DNA Damage Invokes Mismatch Repair-dependent Cyclin D1 Attenuation and Retinoblastoma Signaling Pathways to Inhibit CDK2*

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DNA-damage evokes cell cycle checkpoints, which function to maintain genomic integrity. The retinoblastoma tumor suppressor (RB) and mismatch repair complexes are known to contribute to the appropriate cellular response to specific types of DNA damage. However, the signaling pathways through which these proteins impact the cell cycle machinery have not been explicitly determined. RB-deficient murine embryo fibroblasts continued a high degree of DNA replication following the induction of cisplatin damage, but were inhibited for G2/M progression. This damage led to RB dephosphorylation/activation and subsequent RB-dependent attenuation of cyclin A and CDK2 activity. In both Rb+/+ and Rb−/− cells, cyclin D1 expression was attenuated following DNA damage. As cyclin D1 is a critical determinant of RB phosphorylation and cell cycle progression, we probed the pathway through which cyclin D1 degradation occurs in response to DNA damage. We found that attenuation of endogenous cyclin D1 is dependent on multiple mismatch repair proteins. We demonstrate that the mismatch repair-dependent attenuation of endogenous cyclin D1 is critical for attenuation of CDK2 activity and induction of cell cycle checkpoints. Together, these studies couple the activity of the retinoblastoma and mismatch repair tumor suppressor pathways through the degradation of cyclin D1 and dual attenuation of CDK2 activity.

DNA damage induces checkpoints to prevent damaged cells from progressing deleteriously through the cell cycle (1–5). It is postulated that genetic damage is sensed by specific proteins which initiate signal transduction pathways to inhibit cell cycle progression. Cell cycle transitions are driven by the coordinated activity of cyclin-dependent kinase (CDK) cyclin complexes. Mitogens stimulate the expression of cyclin D and the subsequent activation of CDK4-cyclin D complexes (6). These cyclin D-associated complexes initiate the phosphorylation of RB, which disrupts RB-mediated transcriptional repression of specific target genes allowing progression through G1 (7–9). It is believed that the targets for RB are encompassed by a host of E2F-regulated genes, including metabolic enzymes and cyclins E and A (10, 11). Since the activity of these cyclins is required for cell cycle progression, RB phosphorylation/inactivation is requisite for passage into S-phase. Subsequent activation of CDC2-cyclin B complexes is required for mitotic entry. Importantly, numerous participants in checkpoint processes are implicated in tumor development/progression.

In fact, RB is a critical cell cycle regulator that has recently been shown to participate in the cellular response to DNA damage (12–14). Environmental stresses such as DNA damage prevent RB phosphorylation, thus leading to RB-dependent cessation of cell cycle progression. For example, RB is dephosphorylated/activated when primary fibroblasts are exposed to ionizing radiation, and this event triggers cell cycle arrest (13). Our laboratory has previously shown that the role of RB is also conserved in the replicative checkpoint response to cisplatin (cis-diaminedichloroplatinum-II: CDDP) (13, 14). However, the full spectrum of RB-dependent checkpoints and the mechanism through which RB is activated and then exerts its cell cycle inhibitory effects in response to DNA damage are not clearly delineated.

Here we assessed the mechanism through which CDDP-mediated DNA damage signals through RB to elicit checkpoint responses. We find that RB is required for the cessation of G1- and S-phase progression in primary fibroblasts. However, RB is dispensable for the G2/M block instilled by CDDP. Analysis of the mechanism through which RB inhibits G1/S demonstrated that RB is required for the attenuation of cyclin A expression and CDK2-associated kinase activity following CDDP damage. Analysis of upstream signaling demonstrated that CDDP damage mediates loss of cyclin D1 expression irrespective of RB, suggesting that this response may be critical for the initiation of the RB-dependent checkpoint. Analysis of multiple pathways indicated that the degradation of cyclin D1 is dependent on the mismatch repair complex. This mismatch repair-dependent degradation of cyclin D1 was critical for appropriate checkpoint response, RB activation or reduction in CDK2 activity. Importantly, mismatch repair activities function as tumor suppressors (15–19). Additionally, it is known that loss of mismatch repair contributes to the resistance of cancer cells chemotherapeutically. Together these studies show that mismatch repair and RB-dependent pathways coalesce to facilitate DNA-damage checkpoints.

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† The abbreviations used are: CDK, cyclin-dependent kinase; RB, retinoblastoma tumor suppressor; CDDP, cis-diaminedichloroplatinum II; MEF, murine embryo fibroblasts; BrdUrd, 5-bromodeoxyuridine; LP, large pocket.
**Materials and Methods**

*Cell Culture and Drug Treatment—* Rb+/+ and Rb−/− MEFs were derived from embryos isolated from the mating of mice with Rb+/− (14). The DNA-PK, p21<sup>cip1</sup>, MSH2, PMS-2, MLH-1, and c-Abl-deficient cells have been previously described (14, 25–28). The HCT116 and related cell lines have been previously described (24). Murine fibroblasts were propagated in MEF media (14). Transfections were by calcium phosphate. Adenoviral infections were carried out at a calculated multiplicity of infection of 50–100, actual infection efficiency was greater than 95% as determined by GFP fluorescence. Pharmaceutical cisplatin (Bristol Oncology) was applied at the given concentration for the indicated time period. Nocodazole was used at 0.4 μg/ml. Mitomycin C, etoposide, and doxorubicin were from Sigma.

