RB-Dependent S-Phase Response to DNA Damage

KAREN E. KNUDSEN,¹ DANA BOOTH,^{2,3} SOHEIL NADERI,^{2,3} ZVJEZDANA SEVER-CHRONEOS,¹
ANNE F. FRIBOURG,¹ IRINA C. HUNTON,^{2,3} JAMES R. FERAMISCO,^{3,4}
JEAN Y. J. WANG,^{2,3} AND ERIK S. KNUDSEN^{1*}

*Department of Cell Biology, University of Cincinnati, Cincinnati, Ohio 45267-0521,¹ and Department of Biology,²
Cancer Center,³ and School of Medicine,⁴ University of California, San Diego, La Jolla, California 92093*

Received 22 February 2000/Returned for modification 21 April 2000/Accepted 10 July 2000

The retinoblastoma tumor suppressor protein (RB) is a potent inhibitor of cell proliferation. RB is expressed throughout the cell cycle, but its antiproliferative activity is neutralized by phosphorylation during the G₁/S transition. RB plays an essential role in the G₁ arrest induced by a variety of growth inhibitory signals. In this report, RB is shown to also be required for an intra-S-phase response to DNA damage. Treatment with cisplatin, etoposide, or mitomycin C inhibited S-phase progression in Rb^{+/+} but not in Rb^{-/-} mouse embryo fibroblasts. Dephosphorylation of RB in S-phase cells temporally preceded the inhibition of DNA synthesis. This S-phase dephosphorylation of RB and subsequent inhibition of DNA replication was observed in p21^{Cip1}-deficient cells. The induction of the RB-dependent intra-S-phase arrest persisted for days and correlated with a protection against DNA damage-induced cell death. These results demonstrate that RB plays a protective role in response to genotoxic stress by inhibiting cell cycle progression in G₁ and in S phase.

The retinoblastoma tumor suppressor protein (RB) is a negative regulator of cell proliferation (1, 31, 33, 39, 49). The antiproliferative activity of RB is mediated by its ability to inhibit the transcription of genes that are required for cell cycle progression, e.g., cyclin A (17, 33). This transcriptional regulatory function of RB is achieved through several distinct mechanisms, which are best illustrated by the inhibition of E2F-regulated gene expression. RB binds to the C-terminal transactivation domain of the E2F subunit in E2F-DP heterodimers and therein neutralizes the transactivation function of E2F (10, 53). RB also assembles a repressor complex at promoters containing E2F binding sites through simultaneous binding to both E2F and histone deacetylase (3, 30). Because histone deacetylase modifies chromatin to a closed state through deacetylation, transcription is repressed (22). Additionally, it has recently been reported that the RB-mediated repression of specific cell cycle genes (e.g., the cyclin A gene) is dependent on association with SWI/SNF chromatin remodeling activity (42, 43, 55). The mechanism through which the SWI/SNF complex mediates RB-dependent transcriptional repression is not clearly understood. However, loss of SWI/SNF activity disrupts RB-mediated repression of specific cell cycle targets and renders cells resistant to RB-mediated cell cycle arrest. Lastly, RB may interact with specific components of the basal transcription machinery (e.g., TFIID250) to regulate their activities (41). Through these collective mechanisms of transcriptional regulation, RB exerts its antiproliferative action (56). In general, RB activity is induced in response to environmental signals which favor halting the cell cycle. For example, the antimutagenic activity of transforming growth factor β requires RB activation (15). Similarly, it has been shown that RB activity is required for G₁ arrest in response to DNA damage (13).

The ability of RB to inhibit cellular proliferation is counterbalanced by the action of cyclin-dependent kinases (Cdks). In response to proliferative signals, Cdks are activated by their

cyclin regulatory subunits to phosphorylate RB and thereby inactivate its protein binding function (1, 31, 39, 49). Specifically, when quiescent cells are stimulated to enter the cell cycle, Cdk4/6-cyclin D complexes become active in mid-G₁ and initiate the phosphorylation of RB (31, 39). Later in G₁, RB becomes hyperphosphorylated through the combined actions of Cdk4-cyclin D, Cdk2-cyclin E, and Cdk2-cyclin A (5, 28, 31, 54). The activities of Cdk2-cyclin E and Cdk2-cyclin A are both rate limiting and required for entry into S phase (34, 35, 38). Phosphorylation of RB is maintained throughout S and G₂, until RB is finally dephosphorylated by a phosphatase at the M/G₁ transition (25, 31). The E2F binding function of RB can be inactivated by the phosphorylation of several Cdk phosphorylation sites (6, 20). The binding of RB to c-Abl tyrosine kinase is inactivated by the phosphorylation of two specific serine sites (19), and binding to viral oncoproteins or histone deacetylase is inactivated by two specific threonine sites (12, 19, 20). These data show that phosphorylation of RB is a highly regulated process and that specific phosphorylation events result in distinct outcomes.

The importance of RB phosphorylation is underscored by the prevalence of mutations in cancer that result in deregulation of RB phosphorylation (1, 39). For example, amplification or overexpression of cyclin D and Cdk4/6, or loss of the Cdk4/6 inhibitor p16^{ink4a}, occurs with high frequency in human tumors (1, 39). Each of these types of mutations results in increased RB phosphorylation and inactivation of RB function. Accordingly, RB mutant proteins that lack the Cdk phosphorylation sites which regulate E2F binding are potent inhibitors of the cell cycle (6, 18, 27). These phosphorylation site-mutated RB proteins (PSM-RB) cause a cell cycle arrest in G₁, which can be overridden by the increased expression of cyclin E (6, 18, 27). Interestingly, however, overproduction of cyclin E does not rescue cell cycle inhibition imposed by PSM-RB, as these cells entered but could not progress through S phase (6, 18). The S-phase inhibitory action of RB cannot be mimicked by Cdk inhibitors such as p16^{ink4a}, p21^{Cip1}, or p27^{Kip1} (18). The observation that PSM-RB could inhibit S-phase progression was consistent with the continued phosphorylation of RB throughout S phase and suggested that RB might become

* Corresponding author. Mailing address: Department of Cell Biology, University of Cincinnati, Cincinnati, OH 45267-0521. Phone: (513) 558-8885. Fax: (513) 558-4454. E-mail: Erik.Knudsen@UC.edu.

dephosphorylated under specific conditions, resulting in the inhibition of DNA replication.

In this report we show that several DNA damage inducers, including cisplatin, etoposide, and mitomycin C, can inhibit S-phase progression. The S-phase inhibition induced by these DNA-damaging agents was observed with RB-positive cells but not with RB-negative cells. The inhibition of DNA replication was preceded by RB dephosphorylation, which also occurred in p21^{Cip1}-deficient cells. This RB-dependent S-phase block in damaged cells persisted for days and was correlated with a protection against cell death. These observations demonstrate that RB plays an essential role in the S-phase response to genotoxic stress and suggest that regulation of RB phosphorylation can occur in S phase to inhibit DNA synthesis.

MATERIALS AND METHODS

Cell culture, synchronization, and drug treatment. Mouse embryo fibroblasts (MEFs) were derived from 10- to 12-day embryos isolated from the mating of mice with Rb^{+/+} or p21^{+/+} genotype. The genotype of all MEFs was determined by PCR of head DNA, and the fibroblasts used in this study were between passages 2 and 6. Cells were propagated in MEF medium (Dulbecco modified Eagle medium supplemented with penicillin, streptomycin, and glutamine in 10% fetal bovine serum [FBS] with 0.001% β -mercaptoethanol). To synchronize in quiescence, MEFs were cultured in MEF medium containing only 0.1% FBS for 72 h. To synchronize cells in S phase, cells were stimulated with 10% FBS for 16 h and then blocked with either aphidicolin (Aph; 2 μ g/ml) or hydroxyurea (HU; 1 mM) (Sigma). Cells were cultured in the presence of either drug for an additional 10 h to allow S-phase accumulation. Clinical-grade *cis*-diamminedichloroplatinum II (CDDP; Bristol-Oncology) and reagent-grade etoposide and mitomycin C (both from Sigma) were applied at the given concentrations for the indicated period of time. Release from S-phase synchrony was achieved by washing the cells once with phosphate-buffered saline (PBS) followed by two washes with drug-free medium for 5 min. Bromodeoxyuridine (BrdU; Amersham) was added to the medium upon release, and labeling was carried out for the indicated time. The Rat-1 cells were cultured and synchronized as previously described (18).

