MLH1- and ATM-dependent MAPK Signaling Is Activated through c-Abl in Response to the Alkylator N-Methyl-N'-nitro-N''-nitrosoguanidine

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N-Methyl-N'-nitro-N''-nitrosoguanidine (MNNG) is a DNA-methylating agent, and deficiency in mismatch repair (MMR) results in lack of sensitivity to this genotoxin (termed alkylation tolerance). A number of DNA damage response pathways are activated in a MMR-dependent manner following MNNG, and several also require ATM kinase activity. Here we show that activation of the transcription factor c-Jun is dependent upon both the MMR component MLH1 and ATM, but not ATR, in response to MNNG. In addition to c-Jun, the upstream MAPKs JNK and M KK4 are also activated in a MLH1- and ATM-dependent manner. We document that c-Jun activation is dependent on the MAPK kinase kinase MEKK1. Additionally, the tyrosine kinase c-Abl is required to activate this signaling cascade and forms a complex with MEKK1 and MLH1. This study indicates that an arm of DNA damage-activated MAPK signaling is activated in an MLH1- and ATM-dependent manner in response to MNNG and perhaps suggests that dysregulation of this signaling is responsible, in part, for alkylation tolerance.

DNA damage activates a number of cellular signaling mechanisms that promote DNA repair, halt cell growth, or activate cell death mechanisms. It has been proposed that these damage response mechanisms serve to limit the accumulation of heritable genetic errors thus maintaining genomic homeostasis (1). Unsurprisingly, tumor cells are often defective in one or more of the elements that control such responses, and this contributes to the heightened rates of genomic instability observed in cancer. It is now clear that DNA damage response mechanisms are crucial to limiting the occurrence of somatic mutations, maintaining genomic homeostasis, and limiting cancer development (2, 3).

N-Methyl-N'-nitro-N''-nitrosoguanidine (MNNG) is a monofunctional S-N,1-alkylating (methylating) agent. MNNG and related alkylators are extremely mutagenic and carcinogenic, and they evoke a strong cell cycle arrest and/or apoptotic response. MNNG alkylates multiple nucleophilic centers within DNA with methylation of the N3 position of adenine and the N7 and O6 positions of guanine being the predominant lesions (4). N3MeA and N7MeG lesions are repaired by base excision repair. However, the cytotoxic and mutagenic properties of MNNG principally stem from the methylation of the O6 position of guanine (5, 6). Direct repair of mutagenic O6MeG lesions is primarily accomplished by the repair protein methylguanine-DNA methyltransferase (7). Accordingly, lost or diminished methylguanine-DNA methyltransferase activity results in an increased MNNG-induced lesion load thus significantly raising sensitivity to MNNG and related alkylators (8).

O6MeG lesions are also recognized by the mismatch repair (MMR) system (9, 10). MMR is an evolutionarily conserved DNA repair mechanism that is chiefly responsible for resolving post-replicative base mispairing in DNA (11). In addition to its capacity as a repair mechanism, numerous observations clearly implicate the MMR system as a necessary component in activation of signaling in response to persistent O6MeG lesions (6). Exposure to MNNG and related methylators results in robust establishment of G2 arrest, and MMR-deficient cells are unable to activate either cell cycle arrest and/or apoptosis in response to these drugs (12–15). This phenotype of MMR-deficient cells has been termed alkylation tolerance (13, 16).

A prominent signaling pathway activated in response to cellular stress is mitogen-activated protein kinase (MAPK) signaling. MAPK pathways operate through sequential phosphorylation events that ultimately phosphorylate/activate transcription factors and thus regulate gene expression in response to various cellular stresses and stimuli. At present, three distinct arms of MAPK signaling have been characterized: extracellular signal-regulated kinases (ERKs), p38 MAPKs, and stress-activated protein kinases/Jun-N-terminal kinases (JNKs) (17, 18). A prominent transcription factor activated through MAPK signaling is c-Jun through direct phosphorylation by JNK (19–21). JNKs are activated by cellular stress signals induced by inflammatory cytokines and genotoxic insult (17, 22). Overall, the contribution of JNK and consequential acti-
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vation of c-Jun following genome damage remains unclear; however, JNK is reported to be involved in activating apoptosis in certain cell types, namely lymphoid and neuronal cells (23–25).

Several lines of evidence seemingly support a potential role for both MMR and the DNA damage-responsive kinase ATM in activation of JNK during response to MNNG. ATM, which is activated in response to MNNG (26), is required for the activation of JNK following exposure to ionizing radiation (27). Further, the non-receptor tyrosine kinase c-Abl, which is activated in response to exposure to various genotoxins (28, 29), is required for activation of JNK in response to cellular stress (30). Of note, Nehme et al. (31) found that, in response to cisplatin, c-Abl and JNK activation is dependent on a functional MMR system. Gong et al. (32) also showed that dysregulation of c-Abl activation is responsible, at least in part, for the cisplatin resistance displayed by MMR-deficient cells (33). Finally, several groups have documented that activation of c-Abl is ATM-dependent in response to ionizing radiation (34, 35).

Based on these collective works, we hypothesized that MAPK signaling is possibly dysregulated in ATM- and MLH1-deficient cells in response to MNNG exposure. The results of the present study indicate that c-Jun activation following MNNG exposure is dependent upon ATM and MLH1 as is activation of JNK. Our investigation determined that these defects are attributable to dysregulation of the upstream MAPK signaling cascade, and observations support a role for ATM and MLH1 in the activation of c-Abl in response to MNNG.

