is the DNA synthesis activity of β-pol that is required under these circumstances.

Poly(ADP-ribose) polymerase (PARP)-1 is the first described member of a family of poly(ADP-ribose)lyating enzymes. It comprises an N-terminal DNA binding domain, an automodification domain, and a C-terminal catalytic domain and can detect and bind to nicks and strand breaks in cellular DNA, including those formed during BER. Binding to damaged DNA results in rapid activation of PARP-1 leading to poly(ADP-ribose)ylation of numerous nuclear proteins, including itself, using NAD$^+$ as substrate. As a consequence of self-poly(ADP-ribosylation), PARP-1 is released from its DNA binding site, allowing repair to continue (10, 11). As early as 1980 it was proposed that PARP-1 plays a role in BER (12), but the mechanism of its involvement still remains unclear. In an examination of the interaction of BER proteins with DNA repair intermediates and utilizing a photoaffinity labeling technique, it was revealed that the amount of DNA probe cross-linked to PARP-1 was much greater than that of any other BER protein (13).

Extracts from PARP-1/−/− cells are deficient in BER (14), and PARP-1/−/− mouse embryonic fibroblasts are moderately hypersensitive to methylating agents and hydrogen peroxide (H$_2$O$_2$) (15, 16). In addition, inhibition of PARP activity in mouse fibroblasts by 4-amino-1,8-naphthalimide (4-AN) or 3-aminobenzamide (3-AB) results in tremendous sensitization to MMS (8, 13). The crystal structure of the catalytic domain of PARP-1 with 4-AN shows that the inhibitor binds to the nicotineamide subsite of the NAD$^+$ binding pocket of the enzyme.
(17). Similarly, trans-dominant inhibition of PARP activity by overexpression of the PARP-1 DNA binding domain blocks repair synthesis induced by N-methyl-N'-nitro-N-nitrosoguanidine and results in a marked reduction in cell survival (18, 19). Inhibited PARP-1 protein can still detect and bind to strand breaks in DNA, but it cannot be auto-modified and may remain irreversibly bound to damaged DNA, perhaps entirely preventing repair.

In the present study we extend our initial observations of the enhancement of the growth inhibitory effect of MMS after a block of cellular PARP activity and demonstrate that this effect is S-phase-dependent. We describe perturbations in the cell cycle that occur after exposure of mouse fibroblasts to a sub-lethal concentration of MMS and a non-toxic PARP inhibitor. We then analyze cell signaling proteins in extracts from treated cells and propose that an ATR- and Chk1-dependent signaling pathway is involved in the cellular response to PARP inhibition after DNA damage by MMS.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures—**Clones of the originally characterized wild-type and β-pol null SV40-transformed mouse embryonic fibroblasts (MB163 and MB19.4, respectively) have been described previously (4). Cells were routinely grown at 34 °C in a 10% CO2 incubator in Dulbecco’s modified Eagle’s medium supplemented with Glutamax-I (Invitrogen), 10% fetal bovine serum (FBS; HyClone, Logan, UT), and hygromycin (80 μg/ml; Roche Applied Science). PARP-1+/+ and PARP-1−/− spontaneously immortalsed mouse embryonic fibroblasts were obtained from Dr. Josianne Ménilisier-de Murcia (CNRS, Illkirch-Graffenstaden, France). These cells were cultured at 37 °C in a 10% CO2 incubator in Dulbecco’s modified Eagle’s medium containing t-glutamine and 10% FBS. All cells were routinely tested and found to be free of mycoplasma contamination.

**Cytotoxicity Studies—**Cytotoxicity was determined by trypan blue exclusion (see Materials and Methods) for MMS-exposed cells described previously (4). Wild-type and β-pol null mouse fibroblasts or PARP-1+/+ and PARP-1−/− mouse fibroblasts were seeded at a density of 40,000 cells/well in six-well dishes. The following day they were treated with a range of concentrations of DNA-damaging agent in growth medium, without hygromycin in the case of the β-pol cells. MMS, methylisourourea (MNU), H2O2, bleomycin, and 5-hydroxyethyl-2-deoxyuridine (hmdUrd) were purchased from Sigma-Aldrich, and peroxynitrite was from Tocris (Bristol, NC). Temozolomide (TMZ) was obtained from the Drug Synthesis and Chemistry Branch, NCI, National Institutes of Health, Bethesda, MD. The MMS and H2O2 were added directly to the medium at the time of the experiment, stock solutions of MNU and TMZ were made in dimethyl sulfoxide (Me2SO), bleomycin and hmdUrd were dissolved in phosphate-buffered saline (PBS) before adding to medium, and peroxynitrite in 0.3 N NaOH was added to medium immediately before dosing the cells, and the pH returned to 7.4 by the addition of HCl. After 1 h of exposure to MMS, MNU, or H2O2 or 4 h for TMZ and 24 h for hmdUrd, cells were washed with Hanks’ balanced salt solution (HyClone), and fresh medium was added. For UV irradiation (254 nm), cell monolayers were washed twice with Hanks’ balanced salt solution before UV exposure (0–20 J/m2) in a Stratalinker model 1800 (Stratagene, La Jolla, CA), and then the growth medium was replaced after exposure. Dishes were incubated for 4–5 days in a 10% CO2 incubator until untreated control cells were >80% confluent. Cells (triplicate wells for each drug concentration) were counted by a cell lysis procedure (20), and results were expressed as the number of cells in drug-treated wells relative to control wells (% control growth). In many studies cells were treated with DNA-damaging agents in the presence of the PARP inhibitors 4-AN or 3-AB, and the incubation with inhibitor was continued after washing. In general, cells were treated with 4-AN (10 μM) or 3-AB (10 μM) for a total of 24 h, but for 4-AN, other concentrations and times of exposure were also utilized in the experiments described in Figs. 2, 3, and 8. Stock solutions of the inhibitors were prepared in Me2SO and were diluted to the appropriate concentration in medium just before use. In some studies cells were treated with caffeine (1 mM) as well as 4-AN during the 1-h MMS exposure and for the following 23 h. The IC50 for MMS is the concentration that results in 90% growth inhibition.

