Cdc6 Determines Utilization of p21<sup>WAF1/CIP1</sup>-dependent Damage Checkpoint in S Phase Cells*<sup>S</sup>

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When cells traversing G1 are irradiated with UV light, two parallel damage checkpoint pathways are activated: Chk1-Cdc25A and p53-p21<sup>WAF1/CIP1</sup>, both targeting Cdk2, but the latter inducing a long lasting arrest. In similarly treated S phase-progressing cells, however, only the Cdc25A-dependent checkpoint is active. We have recently found that the p21-dependent checkpoint can be activated and induce a prolonged arrest if S phase cells are damaged with a base-modifying agent, such as methyl methanesulfonate (MMS) and cisplatin. But the mechanistic basis for the differential activation of the p21-dependent checkpoint by different DNA damaging agents is not understood. Here we report that treatment of S phase cells with MMS but not a comparable dose of UV light elicits proteasome-mediated degradation of Cdc6, the assembler of pre-replicative complexes, which allows induced p21 to bind Cdk2, thereby extending inactivation of Cdk2 and S phase arrest. Consistently, enforced expression of Cdc6 largely eliminates the prolonged S phase arrest and Cdk2 inactivation induced with MMS, whereas RNA interference-mediated Cdc6 knockdown not only prolongs such arrest and inactivation but also effectively activates the p21-dependent checkpoint in the UV-irradiated S phase cells.

The replication of cellular DNA is initiated by the formation of pre-replicative complexes that takes place in late M-G1 phases (1–5). In the assembly of pre-replicative complexes, Cdc6 plays a key role in loading the minichromosome maintenance (Mcm) helicase complexes on the origin recognition complex-bound at early and late replication origins. Cdt1 cooperates with Cdc6, whereas Geminin antagonizes Cdt1. After pre-replicative complexes are assembled, several factors, some of which require activation by Cdk2, are further loaded on early origins. Finally, DNA polymerases are recruited to those origins and the Mcm helicase is activated by Cdc7 to begin DNA synthesis. During progression in G1-S phases, Cdk2 activity is negatively controlled by two distinct mechanisms: phosphorylation at Tyr<sup>15</sup> (and Thr<sup>14</sup>) and association with the cyclin-dependent kinase inhibitors p21<sup>WAF1/CIP1</sup> or p27<sup>KIP1</sup> (6–8). Activated Chk1 inactivates Cdc25A phosphatase via phosphorylation-dependent nuclear exclusion and/or proteolytic destruction (9). This rapid reaction causes initial inactivation of Cdk2 as a consequence of accumulation of its Tyr<sup>15</sup> (and Thr<sup>14</sup>) phosphorylated form. In parallel, both Atr and activated Chk1 activate and stabilize p53, which in turn induces a few factors including p21, which are responsible for a long-lasting inactivation of Cdk2 and therefore an extended G1 phase arrest (8, 10, 11).

When S phase-progressing cells are similarly irradiated, the same sensor detects damage and activates Chk1. But only the Chk1-Cdc25A pathway becomes effectively activated (10, 11). Consequently, inactivation of Cdk2 is transient and S phase arrest is short despite that late origin firing could also be blocked via an additional mechanism involving activated Chk1 and Cdc7 (12). The lack of effective utilization of the p53-p21 pathway for S phase arrest has been attributed at least in part to a weak induction of p21 resulting from S phase-specific impairments of its transcription and facilitation of its proteasome-mediated degradation (13–19).

However, poor utilization of the p21-dependent checkpoint is not an innate property of S phase cells. We have recently found that treatment of S phase-progressing cells with a base-modifying chemical, such as methyl methanesulfonate (MMS), effectively activates p21-dependent damage checkpoint and induces a prolonged S phase arrest with closely associated inactivation of Cdk2 by binding p21 despite no obvious differences in the extent of p21 induction and its subcellular localization between the cells treated with MMS and those irradiated with a comparable dose of UV (20). To understand the mechanistic basis for this phenomenon, we have investigated major cell cycle factors controlling the onset and progression of S phase and identified Cdc6 as a key determinant for the utilization of the p21-dependent checkpoint at least for DNA damage-induced S phase arrest.

