Rapid DNA Double-Strand Breaks Resulting from Processing of Cr-DNA Cross-Links by Both MutS Dimers

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Abstract
Mismatch repair (MMR) strongly enhances cytotoxicity of several chemotherapeutic agents and environmental carcinogens. DNA double-strand breaks (DSB) formed after two replication cycles play a major role in MMR-dependent cell death by DNA alkylating drugs. Here, we examined DNA damage detection and the mechanisms of the unusually rapid induction of DSB by MMR proteins in response to carcinogenic chromium(VI). We found that MSH2-MSH6 (MutSα) dimer effectively bound DNA probes containing ascorbate-Cr-DNA and cysteine-Cr-DNA cross-links. Binary Cr-DNA adducts, the most abundant form of Cr-DNA damage, were poor substrates for MSH2-MSH6, and their toxicity in cells was weak and MMR independent. Although not involved in the initial recognition of Cr-DNA damage, MSH2-MSH3 (MutSβ) complex was essential for the induction of DSB, micronuclei, and apoptosis in human cells by chromate. In situ fractionation of Cr-treated cells revealed MSH6 and MSH3 chromatin foci that originated in late S phase and did not require replication of damaged DNA. Formation of MSH3 foci was MSH6 and MLH1 dependent, whereas MSH6 foci were unaffected by MSH3 status. DSB production was associated with progression of cells from S into G2 phase and was completely blocked by the DNA synthesis inhibitor aphidicolin. Interestingly, chromosome 3 transfer into MSH3-null HCT116 cells activated an alternative, MSH3-like activity that restored dinucleotide repeat stability and sensitivity to chromate. Thus, sequential recruitment and unprecedented cooperation of MutSα and MutSβ branches of MMR in processing of Cr-DNA cross-links is the main cause of DSB and chromosomal breakage at low and moderate Cr(VI) doses.

Introduction
The primary function of mismatch repair (MMR) is to correct errors arising during DNA replication (1, 2). Detection of single base mispairs occurs through the binding of the MutSα complex (MSH2-MSH6 heterodimer; refs. 3, 4), which is also capable of recognizing one nucleotide insertion/deletion (4). The less-abundant MutSβ heterodimer (MSH2-MSH3) predominantly recognizes insertion/deletion loops containing two or more nucleotides (3, 4). After recognition and binding to mispaired DNA, MutSα/β recruit MutLα (MLH1-PMS2 heterodimer), and the MutS-MutL complex activates the excision of up to 1 kb of newly synthesized DNA to remove the mispairs (1, 2). Loss of MMR leads to highly elevated rates of spontaneous mutagenesis and is a cause of microsatellite instability found in 15% to 20% of human cancers (5). In addition to their role in correction of replication errors, MMR proteins are known to participate in the cytotoxic responses to several chemotherapeutic drugs including Sn3-type methylating agents (6, 7), cisplatin (8, 9), and halogenated nucleotides (10–12). The activation of cell death by these drugs involved the initial recognition of modified DNA bases by MutSα and a subsequent recruitment of MutLα. MutSβ has not been found to play any significant role in toxicity of alkylating agents or base analogues (13, 14). MMR-dependent processing of DNA damage leads to a delayed formation of DNA double-strand breaks (DSB) and these secondary lesions are important for apoptosis, cell cycle arrest, and cytogenetic damage (15–17). Direct signaling from DNA damage–bound MMR complexes has been proposed as an alternative model for the induction of cell death (5, 18, 19).

