The p38 Mitogen-Activated Protein Kinase Pathway Links the DNA Mismatch Repair System to the G2 Checkpoint and to Resistance to Chemotherapeutic DNA-Methylating Agents

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Although human cells exposed to DNA-methylating agents undergo mismatch repair (MMR)-dependent G2 arrest, the basis for the linkage between MMR and the G2 checkpoint is unclear. We noted that mitogen-activated protein kinase p38α was activated in MMR-proficient human glioma cells exposed to the chemotherapeutic methylating agent temozolomide (TMZ) but not in paired cells made MMR deficient by expression of a short inhibitory RNA (siRNA) targeted to the MMR protein Mlh1. Furthermore, activation of p38α in MMR-proficient cells was associated with nuclear inactivation of the cell cycle regulator Cdc25C phosphatase and its downstream target Cdc2 and with activation of the G2 checkpoint, actions which were suppressed by the p38α/β inhibitors SB203580 and SB202590 or by expression of a p38α siRNA. Finally, pharmacologic or genetic inhibition of p38α increased the sensitivity of MMR-proficient cells to the cytotoxic actions of TMZ by increasing the percentage of cells that underwent mitotic catastrophe as a consequence of G2 checkpoint bypass. These results suggest that p38α links DNA MMR to the G2 checkpoint and to resistance to chemotherapeutic DNA-methylating agents. The p38 pathway may therefore represent a new target for the development of agents to sensitize tumor cells to chemotherapeutic methylating agents.

DNA-methylating agents are among the most diverse and well-studied DNA-damaging agents. In their simplest form, DNA-methylating agents such as methyl methane sulfonate and ethyl methane sulfonate are used as mutagens to randomly introduce genetic alterations, screening of which can help define the functions of affected genes. More complex methylating agents such as temozolomide (TMZ) and dacarbazine are higher order prodrugs that decompose under physiologic conditions (50) or are metabolized (12) to yield reactive intermediates which, like simple methylating agents, lead to methylation of DNA (15, 27). Although complex methylating agents also have mutagenic properties, they are more commonly used as cytotoxic agents for the treatment of a variety of malignancies, including melanoma and brain tumors (5, 46, 55). The clinical and laboratory utility of methylating agents led to extensive investigation of the biologic properties of these agents, which has in turn uncovered a rich web of connections between methylating agent-induced DNA damage, DNA repair, and cell cycle regulation.

The mutagenic and cytotoxic potential of DNA-methylating agents is not a consequence of their inherent ability to alter DNA, but rather of the cellular response to methylating agent-induced DNA damage. All DNA-methylating agents form DNA adducts at the O6 and N7 positions of guanine and the N3 position of adenine, with the relative amounts of these lesions varying by chemical composition of the agent used (43). Although all of these lesions may play some role in the actions of the compounds and the cellular response, O6-methylguanine (O6MG) is believed to be of particular importance. O6MG, unlike other lesioned bases, mispairs with thymine during DNA replication, resulting in guanine-thymine DNA mismatches (30). The mutagenic potential of methylating agent-induced O6MG is opposed by O6MG DNA methyltransferase (MGMT), a DNA repair protein that exclusively removes the adducted methyl group from O6MG in a stoichiometric “suicide” reaction that consumes one MGMT molecule per O6MG lesion repaired (44, 45). In the absence or depletion of MGMT, unrepaired O6MG lesions give rise to guanine-thymine mismatches, which in turn activate the DNA mismatch repair (MMR) system (20, 31). The DNA MMR system in humans consists of at least five homologs of the bacterial protein MutS and at least four homologs of the bacterial MutL protein (3), which function in multimeric MutS/MutL complexes to recognize guanine-thymine mismatches as well as other genetic alterations, including insertions or loops and recombination intermediates (17, 42). The DNA MMR system has evolved to recognize DNA mismatches and to remove DNA from the newly synthesized, mismatched strand, after which repair resynthesis allows for correction of misincorporated nucleotides (22). In the case of guanine-thymine mismatches caused by O6MG mispairing, however, removal of the thymine opposite the O6MG lesions is followed by repair resynthesis of DNA that reinserts thymine opposite to O6MG and triggers the reinitiation of multiple rounds of futile MMR (32). This futile MMR is believed to be linked, by poorly defined means, to the creation of DNA strand breaks and ultimately cytotoxicity (4), all of which does not occur in cells that either have high levels of MGMT (20, 31) or lack a

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functional MMR system (6, 7). The cytotoxic potential of methylating agents is therefore highly dependent not only on the initial DNA damage but also, more importantly, on the cellular processing of this damage.

While the cellular response to methylating agent exposure is highly dependent on DNA repair processes, it has also recently been shown to be influenced by the G2 cell cycle checkpoint (23). A consistent observation in cells exposed to methylating agents, and in particular to cytotoxic methylating agents, is cell cycle arrest at the G2/M boundary (13, 21, 23, 27). While this arrest is transient and is followed closely by the onset of apoptosis in cells such as lymphocytes (13), in other cells such as those derived from gliomas, the arrest can be very prolonged, lasting 7 to 10 days before resolution (23, 24). The purpose of this arrest is unclear, although it has long been suggested that cell cycle arrest provides cells that have incurred DNA damage time in which to reverse the damage (4). Although this suggestion has not been directly tested, it is clear that the prolonged G2 arrest noted in glioma cells exposed to cytotoxic methylating agents alters the cellular outcome. In particular, we noted that cells that underwent a prolonged G2 arrest in response to the methylating agent TMZ had increased survival relative to cells that underwent a more transient arrest and that died by mitotic catastrophe and/or senescence-like growth arrest (23). These results suggested that although DNA repair was a key component in the cellular response to methylating agent-induced DNA damage, the cellular response was also influenced by the linkage of methylating agent-induced DNA damage to cell cycle arrest.

