Rapid Degradation of Cdt1 upon UV-induced DNA Damage Is Mediated by SCF$^{Skp2}$ Complex*

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Takeshi Kondo‡‡, Masanobu Kobayashi‡, Junko Tanaka, Akiko Yokoyama‡, Sachiko Suzuki‡, Naoko Kato*††, Masahiro Onozawa‡, Kohji Chiba‡, Satoshi Hashino‡, Masahiro Imamura**, Yasuhiro Minami†††, Naoto Minamino‡, and Masahiro Asaka‡

From the ‡Department of Gastroenterology and Hematology, Hokkaido University Graduate School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapporo, Hokkaido 060-8638, the †Division of Cancer Pathobiology, Institute for Genetic Medicine, Hokkaido University, Kita 15, Nishi 7, Kita-ku, Sapporo, Hokkaido 060-8638, the ¶National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, the ‡‡Department of Hematology and Oncology, Hokkaido University Graduate School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapporo, Hokkaido 060-8638, and the †††Department of Genome Sciences, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

Cdt1 is a licensing factor for DNA replication, the function of which is tightly controlled to maintain genome integrity. Recent studies have indicated that the cell cycle-dependent degradation of Cdt1 is triggered at S phase to prevent re-replication. In this study, we found that Cdt1 is degraded upon DNA damage induced by either UV treatment or γ-irradiation (IR). Although the IR-triggered degradation of Cdt1 was caffeine-insensitive, the UV-triggered degradation of Cdt1 was caffeine-sensitive. This indicates that the cells treated with UV utilize the checkpoint pathway, which differs from that triggered by IR. A recent study has suggested that Cdt1 is phosphorylated, ubiquitylated, and degraded at the G1/S boundary in the normal cell cycle. Treatment with MG132, a proteasome inhibitor, inhibited the degradation of Cdt1 and resulted in the accumulation of the phosphorylated form of Cdt1 after UV treatment. In the case of UV treatment, phosphorylation of Cdt1 induced the recruitment of Cdt1 to a SCF$^{Skp2}$ complex. Moreover, ectopic overexpression of Cdt1 after UV treatment interfered with the inhibition of DNA synthesis. These results indicate that Cdt1 is a target molecule of the cell cycle checkpoint in UV-induced DNA damage.

Cdt1 is a licensing factor for DNA replication that is developmentally conserved from yeast to mammals (1–3). Cdt1 is required to form pre-replicative complexes (pre-RC) at G1 phase, and the function of Cdt1 is tightly regulated to prevent over-replication of the genome. Thus, Cdt1 is expressed at the exit of G1 phase and degraded at the boundary of G1/S transition, and geminin, an inhibitor of Cdt1, is expressed from S phase to M phase (4–8). Once Cdt1 is aberrantly activated in cells, the cells start to over-replicate the genome, and the integrity of the genome is therefore not preserved (9–11). It has recently been shown that Cdt1 is degraded through a ubiquitin/proteasome pathway. Two E3 ubiquitin ligase complexes have been suggested to play a crucial role in the degradation of Cdt1. One is the E3 ubiquitin ligase, which contains Cul4 and Roc1 (12), and the other is the SCF$^{Skp2}$ complex (Skp1/Cul1/F-box protein, Skp2) (13). Thus, the degradation of Cdt1 can be mediated by either a Cul4-containing complex or the SCF$^{Skp2}$ complex in the progression of the cell cycle.

Accumulating evidence indicates that the cell cycle checkpoint plays an important role in maintenance of the genome integrity (14). When the genome is damaged, the initial step of the checkpoint is recognition of damaged DNA. In this step, ataxia telangiectasia-mutated (ATM) and ATM/Rad3-related kinase (ATR) play crucial roles. Once ATM or ATR is activated by the damaged DNA, these kinases phosphorylate downstream substrates, and the triggered signals induce cell cycle arrest, DNA repair, or apoptosis. ATM is primarily activated by IR, which induces DNA double-strand breaks, whereas ATR responds to UV. Although these kinases are activated by different types of DNA damage, they share common substrates, which give similar biological effects.

