Mismatch repair (MMR) proteins participate in cytotoxicity induced by certain DNA damage-inducing agents, including cisplatin (cis-diaminedichloroplatinum(II), CDDP), a cancer chemotherapeutic drug utilized clinically to treat a variety of malignancies. MMR proteins have been demonstrated to bind to CDDP-DNA adducts and initiate MMR protein-dependent cell death in cells treated with CDDP; however, the molecular events underlying this death remain unclear. As MMR proteins have been suggested to be important in clinical responses to CDDP, a clear understanding of MMR protein-dependent, CDDP-induced cell death is critical. In this report, we demonstrate MMR protein-dependent relocation of cytochrome c to the cytoplasm and cleavage of caspase-9, caspase-3, and poly(ADP-ribose) polymerase upon treatment of cells with CDDP. Chemical inhibition of caspases specifically attenuates CDDP/MMR protein-dependent cytotoxicity, suggesting that a caspase-dependent signaling mechanism is required for the execution of this cell death. p53 protein levels were up-regulated independently of MMR protein status, suggesting that p53 is not a mediator of MMR-dependent, CDDP-induced death. This work is the first indication of a required signaling mechanism in CDDP-induced, MMR protein-dependent cytotoxicity, which can be uncoupled from other CDDP response pathways, and defines a critical contribution of MMR proteins to the control of cell death.

The MMR system of proteins plays a role in diverse cellular processes, perhaps most notably in preserving genomic integrity by recognizing and facilitating the repair of post-DNA replication base pairing errors. Recognition of these errors and recruitment of repair machinery is performed by the MutSα complex (consisting of the MMR proteins MSH2 and MSH6) or MutSβ complex (consisting of MSH2 and MSH3). Defects in MMR proteins render cells hypermutable and promote microsatellite instability, a hallmark of MMR defects. MMR protein defects are found in a wide variety of sporadic cancers, as well as in hereditary non-polyposis colorectal cancer (1).

In addition to their role in DNA repair, MMR proteins also play a role in cytotoxicity induced by specific types of DNA-damaging chemotherapeutic drugs, such as CDDP, which is utilized clinically to treat a number of different cancer types. MutSα recognizes multiple types of DNA damage, including 1,2-intrastrand CDDP adducts and O6-methylguanine lesions (2). Treatment of cells with compounds that induce these types of lesions, including CDDP and methylating agents such as N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), results in MMR protein-dependent cell cycle arrest and cell death (3–7). This suggests that MMR proteins, in addition to their role in DNA repair, are also capable of initiating cell death in response to certain types of DNA damage.

Cells treated with DNA-damaging agents frequently activate an apoptotic cell death pathway mediated by the mitochondria. This intrinsic death signaling pathway predominantly involves the coordinated activity of two groups of proteins: pro-death members of the Bcl-2 family that control the integrity of mitochondrial membranes, and members of the caspase family of cysteiny1 proteases that proteolytically cleave intracellular substrates, giving rise to apoptotic morphology and destruction of the cell (8, 9). Pro-death Bcl-2 family members, such as Bax and Bak, target the outer mitochondrial membrane and cause the cytosolic release of pro-death factors residing within the mitochondria of unstressed cells (8). Predominant among these factors is cytochrome c, whose cytoplasmic localization results in the formation of a caspase-activating platform known as the apoptosome (10). This complex includes the adaptor protein Apaf-1, and when formed the apoptosome promotes the cleavage and activation of caspase-9 (11, 12). Once activated, this apical caspase proceeds to cleave and activate caspase-3, the predominant effector protease of apoptosis.

A significant amount of evidence has been gathered illustrating MMR protein-dependent pro-death signaling in response to methylating agents (13–16, 3). In contrast, the MMR protein-dependent cytotoxic response to CDDP is largely unknown, with only the p53-related transactivator protein p73 and the c-Abl kinase clearly implicated as potential mediators of CDDP/MMR protein-dependent cell death in human cells (17, 18). Interestingly, ATM, Chk1, Chk2, and p53, which are acti-
Mismatch Repair Protein-dependent Response to Cisplatin

