p53 plays an important role in response to ionizing radiation by regulating cell cycle progression and triggering apoptosis. These activities are controlled, in part, by the phosphorylation of p53 by the protein kinase ATM. Recent evidence indicates that the monofunctional DNA alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) also triggers up-regulation and phosphorylation of p53; however, the mechanism(s) responsible for this are unknown. We observed that in MNNG-treated normal human fibroblasts, up-regulation and phosphorylation of p53 was sensitive to the ATM kinase inhibitor wortmannin. ATM-deficient fibroblasts exhibited a delay in p53 up-regulation indicating a role for ATM in triggering the MNNG-induced response. Likewise, a mismatch repair (MMR)-deficient colorectal tumor line failed to show rapid up-regulation of p53. However, unlike ATM-deficient cells, these MMR-deficient cells displayed rapid phosphorylation of the p53 residue serine 15 after MNNG. In vitro kinase assays indicate that ATM is rapidly activated in both normal and MMR-deficient cells in response to MNNG. Using a number of morphological and biochemical approaches, we failed to observe MNNG-induced apoptosis in normal human fibroblasts, suggesting that apoptosis-induced DNA strand breaks are not required for the activation of ATM in response to MNNG. Comet assays indicated that strand breaks accumulated, and p53 up-regulation/phosphorylation occurred quite rapidly (within 30 min) after MNNG treatment, suggesting that DNA strand breaks that arise during the repair process activate ATM. These findings indicate that ATM activation is not limited to the ionizing radiation-induced response and potentially plays an important role in response to DNA alkylation.

Many forces and toxins originating from endogenous and exogenous sources are capable of destroying the phosphodiester bonds comprising the helical backbone and/or altering the chemical structure of individual nucleotide subunits within the DNA molecule. Clearly, such perturbations within the genome present a tangible risk to the maintenance of cellular integrity and the well-being of the host organism. Hence, DNA damage must be rectified to maintain genomic homeostasis. Furthermore, loss of cellular factors that ensure genome integrity is often associated with enhanced mutation rates and, as a frequently observed consequence, tumorigenesis (1). Such findings firmly underscore the requirement for vigilant maintenance of the genome in organismal health and survival.

Important components in the DNA damage response are members of a family of high molecular weight proteins that share catalytic domain homology with the lipid kinase phosphatidylinositol 3-kinase (PIK) $^5$ PIK-like family members identified in humans include ATM (ataxia telangiectasia-mutated), ATR (ATM- and Rad3-related), and the catalytic subunit of DNA-PK. These PIK-like proteins each possess a canonical kinase domain at their carboxyl terminus and possess detectable protein (SerThr) kinase activity (2–4).

The best documented role of a PIK-like protein in the DNA damage response is the function of ATM after exposure of cells to ionizing radiation (IR). The tumor suppressor protein p53 is phosphorylated on a critical serine residue (Ser$^{15}$) by ATM following IR or radiomimetic drugs (2, 3). Further, ATM activates the kinase Chk2 which, in turn, phosphorylates p53 residue Ser$^{383}$ (5). These events appear to be critical for both the accumulation and activation of p53 as a functional transcription factor in response to DNA damage (6–8). Resulting from these phosphorylation events, p53 controls the cellular levels of a cadre of proteins involved in DNA damage response. For example, p53-dependent up-regulation of p21$^{upf1/cip1}$ expression is required to establish the G$\_1$/S cell cycle checkpoint in response to IR-induced DNA damage (9, 10). Further, ATM is critical in the establishment of the p53-independent S phase and G$\_2$/M checkpoints after γ-irradiation (11–14). In addition to activating checkpoints, ATM also appears to play a role in triggering apoptosis after IR (15, 16). Hence, both ATM and p53 are essential components in the cellular response to IR-induced genome damage.

In addition to IR, recent studies have suggested that PIK-like proteins are involved in response to DNA alkylation. For example, it has been shown that treatment of cells with the monofunctional DNA alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) results in phosphorylation of p53 at Ser$^{15}$ and up-regulation of p53 abundance (17). Further, this phosphorylation event requires functional protein complexes involved in mismatch repair (MMR); namely, hMutSa

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$ The abbreviations used are: PIK, phosphatidylinositol 3-kinase; IR, ionizing radiation; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; MMR, mismatch repair; NHF, normal human fibroblasts(s); PBS, fetal bovine serum; pen/strep, penicillin/streptomycin; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose)-polymerase; O$^\text{2-}$MeG, O$^\text{2-}$methylguanine; TUNEL, terminal deoxynucleotidyl-mediated dUTP nick end labeling.
MNNG Exposure Activates ATM

(hMSH2/hMSH6) and hMutLo (hMLH1/hPMS2) (17, 18). Of note, cell lines deficient in hMutLo or hMutLo display lower sensitivity to alkylating agents and fail to elicit a G2/M checkpoint response after treatment with MNNG and other nitrosoarenes, unlike MMR-proficient cells (19, 20). Such findings suggest that, in addition to its role in DNA repair, the MMR system plays a role in triggering signaling events in response to DNA alkylation.