**EXPERIMENTAL PROCEDURES**

**Chemical and Antibodies—**MMS was purchased from Sigma; antibodies αCdc6 (DCS-180) and αMcm7 Ab-2 (47DC141) were from NeoMarkers; αCdk2 (M2), αCdk2(M2)-G, αCdk4 (C-22), αCdk6 (C-21), αCyclin E (C-19), αCyclin A (C-19), αMcm2

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<sup>2</sup>The abbreviations used are: Mcm, minichromosome maintenance; MMS, methyl methanesulfonate; MEF, mouse embryonic fibroblast; MOPS, 4-morpholinepropanesulfonic acid; RNAi, RNA interference.
Inducible for Cdc6

infect p27

packaging cell line. The resulting pRevTet-Off virus was used to

pRevTet-Off vector and the pRevTRE response vector with the

inserted into the pRevTRE response vector (Clontech). The

(25), and 20

(24).

NRK cells progressing in mid-S phase were prepared by G0

embryonic fibroblasts were isolated from C-57 mouse embryos.

ATCC) were maintained in Dulbecco's modified Eagle's

culture medium or irradiated with UV at 7.5 or 15 J/m², washed

tained in Dulbecco's modified Eagle's medium containing 10%

silk fibroblasts and human KB cells (22) were synchronized to early S by a double thymidine block (23)

and then released in fresh medium for 1–2 h to progress into

mid-S phase.

**MMS Treatment and UV Irradiation**—For each time point, cells traversing mid-S phase at a density of 2–5 × 10^5 per 10-cm dish were treated for 2–3 h with MMS at 120–180 μg/ml in culture medium or irradiated with UV at 7.5 or 15 J/m², washed twice with phosphate-buffered saline, and cultured in growth medium containing 15–25 ng/ml colcemid with cell sampling at the specified times.

**Cell Viability**—The % viability of cells after genotoxic treatment was determined from the number of colonies formed after a 1-week culture of 200 cells plated in a 10-cm dish in a triplicate experiment.

**Flow Cytometry**—Flow cytometric analysis was performed with the FACScan flow cytometer (Beckman Coulter ECPI XL) equipped with computer-assisted analysis of cell population in each cell cycle phase according to the manufacturer’s manual.

**Preparation of Whole Cell Extract, Immunoblot, and Cdk2 Kinase Assay**—Harvested cells were lysed as described (24). Lysates were divided into halves. One-half was used for immunoblot analysis and the other half for immunoprecipitation of Cdk2. Cdk2 kinase activity was assayed with a truncated Rb protein (QED Bioscience) as a substrate followed by immunoblot detection of Ser^807/Ser^811-phosphorylated Rb as described (24).

**Northern Blot Analysis**—Total cellular RNA was prepared (25), and 20 μg of aliquots were electrophoresed in a 1.1% agarose gel containing MOPS-buffered 2.2 M formaldehyde. Separated RNAs were transferred to a nylon membrane and hybridized with a random primed 32P-labeled probe of rat Cdc6 or β-actin cDNA.

**Construction of p27^-/- Mouse Embryonic Fibroblast (MEF) Inducible for Cdc6**—A cDNA encoding His-tagged Cdc6 was inserted into the pRevTRE response vector (Clontech). The pRevTet-Off vector and the pRevTRE response vector with the Cdc6 cDNA insert were separately transfected into the EcoPack packaging cell line. The resulting pRevTet-Off virus was used to infect p27^-/- MEF (26) to produce a stable Tet-Off cell clone. This Tet-Off clone was then infected with pRevTRE-Cdc6 to obtain a pool of cell clones inducible for Cdc6. Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 1 μg of doxycycline/ml. Expression was induced by withdrawal of doxycycline.

**RNA Interference**—Dicer-Substrate 27-mer duplexes targeting the Cdc6 gene that were selected from a pre-designed set of duplexes from the RefSeq collection of mouse genes in GenBank™ were purchased from Integrated DNA Technologies. Exponentially proliferating cells were transfected with the universal negative control RNA duplex or the Cdc6-specific duplex at a final concentration of 10 nm according to the vendor’s instruction. Analyses were performed 72 h post-transfection.