**Flow Cytometric Cell Cycle Analysis after Treatment with MMS with and without 4-AN—**Cell cycle and DNA synthesis, as assessed by staining with propidium iodide (PI) and incorporation of bromodeoxyuridine (BrdUrd), respectively, were analyzed simultaneously, as described previously (4). Cells were seeded in 100-mm dishes at a cell density equivalent to that used in the cytotoxicity experiments. The following day cells were treated for 1 h with MMS (0.25 mM for β-pol wild-type cells, 0.5 mM for PARP null cells) with and without 4-AN (10 μM). Incubation with 4-AN was continued for up to 24 h. In some studies cells were treated with 1 mM caffeine in addition to 4-AN during and after the 1-h MMS exposure. Before the times specified (2–24 h) after the beginning of exposure to MMS, 10 μM BrdUrd (Sigma) was added to the dishes for 30 min (β-pol wild-type cells) or 2 h (PARP cells) to pulse-label the cells. Cells were washed with PBS, harvested by trypsinization, and then washed a second time with PBS. The cell pellet obtained after centrifugation was resuspended in 100 μl of cold PBS, and the cells were dropped slowly into 70% ethanol and allowed to fix at 4 °C overnight. The samples were washed then suspended in 2 N HCl containing 0.5% Triton X-100 and incubated for 30 min at room temperature to denature DNA. The cell samples were pelleted, resuspended in 0.1 M sodium citrate/methanol solution, neutralized with 0.3 N NaOH, and then washed twice with 70% ethanol. Cells were then incubated at 4 °C overnight with 20 μl of anti-BrdUrd-fluorescein isothiocyanate (FITC)-conjugated antibody (BD Biosciences) in PBS containing 0.5% Tween 20 and 1% bovine serum albumin and 5 μl of 10 mg/ml RNase (Sigma) stock solution. The following day the cells were pelleted, washed with PBS, and resuspended in 1 ml of PBS containing 5 μg/ml PI (Sigma). The samples were analyzed by flow cytometry using Cell Quest software (BD Biosciences), and histograms of PI fluorescence (DNA content) versus BrdUrd incorporation were generated. Cell cycle populations are designated G0/G1 (2 N DNA content with no BrdUrd incorporation), S (variable DNA content with BrdUrd incorporation), and G2/M (4 N DNA content without BrdUrd incorporation). Data represent the mean of at least two independent experiments. For some samples data were also analyzed using ModFit LT software (Verity Software House, Inc., Topsham, ME).

Alternatively, after appropriate treatment, cells were harvested by trypsinization and washed with PBS then resuspended in 1 ml PBS. An equal volume of Nuclear Isolation and Staining Solution (nuclear isolation media–4’,6-diamidino-2-phenylindole; NPE Systems, Inc., Pembroke Pines, FL) was added. The NPE DNA reference calibrator solution was mixed by gently turning the vial upside down a few times; two microliters (2 μl) were removed from the cell sample with a 25-μL filter tip into another tube and was filtered with a 25-μM NPE filter tip into another tube. The fluorescence peak and volume peak were set, and optical alignments were performed on a NPE Quanta flow cytometer. Analysis was performed using ModFit LT software.

**Cell Synchronization—**Wild-type or β-pol null cells were seeded at equivalent cell density in 100-mm dishes for cell cycle analysis or simultaneous cytotoxicity studies for cytotoxicity determinations as required. The following day the cells were washed one time with Hanks’ balanced salt solution, one time with medium containing 0.2% FBS, and then incubated with medium containing 0.2% FBS for 48 h in a 10% CO2 incubator at 34 °C. The medium was then aspirated, and the cells were incubated with 2.5 μM aphidicolin (APH; Sigma) in growth medium with 10% FBS for 16 h. After 16 h, cells were treated for 4–12 h with DNA-damaging agent and 10 μM aphidicolin in growth medium. For cell cycle analysis, cells were incubated with BrdUrd at the appropriate time to pulse-label the cells, and the samples were analyzed as described above. For cytotoxicity studies cells were treated with MMS with and without 4-AN as described earlier beginning 30 min after release from the APH block.

**Cell Lysate Preparation and Western Blotting—**Wild-type mouse fibroblasts were seeded in 145-mm dishes at a cell density of 6.9 × 106 cells/dish. The following day the cells were treated with MMS (0.25 mM) or 4-AN (10 μM) or a combination of the two agents as described previously. In some studies cells were treated with 1 mM caffeine in addition to 4-AN during and after the 1-h MMS exposure. After 24 h, treated and control untreated cells were harvested by scraping and washed with PBS. Cell lysates were prepared in a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 25 mM NaF, 0.1 mM sodium orthovanadate, 0.2% Triton X-100, 0.3% Nonidet P-40) containing protease inhibitors, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 5 μg/ml leupeptin as described previously (21). Cells in lysis buffer were incubated on ice for 30 min, then centrifuged at 14,000 rpm for 30 min at 4 °C, and the supernatant fraction was transferred to a new tube. The protein concentration in the extract was determined using the Bio-Rad protein assay, with bovine serum albumin as standard.