vated in an MMR protein-dependent manner after treatment of cells with MNNG (3, 13), are not involved in the MMR-dependent response to CDDP (7, 17). In addition, the magnitude of MMR protein-dependent cell death induced by methylating agents and CDDP differs (4). These findings suggest that unique signaling pathways may be engaged by MMR proteins depending upon the type of recognized lesion. As such, there is a requirement for further study of the molecular events underlying MMR protein-dependent cell death and cell cycle arrest for each type of recognized DNA lesion. This is particularly relevant in the case of CDDP, as evidence from a limited number of retrospective clinical studies suggests that MMR proteins play an important role in patient response to CDDP. Several studies examining immunohistochemical staining against MSH2 or MLH1 have demonstrated that levels of these proteins are reduced in ovarian and esophageal tumor samples following CDDP-based chemotherapy (19, 20). Low levels of MMR protein post-chemotherapy seem to be predictive of lower overall survival in a certain subset of tumors (esophageal cancer), but not others (ovarian and non-small cell lung cancer) (19–21). Two recent studies examining MMR protein levels and microsatellite instability in germ cell tumors from patients receiving platinum-based chemotherapy have suggested a prognostic value for pre-chemotherapy MMR protein status in these tumors (22, 23). This potential clinical relevance underscores the need for a greater understanding of MMR protein-dependent mechanisms of CDDP-induced cell death.

In this study, we report that CDDP induces an MMR protein-dependent decrease in cell viability and MMR protein-dependent signaling in the form of cytochrome c release to the cytoplasm and cleavage of caspase-9, caspase-3, and PARP. Chemical inhibition of caspases specifically attenuates CDDP/MMR protein-dependent loss of cell viability, indicating a requirement for caspase activation in this process and uncoupling MMR protein-dependent cytotoxic signaling from other CDDP response pathways. Additionally, the CDDP-induced, MMR protein-dependent cytotoxic response is independent of p53 signaling. Our results demonstrate for the first time an MMR protein-dependent pro-death signaling pathway in cells treated with CDDP.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Chemicals**—Human endometrial carcinoma cell lines HEC59, HEC59(2), HHUA, and HHUA(2) and human colon cancer cell lines HCT116 and HCT116(2) cells were a gift from T. Kunkel (NIEHS, National Institutes of Health). Their establishment and characterization has been previously described (24–27). HEC59, HHUA, and HCT116 cells were maintained in Dulbecco’s modified Eagle’s medium/F-12 media (Invitrogen) containing 10% fetal bovine serum, whereas HEC59(2), HHUA(2), and HCT116(2) were maintained in Dulbecco’s modified Eagle’s medium/F-12 media containing 10% fetal bovine serum and 400 μg/ml G418. All cell lines were maintained at 37 °C in a 5% CO2 atmosphere. For experiments, all cells were plated in growth media without antibiotic. Cisplatin (Sigma) was dissolved in DMSO to a stock concentration of 20 mM and stored in single-use aliquots at −80 °C in the presence of a desiccating agent. Staurosporine was purchased from Sigma and stored at −20 °C. All treatment agents were further diluted in media prior to administration to cells. Propidium iodide (PI) (Sigma) was dissolved in PBS to a concentration of 0.25 mg/ml and stored protected from light at 4 °C.

**Assessment of Cell Viability**—Cell viability was assessed by either 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay or PI exclusion assay. For MTT assays, 3 × 10^5 cells/well were seeded into wells of 96-well plates and incubated overnight before treatment with indicated drugs. At various times following treatment, MTT assays were performed using the Cell Proliferation Kit I (Roche Applied Science) as per manufacturer instructions. Plates were read with a plate reader capable of utilizing wavelengths of 560 nm (MTT formazan product absorbance wavelength) and 650 nm (reference wavelength) and analyzed with SoftMax software. To calculate percent viability, raw absorbance values from 650 nm were subtracted from those obtained at 560 nm, and averaged plate blank (media alone) values were subtracted from these values. Resulting values from triplicate wells were averaged to generate a final raw absorbance value. Untreated cells were considered 100% viable, and untreated final raw values were divided by final raw values obtained from treated wells to yield percent viability. Each experiment was performed at least in triplicate.

For PI exclusion assay, cells were seeded at 2 × 10^5 cells/well into six-well plates and incubated overnight before treatment with drugs. At various times following treatment, cells were harvested by trypsinization, transferred with media into 5-ml round-bottom tubes, and centrifuged at 250 × g. Media was removed, and the pellet was resuspended in PBS containing 2 μg/ml PI. A BD FACSCalibur flow cytometer was used to obtain PI fluorescence (FL3-H channel) and light scatter (FSC-H) values for 2 × 10^4 events/well. WinList (Verity) software was utilized to plot event values and analyze viability. FL3-H values were plotted against FSC-H values and an arbitrary region was drawn around the bulk of events obtained from untreated samples (≥75% of total events). Events within this region were considered viable cells. The region was maintained and applied to events from untreated and treated samples. Resulting values from duplicate wells were averaged to generate an average of events within the “viable region.” Untreated samples were considered 100% viable, and untreated values were divided by values obtained from treated wells to yield percent viability. Each experiment was performed at least in triplicate. Graphing and statistical calculations for all cell survival assays were performed using Prism software (GraphPad).