**Immunofluorescence—* BrdUrd incorporation was determined as previously described (29). In all experiments the percentage of BrdUrd-positive cells was determined as the percentage of Hoechst-stained nuclei which were BrdUrd positive. For the analysis of mitotic chromosome condensation, cells were treated with the indicated dose of cisplatin for 16 h. CDDP was then washed from the media and the cells were allowed to progress for 6–8 h in the presence of nocodazole to increase the numbers of mitotic cells. Both adherent and floating cells were harvested, fixed, and stained with Hoechst. Mitotic nuclei were readily apparent through microscopic analysis of the stained nuclei.

**Immunofluorescence, Immunoprecipitation, and Kinase Reactions—* The detection of RB in MEFs was as previously described (14). The immunoblotting and immunoprecipitations for cyclins, CDKs, and inhibitors were carried out by standard protocols. The CDK4 (H-22), CDK2 (M-2), cyclin E (C-19), and cyclin A (C-19 and H432) antibodies were procured from Santa Cruz. The cyclin D1 (Ab-3) antibody was obtained from Neo-markers. CDK2-kinase assays against histone H1 were carried out by standard protocols. The CDK4 (H-22), CDK2 (M-2), and CDK1 (Y15) antibodies were from New England Biolabs.

**Flow Cytometry Staining and Analysis—* Cells were fixed with 80% ethanol and processed for propidium iodide staining, as previously described (30). For bivariate analyses, cells were fixed with ethanol and stained for BrdUrd incorporation and propidium iodide staining (14). Flow cytometry was performed on a Coulter Epic flow cytometer. Statistical analysis of the flow cytometry data was carried out blind.

**Results**

**RB Specifically Mediates G1 and S-phase Checkpoints—** DNA damage elicits cell cycle checkpoints in multiple phases of the cell cycle. To evaluate the requirement for RB in cell cycle checkpoints elicited upon CDDP-mediated damage, primary murine embryonic fibroblasts (MEFs) with varying RB status were utilized. CDDP induces striking G1, S-phase, and G2/M checkpoints, and thus enables one to investigate a variety of checkpoints through the use of a single agent. Therefore, asynchronously growing wild-type (Rb+/+) or RB-deficient (Rb−/−) MEFs were treated with 32 μM CDDP for 16 h. These cells were then labeled for 8 h with BrdUrd and scored for the ability to incorporate BrdUrd (Fig. 1A). In wild-type MEFs, treatment with CDDP dramatically inhibited BrdUrd incorporation (Fig. 1A). In contrast, CDDP had virtually no influence on BrdUrd incorporation in Rb−/− cells (Fig. 1A). To determine the role of RB on cell cycle phase transitions following CDDP exposure, we employed bivariate flow cytometry (Fig. 1B). Using this approach, incorporation of BrdUrd (an indicator of DNA replication) and DNA content (an indicator of cell cycle phase) were measured concurrently. Asynchronously growing cells were treated with 32 μM CDDP and then pulse labeled with BrdUrd for 1 h. During the pulse labeling, only cells in S-phase of the cell cycle will incorporate BrdUrd. Untreated Rb+/+ and Rb−/− cells readily incorporated BrdUrd in S-phase (Fig. 1B). CDDP treatment inhibited BrdUrd incorporation and therefore DNA replication of Rb+/+ cells. Rb−/− cells that had already entered S-phase (DNA content between 2N and 4N) failed to incorporate BrdUrd and complete DNA replication, thus CDDP damage inhibited S-phase progression. Furthermore, following CDDP treatment Rb+/+ cells fail to accumulate in any phase of the cell cycle, indicating that CDDP initiated arrest in G1, S, and G2/M phases of the cell cycle. In contrast, Rb−/− cells continued to incorporate BrdUrd following CDDP treatment (Fig. 1B). Importantly, the Rb−/− cells were able to complete DNA replication, as indicated by the increase in cells with a 4N DNA content. Strikingly, the Rb−/− cells continue replication without apparently progressing through cytokinesis, as indicated by the accumulation of cells with a DNA content greater than 4N.