MATERIALS AND METHODS

Cell Culture and Drug Treatment—The SV40-immortalized A-T fibroblast line AT22JE-T stably expressing full-length recombinant human ATM (designated YZ-5) or stably transfected with empty vector (designated EBS-7) were cultured as previously indicated (36). The MLH1-deficient human colorectal adenocarcinoma line HCT116 and its MLH1-proficient derivative (HCT116+c3) were cultured with or without 400 µg/ml G418 as previously outlined (14, 37). All cell lines were grown at 37 °C in a humidified 5% CO2 incubator. ATM and MLH1 expression was confirmed by immunoblotting.

MNNG (Aldrich Chemical, Milwaukee, WI) was dissolved in 0.1 M sodium acetate, pH 5.0, at a stock concentration of 10 mM and stored at −80 °C. MNNG treatments were performed by removing media from cultures of logarithmically growing cells and adding serum-free media. MNNG was then added to the indicated final concentration, and cells were returned to the incubator. After a 1-h drug exposure, plates were rinsed extensively with PBS, and cultures were refed on complete growth media and returned to the incubator.

The JNK inhibitor I attractiveness 1,9-cdpyrazol-6(2H-one) one 1,9-pyrazolanthrone (SP600125, Calbiochem) was dissolved in Me2SO to 10 mM as a stock solution and stored at −80 °C. SP600125 was added to cell cultures (10 µM final concentration) 1 h before MNNG treatment. Cells were maintained on SP600125 both during and after MNNG exposure until cells were harvested. STI-571 was obtained from Novartis and prepared as a 10 mM stock solution in Me2SO. Cells were pretreated with 10 µM STI-571 for 24 h before treatment with MNNG and maintained on STI-571 both during and after MNNG exposure.

Immunoblotting—Cells were harvested by scraping in ice-cold PBS, and extracts were formed by the addition of lysis buffer (125 mM Tris-HCl, pH 7.5/5 mM EDTA/5 mM EGTA/10 mM β-glycerophosphate/10 mM NaF/10 mM sodium pyrophosphate/1.0% SDS) for 5 min on ice, and then placed in a 95 °C hot block for 5 min. Lysates were then sonicated and centrifuged at 3000 × g for 3 min.

Protein concentrations were determined using the BCA protein assay (Pierce), and lysates were stored at −80 °C prior to use. 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% bromphenol blue, 0.01% pyronin-Y) and boiled for 5 min. Proteins were resolved on 10% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes overnight at 12 V. After transfer, the membranes were probed with anti-phospho-c-Jun (ser63) II, total c-Jun, phospho-JNK (Thr-183/Tyr-185), total JNK, phospho-MKK4 (Ser-257/Thr-261), phospho-MKK7 (Ser-271/Thr-275), or phospho-p53 (Ser-15). These antibodies were all purchased from Cell Signaling (Beverly, MA). Alternatively, blots were probed with anti-MEK1, c-Abl, MLH1, MSH2, PM52, or lamin A/C. These antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ATM was detected using the monoclonal antibody AM-9 (38) or a rabbit polyclonal antiserum obtained from Bethyl Laboratories (Montgomery, TX). ATR was detected using a rabbit polyclonal antiserum, termed pAb-ATR39, created and characterized in our laboratory as previously described (39). Anti-α-tubulin (DM1A) was a generous gift of Dr. D. W. Cleveland (University of California-San Diego). Peroxidase-conjugated secondary antibodies were obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Immunoreactivity was visualized by chemiluminescence and recorded on x-ray film.

Transfection—A full-length cDNA clone encoding human MLH1 cloned into pBluescript was obtained from Dr. R. Liskay (Oregon Health Sciences University). Subsequently, the MLH1 cDNA was subcloned into the eukaryotic expression vector pcDNA3.1/Hygro (Invitrogen). Following verification by restriction mapping and sequencing, this plasmid, termed pcDNA-MLH1, was used in transfection assays. Prior to transfection, MLH1-deficient HCT-116 cells were seeded at a density of 2 × 104 cells per well in 6-well tissue culture plate. The following day, medium was removed and serum-free culture medium (Opti-MEM, Invitrogen) added. To the cells, 4 µg of pcDNA-MLH1 or empty pcDNA3.1/Hygro and 10 µl of Lipo-fectamine 2000 reagent (Invitrogen) diluted in 500 µl of Opti-MEM was added to the culture medium, and the mixture was incubated with cells for overnight at 37 °C. After transfection, cells were harvested and seeded into 6-well plates. Three days post-transfection, cells were treated with 10 µM MNNG for 1 h and harvested 4 h after drug for analysis.

RNA Interference—RNAi was carried out with a modified version of a procedure described previously by our laboratory (39). Synthetic siRNA duplexes specific for human ATM, ATR,
FIGURE 1. MLH1 is required for the phosphorylation/activation of the transcription factor c-Jun in response to MNNG. A, the MLH1-deficient colorectal tumor line HCT116 (top) and isogenic MLH1-proficient HCT116 +ch3 (bottom) cells were either mock treated (lane 1) or treated with MNNG doses ranging from 1.0 to 50 µM (lanes 2–6). Cells were harvested 4 h after drug, and lysates were immunoblotted with antibodies specific for phospho-ser63 c-Jun or total c-Jun as indicated. Relative immunoblot signal intensity is indicated. B, HCT116 cells were transfected with empty pcDNA3.1 vector (lane 2), a human MLH1 cDNA construct (pcDNA-MLH1; lane 3), and 24 h after transfection these cells and MLH1-positive HCT116 +ch3 (lane 1) cells were harvested and blotted with anti-MLH1 or anti-tubulin. C, HCT116 cells transfected with either empty pcDNA3.1 (lanes 1 and 2) or pcDNA-MLH1 (lanes 3 and 4) and were subsequently either mock treated (lanes 1 and 3) or treated with 10 µM MNNG (lanes 2 and 4). Following this, cells were harvested, lysates formed and immunoblotted with anti-phospho-ser63 c-Jun or anti-total c-Jun.

