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Involvement of the Interaction between p21 and Proliferating Cell Nuclear Antigen for the Maintenance of G_2/M Arrest after DNA Damage*

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Although a major effect of p21, a cyclin-dependent kinase inhibitor, is considered to be exerted during G_1 phase of the cell cycle, p21 gene knock-out studies suggested its involvement in G_2/M checkpoint as well. Here we demonstrate evidence that p21 is required for the cell cycle arrest at G_2 upon DNA damage. We found that expression of wild-type p21 ($p21^{WT}$), not mutant p21 ($p21^{PCNA-}$) lacking the interaction with proliferating cell nuclear antigen (PCNA), caused G_2 cell cycle arrest in p53-deficient DLD1 colon cancer cell line after the DNA damage by treatment with *cis*-diamminedichloroplatinum (II). We also found that $p21^{WT}$ was associated with Cdc2/cyclin B1 together with PCNA. Furthermore, coimmunoprecipitation experiments revealed that PCNA interacted with Cdc25C at the G_2/M transition, and this interaction was abolished when $p21^{WT}$ was expressed presumably due to the competition between $p21^{WT}$ and Cdc25C in the binding to PCNA. These findings suggest that p21 plays a regulatory role in the maintenance of cell cycle arrest at G_2 by blocking the interaction of Cdc25C with PCNA.

The cell cycle is regulated by two major checkpoints at G_1-S and G_2/M transition. The fidelity of genomic replication during DNA synthesis and cell division is ensured by checkpoint controls that prevent cell cycle progression when the DNA damage or incomplete DNA replication is detected (1). Thus, checkpoint loss would result in genomic instability and has been implicated in carcinogenesis (2). In fact, the p53 tumor suppressor gene, a major gatekeeper of cell cycle checkpoints, is mutated in a large fraction of human cancers (3). Cell cycle arrest in G_1 caused by DNA damage or cellular senescence is mediated by p21, a cyclin-dependent kinase inhibitor, that is under the transcriptional control of p53 (4). Interestingly, although cells deficient in p21 proliferate normally, they are unable to maintain stable G_2 arrest and initiate cell death program when exposed to DNA-damaging agents such as irradiation and anticancer drugs (5, 6). Circumstantial evidences indicate the involvement of p21 at the G_2/M transition. For example, p21 mRNA in human fibroblasts show bimodal periodicity with peaks in G_1 and G_2/M (7) and that p21 protein reaccumulates in the nucleus at the onset of mitosis (8). In addition, inducible p21 expression caused cell cycle arrest at G_1 and G_2 (9) and p21 induced G_2 arrest when it was induced at the beginning of S phase (10). These observations have suggested that p21 is also involved in G_2/M checkpoint.

It is well established that p21 consists of at least two functional domains that bind to proliferating cell nuclear antigen (PCNA)[1] and Cdk/cyclins (11–13). PCNA was initially identified as an auxiliary protein for DNA polymerase δ that is essential for DNA replication and repair (14–16). The role of PCNA in cell cycle regulation is suggested by the fact that polymerase δ is regulated by cell cycle proteins (17). In fact, PCNA was shown to interact with various Cdk-cyclin complexes (11, 16, 18). Thus, PCNA may act as a platform for multiple protein-protein interactions involved in replication, repair, recombination, and cell cycle regulation. Interestingly, the PCNA protein levels increased steadily through the entire cell cycle period and remained high at G_2/M (19). Thus, PCNA may also be involved in G_2 cell cycle control.

The G_2 checkpoint has been extensively studied in the fission yeast and is known to involve a number of proteins including Cdc2/cyclin B, Cdc25, 14-2-3, Wee1, Chk1, Cds1/Rad53, and DNA damage sensor proteins (20, 21). Among these proteins, Cdc25, a dual phosphatase for Cdc2, plays a central role in the G_2 checkpoint by controlling the phosphorylation status, thus its kinase activity, of Cdc2 (22–24). In human cells, there are three Cdc25 homologues, Cdc25A, -B, and -C (25–27). Whereas Cdc25A is involved in the G_1 checkpoint, Cdc25B and Cdc25C are involved in the G_2/M transition (28–34). Because the activity of Cdc25C is induced at the onset of mitosis and regulates Cdc2/cyclin B1, it is regarded as a major regulator of the G_2 checkpoint, besides the fact that Cdc25C has the highest homology with the yeast Cdc25. Cdc2 is subject to multiple levels of regulation including periodic association with cyclin B, phosphorylation, dephosphorylation (35–38), and intracellular compartmentation (39–44). Cdc2 associates with cyclin B at the G_2/M transition (38), and this complex is retained in an inactive state throughout S and G_2 phases by phosphorylation of

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[1] The abbreviations used are: PCNA, proliferating cell nuclear antigen; PI, propidium iodide; Tet, tetracycline; Fen1, flap endonuclease 1; WT, wild type; CDDP, *cis*-diamminedichloroplatinum (II); HA, hemagglutinin; PBS, phosphate-buffered saline; Cdk, cyclin-dependent kinase.