**RESULTS**

*p21_{WAF1/CIP1} Is Induced to a Similar Level in UV-irradiated and MMS-treated Cells but Binds the Cdk2 Only in MMS-treated Cells**—To identify the molecular basis for the activation of p21-dependent intra-S checkpoint by base-modifying chemicals, we first compared the cellular and molecular responses elicited by MMS treatment and UV irradiation, particularly the duration of S phase arrest and the status of activation of the Chk1/Chk2-Cdc25A and p53-p21 checkpoint pathways to confirm our recent finding (20). To scientifically validate comparison, the dose of UV irradiation was chosen to give roughly the same cell viability as with MMS treatment.

Cells of the rat fibroblast line NRK-49F (NRK) were synchronized to mid-S phase, treated for 3 h with MMS at 120 μg/ml or irradiated with UV at 7.5 J/m², and cultured further in growth medium containing colcemid, a metaphase blocker, to prevent their return to G1, that would obscure the population of S phase-arrested cells. During treatment with MMS or UV and subsequent culturing, cells were collected every 4 h and analyzed for their cell cycle patterns and the levels of Chk1, Chk1 Ser^345 phosphorylation, Chk2, Chk2 Thr^68 phosphorylation, Cdc25A, p53, p21, and Cdk2 as well as the activity of Cdk2, its Tyr^15 phosphorylation status and the amount of Cdk2-associated p21. In parallel, the treated cells were subjected to a colony formation assay to determine their actual viability. It was 70% for the MMS-treated cells and 78% for the UV-irradiated cells.

As indicated by cytometry, the MMS-treated cells arrested in S phase for more than 20 h, whereas the UV-irradiated NRK arrested for less than 12 h (Fig. 1A, panel a), both accompanied by inactivation of Cdk2 (Fig. 1A, panel b). In these cells, Ser^345 phosphorylation of Chk1 appeared within 4 h and faded out by 8 h with no apparent elevation of Chk2 Thr^68 phosphorylation, consistent with the previous finding that the DNA damage induced by MMS and UV was detected by Atr but not Atm (8). On the other hand, induction of p53 and p21 began within 4 h and continued for more than 20 h in the MMS-treated cells and at least for 12 h in the UV-irradiated cells. Remarkably, there was no significant difference in the levels of induced p21 or p53 between these two cells despite the marked difference in the duration of S phase arrest. However, significantly higher amounts of p21 were associated with the inactivated Cdk2 in the MMS-treated cells, whereas Tyr^15 phosphorylation of Cdk2 was elevated only in the UV-irradiated cells and at 4 and 8 h. In the MMS-treated cells, Cdk2 was reactivated at 20 h concomitant with the reduction of bound p21, whereas in the UV-irradiated cells, Cdk2 was reactivated at 12 h coinciding with the
decrease of Tyr15 phosphorylation. These results are consistent with exclusive utilization of the Chk1-Cdc25A pathway in UV-irradiated S phase cells as well documented (8, 10, 11) and reiterate our recent finding that treatment with base-modifying chemicals activated the p21-dependent damage checkpoint in S phase cells (20).