RESULTS

Phosphorylation of the Transcription Factor c-Jun in Response to MNNG Is ATM- and MLH1-dependent—Based on observations made using MNNG and other genotoxins, we hypothesized that MAPK signaling triggered in response to MNNG was potentially dependent upon a functional MMR system. To address this hypothesis we examined activation-linked phosphorylation of c-Jun using matched MLH1-proficient/deficient colorectal tumor lines. MLH1-deficient HCT116 and MLH1-proficient HCT116 + ch3 were either mock treated (buffer only) or treated with a range of MNNG concentrations from 1.0 to 50 µM. 4 h post-drug, cells were harvested, and extracts were formed and subsequently immunoblotted with an antibody specific for phospho-ser63 c-Jun, because phosphorylation of this residue is linked to activation of this transcription factor (41). When extracts from MNNG-treated HCT116 were probed with anti-phospho-ser63 c-Jun we observed negligible levels of phosphorylation of this molecule in both mock treated and cells treated with all drug concentrations tested at the 4 h time point (Fig. 1A, top). In contrast, when the MLH1-proficient HCT116 + ch3 cell line was treated with MNNG we observed a dose-dependent increase in ser63-phosphorylated c-Jun (Fig. 1A, bottom). Specifically, we measured an increase of ~2-fold in phosphorylated c-Jun in HCT116 + ch3 cells treated with 10 and 20 µM MNNG and an increase of ~4-fold in cells treated with 50 µM MNNG.

To confirm that the defect in c-Jun phosphorylation observed in HCT116 was directly attributable to MLH1 deficiency, we transfected HCT116 with either empty pcDNA3.1/hygro expression plasmid, or plasmid containing a full-length copy of the human MLH1 cDNA (pcDNA-MLH1). Transient transfection of this cell line with pcDNA-MLH1 results in robust expression of MLH1 24 h post-transfection (Fig. 1B). Next, HCT116 cells were transfected with either
empty vector or pcDNA-MLH1, cells treated with 10 µM MNNG 24 h post-transfection, and harvested 4 h after drug treatment. Immunoblot analysis of mock treated cells or MNNG-treated cells transfected with empty vector or pcDNA-MLH1 with phospho-specific c-Jun indicated that HCT116 cells transfected with pcDNA-MLH1 displayed a clear increase in MNNG-induced phosphorylation of c-Jun (Fig. 1C). In contrast, cells transfected with empty vector did not display increased phosphorylation of this molecule in response to MNNG.

ATM is a critical DNA damage-response kinase, and our group has shown this molecule is catalytically activated in response to MNNG (26). To examine a potential role for ATM in activation of c-Jun in response to MNNG, we utilized the isogenic ATM-proficient/deficient human fibroblast lines YZ-5 and EBS-7. These lines were derived from the A-T cell line AT221JE-T, which has been stably transfected with a plasmid encoding full-length human ATM cDNA (YZ-5) or empty vector (EBS-7) (36). Using anti-ser63 c-Jun we observed elevated basal levels of phosphorylated c-Jun in EBS-7 cells, and this level did not increase in response to MNNG doses tested (Fig. 2A, top). Untreated YZ-5 cells do not display a basal level of phosphorylated c-Jun present in EBS-7; however, this ATM-proficient line did show a dose-dependent increase in phosphorylated c-Jun in response to MNNG (Fig. 2A, bottom).

To confirm that the up-regulation of phosphorylated c-Jun observed in YZ-5 cells following MNNG exposure was directly attributable to ATM activity we used RNAi. Transfection of YZ-5 cells with ATM-specific siRNA forced an approximate 2-fold decrease in ATM expression in this cell line when compared with cells transfected with nonspecific control siRNA (Fig. 2B, top). Following transfection with either control or ATM siRNAs, cells were treated with 5 µM MNNG for 1 h. 4 h post-drug, cells were harvested, and extracts were immunoblotted with phospho-Ser-63 c-Jun antibody. We observed increased levels of phosphorylated c-Jun in MNNG-treated cells transfected with control siRNA, whereas YZ-5 cells transfected with ATM-specific siRNA showed a minimal (1.3-fold) increase in c-Jun phosphorylation in response to MNNG (Fig. 2B, bottom).

When parallel RNAi experiments were performed on HCT116+ch3 cells, we observed a 2.4-fold decrease in ATM expression in this cell line (Fig. 2C, top). Similar to results obtained in YZ-5 cells, we found that reduced ATM expression in HCT116+ch3 cells resulted in diminished c-Jun phosphorylation in response to 10 µM MNNG (Fig. 2C, bottom). Based on these results, we conclude that MLH1 and ATM are required to induce rapid phosphorylation of c-Jun in response to MNNG-induced alkylation. We have also examined MNNG-induced phosphorylation of ATM-2 that, like c-Jun, is a member of the AP-1 (activating protein-1) family of transcription factors. Our investigation demonstrated that, in parallel to c-Jun, ATF-2 is also activated in an ATM- and MLH1-dependent manner in response to MNNG (supplemental Fig. S1).

