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# Cytoplasmic Localization of Cyclin Kinase Inhibitor p21 Delays the Progression of Apoptosis

DAISUKE ARAI, NORIHIRO NOMURA, KUNIHIKO FUKUCHI and KUNIHIDE GOMI

*Department of Clinical Pathology, Showa University, School of Medicine, Tokyo 142 8666, Japan*

**Abstract.** *The different functions of the cyclin kinase inhibitor, p21, rely on its localization to either the cytoplasm or nucleus. Phosphorylation at Thr-145 and/or Ser-146 was reported to target p21 to the cytoplasm. To clarify the function of cytoplasmic p21, we constructed non-phosphorylatable mutants, Thr-145 to Ala (T145A) and Ser-146 to Ala (S146A), and phosphorylation mimic mutants, Thr-145 to Asp (T145D) and Ser-146 to Asp (S146D), and the cells stably expressing those mutants were identified. The association of all four mutants with either CyclinA or CDK2 was increased by γ-irradiation, indicating that the mutants functioned as cyclin kinase inhibitors. PCNA binding was detected in T145A and S146A, but not in T145D and S146D. In the stably-expressing cells, T145D and S146D binding was observed in the cytoplasm, while T145A and S146A in the nucleus. Further, lactacystin treatment enhanced T145A and S146A, but not T145D and S146D, which is consistent with the degradation of p21 by proteasome in the nucleus. Apoptosis induced by γ-irradiation was delayed in the cells expressing either T145D or S146D. The activities of caspase 3 were not reduced in mutant-expressing cells. These results suggest that the PCNA-unbound form of the full length p21 in the cytoplasm delays apoptosis through the interaction with caspase 3 or downstream components.*

Progression of the cell cycle is precisely controlled by cyclin dependent kinases (CDKs). The sequential activation of CDKs is accomplished by the association of each CDK with a specific cyclin subunit (1, 2). In the mammalian cell cycle, G1 progression is driven by the association of CyclinD with either CDK4 or CDK6, while the G1-S transition requires both CyclinE-CDK2 and CyclinA-CDK2 associations. As the cell cycle progresses, each cyclin is degraded and the phase-specific CDK activity is diminished. When dividing cells are exposed to genetic stress, the cell cycle must be arrested immediately to ensure the integrity of the DNA and/or the cell cycle control. To prevent an unscheduled entry into S-phase, the activity of the Cyclin-CDK complex is suppressed by an association with CDK inhibitors. Dependent on the extent of the damage, the cell must determine whether to arrest the cell cycle, repair the DNA, or to enter the apoptosis pathway (3).

Recently, evidence has accumulated that the cyclin kinase inhibitor, p21, is a multifunctional cell cycle-regulatory molecule that contributes to the regulation of apoptosis, as well as associating and inhibiting Cyclin-CDKs or PCNA (4). The p53-dependent and p53-independent expression of p21 protects cells from adriamycin-induced apoptosis (5). The overexpression of p21 in human osteosarcoma cells protected the cells from etoposide-induced cell death (6). Radiation therapy of xenograft tumors with intact p21 genes is ineffective, whereas 18-38% of xenograft tumors from p21-deficient cells are reduced (7). In addition, p21 antisense therapy radiosensitizes human colon cancer cells to apoptosis (8). The expression of p21 protects cells exposed to the hydrogen peroxide from apoptosis (9, 10). Further, p21 protects the cells from cytokine-activated apoptosis. In retinal endothelial cells, the up-regulation of p21 inhibits TGF-induced apoptosis (11). Overexpression of p21 in Ewing tumor cells protects the cells from TNF-mediated apoptosis (12).

In contrast, a number of reports suggest that p21 possesses pro-apoptotic functions under certain conditions in specific systems. Overexpression of p21 in thymocytes leads to a hypersensitivity to p53-dependent cell death in response to ionizing radiation and UV (13). Overexpression of p21 in SV40 T-antigen-positive mammary tumor cells increases apoptosis (14), while its overexpression has also been shown to enhance the apoptotic response to the chemotherapeutic agent, cisplatin, in glioma (15) and ovarian carcinoma (16) cell lines. p21 may further play an active role in apoptosis induced by the activation of members of the TNF family of death receptors. A reduction in Fas-

*Correspondence to:* Kunihiko Fukuchi, Department of Clinical Pathology, Showa University, School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142 8666, Japan. e-mail: kfukuchi@med.showa-u.ac.jp

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dependent apoptosis was seen in thymocytes deficient in p21 when compared with wild-type control thymocytes (17).

The mechanism by which p21 can regulate the apoptotic pathway is not well understood. While cell cycle inhibition by p21 is a nuclear event, since p21 binds and inhibits CDKs and/or PCNA in the nuclei, the regulation of apoptosis may be a nuclear or cytoplasmic event, or both. Since there are differing apoptosis signal cascades, one induced by DNA damage and another being the death signal from the cell membrane or cell organelle, various regulatory points in the apoptosis signal cascades exist. Of the above-mentioned reports, three display the nuclear localization of ectopic-expressed full length p21 by immunohistochemistry. Prabhu *et al.* and Lincet *et al.* demonstrated the anti-apoptotic effect of p21, while Shibata *et al.* showed the pro-apoptotic effect (6, 14, 16). Thus, the influence and activity of p21 on the apoptosis signal cascade may depend on the cell type or the cell conditions.