We hypothesized that Cdt1 plays an important role in the cell cycle checkpoint. In this study, we found that Cdt1 is rapidly degraded through a ubiquitin/proteasome pathway upon DNA damage induced by either UV or IR treatment. Caffeine, an ATM/ATR inhibitor (15, 16), only inhibited UV-induced Cdt1 degradation. This finding implies that cells utilize caffeine-sensitive checkpoint pathways upon UV-induced DNA damage, although IR-induced Cdt1 degradation is caffeine-insensitive. After UV treatment, Cdt1 was phosphorylated, and the phosphorylation of Cdt1 triggered the recruitment of Cdt1 to the SCF$^{Skp2}$ complex. We conclude that Cdt1 is rapidly degraded in response to DNA damage to prevent cells from replicating the damaged DNA.

EXPERIMENTAL PROCEDURES

Plasmid Construction—A DNA clone (IMAGE 304853) containing human Cdt1 cDNA was purchased from IncyteGenomics. For constructing various Cdt1 deletion mutants, each cDNA was generated by PCR, and a FLAG tag was attached at the C terminus. These mutants lack the following corresponding amino acids: dc139, amino acids 198–546; dc283, amino acids 283–546; dc445, amino acids 445–546; dn52, amino acids 2–52; dn93, amino acids 2–93. Then each cDNA of full-length and mutant Cdt1 with a FLAG tag was cloned into pBabe-Puro vector, which contains a T7 tag at the N terminus.

Cell Culture, Drug Treatment, and DNA Damage—HeLa cells were...
cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. HCT-15 cells were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University and cultured in RPMI 1640 supplemented with 10% fetal bovine serum. HeLa Tet-On cells were purchased from Clontech and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 100 μg/ml G418. To establish HeLa cells containing a Cdt1 deletion mutant, we used a retrovirus packaging kit Amphi (TaKaRa). HeLa cells were infected with a retrovirus harboring each mutant of Cdt1 and selected with puromycin. HeLa Tet-On/Cdt1 cells were established by co-transfecting pTRE-Cdt1 and a puromycin-resistant gene and selected with both G418 and puromycin. For UV-induced DNA damage, the culture medium was removed, and the cells were treated with 20 J/m² UV. After UV treatment, the culture medium was added back to the cells. For the treatment of inhibitors of checkpoint kinases or proteasome, the following drugs were added to the culture medium 15 min before the induction of DNA damage: caffeine (15 mM, Sigma), wortmannin (10 μM, Sigma), UCN-01 (200 nm, Kyowa Hakko Kogyo) and MG132 (50 μM, Calbiochem).

**Antibodies, Immunoblotting, and Immunostaining**—Polyclonal antibodies against human Cdt1 and geminin were raised against glutathione S-transferase-fused C-terminal 321–546 amino acids of Cdt1 and full-length geminin, respectively. Anti-FLAG M2 antibody, T7 tag antibody, anti-actin antibody (I-19; catalog number SC-1616), and anti-Skp2 antibody (H-435; catalog number SC-7164) were purchased from Sigma, Novagen, and Santa Cruz Biotechnology, respectively. For immunoblotting, the cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% (v/v) Nonidet P-40) supplemented with protease and phosphatase inhibitors (10 μg/ml aprotinin, 50 μg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride, 100 mM NaF, 1 mM Na3VO4) unless otherwise indicated. To analyze the phosphorylation-dependent association between Cdt1 and Skp2, immunoprecipitation was carried out with anti-FLAG M2 antibody, and the immunoprecipitates were treated with λ-phosphatase (New England Biolabs) and then washed with Nonidet P-40 lysis buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell). For immunostaining, the cells were fixed with 3.7% formaldehyde and incubated with anti-Cdt1 antibody as a primary antibody and then incubated with a fluorescein isothiocyanate-conjugated anti-rabbit antibody (Santa Cruz Biotechnology). For DNA staining, the fixed cells were stained with 4',6'-diamidino-2-phenylindole-dihydrochloride.