To explicitly investigate the role of RB in the G2/M transition, we treated asynchronously growing Rb+/+ or Rb−/− MEFs with 32 μM CDDP followed by incubation with nocadazole (0.4 μg/ml) to enrich for mitotic cells (Fig. 1C). Approximately 15% of Rb+/+ and Rb−/− untreated cells were mitotic following enrichment with nocadazole. In contrast, following CDDP treatment, less than 1% of either Rb+/+ or Rb−/− nuclei exhibit mitotic condensation (Fig. 1C). Therefore, CDDP blocks the G2/M transition irrespective of RB status.
Cyclin A and CDK2 Attenuation Is a Critical RB-dependent Response to DNA Damage—While these data demonstrate a role for RB in the G1 and S-phase DNA damage checkpoints, the pathway for this action of RB was not known. As expected, treatment with 32 \( \mu \text{M} \) CDDP leads to the dephosphorylation/activation of the endogenous RB protein present in MEFs (Fig. 2A). The expression of cyclin E, CDK2, and cyclin A is proposed to be regulated by RB/E2F. Therefore, we analyzed the influence of RB status on the levels of these proteins following treatment with CDDP. Consistent with prior results, we observed increased levels of cyclin E protein in the Rb\textsuperscript{-/-} cells (31) (Fig. 2B). Surprisingly, cyclin E protein levels were not altered in Rb\textsuperscript{+/+} cells after CDDP treatment (Fig. 2B). This finding suggested, that the active RB in these cells does not act by further attenuating cyclin E. In contrast, cyclin A protein expression was inhibited in CDDP-treated Rb\textsuperscript{+/+} MEFs, but not in the Rb\textsuperscript{+/+} MEFs (Fig. 2B). The attenuation of cyclin A was also observed at 16 \( \mu \text{M} \) CDDP (Fig. 2C), a dose of CDDP at which RB is dephosphorylated and there is an RB-dependent checkpoint (not shown). Together, these results indicate that the inhibition of cyclin A expression requires functional RB and correlates with the checkpoint elicited by CDDP. We also determined the CDK2 kinase activity in Rb\textsuperscript{+/+} and Rb\textsuperscript{-/-} MEFs, in the presence or absence of CDDP. We found that CDK2 kinase activity is decreased in Rb\textsuperscript{+/+} MEFs after CDDP treatment (Fig. 2D). In Rb\textsuperscript{-/-} cells that express high levels of cyclin E and cyclin A even in the presence of CDDP, CDK2 kinase activity was not changed by CDDP addition (Fig. 2D).

To determine whether the reduction in cyclin A expression was a critical determinant for cell cycle checkpoint in RB positive cells, we attempted to restore cyclin A by ectopic expression. Rb\textsuperscript{+/+} MEFs were infected with recombinant adenovirus expressing either GFP (control) or expressing both human cyclin A and GFP. The expression of the ectopic human cyclin A could be readily detected in infected MEFs using antibodies directed against human cyclin A (Fig. 2E). Treatment with CDDP inhibits the expression of endogenous cyclin A in control infected cells (Fig. 2E), but does not influence the level of ectopically produced cyclin A. Therefore, we could evaluate whether the restoration of cyclin A expression can rescue the RB-mediated inhibition of DNA replication following CDDP damage. Wild-type (Rb\textsuperscript{+/+}) MEFs infected with either GFP or GFP-cyclin A recombinant adenoviruses were treated with 0, 16, or 32 \( \mu \text{M} \) CDDP 24 h post-infections. These cells were then labeled with BrdUrd for 4 h and scored for the ability to incorporate BrdUrd. Untreated Rb\textsuperscript{+/+} MEFs incorporated BrdUrd at approximately the same levels after infection with cyclin A or GFP control adenoviral constructs. However, cells infected with a GFP-control construct showed inhibition of BrdUrd incorporation in a CDDP dose-dependent manner that was similar to the extent of inhibition observed with uninfected cells (Fig. 2F). The infection of Rb\textsuperscript{+/+} cells with cyclin A restored BrdUrd incorporation at 16 \( \mu \text{M} \) CDDP (Fig. 2F). However, in the presence of 32 \( \mu \text{M} \) CDDP, human cyclin A expression only partially restored BrdUrd incorporation (Fig. 2F).

DNA Damage-mediated Attenuation of Cyclin D1 Is Dependent on Multiple Mismatch Repair Proteins—Based on the importance of RB on the CDDP damage response we sought to determine the mechanism through which RB is activated to instill cell cycle inhibition. RB phosphorylation is controlled at numerous levels, including the kinase activity associated with CDK4/cyclin D and CDK2/cyclin E complexes. In general, DNA damage provokes a cellular response leading to increased expression of p53 and p21\textsuperscript{Cip1} and resulting in dephosphorylation/activation of RB. In MEFs, we failed to observe a significant induction of p21\textsuperscript{Cip1} following CDDP damage (Fig. 3A), suggesting that an alternative pathway is mediating RB dephosphorylation. It is well established that the main function of D-type cyclins is functional inactivation of RB by phosphorylation, and it has recently been reported that cyclin D1 degradation participates in the DNA damage checkpoint response (32). We found that the protein levels of cyclin D1 were strongly inhibited in both Rb\textsuperscript{+/+} and Rb\textsuperscript{-/-} MEFs treated with CDDP (Fig. 3B). This result indicated that CDDP is capable of inducing molecular signals in both the Rb\textsuperscript{+/+} and Rb\textsuperscript{-/-} cells, however, only in the presence of RB do these signals (i.e. cyclin D1 attenuation) elicit G1/S inhibition. The loss of cyclin D1 expression occurred within 4 h and could be blocked with proteosome inhibitors (not shown and see below). Under these same conditions CDK4 and p16ink4a protein were not influenced following treatment with CDDP in both wild type and RB\textsuperscript{-/-} cells (Fig. 3B and not shown). Since CDK4 activity is required to initiate RB phosphorylation and cyclin D1 attenuation occurred in an RB-independent manner, this suggested that the attenuation of cyclin D1 may be a critical determinant for RB activation.