Immunofluorescence and microinjection. MEFs were seeded at 0.75×10^5 to 1.0×10^5 cells per well of a six-well plate onto glass coverslips. Aph-synchronized cells were injected using an Eppendorf automatic microinjection system mounted on a Zeiss S100 Axiocvert microscope. Plasmids were diluted to a concentration of 50 ng/ μ l in injection buffer as previously described (18). The PSM-7LP plasmid has been previously described (18). All injections contained a nuclear green fluorescent protein (GFP) expression plasmid (histone H2B fused to GFP) to allow for the identification of productively injected cells. Cells were released from Aph arrest 16 h postinjection and labeled with BrdU for 4 h. BrdU incorporation was determined as previously described (18). In all experiments, the percentage of BrdU-positive cells was determined as the percentage of Hoechst-stained nuclei or GFP-positive nuclei which were BrdU positive.

The ICR4 antibody was kindly provided by Michael Tilby (University of Manchester) (45). For ICR4 staining, cells grown on glass coverslips were fixed in 3.7% formaldehyde in PBS for 15 min at room temperature. Fixed cells were then permeabilized using 0.3% Triton X-100 in PBS for 15 min at room temperature. ICR4 was diluted at 1:10 in IF (immunofluorescence) buffer (PBS, 0.5% NP-40, 5 mg of bovine serum albumin/ml) supplemented with MgCl₂ and DNase I and added to the permeabilized cells for 1 h 37°C. Cells were washed with PBS and then subjected to secondary antibody diluted 1:100 in IF buffer for 1 h at 37°C. Cells were washed again in PBS and mounted using Gelvatol.

Immunoblotting, immunoprecipitation, and kinase assay. For the detection of RB, 2.0×10^6 cells were plated on 15-cm-diameter dishes and subjected to synchronization and drug treatment as described. Cells were lysed in RIPA (radioimmunoprecipitation assay) buffer, and RB was immunoprecipitated with antibody 851 (44) and protein A-Sepharose. Resulting immunocomplexes were recovered by centrifugation and washed four times in RIPA buffer. Proteins were denatured by boiling in sodium dodecyl sulfate (SDS) buffer supplemented with 3% β -mercaptoethanol, resolved by polyacrylamide gel electrophoresis (PAGE) on an SDS-6.5% gel, and transferred to Immobilon-P. RB was detected by immunoblotting with antibody 851.

For detection of cyclin E, cyclin A, p21, and Cdk2, cells were subjected to synchronization and drug treatment as described. Cells were lysed in RIPA buffer, and equal amounts of protein were resolved by SDS-PAGE. Proteins were detected using standard immunoblotting procedures and the following antibodies: for cyclin A, C-19 (Santa Cruz); for cyclin E, M20 (Santa Cruz); for Cdk2, M2 (Santa Cruz); and for p21^{Cip1}, PC55 (Calbiochem).

Cdk2 immunoprecipitation and kinase assays were performed as previously described (17).

Flow cytometry staining and analysis. Cells were fixed with 80% ethanol and processed for propidium iodide staining as described previously (6, 12, 19, 20, 54). For bivariate analyses, cells were fixed with ethanol and stained for BrdU

incorporation and propidium iodide staining. Flow cytometry was performed on a Coulter Epic or Becton Dickinson flow cytometer. The percent BrdU labeling was determined for S-phase cells by gating those cells with greater than 2N but less than 4N DNA content and determining the percentage of these cells which were BrdU positive. All statistical analysis of the flow cytometry data was carried out blind.

Transfection and reporter assays. For transfection, 1.5×10^5 MEFs were seeded on a 6-cm-diameter dish and transfected 24 h later with 8 μ g of total plasmid DNA (1 μ g of CMV-beta-gal, 5 μ g of 3XE2FLUC, and 2 μ g of CMV-NeoBam) by standard calcium phosphate procedures, as previously described (21). After transfection, cells were synchronized in Aph for 24 h, at which time 0 or 32 μ M CDDP was added. After 16 h, the cells were harvested and processed for luciferase activity using the Promega luciferase assay system according to the manufacturer's protocol. β -Galactosidase activity was also quantitated as an internal control for transfection efficiency. Reported relative luciferase activity reflects luciferase activity normalized to β -galactosidase activity. Data shown represent the average of at least four independent experiments.

RESULTS

Cisplatin-induced RB dephosphorylation in S-phase cells.

We have previously shown that PSM-RB, an RB mutant which cannot be inactivated by phosphorylation, inhibits DNA synthesis when expressed in S-phase Rat-1 cells (18). This observation suggested that RB might be involved in the regulation of S-phase progression. Physiological growth factors generally regulate S-phase entry but do not control the progression through S phase. However, intra-S-phase checkpoints can be triggered under stress and by DNA damage (24, 50, 51). To assess the role of RB in the S-phase DNA damage response, we examined whether RB becomes activated or dephosphorylated in S-phase cells after genotoxic stress. Rat-1 cells were synchronized in early S phase with the DNA polymerase inhibitor Aph and subsequently exposed either to the chemotherapeutic agent cisplatin (in the form of CDDP; 50 μ M, 3 h) or to ionizing radiation (IR; 10 Gy). Following treatment, cells were released from the Aph arrest and monitored for progression through S phase. Exposure to IR did not interfere with DNA synthesis following Aph release (data not shown). When released from Aph, Rat-1 cells progressed from S phase through G₂ and into the subsequent G₁ in 6 h (Fig. 1a, left panels), and 80% of the cells incorporated BrdU during this time period (Fig. 1b). In contrast, cells treated with CDDP retained S-phase DNA content (Fig. 1a, right panels) and failed to incorporate BrdU (Fig. 1b). We then examined the status of RB phosphorylation in CDDP-treated cells (Fig. 1c). Hypophosphorylated RB (pRB) was detected in quiescent Rat-1 cells (Fig. 1c, lane 1), and predominantly hyperphosphorylated RB (ppRB) was found in asynchronously growing (lane 2) or Aph-arrested (lane 3) cells. After 3 h of treatment with 50 μ M CDDP, however, ppRB was no longer detectable in the Aph-arrested, S-phase cells (lane 4). These results established a correlation between the dephosphorylation of RB and the inhibition of S-phase progression in cells damaged by CDDP.

RB is required for cisplatin-induced inhibition of DNA synthesis. To further assess the role of RB in CDDP-induced S-phase inhibition, we compared the responses of RB-positive and RB-deficient MEFs to CDDP. As a control, we first examined the G₁ checkpoint response in Rb^{+/+} and Rb^{-/-} MEFs, since a previous report has shown that Rb^{-/-} MEFs do not undergo G₁ arrest following exposure to CDDP (13). To examine the G₁ response to CDDP, both Rb^{+/+} and Rb^{-/-} MEFs were made quiescent by serum starvation. Following serum stimulation, 35 to 45% of the MEFs incorporated BrdU, representing the proliferative index of the MEF culture (data not shown). The proliferative indices of Rb^{+/+} and Rb^{-/-} cultures were similar, and both types of MEFs showed serum dependence for G₁ progression (Fig. 2A, left panel). When quiescent MEFs were treated with CDDP (16 or 32 μ M) and

a. **b.** **c.**

FIG. 1. CDDP inhibits DNA synthesis and induces RB dephosphorylation in S-phase cells. (a) Rat-1 cells were arrested in Aph (2 μ g/ml, 24 h) and then treated with CDDP (50 μ M) or vehicle for an additional 3 h. Cells were washed extensively to remove Aph and CDDP and then collected at 2, 4, or 6 h after washing. The DNA content of each population of cells was determined by flow cytometry. Pretreatment with CDDP blocked S-phase progression in Rat-1 cells. (b) Rat-1 cells were arrested in Aph (2 μ g/ml, 24 h) and then treated with CDDP (50 μ M) or vehicle for an additional 3 h. Cells were washed extensively to remove Aph and CDDP and then labeled with BrdU for 3 h. The cells were then fixed and reacted with anti-BrdU antibody. The percentage of cells that incorporated BrdU was determined by counting at least 100 cells per experiment. Data shown are means and standard deviations from three independent experiments. Representative fields of BrdU-labeled cells are shown. (c) The status of RB phosphorylation in Rat-1 cells that were serum starved for 72 h (Qui; lane 1), asynchronously growing (Asy; lane 2), Aph arrested (2 μ g/ml, 24 h) (APH; lane 3), or Aph arrested and CDDP treated (50 μ M, 3 h) (CDDP + APH; lane 4) was examined by immunoprecipitation of total cell lysate with anti-RB 851 followed by immunoblotting with anti-RB 851.

then stimulated with serum, the Rb^{+/+} MEFs arrested in G₁ (Fig. 2a, right panel). In contrast, the Rb^{-/-} MEFs incorporated BrdU irrespective of CDDP treatment (Fig. 2a, right panel). These results confirmed that RB is required for CDDP to induce a cell cycle arrest in G₁.

