Involvement of a p53-independent and post-transcriptional up-regulation for p21$^{WAF/CIP1}$ following destabilization of the actin cytoskeleton

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Abstract. The tumor suppressor p21$^{WAF/CIP1}$ mediates the proliferation arrest via p53-dependent or -independent gene transactivation following distinct environmental stresses. In this study, we show that direct destabilization of the actin cytoskeleton by actin-targeting reagents leads to a p53-independent up-regulation of p21$^{WAF/CIP1}$. The actin-targeting agent cytochalasin B (10 μM) quickly disrupted the actin cytoskeleton of p53 wild-type and p53-null cells accompanied by up-regulation of p21$^{WAF/CIP1}$. Nevertheless, both total p53 and ser-15 phosphorylated p53 were not accumulated concomitantly, compared to the effect caused by ionizing irradiation. P53-independent up-regulation of p21$^{WAF/CIP1}$ was also observed by two other actin-targeting agents cytochalasin D and latrunculin B, but not by the microtubule inhibitor colcemid. Furthermore, we showed that p21$^{WAF/CIP1}$ mRNA level was not increased, whereas the protein degradation was delayed. A reduction of ubiquitination for p21$^{WAF/CIP1}$ protein was detected using immunoprecipitation/immunoblot analysis. Up-regulation of p21$^{WAF/CIP1}$ was not associated with cytotoxicity induced by cytochalasin B that influenced DNA integrity and plating efficiency only after 24 h of treatment. In addition, up-regulated p21$^{WAF/CIP1}$ was accompanied by reduction of phosphorylation on retinoblastoma (Rb) protein in p53-null cells, implying that p21$^{WAF/CIP1}$ might in part account for the molecular regulation of cytochalasin B induced G1 phase arrest. Together, current results suggest that p21$^{WAF/CIP1}$ level can be mediated by actin organization in the absence of p53 via a post-transcriptional machinery, and it may contribute to the growth ablation by agents targeting the actin cytoskeleton.

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Introduction

P21$^{WAF/CIP1}$ is one of the cell cycle regulators that can bind to cyclin/cyclin-dependent kinase complexes and inhibit their activities in response to DNA damage, anti-mitogenic or differentiating signals. These effects can be mediated through p53-dependent or -independent machinery according to the types of stimuli (1-6). There are two p53-binding elements locating at the p21$^{WAF/CIP1}$ gene promoter that can be transactivated by the nuclear accumulated p53 after DNA damage (7). On the other hand, the underlying mechanisms for p53-independent induction of p21$^{WAF/CIP1}$ are diverse. For instance, differentiating reagents such as butyrate, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and trans-retinoic acid can elevate p21$^{WAF/CIP1}$ mRNA expression in various human cell lines that harbor no or mutant p53 (8-10). Besides, the mitogenic growth factors, serum, carbon tetrachloride or oxidative stress can stimulate p21$^{WAF/CIP1}$ gene expression in the absence of functional p53 (3-6,8). The signal transduction via Ras/Raf/MEK/ERK pathway or activation of protein kinase C may be involved in transactivation of p21$^{WAF/CIP1}$ gene without p53 activation (11-13).

Post-transcriptional regulation of p21$^{WAF/CIP1}$ protein is another important mechanism for determining the p21$^{WAF/CIP1}$ level in cells. P21$^{WAF/CIP1}$ is a short-lived protein that can be degraded via the ubiquitin-dependent or -independent proteasome pathway (14-17). Suppression of proteolysis may also lead to accumulation of p21$^{WAF/CIP1}$ in cells. However, which type of environmental stimuli can elevate p21$^{WAF/CIP1}$ level via post-transcriptional regulation is poorly understood. In addition, no evidence currently shows that p53 is associated with post-transcriptional regulation of p21$^{WAF/CIP1}$.