The pathway coupling DNA damage to cyclin D1 degradation is unknown. To seek the possible candidates for CDDP signaling to cyclin D1, we employed mouse embryo fibroblasts defective in proteins involved in DNA-damage response (p21\textsuperscript{Cip1}, c-Abl, and DNA-PK). Both DNA-PK and c-Abl kinases are implicated in the response to DNA damage (26, 33). However, neither DNA-PK nor c-Abl is necessary for cyclin D1 degradation (Fig. 3C). The CDK4-inhibitor p21\textsuperscript{Cip1}, is also not required for cyclin D1 degradation (Fig. 3C).

The DNA mismatch repair system plays an important role in the recognition of CDDP adducts, additionally cancer cells defective in mismatch repair are less sensitive to CDDP (25, 26, 33–36). Based on these observations, we hypothesized that mismatch repair complexes are involved in signaling cyclin D1 degradation and subsequent RB activation. The genes that encode proteins with roles in DNA mismatch repair include the MutS homologues MSH2, MSH3, MSH6, and MutL homologues MLH1, PMS1, and PMS2 (37). When mouse embryo fibroblast deficient in PMS2 were treated with 32 \( \mu \text{M} \) cisplatin for 16 h, cyclin D1 was not degraded (Fig. 3D). Similarly, cyclin D1 was largely retained in MSH2-deficient cells treated with cisplatin (Fig. 3D). Similar results were achieved with immortalized cells that are MLH1 deficient (MC2--/- cells, data not shown). Collectively our data demonstrate that mismatch repair components PMS2, MSH2, and MLH1 are requisite for cisplatin-mediated cyclin D1 degradation.

To determine if the influence of the mismatch repair on the DNA damage checkpoint response, we evaluated the ability of PMS2\textsuperscript{+/+} or PMS2\textsuperscript{-/-} cells to incorporate BrdUrd in the presence of CDDP damage. The PMS2\textsuperscript{+/+} cells were readily inhibited for BrdUrd incorporation with 32 \( \mu \text{M} \) CDDP (Fig. 4A). In contrast, the PMS2\textsuperscript{-/-} cells incorporated BrdUrd in the presence of CDDP damage (Fig. 4A). Since the behavior of PMS2\textsuperscript{-/-} cells paralleled the behavior of Rb\textsuperscript{-/-} cells, we determined if these cells were compromised for signaling to cyclin A. In PMS2\textsuperscript{+/+} cells, cyclin D1 was attenuated and there is a concomitant decrease in cyclin A levels (Fig. 4B). For the PMS2\textsuperscript{-/-} cells there was no cyclin D1 attenuation or reduction in cyclin A protein levels (Fig. 4B). Thus these data support the model that the mismatch repair-dependent attenuation of cyclin D1 is, in turn, requisite for the RB-dependent attenuation of cyclin A. Similar results were also observed with MSH2--/- MEFs (not shown).

Cyclin D1 Degradation Does Not Occur in Mismatch Repair-deficient Tumor Lines Treated with Divergent DNA-damaging
Agents—Loss of mismatch repair function occurs in tumors where it is believed to promote genomic instability and contribute to drug resistance (22). We therefore determined if these initial findings in murine fibroblasts extended to human tumor systems, wherein we could better assess the functional consequence of cyclin D1 loss. In the hMLH1-deficient cell line HCT116, cyclin D1 levels were unchanged by exposure to cisplatin (Fig. 5A). When hMLH1 is provided by transfer of chromosome 3 (HCT116 3-(6)) (24) cyclin D1 degradation occurred in response to cisplatin (Fig. 5A, compare lanes 7 and 8). This restoration of signaling is specific, since transfer of chromosome 2 (HCT116 2-(1)) failed to facilitate degradation (Fig. 5A). Similarly, in a colorectal cancer cell line proficient in mismatch repair, SW480, cyclin D1 levels were readily attenuated following cisplatin damage (Fig. 5A). The dependence of cyclin D1 degradation on mismatch repair was independent of p53/p21Cip1 induction, since p53 and p21Cip1 were induced in all HCT cell lines by cisplatin (Fig. 5A). Furthermore, the attenuation of cyclin D1 was independent of functional p53, as SW480 cells harboring mutant p53 failed to induce p21Cip1 but were proficient for cyclin D1 attenuation (Fig. 5A). To confirm that the loss of cyclin D1 expression was in fact due to degradation, we employed the proteosome inhibitor Cbz-LLL, which prevented CDDP-mediated attenuation of cyclin D1 in SW480 cells (Fig. 5B). To determine whether a similar dependence on mismatch repair (MLH-1 specifically) was evoked with different forms of DNA damage, HCT-116 or SW480 cells were treated with mitomycin C, doxorubicin, or etoposide. All of these treatments elicited degradation of cyclin D1 that was dependent on mismatch repair activity (Fig. 5C). Together, these data couple mismatch repair to the cell cycle regulatory machinery through the degradation of cyclin D1.