Another chemical that displays a MMR-dependent mechanism of cell death is the potent human carcinogen chromium(VI) (20, 21). Cr(VI) is a procarcinogen activated via reduction to Cr(III) by cellular ascorbate (Asc) and small thiols (22), which results in the formation of weakly mutagenic Cr-DNA adducts and strongly mutagenic reducer-Cr-DNA cross-links (22–25). The importance of MMR in Cr(VI) carcinogenesis is indicated by a very high frequency (>80%) of microsatellite instability in chromosome-induced human lung carcinomas (26, 27), suggested to result from selective outgrowth of resistant cells during chronic exposures to Cr(VI) (refs. 20, 28). Mechanistically, MMR seems to induce cell death in response to Cr-DNA damage differently than in the cases of other DNA-damaging agents. The initiation of apoptosis by MMR of Cr-treated cells was preceded by the formation of poorly repaired DSB that were induced almost immediately after Cr(VI) exposure (20, 29). This rapid DSB production is inconsistent with the model of futile MMR cycling in which DSB are not formed until the second S phase after a generation of single-strand breaks and gaps in the daughter DNA strand after the first S phase (15–17). Thus, in addition to the importance of mechanistic understanding of high genotoxicity of this widespread carcinogen, Cr(VI) can also serve as a tool to uncover novel functions of MMR proteins in responses to DNA damage.

In this work, we identified ternary Cr-DNA cross-links as high affinity substrates for recognition by MSH2-MSH6 dimer. Using an in situ fractionation approach and selective knockdowns of MMR proteins, we discovered the formation of MSH6 and MSH3 nuclear foci and a unique requirement for the MutSβ complex in induction of DSB and apoptosis by Cr(VI).
Materials and Methods

Cells and treatments. Human colon HCT116 (MLH1−/−) and DLD1 (MSH6−/−), lung epithelial H460, and lung fibroblast IMR90 cell lines were purchased from American Type Culture Collection (ATCC). HCT116+chr.3 (MLH1+) and DLD1+chr.2 (MSH6+) were gifts from Dr. T. Kunkel (NIH/NIEHS, Research Triangle Park, NC). Loading with Asc and measurements of its cellular concentrations by high performance liquid chromatography were performed as previously described (29, 30). Exposures to K₂CrO₄ [Cr(VI)] were for 3 h in serum-free medium. For clonogenic experiments, colonies were stained with Giemsa solution and counted 10 to 14 d later.

Cr-DNA modifications. Cr-DNA adducts were formed according to the previously described conditions (24, 25). Binary Cr-DNA adducts were generated by reacting 2 μg DNA with 0 to 80 μmol/L CrCl₃·H₂O in 25 mmol/L MES buffer (pH 6.0). Standard reaction mixtures for Cr-DNA binding during Cr(VI) reduction contained 2 μg DNA, 25 mmol/L MOPS buffer (pH 7.0), 0 to 150 μmol/L K₂CrO₄ and either 1 mmol/L Asc or 2 mmol/L cysteine. Reactions were incubated at 30°C for 30 min (CrCl₃ and Asc reactions) or 1 h (cysteine reactions). Unreacted Cr was removed by two passages through Bio-Gel P-30 (plasmids) or P-6 (oligos) columns. DNA-bound Cr was measured by graphite furnace atomic absorption spectrometry (30).

DNA-protein pulldown assay. Nuclear extracts were prepared as described (31) with the addition of a protease inhibitor cocktail (Roche) to the lysis buffer. The sense oligo, 5'-GGGAGAAGCTCACGCCCCAGTGTTCAAGGGGCTGGCAGCTTC-3', was modified with a 5' biotin label. The antisense sequence for the homoduplex contained normal Watson-Crick base pairing and antisense sequence for the heteroduplex probe contained one G/T mismatch in the center of the sequence, 5'-GAGCATAGGAGGCTGAAGAGAAGGCTGGCAGCTTCCC-3'. Nuclear extracts (100 μg) were preincubated with 0.1 μg of nonbiotinylated homoduplex and 5 μg poly DEDC in 1× DNA binding buffer [12% glycerol, 20 mmol/L HEPES (pH 7.9), 100 mmol/L KCl, 1 mmol/L DTT, 5 mmol/L MgCl₂] for 5 min at 4°C. Next, 100 ng biotinylated duplex were added to the reaction for 30 min. DNA-protein complexes were captured by incubation with 80 μl streptavidinagarose beads for 2 h at 4°C. Beads were washed 4 times with 1 mL of 1× DNA binding buffer, resuspended in 30 μL of 2× SDS loading buffer and bound proteins were released by boiling.