Although $p21^{WAF/CIP1}$ gene is rarely mutant in human tumors, inefficient expression of $p21^{WAF/CIP1}$ has been found in several types of cancers (18-23). In mouse models, $p21^{WAF/CIP1}$ nullizygosity has been reported to increase the incidence of tumorigenesis and metastasis, suggesting that a normal $p21^{WAF/CIP1}$ expression is important for preventing malignancy (24,25). It has been reported that Ras oncoprotein induced $p21^{WAF/CIP1}$ gene expression is suppressed by Rho GTPase protein via transcriptional and post-transcriptional pathways, and inhibition of Rho activity can up-regulate $p21^{WAF/CIP1}$ level thereby promoting cell cycle progression (26,27). Since Rho family GTPase are mainly responsible for actin cytoskeletal assembly in normal and transformed cells (28), the integrity of actin cytoskeleton may be also important for regulation of $p21^{WAF/CIP1}$ levels for cell growth. Nevertheless, such a hypothesis remains to be addressed.

In this study, we investigated whether $p21^{WAF/CIP1}$ expression could be affected by direct destabilization of the actin cytoskeleton. For this purpose, we exposed cultured human cancer cells to actin-targeting reagents and showed that $p21^{WAF/CIP1}$ protein level was up-regulated independent of p53. A delay of protein degradation rate rather than increase of gene transcripts might account for the accumulation of $p21^{WAF/CIP1}$ protein, by which the protein ubiquitination was reduced. Furthermore, up-regulated $p21^{WAF/CIP1}$ in p53-null cells was also in part associated with G1 phase arrest caused by destabilization of actin cytoskeleton. It implies that targeting on the actin cytoskeleton may be important for the development of new therapeutic strategies when treating tumors with mutant or deleted p53 genes.

Materials and methods

Cell culture. Human non-small lung adenocarcinoma A549 cells and H1299 cells, and human osteosarcoma MG63 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamate, 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen Inc., Carlsbad, CA). The stable H1299/p53 cells that can constitutively over-express wild-type p53 was maintained in DMEM containing 10% FBS and 300 μg/ml G418 as described previously (29). The pH of medium was adjusted to 7.0-7.2 by sodium bicarbonate. The cells were maintained in a 37˚C, humidified incubator (5% CO_2 and 95% air) and routinely passaged every two days.

Reagents. Cytochalasin B, cytochalasin D and cycloheximide were purchased from Sigma-Aldrich (St. Louis, MO). Latrunculin B was obtained from Calbiochem (La Jolla, CA). The reagents were dissolved in dimethyl sulfoxide (DMSO) to obtain concentrations of 100 mM (for cytochalasin B) and 10 mM (for cytochalasin D and latrunculin B) in the stock solutions. The working concentration of each actin inhibitor is indicated in the figure legends. The controls were cells treated with 0.01% DMSO. Colcemid solution was purchased from Invitrogen. Cycloheximide was prepared as a 10 mg/ml stock solution in DMSO.

Actin staining. Cells were grown on 2-well chamber slides (Nalge Nunc International Corp., Naperville, IL) for 48 h, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature and then permeabilized with 0.5% Triton X-100 for 5 min. Cells were then stained with 0.2-0.3 μg/ml of fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma Chemical Co., St. Louis, MO) for 15 min and rinsed with PBS at least 4 times. The slide was washed, mounted using 2% anti-fade reagent DABCO (Kodak, Rochester, NY), sealed and examined by confocal fluorescence microscopy (Leica Microsystems Inc, Bannockburn, IL).

Western blot analysis. Cells were lyzed in NP-40 lysis buffer (50 mM Tris-HCl; 120 mM NaCl and 0.5% NP-40) containing 20 μg/ml phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). The crude protein lysates were collected by centrifugation and the protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). Crude proteins lysates (50-100 μg) were separated on 10% or 15% SDS-polyacrylamide gel by electrophoresis. The fractionated proteins were electro-transferred to nitrocellulose membrane, blocked with TBST buffer (0.8% NaCl, w/v; 0.02% KCl, w/v; 25 mM Tris-HCl and 0.1% Tween-20, v/v) plus 4% skim-milk for 1 h and then incubated with primary antibody at 4˚C overnight. The membrane was washed with TBST buffer and re-incubated with horseradish phosphatase (HRP)-conjugated secondary antibody. Protein signals were detected using ECL™ detection reagents (Amersham Bioscience, Buckinghamshire, UK). The primary antibodies used in this study included anti-$p21^{WAF/CIP1}$ (BD Pharmingen, San Diego, CA), anti-p53 (Calbiochem, San Diego, CA), anti-phospho-p53 (ser15-specific) (Cell Signaling Technology, Beverly, MA) and anti-ß-actin (Sigma-Aldrich). The intensity of protein bands was measured by densitometrically using NIH Image for Windows (Beta version 4.02, Scion Corporation, Frederick, MD).