To uncover the mechanism responsible for p53 up-regulation in response to DNA alkylation, we examined MNNG-induced responses in normal diploid human foreskin fibroblast cells (NHF). We observed that wortmannin, a potent inhibitor of PI3K-like protein activity, ablated p53 up-regulation and Ser15 phosphorylation in response to MNNG. ATM- and MMR-deficient cells show a lack of rapid p53 accumulation shortly after MNNG exposure, suggesting that both ATM and MMR are required for a normal response to MNNG. Further, in vitro kinase assays showed that ATM is rapidly activated by MNNG in both MMR-proficient and -deficient cells. Finally, using several independent assays for apoptotic progression, we did not observe the triggering of apoptosis in MNNG-treated NHF, indicating that DNA strand breaks associated with the apoptotic process are not required for the activation of ATM. However, strand breaks were detected by comet assay in MNNG-treated NHF shortly after exposure, suggesting that strand scission that occurs as a result of DNA repair-associated endonuclease activity may trigger ATM activation. These findings clearly indicate that ATM is activated in response to MNNG-induced DNA alkylation and potentially serve to expand our appreciation of the role that this protein plays in the general genome damage response.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Drug Treatment—** Diploid NHF were established from neonatal circumcision specimens collected at the Medical Center of Louisiana-New Orleans. NHF were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS) and 100 units/ml penicillin/streptomycin (pen/strep) and generally used at passages 3 to 6. The nontransformed A-T fibroblast line ATM22 (used at passage 6) were obtained from the Coriell Institute for Medical Research and cultured in minimum essential medium with Earle’s salts supplemented with 20% FBS, 2× essential amino acids, 1× nonessential amino acids, 1× vitamins, and 100 units/ml pen/strep. The SV-40-immortalized A-T fibroblast line ATM22 (used at passage 6) were obtained from the Coriell Institute for Medical Research and cultured in minimum essential medium with Earle’s salts supplemented with 20% FBS, 2× essential amino acids, 1× nonessential amino acids, 1× vitamins, and 100 units/ml pen/strep.

**Drug (MNNG or camptothecin) treatments were performed by removing the medium from cultures of logarithmically growing cells and adding serum-free medium. The drug was then added at the indicated final concentration, and the cells were returned to the incubator. After a 1-h drug exposure, the plates were rinsed with PBS and refed on complete growth medium, and the cells were returned to the incubator.** Where indicated, the cells were pretreated with 10 μM wortmannin in serum-free medium for 1 h at 37 °C before the addition of MNNG. MNNG (Aldrich) was dissolved in 0.1 M sodium acetate (pH 5.0) at a concentration of 10 mM. Wortmannin (Sigma) was dissolved in Me2SO at a stock concentration of 20 mM. Camptothecin (Sigma) was dissolved in deionized H2O at a stock concentration of 10 mM. All of the drug stocks were stored at −20 °C prior to use.

**Clonogenic Survival Assay—** Clonogenic survival was assessed using the method outlined by Ziv et al. (21). The cells were harvested by trypsinization and replated at known density in tissue culture flasks and returned to the incubator for 18–24 h. The cells were exposed to various doses of IR from a 137Cs source (Gammaxell 1000, Atomic Energy of Canada Ltd.; dose rate = 318 rad/min) or MNNG and, following treatment, returned to the incubator. Nontreated cells were cultured in parallel to determine relative plating efficiency. Ten days post-treatment, the medium was removed, the cells were stained with crystal violet, and colonies consisting of ≥50 cells were scored. Three to six individual survival values (percentage of survival) for each dose were plotted.