Western blotting. Cells were collected by scraping, washed twice with cold PBS and then resuspended in a lysis buffer supplemented with protease and phosphatase inhibitors (20). Proteins were separated by SDS-PAGE and electrotransferred onto ImmunoBlot polyvinylidene difluoride membranes. Primary antibodies for MLH1, MSH2, MSH3, and MSH6 were from Pharmingen. Protein bands were visualized using horseradish peroxidase–conjugated secondary antibodies.

Plasmid replication in cells. Control and Cr-modified pSP189 plasmids (M. Seidman, NIH/NIA, Baltimore, MD) were transfected into HCT116 and HCT116+chr.3 and allowed to replicate for 48 h. The replicated plasmids were isolated by the Mini-prep kit from Qiagen. DNA was ethanol precipitated, dissolved in deionized water, and electroporated into Escherichia coli MBL50 strain. Transformed E. coli cells were plated on minimal agar plates containing 30 μg/mL of ampicillin and 0.5 μg/mL of chloramphenicol to assess the yield of replicated plasmids.

Knockdown experiments. Stable depletion of protein levels was achieved by expression of short hairpin RNA (shRNA) from the pSUPER-RETRO vector. The plasmid was linearized with HindIII and BglII to permit the insertion of the annealed oligonucleotides directed toward the mRNA of interest. The targeting vectors for MSH2, luciferase (29, 30), MLH1 (32), and infection conditions (33) have already been described. MSH6- and MSH3-targeting vectors were constructed using 5'-gattccgactctcgtagccccaggaatctagacaagaccttctcagagggatcacctttttggaaa and 5'-gatccgactctcgtagccccaggaatctagacaagaccttctcagagggatcacctttttggaaa oligos, respectively. Vector-expressing cells were selected in the presence of 600 μg/mL G418.

MLH1 complementation. Mllh1 fragment carrying MLH1 gene was excised from pCMV-SPORT6 (ATCC) and inserted into the pQCXIN retroviral vector opened with EcoRI. Viral particles were packaged in 293T cells by cotransfection with plasmids encoding VSV-G envelope protein and MoMuLV gag-pol (33). HCT116 cells were infected with pQCXIN-MLH1 and control vectors and then selected with 600 μg/mL G418.

Microsatellite instability assay. Cells were seeded in 60-mm dishes and allowed to attach overnight prior to retroviral infection with the pCXpar/CA17/out-of-frame plasmid (gift from Dr. N. Kato, Okayama University, Okayama, Japan; ref. 34). Infected cells were cultured for 7 to 10 d (3 passages) and then selected with 2 μg/mL puromycin at a density of 1 × 10⁶ cells per 100-mm dish. Mutation frequency was calculated by dividing the number of pyrimycin resistant colonies per 10⁶ clonable cells.

Microscopy. Micronuclei were scored at 48 h post-Cr as previously described (29). For confocal immunofluorescence, cells were grown on