To examine the intra-S-phase response to CDDP in this system, MEFs were synchronized in early S phase by Aph treatment. Aph synchronization was performed in the presence of serum to allow G₁ progression beyond the restriction point. This was shown by the incorporation of BrdU upon Aph release in the presence or absence of serum (Fig. 2b, left panel). Furthermore, the Aph-synchronized cells incorporated BrdU within 1 h of release from Aph, consistent with a block in early S phase (not shown). The Aph-synchronized S-phase cells were treated with several concentrations of CDDP and washed free of Aph and CDDP; then the incorporation of BrdU was examined. Exposure of the Aph-synchronized Rb^{+/+} MEFs to CDDP (16 or 32 μ M) for 16 h led to an inhibition of DNA synthesis (Fig. 2b, right panel). Thus, CDDP inhibited S-phase progression in Rb^{+/+} MEFs, similar to the response observed with Rat-1 cells (Fig. 1b). Interestingly, Aph-synchronized Rb^{-/-} MEFs were unresponsive to CDDP, as these cells incorporated BrdU upon Aph release irrespective of prior treatment with CDDP (Fig. 2b, right panel). To verify that the CDDP effect was not specific for Aph-synchronized cells, we used HU as an alternative means to block cells in early S phase. Treatment of HU-synchronized Rb^{+/+} cells with CDDP led to an inhibition of BrdU incorporation following HU release, while HU-synchronized Rb^{-/-} MEFs did not respond to CDDP and continued to incorporate BrdU following HU release (Fig. 2c).

CDDP is passively diffused into cells, where it forms adducts with DNA. To verify that adducts were generated in both Rb^{+/+} and Rb^{-/-} cells, we used a specific antibody (ICR4) which reacts with the platinum adducts (45). In Aph-synchronized MEFs not exposed to CDDP, ICR4 immunoreactivity was not detectable above the background level (Fig. 2d, top panel). As expected, exposure to CDDP led to a dose-dependent increase in immunoreactivity toward ICR4 (Fig. 2d, lower panels). Quantitation by digital imaging of ICR4 reactivity showed a similar extent of platinum adduct formation between the Rb^{+/+} and Rb^{-/-} MEFs (not shown).

In both Rb^{+/+} and Rb^{-/-} MEFs, CDDP caused an inhibition of mitosis, assayed by nuclear condensation (data not shown). Thus, the G₂/M checkpoint is intact in Rb^{-/-} cells. In other experiments, we compared the responses of Rb^{+/+} and Rb^{-/-} MEFs to a transient 1-h exposure to CDDP (10 to 50 μ M). At these levels of exposure, CDDP caused only a reduction in the rate of DNA synthesis; this was observed with both Rb^{+/+} and Rb^{-/-} MEFs. This reduction in the rate of DNA synthesis was consistent with comparable platinum adduct formation in these two cell types. Taken together, these results suggest that Rb^{-/-} cells experienced comparable damage by CDDP as the Rb^{+/+} cells. Moreover, RB is required for CDDP to inhibit DNA synthesis, which occurred under conditions of prolonged exposure and possibly irreparable damage.

Dephosphorylated RB is observed prior to the inhibition of DNA replication in S-phase cells. Since RB appeared to be required for the S-phase response to CDDP, RB activity was monitored via examination of its phosphorylation state. Serum-starved MEFs contained predominantly pRB (Fig. 3a, lane 2). After serum stimulation and Aph synchronization, ppRB was observed (Fig. 3a, lane 3). Because a portion of the MEFs were not actively cycling, pRB was also detected in these Aph-synchronized cultures. Further incubation of Aph-syn-

FIG. 2. Inhibition of DNA synthesis by CDDP requires RB. MEFs were prepared by mating Rb-heterozygous mice and harvesting embryos at day 10 to 12 of gestation. The genotype of each embryo was determined by PCR of head DNA. Rb^{+/+} or Rb^{-/-} MEFs from the same litter were used between passages 2 and 6. (a) Rb^{+/+} and Rb^{-/-} MEFs were cultured in 0.1% serum for 72 h. (Left) Quiescent cells were stimulated with 10% FBS for 26 h (10%) or for only 30 min and then transferred into 0% FBS (0%) and labeled with BrdU. The stained cells were counted (see Materials and Methods), and the percentage of BrdU-positive cells in 10% FBS was set at 100. (Right) In parallel, quiescent cells were treated with the indicated concentration of CDDP for 16 h and then stimulated with 10% FBS and labeled

chronized cells did not alter the ratio of ppRB to pRB (lane 4); however, addition of CDDP caused the disappearance of ppRB (lane 5). We often observed the presence of a shorter form of RB in $Rb^{+/+}$ MEFs, and this delta-RB corresponded to the C-terminally truncated RB previously described to be generated by caspase cleavage (44).

As mentioned above, the inhibition of DNA synthesis was observed after a prolonged exposure to CDDP. We therefore compared the kinetics of RB dephosphorylation and the inhibition of DNA synthesis in Aph-synchronized MEF cultures (Fig. 3b and c). With 32 μ M CDDP, little increase in pRB was observed after 4 h of incubation (Fig. 3b, compare lanes 3 to 5). Correspondingly, DNA synthesis was not inhibited when cells were released from the Aph block from a 4-h incubation with CDDP (Fig. 3c). When cells were released after 8 h of exposure to CDDP, ppRB was no longer detected (Fig. 3b, lane 6) and DNA synthesis was inhibited by approximately 50% (Fig. 3c). After 12 h of exposure to CDDP, ppRB had been absent for at least 4 h (Fig. 3b, compare lanes 4 to 7), and DNA synthesis was inhibited in the majority of the cells in the culture (Fig. 3c). Thus, the shift to active RB preceded the inhibition of DNA synthesis in CDDP-treated S-phase cells.

S-phase inhibition and dephosphorylated RB in $p21^{Cip1}$ -deficient cells. The Cdk inhibitor $p21^{Cip1}$ is upregulated by p53 in response to DNA damage (8). Because $p21^{Cip1}$ can inhibit RB phosphorylation by Cdk (5), and because $p21^{Cip1}$ can interact with PCNA to inhibit DNA synthesis (48), we examined whether $p21^{Cip1}$ might be responsible for the CDDP-induced increase in pRB and inhibition of DNA synthesis. To do so, MEFs were prepared from wild-type, $Rb^{-/-}$, and $p21^{Cip1-/-}$ embryos (4). Cells were then synchronized by Aph and subsequently treated with increasing concentrations of CDDP. As can be seen in Fig. 4a, wild-type MEFs arrest DNA synthesis with increasing dose of CDDP (top panel), whereas $Rb^{-/-}$ MEFs exhibit no inhibition of BrdU incorporation, even at relatively high (40 μ M) doses of CDDP (middle panel). By contrast, $p21^{Cip1-/-}$ cells demonstrate an intermediate phenotype, showing no inhibition at low or intermediate doses (0 to 16 μ M) but exhibiting S-phase retardation at higher doses (32 to 40 μ M) (bottom panel). $p21^{Cip1-/-}$ MEFs treated with 32 μ M CDDP contained predominantly the underphosphorylated form of RB only after 12 h of exposure (Fig. 4b), therefore exhibiting a delayed activation of RB compared to wild-type MEFs (Fig. 3). The observation that $p21^{Cip1-/-}$ MEFs exhibited a wild-type (albeit delayed) response to CDDP in S phase is consistent with our previous observation that the expression of $p21^{Cip1}$ in S-phase cells does not inhibit DNA synthesis, whereas the expression of PSM-RB blocks the incorporation of BrdU under identical conditions (18). Moreover, $p21^{Cip1}$ was not induced in S-phase-synchronized $Rb^{+/+}$ MEFs following 16 h of treatment with 32 μ M CDDP (Fig. 4c, compare lanes 1 and 2). However, the presence of $p21^{Cip1}$ did affect Cdk2 activity. As shown in Fig. 4d, Cdk2-associated kinase activity