**Immunoblotting Analysis—** Immunoblotting was conducted as previously outlined (22). Briefly, the cells were harvested by trypsinization, and the extracts were prepared by resuspending cell pellets in SDS solubilization solution (125 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM sodium pyrophosphate, 1% SDS), followed by immersion in a boiling water bath for 5 min. After this, the lysates were briefly sonicated and centrifuged at 1,500 × g for 20 min at room temperature. The protein concentrations were determined using the BCA protein assay (Pierce), and the lysates were stored at −80 °C. Prior to electrophoresis, an appropriate volume of cell lysate was diluted in 3× SDS sample buffer (150 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 20% glycerol, 3% SDS, 0.01% bromophenol blue, 0.01% pyronin-Y) and boiled for 2 min. 25 μg of total cell protein was subjected to SDS-PAGE on 10% polyacrylamide gels and electrotransferred onto nitrocellulose membranes. The membranes were probed with antibodies directed against total p53 (DO-1, Pharmingen), phospho-Ser15 p53 (Cell Signaling Technology), PARP (F-2, Santa Cruz Biotechnology), or tubulin (DM1A, a gift of Dr. D.W. Cleveland). Quantitation of immunoblot signals was performed by densitometry of exposed x-ray films.

**In vitro Kinase Assays—** ATM kinase assays were conducted using the protocol outlined by Shangary et al. (23). Briefly, MNNG-treated and untreated cells (1 × 10⁷) were harvested 3 h post-treatment, washed with ice-cold PBS, and lysed in 500 μl of 1× lysis buffer (20 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM NaF, 1 mM dithiothreitol, 1 mM sodium vanadate) and a protease inhibitor mixture. After clarifying the lysates by centrifugation and adjusting their concentrations for equal protein content, 5 μl of the ATM antisera pAb 132 (24) was added to the lysates and incubated for 2 h on ice. After this step, 100 μl of a 50/50 slurry of protein A/G-Sepharose (Amersham Biosciences) in PBS was added and incubated for another 1 h on an end-over-end rotator at 4 °C. The immune complexes were harvested by centrifugation and washed three times with 1× lysis buffer containing 500 mM NaCl and twice with 1× lysis buffer containing 100 mM NaCl. After washing, the immune complexes were resuspended in 35 μl of kinase buffer (25 mM HEPES, pH 7.5, 500 mM KCl, 5 mM MnCl₂, 0.5 mM EDTA, 5 mM dithiothreitol, 2.5 mM phenylmethylsulfonyl fluoride). 1 μg of bacterially synthesized, purified recombinant GST-p53 fusion protein, 25 μM ATP (unlabeled), and 30 μCi of [γ-32P]ATP were added, and the reaction was incubated at room temperature for 30 min. The reaction was terminated by adding an equal volume of 2× SDS sample buffer followed by immersion in a boiling water bath. The reaction products were resolved on 10% acrylamide gels, electrotransferred onto Immobilon-P membranes, and analyzed by autoradiography. The immunocomplexes were also subjected to immunoblotting with anti-ATM to ensure equivalent kinase abundance.

**DNA alkylation—** The cells were seeded into culture dishes containing presterilized glass coverslips and allowed to attach overnight. Subsequently, the cells were treated with the indicated drug and 48 h after treatment were fixed by immersion in cold (−20 °C) methanol, washed with PBS, and stained with Hoechst 33258 (final concentration, 0.1 μg/ml) diluted in PBS. Coverslips were then mounted onto glass slides, and DNA morphology was assessed. The cells were photographed using a Leitz microscope equipped with epi-fluorescence optics and a SPOT CCD camera.