**Antibodies**—Anti-MSH2 antibodies were purchased from BD Pharmingen (610360) or Santa Cruz Biotechnology (sc-494). Anti-cleaved caspase-3 (9661) and -cleaved caspase-9 (9505) antibodies were purchased from Cell Signaling Technology. Anti-PARP (556494) and -cytochrome c (556433) antibodies were purchased from BD Pharmingen. Anti-β-actin (A2066) antibody was purchased from Sigma. Anti-p53 (OP43T) antibody was purchased from Calbiochem. Anti-HSP60 (sc-13115) antibody was purchased from Santa Cruz.
Biotechnology. Goat anti-mouse (170-6515) and -rabbit (170-6516) secondary antibodies were purchased from Bio-Rad.

Isolation of Whole Cell or Cyttoplasmic Extracts and Immunoblot Analysis—Cells were plated at 4 × 10^5 cells/dish in 60-mm dishes, incubated overnight, and treated with indicated drugs. To obtain whole cell lysates, at various times post drug treatment cells were scraped into media, which was transferred to a conical tube on ice and centrifuged at 250 × g. Cells were then washed with PBS and transferred to a microcentrifuge tube, centrifuged at 1000 × g, and the pellet was stored at −80 °C at least overnight. The cell pellet was resuspended in lysis buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1× Complete Protease Inhibitor (Roche Diagnostics)), sonicated, and centrifuged at 18,000 × g to remove debris. The supernatant containing cellular proteins was then removed to a clean microcentrifuge and stored at −80 °C until use. To obtain cytoplasmic fractions, cells were harvested at various times post drug treatment by trypsinization, transferred with media into a conical tube, and centrifuged at 250 × g. Cells were washed with PBS, transferred to a microcentrifuge tube, and centrifuged at 1000 × g. The pellet was resuspended in extraction buffer (250 mM sucrose, 70 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 200 μg/ml digitonin and 1× Complete Protease Inhibitor, all in PBS). Cells were then incubated on ice 10 min and centrifuged at 1000 × g, and supernatant containing cytoplasmic proteins was removed to a clean microcentrifuge tube and stored at −80 °C until use. For immunoblot analysis, extracts were loaded onto either 4–20% or 16% tris-glycine pre-cast gels (Invitrogen), and proteins were separated by SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membranes (Millipore), and the membranes were incubated in blocking buffer and treated with primary and secondary antibodies, as per manufacturer instructions. Detection of bands was accomplished using ECL Plus Western Blotting Detection Reagents (Amersham Biosciences) and FujiFilm ImageReader LAS-300 exposure/image acquisition hardware and software. Multi Gauge software (FujiFilm) was utilized to perform image preparation and densitometric analysis. All densitometry data presented in the text was acquired using the Bio-Rad Protein Assay and relative fluorescence units/min values were standardized to milligrams of protein per sample.