To assess the functional consequence of mismatch repair-dependent degradation of endogenous cyclin D1, we initially analyzed the G2-checkpoint by determining the ability of cisplatin treatment to inhibit entry into mitosis. For these studies HCT116, HCT116 3-(6), and SW480 cells were treated with 0 or 32 μM CDDP for 16 h followed by incubation with nocadazole for an additional 6 h to enrich for mitotic cells. Approximately 50% of untreated cells were mitotic following enrichment with treated with 0 or 32 μM CDDP for 16 h. Cells were harvested and RB was detected by immunoprecipitation followed by immunoblotting. ppRB, hyperphosphorylated RB; pRB, hypophosphorylated RB. B, Rb−/− (lanes 1 and 2) or Rb+/+ (lanes 3 and 4) cells were treated with 0 (lanes 1 and 3) or 32 (lanes 2 and 4) μM CDDP for 16 h. Cells were harvested and equal total protein was resolved by SDS-PAGE. CDK2, cyclin E, and cyclin A proteins were detected by immunoblotting. C, Rb−/− (lanes 1–3) or Rb+/+ (lanes 4–6) cells were treated with 0 (lanes 1 and 4), 16 (lanes 2 and 5), or 32 (lanes 3 and 6) μM CDDP for 16 h. Cells were harvested and equal total protein was resolved by SDS-PAGE. CDK4 and cyclin A proteins were detected by immunoblotting. D, Rb−/− (lanes 1–3) or Rb+/+ (lanes 4 and 5) cells were treated with 0 (lanes 1, 2, and 4) or 32 (lanes 3 and 5) μM CDDP for 16 h. Cells were harvested, lysed, and 150 μg of total protein was immunoprecipitated with E1A (negative control) (lane 1) or CDK2 (lanes 2–5) antibodies and immune complexes were used in in vitro kinase assays against histone H1. Reactions were resolved by SDS-PAGE and transferred to membrane. Phosphorylated histone H1 was detected by autoradiography and CDK2 protein was detected by immunoblotting. Results are representative of two independent experiments. E, Rb+/+ cells were infected with adenoviruses encoding either GFP (lanes 1 and 2) or GFP + cyclin A (lanes 3 and 4). Twenty-four h post-infection cells were treated with 0 (lanes 1 and 3) or 32 (lanes 2 and 4) μM CDDP for 16 h. Cells were harvested and equal total protein was resolved by SDS-PAGE. Cyclin A proteins were detected by immunoblotting. F, Rb+/+ cells were infected with adenoviruses encoding either GFP or GFP + cyclin A. Twenty-four h post-infection cells were treated with 0, 16, or 32 μM CDDP for 16 h. Cells were then labeled with BrdUrd for 4 h, fixed, and stained for BrdUrd incorporation. Data shows the percentage of cells incorporating BrdUrd, with untreated cells arbitrarily set to 100%. At least 200 infected cells were counted for each experiment.

Fig. 2. RB-dependent attenuation of cyclin A is critical for the CDDP-mediated cell cycle checkpoint. A, Rb+/+ MEFS were

- **A**: Hyperphosphorylated RB (ppRB) and hypophosphorylated RB (pRB) after treatment with 32 μM CDDP.
- **B**: CDK2 and cyclin A proteins detected by immunoblotting.
- **C**: Cyclin A expression in Rb−/− and Rb+/+ MEFS treated with 32 μM CDDP.
- **D**: Phosphorylated histone H1 (H1) and CDK2 expression after 32 μM CDDP treatment.
- **E**: Cyclin A expression in GFP and Cyclin A-infected Rb+/+ cells treated with 32 μM CDDP.
- **F**: BrdUrd incorporation in cells treated with different doses of CDDP.
nocadazole. In contrast, following cisplatin treatment, mitotic index in these cells drops below 1% (Fig. 5D). Therefore, CDDP damage blocks the transition into mitosis irrespective of mismatch repair status. To determine the role of mismatch repair on the ability of cells to undergo DNA synthesis, BrdUrd incorporation was analyzed. As quantified in Fig. 5D, we found that in SW480 and HCT116 3-(6) cells, CDDP treatment led to a significant decrease in BrdUrd incorporation. In contrast,
FIG. 4. Mismatch repair-dependent signaling to cell cycle checkpoints and cyclin A attenuation. A, PMS2+/+ or PMS2−/− MEFs were treated with 0 or 32 μM CDDP for 16 h. Cells were labeled with BrdUrd for 8 h and stained for BrdUrd incorporation. Data shown is the percent of cell staining positively for BrdUrd incorporation, with untreated cells set arbitrarily to 100%. Results are from greater than 200 cells. B, PMS2+/+ (lanes 1 and 2) or PMS2−/− (lanes 3 and 4) MEFs were treated with 0 (lanes 1 and 3) or 32 (lanes 2 and 4) μM CDDP for 16 h. Equal total protein was resolved by SDS-PAGE and the levels of cyclin D1, cyclin A, and CDK4 were determined by immunoblotting.

parental HCT116 CDDP treatment failed to inhibit BrdUrd incorporation (Fig. 5E).