Recently, evidence that the cytoplasmic localization of p21 is a critical determinant for the regulatory function of p21 has accumulated (18). The cytoplasmic p21 is reported to bind and inhibit ASK1, an apoptosis signal-regulating kinase 1 (19). The constitutive cytoplasmic localization of p21 protects etoposide-induced apoptosis of monocytic leukemia cells by the formation of a complex between p21 and ASK1 (20). In contrast, cleavage at the C-terminal Asp-112 of p21 by caspase 3 after DNA damage, in which the nuclear localization signal is removed, does not reduce apoptosis (21, 22).

Taken together, many of the functions of p21 have been considered to be temporally- and, probably, spatially-regulated. The cellular localization of p21 is reported to be regulated by nuclear localization signals at the C-terminal or phosphorylation of Thr-145, since phosphorylation at Thr-145 leads to cytoplasmic localization (23, 24). Furthermore, the significance of Thr-145 and the adjacent Ser-146 in the function of p21 has been suggested, in that the phosphorylation of Thr-145 and/or Ser-146 inhibiting the PCNA binding (25-27). In addition, PCNA association was reported to stabilize p21 from proteasomal degradation (28).

In this study, the function of cytoplasmic p21 in the progression of apoptosis was examined. Since the phosphorylation of p21 at Thr-145 or Ser-146 may induce cytoplasmic localization, we constructed the non-phosphorylatable mutants, Thr-145 to Ala and Ser-146 to Ala, and phosphorylation mimic mutants, Thr-145 to Asp and Ser-146 to Asp. The function of these mutants was then examined in a transient expression system and in stably-expressing cell lines.

## Materials and Methods

*Generation of expression plasmids.* The site-directed mutants of p21 were propagated using a Transformer site-directed mutagenesis kit (BD Bioscience, NJ, USA) with a p21 cDNA clone as a template (29). The generated fragments were inserted into either pcDNA3.1/His or pcDNA3.1/MycHis (Invitrogen, CA,USA), and transfected into JM109 (30). The transfectants were propagated and extracted as described previously (29, 31). A portion of the plasmid DNA (3 μg) was used in DNA sequencing. The sequencing reactions were performed using a Dual CyDye Terminator Sequencing Kit (Bayer HealthCare, CA, USA). The results were analyzed on a Long-Read Tower (Bayer HealthCare) as described previously (31). The sequence obtained was compared with the p21 cDNA sequence to confirm that the correct clone had been constructed.

*Expression of p21 mutants.* The human colon cancer cell line, DLD-1, from the Health Science Research Resource Bank, Japan, was used for expression of the truncated p21 fragments. The cells were cultured in Dulbecco's modified Eagle's essential medium (DMEM) containing 10% fetal calf serum (FCS), at 37˚C in a humidified 5% $CO_2$ incubator. The cells were seeded at $1x10^6$ cells in 10-cm-diameter dishes, and passaged every third day. Exponentially growing cells ($2x10^7$) were harvested, washed with ice-cold phosphate-buffered saline (PBS), and then resuspended in 0.5 ml of DMEM without FCS on ice. The cell suspensions were transferred to electroporation cuvettes (Bio Rad, CA, USA) that were chilled on ice. Supercoiled plasmid DNA (150 μg for the transient expression, and 10 μg for the stable expression) was added to the cell suspension, and the mixture was incubated on ice for 5 min. Electroporation was performed at 250 V with a 1000 μF capacitor. After electroporation, the cuvette was placed on ice for 10 min. Transfected cells were diluted into 10 ml DMEM with 10% FCS, and cultured at 37˚C in a humidified 5% $CO_2$ incubator for 48 h.

To establish the stably-expressing cell lines, after 48-h incubation, 400 μg/ml of G418 was added to the medium for the selection. After 2 weeks of cultivation, colonies were isolated and the expression of site-directed p21 mutant in each clone was examined by Western blotting.

γ-irradiation was performed at room temperature using a Gammacell 3000 Elan (Nordion International, Canada) with a $^{137}Cs$ source emitting at a fixed dose-rate of 2.7 Gy $min^{-1}$.

The proteasome inhibitor, lactacystin (Kyowa Medex Co. Ltd., Tokyo, Japan) was dissolved in ethanol at a concentration of $1x10^{-3}$M.

*Western blot.* For Western blot analysis, a total of $2x10^7$ cells were harvested by trypsinization, washed with PBS and then lysed in 3 ml of $2x10^{-2}$ M sodium phosphate, $5x10^{-1}$ M sodium chloride, pH 7.8, 5 μg/ml leupeptin and 5 μg/ml aprotinin. The lysate was homogenized by sonication at 100 W for 5 sec on ice, centrifuged at 105,000 xg for 30 min, and the supernatant was collected. To the lysate, 1% sodium dodecyl sulfate (SDS) and 2.5% β-mercaptoethanol were added, and the mixture was heated for 5 min at 100˚C. The denatured samples were subjected to 10-20% SDS-polyacrylamide gel electrophoresis using a running buffer of $2.5x10^{-2}$ M Tris-HCl, pH 8.3, $1.92x10^{-1}$ M glycine and 0.1% SDS. The gels were electroblotted (5 $mA/cm^2$, 2 h) onto a polyvinylidene difluoride (PVDF) membrane (Bio Rad) in a transfer buffer of $2.5x10^{-2}$ M Tris-HCl, pH 8.3, $1.92x10^{-1}$ M glycine and 20% methanol. The membranes were incubated with 10% non-fat dry milk in $1x10^{-2}$ M Tris-HCl, pH 8.3, and $5x10^{-1}$ M NaCl (TBS) for 1 h at 25˚C, and then incubated with the first antibody diluted in TBS for 1 h at 25˚C. The blot was washed in TBS with 0.05% Tween 20 (TTBS) to remove unbound antibody and incubated with a biotinylated anti-rabbit IgG or biotinylated anti-mouse IgG in TBS for 1 h at room temperature. The membrane was again washed in TTBS and incubated

in streptavidin-biotinylated alkaline phosphatase complex. After washing thoroughly, the nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate was used to visualize the bands.