FIG. 3. Dephosphorylation of RB in S phase precedes the inhibition of DNA replication. (a) Lysates were prepared from either asynchronously growing $Rb^{-/-}$ 3T3 cells (lane 1) or $Rb^{+/+}$ MEFs which were synchronized in either quiescence (Qui; lane 2) or Aph (lane 3) and then treated with vehicle (lane 4) or treated with 32 μ M CDDP (lane 5) for 12 h. RB was recovered from the lysates by immunoprecipitation, resolved by SDS-PAGE, and detected by immunoblotting. (b) Lysates were prepared from either asynchronously growing $Rb^{-/-}$ 3T3 cells (lane 1) or $Rb^{+/+}$ MEFs that were synchronized in quiescence (lane 2) or S phase using Aph (lane 3) and treated with 32 μ M CDDP for 0 (lane 4), 4 (lane 5), 8 (lane 6), or 12 (lane 7) h. RB was recovered from the lysates by immunoprecipitation, resolved by SDS-PAGE, and detected by immunoblotting. (c) Cells cultured as described for panel b were released from the Aph block, and BrdU incorporation was determined from a 4-h labeling. Data shown are relative percentages of BrdU-positive cells from two independent experiments.

with BrdU for 26 h. The BrdU-positive cells were counted, and the value was normalized to the percentage of BrdU-positive cells in untreated cells, which was set to 100%. (b) (Left) $Rb^{+/+}$ or $Rb^{-/-}$ MEFs were arrested in early S phase by Aph as described in Materials and Methods. The Aph-arrested cells were beyond the G_1 restriction point, as shown by a serum-independent DNA synthesis following release from the Aph block. After the Aph was washed away, BrdU was added to cells with the indicated concentration of FBS for 4 h. BrdU-positive cells were counted, and the percentage of BrdU-positive cells in 10% FBS was set as 100. (Right) Aph-arrested S-phase cells were treated with the indicated concentrations of CDDP for 16 h. Cells were then washed extensively to remove both Aph and CDDP and labeled with BrdU in 10% FBS for 4 h. The BrdU-positive cells were counted, and the value was normalized to that of CDDP-untreated culture, which was set to 100%. (c) MEFs of either $Rb^{+/+}$ or $Rb^{-/-}$ genotype were synchronized in S phase by sequential serum stimulation from quiescence for 16 h followed by incubation in HU (1 mM) for 10 h. The S-phase cells were then subjected to either 0 or 32 μ M CDDP for 16 h and washed extensively in medium for release from the HU block. BrdU was added to the medium, and cells were labeled for 4 h to monitor progression through S phase. Cells were fixed and stained for the incorporation of BrdU. Data shown are relative percentages of cells staining positive for BrdU incorporation from two independent experiments. (d) $Rb^{+/+}$ or $Rb^{-/-}$ MEFs were synchronized in S phase using Aph, treated with 0 to 32 μ M CDDP for 16 h, and then washed for release for 4 h. Cells were fixed and subjected to staining with the ICR4 antibody, which detects platinated DNA. Representative pictures shown were captured with identical exposure times.

FIG. 4. p21-defective cells exhibit a partial response to CDDP. (a) Aph-blocked wild-type (top), Rb^{-/-} (middle), and p21^{Cip1}^{-/-} (bottom) MEFs were subjected to increasing doses (0 to 40 μ M) of CDDP for 16 h. Cells were released from Aph and labeled with BrdU for 4 h. The relative percent BrdU incorporation shown is the average from one to three experiments. (b) Lysates were prepared from either asynchronous Rb^{-/-} 3T3 cells (lane 1) or p21^{Cip1}^{-/-} MEFs that were synchronized in quiescence (lane 2), blocked in Aph (lane 3), and treated with 32 μ M CDDP for 0 (lane 4), 4 (lane 5), 8 (lane 6), 12 (lane 7), or 16 (lane 8) h. RB was recovered using immunoprecipitation, resolved by SDS-PAGE, and detected by immunoblotting. (c) Lysates were prepared from Rb^{+/+} MEFs that were synchronized with Aph (lane 1) and treated with 32 μ M CDDP for 16 h (lane 2). Equal amounts of protein were resolved by SDS-PAGE, and p21^{Cip1} was detected by immunoblotting. (d) Lysates were prepared from Rb^{+/+} and p21^{Cip1}^{-/-} MEFs that were synchronized in quiescence (lanes 1 and 4), blocked in Aph (lanes 2 and 5), and treated with 32 μ M CDDP for 16 h (lanes 3 and 6). Cdk2 complexes were recovered by immunoprecipitation and analyzed in *in vitro* kinase assays using histone H1 as a substrate. Kinase activity was detected by autoradiography, and the immunoprecipitated Cdk2 was detected by immunoblotting.

was attenuated in Aph-synchronized Rb^{+/+} MEFs following a 16-h treatment with 32 μ M CDDP (compare lanes 2 and 3). By contrast, little change in Cdk2 activity was consistently observed in p21^{Cip1}^{-/-} MEFs following identical treatment (compare lanes 5 and 6). Collectively, these observations suggest that the inhibition of S-phase progression by CDDP is partially dependent on p21^{Cip1} function, thus indicating p21^{Cip1}-independent mechanisms of RB dephosphorylation in S-phase cells.

Cisplatin causes an RB-dependent arrest in mid-S phase. To demonstrate that CDDP affects S-phase progression in the

absence of Aph, we examined the effect of CDDP on S-phase progression in asynchronously growing MEF cultures. Titration and time course experiments were performed to identify a CDDP concentration that would induce RB dephosphorylation in asynchronous cells within one S-phase cycle. We found that 64 μ M CDDP caused the dephosphorylation of RB between 4 and 6 h in asynchronously growing cells. Therefore, Rb^{+/+} MEFs were treated with 64 μ M CDDP and pulse-labeled with BrdU for 1 h prior to the collection of samples for RB protein analysis (Fig. 5a) and flow cytometric analysis (Fig. 5b). After 6 h of CDDP treatment, ppRB was no longer de-

tected (Fig. 5a, lane 4). Pulse-labeling between h 5 to 6, however, showed no inhibition of DNA synthesis (Fig. 5b, panel 3). However, pulse-labeling with BrdU between h 7 to 8 showed a strong inhibition of BrdU incorporation (Fig. 5b, panel 4), and only pRB was found in cells harvested at the 8-h time point (Fig. 5a, lane 6). Dephosphorylated RB persisted through 12 h of CDDP treatment (Fig. 5a, lane 8), and DNA synthesis remained inhibited (not shown). Most importantly, cells with S-phase DNA content were present in these CDDP-treated cultures (as detected by propidium iodide staining), but these S-phase cells no longer incorporated BrdU (Fig. 5b, compare panels 2 and 4). Quantitation of the fluorescence-activated-cell sorting (FACS) data showed that greater than 75% of the cells with DNA content between 2N and 4N incorporated BrdU in the absence of CDDP, but only 10% of such cells incorporated BrdU following 7 to 8 h of CDDP treatment (Fig. 5c, panels 1 and 2; Fig. 5d). However, when $Rb^{-/-}$ MEFs were subjected to the same CDDP treatment, DNA synthesis continued, as $Rb^{-/-}$ MEFs treated with 64 μ M CDDP continued to undergo BrdU incorporation (Fig. 5c, compare panels 3 and 4). The relative level of BrdU incorporation was decreased in the CDDP-treated culture, consistent with a reduction in the rate of DNA synthesis due to the formation of platinum adducts in the DNA. Quantitation of the FACS data showed that greater than 75% of the S-phase $Rb^{-/-}$ MEFs incorporated BrdU, whether or not CDDP was present (Fig. 5d). Moreover, the $Rb^{-/-}$ MEFs progressed from S into G_2 and accumulated in G_2 phase (not shown). These results showed that CDDP can inhibit S-phase progression without prior treatment of cells with Aph or HU, and they reaffirmed that the inhibition of DNA synthesis followed RB dephosphorylation by approximately 2 h. The inhibition of DNA synthesis was not observed in asynchronously growing $Rb^{-/-}$ MEFs treated with CDDP. In other experiments, we found that asynchronously proliferating $p21^{Cip1-/-}$ MEFs responded to CDDP by undergoing an S-phase arrest, similar to $p21^{Cip1+/+}$ MEFs (not shown).