FIGURE 1. The prolonged S phase arrest induced by MMS treatment is accompanied by a marked reduction in the Cdc6 level. A, NRK cells were synchronized to mid-S, treated for 3 h with MMS at 120 μg/ml or irradiated with UV at a dose of 7.5 J/m², and incubated in colcemid-containing medium every 4 h for cell sampling (the start of treatment or irradiation as 0 h). The cytometric patterns and populations in each phase of the collected cells were determined (panel a). In parallel, the collected cells were lysed and analyzed for the levels of Chk1 phosphorylated at Ser345 (pCHK1), Chk1, Chk2, Chk2 phosphorylated at Thr68 (pCHK2), Cdc25A, p21, p53, p27, Cdk2 (panel b), Cdc6, Cyclin E (CycE), Cyclin A (CycA), Geminin, Cdt1, Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, Mcm7, Cdk4, Cdk6, and Cyclin D1 (CycD1) (panel c). Furthermore, the amount, activity, and Tyr15 phosphorylation of Cdk2 and the amount of Cdk2-bound p21 were determined following immunoprecipitation of Cdk2 (panel b). β-Actin was used as loading controls. Panel d, Cdc6 is predominantly localized in the nucleus during DNA damage and recovery. The cytosolic (C) and nuclear/chromatin-bound (N) fractions were prepared by digitonin-mediated cell permeabilization (31) and subsequent 0.35 M NaCl/immunoprecipitation buffer extraction of each cell sample and immunoblotted for Cdc6, p21, Cdk2, Histone H1 (H1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the latter two as nuclear and cytoplasmic markers, respectively. B, a higher dose of UV irradiation destabilizes Cdc6 and induces p21-bound Cdk2 inactivation and prolonged S phase arrest. NRK cells were synchronized to mid-S, irradiated with UV at a dose of 15 or 7.5 J/m², and analyzed as in A. C, the decrease of the Cdc6 protein level during MMS-induced S phase arrest is caused by proteasome-mediated degradation. Panel a, Northern blot analysis of Cdc6 mRNA. Mid-S phase-synchronized NRK cells were treated with MMS as in A and harvested every 4 h. Total cellular RNA was prepared from each cell sample and analyzed by Northern blotting with radiolabeled probes. β-Actin mRNA was used as a loading control. Panel b, co-treatment with a specific proteasome inhibitor stabilizes Cdc6 protein. Mid-S phase-synchronized NRK cells were treated with MMS as in A, except that lactacystin was added to the culture medium at a final concentration of 10 μg/ml during MMS treatment and subsequent culturing. The levels of Cdc6 and p53 were determined by immunoblotting. β-Actin was used as a loading control.
The highly selective activation of either one of the two parallel checkpoints in S phase cells by these damaging agents under the current experimental conditions was further confirmed as follows. We previously showed that when two NRK cell clones overexpressing wild-type Cdk2 (NRK-Cdk2<sup>Y15</sup>) and non-phosphorylatable Cdk2<sup>F15</sup> (NRK-Cdk2<sup>F15</sup>) were treated with MMS, they arrested in S phase for virtually the same duration (18–22 h) with inactivation of both Cdk2<sup>Y15</sup> and Cdk2<sup>F15</sup> associated with similar amounts of induced p21 (20).

When the same pair of the cells were irradiated with the same dose of UV as in Fig. 1A during their mid-S progression and analyzed similarly, NRK-Cdk2<sup>Y15</sup> arrested in S phase for >8 h with inactivation of Cdk2 accompanied by elevation of its Tyr<sup>15</sup> phosphorylation just like the Fig. 1A data (supplemental Fig. S1A). On the other hand, NRK-Cdk2<sup>F15</sup> showed a short S phase arrest (<8 h) with a transient inactivation of Cdk2 at 4 h presumably caused by its Thr<sup>14</sup> phosphorylation (6). Again, unlike after MMS treatment, no significant amounts of p21 bound to the Cdk2. Consistent with the lack of p21 involvement, ablation of p21 expression had virtually no effects on the UV-induced Cdk2 inactivation and S phase arrest (supplemental Fig. S1B).

In this experiment, MEF and p21 gene knock-out MEF (p21<sup>−/−</sup>-MEF) responded exactly alike to UV irradiation, being arrested in S phase for the same duration and then recovered at the same time with reactivation of Cdk2.

As shown above, irradiation with 7.5 J/m<sup>2</sup> UV activated only the Cdc25A-dependent checkpoint in S phase. However, this checkpoint selection was not an innate nature of UV irradiation. When NRK cells were irradiated with twice the dose of UV (15 J/m<sup>2</sup>), which lowered the cell viability to 32%, they arrested much longer with inactive Cdk2 bound by p21 but diminished for its Tyr<sup>15</sup> phosphorylation despite a noticeable reduction of the Cdc25A level as reported (9) (Fig. 1B). Thus, a high dose UV irradiation activated the p21-dependent checkpoint and simultaneously suppressed the Cdc25A-dependent checkpoint as if it had been MMS treated. On the contrary, use of a reduced dose of MMS simply resulted in a short S phase arrest and partial Cdk2 inactivation with binding of p21 but no apparent elevation of its Tyr<sup>15</sup> phosphorylation, showing that the p21-dependent checkpoint was weakly activated but the Cdc25A-dependent checkpoint remained suppressed (supplemental Fig. S2).