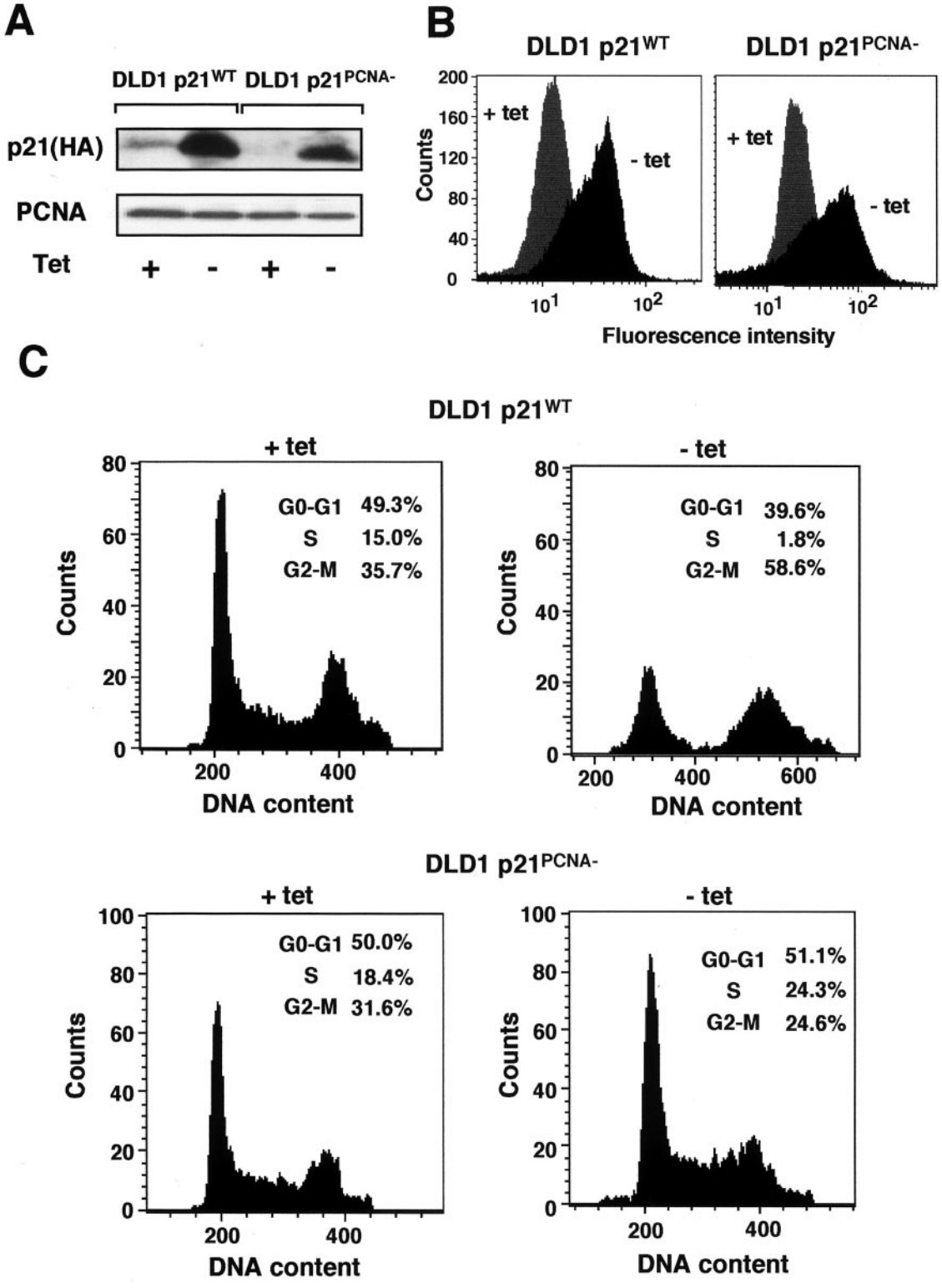

FIG. 1. **Abolishment of cell cycle progression by p21 requires its interaction with PCNA.** *A,* detection of p21 expression in DLD1 $p21^{WT}$ and DLD1 $p21^{PCNA-}$ cells. Cell lysates were prepared from cultured cells in the presence (+ *Tet*) of tetracycline or in the absence of tetracycline for 96 h (− *Tet*) and probed with anti-HA antibody (detecting both wild type and PCNA (−) mutant form p21 proteins) by Western blotting. *B,* quantitation of cells expressing p21 proteins by flow cytometry. *C,* cell cycle analysis. Cells were stained with propidium iodide (*PI*) and subjected to the DNA content analysis using fluorescence-activated cell sorter. The percentage of cells accumulated at each cell cycle stage is indicated. Induction of p21 protein was done by culturing cells in the medium without tetracycline for 96 h. Note that expression of wild-type p21 (DLD1 $p21^{WT}$, − *Tet*) arrested the cell cycle progression at both G_1 and G_2/M with a significant decrease in cells at S phase, whereas expression of mutant $p21^{PCNA-}$ (DLD1 $p21^{PCNA-}$, − *Tet*) failed to do so.