Equal amounts (100 μg) of total protein were loaded on gels and separated by 4–12% SDS-PAGE. The proteins were then transferred onto a nitrocellulose membrane in a transblot apparatus for 3 h or overnight at 25 V. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T) and then
first probed with anti-Chk1 polyclonal antibody (1:1000 dilution; Cell Signaling, Beverly, MA). Goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1000 dilution; Cell Signaling) was used as the secondary antibody, and immobilized horseradish peroxidase activity was detected using either Phototope-horseradish peroxidase (Cell Signaling) or Renaissance (PerkinElmer Life Sciences) chemiluminescence detection kits for Western blotting. The blot was stripped by incubating with buffer containing 62.5 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, and 1% SDS for 30 min at 50 °C followed by 2 washes with TBS-T at room temperature. Detection of phosphorylated Chk1 (P-Chk1) was with rabbit polyclonal anti-P-Chk1 (serine 345) antibody (1:1000; Cell Signaling). The membrane was stripped again and, as a loading control, probed for actin using anti-actin mouse monoclonal antibody (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA); the secondary antibody was goat anti-mouse IgG-horseradish peroxidase conjugate (1:5000, Bio-Rad).

**RESULTS**

**Effect of PARP Inhibitors on MMS-induced Cytotoxicity in Mouse Fibroblasts**—It is well documented that β-pol null cells, deficient in β-pol-dependent BER, are hypersensitive to a wide range of monofunctional methylating and ethylating agents (2, 4). In addition, it is known that both wild-type and β-pol null cells can be extremely sensitized to MMS by the PARP inhibitors 3-AB and, in particular, 4-AN (8, 13). Our observation that treatment of cells with MMS for 1 h along with exposure to 4-AN (10 μM) for 24 h results in extreme sensitization to this methylating agent is illustrated in Fig. 1A. In this experiment, in the presence of 4-AN and, therefore, in the absence of PARP activity, we find that β-pol null cells are still hypersensitive to MMS compared with wild-type cells, as shown in the lower panel of Fig. 1A. Here, when comparing IC_{50} for MMS, an ∼40- and 80-fold sensitization is seen in wild-type and β-pol null cells, respectively. Thus, under conditions where PARP activity is inhibited, β-pol null cells demonstrate twice the hypersensitivity to MMS relative to wild-type cells than under control conditions (5.2- and 2.6-fold, respectively). The results demonstrate that PARP activity is critical in protection of the cells against cytotoxic methyl DNA adducts both in the presence and absence of β-pol-dependent repair pathways. The more widely used PARP inhibitor, 3-AB, can also sensitize both wild-type and β-pol null cells (Fig. 1B), but the effect is not so dramatic even when using a concentration of 10 μM 3-AB (1000 times the maximally effective concentration of 4-AN). Inhibitor studies in vitro had shown the IC_{50} for 4-AN and 3-AB to be 0.18 and 33 μM, respectively (23), and studies in cells demonstrated that 4-AN was 1000-fold more potent than 3-AB at inhibiting PARP activity (24). In the absence of a PARP inhibitor a “shoulder” was seen on both the wild-type and β-pol null MMS survival curves (Fig. 1) showing that even the β-pol null BER-deficient cells demonstrate a degree of MMS resistance at low concentrations of this agent. In the presence of an inhibitor, the shoulder is not seen in either cell line, suggesting that PARP activity is required for resistance to even low dose MMS-induced cytotoxicity.

We next extended these initial observations and documented the effects in more detail. The concentration dependence of the 4-AN-mediated sensitization of wild-type and β-pol null cells to a more limited range of MMS dilutions than used in the pre-
A mouse fibroblasts were treated with a range of concentrations of MMS for 1 h combined with 4-AN at the concentration indicated in the panel, and then the incubation with 4-AN was continued for a further 23 h. B, mouse fibroblasts were treated with dilutions of MMS for 1 h combined with 4-AN (10 μM), and then the incubation with 4-AN was continued for the times indicated in the panel. The IC₅₀ for MMS is the concentration that results in 90% growth inhibition. C, comparison of a 1-h co-treatment or 24-h pretreatment with 4-AN (10 μM) on sensitivity of wild-type (WT) cells to MMS.

Effect of PARP Inhibitors on Sensitivity to Other DNA-damaging Agents—In the next experiments we checked the effect of 4-AN on sensitivity to two other DNA methylating agents, MNU and TMZ. Similar to the results observed for the Sₙ₂ methylating agent MMS, a 24-h incubation with 4-AN resulted in extreme sensitization of both wild-type and β-pol null cells to these Sₙ₂ methylating agents (Fig. 4, A and B). Also, in the presence of 4-AN, β-pol null cells still retained their MNU and TMZ hypersensitivity relative to wild-type cells. Additionally, and in agreement with the MMS data, 4-AN had a greater effect in β-pol null cells such that in the absence of PARP activity β-pol null cells demonstrate more than a doubling in the magnitude of the MNU and TMZ hypersensitivity phenotype.

We reported previously that incubation with 4-AN resulted in only a small sensitization to the reactive oxygen species-generating agent H₂O₂ (8). Now we show a similar result using 3-AB (Fig. 4C). Therefore, neither PARP inhibitor was able to cause a comparable extreme sensitization to the reactive oxygen species-generating agent, H₂O₂, as has been observed for methylating agents. The lack of dramatic sensitization by 4-AN was also observed with two other oxidative DNA damaging agents, peroxynitrite and bleomycin (Fig. 4, D and E). The results suggest that there is some requirement for PARP activity in protection against the cytotoxicity of oxidative DNA damage but also serve to emphasize the differences between repair of methylated and oxidized DNA. 4-AN was able to minimally sensitize both cell lines to UV exposure (Fig. 4F).