Radiation source. The γ-rays were delivered by a Cesium-137 source at a dose rate of 385 cGy/min. Cells were exposed to a single dose of 6 Gy.

Determination of $p21^{WAF/CIP1}$ mRNA expression. Total RNA was prepared according to the manufacturer's manual of TRIzol reagent (Invitrogen). Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) was followed by the C. therm. Polymerase One-Step RT-PCR system (Roche Inc., Mannheim, Germany) to determine the level of $p21^{WAF/CIP1}$ mRNA. The sequences of primer set included forward primer: 5'-ATGTCAGAACCGGCTGGGGA-3', and reverse primer: 5'-CTAGGGCTTCCTCTTGGAGA-3'. For quantitative real-time PCR (qPCR), 5 μg of total RNA was used for obtaining the first strand cDNA by SuperScript II™ reverser transcriptase (Invitrogen). Subsequently, the cDNA products were mixed with SYBR® green PCR Master Mix and the primer mixture for reaction using 7000 Sequence Detection System (Applied Biosystems, Warrington, UK). Primers used for qPCR were 5'-GGACCTGGAGACTCTCA GGG-3' and 5'-CAGGGGACAGCAGAGGAAGAC-3'. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was carried out simultaneously and used as the internal control. The data were collected and normalized

to determine the level change of $p21^{WAF/CIP1}$ mRNA. Each data point is representative of the duplicate experiments.

Plasmids and reporter gene assay. The WWP-Luc reporter plasmid contains a 2.4-kb including the human wild-type $p21^{WAF/CIP1}$ gene promoter, which is fused with a firefly luciferase reporter gene (7). For reporter gene assay, cells were transiently transfected with the plasmids using Superfectin® (Qiagen, Inc., Valencia, CA) according to the manufacturer's manual. In brief, 2 μg of the reporter plasmid and 10 ng of the pRL-SV40 plasmid (Promega Inc., Madison, WI) were mixed with Superfectin and serum-free DMEM for 10 min. The formed DNA-Superfectin complexes were then mixed with 0.6 ml serum-containing DMEM and poured over a monolayer cell culture that have been seeded and grown overnight. The transfection reaction were carried for 3 h at 37˚C and then stopped by replacing the mixture with complete DMEM. After transfection, the cell extracts were harvested with passive lysis buffer and the luciferase activity was analyzed using the dual-luciferase reporter gene assay system (Promega). The pRL-SV40 plasmid that constitutively expressed Renilla luciferase activity was used as internal control. The emitted light intensity was measured using an Optocomp® I luminometer (MGM Instruments, Inc., Hamden, CT).

Detection of ubiquitination on $p21^{WAF/CIP1}$. $P21^{WAF/CIP1}$ ubiquitination was determined as the procedure reported previously (30). In brief, the monolayer was lyzed in NP-40 lysis buffer containing 2% protease inhibitor cocktail set III (Calbiochem). Cell extract (500 μg) was incubated with anti-$p21^{WAF/CIP1}$ antibodies (Santa-Cruz Inc., Santa-Cruz CA) for 2 h at 4˚C with gentle shaking and then mixed with Protein-A/G agarose (Santa-Cruz) overnight. The formed immune complexes were then washed using NP-40 lysis buffer four times and the pellets were collected by centrifugation. The washed immune complexes were added to 2X sample buffer and separated on SDS-PAGE. Western blot analysis was subsequently adopted to detect the level of p21-conjugated ubiquitins using anti-ubiquitin antibody (P4D1) (Cell Signaling Technology, Beverly, MA).

