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Rapid Downregulation of Cyclin D1 mRNA and Protein Levels by Ultraviolet Irradiation in Murine Macrophage Cells

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DNA damage causes G1 cell cycle arrest through stabilization of p53 and its induction. As this process requires transcription, it takes several hours to achieve cell cycle arrest. We observed that ultraviolet (UV) light induces an immediate G1 arrest by rapid clearance of cyclin D1 in the murine macrophage cell line Bac1.2F5. The rapid disappearance of the cyclin D1 protein after exposure to UV was caused by at least two different mechanisms. In the first mechanism, cyclin D1 mRNA promptly disappeared within 1 min after UV irradiation, although cdk4 mRNA levels were unchanged. In the second mechanism, UV irradiation accelerated the degradation of cyclin D1 protein through the proteasome pathway. The half-life of the cyclin D1 protein was measured by pulse chase analysis and was shortened by UV light. These findings suggest that in the UV-irradiated Bac1.2F5 cells the amount of cyclin D1 protein is regulated at both the mRNA and protein levels. These two clearance mechanisms were also observed in murine bone-marrow-derived macrophages from wild type and p53 −/− mice, indicating that cyclin D1 mRNA and protein levels are independent of p53 function. This machinery might contribute to G1 cell cycle arrest and prevent cells from accumulating further DNA damage. © 2001 Academic Press

Key Words: **cyclin D1; cell cycle; ultraviolet; proteasome.**

Cell cycle progressions and transitions in mammalian cells are governed by distinct cyclin-dependent kinases (cdks) whose activities are regulated by binding of their regulatory subunits, called cyclins, or cdk inhibitors and phosphorylation to their critical threonine residues (1). The G1 cyclins consist of D- (D1, D2, and D3) and E-type cyclins, which form enzymatically active complexes with Cdk4 (or Cdk6) and Cdk2, respectively (1–4). Both cyclin D/Cdk4 and cyclin E/Cdk2 complexes induce sequential phosphorylation of the retinoblastoma (Rb) protein, thereby canceling its suppressive function and facilitating the entrance of cells into the S phase. Expression of D-type cyclins depends on various mitogenic stimuli. Growth factor starvation leads to the disappearance of D-type cyclin, which is followed by the reduced kinase activity of the cyclin D/Cdk4 complex, the accumulation of hypophosphorylated Rb, and consequently cell cycle arrest in the G1 phase (1). Recently it has been reported that the phosphorylation of the cyclin D1 protein triggers its degradation through an ubiquitination-mediated proteasome pathway in a point of constitutive turnover (5). In contrast, the mechanism of the rapid disappearance of cyclin D1 protein after ultraviolet (UV) irradiation remains unknown. In this report, we demonstrate that UV irradiation accelerates the turnover of cyclin D1 mRNA and shortens the half-life of the cyclin D1 protein through a proteasome pathway. We also speculate that the 3′-untranslated region of cyclin D1 mRNA contributes to its stability *in vivo.* These regulatory mechanisms of cyclin D1 might contribute to cell cycle arrest after UV irradiation, which could cause severe DNA damage to the cycling cells.

MATERIALS AND METHODS

Cell culture. A murine macrophage cell line, Bac1.2F5, was maintained in Dulbecco's modified Eagle medium (DMEM, Sigma, St. Louis, MO) supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, 100 U of penicillin per ml, 100 mg of streptomycin per ml, and 20% of L-cell conditioned medium as a source of colony-stimulating factor 1 (CSF-1) (6). A Bac1.2F5-derived cell line, designated Bac1.2F5-5D, was engineered to express the murine cyclin D1 mRNA from the methalothionein promoter. Cells were transfected with a plasmid encoding the entire cyclin D1 protein but lacking the 3′-untranslated sequence. To analyze cyclin D1 and Cdk4 expression

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in synchronized cells, the Bac1.2F5 macrophages were arrested in the early G1 phase by CSF-1 starvation for 18 h and were restimulated with CSF-1 to resume the cell cycle (4). The cells were expected to be in the mid-G phase 6 h after restimulation, based on previous reports (4). Cells prepared from the leg bones of B6J-p53 −/− mice and their littermates (wild type) were cultured for 7 days in a serum-containing medium in the presence of CSF-1 as previously reported (2, 7–9). Under these conditions, pure populations of the macrophages that express typical lineage markers, including the CSF-1 receptor, were obtained. The murine macrophages derived from normal or p53 −/− mice were also arrested in the G1 phase by depriving them of CSF-1 for 18 h and were restimulated with CSF-1. A UV (254 nm) irradiation was performed using DNA-Fix AB-1500 crosslinker (ATTO, Japan).