**Marked Decrease of the Cdc6 Level Accompanies the Protracted S Phase Arrest Induced by MMS**—Next, to gain insights into the molecular mechanism for the suppression of the p21-dependent checkpoint in the 7.5 J/m<sup>2</sup> UV-irradiated but not the MMS-treated NRK cells, we compared expression of major cell cycle factors regulating the onset and progression of S phase between them. To this end, the same cell lysates as prepared for Fig. 1A, panel b, were analyzed for Cdc6, Cyclin E (CycE), Cyclin A (CycA), Cdt1, Geminin, Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, Mcm7, Cyclin D1 (CycD1), Cdk4 and Cdk6 (Fig. 1A, panel c). Interestingly enough, of these factors examined, one markedly differed between these two treatments. In the MMS-treated cells, the Cdc6 protein significantly diminished and then re-accumulated at 16 h, closely followed by reactivation of Cdk2 concomitant with a decrease of bound p21 as described above. By contrast, in the UV-irradiated cells where the p21-dependent checkpoint was completely suppressed, the level of Cdc6 remained virtually unchanged (Fig. 1A, panel c, supplemental Fig. S1, A and B). Thus, activation of the p21-dependent checkpoint was closely associated with a decrease of the Cdc6 level. This relationship was retained also in the high dose UV-irradiated cells (Fig. 1B). Unlike with 7.5 J/m<sup>2</sup> UV, irradiation with 15 J/m<sup>2</sup> UV destabilized Cdc6 protein, whose reduction coincided well with the appearance of the binding of p21 to Cdk2.

During S phase arrest and recovery, Cdc6 protein was predominately localized in the nuclear/chromatin-bound fraction, whereas a considerable amount of p21 became re-localized from the cytosol to the nuclear/chromatin-bound fraction regardless of whether cells were treated with MMS or UV (Fig. 1A, panel d). By contrast, the majority of Cdk2 protein remained in the cytosol.

**MMS-induced Reduction of Cdc6 Expression Is Caused by Proteasome-mediated Protein Destabilization**—The reduction of the Cdc6 level caused by MMS treatment was mediated mainly by proteasome-mediated protein degradation and little by transcriptional repression. The level of Cdc6 mRNA was not significantly altered by treatment with MMS (Fig. 1C, upper panel), whereas lactacystin, a specific proteasome inhibitor, effectively blocked the disappearance of Cdc6 protein in MMS-treated NRK cells (Fig. 1C, lower panel), consistent with the recent finding of Cdc6 degradation by Huwel-dependent ubiquitination/proteasome after DNA damage (27).

**Enforced Cdc6 Expression Advances Re-initiation of S Phase Progression in MMS-arrested but Not UV-arrested NRK Cells**—As shown in Fig. 1A, in the MMS-treated NRK cells, re-accumulation of Cdc6 protein was closely followed by a decrease of the Cdk2-bound p21 with concurrent reactivation of Cdk2 and subsequent re-initiation of S phase progression. Moreover, no significant amounts of induced p21 became associated with Cdk2 whenever Cdc6 was stable. Thus, there was a clear inverse correlation between the level of Cdc6 and the binding of p21 to Cdk2. We, therefore, sought to determine the effect of enforced Cdc6 expression on the timing of reactivation of Cdk2 as well as recovery from S phase arrest. NRK-Cdc6<sup>Y15</sup>, a previously established NRK cell clone roughly 2-fold overexpressing Cdc6 from the constitutive cytomegalovirus promoter (24), was subjected to the same analysis as in Fig. 1A. As shown in Fig. 3A, when treated with MMS, NRK-Cdc6#17 arrested briefly and re-initiated S phase progression roughly 12 h earlier than the empty vector-transfected control NRK cell although...
there was no apparent difference in the rate of S phase progression between the two when untreated (the latter data not shown).

Biochemical analysis revealed intriguing results. The timing, extent, and duration of Chk1 phosphorylation and p21 induction were similar between the two. But, in the overexpressor, reactivation of Cdk2 began just before the advanced re-initiation of S phase progression despite a high level of p21, but slightly behind the restoration of the degraded Cdc6 to the original level. Again, the reactivation of Cdk2 was closely associated with the decrease of bound p21. Thus, enforced expression of Cdc6 advanced both recovery from S phase arrest and reactivation of Cdk2 with a concomitant reduction of bound p21, but without shortening the duration of the Chk1 signal and induction of p21.