Mismatch Repair-dependent Attenuation of CDK2 Activity—Since we had earlier linked mismatch repair-dependent cyclin D1 degradation to the RB/cyclin A-pathway, we investigated this pathway in the human tumor cell lines. Initially, HCT116 and SW480 cells were treated with 32 μM CDDP for 16 h and then analyzed the phosphorylation status of RB (Fig. 6A). Surprisingly, no significant dephosphorylation of RB was observed in either cell line (Fig. 6A). To demonstrate that RB can be dephosphorylated in these cells, we employed p16ink4a recombinant adenovirus, which effectively dephosphorylated RB (Fig. 6A). To confirm that a minor pool of RB is not dephosphorylated in response to CDDP, we employed a previously characterized mutant cyclin D1 (R29AT286A) which is refractory to DNA damage-mediated degradation (32). This protein was not degraded when SW480 cells are treated with CDDP, and LP remained hyperphosphorylated (Fig. 6C). Thus, cyclin D1 degradation does impinge on the phosphorylation status of RB. In contrast, treatment with CDDP failed to elicit degradation of the ectopically expressed cyclin D1 in HCT116 cells and LP phosphorylation was unchanged (Fig. 6C).

While these results indicate that mismatch repair-mediated cyclin D1 degradation does contribute to RB activation, RB is not universally invoked during the checkpoint response, and other mechanisms must mediate cell cycle inhibition in the presence of hyperphosphorylated RB (as is observed in SW480 cells). To elucidate this mechanism we investigated the activity associated with CDK2 in the CDDP-sensitive SW480 cells as opposed to the resistant HCT116 cells. As shown in Fig. 6D, CDDP treatment inhibits CDK2 kinase activity in SW480 and HCT116 3-(6) cells, but not HCT116 or HCT116 2-(1) cells. This finding is consistent with the idea that cyclin D1 degradation releases inhibitors associated with CDK4 to mediate inhibition of CDK2-associated (38). In accordance with this model, p27Kip1 interaction with CDK2 was strongly enhanced in specifically SW480 cells following CDDP damage (Fig. 6E). Together these results suggest two pathways through which mismatch repair-dependent degradation of cyclin D1 impacts CDK2 activity to limit DNA replication following CDDP damage (Fig. 6F): one which is through RB-mediated attenuation of cyclin A, and another through CDK-inhibitor switching.

DISCUSSION

In this study, we analyzed the role of RB and mismatch repair in the response to DNA damage. We show that primary murine fibroblast arrest in all phases of the cell cycle following CDDP treatment. Such DNA damage elicits the dephosphorylation/activation of RB, which is required for the inhibition of G1 and S phase. Cyclin A protein levels are attenuated in a RB-dependent manner, and represent a critical target. Conversely, cyclin D1 levels are reduced following CDDP treatment regardless of RB status, indicating that this event acts upstream of RB. We demonstrate that multiple mismatch repair proteins are required for cyclin D1 degradation as was elicited through different forms of DNA damage. The mismatch repair-dependent cyclin D1 degradation was an important determinant of the checkpoint response that can facilitate either activation of the RB pathway to inhibit cyclin A expression or act directly to attenuate CDK2 activity through CDK-inhibitor switching.

Together, these data provide a model, in which DNA damage acts together these results suggest two pathways through which mismatch repair-dependent degradation of cyclin D1 impacts CDK2 activity to limit DNA replication following CDDP damage (Fig. 6F): one which is through RB-mediated attenuation of cyclin A, and another through CDK-inhibitor switching.

RB-dependent Checkpoints—RB is functionally inactivated by CDK/cyclin-mediated phosphorylation (8). Anti-mitogenic signals promote RB dephosphorylation and the resulting cell cycle inhibition. The vast majority of these anti-proliferative signals function in G1 by modulating CDK/cyclin activity, thereby eliciting G1 arrest (39). However, specific forms of DNA control RB phosphorylation (i.e. a lack of dependence on D-type cyclins). Therefore, we used a transient system to assess the role of specifically cyclin D-mediated RB phosphorylation (Fig. 6C). SW480 or HCT116 cells were transfected with the large pocket (LP) fragment of RB, which is efficiently phosphorylated by ectopically expressed cyclin D1. In this system LP hyper-phosphorylation is specifically dependent on the exogenous cyclin D1. In SW480 cells cyclin D1 degradation was readily apparent and LP phosphorylation was diminished, as indicated by the appearance of hypophosphorylated protein (Fig. 6C). To prove that this was dependent on the attenuation of cyclin D1, we employed a previously characterized mutant cyclin D1 (R29AT286A) which is refractory to DNA damage-mediated degradation (32). This protein was not degraded when SW480 cells are treated with CDDP, and LP remained hyperphosphorylated (Fig. 6C). Thus, cyclin D1 degradation does impinge on the phosphorylation status of RB. In contrast, treatment with CDDP failed to elicit degradation of the ectopically expressed cyclin D1 in HCT116 cells and LP phosphorylation was unchanged (Fig. 6C).