Proteasome inhibitors and antibodies. Proteasome inhibitors, MG115, MG132, PSI, and the calpain inhibitor E64d were purchased from the Peptide Institute (Osaka, Japan). LLnL was purchased from Sigma (St. Louis, MO). Lactacystin was a generous gift from K. Tanaka (Tokyo Metropolitan Research Institute, Tokyo, Japan) (10). These inhibitors were dissolved in DMSO at 10 mM. Proteasome inhibitors were added to the culture medium at 50 μM and incubated for 60 min to inhibit proteasomes prior to UV irradiation. A mouse monoclonal antibody to murine cyclin D1 protein was a generous gift from Dr. Charles J. Sherr (St. Jude's Children's Research Hospital, Memphis, TN) (4). An antibody to the cdk4 protein was purchased from Santa Cruz (Santa Cruz, CA).

Immunoprecipitations and immunoblottings. Cells were rinsed three times in an ice-cold phosphate-buffered saline solution (PBS) and lysed in Tween 20 lysis buffer (50 mM Hepes [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20, 10% glycerol) containing protease and phosphatase inhibitors. Sonicated extracts were clarified by centrifugation and the supernatants were immunoprecipitated for 2 h at 4°C with the indicated antibodies (4). Immunoprecipitates were collected with Protein A- or Protein G-Sepharose beads (Pharmacia Biotech) and then the beads were extensively washed with Tween 20 lysis buffer. Immunoblotting analyses were performed using ECL chemiluminescence (Amersham, Sweden) according to the manufacturer's instructions.

Pulse chase analysis. Subconfluent adherent Bac1.2F5 cells were synchronized in the early G1 phase by starvation for 18 h and restimulated with CSF-1 for 6 h (4). The synchronized cells were incubated for 30 min in methionine-free medium and metabolically labeled for 30 min with 200 μCi/ml [^{35}S]-methionine (1000 Ci/mmol; Amersham). After the metabolic labeling, the cells were extensively washed with PBS and re-fed with DMEM containing 15% FBS, 2 mM cold methionine, and 20% L-cell conditioned medium. Then the cells were irradiated with 1000 J/m^2 UV. At various times after the termination of metabolic labeling, equal numbers of cells were lysed; the cyclin D1 protein was immunoprecipitated with the cyclin D1 antibody, and the captured proteins were separated by SDS–PAGE and visualized by autoradiography. When the proteasome inhibitors were used, they were added to the culture medium for 60 min prior to UV irradiation.

Northern blot. Total RNA was extracted according to standard protocols. Ten micrograms of total RNA was applied to each well of an agarose gel and transferred to nitrocellulose membranes after electrophoresis. The membrane was hybridized with specific [^{32}P]-labeled probes against cyclin D1 or cdk4 mRNA (4).

RESULTS

UV irradiation caused the rapid disappearance of cyclin D1 protein in Bac1.2F5 cells. DNA damage upregulates expression of the p21 Cdk inhibitor in a p53-dependent manner and consequently induces cell cycle arrest mainly at the G1 phase (1, 11). UV irradiation causes DNA damage by forming thymidine dimers in the DNA and upregulates p21 expression. Although it has been observed that cyclin D1 protein levels are reduced in UV-irradiated cells (12, 13), the precise mechanisms remain unknown. Therefore, we further investigated the mechanism of immediate reduction of the cyclin D1 protein by UV-irradiation. A murine macrophage cell line, Bac1.2F5, was synchronized in the mid-G1 phase and then irradiated with various doses of UV. The cells were lysed 60 min after UV irradiation, and the amount of cyclin D1 protein was analyzed by Western blot (Fig. 1A). The amount of cyclin D1 protein was apparently constant following irradiation of cells at up to 250 J/m^2; however, protein levels were reduced in the 500 J/m^2-irradiated cells and were undetectable in the 1000 J/m^2-irradiated cells (Fig. 1A). These findings indicate that the amount of cyclin D1 protein is reduced after UV irradiation in a dose-dependent manner.