Figure 1. Recognition of Cr-DNA adducts by MMR proteins. Western blots are representative of three independent experiments for each adduct. Data for Cr-DNA adducts are means from four determinations. A, DNA-protein pulldown assay and its validation with HeLa extracts incubated with normal (G/C) or mismatched (G/T) duplexes. B, binding of MSH2 from HeLa extracts to oligos containing Cr-DNA adducts [Cr(III) reactions] or mixtures of Cr-DNA adducts and reducer-Cr-DNA cross-links [Cr(VI)+cysteine or Cr(VI)+Asc reactions]. C, pulldowns of MSH2 and MSH6 proteins using nuclear extracts from HeLa, DLD1, and DLD1+chr.2 (MSH6−/−) cells. D, replication efficiency of Cr-modified pSP189 plasmids in MLH1−/− and MLH1+ cells. Plasmids were replicated in HCT116 (MLH1−/−) and HCT116+chr.3 (MLH1+) cells for 48 h, and the yield of replicated plasmids was determined by bacterial transformation assay. Cr(III) plasmids were reacted with Cr(III) yielding Cr-DNA adducts; Cr(VI)+Asc plasmids were modified during reduction of Cr(VI) with Asc, which generated both Cr-DNA adducts and Asc-Cr-DNA cross-links. Points, mean of four independent transfections; bars, SD.
Superfrost Plus slides, washed twice with cold PBS and extracted at 4°C for 10 min in a modified cytoskeleton buffer (33). Slides were then fixed with 2% paraformaldehyde in PBS for 15 min, extracted with 1% Triton X-100 for 15 min at room temperature, and incubated with 2% fetal bovine serum for 30 min at 37°C in a humidified chamber. Double labeling was performed by simultaneous incubation of primary antibodies for 2 h at 37°C. Slides were then fixed with 2% paraformaldehyde in PBS for 15 min, extracted with 1% Triton X-100 for 15 min at room temperature, and incubated with 2% fetal bovine serum for 30 min at 37°C in a humidified chamber. Double labeling was performed by simultaneous incubation of primary antibodies for 2 h at 37°C. Slides were washed thrice with PBS for 5 min followed by incubation with Alexa-Flour 488–conjugated anti-mouse IgG and Alexa-Flour 568–conjugated anti-rabbit IgG secondary antibodies for 1 h at room temperature. Slides were washed 4 times with PBS for 5 min and mounted with Vectashield hard set mounting medium containing 4,6-diamidino-2-phenylindole. Fluorescence images were recorded with a Zeiss Axiovert 100 confocal microscope and analyzed by Phoenix and Metamorph software. Slides were always coded and scored in a blind manner.

**Pulsed-field gel electrophoresis.** Pulsed-field gel electrophoresis (PFGE) plugs were prepared using Bio-Rad Mammalian CHEF Genomic Plug kit at 1.0 x 10^6 cells/plug. Cells were digested in proteinase K buffer [100 mmol/L EDTA, 1% N-laurylsarcosyl, 10 mmol/L Tris (pH 8.0), 1 mg/mL, proteinase K] at 20°C for 24 h and then washed 5 times in a detergent-free wash buffer. PFGE was performed using a 1% gel (Pulsefield Certified Megabase Agarose; Bio-Rad) in 0.5X Tris-borate EDTA buffer on a CHEF Mapper XA Pulsed Field Electrophoresis System (Bio-Rad) for 18 h under the following conditions: 120° field angle, 240 s switch time, 4 V/cm, 14°C. Gels were stained with ethidium bromide.

**Results**

**MSH2-MSH6 dimer recognizes Cr-DNA cross-links in vitro.** To examine whether MMR proteins are able to recognize Cr-DNA damage, we devised a DNA-protein pulldown assay. Biotinylated oligos were incubated with nuclear extracts, pulled down with streptavidin beads, and bound proteins were eluted and analyzed by immunoblotting. Validation experiments confirmed the ability of biotinylated DNA containing a single G/T mismatch to pull down both MSH2 and MSH6 from HeLa extracts (Fig. 1A). Cr-DNA binding does not display apparent base or sequence specificity leading to a near random distribution of adducts (23, 35). As adjacent bases exert strong effects on the ability of MMR complexes to bind their substrates (19, 36), analysis of randomly damaged templates offers a benefit of examining multiple sequence contexts. In our initial experiments, we monitored binding of MSH2, a component of both MutSα and MutSβ mismatch-sensing complexes. Oligos containing solely binary Cr-DNA adducts showed reproducible binding of MSH2 but only at a high density of modifications (~7 Cr/oligo; Fig. 1B). However, DNA probes modified in the presence of Cr(VI)-cysteine displayed a strong binding of MSH2 at 2.7 Cr/oligo. Oligos modified in the reaction of Cr(VI) with its dominant cellular reducer Asc pulled down MSH2 at both 1.3 and 3.2 Cr/probe (Fig. 1B). We have previously determined that Cr(VI)-cysteine reactions generated approximately equal numbers of Cr-DNA adducts and cysteine-Cr-DNA cross-links (37). Cr(VI)-Asc reactions yielded ~25% Asc-Cr-DNA cross-links and 75% Cr-DNA adducts (30).