This was anticipated since PARP is thought to play a role in BER but not in the nucleotide excision repair of cytotoxic pyrimidine dimers and 6-4 photoproducts.
In addition to simple methylating agents, β-pol null cells are also hypersensitive to the thymidine analog hmdUrd (4), and we have proposed that the hypersensitivity reflects accumulation of cytotoxic repair intermediates in the absence of β-pol-dependent BER pathways. Now, and in support of this proposal, we show that 4-AN is able to sensitize both cell lines but in particular β-pol null cells to this agent (Fig. 5). Looking at the IC90 for hmdUrd, an ~40- and 130-fold sensitization was observed in wild-type and β-pol null cells, respectively. Interestingly, it was necessary to develop an alternate dosing schedule, utilizing a 24-h 4-AN incubation after the 24-h exposure to hmdUrd to achieve this magnitude of sensitization. When cells were co-exposed to hmdUrd and 4-AN for 24 h, sensitization was not observed (data not shown). This “scheduling requirement” is consistent with the idea that hmdUrd has first to be incorporated into cellular DNA and that cytotoxicity is not observed until the unnatural base is removed by glycosylase (SMUG1)-initiated BER (25).

Sensitizing Effect of 4-AN in PARP-1 −/− Mouse Fibroblasts—PARP-1 −/− cells are known to demonstrate hypersensitivity compared with PARP-1 +/+ cells to methylating agents such as MMS and MNU (13, 14). Here we show that PARP-1 null cells can be further moderately sensitized to MMS by the PARP inhibitor 4-AN (Fig. 6A). In contrast, PARP-1 +/+ wild-type cells are extremely sensitized to MMS by 4-AN, a result similar to our findings in the β-pol wild-type fibroblasts (Fig. 1A). Interestingly, in the presence of 4-AN both PARP-1 +/+ and PARP-1 −/− cells now exhibit a similar sensitivity to MMS. These data suggest a possible role for other proteins with PARP activity in addition to PARP-1 in protection against MMS-induced cytotoxicity. However, among the protein family members, only PARP-1 and PARP-2 have been shown to be activated in response to DNA damage, with PARP-1 being responsible for around 90% of the activity. We also find that PARP-1 −/− cells are extremely hypersensitive to hmdUrd (Fig. 6B), with the level of hypersensitivity greater than for the monofunctional methylating agents studied previously. The observed hypersensitivity is consistent with the idea that PARP-1 is activated in response to DNA strand breaks and with our proposal that sensitivity to hmdUrd results from accumulation of repair intermediates in cells with a deficiency in BER. Lending further support to this hypothesis, a 24-h exposure to 4-AN after the 24-h exposure to hmdUrd results in increased hmdUrd sensitivity in both PARP-1 +/+ and PARP-1 −/− cells (Fig. 6B).

PARP-1 −/− cells also demonstrate hypersensitivity to H2O2 (Fig. 6C). The absence of PARP-1 protein results in a similar degree of hypersensitivity to a DNA-methylating agent, MMS, and an oxidative DNA damaging agent, H2O2. The results suggest that there is a requirement for PARP-1 protein in protection against the cytotoxicity of both methylated and oxidative DNA damage. In contrast to this but in agreement with the data obtained in wild-type and β-pol null cells (Fig. 4, C and E), the addition of the PARP inhibitor 4-AN had only minimal effect on the sensitivity of both PARP-1 +/+ and PARP-1 −/− cells to H2O2 (Fig. 6C) as well as to bleomycin (Fig. 6D). These results again suggest that PARP activity plays only a limited role in protection against cytotoxic oxidative damage. In addition, the data suggest that the absence of PARP-1 protein (i.e. PARP-1 −/− cells) and the presence of inactivated PARP protein (i.e. PARP-1 +/+ in the presence of 4-AN) do not always result in an equivalent hypersensitivity phenotype.

Cell Cycle Arrest in Wild-type Cells after Exposure to MMS and 4-AN—Using flow cytometric analysis of BrdUrd incorpo-

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**Fig. 3. Effect of 4-AN on MMS sensitivity in synchronized cells.** A, wild-type or β-pol null cells were treated for 48 h with medium containing 0.2% FBS followed by APH in complete medium for 16 h to arrest cell growth. Cells were released from the APH block by washing, and BrdUrd-positive cells were detected at the times indicated by flow cytometry as described under “Experimental Procedures.” MMS sensitivity of (B) wild-type and (C) β-pol null cells treated in the absence (Control) or presence of 4-AN (10 μM) for a total of 8 h. Shown are normal unsynchronized cells (circles) or APH-mediated synchronized cells (diamonds) treated with MMS alone or MMS plus 4-AN treated cells (squares) as indicated in the panel. D, comparison of MMS sensitivity in control (no 4-AN) unsynchronized wild-type cells (closed circles), unsynchronized cells with 4-AN exposures of 16 and 24 h (triangles), and synchronized cells with a 4-AN exposure of 8 h (squares).
ration and PI staining, we next investigated the effects of MMS and 4-AN alone and in combination on DNA synthesis and cell cycle progression. Because initially, similar data were generated in both wild-type and \( \beta \)-pol null cells, later experiments and the results presented here are those obtained in the wild-type cells only. Cells were treated for 1 h with MMS (0.25 mM)
and/or continuously with 4-AN (10 μM) and analyzed initially at 2 and 4 h after the beginning of MMS exposure. The cell cycle distributions obtained at 4 h are presented in Fig. 7. In control cells, 34% of cells are in G0/G1, 56% of cells are in S-phase, and 10% are in G2/M (Fig. 7A). The non-toxic exposures of cells to either MMS, or 4-AN alone had no effect on BrdUrd incorporation or cell cycle distribution compared with control, untreated cells (Fig. 7B and C). In contrast, when the agents are combined, there is some inhibition of DNA synthesis at 2 h (data not shown), but an almost complete absence of BrdUrd incorporation becomes apparent by 4 h (Fig. 7D).