Despite the importance of cell cycle arrest to the action of cytotoxic methylating agents, remarkably little is known about how methylating agent-induced MMR is linked to cell cycle regulation. It has been appreciated for some time that cells lacking a functional MMR system undergo neither G2 arrest nor cytotoxicity in response to methylating agents (6, 7, 21, 31, 32), clearly suggesting a link between MMR and G2 arrest. More recent studies have shown that a functional MMR system is critical for phosphorylation and activation of ataxia telangiectasia mutated (ATM) and p53, although the linkages of these phosphorylation events to the cellular response to methylating agents were not described (1). Furthermore, Chk1 may also play some role, as MMR-proficient cells in which Chk1 is inhibited pharmacologically are unable to undergo G2 arrest in response to TMZ (24). Although several potential links connecting MMR to cell cycle regulation have been suggested, a clear picture of this important pathway remains elusive.

A potential signaling pathway that might help connect activation of the MMR system to G2 arrest is that controlled by the p38 mitogen-activated protein kinase (MAPK) family. The p38 family of stress kinases contains four members: α, β, γ, and δ (41). These MAPKs are activated by the upstream kinases MKK3 and/or MKK6 in response to diverse stimuli, including environmental stress and growth factors (18, 41). p38 kinases in turn activate a variety of downstream targets, including MAPKAP2, the C/EBP family of transcription factors, p53, and various other transcription factors (39, 53). In this manner, p38 is believed to play an important, although not well defined, role in coordinating a variety of cellular events, including cell growth, cell differentiation, and cell death. At least two recent studies have suggested that the ability of the p38 pathway to coordinate cell growth and cell death might also extend to actions on cell cycle progression. Cells exposed to ionizing radiation underwent both G1 and G2/M arrest, the latter being associated with activation of the pathways controlled by p38γ (52). In contrast, in fibroblasts exposed to UV, a transient G2 arrest was associated with activation of p38α (9). While these results suggest that various forms of p38 help to control activation of the G2 checkpoint, they do not address how MMR may fit into the pathway, nor do they address how methylating agents that only indirectly cause DNA damage potentially activate the G2 checkpoint. In the present study, we address the potential role of the p38 pathway in methylating agent-induced G2 arrest, and using both pharmacologic and genetic approaches, we demonstrate that the p38 pathway is a key link between activation of MMR and G2 arrest in response to methylating agents. Furthermore, we show that manipulation of the p38 pathway alters not only G2 arrest but also the sensitivity of cells to cytotoxic methylating agents, suggesting that the p38 pathway may represent a key sensitization for tumors to the cytotoxic actions of chemotherapeutic methylating agents.

MATERIALS AND METHODS

Cell lines and cell culture. U87MG human glioblastoma cells were obtained from the Brain Tumor Research Center, University of California, San Francisco. A pooled population of U87MG cells expressing human papilloma virus E6 (U87/ME6) was a generous gift from Daphne Haas-Kogan, Brain Tumor Research Center, University of California, San Francisco. DNA MMR-deficient HCT116 human colorectal adenocarcinoma cells and MMR-proficient HCT116/3-6 human colorectal adenocarcinoma cells were obtained from American Type Culture Collection (Rockville, Md.). All cells were cultured in Dulbecco’s modified Eagle’s H-21 medium supplemented with 10% fetal calf serum (GIBCO/BRL) at 37°C in a 5% CO2 atmosphere. Cells were plated at least 2 days prior to TMZ treatment.

Constructs, drugs, and drug treatment. A retroviral vector encoding short inhibitory RNA (siRNA) targeting human Mlh1 was constructed by first annealing oligonucleotides 5'-TTATAGATCAGCCAGGTAGCAAGCTGCAGAAAGCTTGAGCTGTCTAATGCCTGTGATCTGTATAATTTTTT-3' and 5'-GAATTGAGCCAAGGCTGCTCTCGAGAAGCTGGTGTAGGTGAGTAAAGCTTCTGGGTTCCAATTCCTTTTT-3', which were designed by use of online software provided by Gregory Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) to target hMlh1 (GenBank accession number U07343; nucleotide positions 2245 to 2270). Duplex oligonucleotides were phosphorylated with T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.) and subcloned into the BsrFI-BamHI sites of pSHG-1 vector (provided by Gregory Hannon), which contains the U6 promoter upstream of the BsrFI-BamHI sites and attL1-attR2 sites. The siRNA-encoding fragment was then subcloned into the gWatz-Hygro retroviral construct (obtained from Martin McMahon, UCSF Cancer Center), which contains attR1-attR2 sites, by recombination using LR clonase (Invitrogen, Carlsbad, Calif.). A pooled population of cells expressing the Mhl1 siRNA was obtained after retroviral infection and hygromycin selection as previously described (49). siRNA targeting p38 (SMARTPool, p38MAPK) was purchased from Dharmacon (Lafayette, Colo.). TMZ and β-phenylalanine (BG) were supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. TMZ was dissolved in dimethyl sulfoxide (DMSO; Sigma, St. Louis, Mo.), while BG was dissolved in ethanol. The p38 inhibitors SB202190 and SB203580 were purchased from Calbiochem (San Diego, Calif.) and dissolved in DMSO.