**Terminal Deoxynucleotidyl-mediated dUTP Nick End Labeling (TUNEL) Analysis—** DNA strand breaks associated with apoptosis were measured in indicated cell populations quantitatively by TUNEL assay using an in situ cell death detection kit (Roche Molecular Biochemicals). Briefly, the cells were treated as outlined, harvested by trypsinization, washed in PBS, and fixed for 1 h at room temperature in 4% paraformaldehyde in PBS. Following this, the cells were permeabilized for 2 min at 4 °C (0.1% sodium citrate, 0.1% Triton X-100). The cells were then washed twice in PBS containing 1% bovine serum albumin and resuspended in 50 μl of deoxynucleotidyl transferase reaction buffer (40 mM potassium cacodylate, 40 mM KCl, 0.2 mM EDTA, 0.8 mM 2-mercaptoethanol, 10% glycerol) containing 1.2 μM fluorescently-labeled dUTP, 3 μM dATP, 1 mM CoCl₂, and 6.25 units of deoxynucleotidyl transferase. This reaction was subsequently incubated for 1 h at 37 °C. Finally, the cells were washed once in PBS with 1% bovine serum albumin and analyzed quantitatively for fluorescein isothiocyanate incorporation using a Becton-Dickinson FACScalibur flow cytometer and data analyzed using CellQuest software. TUNEL-positive staining was...
finally subjected to electric current (300 mA) for 1 h. The slides were
lanes 2 and 18 h after MNNG (lane 3) and treated NHF 3 h
growth medium was added, and the cells were returned to the incubator. The extracts were formed from untreated (lane 1) and
pretreated with wortmannin (10 μM) for 1 h before IR exposure and harvested 3 h post-treatment (lane 4). These extracts were subjected to
immunoblotting with anti-p53 (top panel), phospho-Ser15-specific anti-p53 (middle panel), or anti-tubulin (bottom panel) to assure equal loading.
The anti-p53 immunoblot signal intensity is indicated. D, NHF were treated with 25 μM MNNG for 1 h at 37 °C and washed with PBS, complete
growth medium was added, and the cells were returned to the incubator. The extracts were formed from untreated (lane 1) and treated NHF 3 h
and 18 h after MNNG (lanes 2 and 3, respectively). NHF were also pretreated with wortmannin (10 μM) for 1 h before MNNG exposure and
harvested 3 h post-treatment (lane 4). The cells were harvested and analyzed as outlined in C. The relative signal intensity of total p53 immunoblot
signal is given.

established by setting a gate at the highest staining 5% of the population
of untreated cells as previously outlined (25).

Comet Assay—The comet assay procedure used is based on the
method outlined by Singh et al. (26). A base layer of 1.0% agarose was
placed on microscope slides and allowed to harden. 75 μl of 1% low
melting point agarose (37 °C) diluted in deionized H2O was mixed with
−1.0 × 10^4 treated or untreated cells (5–10 μl volume) and applied to the
coated slide. A glass coverslip was then overlaid on the cell layer,
and the agarose was allowed to solidify. The coverslip was then removed,
and a third layer of low melting point agarose (75 μl) was added to
the slide. Again, a coverslip was overlaid, and the agarose was
allowed to solidify. After this, the coverslip was removed, and the slides
were placed in a lysis solution (10 mM Tris, pH 10.0, 2.5 mM NaCl, 100 mM
EDTA, 1% Triton X-100, 10% Me2SO) at 4 °C for 1 h. The slides were
then transferred to an electrophoresis apparatus containing an alkaline
solution consisting of 300 mM NaOH and 1 mM EDTA. The slides
remained in this solution for 1 h to promote DNA unwinding and were
finally subjected to electric current (300 mA) for 1 h. The slides were
removed, washed three times for 5 min in neutralizing buffer (0.4 mM
Tris-HCl, pH 7.5) at room temperature, and stained in 1:10,000 dilution
of Sybr Gold (Molecular Probes). The stained nuclei were subsequently
viewed and photographed.

RESULTS

p53 Is Up-regulated after MNNG-induced DNA Alkylation
and IR in a Wortmannin-sensitive Manner—Exposure of mammalian cells to a variety of DNA-damaging agents results in
up-regulation of cellular p53 levels [27]. This process is mediated by the PIK-like protein kinase ATM in response to IR [2, 3, 7]. The principal focus of this study was to determine
whether a similar mechanism underlies the response to the monofunctional alkylating agent MNNG. To compare responses to these two distinctly different types of genotoxic events, we initially determined doses of IR and MNNG that are
equitoxic in NHF. Hence, we performed clonogenic survival
assays on NHF treated with various doses of IR or MNNG. This
analysis indicated that 10 grays of IR (137Cs source) (Fig. 1A),
commonly employed high dose of γ-irradiation, resulted in a
mean cell survival of 0.036% (± 0.011, n = 5). Interpolation
from the survival curve generated from MNNG-treated cells
(Fig. 1B) indicated that a 1-h exposure to 25 μM MNNG corresponded to a similar rate of survival.

To document the profile of p53 up-regulation after IR, we
subjected logarithmically growing cultures of NHF to 10 grays
of IR and determined total p53 levels and Ser15 phosphorylation
both 3 and 18 h after exposure. In NHF extracts, we observed a −5-fold increase in p53 levels 3 h after treatment
and −9-fold increase at 18 h after IR (Fig. 1C, lanes 1–3, top
panel). Consistent with previous reports (8, 28), we found that
phosphorylation of the p53 residue Ser15 occurs after IR as
judged by immunoblotting with a phoso-specific antibody (Fig. 1C, lanes 1–3, middle panel). To confirm that p53 up-regulation following IR is attributable to PIK-like kinase activity, we pretreated NHF with 10 μM wortmannin prior to IR exposure. This concentration of wortmannin potently inhibits ATM activity but has little effect on the activity of the ATM-related kinase ATR (4). In agreement with the documented role of ATM in response to irradiation, we found that a 1-h pretreatment with 10 μM wortmannin significantly reduced the up-regulation of p53 3 h after IR exposure (Fig. 1C, lane 4, top panel). We also observed that pretreatment with wortmannin decreased phosphorylation of p53 at Ser15 (Fig. 1C, lane 4, middle panel).