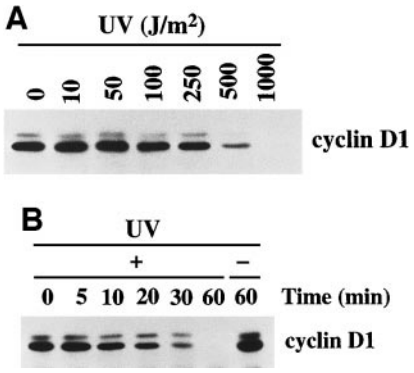

FIG. 1. Expression of cyclin D1 protein was greatly reduced in Bac1.2F5 cells in a dose-dependent and time-dependent manner after exposure to UV light. (A) Bac1.2F5 cells were synchronized in the mid-G1 phase by CSF-deprivation for 18 h and restimulation with CSF-1 for 6 h. The cells then were treated with UV irradiation at various doses (J/m^2). The cells were lysed 60 min after irradiation, and cyclin D1 protein was immunoprecipitated and detected by Western blotting with the appropriate antibody. (B) Bac1.2F5 cells synchronized in the mid-G1 phase were treated with (+) or without (−) 1000 J/m^2 of UV irradiation, harvested at the indicated time, and then subjected to Western blot analysis to detect cyclin D1 protein levels as described above.

In order to investigate the kinetics of the disappearance of the cyclin D1 protein in 1000 J/m^2-irradiated cells, the amount of the cyclin D1 protein was determined up to 60 min following UV irradiation (Fig. 1B). The levels of cyclin D1 protein were reduced after UV irradiation in a time-dependent manner.

UV irradiation caused the rapid disappearance of cyclin D1 mRNA in Bac1.2F5 cells. In order to investigate the mechanism of the disappearance of the cyclin D1 protein after UV irradiation, we also analyzed cyclin D1 mRNA levels by Northern blotting. After 1000 J/m^2 UV irradiation, cyclin D1 mRNA in Bac1.2F5 cells was almost undetectable within 1 min

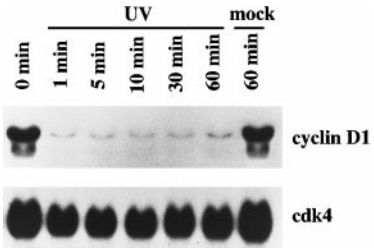

FIG. 2. Northern blot analysis of cyclin D1 and cdk4 mRNA in UV-irradiated Bac1.2F5 cells. Bac1.2F5 cells synchronized in the mid-G1 phase were treated with 1000 J/m^2 of UV irradiation (UV) or without (mock) and harvested at the indicated times. The level of cyclin D1 mRNA was analyzed by Northern blotting (upper panel). The same membrane was reprobed with the [^{32}P]-labeled murine cdk4 cDNA (lower panel).

and stayed at low levels with a slight increase after 60 min (Fig. 2). In contrast, the amount of cdk4 mRNA remained virtually unchanged after UV irradiation. These results were consistent with the disappearance of the cyclin D1 protein in Bac1.2F5 cells and suggest that there could be a specific pathway to downregulate cyclin D1 mRNA.

The 3′-untranslated sequence of cyclin D1 mRNA might play a role in its stability following UV irradiation. We further investigated the mRNA levels of cyclin D1 and cdk4 in Bac1.2F5 transfectant cells constitutively expressing the murine cyclin D1 mRNA (designated as Bac1.2F5-5D). When Bac1.2F5-5D cells were deprived of CSF-1 for 18 h, the endogenous cyclin D1 mRNA levels, which are 4.5 and 3.8 kilobase pairs (kb) long, respectively, were extremely reduced (the closed triangle points at two bands in Fig. 3A). In contrast, the mRNA levels of the exogenously introduced cyclin D1 gene (1.5 kb), which lacked 3 kb of 3′-untranslated sequences, was unchanged since it was driven by methalothionein promoter (the open triangle at 0 h in Fig. 3A). Six hours after the re-stimulation with CSF-1, the Bac1.2F5-5D cells resumed G1 progression and the mRNA of the intrinsic cyclin D1 was detected (6 h in Fig. 3A). The level of the intrinsic cyclin D1 mRNA (the closed triangle in Fig. 3A, lane 3) in both Bac1.2F5-5D and parental Bac1.2F5 cells was clearly reduced 1 h after UV irradiation. However, the level of the exogenously introduced cyclin D1 mRNA (1.5 kb) was apparently not reduced after UV irradiation (the open triangle in Fig. 3A, lane 3). The exogenously introduced cyclin D1 gene contained the entire coding sequence but lacked the last 3 kb of the 3′-untranslated sequence. These results suggest that the last 3 kb of cyclin D1 mRNA may contribute to its stability after UV irradiation. The level of cdk4 mRNA was barely changed after UV irradiation (bottom panel in Figure 3A) compared to cyclin D1.