For all studies using TMZ, unsynchronized cells were exposed to TMZ (0 or 100 μM) for 3 h, after which the cells were washed and placed in TMZ-free medium. For studies that included pharmacologic p38 inhibitors, cells were pretreated with 0 or 20 μM SB202190 or SB203580 for 1 h, after which the cells were exposed to TMZ (0 or 100 μM) for 3 h in the presence of p38 inhibitors. The final DMSO concentration did not exceed 0.1% (vol/vol). After TMZ treatment, cells were gently washed and incubated in fresh medium containing 0 or 20
μM SB202190 or SB203580 for up to 3 days, at which time cells were washed and placed in TMZ-free, SB compound-free medium. For experiments in MGMT-proficient cells (HCT116 and HCT116-6-3), unsynchronized, subconfluent cells were pretreated with 10 μM BG for 2 h, followed by incubation with 100 μM TMZ for 3 h in the presence of BG (10 μM). For studies in p38α siRNA-expressing cells, U87 cells (106 cells) were plated on 10-cm-diameter culture dishes 24 h prior to infection and were incubated in Dulbecco’s modified Eagle’s H-21 medium with 10% fetal calf serum. The cells were transfected with p38 siRNA using Lipoconnectamine 2000 (Invitrogen) according to the manufacturer’s protocol, incubated in the culture medium containing the siRNA for 48 h, and treated with TMZ for 3 h in the presence of p38α siRNA. The cells were then washed, incubated in medium free of siRNA or TMZ, and harvested at the times indicated.

Protein extracts. For experiments using whole-cell lysate samples, cells were washed with ice-cold phosphate-buffered saline (PBS), scraped from the culture dish, and incubated in tissue lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Igepal CA-630, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 1 mM dithiorthioleitol, 10 mM β-glycerophosphate, 1 mM Na3VO4, 100 mM NaF, 100 mg of phenylmethylsulfonyl fluoride per ml, and 10 mg of aprotinin per ml (all reagents were purchased from Sigma) for 30 min on ice. For experiments using subcellular fractionation, cells were scraped and washed as described above, incubated in hypotonic protein extract buffer (10 mM HEPES [pH 7.9], 10 mM NaCl, 0.5% Igepal CA-630, 1 mM EDTA, 1 mM EGTA, 1 mM dithioleitol, 10 mM β-glycerophosphate, 1 mM Na3VO4, 10 mM NaF, 100 mg of phenylmethylsulfonyl fluoride per ml, and 10 mg of aprotinin per ml) for 10 min on ice, and lysed by the addition of Igepal CA-630 (final concentration, 0.4%) with vigorous mixing on a vortex machine for 10 s. The cell lysate was centrifuged at 12,000 × g for 5 min, and the supernatant was used as cytoplasmic protein extract. The pellet was sonicated and incubated in hypertonic protein extract buffer (10 mM Tris [pH 7.4], 400 mM NaCl, 0.5% Igepal CA-630, 1 mM EDTA, 1 mM EGTA, 1 mM dithioleitol, 10 mM β-glycerophosphate, 1 mM Na3VO4, 100 mM NaF, 100 mg of phenylmethylsulfonyl fluoride per ml, 10 mg of aprotinin per ml) for 30 min on ice and centrifuged at 12,000 × g for 5 min, and the supernatant was used as nuclear protein extract. Protein samples were stored at −80°C until use. The protein concentration of extracts was measured by use of protein assay reagent (Bio-Rad Laboratories, Hercules, Calif.).

Immunoblot analyses. Forty micrograms of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto an Immunoblot-M membrane (Millipore, Bedford, Mass.). The membrane was blocked in 5% nonfat skim milk (Bio-Rad Laboratories)-TBST (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) at 4°C overnight and was probed with antibody against phosphorylated p38 (Thr180/Tyr182; Cell Signaling Technology, Beverly, Mass.), phosphorylated Cdc2 (Tyr15; Cell Signaling Technology), phosphorylated p38 (Ser185/187; Cell Signaling Technology), phosphorylated Chk1 (Ser345; Cell Signaling Technology), phosphorylated Chk2 (Thr68; Cell Signaling Technology), p38α (Santa Cruz Biotech, Santa Cruz, Calif.), p38β (Santa Cruz Biotech), p38γ (Upstate Biotechnology), p38δ (Upstate Biotechnology), SAPK4 (Santa Cruz Biotech), human MLH1 (Santa Cruz Biotech), histone H1 (Santa Cruz Biotech), or α-tubulin (Santa Cruz Biotech) for 16 h at 4°C. Bound antibody was detected with horseshadish peroxidase-conjugated secondary immunoglobulin G (IgG) (Santa Cruz Biotech) and ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.).