Next, we examined p53 accumulation/phosphorylation in NHF cells after treatment with an equitoxic dose of MNNG. We exposed logarithmically growing NHF cells to 25 μM MNNG and determined the total p53 levels and Ser15 phosphorylation both 3 and 18 h after exposure. We observed a ~4-fold increase 3 h and an ~9-fold increase in p53 18 h after treatment with 25 μM MNNG (Fig. 1D, lanes 1–3, top panel). Concordant with previous findings (17), we detected phosphorylation of p53 at Ser15 after treatment with MNNG (Fig. 1D, lanes 1–3, middle panel). Pretreatment with 10 μM wortmannin completely abrogated both the up-regulation of p53 (Fig. 1D, lane 4, top panel) and Ser15 phosphorylation (Fig. 1D, lane 4, middle panel) 3 h after MNNG treatment. Taken together, these findings indicate that MNNG and IR elicit similar effects on p53 abundance/phosphorylation and that these effects are sensitive to wortmannin.

A-T and MMR-deficient Cells Fail to Display Rapid p53 Up-regulation after MNNG Exposure—To test a potential role for ATM in MNNG-induced p53 up-regulation and phosphorylation, we exposed nontransformed human A-T fibroblasts (GM1558A and GM02530) to 25 μM MNNG and observed neither p53 up-regulation nor Ser15 phosphorylation in these cells 3 h after MNNG treatment (Fig. 2A and B, lanes 2, top panels). However, ~6–8-fold up-regulation in total p53 (Fig. 2A and B, lanes 3, top panels) was observed along with detectable Ser15 phosphorylation 18 h after MNNG exposure. To assure that the defective rapid p53 up-regulation/phosphorylation observed in untransformed A-T fibroblasts was directly attributable to lost ATM function, we assessed complementation of these defects in A-T cells expressing recombinant human ATM. The ATM-reconstituted cell line (designated YZ-5) and its isogenic ATM-deficient control (designated EBS7) are derivatives of the SV-40 transformed A-T line AT22LJE-T (21); hence, assessment of total p53 up-regulation after MNNG was rendered uninformative because of large T antigen-induced stabilization of p53 (data not shown). However, similar to untransformed A-T fibroblasts, no notable Ser15 phosphorylation was observed in the ATM-deficient EBS-7 line during rapid (1 h) response to MNNG treatment (Fig. 2C, lanes 1 and 2). Conversely, the ATM-proficient YZ-5 line displayed phosphorylation of Ser15 in response to MNNG at this early time point (Fig. 2C, lanes 3 and 4). Taken together, these results indicate that ATM is required for rapid up-regulation and Ser15 phosphorylation of p53 after MNNG but is not required for p53 up-regulation/phosphorylation 18 h after MNNG.

Duckett et al. (17) and Hickman and Samson (18) observed that low doses of MNNG (5 μM) resulted in p53 up-regulation/Ser15 phosphorylation and that this phenomenon was dependent on a functional MMR system. To test a potential role for the MMR system in MNNG-induced p53 up-regulation and phosphorylation at the higher dose of drug used in this study, we tested the MMR-deficient colorectal tumor line HCT-116. We treated HCT-116 cells with 25 μM MNNG and determined p53 levels 3 and 18 h after exposure (Fig. 3, top panel). These results showed that at this dose, similar to ATM-deficient cells, HCT-116 display no p53 up-regulation 3 h after exposure but notable p53 accumulation 18 h after exposure. However, unlike A-T cells, we observed detectable Ser15 phosphorylation 3 h after MNNG that persisted to the 18-h time point in HCT-116 cells (Fig. 3, middle panel). Our findings suggest that although MMR and ATM-deficient cells possess a common phenotype (no p53 up-regulation at 3 h), the molecular lesions in these cells are likely distinct because of the observed differences in rapid (3 h after MNNG) Ser15 phosphorylation.