The primary antibodies were anti-p21 rabbit polyclonal antibody (1:200; H164, Santa Cruz Biotechnology, CA, USA), anti-CyclinA monoclonal antibody (1:1000; BF683, Upstate Biotechnology, NY, USA), anti-CDK2 polyclonal antibody (1:200; H298, Santa Cruz) and anti-β actin monoclonal antibody (1:1000; AC-15, Abcam Ltd., Cambridge, UK)

Metal affinity chromatography using TALON (BD Bioscience) was performed under native conditions, as described previously (31). Approximately 100 μl of a 50% slurry TALON was added to the lysate, and the mixture was rocked gently for 1 h at 4˚C. The resin was subjected to the following series of washes: twice with $2x10^{-2}$ M sodium phosphate and $5x10^{-1}$ M sodium chloride, pH 7.8, and twice with $2x10^{-2}$ M sodium phosphate and $5x10^{-1}$ M sodium chloride, pH 6.0. After washing the resin, the recombinant protein was eluted using $1.5x10^{-1}$ M imidazole, $2x10^{-2}$ M sodium phosphate and $5x10^{-1}$ M sodium chloride pH 6.0. The eluents were dialyzed against $5x10^{-3}$ M Tris-HCl pH 8.0, and vacuum concentrated.

Immunoprecipitation was performed as described previously (32). Anti-p21 monoclonal antibody (1:1000; 2G12, BD Bioscience) and Protein G Sepharose (GE Healthcare, CT, USA) were added to the lysate and the mixture was rocked gently for 1 h at 4˚C. In order to elute the precipitated samples, after washing the resin thoroughly, 1% SDS and 2.5% β-mercaptoethanol were added to the resin and the mixture was heated for 5 min at 100˚C.

*Immunohistochemistry.* The cells were cultured in Lab-Tek chamber slides (Nalige Nunc, IL, USA) and fixed with 4% paraformaldehyde. Cells were incubated with anti-p21 polyclonal antibody (1:100; H164) or pre-immune rabbit Ig for the negative control, and then incubated with a horseradish peroxidase conjugated goat anti-rabbit Ig to detect p21. The antibody reactivity was visualized with 3,3-diaminobenzidine as the chromogen.

*FACS analysis.* The cells were harvested, washed with PBS and then $1x10^6$ cells were resuspended in 1 ml of PBS with 1% Triton X-100 and 50 μg/ml propidium iodide. Fluorescence was analyzed on a flow cytometer using 488 nm excitation and a 600 nm bandpass filter.

*Determination of caspase 3 activities.* The caspase colorimetric protease assay kit (Sigma-Aldrich, MO, USA) was used for the determination of caspase 3 activity. The cells were cultured in DMEM containing 10% FCS at 37˚C in a humidified 5% $CO_2$ incubator. Each cell line was seeded at $1x10^6$ cells in two 10-cm-diameter dishes, and one dish was irradiated with 10 Gy γ-ray, and the other dish was not irradiated. After 24 h of irradiation, $2\text{-}5x10^6$ exponentially-growing cells were harvested and pelleted. The cells were resuspended in 50 μl of chilled cell lysis buffer, $5x10^{-2}$ M HEPES pH7.4, $5x10^{-3}$ M CHAPS and $5x10^{-3}$ M DTT, and incubated on ice for 10 min before centrifugation for 1 min in a microcentrifuge (10,000 xg). Supernatants (cytosolic extract) were transferred to fresh tubes and put on ice, and assayed for protein concentration. The protein concentration was adjusted with the cell lysis buffer, then 50 μl of sample were mixed with 50 μl of 2 x reaction buffer ($4x10^{-2}$ M HEPES pH7.4, $3x10^{-3}$ M CHAPS, $1x10^{-2}$ M DTT and $4x10^{-3}$ M EDTA) and 5 μl of the $4x10^{-3}$ M DEVD-pNA, and incubated at 37˚C for 24 h. After incubation, every sample was read at 405 nm in a microtiter plate reader.

## Results

*Expression of the non-phosphorylatable mutant and phosphorylation mimic mutant at Thr-145 or Ser-146.* To elucidate the function of cytoplasmic p21 and since phosphorylatable at Thr-145 or Ser-146 is known to determine cytoplasmic localization, we constructed the nonphosphorylative mutants, Thr-145 to Ala (T145A) and Ser-146 to Ala (S146A), and the phosphorylation mimic mutants, Thr-145 to Asp (T145D) and Ser-146 to Asp (S146D). The mutants, T145A/D and S146A/D, were cloned into the N-terminal Xpress-His tag vector, pcDNA3.1/ His, or the C-terminal Myc-His tag vector, pcDNA3.1/MycHis, and expressed in DLD-1 cells transiently. The exogenously expressed His tagged mutant p21s were purified from the cell lysate using metal affinity resin, and subjected to Western blot analysis using anti-p21 antibody. Every construct produced the expected sized products, while empty vector did not, thus the present expression system was judged to function properly (Figure 1A top).