We also analyzed the amount of cyclin D1 protein in Bac1.2F5-5D cells by Western blotting. Although the level of the introduced cyclin D1 mRNA (1.5 kb) remained unchanged after UV irradiation of Bac1.2F5-5D cells as well as parental Bac1.2F5 cells, the level of cyclin D1 protein was greatly reduced after 1000 J/m^2 UV irradiation in both cell lines (Fig. 3B). Together, these results suggest that the expression level of cyclin D1 protein is regulated at the protein level (perhaps by degradation) as well as at the mRNA level after exposure to UV light.

The proteasome pathway was involved in the UV-induced degradation of the cyclin D1 protein. Recently, it has been reported that the turnover of the cyclin D1 protein is regulated by the proteasome pathway during steady state growth. Therefore, we further investigated whether the rapid disappearance of the cyclin D1 protein after UV irradiation was regulated by the proteasome pathway. The synchronized Bac1.2F5 cells in the mid-G1 phase were pretreated with proteasome inhibitors, such as MG115, MG132, PSI, or lac-

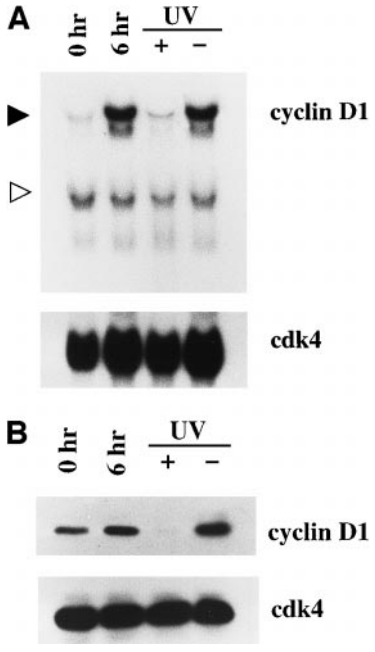

FIG. 3. The stability of cyclin D1 mRNA and protein in the Bac1.2F5-5D transfectant cells after exposure to UV light. (A) Bac1.2F5-5D cells, engineered to overexpress cyclin D1, were synchronized in the mid-G1 phase by the deprivation of CSF-1 for 18 h (0 h) and then restimulated with CSF-1 for 6 h (6 h). The restimulated cells were treated with (+) or without (−) 1000 J/m^2 of UV irradiation and their RNA was obtained 60 min later. Cyclin D1 (top panel) and cdk4 (bottom panel) mRNA levels were analyzed by Northern blotting. The [^{32}P]-labeled cyclin D1 cDNA fragment containing its entire coding sequence was hybridized to the intrinsic 4.5 and 3.8 kb mRNAs (closed triangle) and to the shortest mRNA (1.5 kb) transcribed from the exogenously introduced cyclin D1 gene (open triangle). (B) The synchronized Bac1.2F5-5D transfectants were subjected to Western blot analysis to detect cyclin D1 (top panel) and cdk4 protein levels (bottom panel) 60 min after exposure to 1000 J/m^2 of UV light. The expression of cyclin D1 protein was also greatly reduced in UV-irradiated Bac1.2F5-5D transfectants.

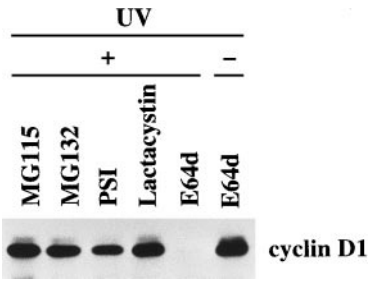

FIG. 4. Proteasome inhibitors suppressed the UV irradiation-induced cyclin D1 degradation in Bac1.2F5 cells. Bac1.2F5 cells synchronized in the mid-G1 phase were pre-treated with the indicated proteasome inhibitors for 60 min prior to UV irradiation (1000 J/m^2). The UV-irradiated (+) or nonirradiated (−) cells were harvested 60 min later and then subjected to Western blot analysis to detect the cyclin D1 protein.

tacystin, for 60 min prior to UV irradiation. Pretreatments with any of these proteasome inhibitors suppressed the UV-induced degradation of the cyclin D1 protein. However, as a negative control, E64d, a cysteine protease inhibitor that does not inhibit the proteasome, did not affect cyclin D1 protein levels (Fig. 4). Although pretreatment with these proteasome inhibitors stabilized the cyclin D1 protein in UV-irradiated Bac1.2F5 cells, cyclin D1 mRNA was undetectable in cells treated with proteasome inhibitors (data not shown), indicating that the proteasome is not involved in the stability of cyclin D1 mRNA. These results indicate that cyclin D1 protein in UV-irradiated Bac1.2F5 cells is degraded through the proteasome pathway.