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FIG. 5. Cyclin D1 degradation is mismatch repair dependent in human tumor cells. A, HCT116 (lanes 1 and 2), SW480 (lanes 3 and 4), HCT116 2-(1) (lanes 5 and 6) and HCT116 3-(6) (lanes 7 and 8) were treated with 0 (lanes 1, 3, 5, and 7) or 32 (lanes 2, 4, 6, and 8) μM CDDP. Cell lysates were resolved and cyclin D1, p53, p21\textsuperscript{CIP1}, and β-tubulin were detected by immunoblotting. B, SW480 cells were treated with 0 (lane 1) or 2.5, 5, 10, or 15 μM CDDP. Cell lysates were resolved and cyclin D1 and β-tubulin were detected by immunoblotting. C, DOX and ETO were treated with 0 (lanes 1 and 3) or 32 μM CDDP. Cell lysates were resolved and cyclin D1 were detected by immunoblotting. D, E, BrdU incorporation was measured in cells treated with 0 μM CDDP (lanes 1 and 3) or 32 μM CDDP (lanes 2 and 4).
32 (lanes 2–6) μM CDDP in the presence of the indicated dose of Cbz-LLL for 16 h. Cells lysates were resolved and cyclin D1 and β-tubulin were detected by immunoblotting. C, HCT116 (lanes 1 and 2) and SW 480 (lanes 3 and 6) cells were treated with 0 (lanes 1, 3, and 6) or 32 μM CDDP (lanes 2, 4, and 5) for 16 h. To induce RB dephosphorylation SW480 cells were infected with p16ink4a recombinant adenovirus (lane 6). Cell lysates were resolved by SDS-PAGE. Cyclin A expression and RB phosphorylation status were determined by immunoblotting. D, TSU-PR1 cells were treated with 0 (lane 1) or 32 (lane 2) μM CDDP for 16 h. Lysates were resolved by SDS-PAGE and RB, cyclin A, cyclin D1, p53, and β-tubulin were detected by immunoblotting. Cells were also labeled with BrdUrd and fixed and stained for BrdUrd incorporation (right panel). E, lysates were prepared from HCT116 (lanes 1 and 2), SW480 (lanes 3 and 4), HCT116 2-(1) (lanes 5 and 6), and HCT116 3-(6) (lanes 7 and 8) treated with 0 (lanes 1, 3, 5, and 7) or 32 (lanes 2, 4, 6, and 8) μM CDDP for 16 h. CDK2 complexes were recovered by immunoprecipitation and analyzed by in vitro kinase assay. F, lysates were prepared from HCT116 (lanes 1 and 2) or SW480 (lanes 3 and 4) cells treated with 0 (lanes 1 and 3) or 32 (lanes 2 and 4) μM CDDP and subjected to immunoprecipitation with CDK2 antibody, recovered complexes were denatured and resolved by SDS-PAGE. CDK2 and p27Kip1 were detected by immunoblotting. F, model for CDDP signaling.
damage can also elicit an RB-dependent S-phase checkpoint (14). Here we find that RB is only a requisite determinant for the halt of replication both in G1/S and S-phase, but is dispensable for the G2/M block. Continued replication with a failure to undergo mitosis and cytokinesis likely underlies the increased DNA content observed in Rb−/− cells treated with CDDP. Based on our findings we would predict that RB-deficient cells are particularly prone to mutations arising from inappropriate DNA-replication under conditions of DNA damage.

RB elicits inhibition of DNA replication following CDDP damage. To evaluate the mechanism through which RB functions, we sought to identify changes in CDK/cyclin expression or activity that were specifically dependent on RB. It is established that cyclin E is overproduced in Rb−/− MEFs suggesting that a failure to regulate cyclin E expression may contribute to checkpoint abrogation (31). Surprisingly, we found that CDDP treatments do not target cyclin E, but does target the expression of cyclin A in an RB-dependent manner. This effect is functionally significant, since restoration of cyclin A antagonizes the checkpoint response.