Next, in order to assess whether the tagged mutant p21 works as a cyclin kinase inhibitor, we tested the capability of the tagged mutant p21 to bind to CyclinA or CDK2. Previously, we reported that the association of CyclinA or CDK2 with tagged full length p21 was induced after γ-irradiation (31), therefore we examined the binding of CyclinA or CDK with the mutant p21s.

Initially, we estimated the expression level of the mutants after 10 Gy γ-irradiation. The expression levels of tagged wild-type full length p21, Myc 164, as well as MycT145A, T145D, S146A or S146D, were not affected by γ-irradiation (Figure 1A top). Again the empty vector did not produce a detectable band (Figure 1A top). In this system, the expression of the mutant was driven by the CMV promoter, thus, the transcriptional activation of mutant p21 through p53 stabilization by irradiation was not observed.

The association of CyclinA or CDK2 to mutant p21 was examined by Western blot analysis of the metal affinity purified sample, which contained the tagged mutant p21 and its associated protein. The associated CyclinA or CDK2 increased after irradiation in Myc164, T145A, T145D, S146A and S146D (Figure 1A middle and bottom). The transfection of empty vector did not show p21, CyclinA or CDK2 bands, thus, the observed bands were considered to result from exogenously-expressed p21 (Figure 1A middle and bottom). Since the expression levels in every construct were not affected by 10 Gy γ-irradiation, the mutants were considered to have a binding competence similar to wild-type p21.

Recent studies reported that Thr-145 and/or Ser-146 are critical for PCNA binding, because phosphorylation of these residues inhibit p21-PCNA binding (25-27). In the expression system, N-terminal Xp tagged wild type p21 (Xp164), XpT145A and S146A bound PCNA, while the phosphorylation

mimic mutant of XpT145D, XpS146D did not bind PCNA (Figure 1B). In addition, the transfection of the empty vector did not show the PCNA band. Therefore, phosphorylation at these residues inhibited the binding of PCNA.

We obtained similar results in the binding assay of CyclinA, CDK2 and PCNA by using C-terminal Myc-tagged and N-terminal Xpress-tagged mutants.

*Stability of T/S to A or T/S to D mutants.* The p21 protein is a short-lived protein that is degraded by the ubiquitin-proteasome protein degradation system in the nucleus. To verify that the exogenously-expressed p21 mutants were stably expressed, 2 μM lactacystin, a proteasome inhibitor, was added to the medium at 24 h after transfection and incubated for 24 h. The expressions of the mutant p21s were compared by Western blot analysis using anti-p21 antibody (Figure 2 top). The optical density of the bands, which were used to indicate expression levels, were indicated as the ratio of p21 to β-actin (Figure 2 bottom). An increase in the optical density with the addition of lactacystin implies that the exogenously-expressed mutant p21 is degraded with proteasome. The expression of wild-type p21 was increased with lactacystin by 82%. Alanine mutants, T145A and S146A, were increased by 80% and 64%, respectively. However, the increase of Asparate mutants was slight, with T145D increasing 25% and S146D increasing 24% (Figure 2).

*T145A/D or S146A/D stable transformants.* To assess the function of Thr-145 and Ser-146, C-terminal Myc-tagged T145A, T145D, S146A and S146D were transfected into DLD-1, and clones stably expressing these mutants were isolated (DLD-T145A, DLD-T145D, DLD-S146A and DLD-S146D).

The expression of mutant p21s in the stable transformants was analyzed by Western blot and immunohistochemistry. To determine whether the mutant p21s were degraded by proteasome, the effect of a proteasome inhibitor, lactacystin, on the expression levels was examined. Before harvesting, cells were incubated with 2 μM lactacystin for 24 h. If the lactacystin treatment increased the expression of a mutant p21, then that mutant was considered to be degraded by proteasome. Expression was examined by Western blotting using whole cell lysates, and the expression levels were indicated as the ratio of p21 to β-actin (Figure 3A). The expression of the mutant p21 was enhanced by lactacystin treatment in DLD-T145A and DLD-S146A, while the treatment with lactacystin did not affect the expression level in DLD-T145D and DLD-S146D (Figure 3A). To confirm that the faint band in T145D was the p21 mutant, DLD-T145D cell lysate was immunoprecipitated with anti-p21 monoclonal antibody (2G12) and probed with anti-p21 polyclonal antibody. A band similar in size to the cell lysate was detected, therefore, the faint band observed in the whole cell lysate was T145D (Figure 3A right).