Pulse chase analysis of cyclin D1 protein in UV-irradiated Bac1.2F5 cells. To estimate the half-life of the cyclin D1 protein in Bac1.2F5 cells with or without UV irradiation, pulse chase analysis was performed. The metabolically labeled Bac1.2F5 cells, synchronized in the mid-G1 phase, were treated with 1000 J/m^2 of UV irradiation in the absence or presence of proteasome inhibitors (MG115 or MG132) followed by pulse chase analysis (Fig. 5). UV irradiation shortened the half-life of the cyclin D1 protein ($t_{1/2}$; approximately 27 min without the UV irradiation, $t_{1/2}$; approximately 15 min with UV irradiation). The proteasome inhibitors

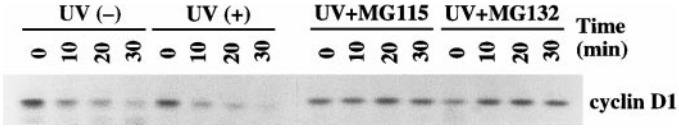

FIG. 5. Pulse chase analysis of cyclin D1 protein in Bac1.2F5 cells. Bac1.2F5 cells synchronized in the mid-G1 phase were metabolically labeled with [^{35}S]-methionine and subjected to pulse chase analysis. In some experiments, the cells were pre-treated with proteasome inhibitors (MG115, MG132) for 60 min before exposure to 1000 J/m^2 of UV. The half-life of the cyclin D1 protein was shortened by exposure of cells to UV but prolonged by proteasome inhibitors.

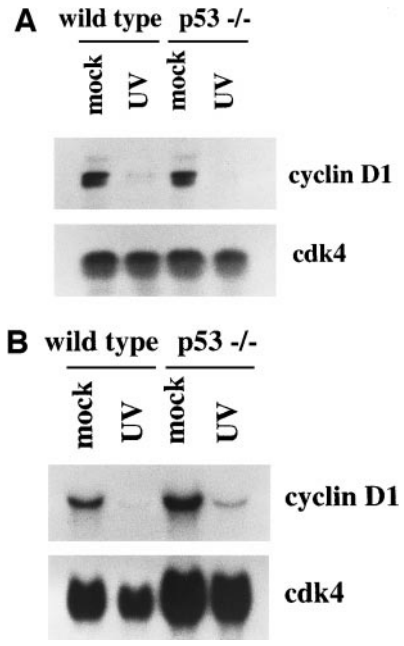

FIG. 6. The UV-induced rapid disappearance of cyclin D1 protein and mRNA was independent of p53. (A) The murine bone marrow-derived macrophages prepared from wild type and p53 −/− mice were synchronized in the mid-G1 phase. Cyclin D1 (top panel) and cdk4 protein (bottom panel) was detected by Western blotting 60 min after irradiation with (1000 J/m^2 of UV) or without (mock). (B) The synchronized and UV-irradiated cells prepared as described above were subjected to Northern blot analysis to detect cyclin D1 and cdk4 mRNA levels.

clearly inhibited the UV-induced degradation of cyclin D1 protein. These results indicate that UV irradiation could facilitate the degradation of cyclin D1 protein through the proteasome pathway.

The UV-induced degradation of both the cyclin D1 protein and mRNA was independent of p53 function. Evidence from numerous cell lines indicated that p53 is involved in the DNA damage-induced cell cycle arrest in the G1 phase (14–16). Consequently, we attempted to elucidate whether the UV-induced rapid disappearance of both cyclin D1 mRNA and protein was regulated by p53. Bone marrow-derived murine macrophages prepared from wild type or p53 −/− mice were synchronized in the mid-G1 phase by cytokine deprivation. The G1-progressing cells were treated with 1000 J/m^2 of UV irradiation and 60 min later the cells were harvested to analyze the expression of cyclin D1 protein and mRNA. Ultraviolet irradiation caused the rapid disappearance of the cyclin D1 protein (Fig. 6A) and mRNA (Fig. 6B) in synchronized macrophages prepared from both wild type and p53 −/− mice as well as in the murine macrophage cell line Bac1.2F5. The levels of the cdk4 protein and mRNA were stable. These results reveal the first evidence that the expression of cyclin D1 is regulated at the mRNA and protein

levels in primary murine macrophages, independent of p53 function.