The cell cycle distribution after MMS and 4-AN did not change significantly during the next 4 h (Fig. 8A), but by 16 h after treatment, cells had progressed through the cell cycle so now there was a greater proportion of cells in G1-phase and fewer cells in G2/M (Fig. 8D). By 24 h after MMS treatment with continuous exposure to 4-AN, greater than 90% of the cells (in some cases 100%) were in S-phase as analyzed by Cell Quest (Fig. 8C) or ModFit LT software. The S-phase arrest achieved in cells treated with MMS and 4-AN was confirmed by staining with nuclear isolation media-4′,6-diamidino-2-phenylindole and analysis by ModFit LT (data not shown). S-phase arrest is seen in cells at 24 h only where there is continuous exposure to 4-AN. If 4-AN is removed at 4 h, at the time when BrdUrd incorporation is completely inhibited, the cell cycle arrest can be reversed (Fig. 8D). At 24 h after MMS exposure but with 4-AN present for only the first 4 h, 26% of cells were in G0/G1, 42% of cells were in S-phase, and 32% were in G2/M (Fig. 8D). The reversal of cell cycle arrest is also apparent as a considerably lower level of growth inhibition achieved after MMS exposure when 4-AN is present for only the first 4 h rather than continuously (Fig. 8F). These data are consistent with the inability of a 4-h exposure to 4-AN to dramatically sensitize cells to MMS (Fig. 2B).

**Effect of Caffeine on Wild-type Cells Treated with MMS and 4-AN**—The cell cycle arrest observed after exposure of cells to a combination of MMS and 4-AN might be caused by checkpoint signaling in response to DNA damage. Alternatively, it may result from a passive mechanical block of DNA replication by inactive PARP-1 protein irreversibly bound to damaged DNA; replication forks are known to be slowed or arrested by specific protein-DNA complexes (26) and to initiate checkpoint signaling.
activation. Caffeine has been shown to inhibit the activity of both ataxia telangiectasia-mutated (ATM) and ATM and Rad3-related (ATR) upstream checkpoint kinases and to override ATM- and ATR-dependent replication and DNA damage checkpoints (27, 28). In the next experiments cells were treated for 24 h with caffeine (1 mM) in addition to MMS and 4-AN with the aim of determining whether the cell cycle arrest we observe requires active ATM- or ATR-dependent signaling. The results are presented in Fig. 9. Treatment with caffeine alone had minimal effect on cell cycle distributions (data not shown). Inhibition of DNA synthesis 4 h after treatment with MMS combined with 4-AN was seen both in the absence and presence of caffeine (Fig. 9, A and B). These data suggest that this early block of DNA synthesis does not utilize a caffeine-sensitive pathway, i.e. that checkpoint signaling by ATM or ATR is most likely not involved. By 8 h after exposure, the cell cycle distribution in the absence of caffeine had not changed (Fig. 9C); however, in the presence of caffeine some of the cells had escaped the DNA synthesis block, and BrdUrd-incorporating cells were now apparent (Fig. 9D). The results implicate the involvement of a caffeine-sensitive signaling pathway in maintenance of the S-phase arrest after the early inhibition of DNA synthesis. The induction of a replication arrest after exposure to MMS + 4-AN is consistent with the requirement for S-phase cells for the 4-AN-mediated sensitization to MMS. Analysis by ModFit LT software revealed that, whereas the majority of the cell population (96.9 ± 1.3%) was arrested in S-phase by 24 h after MMS and 4-AN (Fig. 9E), cells were almost entirely arrested in G2/M (99.2 ± 0.3%) in the presence of caffeine (Fig. 9F). Therefore, although cells were now arrested in a different phase of the cell cycle after the addition of caffeine, both treatment conditions resulted in cell cycle arrest. Furthermore, as shown in Fig. 10, caffeine had only a minimal effect on the cytotoxicity of the MMS plus 4-AN combination.

Cell Cycle Arrest in PARP-1 +/- and PARP-1 +/- Cells after Exposure to MMS and 4-AN—Treatment of PARP-1 +/- cells with a combination of MMS and 4-AN had a similar effect on cell cycle as has been described above in β-pol wild-type mouse fibroblasts. By 4 h, a complete inhibition of BrdUrd incorporation was apparent (Fig. 11C). At 6 h, some of the cells originally in G1-phase had progressed into S-phase (data not shown), and at later times, all cells were in early (16 h) or late (24 h) S-phase of the cell cycle (Fig. 11, E and G). Therefore, by 24 h after exposure to combined MMS + 4-AN, both of the wild-type cell variants (β-pol and PARP) display an S-phase arrest. In contrast to the results obtained in PARP-1 expressing fibroblasts, treatment of PARP-1 +/- cells with a combination of MMS and 4-AN did not result in inhibition of BrdUrd incorporation at 4 h (Fig. 11D). This observation suggests that activity-inhibited PARP-1 protein may be required for the early arrest of DNA synthesis. At later time points (> 16 h) after exposure to MMS plus 4-AN, the entire cell population was arrested in G2/M (Figs. 11F). This result contrasts with the S-phase arrest observed in PARP-1 expressing cells at 24 h (Figs. 9E and 11G). It is more similar to the G2/M arrest observed in wild-type cells treated with caffeine in addition to MMS plus 4-AN (Fig. 9F) and suggests that the caffeine-sensitive signaling pathway for S-phase arrest also requires activity-inhibited PARP-1 protein. When PARP-1 +/- cells were treated with a cytotoxic dose of MMS alone, arrest in G2/M was observed at 24 h (data not shown), and the effects on the cell cycle were...
Activation of Chk1 after Combined Exposure to MMS and 4-AN—