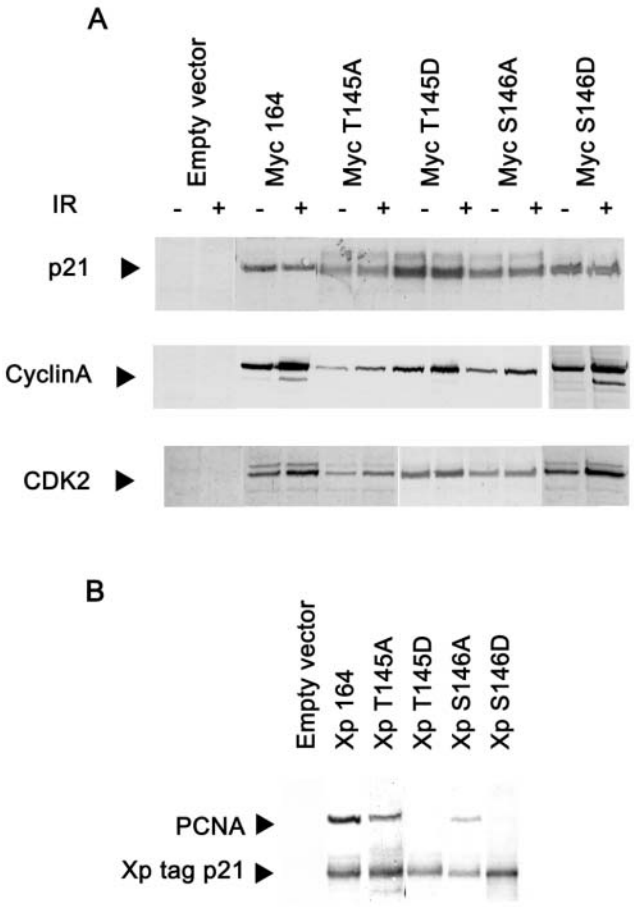



Figure 1. *CyclinA , CDK2 or PCNA associated with the site-directed mutants. CyclinA and CDK2 binding by the C-terminal Myc-His-tagged mutants (A). PCNA binding by the N-terminal Xpress-His-tagged mutants (B). For transient expression, 150 μg of supercoiled DNA was electroporated into exponentially-growing 2x10$^7$ DLD-1 cells. After 24-h cultivation, the cells were γ-irradiated for 10 Gy, and then incubated for 24 h. The cells were lysed in native conditions, and the recombinant proteins were purified by metal affinity chromatography using TALON, as described in Materials and Methods. Concentrated purified protein was subjected to SDS-PAGE and Western blot analysis using anti-p21, anti-CyclinA, anti-CDK2 or anti-PCNA antibodies.*

Immunohistochemical analysis determined the cellular localization of the mutant p21s, with or without lactacystin treatment. The distinct nuclear localization was observed in DLD-T145A and DLD-S146A and the nuclear localization was enhanced by lactacystin treatment (Figure 3B). In DLD-T145D and DLD-S146D, the mutant p21s were detected in the nucleus and cytoplasm and were not affected by lactacystin (Figure 3B). The responses to lactacystin observed by immunohistochemistry were consistent with that observed using Westernblot analysis.

*DNA histogram after γ-irradiation of stably-mutant-expressing cell lines.* In order to analyze the effect of phosphorylation at Thr-145 or Ser-146 on the mechanism of apoptosis, DLD-T145A, DLD-T145D, DLD-S146A and DLD-S146D were exposed to γ-irradiation and their apoptotic responses were estimated by DNA histograms. The exponentially-growing cells were γ-irradiated at a dose of 10 Gy or 30 Gy, then cultivated for 12, 24, 36 or 40 h. Cells were harvested at each time-point and stained with propidium iodide, followed by flow-cytometric analysis to obtain the DNA histogram. Gate was set for the region representing single nuclei on the scattergram based on forward scatter and side scatter of control cells (Figure 4A). The DNA content was measured for a total of 10,000 events per sample. The DNA histogram of the parent DLD-1 cells was not affected at 12, 24 or 36 h, while an apoptotic peak, sub2n peak in the DNA histogram, appeared after 40-h cultivation. Therefore, we compared the DNA histograms of mutant-expressing cell lines at 40 h. In every cell line, γ-irradiation resulted in an accumulation into the G2M region containing 4n DNA (Figure 4B). High sub2n peaks were clearly seen in the parent DLD-1 (40.0% at 10 Gy, 33.0% at 30 Gy), empty vector transfected DLD-1 (33.1% at 10 Gy, 28.2% at 30 Gy), DLD-T145A (64.0% at 10 Gy, 56.0% at 30 Gy), and DLD-S146A (26.5% at 10 Gy, 27.3% at 30 Gy). The sub2n peaks were much smaller (less than 6%) in DLD-T145D and DLD-S146D.

*Caspase 3 activity.* In order to clarify the point of inhibition, the caspase 3 activity, an apoptosis execution enzyme was assayed, in the mutant-p21-expressing cells before and after γ-irradiation. Cells were harvested at 24 h after 10 Gy γ-irradiation, and assayed for the caspase 3 activity of the cell lysate. Caspase 3 activities in each mutant-p21-expressing cell line was increased by γ-irradiation to the same extent as the parent cell line (Figure 5). Therefore, the apoptosis-inhibiting activity of T145D or S146D was mediated by caspase 3 itself or its downstream components.

## Discussion

In this study, we constructed non-phosphorylatable mutants, Thr-145 to Ala and Ser-146 to Ala, or phosphorylation mimic mutants, Thr-145 to Asp and Ser-146 to Asp, to analyze the significance of Thr-145 and Ser-146 on the p21 function in apoptosis. In addition, cell lines were established that stably-expressed these mutants. Apoptosis induced by γ-irradiation was delayed in cells expressing T145D and S146D, and the expression of T145D and S146D was localized to the cytoplasm. Since the activity of caspase 3 was not reduced in the mutant-expressing cells, a cytoplasmic PCNA-unbound form of p21 may interact with the activated caspase 3 or its downstream components.