In vitro p38 kinase assay. Nuclear extracts from U87MG cells were immunoprecipitated by use of anti-p38α, -p38β, -p38γ/SAPK3, or -p38δ/SAPK4 antibody and protein G-Plus-agarose (Santa Cruz Biotech). Immunoprecipitates were washed five times with kinase reaction buffer (25 mM Tris [pH 7.5], 1 mM dithioleitol, 10 μM MgCl2), and equal protein amounts were incubated (30°C, 30 min) with kinase buffer containing ATF2 or MAPKAP2 fusion proteins (1 μg/reaction sample; Cell Signaling Technologies) and ATP (final concentration, 200 μM). Supernatants were subjected to immunoblot analysis using antibody raised against phosphorylated ATF2 (Thr 71; Cell Signaling Technology) or MAPKAP2 (Thr358; Cell Signaling Technology). Equal amounts of protein in each kinase reaction were confirmed by staining the sodium dodecyl sulfate-polyacrylamide gel with Coomassie brilliant blue solution.

Cell cycle studies. At various time points following TMZ exposure, cells attached to culture dishes were digested with trypsin and collected, together with the cells floating in the medium. The cells were then washed in PBS, fixed in 70% (vol/vol) ethanol, and stored for up to 2 weeks at −20°C. The cells were then washed once with PBS, followed by incubation in PBS containing 40 μg of propidium iodine (Sigma) per ml and 200 μg of RNase A (Sigma) per ml for 1 h at room temperature in the dark. Stained nuclei were then analyzed on a FACScan machine (Becton-Dickinson, San Jose, Calif.), with 20,000 events per determination.

FIG. 1. TMZ exposure activates p38. U87 human glioma cells were incubated with TMZ (3 h, 100 μM), after which cells were fractionated and nuclear extracts were immunoprecipitated with anti-p38α, -p38β, -p38γ/SAPK3, or -p38δ/SAPK4 antibody. Kinase activities of the various p38 isoforms were determined by incubating equal amounts of p38 immunoprecipitates with kinase buffer containing ATF2 fusion protein and ATP (200 μM) at 30°C for 30 min, after which reactions were subjected to Western blotting using a phospho-ATF2 (Thr 71)-specific antibody. The data shown are representative of at least three experiments. UT, untreated.

Colonies formation efficiency. U87 cells or U87 cells transfected with p38α siRNA were plated at a concentration of 50 cells/well into 6-well culture plates and were allowed to attach to the plates by overnight incubation at 37°C. These cultures were then treated with p38 inhibitors and/or TMZ as described above and allowed to form colonies. Seventeen days after TMZ exposure, cells were stained with methylene blue (Sigma) and colonies of over 50 cells were counted. The experiments were repeated at least four times.

SA-β-Gal staining. Senescence-associated β-galactosidase (SA-β-Gal) staining was performed as described by Dimri et al. (16). Briefly, at various time points after TMZ exposure, cells were washed in PBS, fixed with 2% formaldehyde–0.2% glutaraldehyde in PBS for 5 min at room temperature, and incubated overnight at 37°C in fresh X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution (1 mg of X-Gal per ml, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl2 in 40 mM citric acid–sodium phosphate buffer, pH 6.0). On the next day, cells were rinsed with PBS and the percentages of SA-β-Gal-positive (blue) cells were determined after scoring 500 cells for each sample with a bright-field microscope.

DAPI staining. Control and TMZ-treated cells were fixed with 4% paraformaldehyde–PBS for 1 h at room temperature, rinsed with PBS, incubated with 1.5% Igepal CA-630–PBS for 1 h at room temperature, and stained with 4,6-diamidino-2-phenylindole (DAPI). Nuclei were visualized by fluorescence microscopy, and more than 100 nuclei were examined for each assessment of the extent of mitotic catastrophe.

RESULTS

p38 activation following exposure of human glioma cells to the methylating agent TMZ. To begin to address the possible role of the p38 pathway in the response of cells to methylating agents, human U87 glioblastoma cells were exposed to the clinically used methylating agent TMZ (100 μM, 3 h). Cells were collected 0 to 24 h after drug removal and separated into nuclear and cytoplasmic fractions. The various p38 isoforms were then immunoprecipitated from the nuclear fractions, and equal amounts of the immunoprecipitated p38 isoforms from each time point were incubated with the p38 substrate ATF2 in the presence of ATP. After electrophoresis, phosphorylated ATF substrate was detected by immunoblotting with a phospho-ATF2-specific antibody and was quantitated. As shown in Fig. 1, TMZ exposure stimulated p38α kinase activity in a
biphasic manner, increasing the amounts of phosphorylated ATF approximately sevenfold at 3 and 24 h after drug exposure. p38β kinase activity was also modestly (threefold) stimulated 3 and 6 h after TMZ exposure, while the activities of p38γ and -δ were not significantly affected. The increase in p38α activity was mirrored by an increase in levels of phospho-p38α, but not Jun N-terminal kinase, in the same nuclear extracts (data not shown), showing that p38 activation is an early and specific response of cells to exposure to the DNA-methylating agent TMZ.