ATM and the structurally related protein ATR are both DNA damage-responsive kinases that share significant overlap in their substrate specificities (42, 43). Because of this overlap, we examined the requirement for ATR in c-Jun phosphorylation in response to MNNG (Fig. 2D). Untreated YZ-5 cells do not display basal levels of phosphorylated c-Jun, and this level did not increase in response to MNNG doses ranging from 1.0 to 50 µM (lanes 2–6). 4 h post-drug cells were harvested, and lysates were formed and immunoblotted with phospho-ser63 c-Jun antibody (Fig. 2D, bottom). HCT116+ch3 transfected with control siRNA (lanes 1 and 2), or cells transfected with ATM-specific siRNA (lanes 3 and 4) were mock treated (lanes 1 and 3) or treated with 10 µM MNNG (lanes 2 and 4) 24 h post-transfection. 4 h post-drug cells were harvested, and following this, lysates were formed and immunoblotted with anti-phospho-ser63 c-Jun or anti-total c-Jun as indicated. D. YZ-5 cells were transfected with either control or ATR-specific siRNA and extracts immunoblotted with anti-ATM or anti-tubulin (top). YZ-5 transfected with control siRNA (lanes 1 and 2), or cells transfected with ATR-specific siRNA (lanes 3 and 4) were mock treated (lanes 1 and 3) or treated with 5 µM MNNG (lanes 2 and 4) 24 h post-transfection. 4 h post-drug cells were harvested, and following this, lysates were formed and immunoblotted with anti-phospho-ser63 c-Jun or anti-total c-Jun as indicated. D. YZ-5 cells were transfected with either control or ATR-specific siRNA and extracts immunoblotted with anti-ATM or anti-tubulin (top). YZ-5 transfected with control siRNA (lanes 1 and 2), or cells transfected with ATR-specific siRNA (lanes 3 and 4) were mock treated (lanes 1 and 3) or treated with 10 µM MNNG (lanes 2 and 4) 24 h post-transfection. 4 h post-drug cells were harvested, and following this, lysates were formed and immunoblotted with anti-phospho-ser63 c-Jun or anti-total c-Jun as indicated. D. YZ-5 cells were transfected with either control or ATR-specific siRNA and extracts immunoblotted with anti-ATM or anti-tubulin (top). YZ-5 transfected with control siRNA (lanes 1 and 2), or cells transfected with ATR-specific siRNA (lanes 3 and 4) were mock treated (lanes 1 and 3) or treated with 10 µM MNNG (lanes 2 and 4) 24 h post-transfection. 4 h post-drug cells were harvested, and following this, lysates were formed and immunoblotted with anti-phospho-ser63 c-Jun or anti-total c-Jun as indicated.
response to MNNG. Again, siRNA was used to knock down ATR expression in YZ-5 (Fig. 2D) and HCT116+ch3 (Fig. 2E) cells. We measured an approximate 2-fold decrease in ATR expression in each cell line when transfected with ATR-specific siRNA (Fig. 2, D and E, top). In YZ-5 cells, we observed no difference in MNNG-induced phosphorylation of c-Jun when cells were transfected with either ATR siRNA or control siRNA (Fig. 2D, bottom). Likewise, knock-down of ATR in HCT116+ch3 cells had no observable effect on c-Jun phosphorylation in response to MNNG (Fig. 2E, bottom). These findings indicate, in contrast to MLH1 and ATM, which are required for c-Jun phosphorylation in response to MNNG, that ATR does not appear to be necessary to trigger this biochemical response following MNNG exposure.

The MAPK JNK and the MAPK Kinase MKK4 Are Activated in Response to MNNG in an MLH1- and ATM-dependent Manner—c-Jun is phosphorylated/activated via direct phosphorylation by JNK, also termed stress-activated protein kinase (21). To test if JNK is activated in response to MNNG and, if so, through a MLH1- and ATM-dependent mechanism we treated HCT116/HCT116+ch3 and EBS-7/YZ-5 cells with MNNG or vehicle alone (mock treated) and subjected extracts to immunoblot analysis with anti-phospho Thr-183/Tyr-185 JNK antibody. Dually phosphorylated (Thr-183/Tyr-185) JNK has been shown to be the catalytically active form of this kinase (44). Extracts of MNNG or mock treated EBS-7 and YZ-5 cells were immunoblotted with phospho-specific anti-JNK. This analysis indicated that a basal level of phosphorylated JNK was detectable in mock treated EBS-7 (Fig. 3A, lanes 1 and 2) cells and that this level did not increase upon MNNG exposure. This detectable basal level of phosphorylated/activated JNK is in agreement with the increased basal levels of phosphorylated c-Jun observed in this ATM-deficient cell line (see Fig. 1D). In YZ-5 (Fig. 3A, lanes 3 and 4) cells we detected low levels of phosphorylated JNK in mock treated cells, but phosphorylated JNK increased 8.5-fold in this cell line following MNNG exposure. Analysis of HCT116 (Fig. 3A, lanes 5 and 6) and HCT116+ch3 (Fig. 3A, lanes 7 and 8) cells indicated that MNNG treatment results in an approximate 4.0-fold increase in JNK phosphorylation only in the MLH1-proficient HCT116+ch3 line.