In Vivo Ubiquitylation of Cdt1—Recombinant Cdt1 with a FLAG tag was collected by anti-FLAG M2 affinity gel (Sigma) from HeLa-derived stable cells as described above. Recombinant Cdt1 was eluted with 0.1 M glycine (pH 2.5) and concentrated by Centricon YM10 (Millipore). pCGT-Skp2 was transfected into 293 cells, and the cell lysates were collected after 48 h. A pCGT empty vector was used as a negative control. To collect the cell lysates from UV-treated cells, the transfected cells were harvested 15 min after UV treatment. The SCF<sup>Skp2</sup> complex was immunoprecipitated with a T7 tag antibody. An in vitro ubiquitylation assay was carried out with the SCF<sup>Skp2</sup> complex and recombinant Cdt1 as described previously (17). To analyze the phosphorylation-dependent ubiquitylation, Cdt1 was pretreated with λ-phosphatase for 30 min before the in vitro ubiquitylation reaction. After the in vitro ubiquitylation reaction, the reaction mixture was analyzed by immunoblotting with an anti-Cdt1 antibody. To confirm that the SCF<sup>Skp2</sup> complex was immunoprecipitated, the same membrane was reprobed with anti-Skp2 antibody.

Inhibition of DNA Synthesis after DNA Damage—Either HeLa Tet-On or HeLa Tet-On/Cdt1 cells were cultured in a culture medium without doxycycline and irradiated with UV. After UV treatment, the cells were cultured with or without 1 μg/ml doxycycline. The expression level of Cdt1 after UV irradiation in either HeLa Tet-On or HeLa Tet-On/Cdt1 was examined by immunoblotting. The cells were labeled for 6 h with IdU, and the incorporated 5-ido-2'-deoxyuridine was analyzed by using a DNA/IdU labeling and detection kit (TaKaRa).

**RESULTS**

Cdt1 Is Rapidly Degraded through a Ubiquitin/Proteasome Pathway upon UV Irradiation—To determine whether Cdt1 plays a role in the cell cycle checkpoint, we carried out an experiment to determine whether the expression of Cdt1 is affected by genotoxic stress. Exponentially growing HeLa cells were treated with UV, and the level of Cdt1 was checked by immunoblotting. Cdt1 disappeared upon UV treatment, and this cellular response was observed within 15 min. We also examined the expression of geminin. Although Cdt1 rapidly disappeared upon UV treatment, the expression level of geminin did not change (Fig. 1A). Thus, the disappearance of Cdt1 is a specific response to UV treatment. By immunostaining with an anti-Cdt1 antibody, Cdt1 was homogeneously stained throughout the nucleus in most of the exponentially growing cells. After UV irradiation, Cdt1 had mostly disappeared (Fig. 1B). Since Cdt1 is degraded through the ubiquitin/proteasome pathway at the entry into S phase in the normal cell cycle (8, 12, 13), we assumed that Cdt1 was degraded through the ubiquitin/proteasome pathway upon UV treatment. When cells were treated with MG132, a proteasome inhibitor, the degradation of Cdt1 was completely inhibited. Interestingly, the extracts of cells treated with both UV and MG132 showed not only stabilized Cdt1 but also slower migrating forms of Cdt1 (Fig. 1C). These results prompted us to check whether the alteration in electrophoretic mobility is attributed to the phosphorylation of Cdt1. HeLa cells were treated with or without UV in the presence of MG132. Then the cell extracts were subjected to phosphatase treatment. The results showed that the mobility shift of Cdt1 was dependent on UV irradiation and sensitive to λ-phosphatase (Fig. 1D). These results suggest that the DNA
damage induced by UV triggers phosphorylation and subsequent degradation of Cdt1. We also examined the effect of IR upon Cdt1 stabilization. Cdt1 was degraded upon IR, and MG132 inhibited Cdt1 degradation induced by IR, but an electrophoretic mobility shift was not observed (Fig. 1E).