Cdc2 at Thr-14 and Tyr-15 by another kinase Wee1 (22, 36). Wee1 is activated by upstream kinases, Chk1 or Cds1 (45, 46), that are activated by damaged DNA (47–49) and unreplicated DNA (45, 50, 51), respectively. Although Cdc25 activates Cdc2/cyclin B by reversing the Wee1-mediated phosphorylation of Cdc2 (22, 23), this activity is suppressed by Chk1 and Cds1

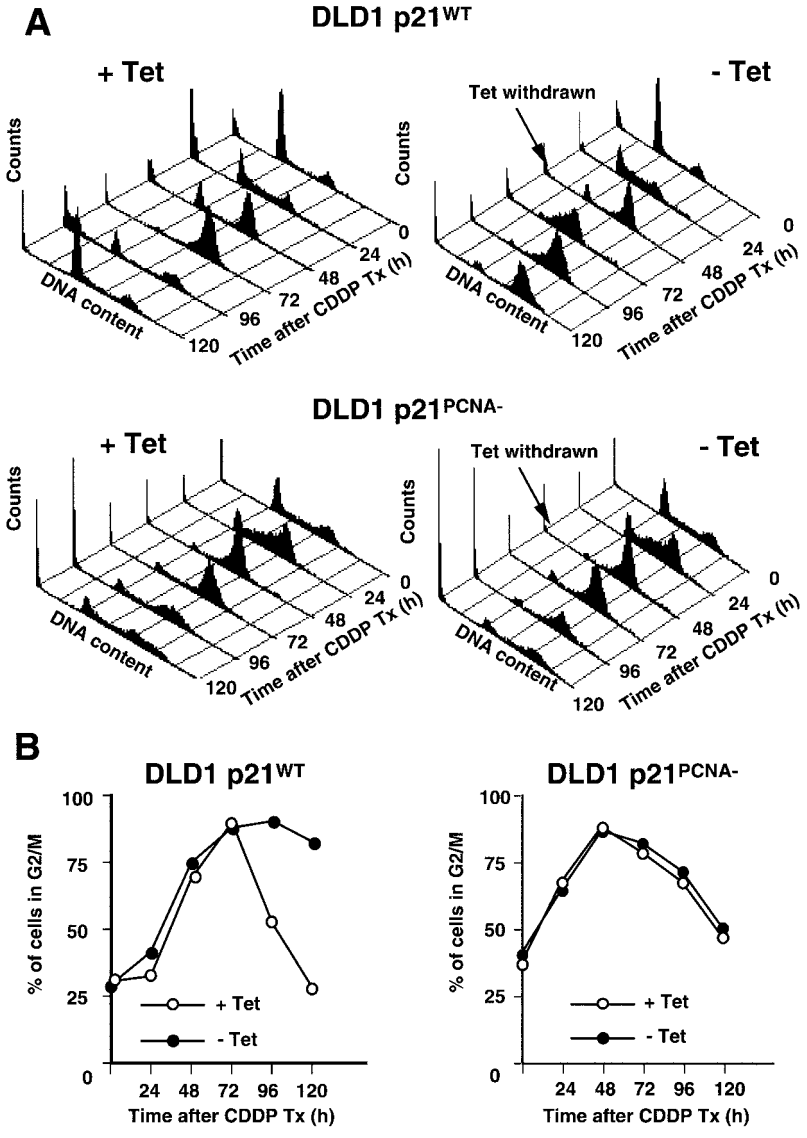

FIG. 2. **Requirement of p21 interaction with PCNA in the maintenance of the G_2/M arrest after DNA damage.** *A,* cell cycle progression of DLD1 $p21^{WT}$ and DLD1 $p21^{PCNA-}$ cells after DNA damage. Cells were treated with CDDP (12 μg/ml) for 1 h, washed, and further cultured. At 48 h after the DNA damage induced by CDDP treatment (*Tx*), Tet was eliminated from the culture medium to induce p21 expression (− *Tet*). They were harvested at various time points (indicated as *h* after *CDDP Tx*). DNA profiles were obtained by flow cytometry after stained with PI. *B,* failure of $p21^{PCNA-}$ in preventing cell cycle progression upon DNA damage. Percentages of DLD1 cells expressing either $p21^{WT}$ or $p21^{PCNA-}$ at the indicated time points after the DNA damage are shown. Note that the cells expressing $p21^{WT}$ were arrested at G_2/M transition, whereas the cells expressing $p21^{PCNA-}$ did not maintain the G_2/M arrest (both *A* and *B*). Those cells that did not accumulate at G_2/M underwent cell death.

through phosphorylation of Cdc25 at Ser-216 in the yeast (50, 52, 53). The phosphorylated Cdc25 is sequestered in the cytoplasm by the interaction with 14-2-3 (54–59). However, a small amount of Cdc25 was shown to reside in the nucleus at interphase, and moreover, most of Cdc25 stays in the nucleus throughout cell cycle in some cells (46, 47). Therefore, an additional mechanism is required to ensure the inactivation of Cdc2-cyclin B complex by preventing coincidental contacts with Cdc25.

In this study, we attempted to examine the role of p21 in G_2/M transition particularly in G_2 DNA damage checkpoint by utilizing the p53-deficient human colon cancer cell lines inducibly expressing the wild-type or the mutant $p21^{PCNA-}$ lacking the interaction with PCNA. We demonstrate evidence that $p21^{PCNA-}$ cannot confer cell cycle arrest at G_2 upon DNA damage, and we suggest that the p21 may interfere with the interaction between PCNA and Cdc25 thus preventing Cdc25 from the activation of Cdc2. The possible role of PCNA as a platform for regulatory protein-protein interactions for the cell cycle regulation is discussed.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Most of the chemicals including hygromycin, tetracycline, and *cis*-diamminedichloroplatinum (II) (CDDP) were purchased from Sigma. Fluorescein isothiocyanate-conjugated anti-HA (F-7), anti-PCNA (PC-10), anti-cyclin B1 (GNS1), anti-Cdc2 p34 (clone 17), anti-Cdk7 (C-14), and anti-cyclin H (FL-323) mouse monoclonal antibodies and anti-Cdc25C rabbit polyclonal antibody (C-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated anti-HA mouse monoclonal antibody (3F10) was purchased from Roche Molecular Biochemicals. Biotin-conjugated anti-PCNA mouse monoclonal antibody was purchased from PharMingen (San Jose, CA). Secondary antibodies and horseradish peroxidase-conjugated goat anti-rabbit and sheep anti-mouse antibodies were purchased from Santa Cruz Biotechnology and Amersham Pharmacia Biotech, respectively.