Measurement of plating efficiency. Exponentially growing monolayer cell cultures were treated with 10 μM cytochalasin B at the time-points described in the results. The cells were then trypsinized and seeded in 60-mm dishes (25, 50 and 100 cells per dish) for colony formation assays. After incubation for 14 days, the colonies (>50 cells) on the dishes were visualized by staining with 1x crystal violet (1.25 g in 1 l 75% ethanol, w/v; Sigma-Aldrich). The plating efficiency was determined as the ratio of the number of formed colonies divided by the number of seeded cells multiples 100.

DNA degradation assay. Cells were treated with cytochalasin B for 0, 6 and 12 h and then harvested by trypsinization. Collected cells were lyzed with lysis buffer (0.5% Sarkosyl; 50 mM Tris-HCl/pH 8.0; 10 mM EDTA, 1 mg/ml proteinase K) at 55˚C for 24 h. The cell lysates were then treated with 500 μg/ml RNase A and kept at room temperature for additional hour. After extraction with phenol/chloroform, the samples were mixed with running buffer and loaded onto a 1.5% agarose gel for separation by electrophoresis. The DNA was stained with 10 μg/ml ethidium bromide and visualized by UV illumination.

Flow cytometric analysis of cell cycle. The cells after various treatments were trypsinized and fixed in 75% ethanol ($1x10^6$ cells/3 ml) at 4˚C overnight. The fixed cells were centrifuged and the pellet was treated with 1 ml RNase A (1 mg/ml) at room temperature for 30 min. Cells were then spun down, treated with 1 ml of propidium iodide (Sigma-Aldrich) and transferred to 5 ml polypropylene round-bottom tubes (Becton-Dickinson Labware, Franklin Lakes, NJ) through a 37 μM mesh. The DNA histogram was created using a flow cytometer (EPICS Elite ESP, Coulter Electronics) and the cell cycle distribution was determined using MCYCLE software (version 3.01, Phoenix Flow System, San Diego, CA).

Statistical analysis. Independent experiments were conducted for comparison of the statistical difference between the control groups and experimental groups. Statistical differences were determined by the Student's t-test. The significantly different results were defined by $p<0.05$.

Results

D*estabilization of the actin cytoskeleton by cytochalasin B leads to $p21^{WAF/CIP1}$ up-regulation prior to p53 activation*. $P21^{WAF/CIP1}$ is a cell cycle regulator that is up-regulated via the p53-dependent or -independent pathway under various environmental stresses. To investigate whether the expression of $p21^{WAF/CIP1}$ is associated with the p53 state after destabilization of actin cytoskeleton, we exposed the p53 wild-type human A549 epithelial cells to the actin inhibitor cytochalasin B. Destabilization of actin cytoskeleton was visualized as loss of the cortical stress fibers and the pericellular microspikes, while the punctuated actin foci were formed in the presence of 10 μM cytochalasin B for 1 h (Fig. 1A). Such an effect was sustained during the treatment (data not shown). We next examined the expression of $p21^{WAF/CIP1}$ protein, and the result showed that the $p21^{WAF/CIP1}$ protein level was significantly up-regulated, whereas the levels of total and ser-15 phosphorylated p53 were not affected up to 4 h of cytochalasin B treatment (Fig. 1B). In contrast, exposure of A549 cells to γ-rays led to concomitantly up-regulation of $p21^{WAF/CIP1}$ and p53, indicated that the p53-$p21^{WAF/CIP1}$ regulatory pathway remained functional (Fig. 1B). These results suggest that destabilization of actin cytoskeleton can up-regulate $p21^{WAF/CIP1}$ without activation of p53.