**Methylation agent-induced p38 activation is dependent on a functional MMR system.** Human glioma cells are known to undergo a prolonged G2 arrest after exposure to cytotoxic methylating agents, an action that is dependent on a functional MMR system (13, 23). To address the potential role of the p38 pathway in MMR-dependent, methylating agent-induced G2 arrest, we first exposed MMR-deficient HCT116 human colorectal adenocarcinoma cells and paired MMR-proficient HCT116 cells containing a copy of Mlh1 on chromosome 3 (HCT116/3-6 cells) (33) to TMZ (100 µM, 3 h), after which the cells were collected and analyzed for levels of p38α phosphorylation-activation and the extent of G2 arrest by Western blotting and FACS analysis, respectively. Neither HCT116 nor HCT116/3-6 cells exhibited p38 induction or G2 arrest in the first 2 days after TMZ exposure (data not shown), consistent with previous studies showing that both of these cell lines are MGMT proficient and presumably repair TMZ-induced O6MG before these lesions mispair with thymine and trigger downstream consequences (37). After exposure to the highly specific MGMT-depleting agent O6BG prior to and after TMZ exposure, however, the MMR-proficient HCT116/3-6 cells exhibited TMZ-induced p38α activation in a biphasic manner, similar to that noted in U87 glioma cells (Fig. 2A), and underwent G2 arrest (here defined as a greater percentage of cells arrested at the G2/M boundary with 4n DNA content than are present in the G1 phase with 2n DNA content) 2 days after TMZ exposure (Fig. 2B). BG-exposed, TMZ-treated, MMR-deficient HCT116 cells, however, exhibited neither p38 activation nor G2 arrest (Fig. 2). These results suggest in a preliminary manner that p38 activation in response to TMZ is dependent on O6MG lesions and on the recognition and/or processing of these lesions by the DNA MMR system.

While studies in HCT cells suggested an association between the DNA MMR system and TMZ-induced G2 arrest, the MMR-corrected HCT116/3-6 cell line that we used was derived from a clone that contains a large portion of human chromosome three (33) and which therefore could differ from the MMR-proficient HCT116 cells in ways that are more dependent on clonal selection and differences in gene expression than on direct correction of the MMR defect. We therefore also examined TMZ-induced p38 activation and G2 arrest in MGMT-deficient, MMR-proficient human U87 glioblastoma cells and in paired cell lines that differ in MMR proficiency by expression of a retrovirally encoded siRNA targeted to the MMR protein MLH1. As shown in the Western blot in Fig. 3A, expression of the siRNA targeted to MLH1 reduced expression of MLH1 fivefold relative to that noted in cells infected with the blank gWzl-Hygro vector control. Furthermore, expression of the retrovirally encoded MLH1 siRNA also blocked TMZ-induced G2 arrest such that cells expressing the MLH1 siRNA, but not cells expressing the blank vector, avoided TMZ-induced G2 arrest in a manner identical to that noted for HCT116 cells from which MLH1 was eliminated by mutation. More importantly, siRNA-mediated reduction of MLH1 levels in U87MG-iMLH1 cells also blocked the ability of these cells to activate the p38 pathway, as shown in the Western blot analysis of phospho-p38α levels in Fig. 3C. These results, in connection with those derived from the studies with HCT cells, suggest that p38 activation is a common response of human cells to the methylating agent TMZ, that this activation is dependent on a functional MMR system, and that p38 activation is associated with methylating agent-induced G2 arrest.

**Pharmacologic or genetic inhibition of p38α blocks methylating agent-induced effects on G2 checkpoint proteins and G2 arrest.** Having demonstrated the linkage between DNA MMR and p38 activation, we wished to more clearly define the potential linkage between p38 activation and G2 arrest. To do so, we used both pharmacologic and genetic inhibitors of p38α and monitored the effects of these inhibitors on G2 checkpoint proteins and on TMZ-induced G2 arrest. For pharmacologic inhibition studies, MGMT-deficient, MMR-proficient U87 cells were exposed to the p38α/β selective inhibitor SB203580 or SB202190 (14, 35) prior to and following TMZ exposure,
after which the effects of p38 inhibition on TMZ-induced p38 activation, activation of the G2 checkpoint pathway, and activation of the G2 checkpoint itself were examined. As shown in the Western blot in Fig. 4A, TMZ exposure activated p38α, increasing the level of phosphorylation of the p38 target MAPKAP2 in TMZ-treated cells. This effect was blocked by the p38α/β-selective inhibitors SB203580 (Fig. 4A) and SB202190 (data not shown), suggesting that TMZ-induced p38 activation involves p38α and/or p38β. Both SB compounds not only blocked TMZ-induced p38 activation but also dramatically inhibited the ability of U87 cells to undergo G2 arrest 2 days after TMZ exposure (Fig. 4B). Because activation of the G2 checkpoint is associated with nuclear phosphorylation-activation of Chk1 and Chk2, inactivating Ser 216 phosphorylation of the Cdc25C phosphatase and subsequent accumulation of inactive Tyr 14- and Thr 15-phosphorylated Cdc2 (19, 38, 40, 47), we also examined the effects of p38 inhibition on these G2 checkpoint control proteins in the nuclei of U87 cells 1 and 2 days after TMZ exposure. As shown in the Western blot in Fig. 4C, phosphorylated active Chk1 and Chk2, phosphorylated inactive Cdc25C, and phosphorylated inactive Cdc2 accumulated in the nuclear (alpha-tubulin-lacking, histone H1-containing) fraction of TMZ-treated cells in a time frame consistent with the onset of G2 arrest. Exposure of cells to SB203580 prior to and following TMZ exposure blocked the inactivation of both Cdc25C and Cdc2, consistent with the inability of SB compound-treated cells to undergo G2 arrest following TMZ exposure. SB203580 exposure, however, had no significant effect on TMZ-induced Chk1 or Chk2 activation. These results suggest that the p38 pathway, specifically p38α/β, is linked not only to MMR but also to activation of the G2 checkpoint through Ser 345-Chk1- and Thr 68-Chk2-independent actions on Cdc25C and/or Cdc2.