We next examined the role of MSH2-MSH6 and MSH2-MSH3 complexes in recognition of Cr-DNA adducts. Oligos modified during Cr(VI) reduction with cysteine or Asc pulled down MSH6
from HeLa nuclear extracts (Fig. 1C). We were unable to detect the presence of MSH3 in HeLa pulldowns (data not shown). To further test a potential involvement of MSH3, we analyzed DNA adduct–binding activity of nuclear extracts from DLD1 (MSH6+/−) and DLD1+chr.2 (MSH6+/+) cells. Although MSH2 was pulled down by Cr-DNA damage from MSH6+ extracts, there was no MSH2 binding in MSH6−/− extracts (Fig. 1C). Similarly to detection of base mismatches, recognition of Cr-DNA damage by MutSα was independent of MLH1 as nuclear extracts from MLH1-null HCT116 cells showed normal binding of MSH2 to Cr/Asc-modified DNA probes (Fig. 1C). Because lack of MLH1 makes cells resistant to Cr(VI) cytotoxicity (20, 21), this protein seems to act in the genotoxic responses downstream of MutSα-mediated recognition of Cr-DNA adducts. To test whether the differences in binding by MSH2-MSH6 accurately predict genotoxic responses in cells, we examined replication inhibition by Cr-DNA modifications at levels equivalent to 0.8 to 2.2 Cr/pulldown oligo. In agreement with MutSα binding affinity, plasmids treated with Cr(VI)+Asc but not with Cr(III) displayed a strong MMR-dependent replication inhibition (Fig. 1D).

**Both MutS dimers are required for the induction of cell death by Cr-DNA damage.** To test the importance of MMR complexes in cellular responses to Cr(VI), we created stable knockdowns of different MMR proteins in H460 cells (Fig. 2A). shRNA-mediated depletions of MLH1, MSH3, and MSH6 were highly specific, whereas knockdown of MSH2 was also accompanied by decreased stability of its partners MSH3 and MSH6. Surprisingly, loss of MSH3 suppressed clonogenic lethality of Cr(VI) to the same degree as did loss of MSH6, MSH2, or MLH1 (Fig. 2B). The same result was observed in cells supplemented with physiologic 1 mmol/L Asc, the most important Cr(VI) reducer in vivo (22) with barely detectable concentrations in H460 (21) and other cultured human cells (29, 30). Potentiating effects of Asc on Cr(VI) toxicity are completely blocked by MMR
inactivation (21, 29), which makes preloading of cells with this vitamin a very useful tool for a selective enhancement of the MMR-dependent toxic responses. Silencing of MSH3 or other MMR proteins all inhibited Cr(VI)-induced apoptosis as evidenced by decreased PARP cleavage (Fig. 2C). MSH3 depletion had no effect on clonogenic lethality of two chemicals with MSH6- but not MSH3-dependent toxicity (13, 14), N'-methyl-N'-nitrosoguanidine (MNNG) and 6-thioguanine (Fig. 2D), demonstrating that the involvement of MSH3 in MMR-activated cell death was specific to Cr-DNA damage. Stable knockdowns of MSH3 and MSH6 in primary lung IMR90 fibroblasts were also equivalent in suppression of all Cr(VI)-induced cytotoxic effects (data not shown).

Both MSH6 and MSH3 are involved in DSB formation. The inhibition of all forms of cytotoxicity by silencing of MSH3 or MSH6 suggested that both proteins acted very early in toxic processing of Cr-DNA damage. Therefore, we examined the role of MSH6 and MSH3 in the generation of a DSB-specific marker γH2AX foci (38) that appear early after Cr-DNA damage and precede the activation of apoptotic responses (20, 29). We found that knockdowns of MSH3, MSH6, or MLH1 all abrogated the formation of γH2AX foci in Cr(VI)-treated IMR90 cells (Fig. 3A). As expected, supplementation of IMR90 cells with 1 mmol/L Asc strongly enhanced the production of γH2AX foci by Cr(VI) but silencing of MSH3 or MSH6 eliminated the induction of this DSB marker. Knockdown of MSH3 or MSH6 also completely suppressed the formation of micronuclei by 0.5 and 1 μmol/L Cr(VI; Fig. 3B), indicating that processing of Cr-DNA damage by MutS dimers was responsible for chromosomal breakage by doses below the current US-EPA water standard of 100 ppb (1.92 μmol/L) chromium. PFGE further confirmed that both MSH6 and MSH3 were required for DSB induction by Cr(VI; Fig. 3C).