RB-dependent S-phase arrest by other DNA-damaging agents. To assess the effect of other genotoxic agents on S-phase progression, $Rb^{+/+}$ and $Rb^{-/-}$ MEFs were treated with IR, etoposide, and mitomycin C. IR causes both single- and double-strand breaks in DNA, etoposide inhibits topoisomerase II activity and results in predominantly double-strand breaks in late S-phase, and mitomycin C is an alkylating agent which causes DNA cross-links and double-strand breaks, similar to the effects of CDDP (32). Asynchronously growing cells were either (i) treated with 50 Gy of IR and cultured for an additional 8 h or (ii) exposed for 14 h to 5 μ M etoposide or 4 μ M of mitomycin C per ml. Cells were then pulse-labeled with BrdU for 1 h, fixed, and processed for bivariate flow cytometry. For all treatments, the percentage of S-phase cells (DNA content greater than 2N but less than 4N) incorporating BrdU was determined. Eight hours following IR, both $Rb^{+/+}$ and $Rb^{-/-}$ MEFs incorporated BrdU similarly to unirradiated controls. In both cell types, there was an accumulation of cells in G_2 . By contrast, $Rb^{+/+}$ MEFs showed a strong S-phase inhibition following treatment with either etoposide or mitomycin C (Fig. 5e). Again $Rb^{-/-}$ cells did not show a significant reduction in the percentage of cells incorporating BrdU following treatment with either etoposide or mitomycin C. As with CDDP treatment, there was a reduction in the relative amount of BrdU incorporated (not shown). However, the $Rb^{-/-}$ cells accumulated with a 4N DNA content, indicating that they were capable of progressing through S phase. These observations showed that the S-phase arrest could be induced by CDDP, etoposide, and mitomycin C but not by IR. Importantly, the

induction of S-phase arrest by these specific DNA-damaging agents was dependent on RB.

PSM-RB inhibits DNA synthesis in $Rb^{-/-}$ MEFs. Since the data presented suggest that CDDP-, mitomycin C-, and etoposide-induced inhibition of S phase is dependent on RB, we verified that activation of RB is sufficient to inhibit S-phase progression in the Rb -deficient cells. To do so, active RB (PSM-RB) was expressed in $Rb^{-/-}$ MEFs. PSM-RB was chosen for these experiments, as wild-type RB is inactivated by endogenous Cdk-cyclin complexes in S-phase cells (18). $Rb^{-/-}$ MEFs were first synchronized by Aph in S phase and then microinjected with both a PSM-RB expression vector and a plasmid expressing a fusion protein of histone H2B and GFP, which is localized to the nucleus. Sixteen hours postinjection, cells were released from Aph and labeled with BrdU for 4 h. Productively injected cells were identified by the nuclear GFP fluorescence, and the BrdU-positive cells were detected by immunostaining with anti-BrdU antibodies (Fig. 6a). Quantitation of these fluorescence images showed that the expression of H2B-GFP alone did not inhibit BrdU incorporation (Fig. 6b, vector). By contrast, coexpression with PSM-RB completely inhibited DNA synthesis in the Aph-synchronized $Rb^{-/-}$ cells (Fig. 6b). Thus, active RB is sufficient to inhibit S-phase progression.

S-phase arrest coincides with inhibition of cyclin A but not E2F. Since the data presented demonstrate that CDDP initiates an RB-dependent S-phase arrest, we probed the mechanism by which this arrest is induced. The ability of RB to inhibit G_1 /S progression has been attributed to its function as a transcriptional repressor (33, 56). Initially, we investigated the effect of CDDP treatment on E2F activity. For these experiments, $Rb^{+/+}$ and $Rb^{-/-}$ MEFs were transfected with the 3 $\times$ E2FLUC reporter and synchronized in S-phase with Aph. Transfected cells were treated with either 0 or 32 μ M CDDP for 16 h, at which time the cells were harvested for reporter assay. As shown in Fig. 7a, E2F transcriptional activity was inhibited approximately twofold in both $Rb^{-/-}$ and $Rb^{+/+}$ cells following CDDP treatment. These data indicate that CDDP damage can down-regulate E2F activity, but this inhibition occurs in $Rb^{-/-}$ MEFs.

To examine whether histone deacetylase activity is required for CDDP to inhibit S-phase progression, we added an inhibitor of histone deacetylase (trichostatin A [TSA]) to CDDP-treated cells (3, 30). Addition of TSA at concentrations that can block the transcription repression function of RB (3, 30) (data not shown) did not affect the ability of CDDP-treated cells to arrest in S phase (Fig. 7b). This result suggests that histone deacetylase activity is dispensable for the S-phase arrest. However, this result does not rule out gene repression as the cause of RB-mediated S-phase arrest, since RB can inhibit transcription in a histone deacetylase-independent manner (23, 29, 56).

Cyclin E, cyclin A, and Cdk2 are all important activators of DNA replication which are regulated by RB and E2F. We therefore examined the levels of these proteins in Aph-synchronized $Rb^{-/-}$ and $Rb^{+/+}$ MEFs treated for 16 h with 0 or 32 μ M CDDP (Fig. 7c). The Cdk2 protein levels were not affected in either cell type following CDDP treatment (middle panel, compare lanes 1 and 2 and lanes 3 and 4). The cyclin E protein level was significantly higher in $Rb^{-/-}$ MEFs than in $Rb^{+/+}$ MEFs (16), but CDDP treatment had no effect on cyclin E expression in either cell type (top panel, compare lanes 1 and 2 and lanes 3 and 4). By contrast, cyclin A protein levels were specifically attenuated in $Rb^{+/+}$ cells after CDDP treatment (lower panel, compare lanes 3 and 4), whereas cyclin A protein levels were unaffected by CDDP in the absence of

FIG. 5. CDDP inhibits DNA synthesis in mid-S-phase cells. (a) Asynchronously proliferating Rb^{+/+} MEFs were subjected to vehicle (lanes 1, 3, 5, and 7) or 64 μ M CDDP (lanes 2, 4, 6, and 8) for the indicated time. Cells were harvested, lysates were prepared, and Rb was immunoprecipitated, resolved by SDS-PAGE, and detected by immunoblotting. (b) Asynchronously growing Rb^{+/+} MEFs were treated with CDDP for the indicated times (panel 1, no BrdU; panel 2, 0 h; panel 3, 6 h; panel 4, 8 h) with BrdU pulse-labeling during the final hour of treatment. Cells were fixed and processed for BrdU incorporation to detect DNA synthesis and stained with propidium iodide (PI) to determine cell cycle phase by flow cytometry. Shown are representative scatter plots with the log fluorescein isothiocyanate (FITC) anti-BrdU staining versus propidium iodide staining. (c) Asynchronously growing Rb^{+/+} (panels 1 and 2) or Rb^{-/-} (panels 3 and 4) MEFs were treated with vehicle (panels 1 and 3) or 64 μ M CDDP (panels 2 and 4) for 8 h with BrdU pulse-labeling during the final hour of treatment. Cells were fixed and processed for BrdU incorporation to detect DNA synthesis and stained with propidium iodide to determine cell cycle phase by flow cytometry. Shown are representative scatter plots with the log FITC anti-BrdU staining versus propidium iodide staining. (d) The percentage of S-phase cells (greater than 2N but less than 4N DNA content) exhibiting DNA synthesis (BrdU positive) was determined. Data shown are from three independent experiments. (e) Asynchronously growing wild-type and Rb^{-/-} MEFs were treated with either IR (50 Gy), 5 μ M etoposide, or 4 μ g of mitomycin C (MMC) per ml. Following treatment, cells were pulsed with BrdU for 1 h and subsequently fixed and processed for bivariate flow cytometry. The percentage of S-phase cells (with DNA content between 2N and 4N) that incorporated BrdU was determined.

e.

FIG. 5—Continued.