Although SB203580 and SB202190 are relatively selective inhibitors of p38α/β, reports have suggested that these compounds also interfere with other proteins, which could conceivably play a role in activation of the G2 checkpoint (34). We therefore also employed genetic means to selectively inhibit p38α and to assess the consequences of p38α inhibition on the G2 arrest pathway. To do so, MGMT-deficient, MMR-proficient U87 human glioma cells were retrovirally infected with a blank vector or a vector expressing an siRNA targeted to the MMR protein MLH1, after which expression of MLH1 was assessed by Western blotting (A). Control and MLH1 siRNA-expressing U87 cells were incubated with TMZ (3 h, 100 μM), after which the cells were collected and analyzed for extent of G2 arrest (B) and levels of p38α phosphorylation-activation (C) by FACS analysis and Western blotting, respectively. Data shown are representative of three independent experiments.

**FIG. 3.** MMR-deficient human glioma cells do not undergo p38-associated G2 arrest in response to TMZ. MMR-proficient U87 human glioma cells were retrovirally infected with a blank vector or a vector expressing an siRNA targeted to the MMR protein MLH1, after which expression of MLH1 was assessed by Western blotting (A). Control and MLH1 siRNA-expressing U87 cells were incubated with TMZ (3 h, 100 μM), after which the cells were collected and analyzed for extent of G2 arrest (B) and levels of p38α phosphorylation-activation (C) by FACS analysis and Western blotting, respectively. Data shown are representative of three independent experiments.

**Inhibition of the p38 pathway enhances mitotic catastrophe and sensitizes glioma cells to cytotoxic methylating agents.** We previously showed that glioma cell lines respond to cytotoxic methylating agents by undergoing prolonged G2 arrest, the resolution of which is either permanent growth arrest and a senescence-like state, death by entry into mitosis and subsequent mitotic catastrophe, or survival (23). The ability of both pharmacologic and genetic inhibitors to suppress methylating agent-induced activation of the G2 checkpoint led us to assess the fate of cells incapable of undergoing arrest. To address this question, the same p38α-inhibited cells used for the previous studies were exposed to 0 or 100 μM TMZ for 3 h and were
subsequently analyzed for the extent of mitotic catastrophe, the extent of SA β-Gal expression, and the ability to form colonies. Although pharmacologic or genetic inhibition of p38 did not alter its clonagenicity (Fig. 6A), extent of SA β-Gal expression, or extent of mitotic catastrophe (data not shown), it did enhance the sensitivity of U87 cells to TMZ-induced cytotoxicity, reducing the colony-forming ability of these cells approximately 10-fold relative to TMZ-only treated cells (Fig. 6A). The enhanced cytotoxicity of TMZ in cells in which the p38 pathway was suppressed was not a consequence of increased entry of cells into a senescence-like state, as p38 inhibition significantly reduced the percentage of cells exhibiting TMZ-induced SA β-Gal expression 10 days after TMZ exposure (Fig. 6B). P38 inhibition did, however, increase the percentage of TMZ-treated cells that died by mitotic catastrophe (Fig. 6C), consistent with the idea that cells incapable of undergoing G2 arrest enter mitosis with damaged DNA and die by mitotic catastrophe. These results suggest that p38 not only links MMR to the G2 checkpoint, but also influences the response of cells to cytotoxic methylating agents, and may therefore serve as a novel therapeutic target.

DISCUSSION

p38 links MMR to methylating agent-induced G2 arrest. Human cells activate the G2 checkpoint following exposure to DNA-methylating agents. The lack of G2 checkpoint activation in cells with a nonfunctional MMR system suggests that MMR is linked to G2 arrest, although the exact means by which this occurs have not been defined. For this study, we used both pharmacologic and genetic approaches to show that p38 is necessary for methylating agent-induced G2 arrest and that p38, through a pathway associated with Cdc25C and Cdc2, is a critical molecule for the linkage of MMR to the G2 checkpoint.