To determine if JNK was required for the activation of c-Jun in response to MNNG we utilized the compound SP600125, a potent and selective inhibitor of JNK kinase activity (45). We observed YZ-5 cells treated with MNNG and SP600125 displayed blunted phosphorylation of c-Jun compared with MNNG-treated cells not treated with SP600125 (Fig. 3B, lanes 1–4). Similarly, we observed that SP600125 markedly reduced MNNG-induced c-Jun phosphorylation in HCT116+ch3 cells compared with cells not treated with SP600125 (Fig. 3B, lanes 5–8). These observations support our conclusion that JNK is activated in response to MNNG through an MLH1- and ATM-dependent mechanism and that activation of JNK is critical for c-Jun phosphorylation following MNNG exposure.

In response to various genotoxic insults, JNK is activated by the MAPK kinases MKK4 and MKK7 (44, 46). To examine activation of these two kinases in response to MNNG, ATM-proficient/deficient fibroblasts were either mock treated or treated with MNNG. Cells were harvested 3 h after drug addition, and extracts were immunoblotted with phospho-Ser-271/Thr-275 anti-MKK7 (Fig. 3A). Similarly, we observed that SP600125 markedly reduced phosphorylation of MKK7 in MNNG-treated cells compared with cells not treated with SP600125 (Fig. 3B, lanes 5–8). These observations support our conclusion that JNK is activated in response to MNNG through an MLH1- and ATM-dependent mechanism and that activation of JNK is critical for c-Jun phosphorylation following MNNG exposure.

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![FIGURE 3. The MAPKs JNK and MKK4 are activated in a MLH1- and ATM-dependent manner in response to MNNG. A, EBS-7 (lanes 1 and 2) and YZ-5 (lanes 3 and 4) cells were either mock treated (lanes 1 and 3) or treated with 5 μM MNNG (lanes 2 and 4). In addition, HCT116 (lanes 5 and 6) and HCT116+ch3 (lanes 7 and 8) cells were either mock treated (lanes 5 and 7) or treated with 10 μM MNNG (lanes 6 and 8). 3 h after drug, cells were harvested, and lysates were formed and immunoblotted with anti-phospho Thr-183/tyr185 JNK (top) or anti-total JNK bottom). B, YZ-5 (lanes 1–4) cells were either untreated (lanes 1 and 2) or pretreated with the compound SP600125 (lanes 3 and 4), and mock treated (lanes 1 and 3) or treated with 5 μM MNNG (lanes 2 and 4). Similarly, HCT116+ch3 (lanes 5–8) cells were cultured in the presence or absence of SP600125, treated with 10 μM MNNG or mock treated and harvested 4 h after drug. Lysates were subsequently immunoblotted with anti-phospho-ser63 c-Jun (top) or anti-total c-Jun (bottom). C, EBS-7 (lanes 1 and 2) and YZ-5 (lanes 3 and 4) cells were either mock treated (lanes 1 and 3) or treated with 5 μM MNNG (lanes 2 and 4). HCT116 (lanes 5 and 6) and HCT116+ch3 (lanes 7 and 8) cells were either mock treated (lanes 5 and 7) or treated with 10 μM MNNG (lanes 6 and 8). 2 h after drug, cells were harvested, lysates were formed and immunoblotted with anti-phospho-ser257/thr261MKK4 (top) or anti-total MKK4 (bottom). D, EBS-7 (lanes 1 and 2) and YZ-5 extracts (lanes 3 and 4) and HCT116 (lanes 5 and 6) and HCT116+ch3 (lanes 7 and 8) extracts were either mock treated (lanes 1, 3, 5, and 7) or treated with 5 μM (lanes 2 and 4) or 10 μM (lanes 6 and 8) MNNG and analyzed by immunoblot analysis with anti-phospho-Ser-271/Thr-275 anti-MKK7 (top) or anti-total MKK7 (bottom).]
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Ser-257/Thr-261 phosphorylation of the MKK4 molecule in MLH1-proficient HCT116+ch3 cells (Fig. 3C, lanes 7 and 8), whereas no increase in phosphorylated MKK4 was observed in HCT116 following MNNG treatment (Fig. 3C, lanes 5 and 6).

A marginal increase in MKK7 Ser-271/Thr-275 phosphorylation was observed in YZ-5 (Fig. 3D, lanes 3 and 4) cells, whereas no increase in phosphorylated MKK7 was noted in MNNG-treated EBS-7 (Fig. 3D, lanes 1 and 2). Additionally, we did not observe increased levels of phospho-Ser-271/Thr-275 MKK7 in either HCT116 (Fig. 3D, lanes 5 and 6) or HCT116+ch3 (Fig. 3D, lanes 7 and 8) following treatment with MNNG. When taken together, these findings indicate that MKK4 is phosphorylated/activated in response to MNNG exposure and that this signaling event occurs in an MLH1- and ATM-dependent manner.

The MAPK Kinase Kinase MEKK1 and the Non-receptor Tyrosine Kinase c-Abl Are Required for Activation of c-Jun and JNK in Response to MNNG—The upstream kinase responsible for the activation of MKK4 in response to DNA damage is MEKK1 (48). To examine the dependence of MNNG-induced MAPK signaling on MEKK1, we transfected YZ-5 cells with control or MEKK1-specific siRNA. Immunoblot analysis indicated that siRNA transfection resulted in an approximate 5-fold knockdown of MEKK1 expression in this cell line (Fig. 4A, top). YZ-5 cells transfected with control or MEKK1-specific siRNA were either mock treated or treated with 5 μM MNNG for 1 h. 3 h after drug exposure cells were harvested and immunoblotted for phosphorylated c-Jun. This experiment revealed that knock-down of MEKK1 reduced MNNG-induced c-Jun phosphorylation by ~3-fold compared with MNNG-treated cells transfected with control siRNA (Fig. 4A, bottom).