Cell Lines and Culture Conditions—Parental DLD1 human colon carcinoma cell line constitutively expresses very low levels of endogenous p21 because of mutations of both alleles of p53 (p53−/−) (60). DLD1 cell lines containing exogenous genes for wild-type (WT) or mutant (PCNA−) p21 proteins tagged with HA epitope were as described (9). The $p21^{PCNA-}$ mutant contains amino acid substitutions (M147A, D149A, and F150A) to abolish the interaction with PCNA specifically. Expression of p21 in these cells is under the tight control of a tetracycline-regulated promoter and can be induced by eliminating tetracycline (Tet) from the culture medium ("Tet-OFF" system). These cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (IBL, Maebashi, Japan), 4 mM L-glutamine (Life Technologies, Inc.), 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.), and 2 μg/ml tetracycline in a 5% CO_2 incubator. Subcloning of these cell lines was repeatedly done in selective medium containing 50 μg/ml hygromycin. Western blotting and flow cytometry was performed to ensure that ~90% of the cells expressed p21 when tetracycline was eliminated. There was no significant difference in the sensitivity to CDDP among parental DLD1, DLD1 $p21^{WT}$, and $DLD1^{PCNA-}$ cells.

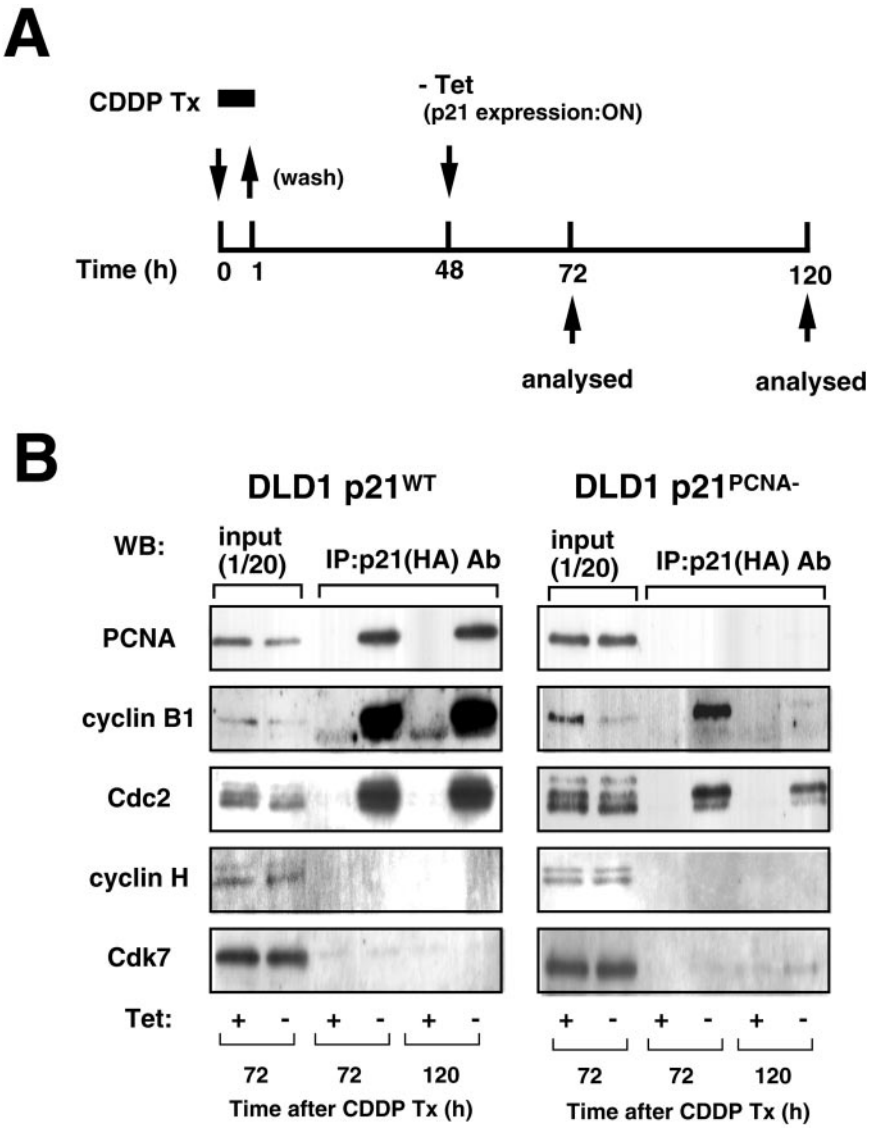

FIG. 3. **Formation of protein complex containing p21^{WT}, Cdc2, cyclin B1, and PCNA during G_2/M phase.** *A,* diagram of the experimental procedure. To induce p21 proteins, DLD1 p21^{WT} and DLD1 p21^{PCNA-} cells were treated with CDDP (12 μg/ml) for 1 h, and tetracycline was eliminated (− *Tet*) from the culture at 48 h after CDDP treatment (*Tx*). Cells were washed and further cultured for additional 24 (*72* h) or 72 h (*120* h). At 72 h, both − *Tet and* + *Tet* cells entered G_2. At 120 h, whereas − *Tet* DLD1 p21^{WT} cells remained at G_2, + *Tet* cells and − *Tet* DLD1 p21^{PCNA-} cells entered G_1 because of the lack of functional p21 (for the details of cell cycle status, see Fig. 2). *B,* detection of proteins co-immunoprecipitated with p21^{WT} (*left panel*) or p21^{PCNA-} (*right panel*) by Western blot (*WB*). Cells were harvested, and the protein complexes containing p21 were immunoprecipitated (*IP*) from the total cell lysate by anti-HA (epitope for the exogenous p21^{WT} and p21^{PCNA-}) antibody followed by Western blot analyses as described under "Experimental Procedures" using the indicated antibodies including anti-PCNA, anti-Cdc2, anti-Cdk7, anti-cyclin B1, and anti-cyclin H antibodies. Equal amounts of cell lysates were used in each immunoprecipitation. The similar experiments were carried out for three times with essentially the same results. The representative results are demonstrated. Note that cyclin B1 was degraded in DLD1p21^{PCNA-} cells (− *Tet*) at 120 h after the DNA damage.