The finding that p38 is critical for activation of the G2 checkpoint after methylating agent exposure differs in key aspects from previous examinations of the role that various p38 isoforms play in activation of the G2 checkpoint following exposure to DNA-damaging agents. Activation of the G2 checkpoint following ionizing radiation was shown to involve activation of p38, but not p38, and the dependence of this activation on DNA damage was not examined (52). In contrast, activation of the G2 checkpoint following UV radiation was shown to involve activation of cytoplasmic p38 in a manner that was suggested to be independent of DNA damage (9). The present study clearly shows that p38 is necessary for methylating agent-induced DNA damage to activation of the G2 checkpoint. Of the four common p38 isoforms, p38 was most strongly activated following methylating agent exposure, and selective pharmacologic and genetic inhibition of p38 blocked activation of the G2 checkpoint. The present studies do not exclude a role for other p38 isoforms in methylating agent-induced G2 arrest, nor do they prove that p38 is sufficient for the process. They do, however, clearly show that p38 plays a key role in, and is necessary for, the linkage between MMR and G2 arrest. The present studies also show that p38 activation in response to methylating agent exposure is directly linked to DNA damage and MMR processing of this damage. O6MG lesions and their processing

FIG. 4. Pharmacologic inhibition of p38 blocks TMZ-induced p38 activity, G2 arrest, and activation of G2 checkpoint proteins. U87 human glioma cells were pretreated with SB202190 or SB203580 (20 μM, 1 h), after which the cells were incubated with TMZ (3 h, 100 μM) in the presence of SB compounds, washed, and plated in SB compound-containing medium. Cells were then either fractionated and assayed for levels of phosphorylation of the p38 substrate MAPKAP2 (A), analyzed for extent of G2 arrest by FACS analysis (B), or analyzed for nuclear activation of the G2 checkpoint proteins Chk1, Chk2, Cdc25C, or Cdc2 by Western blotting using phospho-specific antibodies (C). In panel C, the mean fold induction of protein levels based on densitometric analysis is displayed below each immunoreactive band. Histone H1 and tubulin were used as loading controls and as controls for cell fractionation. The data shown are representative of three experiments. * values differing statistically (P < 0.05; Student’s t test) from controls.
by the MMR system were critical for p38 activation in response to methylating agents, because only MMR-proficient cells exhibited TMZ-induced p38 activation and G2 arrest, and then only after selective depletion of O6MG repair capacity. The exact type of DNA damage that triggers the biphasic p38 activation following TMZ exposure and the pathways that link this damage to p38 activation remain unclear. TMZ induces DNA single-strand breaks which form within 24 h of TMZ exposure (25), DNA double-strand breaks which appear 2 to 3 days after TMZ exposure (25), and stalled replication forks, any of which could contribute to p38 activation. This damage in turn could be sensed by ATM, ATR, and/or c-Abl, which in turn could pass the signal to MKK3 and/or MKK6, common activators of the p38 pathway. While preliminary studies suggest that ATM, c-Abl, and MKK6 are not necessary for TMZ-induced p38 activation and G2 arrest (Hirose et al., unpublished data), more detailed studies will be required to piece together the undoubtedly complicated linkage between MMR-induced DNA damage and p38 activation as well as to examine whether the signal for p38 activation emanates exclusively from the nucleus. Finally, the present studies suggest that the contribution of p38α to MMR-induced G2 arrest relies on phosphorylation of Cdc25 and Cdc2, but not necessarily on phosphorylation-activation of Chk1 or Chk2, both of which are phosphorylated at known sites of activation in the absence or presence of siRNA targeted to p38α. While these findings suggest that p38α may have direct effects on the downstream components of the G2 checkpoint, an equally valid explanation is that p38 may also stimulate Chk1/Chk2 activation by phosphorylation at other as yet undefined sites. As a whole, however, the cited studies suggest that the p38 pathway is a common route by which DNA damage and/or cellular perturbation is linked to cell cycle control, although it is clear from this and other studies that multiple variations of the pathway exist to deal with different types of cellular insults.

Unique pathways link MMR to different cell cycle checkpoints. While the present study has shown that MMR is linked to the G2 checkpoint via p38α, other studies have shown that the MMR system is also linked to other DNA damage-respon-
sive checkpoints. Most notable in this regard is the study of Brown et al. (8), in which a functional MMR system was shown to be critical for S-phase arrest initiated by infrared radiation. In that study, MMR was shown to be linked to activation of the S-phase checkpoint not by p38 but rather by DNA damage-associated activation of Chk2 and ATM. Specifically, the MMR protein MLH1 was shown to associate with ATM, an association that allowed phosphorylation of Chk2, which was in turn brought to the ATM/MLH1 complex by its association with the MLH1 partner MSH2. ATM is activated by methylating agent exposure (1), as are both Chk1 and Chk2 (24), suggesting that perhaps the same type of MLH-MSH-ATM-Chk2 associations that mediate infrared radiation-induced S-phase arrest also mediate G2 arrest following methylating agent exposure. We previously showed that pharmacologic inhibition of Chk1/2 with UCN-01 blocked TMZ-induced G2 arrest in a manner similar to that described in the present study for SB compounds (24), suggesting that an MMR-Chk connection might indeed contribute to methylating agent-induced G2 arrest. As shown in the present study, however, TMZ-induced Chk1 and Chk2 activation was not affected by SB compounds or by p38α siRNA, although the SB compounds and p38α siRNA were both independently capable of suppressing TMZ-induced activation of the G2 checkpoint. The p38 pathway that links MMR to G2 arrest therefore clearly differs from the pathway previously described to link MMR to S-phase arrest, although it remains possible that p38, like Chk2, is brought to sites of DNA damage for activation by other DNA damage-sensing proteins. Whether the Chk1-associated pathway that is activated after methylating agent exposure resembles the pathway that links MMR to S-phase arrest also remains unclear, as does whether the p38 pathway described in this study is also involved in the linkage of MMR to S-phase arrest. The answer to these questions undoubtedly lies in a better understanding of the interconnections between Chk2 and p38 pathways.