Cdt1 Degradation Induced by UV Irradiation Is Caffeine-sensitive—DNA damage activates the cell cycle checkpoint, in which ATM or ATR triggers the signaling cascade. Chk1 and Chk2 are downstream kinases of ATM and ATR that mediate signals to the effectors (18, 19). Since Cdt1 was phosphorylated by UV irradiation and the degradation of Cdt1 was rapid, we assumed that this response involves the activation of checkpoint kinases. We treated the cells with UV in the presence of inhibitors of checkpoint kinases and examined the effects of the inhibitors. Caffeine is an inhibitor of ATM and ATR, wortmannin is an inhibitor of ATM and DNA-dependent protein kinase (DNA-PK) (20), and UCN-01 is an inhibitor of Chk1 (21, 22). It was found that caffeine inhibited Cdt1 degradation but that wortmannin had no effect (Fig. 2A). In addition, extracts of cells treated with caffeine did not show slower migrating forms (Fig. 2A, lane 3), suggesting that a caffeine-sensitive and wortmannin-insensitive molecule, possibly ATR, plays a role in the modification of Cdt1. These results indicate that Cdt1 is degraded upon UV-induced DNA damage, possibly through the ubiquitin/proteasome pathway, and that the activities of ATM, DNA-PK, and Chk1 are dispensable for this cellular response. Previous studies have shown that there is redundancy between Chk1 and Chk2 functions and that these kinases share common substrates in the signaling cascade of the cell cycle checkpoint (18, 19). To check the possibility that the activity of either Chk1 or Chk2 is necessary for the degradation of Cdt1, we used HCT-15 cells, which lack functional Chk2 activity (23, 24). We examined the level of Cdt1 in HCT-15 cells after UV treatment in the presence of UCN-01. We found that Cdt1 was still degraded upon UV treatment without the activity of either Chk1 or Chk2 (Fig. 2B). Thus, neither Chk1 nor Chk2 seems to be involved in the pathway for the degradation of Cdt1. We also examined the effect of kinase inhibitors upon IR-induced Cdt1 degradation. All of the kinase inhibitors used, including caffeine, had no effect on the degradation of Cdt1 (Fig. 2C). These results suggest that the cells utilize caffeine-insensitive checkpoint pathways upon IR-induced DNA damage.

Cdt1 Is Recruited to E3 Ubiquitin Ligase, SCFSkp2 Complex, after UV Treatment in a Phosphorylation-dependent Manner—A recent study has suggested that Cdt1 is phosphorylated, ubiquitylated, and subsequently degraded at the G1/S boundary through the SCFSkp2 complex (13). Since UV treatment induced the phosphorylation of Cdt1, we assumed that
Cdt1 Degradation Caused by UV-induced DNA Damage

Cdt1 is recruited to the SCF<sup>Skp2</sup> complex upon UV treatment. Since our anti-Cdt1 antibody was not suitable for immunoprecipitation analysis, we generated a HeLa-derived cell line, HeLa/Cdt1FLAG, which expressed the full length of Cdt1 with a FLAG tag at the C terminus. To prevent the accumulation of the phosphorylated form of Cdt1 in the normal cell cycle, we treated the cells with MG132 for 10 min prior to UV treatment and harvested the cells 10 min after UV treatment. Immunoprecipitation analysis with anti-FLAG M2 antibody showed that UV treatment augmented the association between Cdt1 and Skp2 (Fig. 3A). In addition, λ-phosphatase treatment of the immunoprecipitates abrogated the association, suggesting that the association of Cdt1 and Skp2 is phosphorylation-dependent (Fig. 3B). We also performed an in vitro ubiquitylation assay. The SCF<sup>Skp2</sup> complex was immunoprecipitated, and we checked that the SCF<sup>Skp2</sup> complex ubiquitylates Cdt1 (Fig. 3C). A high molecular weight form of Cdt1 was generated by the SCF<sup>Skp2</sup> complex (Fig. 3C, lane 3). Pretreatment of Cdt1 with λ-phosphatase decreased the generation of the high molecular weight form of Cdt1 (Fig. 3C, lane 4). We also checked that UV treatment affects the activity of SCF<sup>Skp2</sup> ubiquitin ligase. The SCF<sup>Skp2</sup> complex from the UV-treated cells showed activity of the ubiquitin ligase similar to that of the SCF<sup>Skp2</sup> complex from the untreated cells (Fig. 3C, lane 5). Collectively, the results suggest that UV-induced phosphorylation of Cdt1 is crucial for the recognition and subsequent ubiquitylation by the SCF<sup>Skp2</sup> complex. To determine which portion of Cdt1 is important for degradation induced by UV treatment, we established HeLa-derived stable cell lines that expressed a series of Cdt1 deletion mutants (Fig. 4A). As was reported previously (13), it was found that the N-terminal region of Cdt1 is necessary for association with Skp2. All of the C-terminal deletion mutants retained the ability to associate with Skp2, but two N-terminal deletion mutants lost the ability (Fig. 4B). Immunoblotting analysis showed that the N-terminal deletions, as small as 52 amino acids, totally abolished the UV-induced degradation of Cdt1. In addition, a series of C-terminal deletions showed that 138 N-terminal amino acids is sufficient for the UV-induced degradation of Cdt1 (Fig. 4C). These results are consistent with the ability of the Cdt1 mutants to associate with Skp2.