MNNNG Exposure Activates ATM in Both Normal and MMR-deficient Cells—To directly test whether ATM was activated in human NHF following MNNG exposure, we conducted in vitro kinase assays using recombinant p53 as the kinase substrate. Further, we scored ATM activity in MNNG-treated HCT-116 cells to determine whether activation of ATM occurs in this MMR-deficient cell line. We observed a 2.2-fold increase in ATM kinase activity 3 h after treatment in HCT-116 cells (Fig. 4, lanes 1 and 2) and a 2.8-fold increase in NHF (Fig. 4, lanes 3 and 4). Although the observed fold increase in ATM catalytic activity was modest, it is consistent with ATM activity in irradiated cells (2, 3). These results indicate that ATM is catalytically activated following MNNG exposure and that this activation occurs in a MMR-independent manner.

Normal Human Fibroblasts Do Not Trigger Apoptosis Following MNNG Treatment—MNNG has been shown to induce
apoptosis and concomitant generation of DNA strand breaks because of intranucleosomal digestion of genomic DNA (29, 30). Further, DNA strand breaks induced by ionizing radiation are thought to be a key feature in activation of ATM (31, 32). Therefore, it was reasonable to examine whether apoptosis-associated DNA degradation triggers activation of ATM.

To test this notion, we exposed NHF to 25 \( \mu \)M MNNG or 10 \( \mu \)M camptothecin (a topoisomerase I inhibitor and potent apoptogenic drug (33)), and 48 h post-treatment fixed and stained these cells and untreated cells with the DNA stain Hoescht 33258. The cells were then viewed in a microscope to assess morphology consistent with apoptosis. In a population of camptothecin-treated NHF, we noted that a modest number of cells (5–10%) showed the presence of fragmented nuclei indicating apoptotic onset (Fig. 5C). We did not observe apoptosis in a similar nuclear morphology in populations of untreated NHF (Fig. 5A) or cells treated with MNNG (Fig. 5B). Of note, we observed that MNNG-treated cells displayed a notably larger nuclear volume, consistent with drug-induced growth arrest.

Cleavage of the 120-kDa PARP protein into an 85-kDa carboxy-terminal fragment and a 25-kDa amino-terminal fragment occurs during apoptosis (34). In NHF treated with 25 \( \mu \)M MNNG, we did not observe PARP cleavage 48 h after treatment (Fig. 5D, lane 2). However, accumulation of the 85-kDa fragment was readily observed in camptothecin-treated fibroblasts at the same time point (Fig. 5D, lane 3). As a positive control for apoptosis, we also subjected the T-cell leukemia line Jurkat to 25 \( \mu \)M MNNG and 10 \( \mu \)M camptothecin. Jurkat cells treated with either of these DNA-damaging agents lead to notable accumulation of the 85-kDa PARP fragment (Fig. 5E, lanes 1–3), indicating that 25 \( \mu \)M MNNG induces apoptosis in this cell line.

To directly test for apoptotic-induced DNA fragmentation in NHF and Jurkat cells following MNNG treatment, we performed a fluorescence-activated cell sorter-based TUNEL assay (Fig. 5F). Specifically, 25 \( \mu \)M MNNG-treated NHF and Jurkat cells as well as untreated controls were harvested 48 h post-treatment, fixed, and analyzed by TUNEL assay. We observed no quantitative difference in TUNEL staining between MNNG-treated and untreated NHF, whereas TUNEL positive Jurkat cells accumulated 48 h post-MNNG treatment. Quantitative analysis indicated that 47% of the Jurkat cells were positive for TUNEL staining 48 h post-treatment, whereas 1.2% of the MNNG-treated fibroblasts were TUNEL-positive at the same
time point. Based on results using a number of independent approaches, we have not observed that NHF undergo MNNG-induced apoptosis. This result seemingly indicates that apoptosis-induced DNA fragmentation is not required for ATM activation in response to MNNG treatment.

Both p53 Up-regulation/Ser\(^{15}\) Phosphorylation and DNA Strand Breaks Occur Rapidly after Exposure of NHF to MNNG—Although not dependent upon apoptosis, the question remained as to the nature of the DNA lesion(s) that triggers ATM activation in NHF after exposure to MNNG because monofunctional alkylators do not directly induce DNA strand breaks (35). However, many repair processes (e.g. base excision repair), result in strand scission caused by activation of repair-associated endonuclease(s) (44). To test this hypothesis, we performed comet assays on both untreated NHF and NHF collected 30 min after exposure to 25 \(\mu\)M MNNG (the comet assay is a sensitive technique for the detection of both double- and single-stranded breaks (26)). We observed no migration of DNA in the untreated cells (Fig. 6A, top panel) but observed notable DNA migration emanating from nuclei in NHF exposed to 25 \(\mu\)M MNNG (Fig. 6A, bottom panel). Such characteristic “comet tails,” indicative of DNA strand breaks, were observed in all cells viewed, implying that introduction of these strand breaks in MNNG-treated NHF was independent of the position of the cell in the cell cycle.