When these cells were irradiated with UV, however, they arrested with Cdk2 inactivation for the same duration (Fig. 3B). Thus, enforced Cdc6 expression had virtually no effects on recovery from UV-induced S phase arrest, in which p21 was little involved in inactivation of Cdk2. Consequently, Cdc6-induced facilitation of both S phase arrest recovery and Cdk2 reactivation in the MMS-treated cells was unlikely to be a consequence of enhanced DNA repair or an unusual property of this particular cell line unrelated to enforcedly expressed Cdc6.

To confirm the generality of this finding with a cell closer to primary cells, we used a p27−/− MEF engineered to be inducible for Cdc6 with a Tet-Off induction system because MEF lacking p27 with such an induction system propagated more robustly and therefore better synchronized to mid-S phase than the wild-type MEF counterpart, whereas p27KIP1 was little involved in DNA damage-responsive regulation of Cdk2 at least in S phase cells as shown in Fig. 1A and as well established (8, 11). The inducible p27−/− MEF cells were synchronized to mid-S phase, treated with MMS, and monitored for their S phase progression and reactivation of Cdk2 as above. Meanwhile, doxycycline was removed to induce Cdc6 at around the time of the treatment, whereas in control this drug continued to be added. Without treatment, they traversed the S phase and reached G2/M within 4 h irrespective of induction of exogenous Cdc6 or not (data not shown). When treated with MMS, uninduced MEF arrested in S phase at least for 16 h accompanied by a marked reduction of the Cdc6 level and complete inactivation of Cdk2 with binding of p21. By contrast, Cdc6-induced MEF had a short arrest (<12 h), again accompanied by advanced reactivation of Cdk2 with a simultaneous decrease of bound p21 (Fig. 4). Thus, in this MEF, enforced expression of Cdc6 facilitated re-initiation of S phase progression and reactivation of Cdk2 roughly to the time point of recovery from the UV-induced arrest in which p21-dependent inactivation of Cdk2 was largely suppressed, confirming the data of Fig. 3A.

Cdc6 Knockdown Retards Arrest Recovery and Cdk2 Reactivation after MMS Treatment in a p21-dependent Manner—By contrast, RNAi-mediated repression of Cdc6 expression hindered re-initiation of S phase progression as well as reactivation of p21-bound Cdk2. In this assay, MEF was used instead of NRK cells because RNAi did not work well for this rat cell line. Seventy-two hours prior to mid-S synchronization, rapidly proliferating MEF was transfected with a Cdc6 oligo-RNA duplex or a universal control RNA duplex as a negative control. Both MEFs were then synchronized to mid-S, treated for 2 h with MMS at 150 μg/ml, and analyzed as described in the legend to Fig. 1A (Fig. 5A). Control RNAi-treated MEF arrested in S phase with inactivation of Cdk2 bound by p21 until it resumed S phase progression at 12 h with reactivation of Cdk2 and re-accumulation of Cdc6, whereas Cdc6 RNAi-treated MEF, being blocked for re-accumulation of Cdc6, remained arrested with inactivated Cdk2.

This knockdown effect absolutely depended upon p21 and completely disappeared in MEF lacking p21. In the same experiment but with p21−/− MEF, both control RNAi-treated and Cdc6 RNAi-treated cells underwent an 8-h S phase arrest with no apparent inactivation of Cdk2 despite a drastic decrease of the Cdc6 level in the knockdown cells (Fig. 5B). In MEF lacking p21, Cdc6 was considerably more stable against MMS treatment despite activation of CHK1 to a comparable level.