To investigate whether MMR proteins were present at the sites of DNA breaks, we examined the colocalization of MSH3 and MSH6 with γH2AX foci. After testing several different extraction conditions, we found that permeabilization in the CSK buffer before paraformaldehyde fixation was effective in removal of nucleoplasmic MMR proteins and it also revealed the formation of MSH3 and MSH6 foci in Cr(VI)-treated but not control IMR90 cells (Fig. 3D). Approximately 30% to 40% of γH2AX foci-containing cells showed colocalization with MSH3 foci. The frequency of γH2AX foci–positive cells displaying colocalization with MSH6 foci averaged 85% to 90%. We also found a high degree of colocalization of MSH6 foci with 53BP1 foci (data not shown), another marker of DSB (39). The colocalization of MSH3 and MSH6 to DSB foci further supports a causal role of both proteins in breakage of Cr-adducted DNA.

Dependence of MSH3 and MSH6 foci on other MMR proteins. Consistent with its lack of participation in recognition of Cr-DNA damage by MutSα in pulldown experiments, MSH3 depletion had no significant effect on the formation of MSH6 foci in Cr(VI)-treated IMR90 cells (Fig. 4A). In contrast, MSH6 was absolutely required for the production of MSH3 foci in Cr(VI)-treated cells (Fig. 4B). These results indicate that MutSα complex is involved in the initial detection of Cr-DNA damage in cells and MSH3-containing MutSβ complex is recruited later. The majority of MSH6 foci-containing cells also had MSH3 foci and vice versa. For example, 80.9% of MSH6 foci–containing cells contained MSH3 foci and 90.4% of MSH3 foci–containing cells had MSH6 foci at 6 hours after 10 μmol/L Cr exposure of 1 mmol/L Asc-preloaded IMR90 cells. MLH1 depletion in primary IMR90 cells resulted in a moderately lower frequency of MSH6 foci-containing cells (Fig. 4C) and completely abrogated MSH3 foci (Fig. 4D).

Cell cycle specificity and effect of replication on MSH3 and MSH6 foci. Because the majority of Cr-induced DSB are found in G2 cells (20, 29), we asked whether the formation of MMR foci also exhibits cell cycle specificity. S phase cells were labeled by BrdUrd incorporation, whereas G2 cells were identified by cyclin B1 staining. Our detergent extraction procedure retained cytoplasmic cyclin B1 due to the stability of its binding to microtubules (40). Essentially no cells displayed both BrdUrd incorporation and MSH6/MSH3 foci either immediately or 6 hours after Cr(VI) exposure (Fig. 5A). A large percentage of cells containing MSH6

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Effect of MMR proteins on the induction of MSH3 and MSH6 foci by Cr-DNA damage in IMR90 cells. A, frequency of MSH6 foci-containing cells expressing non-specific (Luc) or MSH3-targeting shRNA. Cells were analyzed at 6 h post-Cr exposure (−Asc, no Asc loading; +Asc, 1 mmol/L Asc supplementation before Cr treatments). Points, means for 3 slides with >100 cells counted per slide; bars, SD. B, frequency of MSH3 foci–containing cells expressing non-specific or MSH6-targeting shRNA. Points, means for 3 slides with >100 cells counted per slide; bars, SD. C, formation of MSH6; D, MSH3 foci in MLH1–depleted cells (1 mmol/L Asc-supplemented cells; points, mean for three slides; bars, SD).
foci and MSH3 foci also expressed the G2 phase marker cyclin B1 (Fig. 5D).