RESULTS

Cisplatin Induces Loss of Cell Viability in an MSH2/MSH6-dependent Manner—To assess MMR protein-dependent cytotoxicity and pro-death signaling events in cells treated with CDDP, we utilized the paired endometrial carcinoma cell lines HEC59/HEC59(2) and HHUA/HHUA(2). HEC59 is heterozygous for two nonsense mutations in the MSH2 gene, rendering the cells deficient for MSH2 protein (24). HHUA contains homozygous missense mutations in MSH6, which render MSH6 inactive (25). Additionally, HHUA cells contain a nonsense mutation in MSH3, such that the cells are deficient for MSH3 (26). HEC59(2) and HHUA(2) contain wild-type copies of MSH2 and MSH6, which were stably restored via chromosome 2 transfer to HEC59 and HHUA cells (24, 25). HEC59(2) and HEC59 cell lines will henceforth be referred to in the text as MSH2-proficient and -deficient, respectively, while HHUA(2) and HHUA cells will be referred to as MSH6-proficient and -deficient, respectively. To confirm previously described MMR protein-dependent sensitivity to CDDP, we examined MMR protein-dependent decrease in cell viability in cells treated with CDDP using two methods: the MTT assay, and the propidium iodide (PI) exclusion assay. Fig. 1 and Table 1 show the results of MTT assays in which cell lines were treated with CDDP and staurosporine (STS). In MSH2-proficient and -deficient cells treated with 25 μM CDDP an MSH2-dependent decrease in cell viability was observed beginning at 48 h and continuing at 96 h (Fig. 1A). Concentration-dependent exposure to CDDP, assayed at 96 h, resulted in a CDDP dose-dependent, MSH2-dependent decrease in cell viability after treatment of MSH2-proficient and -deficient cells (IC_{50} 12 ± 6.6 versus 2.9 ± 1.2, respectively, p < 0.05, Table 1). MSH6-proficient and -deficient cells behaved similarly (Fig. 1B). An MSH6-dependent decrease in cell viability was noted, beginning at 48 h and continuing at 96 h when MSH6-proficient and -deficient cells were treated with 10 μM CDDP. MSH6-deficient cells were more tolerant than MSH6-proficient cells (IC_{50} 5.1 ± 1.9 versus 2.3 ± 0.24, respectively, p < 0.05, Table 1) when cells were treated with increasing doses of CDDP and assayed at 96 h post-treatment. STS, a drug that induces cell death via DNA damage-independent mechanisms, caused an MMR protein-independent decrease in cell viability in both paired cell lines (Fig. 1, A and B), demonstrating the specificity of the MMR protein-dependent response for CDDP. As a control for the addition of chromosome 2, which transfers a number of additional genes into the complemented cells, we performed MTT assays utilizing HCT116 and HCT116(2) cells. HCT116 is a human colon cancer cell line that is wild type for MSH2 and MSH6 (but contains an MLH1 defect). HCT116(2) cells contain a restored copy of chromosome 2, as in HEC59(2) and HHUA(2) cells, which does not complement the MLH1 defect (27). When we treated these cells with increasing doses of CDDP and performed MTT assays at 96 h post-treatment, we found no significant difference in viability between HCT116...
Mismatch Repair Protein-dependent Response to Cisplatin

and HCT116(2) cells (IC\textsubscript{50} 6.2 ± 2.4 versus 5.3 ± 1.5, respectively, p > 0.05, Table 1). This demonstrates that MMR protein-dependent effects, not chromosome 2-dependent effects, are most likely responsible for the decreased viability in HEC59(2) and HHUA(2) cells treated with CDDP. To further confirm our cell viability data, we performed PI exclusion assays after treating our cell lines with CDDP. Fig. 1c shows MMR protein-dependent decreases in cell viability in a CDDP dose-dependent manner, such that at 96 h, MSH2-deficient cells were more tolerant to the cytotoxic effects of CDDP than MSH2-proficient cells (IC\textsubscript{50} 17.2 ± 2.9 versus 11.1 ± 1.6, p < 0.05, Table 1). Similarly, MSH6-deficient cells were more tolerant of CDDP than MSH6-proficient cells (IC\textsubscript{50} 9.8 ± 2.4 versus 6.1 ± 0.4, p < 0.05, Table 1). These results demonstrate a temporal and concentration-dependent MMR protein-dependent decrease in cell viability in cells treated with CDDP.

**CDDP Induces MSH2/MSH6-independent p53 Up-regulation**—We next sought to determine the molecular mechanism of CDDP/MMR protein-dependent cell death. The tumor suppressor p53 functions as an inducer of cell death in response to a number of different types of DNA-damaging agents, including CDDP (17, 28). Following DNA damage, p53 levels increase and p53 becomes active (17, 28). p53 had been previously shown to be induced in cells treated with CDDP independently of the MMR status of these cells (17). To explore the potential involvement of p53 signaling in CDDP-induced, MMR protein-dependent cell death, we examined p53 levels by

![Figure 1](https://example.com/figure1.png)

**TABLE 1**

| Cell Line IC\textsubscript{50} values in CDDP-treated cells |
|---------------------------------|
| Calculations were performed using the results of at least three independent experiments performed in triplicate (MTT) or duplicate (PI exclusion). |

| Cell Line | IC\textsubscript{50} (95% CL) | Fold Difference | p value |
|-----------|-------------------------------|-----------------|---------|
| MTT assay |
| msh2      | 12 ± 6.6 (9.2–12)             | 4.1 (0.05)      |
| MSH2      | 2.9 ± 1.2 (2.5–3.1)           | <0.05           |
| msh6      | 5.1 ± 1.9 (3.2–7.6)           | <0.05           |
| MSH6      | 6.2 ± 0.2 (4.8–7.2)           | 1.2 (0.25)      |
| mlh1      | 6.1 ± 0.4 (5.0–7.2)           | <0.05           |
| mlh1 + chr.2 | 6.1 ± 0.4 (5.0–7.2) | <0.05           |

| PI exclusion assay |
|---------------------|
| msh2    | 8.2 ± 2.4 (5.8–12) | 1.2 (0.25) |
| MSH2    | 11 ± 1.5 (9.5–13)  | <0.05      |
| msh6    | 8.8 ± 2.4 (6.8–12) | 1.2 (0.25) |
| MSH6    | 6.1 ± 0.4 (5.0–7.2) | <0.05      |