Because DNA strand breaks were observed very rapidly after exposing NHF cells to MNNG, we next determined how rapidly p53 is up-regulated and Ser\(^{15}\) is phosphorylated after MNNG treatment. To test this, we exposed NHF to 25 \(\mu\)M MNNG and scored p53 abundance at 30, 60, and 120 min after treatment (Fig. 6B, top panel). We observed a rapid 2.2-fold increase in p53 abundance at 30 min after MNNG exposure. Similarly, Ser\(^{15}\) phosphorylation was observed 30 min after exposure, and no quantitative increase in signal intensity was observed following this time point (Fig. 6B, middle panel). Taken together, these findings indicate that MNNG exposure rapidly results in DNA strand breaks and up-regulation/phosphorylation of p53. Moreover, these events are consistent with the activation of ATM and suggest that strand scission that occurs during the repair of MNNG-induced alkylation may be responsible for activation of this kinase.

**DISCUSSION**

Genotoxic events activate a number of signaling pathways that serve, for example, to activate DNA repair mechanisms, halt cell cycle progression and/or trigger advancement into apoptosis (10, 37, 38). Although all genotoxins produce such general responses, the mechanisms that govern response to divergent forms of DNA damage are potentially diverse themselves. In this report, we show that equitoxic doses of IR and the monofunctional alkylating agent MNNG elicit similar effects upon the up-regulation and Ser\(^{15}\) phosphorylation of p53, although each produces different types of DNA damage (\(\gamma\)-irradiation produces double and single strand breaks as well as numerous oxidative changes to bases and deoxyribose moieties, whereas MNNG alkylates (methylates) several nucleophilic centers in bases but does not directly induce strand breaks (35)). Moreover, we observed that these effects on p53 were sensitive to wortmannin pretreatment in both irradiated and MNNG-treated human fibroblasts. Because wortmannin is a potent inhibitor of many PIK-like protein kinases (4), this finding serves to underscore the importance of PIK-like protein(s) in response to these genotoxic agents. In sum, these findings indicate that, insofar as effects on p53 abundance and Ser\(^{15}\) phosphorylation, IR and MNNG elicit similar effects through a PIK-like protein(s)-dependent mechanism(s).

We have documented that, like IR, MNNG exposure results in the activation of ATM. Although the role that ATM plays in MNNG-induced responses remains unknown, given the prominent role that ATM plays in activating cell cycle checkpoints and apoptosis in response to IR, it is reasonable that this molecule performs similar functions in response to MNNG-induced alkylation. We and others (39, 40) have found that ATM-deficient cells show no enhanced sensitivity to MNNG and related nitrosoureas when compared with normal cells. Whereas ATM deficiency results in severe radiosensitivity (41), it is reasonable that IR- and MNNG-induced ATM-dependent responses may not be completely overlapping.

Of note, we found wortmannin pretreatment to have dramatic effects upon p53 up-regulation/phosphorylation 3 h after irradiation or MNNG. However, continuous exposure of cells to 10 \(\mu\)M wortmannin had little or no effect on p53 up-regulation/phosphorylation at the 18 h post-treatment time point (data not shown). One potential explanation for this finding is that ATM activity, as judged by in vitro kinase assays, is ~60% inhibited by 10 \(\mu\)M wortmannin, but the related PIK-like protein ATR displays minimal sensitivity to this dose (4). In addition, we observed that ATM deficiency led to an inability to up-regulate p53 abundance rapidly after MNNG but was not required for long term (18 h post-treatment) p53 up-regulation and Ser\(^{15}\) phosphorylation. Because the Ser\(^{15}\) site is a target for ATR activity in vitro (2, 42), these findings suggest that ATR may play a role in response to MNNG-induced DNA damage in addition to the activation of ATM outlined in this report. If true, such observations seemingly support the notion that ATM activation

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2 A. W. Adamson and K. D. Brown, unpublished data.
proposed by Sarkaria et al. (4) that ATM plays a prominent role in rapid response to DNA damage, whereas ATR performs a more measured, long term role in genome damage response.