RB (compare lanes 1 and 2). Thus, the RB-mediated S-phase inhibition correlated with down-regulation of cyclin A. These results are also consistent with previous reports that RB inhibits cyclin A expression through a mechanism that does not require the action of histone deacetylase (56).

Rb^{-/-} MEFs are hypersensitive to CDDP-induced cytotoxicity. Since it is known that checkpoint defects often render tumor cells hypersensitive to antineoplastic agents, we assessed the long-term consequence of RB-mediated arrest. To do so, asynchronously proliferating Rb^{+/+} MEFs were treated with 16 or 32 μM CDDP for 16 h to induce RB dephosphorylation, after which the cells were placed in fresh medium without CDDP. Samples were collected 36 h to 144 h following CDDP addition for FACS analysis and live-cell counting. In Rb^{+/+} MEFs, no significant alteration in the cell cycle distribution was observed by CDDP treatment or after CDDP withdrawal (Fig. 8a), except that the CDDP-treated cultures were inhibited for DNA synthetic activity during the entire 144-h experimental time course (not shown). Thus, CDDP arrested Rb^{+/+} cells in G₁, S, and G₂ phases of the cell cycle, and the arrest appeared to be irreversible. Consistent with an irreversible arrest in G₁, S, and G₂, there was no increase in cell number from 36 to 144 h (Fig. 8b). The maintenance of live cells was not due to a balance between cell proliferation and cell death, because these Rb^{+/+} MEFs did not incorporate BrdU and did not contain sub-G₁ DNA during the experimental time course. By contrast, Rb^{-/-} MEFs did not survive following the withdrawal of CDDP. A dramatic reduction in live cells (up to 90%) was observed following transfer into fresh medium (Fig. 8b). The Rb^{-/-} MEFs underwent at least another round of DNA replication following the withdrawal of CDDP to contain 8N DNA (not shown). At the same time, a large percentage of Rb^{-/-} MEFs showed sub-G₁ DNA content, indicative of apoptosis (not shown). Therefore, the RB-dependent arrest plays an important role in protecting cells from CDDP-induced apoptosis. Without RB, CDDP did not cause G₁ or S-phase arrest. In addition, the Rb^{-/-} MEFs bypassed the G₂ checkpoint and underwent a subsequent round of replication. The continued DNA synthesis following CDDP-induced DNA damage in Rb^{-/-} MEFs is associated with an increased sensitivity to killing by CDDP.

DISCUSSION

DNA damage is known to activate regulatory mechanisms that stop a proliferating cell in the G₁ or G₂ phase of the cell cycle (9, 14, 50, 51). The induction of G₁ or G₂ arrest prevents replication or segregation of damaged DNA and hence contributes to the maintenance of genome integrity. DNA damage can also affect the progression through S phase. In previous studies, DNA damage has been shown to cause a transient reduction in the rate of DNA synthesis (24, 36, 46). In this study, we have identified an additional intra-S-phase response that is activated by a prolonged exposure to CDDP. We demonstrate that this S-phase response requires RB activation, and dephosphorylation or activation precedes S-phase inhibition. The RB-dependent intra-S-phase response to CDDP appeared to be a long-term arrest in S-phase of the cell cycle. RB also mediated the G₁ arrest in CDDP-damaged cells (13), but the G₂ checkpoint response to CDDP did not require RB. Similar RB-dependent S-phase inhibition was observed in response to mitomycin C and etoposide exposure. The G₁ and S-phase arrest induced by RB had a protective effect against CDDP-induced death, because Rb^{-/-} cells could not undergo either the G₁ or the S-phase arrest and were hypersensitive to the cytotoxic effect of CDDP.

It is known that cells respond in G₁ to DNA damage by p53-mediated induction of p21^{Cip1} (5). Increased p21^{Cip1} serves to attenuate Cdk2 activity, thus preventing RB phosphorylation and inhibiting progression into S phase. The data presented herein demonstrate that the S-phase response to CDDP can be only partially attributed to p21^{Cip1} function, since p21^{Cip1} MEFs demonstrated a delayed response to CDDP compared to their wild-type counterparts. Moreover, p21^{Cip1} was not induced in S-phase Rb^{+/+} MEFs after CDDP treatment. However, the data presented indicate that p21^{Cip1} activity is required for down-regulation of Cdk2 in response to CDDP. These observations support a model wherein both p21^{Cip1}-dependent down-regulation of Cdk2 and p21^{Cip1}-independent mechanisms are important for RB-mediated arrest in response to CDDP.

It is possible that RB may be activated by a phosphatase in S phase after DNA damage. Other studies have reported the activation of RB phosphatase activities by stress. For example, in p53-deficient cells (which cannot induce p21^{Cip1} expression), the dephosphorylation of RB following DNA damage is me-

FIG. 6. Active RB is sufficient to block recovery from S-phase block. (a) $Rb^{-/-}$ MEFs were synchronized in S phase by sequential serum stimulation from quiescence for 16 h followed by incubation in Aph for 10 h. These S-phase cells were then microinjected with the indicated plasmids (50 ng/ μ l) and a plasmid encoding nuclear GFP (50 ng/ μ l). After 16 h (to allow for expression of the plasmid-encoded protein), the cells were washed extensively in medium for release from Aph block. BrdU was added to the medium, and cells were labeled for 4 h to monitor progression through S phase. Cells were fixed and stained for the incorporation of BrdU. Representative images are shown. (b) Data shown are percentages of GFP-positive cells staining positive for BrdU incorporation from three independent experiments with more than 40 injected cells per experiment. Control reflects the percentage of uninjected cells staining positively for BrdU incorporation from the same coverslips.

diated by the activation of RB phosphatase (7). Exposure to UV has recently been shown to invoke phosphatase activity, and this was correlated with the dephosphorylation of the RB-related protein p107 (47). In addition, exposure of cells to conditions of hypoxia also caused the dephosphorylation of RB in S-phase cells, and this was attributed to phosphatase activity (26). Under hypoxic conditions, the S-phase dephosphorylation of RB also correlated with an inhibition of DNA synthesis (26). Although these issues will be difficult to resolve until the mechanisms governing RB phosphorylation in S phase are delineated, the contribution of RB phosphatase activity and Cdk inhibition in the S-phase response to DNA damage are of obvious relevance.

The mechanism by which RB inhibits DNA synthesis must also be considered. Several possibilities can be envisioned, including (i) direct inhibition of the DNA replication enzymes,

(ii) disruption of the prereplication complex (pre-RC) at origins that have not been activated in mid-S phase, or (iii) destruction of a labile factor that is required for origin firing. The direct inhibition of replication enzymes by dephosphorylated RB is not likely, since DNA synthesis is not inhibited until approximately 2 h after the dephosphorylation of RB. RB is also not likely to inhibit the formation of pre-RC, because this complex is assembled during early G_1 prior to the activation of Cdks and RB phosphorylation (2). Furthermore, if RB were to directly disrupt the pre-RC, we would again expect RB dephosphorylation and the inhibition of DNA synthesis to occur simultaneously. At present, we favor the third hypothesis, in which RB inhibits the expression of a labile factor that is required for activation of the pre-RC. This mechanism is consistent with the 2-h delay observed between RB dephosphorylation and the S-phase arrest. It is also supported by our

FIG. 7. RB-dependent S-phase arrest correlates with inhibition of cyclin A but not E2F. (a) Rb^{+/+} and Rb^{-/-} MEFs were cotransfected with the 3×E2FLUC and CMV-betagal reporter constructs. Transfected cells were synchronized posttransfection with Aph and treated with 0 or 32 μ M CDDP. Cells were harvested after 16 h of treatment, and reporter assays were performed. Data shown are averages of at least four independent experiments. (b) Asynchronously growing Rb^{+/+} MEFs were treated with 0 or 64 μ M CDDP for 8 h; TSA (200 ng/ml) or vehicle was also added for the indicated times. BrdU was added to the cells for the last hour of treatment, and the incorporation of BrdU was determined by staining with anti-BrdU antibody followed by FACS analysis (Materials and Methods). The percent of S-phase cells (with DNA content between 2N and 4N) that incorporated BrdU was determined and shown for each of the experimental time points. (c) Rb^{+/+} and Rb^{-/-} MEFs were synchronized in Aph and treated with 0 or 32 μ M CDDP for 16 h. Lysates were harvested, equal amounts of protein were resolved by SDS-PAGE, and cyclin A, cyclin E, and Cdk2 were detected by immunoblotting.

previous studies showing that the simian virus 40 T antigen, the adenovirus E1A protein, or the overproduction of E2F1 can reverse the S-phase inhibitory function of PSM-RB (18). However, we find that TSA does not reverse the effect of CDDP on S-phase progression. Thus, the RB-mediated S-phase arrest may be due to gene repression that is not dependent on histone deacetylase.