Flow Cytometric Analysis—Expression of p21 and cell cycle analysis were performed by flow cytometry with FACScan (Becton Dickinson, Mountain View, CA) with the program CELLQuest (Becton Dickinson) (61). For detection of p21 protein expression, DLD1 cells (5×10^6) were trypsinized, washed with PBS, fixed in 70% ethanol, resuspended in TPBS (PBS containing 0.1% Tween 20), and incubated with fluorescein isothiocyanate-conjugated anti-HA mouse monoclonal antibody (F-7) for 60 min at room temperature in the presence of 0.1% bovine serum albumin (Sigma) and 0.5 μg/ml ribonuclease A (Roche Molecular Biochemicals). The cells were then washed three times with TPBS, stained with propidium iodide (Sigma), and incubated overnight at 4 °C in TPBS containing 200 μg/ml propidium iodide (PI). The cell cycle status of the cells was determined as described previously (24). Briefly, cells (5×10^6) were collected, washed twice with cold PBS, and resuspended in Krisham's solution (0.1% sodium citrate, 50 μg/ml PI, 20 μg/ml ribonuclease A, and 0.3% Nonidet P-40) prior to flow cytometric analysis.

Co-immunoprecipitation and Immunoblotting—DLD1 cells (2×10^6) were collected, washed twice with PBS, and suspended in 200 μl of lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM EDTA, 10 μM NaF, 1 mM Na_3VO_4, 1 μM okadaic acid, 2 mM dithiothreitol, 0.25% Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride) according to the method described previously (62). The lysate was clarified by centrifugation at 15,000 × *g* for 20 min. The supernatant was collected and incubated with either biotin-conjugated anti-PCNA antibody or anti-HA antibody (for detecting p21) for immunoprecipitation at 4 °C for 1.5 h with gentle rotation. Twenty μl of streptavidin-Sepharose beads (Amersham Pharmacia Biotech) or protein G-Sepharose beads (Amersham Pharmacia Biotech) were added and further incubated for 1 h. The beads were washed 5 times with 1 ml of lysis buffer. Antibody-bound complexes were eluted by boiling in 2× Laemmli sample buffer and resolved by 12% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Hybond-C, Amersham Pharmacia Biotech). Membranes were blocked in TPBS including 2% non-fat milk 4 °C overnight, probed with various primary antibodies for 1 h at room temperature, washed three times with TPBS, and probed with the secondary antibody for 40 min at room temperature. The immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal; Pierce).

RESULTS

Conditional Expression of Wild-type and Mutant p21 Proteins in p53-deficient Human Colon Cancer Cell—Repeated subcloning of cells, DLD1 p21^{WT} and DLD1 p21^{PCNA-}, was carried out to enrich cell lines conditionally expressing wild-type or mutant (PCNA−; in which the PCNA-interacting amino acid residues were mutated) p21, respectively. As shown in Fig. 1, *A* and *B*, levels of p21 protein expression in these cells were virtually null in the presence of tetracycline but became readily detectable when tetracycline was eliminated from the culture medium. In Fig. 1*B*, cells were fixed and p21 expression was examined by flow cytometry. Upon induction, p21 protein expression was detected in 94% of DLD1p21^{WT} and in 87% of DLD1p21^{PCNA-} cells.