Whereas ATM is primarily activated by DNA double-strand breaks, one of the functions of the transducer protein ATR is to amplify and relay signals resulting from detection of stalled replication forks (29). Chk1 is an essential downstream effector kinase that is regulated by ATR and plays a key role in DNA damage checkpoints (30). In the next experiments we looked for evidence of Chk1 activation by phosphorylation at serine 345 in extracts prepared from wild-type cells treated with the MMS plus 4-AN combination. The phosphorylated protein has reduced gel mobility as compared with the unmodified protein; also, it can be detected by a phospho-specific antibody. P-Chk1 was not detected in control untreated wild-type cells (Fig. 12A, left panel) or in cells treated with 4-AN alone (data not shown). At 2 h after exposure to MMS alone, a low level of P-Chk1 was observed; however, the level of phosphorylation was considerably higher in MMS plus 4-AN-

**Fig. 9.** Effect of caffeine on combined MMS and 4-AN-mediated cell cycle arrest. Wild-type cells were treated with MMS (0.25 mM) and 4-AN (10 μM) for 1 h, and then the 4-AN incubation continued for up to 24 h in the absence (A, C, and E) or presence of caffeine (1 mM) (B, D, and F). For reference, untreated control wild-type cells are shown in panel G. At the time indicated, cells were pulsed with BrdUrd then harvested for flow cytometry.

**Fig. 10.** Effect of caffeine on sensitivity of wild-type cells to MMS and 4-AN in combination. Wild-type cells were treated with a range of concentrations of MMS together with 4-AN (10 μM) for 1 h in the absence (squares) or presence of 1 mM caffeine (diamonds). Incubations with 4-AN and caffeine were continued for a total of 24 h. Growth inhibition assays were carried out as described under “Experimental Procedures.” Data represent the mean ± S.E. of four independent experiments.
treated cells. Phosphorylation could no longer be detected by 24 h after treatment and, therefore, is an immediate early response to DNA damage. Chk1 is known to be an unstable protein with a half-life of less than 2 h (31). The level of unmodified Chk1 protein remained constant in all the extract samples analyzed (Fig. 12A). When cells were treated with caffeine in addition to MMS plus 4-AN, Chk1 phosphorylation levels were reduced to that seen after exposure to MMS alone (Fig. 12A, right panel). Taken together, the results suggest that the caffeine-sensitive pathway responsible for the MMS plus 4-AN-induced S-phase arrest involves activation of both ATR and Chk1 checkpoint kinases.

Next, we confirmed that there was a physical interaction between ATR and Chk1 in extract from the wild-type mouse fibroblasts studied here. Extracts were immunoprecipitated with anti-ATR antibody and then probed by immunoblotting to detect Chk1 (Fig. 12B, left panel). Co-immunoprecipitation between the two proteins was seen in control untreated cells as well as in cells treated with MMS alone or MMS plus 4-AN. To establish the specificity of the interaction, negative control immunoprecipitations using IgG or no antibody were conducted in extracts from MMS plus 4-AN-treated cells. Other samples were immunoprecipitated with anti-ATR antibody then probed for P-Chk1. An interaction between ATR and P-Chk1 was seen in both MMS and MMS plus 4-AN-treated cells (Fig. 12B, right panel). As expected, no phosphorylated protein was detected in control untreated cells. To further verify that the protein band assigned as P-Chk1 is the phosphorylated protein, a lysate was prepared from MMS plus 4-AN-treated cells in the absence of phosphatase inhibitors, immunoprecipitated with anti-ATR antibody, and finally, digested with the protein phosphatase PP1 before SDS-PAGE. The P-Chk1 band was no longer seen after the PP1 digestion (Fig. 12B, right panel). These co-immunoprecipitation experiments demonstrate complex formation between ATR and Chk1 even in untreated control cells and also an interaction between ATR and P-Chk1 after treatment with MMS alone or MMS plus 4-AN.

DISCUSSION

Effect of PARP Inhibition on Sensitivity to DNA-damaging Agents—We and others have demonstrated the importance of PARP activity for protection of mammalian cells against the cytotoxicity of DNA damaging agents. Specifically, we have reported on the extreme hypersensitivity of cells to MMS when combined with an inhibitor of PARP activity (8, 13). Now we again demonstrate the tremendous potency of 4-AN in mouse fibroblasts (Fig. 2A) but also find that it is necessary to incubate the cells with 4-AN for a minimum of 12 h and preferably
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16 h or longer to achieve the maximal sensitization (Fig. 2B). It is also required that the incubation with 4-AN follows exposure to MMS, since pre-exposure had only a minimal sensitizing effect (Fig. 2C). This is consistent with the idea that PARP protein binds to nicks and breaks in DNA formed as a result of repair of methylation damage and that it is this binding that results in stimulation of PARP activity.

We observe an extreme sensitization of both wild-type and β-pol null mouse fibroblasts to the S<sub>α</sub>-type methylating agents MNU and TMZ (Fig. 4, A and B) as well as to the S<sub>α</sub>,<sub>β</sub>-type methylating agent MMS (Fig. 1A). Potentiation of TMZ-induced cytotoxicity by PARP inhibitors has been reported previously (32, 33). For each of these agents, exposure to 4-AN resulted in a larger potentiation of cytotoxicity in β-pol null compared with wild-type cells (Figs. 1A, 4, A and B). This is consistent with our hypothesis that there is a greater accumulation of intermediates of N-methyl DNA adduct repair in β-pol null cells responsible for the observed hypersensitivity to monofunctional methylating agents. Toxicity of the clinically utilized methylating agent TMZ as well as other S<sub>α</sub>,<sub>β</sub>-methyltransferases is usually attributed to mismatch repair of O6-methylguanine (34, 35). However, we have observed hypersensitivity to TMZ in β-pol null BER-deficient cells (4) as well as a potentiation of cytotoxicity in the absence of PARP activity (Fig. 4B). Clearly, in our cell lines as well as in others (32, 33, 36), the repair of N-methyl DNA adducts by BER is an important determinant of TMZ-mediated cytotoxicity.