Ectopic Expression of Cdt1 Interfered with the Inhibition of DNA Synthesis by UV-induced DNA Damage—Once cells suffer genotoxic stress, the cell cycle checkpoint inhibits the synthesis of DNA to prevent the accumulation of mutations. Since Cdt1 is an essential factor for the formation of pre-RC, which is necessary to start DNA synthesis, we assumed that degradation of Cdt1 leads to inhibition of DNA synthesis. Finally, we investigated whether the rapid degradation of Cdt1 upon DNA damage has any physiological effect on DNA synthesis. We established a stable cell line, HeLa Tet-On/Cdt1, in which the expression of exogenous Cdt1 is inducible by the addition of doxycycline (25). Using these cells, the level of inhibition of DNA synthesis after DNA damage was examined by determining 5-iodo-2′-deoxyuridine incorporation into the cells. After
UV treatment, the expression of Cdt1 was induced by doxycycline (Fig. 5A). In the absence of doxycycline, parental HeLa Tet-On cells and HeLa Tet-On/Cdt1 cells showed the same extent of inhibition of DNA synthesis after DNA damage. However, in the Cdt1-induced condition, the level of inhibition of DNA synthesis induced by DNA damage was inhibited (Fig. 5B). These results indicate that degradation of Cdt1 is important for the inhibition of DNA synthesis induced by UV-induced DNA damage.

**DISCUSSION**

The expression of Cdt1 is regulated in the cell cycle, in which Cdt1 is expressed mainly in the G1 phase and degraded through the ubiquitin/proteasome pathway after the onset of S phase (7, 12, 13). A recent study has suggested that Cdt1 is phosphorylated and recruited to the SCFSkp2 complex at the G1/S transition (13). Since our results suggest that the phosphorylation and degradation of Cdt1 are also induced by UV-induced DNA damage, the expression level of Cdt1 seems to be regulated by both the cell cycle stage and the cell cycle checkpoint. Once Cdt1 is degraded, it is thought that the cells fail to form pre-RC. Thus, DNA damage impairs the formation of pre-RC and results in inhibition of DNA synthesis. The SCFSkp2 complex was initially characterized as promoting the transition of G1 phase to S phase, which mediates the ubiquitylation of the cell cycle checkpoint. The SCFSkp2 complex not only for the cell cycle progression but for the checkpoint response as well. It would be interesting to check the possibility that there is a functional relationship and coordination between the SCFSkp2 complex and Cdt1 for the checkpoint response.

Upon genotoxic stress, ATM and ATR usually act as primary sensors for the damaged DNA (14). However, from our results, although the degradation of Cdt1 induced by UV treatment was inhibited by an ATM/ATR inhibitor, caffeine, it seems that the activities of ATM, ATR, and DNA-PK are not essential for the degradation of Cdt1 induced by IR. A recent study has suggested that the Cul4/Roc1 complex plays a crucial role in the IR-induced ubiquitylation of Cdt1 and that degradation of Cdt1 is caffeine-insensitive (35). At this point, it is thought that there is an independent pathway for IR-induced DNA damage of ATM, ATR, and DNA-PK to induce the degradation of Cdt1, which should be further characterized. It is also possible that there is a functional relationship and coordination between the SCFSkp2 complex and Cul4/Roc1 complex for Cdt1 degradation in response to DNA damage. It has been reported that overexpression of Cdt1 has oncogenic potential (36). Thus, the aberration of Cdt1 degradation may contribute to the cancer development by promoting cell cycle progression and genetic instability.

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