Fig. 1*C* demonstrates the effects of the p21 expression on cell

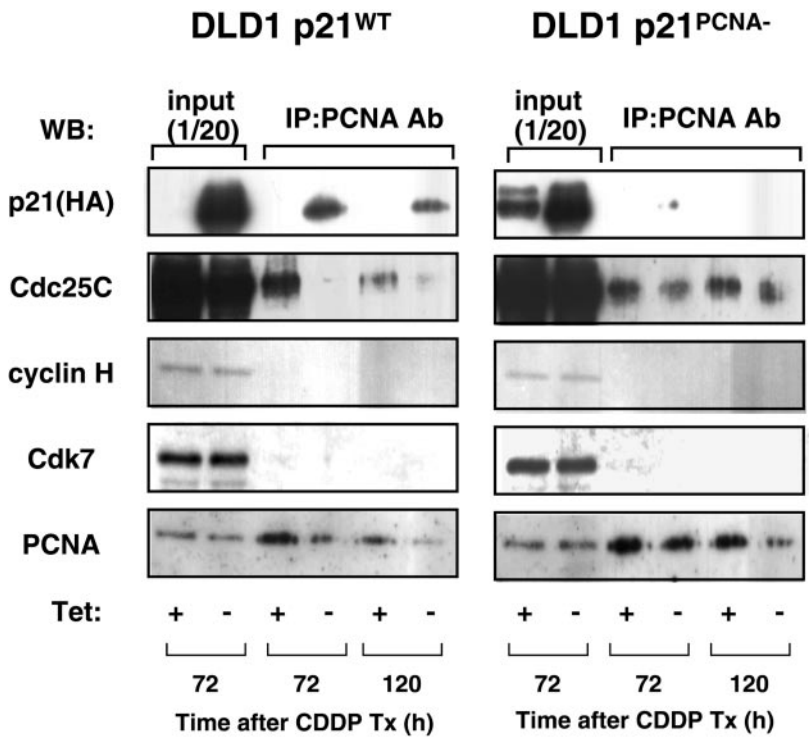

FIG. 4. **Alternative binding of PCNA to p21 and Cdc25C during G_2/M progression.** Similar co-immunoprecipitation and Western blot (*WB*) determination was performed with DLD1 $p21^{WT}$ (*left panel*) and DLD1 $p21^{PCNA-}$ (*right panel*) after the treatment with CDDP as in Fig. 3. The cell lysate was immunoprecipitated with anti-PCNA antibody, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to a membrane. Western blot determination of the PCNA precipitates was performed with antibodies to p21 (*HA*), Cdc25C, cyclin H, Cdk7, and PCNA. Equal amounts of cell lysates were used in each immunoprecipitation. The similar experiments were carried out for three times with essentially the same results. The representative data are demonstrated here.

proliferation and cell cycle progression by flow cytometry. In the absence of p21 expression, the cell cycle distribution was similar for both cell lines. For example, in DLD1$p21^{WT}$ ~49.3, 15.0, and 35.7% of cells were in G_1, S, and G_2 phases, respectively (Fig. 1*C*). When $p21^{WT}$ expression was induced in DLD1 cells, a striking decrease in the number of cells in S phase (1.8%) was observed, whereas the majority of cells accumulated at G_1 (39.6%) or G_2/M (58.6%). On the other hand, $p21^{PCNA-}$ expression did not significantly change the cell distribution (Fig. 1*C, lower panel*). These findings confirmed those by Cayrol *et al.* (9) that p21 was involved in the cell cycle arrest at G_1 and G_2/M through binding to PCNA.

Requirement of Wild-type p21 in the G_2 DNA Damage Checkpoint—To examine the effects of p21 expression on the DNA damage-induced G_2 cell cycle arrest, DLD1 $p21^{WT}$ and DLD1 $p21^{PCNA-}$ cells were treated with CDDP (12 μg/ml) for 1 h, and p21 proteins were induced by removing Tet, and the cell cycle analysis was carried out by flow cytometry. As shown in Fig. 2*A*, because most of the cells were accumulated at G_2/M 48–72 h after the DNA damage induced by CDDP, we induced p21 expression by Tet withdrawal ("− Tet") at 48 h after the CDDP treatment. Cells not expressing p21 or expressing p21 mutant progressed into G_1, and significant numbers of cells underwent cell death (detected as cells in sub-G_1). Fig. 2*B* indicates that 48 h after the DNA damage ~74% of cells expressing $p21^{WT}$ entered G_2/M, and these cells remained in G_2/M at 120 h. On the other hand, cells not expressing p21 or expressing p21 mutant immediately progressed into G_1. For example, although ~87% of DLD1 cells expressing $p21^{PCNA-}$ entered G_2/M at 48 h after the DNA damage, only 50% of the cells were detected at G_2/M at 120 h. Similar observations were obtained with cells not expressing p21. These observations indicate that p21 is involved in the DNA damage-induced G_2 cell cycle arrest and that the ability of p21 to bind PCNA is crucial.

Binding of $p21^{WT}$ to the PCNA-Cdc2-Cyclin B1 Complex in the DNA Damage G_2 Checkpoint—It is known that p21 not only inhibits Cdk4/cyclin D1 and Cdk6/cyclin D2 in G_1 checkpoint but also inhibits Cdc2/cyclin B1 during the G_2/M transition at least *in vitro* (63). We then examined if p21 associates with PCNA at the G_2/M transition together with Cdc2/cyclin B1. In Fig. 3, DLD1 cells were treated with CDDP for 1 h, and expression of either wild type ($p21^{WT}$) or mutant ($p21^{PCNA-}$) was induced at 48 h after the DNA damage. The interaction of p21 with the Cdc2-cyclin B1 complex and PCNA at the DNA damage G_2 checkpoint was examined by immunoprecipitation (with anti-HA antibody detecting $p21^{WT}$ and $p21^{PCNA-}$) followed by Western blotting (with antibodies to PCNA, cyclin B1, and Cdc2).