\*95% confidence limits.
\*Fold difference comparing MSH2- or MSH6-proficient and -deficient cells, or MLH1-deficient cells complemented and uncomplemented with chromosome 2.
\*p values indicate results of a two-sided paired t-test.
immunoblot in cells that had been treated with CDDP for 12,
24, 48, or 72 h. Fig. 2 shows that, in all cell lines tested, p53 levels
were increased from 12 to 72 h following treatment with CDDP,
compared with untreated cells; however, this up-regulation
occurred independently of MMR protein status. These results
suggest that, while possibly playing a role in the MMR protein-
independent cell death induced by CDDP, it is unlikely that p53
is involved in MMR protein-dependent response to CDDP.

**CDDP Induces MSH2/MSH6-dependent Cytochrome c Relocalization to the Cytoplasm**—We next sought to investigate the
potential involvement of the mitochondrial pro-death signaling
pathway in CDDP-induced, MMR protein-dependent cytotoxicity.
DNA-damaging drugs, including CDDP, frequently
induce the relocalization of cytochrome c from the mitochondria
to the cytoplasm, where it promotes a caspase signaling cascade (29).

The cytoplasmic presence of cytochrome c was
analyzed by immunoblot after treatment of cell lines with
CDDP for 24, 48, or 72 h. Fig. 3 shows that cytochrome c relocalized
to the cytoplasm in an MSH2- and MSH6-dependent manner,
beginning at 48 h after cells were treated with CDDP
and increasing to 72 h post-treatment. Densitometric analysis
indicated a 2.0- and 17-fold increase in cytoplasmic cyto-

![Figure 2](image2.png)

**FIGURE 2.** p53 induction in cells treated with CDDP occurs independently of MMR protein status. Cells were treated with CDDP as indicated, and whole cell lysates were prepared for immunoblot analysis at indicated times post-drug treatment. U, untreated.

![Figure 3](image3.png)

**FIGURE 3.** Cisplatin induces MMR protein-dependent relocalization of cytochrome c to the cytoplasm. Cells were treated with CDDP (A and B) or STS (C), as indicated, and cytoplasmic isolates or whole cell lysates were prepared for immunoblot analysis at indicated times post-drug treatment. U, untreated.
quality of cytoplasmic extracts. STS was used as a control to demonstrate specificity of the MMR protein-dependent response for CDDP and to demonstrate the ability of cytochrome \(c\) to be relocalized to the cytoplasm in all cell lines after cellular insult. Treatment of cell lines with STS induced MMR protein-independent cytoplasmic localization of cytochrome \(c\) (Fig. 3C). These results indicate that cytochrome \(c\) is relocalized to the cytoplasm in cells treated with CDDP in an MMR protein-dependent manner, implicating intrinsic mitochondrial pro-death signaling as the mechanism of CDDP-induced, MMR protein-dependent cell death.

CDDP Induces Cleavage of Caspase-9, Caspase-3, and PARP in an MSH2/MSH6-dependent Manner—Localization of cytochrome \(c\) to the cytoplasm of cells promotes the cleavage and activation of caspase-9, which in turn can cleave and activate caspase-3. Cleaved caspase-3 can cleave a number of substrates (including PARP), enforcing ultimate destruction of the cell.

We examined the potential involvement of caspase-9 in CDDP/MMR protein-dependent signaling by analyzing cleavage of the protein by immunoblot analysis. Following treatment with CDDP, cleavage of caspase-9 showed dependence upon both MSH2 and MSH6 beginning at 48 h and increasing to 72 h post-treatment (Fig. 4). Quantification of caspase-9 processing by densitometry revealed that MSH2-proficient cells display a 1.4- and 2.7-fold increase in caspase-9 cleavage at 48 and 72 h, respectively, when compared with MSH2-deficient cells (Fig. 4A). Similarly, MSH6-proficient cells showed a 1.5- and 2.3-fold increase at 48 and 72 h, respectively, when compared with MSH6-deficient cells (Fig. 4B). Treatment of cells with STS induced MSH2- and MSH6-independent cleavage of caspase-9 (Fig. 4C).