Experiments conducted in HCT116+ch3 cells showed that transfection with MEKK1-specific siRNA resulted in a decrease of ~7-fold in MEKK1 expression (Fig. 4B, top). In close agreement with observations in YZ-5 cells, we observed that HCT116+ch3 cells expressing diminished levels of MEKK1 also display ~3-fold reduced levels of c-Jun phosphorylation in response to MNNG compared with controls (Fig. 4B, bottom). These experiments clearly indicate that MEKK1 is a required component in the MAPK cascade that activates c-Jun in response to MNNG.

Kharbanda et al. (49) showed that DNA damage-induced activation of MEKK1 is dependent upon the catalytic activity of the non-receptor tyrosine kinase c-Abl. To test the requirement for c-Abl to activate MAPK signaling during response to MNNG we used STI-571 (Gleevec®), a potent pharmacological inhibitor of c-Abl kinase activity. HCT116+ch3 cells were either preincubated in STI-571 followed by a 24 h treatment with MNNG (Fig. 5A, lanes 1 and 3) or vehicle only (Fig. 5A, lanes 2 and 4) for 24 h and subsequently either mock treated (Fig. 5A, lanes 1 and 3) or treated with 10 μM MNNG for 1 h (Fig. 5A, lanes 2 and 4). 4 h after MNNG, cells were harvested and immunoblotted with anti-phospho MKK4, JNK, or c-Jun. These experiments showed that, although STI-571 alone had a negligible stimulating effect on the phosphorylation of each of these proteins, this inhibitor significantly blunted MNNG-induced phosphorylation of this signaling cascade when compared with MNNG-treated cells not pretreated with STI-571.

To independently confirm the role for c-Abl in activation of MAPK signaling in response to MNNG, we used RNAi to reduce c-Abl expression in YZ-5 and HCT116+ch3 cells. YZ-5 cells transfected with c-Abl-specific siRNA (Fig. 5B, top) displayed an approximate 2-fold decrease in c-Abl expression compared with cells transfected with control siRNA. YZ-5 cells transfected with c-Abl-specific siRNA were either mock treated or treated with 5 μM MNNG, and extracts were immunoblotted with anti-phospho c-Jun. This analysis indicated that knockdown of c-Abl resulted in a 2-fold reduction in c-Jun phosphorylation when compared with MNNG-treated cells transfected with control siRNA (Fig. 5B, bottom).

Parallel experiments conducted in HCT116+ch3 cells also showed an approximate 2-fold decrease in c-Abl expression in cells transfected with c-Abl-specific siRNA (Fig. 5C, top). In accordance with findings in YZ-5 fibroblasts, siRNA-induced knockdown of c-Abl resulted in an approximate 2-fold reduction in c-Jun phosphorylation in response to MNNG when compared with controls (Fig. 5C, bottom). Thus, this work using both pharmacological and RNAi-mediated inhibition of c-Abl agrees with previous work (49) and indicates that c-Abl is required for activation of MAPK signaling in response to MNNG.

Kharbanda et al. (49) also showed that c-Abl associates with MEKK1 in response to γ-irradiation. To determine if this signaling complex is also formed during response to MNNG, we conducted immunoprecipitation/immunoblotting experiments. Specifically, YZ-5 (Fig. 5D, lanes 1 and 2) or HCT116+ch3 (Fig. 5D, lanes 3 and 4) cells were either mock...
treated or treated with MNNG and 3 h after drug, extracts were formed, and immunoprecipitations were conducted with either nonspecific purified mouse IgG (Fig. 5D, top) or anti-c-Abl (Fig. 5D, bottom). Control and c-Abl immunoprecipitations were subsequently immunoblotted with anti-MEK1 or c-Abl. Non-specific mouse IgG precipitated neither MEK1 nor c-Abl from HCT116+ch3 or YZ-5 extracts. In both cell lines, we observed co-precipitation of MEK1 with c-Abl in the absence of MNNG and, moreover, no observable difference in complex abundance was noted in response to MNNG. Further, reciprocal experiments (immunoprecipitated MEK1, immunoblot for c-Abl) yielded similar results (data not shown). These results indicate that, in both YZ-5 and HCT116+ch3 cells, MEK1 and c-Abl form a constitutive signaling complex. Parallel experiments conducted on EBS-7 and HCT-116 cells indicate that this MEK1-c-Abl complex is present in extracts derived from these lines (data not shown), indicating that complex formation is neither ATM- nor MLH1-dependent.