As demonstrated in Fig. 3*B* (*left panel*), $p21^{WT}$ coimmunoprecipitated PCNA, Cdc2, and cyclin B1 when cells were arrested at G_2/M transition due to CDDP-induced DNA damage, indicating the formation of $p21^{WT}$-PCNA-Cdc2-cyclin B1 complex in cells arrested at G_2/M. Interestingly, although Cdc2-cyclin B1 complex interacts with $p21^{PCNA-}$ at the G_2/M transition (72 h after DNA damage) (Fig. 3, *right panel*), cells could not maintain the G_2/M arrest (Fig. 2*B*). In these cells, cyclin B1 was proteolytically degraded and cells entered M and then progressed to G_1 (120 h after DNA damage) (Fig. 3*B, right panel*). These findings suggest that in order for p21 to induce the cell cycle arrest at G_2 in response to DNA damage, the interaction of p21 with PCNA is crucial. Because p21 was previously shown to inhibit CAK-mediated Cdc2 phosphorylation and promote cell cycle arrest at G_2/M (10), we also examined the interaction of p21 with cyclin H and Cdk7 (CAK). However, neither cyclin H nor Cdk7 was coimmunoprecipitated with p21 (Fig. 3*B*).

Involvement of $p21^{WT}$ in G_2/M Checkpoint, Alternative Binding of PCNA to $p21^{WT}$ and Cdc25C—During the cell cycle interphase, Wee1HU inactivates Cdc2-cyclin B1 complex by phosphorylation of Cdc2 at Tyr-15 (64), which is subsequently activated by Cdc25 through dephosphorylation at Tyr-15 when cells enter mitosis (65). We thus hypothesized that, in human cells, the Cdc25C-mediated activation of Cdc2/cyclin B1 would occur at the nascent DNA following the completion of DNA synthesis or DNA repair, and actions of p21 and Cdc25C would be exclusive. We then examined whether Cdc25C associates with PCNA in the absence of p21, and the PCNA binding of p21 and that of Cdc25C are mutually exclusive (Fig. 4). This pos-