Caspase-3 is the predominant downstream effector of caspase-9. Therefore we also examined processing/activation of caspase-3 in our cells. Immunoblot analysis revealed that in a manner similar to that for caspase-9, caspase-3 cleavage was impaired in both MSH2- and MSH6-deficient cells, beginning at 48 h and increasing to 72 h post-CDDP treatment. Quantification of these data demonstrated that MSH2-proficient cells display 1.7- and 3.4-fold increases in caspase-3 cleavage at 48 and 72 h, respectively, when compared with MSH2-deficient cells (Fig. 5A). MSH6-proficient cells showed that the amount of cleaved caspase-3 increased 3.2- and 4.3-fold at 48 and 72 h, respectively, compared with MSH6-deficient cells (Fig. 5B). As was the case for caspase-9 processing, STS treatment resulted in MMR protein-independent cleavage of caspase-3, as shown in Fig. 5C.

To corroborate our findings for the MMR-dependent activation of caspase-3, we assessed cleavage of PARP as an indication of caspase-3 activity. Quantification of immunoblot analysis by densitometry revealed that MSH2-proficient cells displayed 2.0- and 3.6-fold increases in PARP cleavage at 48 and 72 h, respectively, compared with MSH2-deficient cells (Fig. 6A). Similarly, MSH6-proficient cells exhibited 7.0- and 4.3-fold increases at 48 and 72 h, respectively, compared with MSH6-deficient cells (Fig. 6B). Treatment of cell lines with STS induced cleavage of PARP that was MMR protein-independent (Fig. 6C).

These data demonstrate that caspase-9/caspase-3 signaling is activated specifically in an MMR protein-dependent manner following treatment of cells with CDDP, further implicating intrinsic pro-death signaling as the mechanism of CDDP-induced, MMR protein-dependent cell death.
Caspase Inhibition Attenuates CDDP-induced, MSH2-dependent Loss of Cell Viability—To assess the requirement for caspase signaling in MSH2-dependent sensitivity to CDDP, we utilized the pan-caspase inhibitor z-VAD-FMK. We first tested the efficacy of this inhibitor in blocking caspase-3 activity, utilizing a synthetic caspase-3 substrate, which, upon cleavage, 

**FIGURE 5.** Cisplatin induces MMR protein-dependent caspase-3 cleavage. Cells were treated with CDDP (A and B) or STS (C), as indicated, and whole cell lysates were prepared for immunoblot analysis at indicated times post-drug treatment. U, untreated. DMSO (CDDP solvent) alone had no effect on caspase-3 cleavage (supplemental Fig. S1). 

**FIGURE 6.** Cisplatin induces MMR protein-dependent PARP cleavage. Cells were treated with CDDP (A and B) or STS (C), as indicated, and whole cell lysates were prepared for immunoblot analysis at indicated times post-drug treatment. U, untreated.
Mismatch Repair Protein-dependent Response to Cisplatin

shows a measurable increase in fluorescence intensity. Fig. 7A demonstrates that caspase-3 activity was low in untreated MSH2-proficient and MSH2-deficient cells; after treatment with CDDP, an increase in caspase-3 activity was observed in both cell lines, with a 2.0-fold increase in caspase-3 activity observed in MSH2-proficient cells compared with MSH2-deficient cells. These data confirmed results obtained in immunoblot experiments examining cleavage of caspase-3 and PARP (Figs. 5 and 6). Upon pretreatment of cells with z-VAD-FMK prior to CDDP treatment, a robust inhibition of caspase-3 activity was observed in both MSH2-proficient and -deficient cell lines, indicating the efficacy of the inhibitor in blocking caspase-3 activity (Fig. 7A). We next performed PI exclusion assays to determine the effects of caspase inhibition upon CDDP-induced, MSH2-dependent cell death. Pre-treatment of MSH2-proficient cells with z-VAD-FMK resulted in a substantial rescue from CDDP cytotoxicity (Fig. 7B, black bars, 28% versus 44% viability), suggesting that a significant portion of CDDP-induced cell death is caspase-dependent. By comparison, in the absence of z-VAD-FMK treatment, viability of MSH2-deficient cells following CDDP was similar to those of z-VAD-FMK-treated MSH2-proficient cells (42% versus 44% viability). The viability of MSH2-deficient cells remained essentially unchanged following chemical caspase inhibition (Fig. 7B, white bars, 42% versus 48% viability). These data suggest that MSH2 directly contributes to a CDDP-dependent cell death pathway that requires the involvement of caspase activation for its completion and further indicate that engagement of a pro-death signaling pathway by MMR proteins can be uncoupled from other mechanisms of CDDP cytotoxicity.

**DISCUSSION**

In this report, we provide the first evidence of a pro-death MMR protein-dependent signaling mechanism in cell lines that have been treated with the chemotherapeutic drug CDDP. This MMR protein-dependent pathway can be specifically attenuated and separated from other CDDP response pathways and functions independently of p53 signaling.