**MLH1 Complexes with c-Abl**—It has been previously reported that c-Abl activation in response to cisplatin is an MMR-dependent process (31). Similarly, we and others observed that the DNA damage-activated kinases Chk2 and Chk1 are activated in an MMR-dependent manner in response to MNNG (39, 50–52). Wang and Qin (52) and our group (50, 53) observed co-precipitation of Chk1 and Chk2 with MSH2, suggesting that formation of an MMR complex may facilitate activation of these kinases. To determine if c-Abl also complexes with the MMR system, HCT116+ch3 cells either mock treated or treated with 10 μM MNNG were fractionated into cytoplasmic and nuclear fractions (Fig. 6A), because both MMR proteins and c-Abl have been shown to be located in both compartments (54–56). Subsequently, immunoprecipitation was performed using anti-MLH1 or anti-MSH2. As a control, we observed that MLH1 co-precipitated with PMS2 in both nuclear and cytoplasmic fractions as determined by immunoblotting (Fig. 6B). PMS2 binds to MLH1 to form the heterodimeric MutLα MMR complex (11). Further, MLH1 antibody co-precipitated c-Abl in both nuclear and cytoplasmic extracts, and the abundance of this complex within the cytoplasmic fraction seemingly decreased with exposure to 10 μM MNNG (Fig. 6B). In nuclear extracts, we also observed an MLH1-c-Abl complex, but the abundance of this complex increased in response to MNNG. Conversely, we did not observe co-precipitation of c-Abl with MSH2 in these extracts (Fig. 6C). These findings indicate that the MMR proteins MLH1 or PMS2 (or the MutLα complex) form a complex, either directly or indirectly, with c-Abl.

**DISCUSSION**

Several groups documented that genotoxic stress activates MAPK signaling (18, 22, 57). The focus of this study was to examine MAPK signaling in response to the S_{51} methylating compound MNNG. We have documented the rapid activation of the transcription factor c-Jun. This transcription factor is, in turn, activated via phosphorylation through a MAPK cascade consisting of JNK, MKK4, and MEK1. Further, activation of this cascade is c-Abl-, MLH1-, and ATM-dependent. Taken together, these findings lead us to develop a model highlighting
MAPK Signaling in Response to MNNG

It was recently reported that closely spaced alkylated bases present on opposite DNA strands can generate double strand breaks due to endonuclease activity during base excision repair-mediated processing of these lesions (59). This finding provides mechanistic evidence that MNNG indirectly introduces double strand breaks into cells and agrees with work from our group that showed, in response to a high dose of MNNG (25 μM), that ATM was rapidly activated in normal human foreskin fibroblasts through a MMR-independent mechanism (26). We have also documented that ATM is rapidly activated in both YZ-5 and HCT116 + ch3 cells using doses of MNNG (5 and 10 μM, respectively) that rapidly activate MAPK signaling (data not shown). Several groups determined that, in response to ionizing radiation, which directly induces double strand breaks, ATM is required for c-Abl activation (34, 35, 60), and catalytic activation of c-Abl stems from direct phosphorylation of c-Abl by ATM (35). In this study we have shown defective activation of a c-Abl-dependent pathway in ATM-deficient cells in response to MNNG, indicating that activation of c-Abl in response to MNNG is ATM-dependent as well.

The nature of MMR-dependent signaling that occurs in response to MNNG has been the subject of much recent attention. We and others have documented that the kinases Chk1 and Chk2 are activated in response to MNNG through a MMR-dependent mechanism (39, 50–52, 61). Collectively, these works support the conclusion that dysregulation of Chk1/Chk2 activation in MMR-deficient signaling is responsible, in part, for the lack of G2/M cell cycle checkpoint activation observed in MMR mutants in response to MNNG and related alkylators. The present study establishes that activation of the kinase c-Abl, in addition to Chk1 and Chk2, is dependent upon one or more components of the MMR system in response to MNNG. At present, we have not established whether MLH1-dependent c-Abl activation contributes to either triggering of the G2/M cell cycle checkpoint or Chk1/Chk2 activation or, alternatively, if c-Abl activation represents another independent arm of MNNG-activated damage signaling.

We also determined that MLH1 complexes with c-Abl in both the nuclear and cytoplasmic compartments. In the nuclear fraction, the MLH1-c-Abl complex is seemingly enhanced in MNNG-activated damage signaling. The present study establishes that activation of the kinase c-Abl, in addition to Chk1 and Chk2, is dependent upon one or more components of the MMR system in response to MNNG. The nature of MMR-dependent signaling that occurs in response to MNNG has been the subject of much recent attention. We and others have documented that the kinases Chk1 and Chk2 are activated in response to MNNG through a MMR-dependent mechanism (39, 50–52, 61). Collectively, these works support the conclusion that dysregulation of Chk1/Chk2 activation in MMR-deficient signaling is responsible, in part, for the lack of G2/M cell cycle checkpoint activation observed in MMR mutants in response to MNNG and related alkylators. The present study establishes that activation of the kinase c-Abl, in addition to Chk1 and Chk2, is dependent upon one or more components of the MMR system in response to MNNG. At present, we have not established whether MLH1-dependent c-Abl activation contributes to either triggering of the G2/M cell cycle checkpoint or Chk1/Chk2 activation or, alternatively, if c-Abl activation represents another independent arm of MNNG-activated damage signaling.

One principal advance stemming from this investigation is that this MAPK signaling cascade is activated through an ATM- and MLH1-dependent mechanism in response to MNNG. Preliminary findings suggest that MSH2 may be required to trigger MAPK signaling in response to MNNG as well, supporting the idea that triggering of this pathway is generally dependent upon an intact MMR system. However, at present, we have only thoroughly documented the MMR protein MLH1 as a required element in this signaling cascade. Nehme et al. (31), found that c-Abl is catalytically activated through an MLH1-dependent mechanism following cisplatin exposure. This finding is supported by others who showed that dysregulated activation of c-Abl is responsible, at least in part, for defective triggering of apoptosis in MMR-deficient cells (32), and increased MLH1 expression results in increased c-Abl activity in response to cisplatin (58). When interpreted in light of the present study, these findings indicate that MLH1 is required for c-Abl activation in response to either cisplatin or MNNG.