Cdc6 Knockdown Induces a Prolonged S Phase Arrest with p21-mediated Cdk2 Inactivation to UV-irradiated Mouse Embryonic Fibroblast—To further confirm Cdc6 as a key determinant for the utilization of the p21-dependent damage checkpoint in S phase cells, we finally investigated the effect of Cdc6 knockdown on UV-induced S phase arrest and inactivation of Cdk2. Rapidly proliferating p27−/− MEF was transfected with a Cdc6 oligo-RNA duplex or a universal control RNA duplex as a negative control and 72 h later synchronized to mid-S as described in the legend to Fig. 5. Both MEFs were then irradiated with 7.5 J/m2 UV, cultured in colcemid-containing medium at every 4-h cell harvest, and analyzed (Fig. 6).
Control RNAi-treated MEF arrested for 12 h with an elevation of Tyr\textsuperscript{15} phosphorylation of Cdk2 and resumed S phase progression with concurrent reactivation of Cdk2, which coincided with the fadeout of Tyr\textsuperscript{15} phosphorylation, just like the UV-irradiated NRK cells shown in Fig. 1A. Consistent with the NRK data, there was no significant binding of induced p21 to Cdk2 throughout the experiment. By contrast, Cdc6 RNAi-treated MEF arrested with full inactivation of Cdk2 accompanied by both p21 binding and elevated Tyr\textsuperscript{15} phosphorylation at least for 16 h, during which Tyr\textsuperscript{15} phosphorylation returned to the original level but considerable amounts of p21 still remained bound to the Cdk2. Cdc6 expression in this MEF diminished roughly to one-fifth the level in the control MEF. The diminished Cdc6 expression alone did not cause S phase arrest because without UV irradiation, this MEF progressed to G\textsubscript{2}/M phases within 4 h like others and as demonstrated in Fig. 5B. Thus, repression of Cdc6 expression additionally activated the p21-dependent checkpoint in the UV-irradiated cells and induced a prolonged S phase arrest with inactivation of Cdk2 as if they had been treated with MMS. All the data presented collectively led us to conclude that regardless of how it was brought about, reduction of the Cdc6 level allowed damage-induced p21 to bind Cdk2, thereby turning on the p21-dependent checkpoint at least in S phase cells.

**DISCUSSION**

Unlike irradiation with a comparable dose of UV (7.5 J/m\textsuperscript{2}), treatment of S phase cells with MMS effectively activated the p21-dependent damage checkpoint (20), but the mechanistic basis for this phenomenon was unclear. In this study, we addressed this question and have found that Cdc6, known as the assembler of pre-replicative complexes, is actually a key determinant for this checkpoint selection. As presented here, there was a strictly inverse and causal relation between the level of Cdc6 and the binding of p21 to Cdk2. Whenever the level of Cdc6 protein was reduced, damage-induced p21 was allowed to bind and inactivate Cdk2 regardless of how it was brought about. Unlike 7.5 J/m\textsuperscript{2} UV, MMS treatment and high dose UV irradiation (15 J/m\textsuperscript{2}) led Cdc6 degradation with
the binding of p21 to Cdk2. Furthermore, RNAi-mediated Cdc6 knockdown allowed p21 to bind Cdk2 in addition to its phosphorylation-dependent inactivation in 7.5 J/m² UV-irradiated cells or retarded reactivation of p21-inactivated Cdk2 in MMS-treated cells. By contrast, enforced expression of Cdc6 strongly counteracted the binding of p21 to Cdk2 and eliminated the prolonged inactivation of Cdk2 induced by MMS treatment.

Based on these results, we conclude that Cdc6 primarily controls the interaction between p21 and Cdk2 including removal of p21 from the inactive p21-Cdk2 complex and that this control is a key mechanism underlying the differential utilization of the p21-dependent damage checkpoint at least in S phase cells responding to base-modifying chemicals or different UV doses because Cdk2, still required for firing late replication origins in mid-S phase cells, is the major target for p21 to retard S phase progression (28), although it has been reported that p21 additionally modulates the function of proliferating cell nuclear antigen vital to polymerase δ (29).

How does Cdc6 control the interaction between p21 and Cdk2? We have recently discovered that Cdc6 can directly activate p21-associated Cdc2 by removing the bound p21 likely via utilizing ATP hydrolysis energy (30). This newly identified function appears to account for all the aspects of the Cdc6-controlled p21-Cdk2 interaction observed in this study. Whether this is the sole mechanism for this control, however, awaits further studies.