We report an MMR protein-mediated activation of mitochondrial pro-death signaling involving cytochrome c, caspase-9, and caspase-3 after treatment of cells with CDDP, beginning at 48 h and continuing at 72 h post-treatment of cells with CDDP (Figs. 3–6). The timing of the relocalization or activation of each of these molecules is consistent with their involvement in a common pathway, and with the timing of MMR protein-dependent sensitivity to CDDP (Fig. 1). This sensitivity is blocked by the pan-caspase inhibitor z-VAD-FMK (Fig. 7), underscoring the requirement for caspase signaling in MMR protein-dependent cytotoxicity induced by CDDP. As predicted by our immunoblot data, employment of the caspase inhibitor had no significant effect on the sensitivity of MSH2-deficient cells to CDDP, suggesting that this inhibitor specifically attenuates the MSH2-dependent response. This result confirms the existence of an
Mismatch Repair Protein-dependent Response to Cisplatin

MMR protein-dependent signaling pathway engaged by MMR proteins in cells treated with CDDP that is independent of other mechanisms of CDDP cytotoxicity.

This is the first report to demonstrate signaling components required for CDDP-induced, MMR protein-dependent cytotoxicity. It had been previously demonstrated that there is an MSH6- and MLH1-dependent activation of caspase-9, caspase-3, and PARP in cells treated with the methylating agent MNNG, and that this MMR protein-dependent death is at least partially blocked by pretreatment of cells with z-VAD-FMK (3, 14). Thus, it would appear that the execution stage of MMR protein-dependent cell death is dependent upon the activity of caspases regardless of the nature of the lesion recognized by MMR proteins; however, upstream events may differ depending upon the nature of the MMR protein-recognized lesion. Presently, p73 and c-Abl are the only proteins implicated as potential mediators of CDDP/MMR protein-dependent cell death (17, 18). A recent study and preliminary findings in our laboratory have suggested ATM and Chk1 are activated independently of MMR protein status upon treatment of cells with CDDP (Ref. 7, data not shown), a finding in opposition to what is noted after treatment of cells with the methylating agent MNNG, which induces MMR protein-dependent activation of these proteins (13). These findings suggest that differing signaling cascades may be activated by MMR proteins dependent upon the nature of the recognized lesion. As such, further study into CDDP/MMR protein-dependent cytotoxic signaling events preceding engagement of mitochondrial pro-death signaling is required.

Mechanistic details about MMR protein-dependent initiation of cell death remain unclear. Two hypotheses are predominant in the field. The first is the "repair-dependent" hypothesis, in which translesion DNA synthesis past a site of DNA damage results in a mispairing of the damaged base. This mispair is a substrate for MMR; however, because the damage persists in the parent DNA strand, iterative "futile" cycles of repair excision and resynthesis of the daughter strand occur. This response is expected to result in the generation of DNA double strand breaks, which, in turn, could lead to cell death. The second hypothesis suggests a direct signaling mechanism of MMR protein-mediated initiation of a DNA damage signal, in which MMR proteins bind DNA damage and via direct interaction with downstream pro-death molecules or blockage of lesion repair initiate a cytotoxic signaling cascade. The former hypothesis assumes that the repair capacity of MMR proteins is required for MMR protein-dependent cell death, while the latter suggests that MMR capacity of these proteins may not necessarily be required in damage signaling. We and others have demonstrated that point mutations affecting MMR proteins in yeast (30–32) or mouse cells (33–35) can separate MMR protein-mediated repair and CDDP-induced cell death, suggesting that the repair function of the MMR proteins is not required for MMR proteins to induce cell death in response to CDDP. Additionally, recent data from Pani and colleagues and preliminary work in our laboratory (Ref. 7, data not shown) has shown that the double strand break responders ATM and H2AX are phosphorylated in an MMR protein-independent manner upon treatment of cells with CDDP, suggesting MMR proteins do not promote the formation of double strand breaks in cells treated with CDDP. Furthermore, we and others have demonstrated that requirements for ATP and protein-lesion interactions of MutSα are altered in response to a CDDP adduct, in comparison with the MutSα response to a mismatch (30–32, 36). These data support the idea that a repair-independent, direct signaling mechanism is utilized by MMR proteins in initiating death in response to CDDP. Further study will be required to elucidate the precise mechanisms of MMR protein-dependent initiation of death signaling.