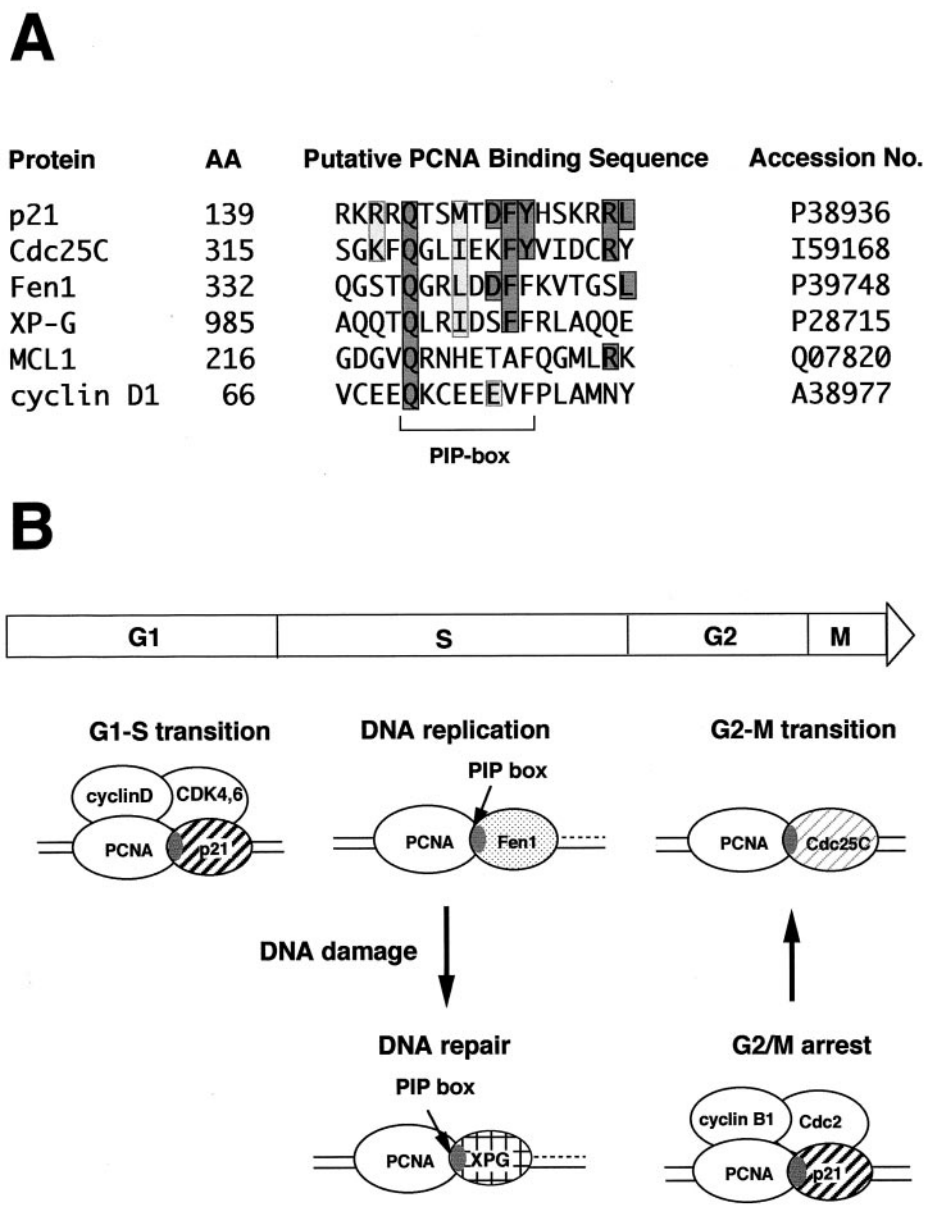

FIG. 5. **PCNA-binding proteins and the model for cell cycle regulation.** *A,* protein sequence alignment of p21 and other PCNA-binding proteins. These proteins contain the characteristic PCNA-binding motif (*PIP box*) (74). *AA* denotes the position of the starting amino acid residues. *Dark boxes* represent identical amino acid residues of previously described PCNA-binding proteins to p21. *Shaded boxes* represent homologous amino acid residues to those of p21. *B,* involvement of PCNA and its binding proteins in cell cycle and DNA damage checkpoints. From our data presented in this study, p21 appears to participate in G_2 checkpoint upon DNA damage by the interaction with PCNA. During normal S-G_2/M transition, PCNA interacts with Fen1 (during S phase) and subsequently with Cdc25C. However, upon the DNA damage, PCNA interacts with p21 instead of Cdc25C and induced cell cycle arrest at G_2/M by recruiting Cdc2/cyclin B1. PCNA may act as a platform for protein-protein interactions for the cell cycle regulation and checkpoints.

sibility was also prompted by a previous report by Saha *et al.* (66) that p21 and Cdc25A competitively bind to Cdk2 presumably at the G_1-S transition.

In Fig. 4, DNA damage was induced in DLD1 cells, and expression of either wild type ($p21^{WT}$) or mutant ($p21^{PCNA-}$) was induced as in Fig. 3. The cell lysate was analyzed for the protein-protein interaction by immunoprecipitation (with anti-PCNA antibody) followed by Western blotting to detect the PCNA-associated p21 and Cdc25C. As demonstrated in Fig. 4 (*left panel*), Cdc25C was detected in the PCNA complex when p21 was not induced (+ *Tet*). Cdc25C was coimmunoprecipitated with PCNA in the absence of DNA damage, *i.e.* in the absence of p21 (data not shown). In the presence of $p21^{WT}$, PCNA coimmunoprecipitated $p21^{WT}$ but not Cdc25C. However, when $p21^{PCNA-}$ was expressed, PCNA coimmunoprecipitated Cdc25C irrespective of the presence of $p21^{PCNA-}$, indicating that p21 and Cdc25C interact with the same region of PCNA.

DISCUSSION

We have explored the role of p21 in the G_2 DNA damage checkpoint using p53-deficient cells in which $p21^{WT}$ or $p21^{PCNA-}$ was complemented. Our findings clearly indicate the crucial role of p21 in the G_2 checkpoint upon DNA damage and that the interaction of p21 with Cdc2-cyclin B1 complex is mediated by PCNA. We assume that the p21-PCNA interaction is probably required for recognition of the repaired DNA. Because the p21-PCNA interaction appeared to be mutually exclusive with the Cdc25C-PCNA interaction at G_2/M transition, p21 may prevent the incorporation of Cdc25C into the Cdc2-cyclin B1 complex and thus induce cell cycle arrest at G_2. In fact, the formation of ternary complex involving p21, PCNA, and Cdc2/cyclin B1 at G_2 was previously demonstrated (7, 19, 63). In addition, Dulic *et al.* (8) and Medema *et al.* (67) independently showed the formation of p21-Cdc2-cyclin B1 complex and inhibition of Cdc2 kinase activity at G_2. Moreover, we observed that cells failed to arrest at G_2/M because of the absence of functional p21 and underwent cell death (Fig. 2). In agreement with this finding, Bunz *et al.* (5) demonstrated with cells defective for p53 or p21 genes that γ-irradiation induced cell death associated with the lack of cytokinesis after entering into M phase, again indicating the crucial importance of p21 for the maintenance of the G_2/M arrest.

Expression of p21 is transcriptionally regulated by p53 upon DNA damage or cellular senescence and is known to induce cell cycle arrest at G_2 as well as at G_1 by inhibiting Cdc2 (4, 68, 69).

In addition to the binding with Cdc2-cyclin B1 complex, p21 is also shown to bind PCNA (70). PCNA acts as an auxiliary factor for DNA polymerase δ and stimulates DNA replication (71). It was shown that p21 inhibited PCNA-dependent DNA replication *in vitro* by binding to PCNA through its PCNA-interacting region (11, 12, 72, 73). We found that DLD1 $p21^{PCNA-}$, expressing a mutant p21 defective for the interaction with PCNA, could not arrest at G_2/M checkpoint even after DNA damage (Fig. 2). Thus, it is possible that, through the interacting with PCNA, p21 inhibits DNA synthesis and maintains G_2/M arrest.

These findings also suggest an important role for PCNA as a platform for the interaction of various cell cycle regulator proteins that occur adjacently to the nascent DNA or the repaired DNA. Among these proteins, p21, Fen1 (flap endonuclease 1), and xeroderma pigmentosum G are known to contain similar sequences (called PIP box) that interact with PCNA (74). It was shown that Fen1 and p21 compete for binding to the same site of PCNA (75). Similarly, xeroderma pigmentosum G and p21 were shown to compete for the PCNA binding at least *in vitro* (76). We also found a similar PCNA-binding motif in Cdc25C (Fig. 5*A*). It is thus conceivable that competitive binding among PCNA-interacting proteins plays an important role in the coordinated DNA replication and repair. Although further studies are needed, it is likely that p21 inhibits cell cycle progression to mitosis by regulating the Cdc25C interaction with the Cdc2/cyclin B1 (Fig. 5*B*). Considering that fact that PCNA forms a stable homotrimer when it binds to DNA upon DNA synthesis and possibly DNA repair, it remains to be clarified whether these protein-protein interactions with PCNA can occur concomitantly to some extent or are mutually exclusive.

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