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tion of c-Abl with MLH1 may be important in the catalytic ATM-dependent activation of c-Abl; however, how MLH1 mechanistically participates in this event as well as which types of DNA damage trigger activation of c-Abl through this potential mechanism have yet to be determined. We also observed c-Abl-MLH1 complex in the cytoplasmic fraction of mock treated but not MNNG-treated cells. Because both c-Abl and MMR proteins undergo nuclear translocation in response to genotoxic stress (54, 55), our findings may indicate that the cytoplasmic MLH1-c-Abl complex that exists prior to MNNG exposure may be translocated to the nucleus in response to damage.

Numerous studies indicate that both ATM- and ATR-dependent signaling cascades are active following exposure to S_{\text{s1}} methylyating compounds (26, 39, 50, 51, 63, 64). However, we demonstrated that ATM but not ATR is required for the rapid activation of MAPK signaling stemming from c-Abl activation in response to MNNG. This is consistent with previous findings that UV irradiation, a potent activator of ATR-dependent signaling (65–67), does not catalytically activate c-Abl (29). Taken together, these facts seemingly indicate that c-Abl is not a catalytic target of ATR during DNA damage response. This finding is somewhat surprising given that ATM and ATR are structurally related protein kinases that share considerable overlap in their substrate specificities (42, 43); however, recent proteomic profiling efforts indicate considerable complexity in ATM/ATR-dependent signaling networks (68, 69).

The downstream event resulting from the activation of the MAPK signaling outlined in this work is the activation of the transcription factor c-Jun (and ATF-2) in response to MNNG. Both c-Jun and ATF-2 are members of the AP-1 family of basic-leucine zipper transcription factors (21). At least two members of this family, c-Jun and c-fos, are proto-oncogenes owing to their ability to activate pro-growth genes such as cyclin D1 (70). The fact that activation of AP-1 members is a common response to DNA damage has lead to a longstanding conundrum within the field; specifically, why is pro-growth signaling activated in cells that principally respond to DNA damage by repressing growth through activation of cell cycle checkpoints? Several recent studies have shed needed light on this issue. Shaulian et al. (71) showed that c-Jun is necessary for cell-cycle re-entry of UV-irradiated cells by negatively regulating p53-dependent expression of the p21_{waft/cip1} gene. Others showed that ATF-2 is itself directly phosphorylated by ATM in response to \gamma-irradiation and that this event is both required to activate checkpoints and is independent of ATF-2 transcriptional activity (72). Finally, Hayakawa et al. (73) showed that blocking ATF-2 activity resulted in heightened sensitivity to a range of genotoxic agents. This group went on to later show, using comprehensive promoter arrays, that following cisplatin exposure both ATF-2 and c-Jun bound the promoters of numerous DNA repair genes (74). Given the fact that MMR deficiency leads to a strong mutator phenotype (11), it is tempting to speculate that dysregulation of MAPK signaling may contribute to this phenotype.

At present, the alklylation tolerant phenotype is principally defined as a general failure to activate cell cycle arrest or cell death in response to S_{\text{s1}} alkylators (6). Several reports have demonstrated that the DNA damage-responsive protein, GADD45\alpha, is up-regulated by JNK activity (75–77), and GADD45\alpha has been shown to bind cdc2 (78), the cyclin-dependent kinase principally responsible for progression from G2 into mitosis (79). We investigated if GADD45\alpha was up-regulated in response to MNNG and found that although it is increased in an ATM-dependent manner in YZ-5 cells, pharmacological inhibition of JNK had no effect on G2 arrest (supplemental Fig. S2). Because we did not observe a perturbation in G2/M checkpoint response when JNK activity was blocked, this finding casts doubt on the impact of this specific signaling pathway as critical for establishment of a G2 arrest in response to MNNG. However, Hirose et al. (80) previously found that the MAPK p38\alpha is activated in a MMR-dependent manner in response to the S_{\text{s1}} methylyator temozolomide and that p38\alpha activation was required for G2 checkpoint activation. Similar to JNK activation in response to MNNG, p38 activation is c-Abl-dependent in response to cisplatin (81). It is tempting to speculate that MMR-dependent activation of c-Abl impacts the G2/M checkpoint through the p38 arm of MAPK signaling rather than the JNK arm.

In various cell types, such as human lymphoblastoid cells and rodent fibroblasts, MNNG can trigger a robust apoptotic response (15, 82–84). Moreover, several studies have linked JNK activity to the triggering of apoptosis in diverse cell types, including lymphocytes (23–25, 85, 86). Further, the previous finding, that c-Abl dysregulation in MMR-deficient cells is responsible for the lack of apoptosis in cells treated with cisplatin (32), supports the idea that this MAPK pathway may control apoptosis in response to this alkylator. However, it remains undetermined if MAPK signaling is required to trigger programmed cell death in response to MNNG.

In conclusion, compelling evidence indicates that a branch of MAPK signaling induces rapid activation of JNK and the consequential activation of the downstream transcription factor c-Jun in response to the alkylating agent MNNG. Furthermore, this MAPK signaling is activated in a MLH1- and ATM-dependent manner and appears to stem from the ATM- and MLH1-dependent activation of c-Abl. Although this pathway does not impact G2 checkpoint response following MNNG exposure, it is possible that dysregulation of this signaling cascade impacts on the alklylation-tolerant phenotype of MMR-deficient cells.

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