The results shown here demonstrating that MMR proteins engage the pro-death mitochondrial signaling pathway in cells treated with CDDP have clinical implications. CDDP is utilized to treat a number of cancer types; despite this, mechanistic details of CDDP-induced cytotoxicity are not yet fully understood. A more advanced understanding of the mechanisms of CDDP-induced cytotoxicity will translate into improved predictions of patient response based on the presence/activity of pro-death signaling components; this, in turn, will allow the drug to be administered to patients in such a manner that its full benefits can be realized. Our data show that CDDP/MMR protein-dependent cell death proceeds independently of p53 signaling (Fig. 2). p53 is frequently mutated in human tumors; our data suggest that exploiting the MMR protein-dependent cytotoxic signaling pathway may be a strategy for attacking tumors with inactive p53 signaling.

We demonstrate the activation of a pro-death mitochondrial signaling cascade in a MMR protein-dependent manner in cells treated with CDDP. Overexpression of pro-survival Bcl-2 family members prevents the release of mitochondrial factors, such as cytochrome c, from the mitochondria and can inhibit intrinsically mediated signaling and death (29). Interestingly, two recent reports have suggested that overexpression of pro-survival Bcl-2 may result in the inhibition of MMR protein functions via binding to MSH6 and disruption of the MutSα complex after cells are treated with a DNA-damaging agent (37) or via a decrease in MSH2 mRNA/protein via Bcl-2-mediated decrease in E2F1 activity (38). Coupled with our findings, this suggests the possibility that overexpression of pro-survival Bcl-2 family members may inhibit MMR protein-dependent cell death in cells treated with CDDP, via inhibition of MMR proteins directly, or via blockade of MMR protein-dependent pro-death signaling at the mitochondria. We found that overexpression of Bcl-2 attenuated CDDP-induced MMR protein-dependent cytotoxicity in both of our sets of paired cell lines (data not shown). It is unclear if this is due to direct inhibition of MMR proteins, a blockage of pro-death signaling at the mitochondria, or a combination of both. This latter possibility has certain merit, as the level of Bcl-2 overexpression required to inhibit MMR protein function versus prevent the release of pro-death factors from the mitochondria has not been rigorously tested. Up-regulation of pro-survival Bcl-2 family members and down-regulation of pro-death family members has been observed in a number of cancer types, and alterations in the levels of these proteins can affect clinical response to CDDP; for example, high levels of Bax have been demonstrated to be a favorable prognostic indicator for ovarian cancer patients receiving CDDP-based chemotherapy (39), and high levels of
Mismatch Repair Protein-dependent Response to Cisplatin

pro-survival Bcl-xL have been associated with recurrent disease and shorter disease-free survival in ovarian cancer patients (40). Retrospective analysis of clinical tumor tissue samples has suggested the possibility that MMR protein levels and degree of microsatellite instability may be indicative of response and overall survival in patients receiving CDDP-based chemotherapy in a specific subset of cancer types. A combined examination of MMR and Bcl-2 family member protein levels may yield a useful prognostic signature in certain tumor types that could better predict response and overall survival in patients treated with CDDP.

This work suggests therapeutic strategies that would lead to the more efficient elimination of both MMR-proficient and -deficient tumor cells. With regard to the findings reported in this study, the most appropriate CDDP-based drug combinations would include a drug that exerts cytotoxic activity independently of MMR proteins and enforces cell death via mitochondrial/caspase-dependent signaling, so as to offset the absence of activation of this signaling pathway in MMR protein-defective cells, and possibly enhance the intensity of signaling in MMR protein-proficient cells. It has been demonstrated that the microtubule inhibitor paclitaxel induces cell death independently of MMR protein status (41), and this drug has been demonstrated to induce pro-death mitochondrial signaling and cell death (42). Where possible, this drug might be appropriate in combination with CDDP to prevent the potential survival and expansion of tumor cells with MMR protein defects.

In this report, we demonstrate that MMR protein-dependent cytotoxic signaling in cells treated with CDDP proceeds via cytochrome c, caspase-9, and caspase-3. Further study into potential MMR protein-dependent cytotoxic signaling mechanisms upstream of the mitochondria in cells treated with CDDP is needed. In addition, our findings strongly advise a thorough investigation into the prognostic value of pre- and post-chemotherapy MMR protein status in patients treated with CDDP, with emphasis placed on patient response, recurrence, survival benefit, and the study of a spectrum of tumors currently being treated with a variety of CDDP-based regimens. The examination of the status of other proteins in combination with MMR proteins (such as Bcl-2 family members) may serve to enhance the predictive value of MMR protein status in CDDP-based therapy, and may provide a prognostic signature to further predict the benefits of CDDP administration to specific patients.

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