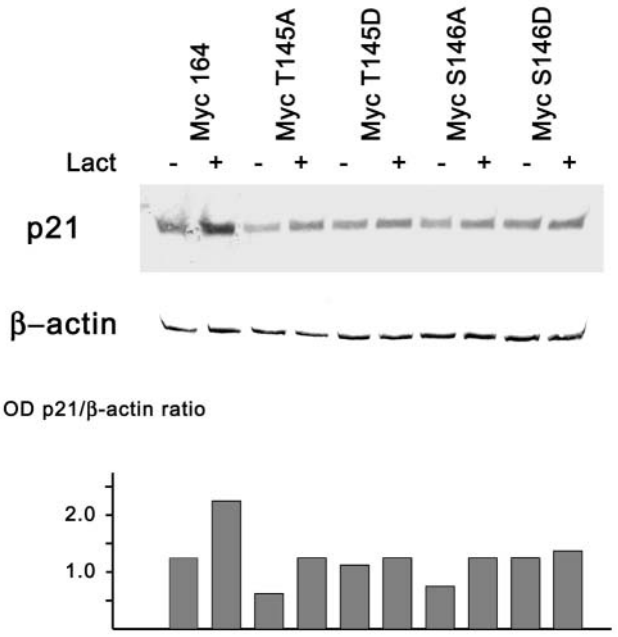
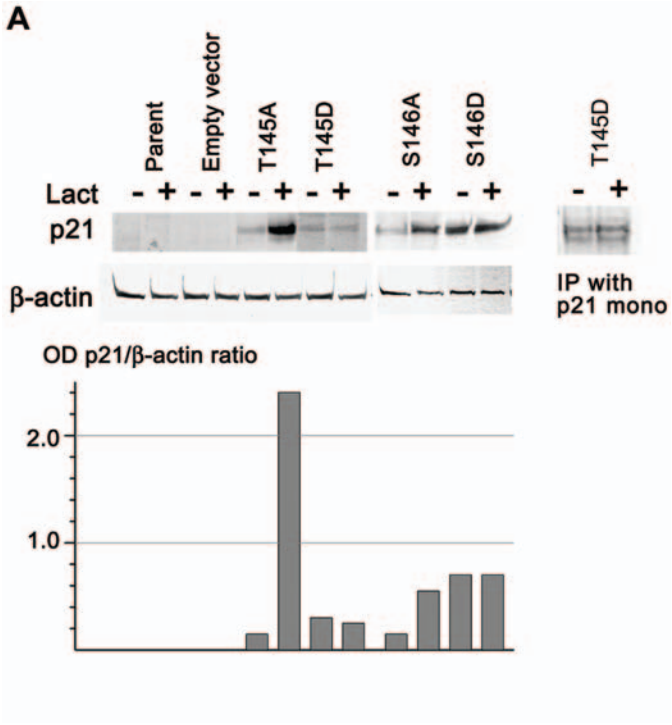



Figure 2. *Effect of lactacystin on the expression of mutant p21. Supercoiled DNA (150 μg) was electroporated into exponentially-growing 2x10$^7$ DLD-1 cells. After 24-h cultivation, 2 μM lactacystin was added to the medium, which was incubated for 24 h. The cells were lysed in the native conditions, and were subjected to SDS-PAGE and Western blot analysis using anti-p21 or anti-β-actin. The ratios of band intensities as optical density (OD) of p21 to β-actin are indicated as a graph.*

In the transient expression system, the association of mutants, T145A, T145D, S146A and S146D, with CyclinA or CDK2 was increased after γ-irradiation, as observed in wild-type p21, confirming that these mutant p21s were functional. However, binding with PCNA was detected in wild-type p21, T145A and S146A, but not in T145D and S146D. This result agreed with a report that phosphorylation at Thr-145 or Ser-146 inhibited PCNA binding (26). Treatment with lactacystin, a proteasome inhibitor, induced the expression of T145A and S146A, but not T145D and S146D, which suggests that T145A and S146A are degraded by proteasome.

Using the stable mutant-expressing cells, we showed that T145A and S146A localized to the nucleus and were induced by lactacystin, while T145D and S146D localized to the cytoplasm and were not affected by lactacystin. Those results are consistent with that obtained in the transient expression system. While the stably-expressing cells were cloned cell lines, there positively- and negatively-stained cells were present. To obtain the stably-expressing cell line, we repeated the cloning procedure and selected highly-expressing sub-clones by Western blotting. However, we were not able to obtain cell lines in which every cell was positive by immunohistochemistry. In the case of DLD-