The p38 pathway influences cell fate and sensitivity to cytotoxic methylating agents. While p38α plays a key role in linking MMR to G2 arrest, the present results also show that the p38 pathway has a significant role in the ultimate response of human cells to methylating agent exposure. We previously reported that human glioma cells exposed to TMZ resolved G2 arrest in one of three ways. Prolonged G2 arrest is associated with the appearance of flattened cells that express SA-β-Gal activity and have lost the ability to reenter the cell cycle (23). Therefore, the longer the cells remain arrested, the less likely they are to resume growth. A percentage of cells, however, move past the G2 checkpoint and into mitosis prior to the onset of permanent growth arrest. The movement of these cells into mitosis is associated with the appearance of cells with multilobulated nuclei and a 4n DNA content characteristic of mitotic catastrophe (10, 26). As a third possible option, some cells were incubated with TMZ (3 h, 100 μM), after which cells were washed, plated, and monitored 10 days later for colony formation efficiency (A), the percentage of cells expressing SA-β-Gal activity (B), and the percentage of cells exhibiting aberrant nuclei characteristic of mitotic catastrophe (C). Values are means ± standard deviations derived from at least four independent experiments. **, values are statistically significantly different (P < 0.05) from TMZ-only values.

**FIG. 6.** Pharmacologic or genetic suppression of p38α sensitizes cells to the cytotoxic potential of TMZ, suppresses TMZ-induced senescence, and enhances TMZ-induced mitotic catastrophe. U87 cells and U87 cells in which p38α activity was suppressed by expression of p38α siRNA or by pre- and postincubation with SB203580 (20 μM) were incubated with TMZ (3 h, 100 μM), after which cells were washed, plated, and monitored 10 days later for colony formation efficiency (A), the percentage of cells expressing SA-β-Gal activity (B), and the percentage of cells exhibiting aberrant nuclei characteristic of mitotic catastrophe (C). Values are means ± standard deviations derived from at least four independent experiments. **, values are statistically significantly different (P < 0.05) from TMZ-only values.
appeared to be able to resolve G2 arrest and to go on to form viable colonies. The results of the present study show that inhibition of p38α suppressed the incidence of senescence, increased the incidence of mitotic catastrophe, and had a net effect of decreasing the percentage of cells that were able to successfully resolve G2 arrest. The effects of p38α inhibition on the incidence of senescence and mitotic catastrophe appeared to be direct consequences of the inability of the cells to undergo G2 arrest and avoid premature entry into mitosis. It may be possible, however, that p38-dependent pathways also control activation of the senescence program and that suppression of p38α directly suppresses senescence pathways. Similarly, p38 may influence the ability of cells with damaged DNA to survive mitosis. Enhancement of TMZ-mediated mitotic catastrophe following p38 inhibition may therefore be a consequence of both an increase in cells entering mitosis and a suppression of the ability of these cells to survive abnormal mitoses. In this regard, it is worth noting that Akt activation has been shown to suppress both activation of the G2 checkpoint and apoptosis following exposure of cells to 6-thioguanine (29). The p38 pathway has been suggested to interact with the Akt pathway in numerous ways (2, 11, 54), and it may be that suppression of p38α not only influences G2 checkpoint proteins, but also supresses the cytostrophic effects of Akt required for survival in mitosis. These possibilities warrant further investigation.

Perhaps the most significant aspect of the finding that p38 links MMR to the G2 checkpoint and cell survival in glioma cells exposed to cytotoxic methylating agents relates to the fact that cytotoxic methylating agents such as dacarbazine and TMZ itself are used for the therapy of a variety of solid tumors, including gliomas. Although TMZ is among the most effective agents for the treatment of gliomas, the response of these tumors varies widely and in ways that are not currently predictable. MMR mutations are rare in gliomas (28, 36), and most gliomas have modest levels of MGMT that can be depleted by TMZ alone (48), suggesting that resistance to cytotoxic methylating agents in gliomas is not related to lack of DNA damage, excessive repair of damage, or lack of MMR processing of O6MG. Rather, resistance may be a consequence of alterations of pathways that link DNA damage to the cellular response. There is no published literature with regard to p38 activation in gliomas, although it would be reasonable to suspect that tumors capable of activating the G2 checkpoint via the p38α pathway would be less sensitive than those that are incapable of doing so. Conversely, because inhibition of p38α sensitizes cells to TMZ, the present studies suggest that p38α may be a reasonable therapeutic target. In addition, because TMZ is frequently combined with other agents, including novel small-molecule inhibitors of tyrosine kinase receptors (51), interactions of the p38 pathway with other signaling pathways will also be critical for understanding the response of tumors to TMZ and to TMZ-based regimens. The present studies, by contributing to our understanding of the linkages between MMR and the G2 checkpoint, should not only facilitate our understanding of the p38 pathway, but also should prove useful in developing better cytotoxic methylating agent-based therapies for gliomas and other malignancies.

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