To address this issue, we investigated the effect of CDDP on known RB targets in S phase. The data presented demonstrate that E2F activity is inhibited equally in Rb^{+/+} and Rb^{-/-} cells after CDDP treatment, suggesting that the ability of RB to regulate E2F reporter activity is distinct from the CDDP response. Similarly, cyclin E levels were unchanged by CDDP treatment in both Rb^{+/+} and Rb^{-/-} cells. Since RB-mediated regulation of cyclin E is dependent on histone deacetylase (55), these data are consistent with the failure of TSA to prevent the RB-mediated DNA damage response. By contrast, cyclin A was attenuated only in Rb^{+/+} cells after DNA damage, suggesting that the ability of RB to regulate cyclin A may contribute to the CDDP response (21). Importantly, RB regulation of cyclin A is known to be independent of histone deacetylase (55). We and others have recently shown that the ability of RB to regulate cyclin A is dependent on activity of the SWI/SNF chromatin remodeling protein, Brg-1 (42, 43, 55). In these studies, we showed that dominant negative Brg-1 blocks cyclin

A attenuation and cell cycle arrest after CDDP treatment in immortalized rodent fibroblasts (43). Furthermore, Zhang et al. showed that the RB/Brg-1-mediated down-regulation of cyclin A correlated with S-phase arrest (56). Together, these observations suggest that the ability of RB to regulate cyclin A likely contributes to the S-phase DNA damage response.

The current concept of cell cycle checkpoint in response to DNA damage is based on the yeast paradigm. In yeast, DNA damage induces a transient inhibition of G₁, S, and/or G₂ (9, 14, 50, 51). Genetic evidence suggests that these responses are to provide time for DNA repair, and proper repair increases the chance for survival (37, 40, 52). This current concept implies that the DNA damage-induced checkpoint is not permanent, and cells will progress through the checkpoint once DNA is repaired. The role of RB in mediating the G₁ response to DNA damage is well established (5, 13). However, whether RB fulfills a classical checkpoint role in G₁, by allowing damaged cells to ultimately reenter the cell cycle, has not been determined. Here we show that RB is required for an intra-S-phase cell cycle arrest in response to CDDP-mediated damage. This cell cycle arrest is maintained for a period of at least 5 days. Thus, the RB-mediated long-term arrest in S phase does not appear to be a reversible checkpoint response but may be the last resort for cells to survive irreparable damage caused by CDDP.

FIG. 8. RB prevents cell death following DNA damage. (a) Rb^{+/+} MEFs either asynchronously proliferating (Asy; panel 1) or treated with 32 μ M CDDP for 16 h and then cultured for a total 36 (panel 2) or 144 (panel 3) h after CDDP addition were harvested, fixed, and stained with propidium iodide. Shown are representative histograms of two independent experiments. (b) Rb^{+/+} or Rb^{-/-} MEFs were treated with 0, 16, or 32 μ M CDDP. After 16 h of treatment, CDDP was washed from the cells. The cells were then cultured for the indicated times after CDDP addition, at which point they were fixed and stained with crystal violet (left panel). The relative number of cells was determined by counting random fields of crystal violet stained dishes (right panels). The data are from duplicate plates and representative of three independent experiments.

The ability of RB to regulate both the G₁/S transition and the S-phase progression suggests that RB-deficient cells have an increased capacity to undergo DNA replication under conditions of genotoxic stress. Such a defect could lead to genome instability and tumor progression in tumors that exhibit RB loss through mutation (e.g., small cell lung cancer) or viral oncoprotein binding (e.g., cervical cancer). However, in terms of cancer treatment, such tumors would be expected to respond favorably to CDDP. Although the RB gene itself is mutated at a relatively low frequency in tumors, it is inactivated in the majority of tumor types due to the increased activity of Cdk and decreased activity of Cdk inhibitors (39). The data presented herein predict that RB-positive tumors should be less sensitive to CDDP than tumor cells lacking the RB protein. Moreover, these data demonstrate that the combined regulation of S-phase entry and S-phase progression likely underlie the function of RB as a tumor suppressor.

ACKNOWLEDGMENTS

We are indebted to the Knudsen and Wang laboratories for technical, administrative, and logistical support. We are grateful to Yolanda Sanchez (University of Cincinnati) for critical reading of the manuscript. Mice with targeted disruption of p21^{Cip1} were from Tyler Jacks (MIT). We thank Steven Howell (University of California, San

Diego) and Lyon Gleich (University of Cincinnati) for provision of CDDP. We are indebted to Mike Tilby (University of Manchester) for supplying the ICR4 antibody and Geoff Wahl (Salk Institute) for the H2B-GFP expression plasmid. We also thank George Babcock and Jim Cornelius (Shriners Hospital for Children, Cincinnati, Ohio) for expert flow cytometric analysis.

K.E.K. is supported by an NRSA award from the NIH (CA82034). S.N. is supported by the Norwegian Cancer Society. E.S.K. is a Kimmel Scholar. This work was supported by grants to J.Y.J.W. from the NCI/NIH (CA58320) and to E.S.K. from NCI/NIH (CA82525) and the Ohio Cancer Research Associates.

REFERENCES

- Bartek, J., J. Bartkova, and J. Lukas. 1997. The retinoblastoma protein pathway in cell cycle control and cancer. *Exp. Cell Res.* **237**:1-6.
- Bik, T. 1999. MCM proteins in DNA replication. *Annu. Rev. Biochem.* **68**:649-686.
- Brehm, A., E. A. Miska, D. J. McCance, J. L. Reid, A. J. Bannister, and T. Kouzarides. 1998. Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature* **391**:597-601.
- Brugarolas, J., C. Chandrasekaran, J. I. Gordon, D. Beach, T. Jacks, and G. J. Hannon. 1995. Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* **377**:552-557.
- Brugarolas, J., K. Moberg, S. D. Boyd, Y. Taya, T. Jacks, and J. A. Lees. 1999. Inhibition of cyclin-dependent kinase 2 by p21 is necessary for retinoblastoma protein-mediated G1 arrest after gamma-irradiation. *Proc. Natl. Acad. Sci. USA* **96**:1002-1007.
- Chew, Y. P., M. Ellis, S. Wilkie, and S. Mittnacht. 1998. pRB phosphorylation mutants reveal role of pRB in regulating S phase completion by a