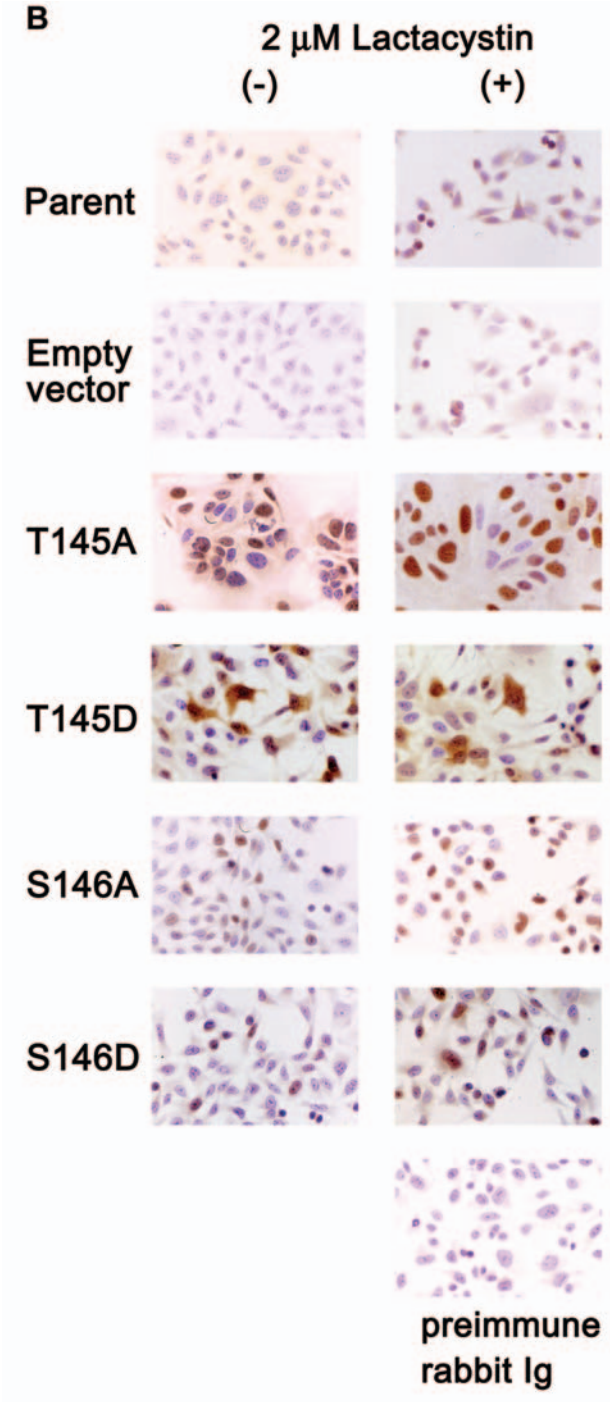




Figure 3. *Establishment of cell lines stably expressing mutant p21. (A). Expression of mutant p21s in stably-expressing cells, with or without lactacystin, were confirmed by Western blot analysis. For lactacystin treatment, cells were incubated with 2 μM lactacystin for 24 h before harvest. The ratios of band intensities as optical density (OD) of p21 to β-actin are indicated as a graph. To confirm the faint band in T145D-expressing cells, the lysate was immunoprecipitated with anti-p21 monoclonal antibody (2G12) followed by Western blot analysis probed with anti-p21 polyclonal antibody, as indicated (right). (B). Localization of the mutant p21, with or without the treatment of lactacystin, was determined by immunohistochemistry, using anti-p21 polyclonal antibody. The antibody reactivity was visualized with 3,3'-diaminobenzidine as the chromogen. The nuclei were stained with hematoxylin. For the negative control, preimmune rabbit Ig was used as a primary antibody.*

T145A and DLD-S146A, the expression of mutant p21 was induced by lactacystin, suggesting that the expressed mutant p21 was degraded properly in these cells. In the case of DLD-T145D and DLD-S146D, the level of degradation by other than proteasome may vary in each cell. Therefore, the existence of positive and negative cells were thought to be dependent on the level of degradation in each cell.

Our immunohistochemical results agreed with findings that the localization of p21 is regulated by the status of Thr-145 and Ser-146 (23, 24, 27). Induction of nuclear T145A and S146A with lactacystin is consistent with the report that p21 is degraded by proteasome in the nucleus (33). Consequently, the cytoplasmic localization made p21 resistant to proteasomal degradation and may allow interaction with elements of the apoptosis mechanisms other than the inhibition of cyclins-CDKs.

It has been reported that cytoplasmic p21 binds to and inhibits ASK1, an apoptosis signal-regulating kinase 1 (19, 20). However, apoptosis is not reduced by p21 with a C-terminal region deleted by cleavage with caspase 3 at Asp-112 (21, 22). In our recent study, the progress of