DISCUSSION

We observed that the levels of cyclin D1 protein are sharply reduced within 60 min after exposure to high doses of UV irradiation in the murine macrophage cell line Bac1.2F5 as well as in primary murine bone-marrow-derived macrophages. Strikingly, cyclin D1 mRNA levels in UV-irradiated Bac1.2F5 cells were extremely reduced within 1 min after UV irradiation. When Bac1.2F5 cells are deprived of CSF-1, the cyclin D1 mRNA exists for more than 2.5 h (2). These findings suggest that the rapid disappearance of cyclin D1 mRNA in UV-irradiated Bac1.2F5 cells might be caused by targeted mRNA degradation. In Bac1.2F5-5D cells constitutively expressing cyclin D1 mRNA, the amount of cyclin D1 mRNA derived from the introduced cyclin D1 gene (1.5 kb) were unchanged after UV irradiation, while mRNA levels of the intrinsic cyclin D1 (4.5 kb) were drastically reduced. Expression of the introduced full length cyclin D1 gene was driven by the metalothionein promoter but lacked the terminal 3 kb of the 3′-untranslated sequence. It is known that the AU-rich elements in the 3′-untranslated region and the polyadenylated tail contribute to the stability of mRNAs (17–19). Given the role that the 3′ untranslated region plays in mRNA stability and our experimental results, it is likely that the last 3 kb of the 3′-untranslated sequence of the cyclin D1 gene plays an important role in UV-induced rapid degradation of cyclin D1 mRNA. Recently, Chang *et al.* reported that UV light directly inhibits mRNA synthesis and abolishes cyclin E expression (20). In our study, we believe that reduced levels of cyclin D1 mRNA are due to RNA degradation rather than inhibition of new mRNA synthesis, since cyclin D1 mRNA disappeared within 1 min after UV exposure. However, we cannot exclude the possibility that cyclin D1 is also regulated at the level of transcription initiation.

Recently, it has been reported that phosphorylation of the murine cyclin D1 protein at threonine 286 triggers its multi-ubiquitination and proteasome-dependent degradation during steady state growth (5). Although protein degradation of cyclin A or B requires its amino-terminal destruction box (21, 22), cyclin D1 protein has no obvious destruction motif but instead has a PEST sequence in its C-terminal domain (22, 23). In the UV-irradiated macrophages, we observed that cyclin D1 was destroyed through the proteasome-dependent pathway. It is not clear that the accelerated phosphorylation of the cyclin D1 protein on its threonine residue was involved in its enhanced destruction.

p53 plays a crucial role in cell cycle arrest induced by DNA damage in the G1 phase (14). In eukaryotic cells, ionizing radiation and UV irradiation damages DNA, thereby inducing cell cycle arrest (15, 16). In our study, p53 was not involved in the rapid down-regulation of cyclin D1 mRNA and protein following UV-irradiation in primary macrophages.

Mailand *et al.* reported that the cdc25A protein was rapidly destroyed in response to ionizing radiation and UV light (24). Recently, it was reported that ionizing radiation induced cyclin D1 protein degradation and that transcriptional regulation was not responsible for decreased protein levels in MCF-7 cells (25). In our hands, ionizing radiation did not affect cyclin D1 protein levels in Bac1.2F5 cells synchronized in the mid-G1 phase (data not shown). The difference in the mechanisms of G1 arrest by ionizing radiation and UV light in Bac1.2F5 cells remains unclear. We demonstrated the rapid proteolysis of the cyclin D1 protein and prompt disappearance of cyclin D1 mRNA after UV irradiation in Bac1.2F5 cells. These results suggest that cell cycle arrest after UV irradiation is caused by many pathways. In addition, UV sensitivity may differ among different cell types, and it is worth noting that in this study we irradiated macrophages at higher doses than previous work using fibroblasts (12). In conclusion, this study provides further insights into the regulatory mechanisms of cell cycle arrest following UV irradiation by demonstrating that cyclin D1 is regulated at its mRNA and protein levels after UV exposure independently of p53 function.

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