Reports from the Modrich and Samson groups showed that a functional MMR system was required for up-regulation of p53 in response to DNA alkylation (17, 18). We also found that MMR-deficient colorectal tumor cells show a defect in p53 up-regulation 3 h following MNNG treatment. However, we observed, at the 25 μM MNNG dose used in our study, no apparent impairment in the ability of these cells to up-regulate p53 at the 18 h post-treatment time point. We hypothesized that the discrepancy between this and the aforementioned studies could be simply explained by a dose-dependent response to MNNG in HCT-116 cells. To test this, we exposed HCT-116 cells to a range of MNNG doses and found no p53 up-regulation in these cells at doses of < 10 μM at the 18-h time point (data not shown). Nevertheless, it is clear that the MMR system is required for rapid increases in p53 abundance in response to MNNG.

We found that MMR deficiency does not adversely affect the catalytic activation of ATM in response to MNNG as judged by in vitro kinase assays. Consistent with the activation of ATM, we detected Ser15 phosphorylation 3 h after MNNG treatment of HCT-116 cells but not ATM-deficient fibroblasts. These observations lead us to conclude that ATM activity is necessary, but not sufficient, to drive the up-regulation of p53 abundance following MNNG exposure. Chehab et al. (43) found that phosphorylation of the p53 residue Ser20 was critical in promoting increased p53 abundance in IR- and UV-irradiated cells. Ser20 is phosphorylated by the kinase Chk2 (5), which itself is catalytically activated by ATM-dependent phosphorylation (11, 12). Although the mechanistic defect in MMR-deficient cells resulting in an inability to up-regulate p53 levels following MNNG exposure is unknown, it is feasible that the MMR system is essential for the activation of Chk2 (or a complementary kinase) required to drive increases in p53 abundance following MNNG.

It is widely believed that it is the presence of broken DNA strands that activates the catalytic activity of ATM (31, 32). However, MNN and other monofunctional alkylating agents do not appear to directly induce scission of the DNA backbone (35, 44). Based on several reports that nitrourourea induce apoptosis (29, 30, 45), we tested whether strand breaks that occur during programmed cell death could potentially induce ATM activation. However, by examining several criteria consistent with apoptotic onset, we failed to observe MNNG-induced apoptosis in treated NIH. Nevertheless, we observed, using the sensitive comet assay, the presence of significant levels of strand breaks in MNNG-treated cells soon after drug exposure. It is well established that MNNG exposure produces the mutagenic and cytotoxic O6-methylguanine (O6MeG) adduct that can force O6MeG-T mispairing following replication (19). O6MeG is primarily repaired by the protein methylation-nineteenth transferase in a reaction that does not result in strand scission (44). In addition to O6MeG, MNNG causes base alkylation at numerous nucleophilic centers within DNA, such as the N3 position of adenine (44). The presence of such adducts triggers DNA glycosylases (i.e. 3-methyldene glycosylase (46, 47)) to generate apurinic/apyrimidinic sites during repair. Apurinic/apyrimidinic sites, in turn, activate an apurinic/apyrimidinic-specific endonuclease (36) resulting in cleavage of the DNA. Hence, it is reasonable that this base excision repair-specific endonuclease generates the strand breaks observed in MNNG-treated fibroblasts.

Although we cannot rule out that alkylated bases themselves serve to trigger activation of ATM, it is feasible that the accumulation of strand breaks caused by the activity of repair-associated endonucleases result in activation of ATM. Moreover, replication through damaged DNA generally stalls DNA synthesis, which results in the generation of free DNA ends. Thus, it is possible that most, if not all, forms of DNA damage could potentially activate ATM in response to the accumulation of free DNA ends resulting from repair-associated endonuclease activity and/or arrested replication. In support of this notion is a recent finding that UV radiation (which does not directly result in ATM activation (2)) results in phosphorylation of the p34 subunit of replication protein A and that this phenomenon is dependent upon DNA replication and ATM activity (48). These investigators proposed that ATM-dependent phosphorylation of replication protein A was triggered by the presence of free DNA ends that accumulate as a result of stalled replication at sites of UV-generated damage. Clearly, a better understanding of the mechanisms that control ATM activation is required to resolve this issue.

In conclusion, we have documented that DNA alkylation stemming from the drug MNNG leads to activation of the PI3K-like protein kinase ATM. In light of the important roles that ATM plays in response to ionizing radiation, it is likely that this protein plays a parallel role in MNNG-activated signal transduction mechanisms that lead to cell cycle arrest and/or apoptosis.

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