**Mismatch Repair Links DNA Damage to Cyclin D1**—Mismatch repair activities play a critical role in maintaining genomic stability and suppressing tumor development. This tumor suppressive role for mismatch repair was first indicated by the finding that loss of mismatch repair factors is a relatively common event in specific human tumor types (15–17). Subsequently, it was observed that inactivation of components of the mismatch repair complex in mice leads to a tumor-prone phenotype (18, 19). Clearly, mismatch repair factors are involved in repairing damaged DNA. However, it is increasingly apparent that mismatch repair complexes are required for specific signals to be processed in response to DNA damage. This was first supported by the findings that mismatch repair-deficient cell lines are resistant to DNA damage-induced cell cycle checkpoints and apoptosis (25, 26, 33–36). Only recently have signaling pathways been described for which mismatch repair is a requisite factor. For example, the activation of c-Abl and subsequent accumulation of p73 following DNA damage is dependent on mismatch repair (26).

That cyclin D1 degradation represents a principal target of the DNA damage response has been recently reported (32). In those studies a critical region in the N-terminal of cyclin D1 was shown to direct the degradation of cyclin D1 following ionizing radiation (32). However, a signaling pathway through which cyclin D1 degradation was triggered had not been demonstrated. In other studies cyclin D1 degradation was found to occur in cells deficient in ATM, p53, RB, and ARF (32). We similarly found that p53 and RB were dispensable for cyclin D1 degradation. Additionally, c-Abl, p21Cip1, and DNA-PK were not required for cyclin D1 attenuation. Thus cyclin D1 degradation occurs via a pathway distinct from those involving p21Cip1 stimulation (p53 pathway) or Cdc25A degradation (ATM pathway) (5). Our analyses show that in the CDDP response cyclin D1 degradation is dependent on the mismatch repair complex. This result is intrinsically consistent, since the p53/p21Cip1 response is mismatch repair independent. Importantly, the dependence on mismatch repair is manifest not only following CDDP damage but also etoposide, mitomycin C, and doxorubicin. Thus the requirement for mismatch repair is general to diverse damage targeting cyclin D1.

In our studies we found that mismatch repair-deficient cells, which failed to degrade endogenous cyclin D1 were compromised for checkpoint responses. This result suggested that down-regulation of endogenous cyclin D1 was important for signaling for the checkpoint. This supposition is supported by data that showed that ectopic expression of a nondegradable cyclin D1 abrogates checkpoints induced by ionizing radiation (32).

**Distinct Targets of Cyclin D1 Attenuation**—The dephosphorylation of RB following stresses is principally attributed to the attenuation of the kinases that phosphorylate RB (8, 39). For example, ionizing radiation leads to RB dephosphorylation in part through the induction of p21Cip1 (12). Here we find that CDDP damage in murine embryo fibroblasts does not significantly alter p21Cip1 levels, but does lead to the attenuation of cyclin D1 expression. Cyclin D1 is typically required for RB phosphorylation in G1, and in the absence of cyclin D1 RB will not be phosphorylated (40). Consistent with such a hypothesis, both murine embryo fibroblasts or tumor cell lines that fail to degrade cyclin D1 retain RB phosphorylation and the downstream target of RB, cyclin A. Conversely, using a nondegradable mutant of cyclin D1 (32), RB hyperphosphorylation was maintained in the presence of CDDP damage. Thus in primary cells and specific tumor cell lines RB-dependent signaling is elicited in a manner dependent on cyclin D1 degradation.

Surprisingly, in specific cells exposed to CDDP where cyclin D1 is efficiently degraded (e.g. SW480) RB remains phosphorylated. This was illustrated both through direct analysis of RB and through the analysis of cyclin A expression as a readout for RB activation. This unexpected finding challenges the idea that attenuation of cyclin D1-associated kinase activity invariably leads to RB dephosphorylation, and supports the idea that other kinases may maintain RB phosphorylation in tumor cells. Interestingly, such a phenomenon is only observed in specific tumor cell lines, suggesting that this defect in RB regulation is genetically tractable and may represent an important feature of tumor progression.

Based on our data cyclin D1 degradation elicits cell cycle inhibition with a degree of cell preference. Cyclin D1 degradation frees CDK-inhibitors from CDK4-cyclin D1 complexes, resulting in the formation of inactive CDK2 complexes. This model is consistent with other studies demonstrating a requirement for cyclin D1 in the association of p27Kip1 with CDK4 (38, 41–47). In the absence of cyclin D1 the CDK inhibitor sequesters CDK2-cyclin complexes to inhibit their activity. We observe this phenomenon in tumor cell lines that fail to dephosphorylate RB in response to DNA damage (e.g. SW480 cells). Additionally, in other cell types wherein RB is activated (e.g. TSU-PR1, MEFs, and 3T3 cells), we observe the down-regulation of cyclin A, which similarly impinges on CDK2 activity.

In summary, we demonstrate that the cellular response to CDDP is dependent on functional mismatch repair and RB pathways. The mismatch repair proteins couple DNA damage to cyclin D1 degradation; thereby impinging on the RB growth suppressive pathway or directly influencing CDK2 activity by mobilizing CDK inhibitors. The net action of invoking these pathways is G1 and S phase cell cycle inhibition mediated through CDK2 inhibition.

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