The role of replication in the formation of MMR foci was examined in two series of experiments with the DNA polymerase inhibitor aphidicolin. In the first set, there was no replication of Cr-adducted DNA at any time due to the presence of aphidicolin during and after Cr(VI) treatments. In the second set, replication was only inhibited during Cr exposure and then the aphidicolin block was removed. We found that even in the absence of any replication, Cr-DNA damage resulted in a significant formation of MSH6 and MSH3 foci immediately after Cr(VI) exposure and their levels remained constant during 4 hours post-Cr incubations (Fig. 5C). Release of the replication block after Cr exposure resulted in a progressively higher number of MSH6/3 foci–containing cells with time and a parallel increase in the number of MMR foci/cyclin B1–double positive cells (Fig. 5C). Unlike MSH6/3 foci, replication arrest by aphidicolin completely blocked the formation of DSB (Fig. 5D), pointing to the importance of the completion of DNA replication and progression from S into G2 for DNA breakage by MMR.

Different responses in HCT116 cells expressing MLH1 via vector or chromosome 3 complementation. We have previously found that MLH1-null HCT116 cells had much greater resistance to Cr(VI) and lower DSB induction than their counterparts complemented with MLH1 via chromosome 3 transfer (20). However, HCT116 cells have also been reported to lack functional MSH3 due to a −1 truncation mutation in the (A)8 repeat of exon 7 (41). This raised the important question of how chromosome 3 transfer increased sensitivity of HCT116 cells to Cr(VI) because MSH3 was clearly required for Cr-induced cytotoxicity and DSB production in IMR90 and H460 cells. Formation and repair of Cr-DNA adducts were identical in HCT116 and HCT116+chr.3 cells (20), excluding the possibility that chromosome complementation altered Cr(VI) metabolism or persistence of adducts. We sequenced exon 7 of MSH3 gene from our HCT116 lines and confirmed the presence of the previously reported truncation mutation in the (A)8 repeat. MLH1 silencing in HCT116+chr.3 cells restored their resistance to Cr(VI) (Fig. 6A), confirming MMR-dependence of cell death in Cr-treated HCT116+chr.3 cells. Because chromosome 3 introduced more genes than just MLH1, we infected HCT116 cells with the pQCXIN-MLH1 retroviral vector and selected several clones that expressed MLH1 at the same level as HCT116+chr.3 cells. As expected, expression of functional MLH1 in HCT116 cells was accompanied by stabilization of its partner PMS2 (Fig. 6B). Although all MLH1-expressing clones were highly sensitive to MNNG, a chemical that induces MMR-dependent toxicity without MSH3 involvement (14), they showed no sensitization to Cr(VI) (Fig. 6C). Because repair of 2 nt-long insertion/deletion loops is primarily dependent on MutSα complex would be expected to display a weak suppression of frameshift mutagenesis at dinucleotide repeats. We found that two tested clones complemented with pQCXIN-MLH1 showed only modest

Figure 5. Cell cycle specificity of MSH3 and MSH6 foci formation in primary IMR90 cells. Cells were preloaded with 1 mmol/L Asc before Cr(VI) treatments. Points, mean for at least 3 slides with >100 cells scored per slide; bars, SD. A, absence of MSH6 and MSH3 foci in BrdUrd-labeled cells at 0 and 6 h post-Cr exposure. MSH6 (MSH3), total frequency of cells with MSH6 (MSH3) foci; dual, BrdUrd-labeled cells with MSH6 or MSH3 foci. B, frequency of MSH6 and MSH3 foci-containing cells expressing cyclin B1. MSH6 (MSH3), total number of cells with MSH6 (MSH3) foci; dual, cells with foci and cyclin B1 expression. C, effect of replication inhibition on the formation of MSH6 and MSH3 foci in Cr-treated cells. Cells were preloaded with 1 mmol/L Asc and then treated with Cr(VI) in the presence of 1 μmol/L aphidicolin (added 15 min before Cr). After Cr exposure, aphidicolin was either removed or added again (+Aph). MSH6/MSH3, total frequency of foci-containing cells; dual, foci-containing cells with cyclin B1 expression. D, PFGE of DNA from IMR90 cells collected at different times after exposure to 10 μmol/L Cr(VI). Cells were preloaded with 1 mmol/L Asc and exposed to Cr(VI) in the presence (+Aph) or absence (−Aph) of aphidicolin. In +Aph samples, aphidicolin was also present during post-Cr incubations.
2.5- and 6.7-fold reductions in frameshift mutagenesis at CA repeats relative to the vector control (Fig. 6B, right). CA dinucleotide instability in two other clones was as high as in parental HCT116 cells (not shown). In contrast, chromosome 3 transfer completely restored CA repeat stability, with the frequency of puromycin-resistant clones decreasing 190-fold from 38.2/C210/C2 to 0.2/C210/C2 for HCT116 to HCT116+chr.3 cells. Thus, HCT116+chr.3 cells seemed to acquire the ability to compensate for the loss of functional MSH3, as evidenced by their sensitivity to Cr(VI) and highly efficient repair of slippage errors in dinucleotide sequences.