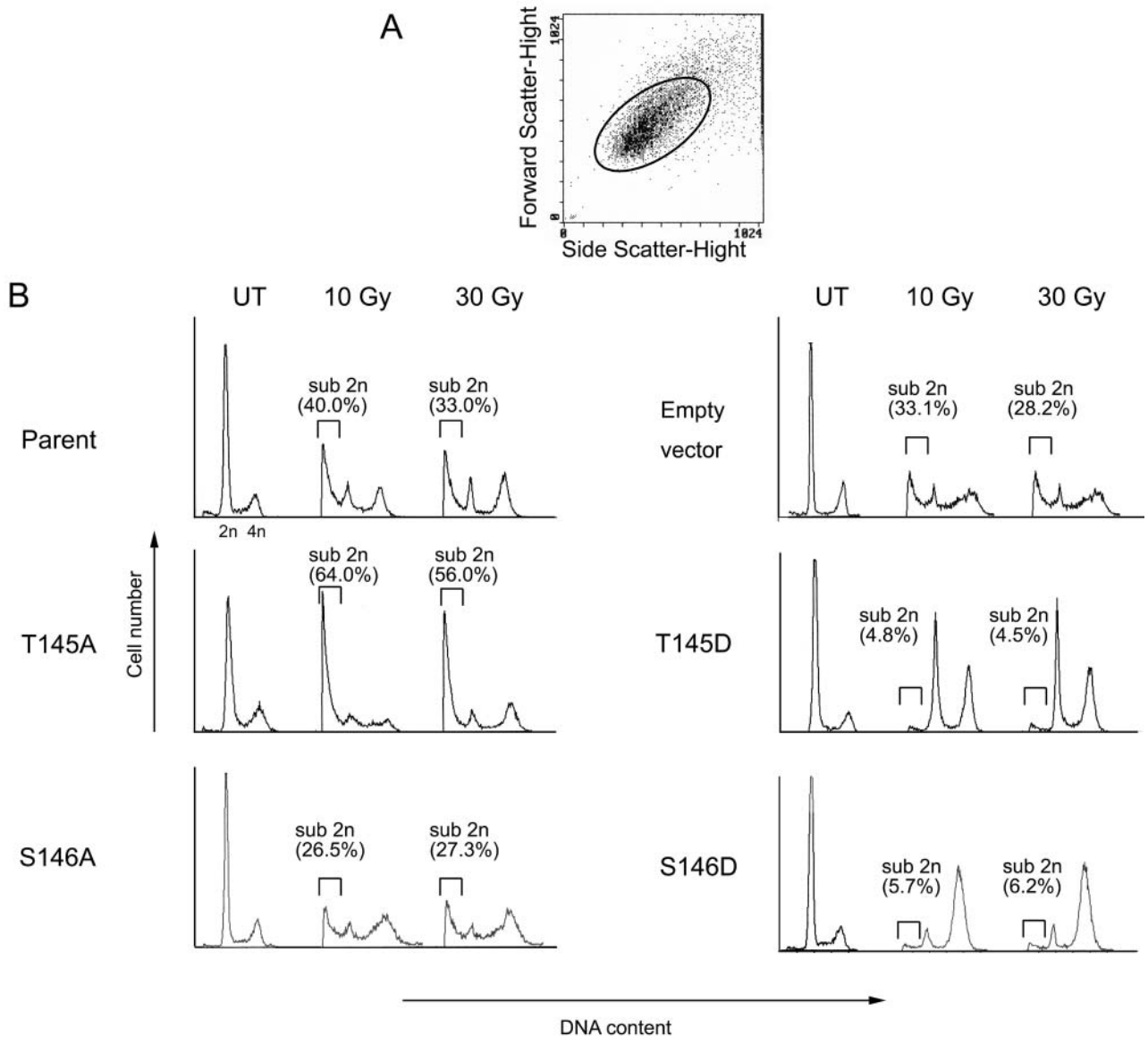



Figure 4. *DNA histogram of γ-irradiated cells stably expressing mutant p21. Exponentially-growing cells were γ-irradiated for 10 or 30 Gy. After 40-h cultivation, the cells were harvested and stained with propidium iodide then analyzed by flow cytometry. (A). Gate was set for the region representing single nuclei on the scattergram based on forward scatter and side scatter of control cells. (B). DNA content was measured for a total of 10,000 events per sample. The percentage of sub 2n region is indicated.*

γ-irradiation-induced apoptosis was not inhibited in HeLa cells that stably expressed C-terminal NLS deletion mutants, p21 1-128 or p21 1-147 (34). In contrast, p21 1-157, in which one NLS at the very end of the C-terminal was removed, inhibited γ-irradiation-induced apoptosis (34). Taken together, p21 1-157 or full length cytoplasmic p21 can inhibit the progress of apoptosis, but C-terminal-deleted fragments shorter than 157 bases have no inhibitory effect on apoptosis. In the present study, the site-directed mutants of p21, T145D and S146D, had intact C-terminal regions and inhibited apoptosis, suggesting that the C-terminal of p21 is required for the inhibition of apoptosis. Further, the anti-apoptotic function of cytoplasmic p21 is consistent (a) with the activation of AKT/PKB kinase by extracellular growth signal phosphorylated Thr-145 and/or Ser-146 resulting in cytoplasmic localization and stabilization (23, 27), and (b) with the cytoplasmic existence of p21 during monocytic or neural differentiation (19, 35).

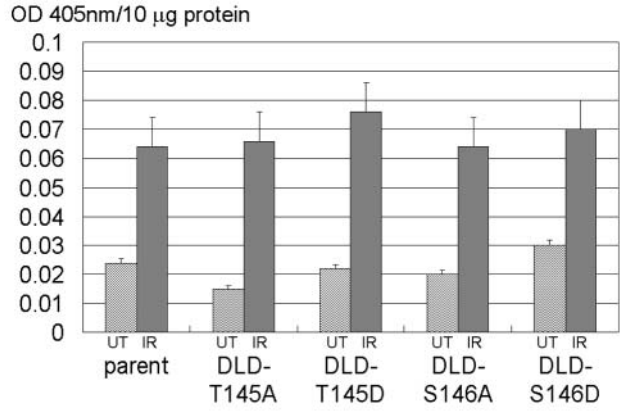



Figure 5. *Caspase 3 activity after 10 Gy γ-irradiation. Twenty-four h after irradiation, exponentially-growing cells were harvested, and 2-5x10$^6$ cells were pelleted. Cells were lysed and equal amounts of cytosolic extract were incubated with caspase 3 substrate, DEVD-pNA, at 37˚C for 24 h. After incubation, every sample was read at 405 nm in a microtiter plate reader. OD readings per 10 μg protein were plotted. Reproducible results are presented in the graph with error bars.*

In our experimental design, T145D and S146D lost the competence to associate with PCNA. The inhibitory effect on apoptosis is related to the PCNA-unbound form of p21.

Association and inhibition of caspase 3 by p21 has been implicated in Fas-mediated apoptosis (36). The N-terminal 15-33 aa of p21 is associated with pro-caspase 3 and suppresses its activation by masking the Serine protease-cleavage site (36).

In the present study, caspase 3 activity was not inhibited in the T145D- and S146D-expressing cells. Therefore, we conclude that the PCNA-unbound form of full length p21 in the cytoplasm inhibits apoptosis by interacting with the downstream components of caspase 3 or the active form of caspase 3.

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