- mechanism independent of E2F. *Oncogene* **17**:2177–2186.
7. Dou, Q. P., B. An, and P. L. Will. 1995. Induction of a retinoblastoma phosphatase activity by anticancer drugs accompanies p53-independent G1 arrest and apoptosis. *Proc. Natl. Acad. Sci. USA* **92**:9019–9023.
 8. el-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**:817–825.
 9. Elledge, S. J. 1996. Cell cycle checkpoints: preventing an identity crisis. *Science* **274**:1664–1672.
 10. Flemington, E. K., S. H. Speck, and W. G. Kaelin, Jr. 1993. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. *Proc. Natl. Acad. Sci. USA* **90**:6914–6918.
 11. Grunstein, M. 1997. Histone acetylation in chromatin structure and transcription. *Nature* **389**:349–352.
 12. Harbour, J. W., R. X. Luo, A. Dei Santi, A. A. Postigo, and D. C. Dean. 1999. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell* **98**:859–869.
 13. Harrington, E. A., J. L. Bruce, E. Harlow, and N. Dyson. 1998. pRB plays an essential role in cell cycle arrest induced by DNA damage. *Proc. Natl. Acad. Sci. USA* **95**:11945–11950.
 14. Hartwell, L. H., and M. B. Kastan. 1994. Cell cycle control and cancer. *Science* **266**:1821–1828.
 15. Herrera, R. E., T. P. Makela, and R. A. Weinberg. 1996. TGF beta-induced growth inhibition in primary fibroblasts requires the retinoblastoma protein. *Mol. Biol. Cell* **7**:1335–1342.
 16. Herrera, R. E., V. P. Sah, B. O. Williams, T. P. Makela, R. A. Weinberg, and T. Jacks. 1996. Altered cell cycle kinetics, gene expression, and G₁ restriction point regulation in Rb-deficient fibroblasts. *Mol. Cell. Biol.* **16**:2402–2407.
 17. Kaelin, W. G., Jr. 1999. Functions of the retinoblastoma protein. *Bioessays* **21**:950–958.
 18. Knudsen, E. S., C. Buckmaster, T. T. Chen, J. R. Feramisco, and J. Y. Wang. 1998. Inhibition of DNA synthesis by RB: effects on G1/S transition and S-phase progression. *Genes Dev.* **12**:2278–2292.
 19. Knudsen, E. S., and J. Y. Wang. 1996. Differential regulation of retinoblastoma protein function by specific Cdk phosphorylation sites. *J. Biol. Chem.* **271**:8313–8320.
 20. Knudsen, E. S., and J. Y. Wang. 1997. Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. *Mol. Cell. Biol.* **17**:5771–5783.
 21. Knudsen, K. E., A. F. Fribourg, M. W. Strobeck, J. M. Blanchard, and E. S. Knudsen. 1999. Cyclin A is a functional target of retinoblastoma tumor suppressor protein-mediated cell cycle arrest. *J. Biol. Chem.* **274**:27632–27641.
 22. Kouzarides, T. 1999. Histone acetylases and deacetylases in cell proliferation. *Curr. Opin. Genet. Dev.* **9**:40–48.
 23. Lai, A., J. M. Lee, W. M. Yang, J. A. DeCaprio, W. G. Kaelin, Jr., E. Seto, and P. E. Branton. 1999. RBP1 recruits both histone deacetylase-dependent and -independent repression activities to retinoblastoma family proteins. *Mol. Cell. Biol.* **19**:6632–6641.
 24. Larner, J. M., H. Lee, and J. L. Hamlin. 1997. S phase damage sensing checkpoints in mammalian cells. *Cancer Surv.* **29**:25–45.
 25. Ludlow, J. W., C. L. Glendening, D. M. Livingston, and J. A. DeCaprio. 1993. Specific enzymatic dephosphorylation of the retinoblastoma protein. *Mol. Cell. Biol.* **13**:367–372.
 26. Ludlow, J. W., R. L. Howell, and H. C. Smith. 1993. Hypoxic stress induces reversible hypophosphorylation of pRB and reduction in cyclin A abundance independent of cell cycle progression. *Oncogene* **8**:331–339.
 27. Lukas, J., T. Herzinger, K. Hansen, M. C. Moroni, D. Resnitzky, K. Helin, S. I. Reed, and J. Bartek. 1997. Cyclin E-induced S phase without activation of the pRB/E2F pathway. *Genes Dev.* **11**:1479–1492.
 28. Lundberg, A. S., and R. A. Weinberg. 1998. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol. Cell. Biol.* **18**:753–761.
 29. Luo, R. X., A. A. Postigo, and D. C. Dean. 1998. Rb interacts with histone deacetylase to repress transcription. *Cell* **92**:463–473.
 30. Magnaghi-Jaulin, L., R. Groisman, I. Naguibneva, P. Robin, S. Lorain, J. P. Le Villain, F. Troalen, D. Trouche, and A. Harel-Bellan. 1998. Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature* **391**:601–605.
 31. Mittnacht, S. 1998. Control of pRB phosphorylation. *Curr. Opin. Genet. Dev.* **8**:21–27.
 32. Mycek, M., R. Harvey, and P. Champe. 1997. Anticancer drugs, p. 373–400. In R. Harvey and P. Champe (ed.), *Pharmacology*, 2nd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
 33. Nevins, J. R., G. Leone, J. DeGregori, and L. Jakoi. 1997. Role of the Rb/E2F pathway in cell growth control. *J. Cell Physiol.* **173**:233–236.
 34. Ohtsubo, M., A. M. Theodoras, J. Schumacher, J. M. Roberts, and M. Pagano. 1995. Human cyclin E, a nuclear protein essential for the G₁-to-S phase transition. *Mol. Cell. Biol.* **15**:2612–2624.
 35. Pagano, M., R. Pepperkok, F. Verde, W. Ansorge, and G. Draetta. 1992. Cyclin A is required at two points in the human cell cycle. *EMBO J.* **11**:961–971.
 36. Painter, R. B., and B. R. Young. 1980. Radiosensitivity in ataxia-telangiectasia: a new explanation. *Proc. Natl. Acad. Sci. USA* **77**:7315–7317.
 37. Paulovich, A. G., and L. H. Hartwell. 1995. A checkpoint regulates the rate of progression through S phase in *S. cerevisiae* in response to DNA damage. *Cell* **82**:841–847.
 38. Resnitzky, D., L. Hengst, and S. I. Reed. 1995. Cyclin A-associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G₁ by p27^{Kip1}. *Mol. Cell. Biol.* **15**:4347–4352.
 39. Sherr, C. J. 1996. Cancer cell cycles. *Science* **274**:1672–1677.
 40. Siede, W., A. S. Friedberg, and E. C. Friedberg. 1993. RAD9-dependent G1 arrest defines a second checkpoint for damaged DNA in the cell cycle of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **90**:7985–7989.
 41. Siegart, J., and P. Robbins. 1999. Rb inhibits the intrinsic kinase activity of the TATA-binding protein-associated factor. *Mol. Cell. Biol.* **19**:846–854.
 42. Strobeck, M. W., A. F. Fribourg, A. Puga, and E. S. Knudsen. 2000. Restoration of retinoblastoma mediated signaling to Cdk2 results in cell cycle arrest. *Oncogene* **19**:1857–1867.
 43. Strobeck, M. W., K. E. Knudsen, A. F. Fribourg, M. F. DeCristofaro, B. E. Weissman, A. N. Imbalzano, and E. S. Knudsen. 2000. BRG-1 is required for RB-mediated cell cycle arrest. *Proc. Natl. Acad. Sci. USA* **97**:7748–7753.
 44. Tan, X., S. J. Martin, D. R. Green, and J. Y. J. Wang. 1997. Degradation of retinoblastoma protein in tumor necrosis factor- and CD95-induced cell death. *J. Biol. Chem.* **272**:9613–9616.
 45. Tilby, M. J., C. Johnson, R. J. Knox, J. Cordell, and C. J. Dean. 1991. Sensitive detection of DNA modifications induced by cisplatin and carboplatin in vitro and in vivo using a monoclonal antibody. *Cancer Res.* **51**:123–129.
 46. Vincent, R. A., Jr., A. J. Fink, and P. C. Huang. 1980. Unscheduled DNA synthesis in cultured ataxia telangiectasia fibroblast-like cells. *Mutat. Res.* **72**:245–249.
 47. Voorhoeve, P. M., R. J. Watson, P. G. Farlie, R. Bernards, and E. W. Lam. 1999. Rapid dephosphorylation of p107 following UV irradiation. *Oncogene* **18**:679–688.
 48. Waga, S., G. J. Hannon, D. Beach, and B. Stillman. 1994. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* **369**:574–578.
 49. Wang, J. Y., E. S. Knudsen, and P. J. Welch. 1994. The retinoblastoma tumor suppressor protein. *Adv. Cancer Res.* **64**:25–85.
 50. Weinert, T. 1998. DNA damage and checkpoint pathways: molecular anatomy and interactions with repair. *Cell* **94**:555–558.
 51. Weinert, T. 1998. DNA damage checkpoints update: getting molecular. *Curr. Opin. Genet. Dev.* **8**:185–193.
 52. Weinert, T. A., and L. H. Hartwell. 1988. The RAD9 gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. *Science* **241**:317–322.
 53. Weintraub, S. J., K. N. Chow, R. X. Luo, S. H. Zhang, S. He, and D. C. Dean. 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature* **375**:812–815.
 54. Zarkowska, T., and S. Mittnacht. 1997. Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. *J. Biol. Chem.* **272**:12738–12746.
 55. Zhang, H. S., M. Gavin, A. Dahiya, A. A. Postigo, D. Ma, R. X. Luo, J. W. Harbour, and D. C. Dean. 2000. Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. *Cell* **101**:79–89.
 56. Zhang, H. S., A. A. Postigo, and D. C. Dean. 1999. Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFbeta, and contact inhibition. *Cell* **97**:53–61.