Introduction of one extra chromosome into diploid human cells has been found to induce large alterations in the transcriptome of the trisomic and other cellular chromosomes (43), and this could have created a new loop-processing activity or somehow enhanced affinity of MutSα to the substrates that are typically recognized by MutSβ. Despite the involvement of the alternative MSH3-like activity, HCT116+chr.3 cells (20) faithfully recapitulated toxic responses of H460 and primary human cells to Cr-DNA damage (21, 29).

Discussion

Pulldown experiments with Cr-modified probes and formation of MSH6 foci in Cr-treated cells in the absence of DNA replication indicated that MSH2-MSH6 dimer acted as a sensor of Cr-DNA damage. Binding of MutSβ complex to Cr-adducted DNA was independent on MSH3 and MLH1, demonstrating that the initial recognition of Cr-DNA damage was very similar to the detection of single base mismatches (1, 2). Reduction of Cr(VI) by Asc or cysteine created a combination of Cr-DNA adducts and...
dependent recruitment of endonucleases could lead to the recruitment of endonucleases (46). In the case of Cr-damaged DNA, MSH3 dimer is known to participate in rejection of imperfectly matched DNA loops with stranded gaps generated by MutSβ in single-stranded regions containing repetitive sequences could lead to inappropriate cleavage of recombination products and formation of toxic DSB.

A combination of our findings with phase-specific markers and replication-arrested cells indicates that the formation of Cr-DNA damage–processing MMR complexes occurs in late S phase. Normal initial formation of MSH6 foci in replication-arrested cells and then parallel increases in the total number of MSH6 foci and MSH6 foci/cyclin B1–double positive cells after removal of replication block are indicative of a relatively rapid progression of MSH6 foci–containing cells into G2 phase. MMR-promoted DSB foci were predominantly found in G2 cells but inhibition of DNA synthesis by aphidicolin completely blocked DSB induction. Thus, accumulation of MSH6 foci and DSB after Cr(VI) exposure seems to require not replication but the progression of cells through late S into early G2 phase, which creates the permissible conditions for cleavage of both DNA strands. S phase cells display higher thresholds for checkpoint activation relative to G2 cells (47) and increased sensitivity to DNA damage–induced signaling in the late S phase could be one contributing factor to the formation of MSH6 foci at this stage of cell cycle. Another important event occurring in late S phase is the induction of exonuclease-1 expression (48), which is a critical MMR component that has recently been found to be necessary for cell death by the MMR-dependent toxicant 6-thioguanine (49).

Recent risk assessment studies have shown that even Cr(VI) exposures not exceeding a new, 10 times lower permissible limit could result in as many as 45 additional cancer deaths per 1,000 workers (50). These risks are striking considering relatively modest amounts of inhaled Cr(VI) and high rates of Cr-DNA damage removal by nucleotide excision repair (30). A rapid activation of both MMR branches apparently outcompetes beneficial repair processes and is the principal cause of chromosomal breaks at low chromate doses. Drugs producing DNA adducts with similarly rapid MMR processing into toxic DSB should offer greater clinical efficacy due to less dependence on repair deficiency of cancer cells and no need for persistence of adducts until second S phase, which is a requirement for the currently used S1 alkylating agents (17).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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