Given that the time of exposure to 4-AN required for maximal sensitization to MMS and the doubling time of our cells is similar, we considered the possibility that incubation with 4-AN for an entire cell cycle was required. However, it had been proposed that another PARP inhibitor, 3-AB, potentiates the effects of MMS primarily during S-phase of the cell cycle (37). Synchronization had no effect on sensitivity to MMS alone in either wild-type (Fig. 3B) or β-pol null cells (Fig. 3C), but exposure to 4-AN during S-phase enhanced its ability to sensitize cells to MMS. Our data suggests that, similar to the results obtained with 3-AB, the S-phase of the cell cycle is required for the effective enhancement of MMS-induced cytotoxicity by 4-AN.

Because oxidized bases are repaired by single-nucleotide BER (7, 38) and it is generally considered that PARP-1 is involved in BER, it is surprising that 3-AB and 4-AN are able to only minimally sensitize cells to H<sub>2</sub>O<sub>2</sub>, peroxynitrite, and bleomycin, agents that result in oxidized DNA damage in cells (Fig. 4, C, D, and E and Ref. 8). One explanation is that methylated and oxidative base damage are repaired by distinct sub-pathways of BER (39). Whereas methylated bases are removed from DNA by a monofunctional glycosylase, repair of oxidized bases is generally initiated by a damage-specific bifunctional glycosylase. Only in the case of repair by a monofunctional glycosylase will there be production of the 5' dRP group as an intermediate of repair. Using photoaffinity labeling, we have shown that PARP-1 binds strongest to DNA representing such an intermediate (13). Interestingly, 4-AN also sensitizes cells to hmdUrd (Fig. 5), which is removed from DNA by another monofunctional glycosylase, SMUG1. Similar to MMS, a greater potentiation of hmdUrd cytotoxicity by 4-AN is seen in β-pol null compared with wild-type cells (Fig. 5). It is likely that the hypersensitivity to hmdUrd reflects a greater accumulation of cytotoxic repair intermediates (4), and this results in increased PARP binding and activation in the absence of β-pol-mediated BER.

Taken together our results suggest that generation of a specific DNA repair intermediate is required for the 4-AN-mediated sensitization.

**DNA-damaging Agent Sensitivity in PARP-1-deficient Cells**—The observed moderate hypersensitivity of PARP-1−/− fibroblasts to agents that produce cytotoxic lesions repaired by BER, including hmdUrd, is expected given that PARP-1 has a putative role in BER (Fig. 6). Indeed, hypersensitivity of PARP-1−/− cells to MMS, MNU, and H<sub>2</sub>O<sub>2</sub> has been reported previously (14–16). Now we demonstrate that PARP-1−/− cells as well as wild-type cells can be further sensitized to MMS and hmdUrd by incubation with 4-AN (Fig. 6A and B). PARP-1 is only one of a family of 18 poly(ADP-ribos)ylating proteins containing a conserved catalytic domain sequence (40, 41). It is possible that 4-AN can inhibit other catalytically active "PARP" proteins, therefore resulting in further sensitization beyond that seen in PARP-1−/− cells. Then again, only PARP-1 and PARP-2 are known to be activated in response to DNA damage with PARP-1 being responsible for 90% of this activity.

A moderate hypersensitivity to H<sub>2</sub>O<sub>2</sub> comparable with the
degree of MMS hypersensitivity is observed in PARP-1−/− cells (Fig. 6C). However, similar to the result obtained in wild-type and β-pol null cells, 4-AN does not further sensitize either PARP-1+/+ or −/− cells to this agent (Fig. 6C). It seems that the absence of PARP-1 protein affects H$_2$O$_2$ cytotoxicity in our cells but that PARP activity is not a determinant of cellular sensitivity. In contrast, since PARP-1−/− cells demonstrate moderate hypersensitivity to MMS (Fig. 6A) and 4-AN can sensitize PARP-expressing cells, both the presence of PARP-1 protein and cellular PARP activity regulate sensitivity to this methylating agent. Our results demonstrate that the absence of PARP-1 protein (i.e. PARP-1−/− cells) and the presence of activity-inhibited protein (i.e. wild-type cells plus 4-AN) do not always result in the same hypersensitivity phenotype. This interpretation is unequivocally supported by the results obtained with H$_2$O$_2$ (Fig. 6C) but might also apply to other DNA-damaging agents. In the absence of PARP-1, BER may be less efficient but can still take place, resulting in the observed moderate hypersensitivity phenotype of the PARP-1−/− cells (Fig. 6). In the presence an inhibitor, PARP-1 can still bind damaged DNA and with selectively high affinity for intermediates of monofunctional glycosylase-initiated repair, but now there will be no self-poly(ADP-ribosyl)ation, and the protein remains bound to damaged DNA. Under these circumstances repair may be prevented entirely and might result in the dramatic sensitization to methylating agents that we observe in the presence of 4-AN (Figs. 1 and 4).