Another important finding made in this study is suppression of the Cdc25A-dependent checkpoint in cells treated with MMS or high dose UV. As described, despite producing similar extents of cytotoxicity with roughly comparable levels of Chk1 activation and without Chk2 activation, MMS and a comparable dose of UV (7.5 J/m²) induced entirely opposite cellular responses in terms of S phase checkpoint selection. MMS activated p21-dependent checkpoint but suppressed Cdc25A-dependent checkpoint, whereas treatment with UV suppressed the former and activated the latter. Although the mechanistic basis for the suppression of the p21-dependent checkpoint in the UV-irradiated cells is now understood as discussed above, the reason for the latter suppression in the MMS-treated cells is unknown. However, at least p21 and Cdc6, both of which behaved differently to these two genotoxic agents, are unlikely
FIGURE 6. Repression of Cdc6 expression via RNAi enables p21-mediated inactivation of Cdk2 and induces a prolonged S phase arrest to UV-irradiated cells. Logarithmically proliferating p27 \(^{-/}\)/MEF cells were transfected with the universal negative control RNA duplex or the Cdc6-specific duplex, 72 h later synchronized to mid-S phase, irradiated with UV at a dose of 7.5 J/m\(^2\), and cultured further in colcemid-containing growth medium every 4 h for cell harvests. In parallel, the p27 \(^{-/}\)/MEF cells were transfected with the Cdc6-specific duplex and synchronized to mid-S phase were cultured without UV irradiation as a control. Each harvested cell was analyzed for cell cycle patterns and the levels of Cdc6, Ser345-phosphorylated Chk1, Chk1, p21, and Cdk2 as well as for Cdk2 activities and the amounts of Cdk2, its Tyr\(^{15}\)-phosphorylated form, and associated p21 after immunoprecipitation of Cdk2. \(\beta\)-Actin was used as a loading control.

to be involved in this process because as shown in Fig. 6, the elevated Tyr\(^{15}\) phosphorylation of Cdk2 in the UV-irradiated MEF was not influenced by the Cdc6 knockdown that effectively lowered the level of Cdc6 protein and thereby allowed p21 to bind the Cdk2. Interestingly enough, suppression of the Cdc25A-dependent checkpoint, which was observed even with a reduced dose of MMS (supplemental Fig. S2), is not specific to base-modifying chemicals. As shown in Fig. 1B, the higher dose of UV invoked the same cellular responses as with MMS. These results suggest that some cellular signaling other than Atr-Chk1 and Atm-Chk2, which might mediate different kinds or severity of DNA damage or cellular stress, being more specifically activated by MMS seemingly in close association with degradation of Cdc6, may be involved in the apparent selection of either one of the two parallel checkpoints for utilization in S phase arrest although the mechanism for the suppression of the Cdc25A-dependent checkpoint is entirely unknown at present.

Although disruption of the interaction between p21 and Cdk2 by Cdc6 effects advanced reactivation of Cdk2, it is unknown whether this function is solely responsible for the Cdc6-driven facilitation of recovery from S phase arrest or not. The pre-replicative complexes that had been assembled on replication origins in M/G\(_1\) phases might be destroyed on some late replication origins by damage to DNA itself or intentionally removed by its repair machinery during arrest. If this is the case, Cdc6 would be required for re-assembling pre-replicative complexes on those origins for cells to resume and complete S phase progression. This scenario is well conceivable but unlikely to be the case. As shown in Fig. 5B, in MEF lacking p21, Cdc6 knockdown had no detectable adverse effects on recovery from S phase arrest after MMS treatment despite a marked reduction in its level.

The importance of the regulation of Cdk2 in damage-responsive cell cycle checkpoints in S phase cells has well be established (8, 11) and is highlighted in the current study. However, this regulation is by no means the sole mechanism for the intra-S phase damage checkpoint control. We observed that MMS-treated p21\(^{-/}\)/MEF still arrested in S phase without detectable inactivation of Cdk2 (Fig. 5B) (20). This arrest could be attributable at least to Chk1-mediated inactivation of Cdc7, another factor essential for firing replication origins (12) because Chk1 was similarly activated in the p21\(^{-/}\)/MEF.

Finally, it may not be irrelevant to discuss about the potential collapse of the DNA replication processes of the cell during a prolonged S phase arrest. As described, the viability of the MMS-treated cells that arrested in mid-S for nearly 24 h was unexpectedly high and similar to that of the UV-irradiated cells despite comparable levels of the damage checkpoint signals (Chk1 Ser\(^{345}\) phosphorylation and induced p21), yet a much shorter S phase arrest. This observation indicates that even such a long S phase arrest can be tolerated by cells no matter whether such a collapse would actually occur during the arrest or not.

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