**Cell Cycle Arrest and Cell Signaling in MMS Plus 4-AN-treated Cells**—The rapid and complete inhibition of DNA synthesis observed in PARP-1-expressing cells after the MMS and 4-AN combination (Figs. 7D and 11C) was seen previously using a highly cytotoxic concentration of MMS alone (4). However, by 24 h most of the cells treated with MMS alone had arrested in G$_2$/M (4), whereas the MMS and 4-AN combination resulted in S-phase arrest (Fig. 6C and 11G). Therefore, the cytotoxic combination of MMS and 4-AN and conditions where PARP activity is inhibited result in a different effect on cell cycle than a cytotoxic combination of MMS alone and conditions of activated PARP. In PARP-1−/− cells treated with MMS alone, a significant reduction in intracellular protein poly(ADP-ribosyl)ation is expected compared with that occurring in PARP-1-expressing cells. Yet, similar to data obtained in previous experiments using another methylating agent, MNU (42), MMS-treated PARP-1−/− cell arrest in G$_2$/M-phase of the cell cycle. Taken together, our data suggest that the 4-AN-induced S-phase arrest is not due to a generalized absence of protein poly(ADP-ribosyl)ation but also requires PARP-1 protein. Consistent with this hypothesis, neither the early inhibition of DNA synthesis nor the S-phase arrest at 24 h occurs after treatment of PARP-1-deficient cells with MMS and 4-AN (Fig. 11). Therefore, the S-phase checkpoint pathway requires inactivated PARP-1 protein in addition to MMS-induced DNA damage.

Our results demonstrate that treatment of mouse fibroblasts with a combination of a sub-lethal concentration of MMS and a non-toxic 24-h exposure to 4-AN results in an extreme growth inhibition (Figs. 1A and 8F). One possibility is that the irreversible association of PARP-1 protein with damaged DNA in the absence of self-poly(ADP-ribosyl)ation results in stalling of replication forks and replication fork collapse, which is lethal to cells. Replication forks are known to arrest when encountering certain DNA lesions or tightly bound proteins (43). Both the early inhibition of DNA synthesis and the later S-phase arrest could result from either a passive stalling of replication by damaged DNA or by a checkpoint signaling cascade. When caffeine was added to the MMS and 4-AN combination, the early inhibition of DNA synthesis was not reversed (Fig. 9B), but importantly, it was not maintained until 8 and 16 h after treatment as occurred in the absence of caffeine (compare Fig. 9, C with D). These results suggest that, after the initial early inhibition of BrdUrd incorporation, a caffeine-responsive pathway acts to maintain the S-phase arrest. Cells treated with caffeine now arrest in G$_2$/M rather than S-phase of the cell cycle at 24 h (compare Fig. 9, E and F), but this results in only a small decrease in the cytotoxicity of the MMS and 4-AN combination (Fig. 10). Previously, caffeine has been shown to reverse the S-phase checkpoint response after exposure to UVC but to produce very little enhancement of the UVC-induced inhibition of cellular proliferation of normal human fibroblasts (44).

Caffeine is known to abrogate both ATM- and ATR-dependent signaling pathways. Because ATM activation is generally associated with double-strand breaks resulting from irradiation damage, we hypothesized that ATR might be the signaling protein involved in the recognition of DNA damage associated with MMS treatment and inhibition of PARP activity. Activation of ATR to trigger an ATR-mediated checkpoint cascade is known to occur only in replicating cells (45), and the involvement of ATR in monitoring genome integrity during S-phase has been proposed (29, 46). A similar role for ATR after treatment of cells with MMS and a PARP inhibitor could explain the increased effectiveness of 4-AN in S-phase cells (Fig. 3). ATR regulates the phosphorylation and activation of Chk1 (47), an essential effector kinase that plays a key role in both G$_2$/M and S-phase DNA damage and replication checkpoints (30, 48). Treatment of cells with MMS plus 4-AN results in the appearance of an elevated level of P-Chk1 compared with treatment with MMS alone, an effect than can be abrogated by caffeine (Fig. 12A). These results strongly suggest that the caffeine-sensitive pathway responsible for maintenance of the MMS plus 4-AN-induced S-phase arrest is dependent on both ATR and Chk1 checkpoint kinases. Consistent with the data reported here, an ATR/Chk1-dependent and caffeine-sensitive S-phase checkpoint has been demonstrated after exposure of cells to UVC, the carcinogen benzo(a)pyrene dihydrodiol epoxide, and psoralen plus UVA radiation (49–51). Similarly, S-phase arrest of cells by hydroxyurea or APH treatment results in Chk1 activation that is abrogated by caffeine (52).

Co-immunoprecipitation experiments reveal a complex containing ATR and Chk1 in mouse cell extracts. The association is seen in both control and DNA-damaging agent-treated cells (Fig. 12B, left panel). Such an association may facilitate activation of Chk1 by ATR as an early response to DNA damage. Interestingly, once Chk1 is activated in response to combined MMS and 4-AN, the interaction between ATR and P-Chk1 persists (Fig. 12B). Activation of the Chk1 signaling cascade requires many factors in addition to ATR (48, 53, 54). The adaptor protein, Claspin, which is cell cycle-regulated and expressed in S-phase cells, is necessary to promote and regulate Chk1 phosphorylation and physically associates with Chk1 (55–57). It has been suggested that Claspin is phosphorylated by ATR, and Claspin is known to interact with ATR in immunoprecipitation experiments (57). It is possible that the complex we observe between ATR and Chk1 occurs by the known association of both proteins with Claspin. However, we note that, in a recent publication describing co-immunoprecipitation experiments in human cells, an association between ATR and Chk1 was not detected in extracts from either untreated or hydroxyurea-exposed samples (57). We have not evaluated a direct protein-protein interaction between ATR and Chk1.

In summary, when PARP activity is inhibited, exposure to an ordinarily sub-lethal dose of MMS results in an arrest of cells in the S-phase of the cell cycle from which they fail to recover. The
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checkpoint kinase Chk1 becomes phosphorylated as a result of treatment with the MMS plus 4-an combination, and both the cell cycle arrest and the appearance of P-Chk1 are abrogated by caffeine. The results are consistent with a role for an ATR- and cell cycle arrest and the appearance of P-Chk1 are abrogated by treatment with the MMS plus 4-AN combination, and both the checkpoint kinase Chk1 becomes phosphorylated as a result of

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