Destabilization of actin cytoskeleton can up-regulate $p21^{WAF/CIP1}$ in p53-null cell lines. To further demonstrate that destabilization of actin cytoskeleton can induce $p21^{WAF/CIP1}$ expression through a p53-independent pathway, we exposed p53-null cell lines human H1299 non-small lung carcinoma cells to cytochalasin B. H1299 cells expressing exogenous wild-type p53 cDNA (H1299/p53) was used as a positive control to demonstrate that $p21^{WAF/CIP1}$ can be induced by p53

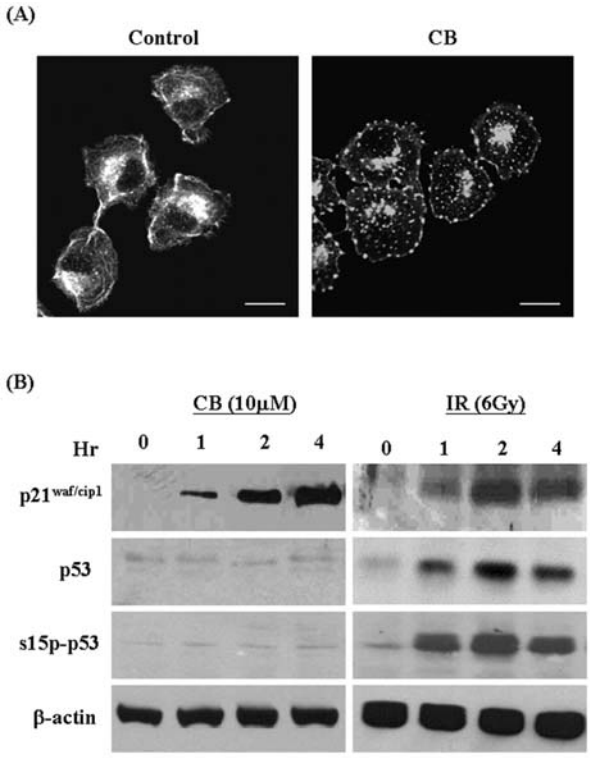


Figure 1. Destabilization of actin cytoskeleton induced p21$^{WAF/CIP1}$ up-regulation without accumulation of p53. (A) Actin staining demonstrated that actin cytoskeleton was disrupted in A549 cells using actin inhibitor cytochalasin B for 1-h treatment. Bar, 40 μm; (B) Both cytochalasin B and ionizing radiation could up-regulate p21$^{WAF/CIP1}$, whereas p53 states were not affected by the former treatment compared to the latter one. The source of ionizing radiation is described in Materials and methods. The results are a representative of duplicate experiments.

over-expression in this cell line. Compared to the untreated controls, significant up-regulation of p21$^{WAF/CIP1}$ was detected after 4 h of cytochalasin B, and the levels were sustained thereafter (Fig. 2A). No p53 expression was detectable in H1299 cells, and p21$^{WAF/CIP1}$ gene transcription could not be induced by γ-ray exposure. Moreover, the same result was obtained by exposing other p53-null MG63 human osteosarcoma cells to cytochalasin B (Fig. 2B).

To test if other actin inhibitors can also up-regulate p21$^{WAF/CIP1}$ via a p53-independent pathway, we exposed H1299 cells to cytochalasin D and latrunculin B. The former can bind to the fast growing ends of actin microfilaments as cytochalasin B, and the latter is able to sequester monomeric actins to block the actin polymerization. The results showed that both cytochalasin D and latrunculin B were able to up-regulate p21$^{WAF/CIP1}$ of H1299 cells after 4 h of treatment (Fig. 2C). To test if microtubule, another dynamic cytoskeleton, can affect p21$^{WAF/CIP1}$ expression, we used the microtubule inhibitor colcemid to treat H1299 cells. The result showed that p21$^{WAF/CIP1}$ level was not significantly influenced by colcemid (Fig. 2D). These results suggest that the actin cytoskeleton is important for regulation of p21$^{WAF/CIP1}$ expression regardless of p53 status.

p21$^{WAF/CIP1}$ transcripts was not up-regulated after destabilization of actin cytoskeleton. P53 is known to transactivate p21$^{WAF/CIP1}$ gene after DNA damage. Since destabilization of actin cytoskeleton mediated up-regulation of p21$^{WAF/CIP1}$ was not dependent on p53 state, we asked whether gene transcription was still associated with such a phenomenon. For this purpose, we detected the p21$^{WAF/CIP1}$ mRNA levels in

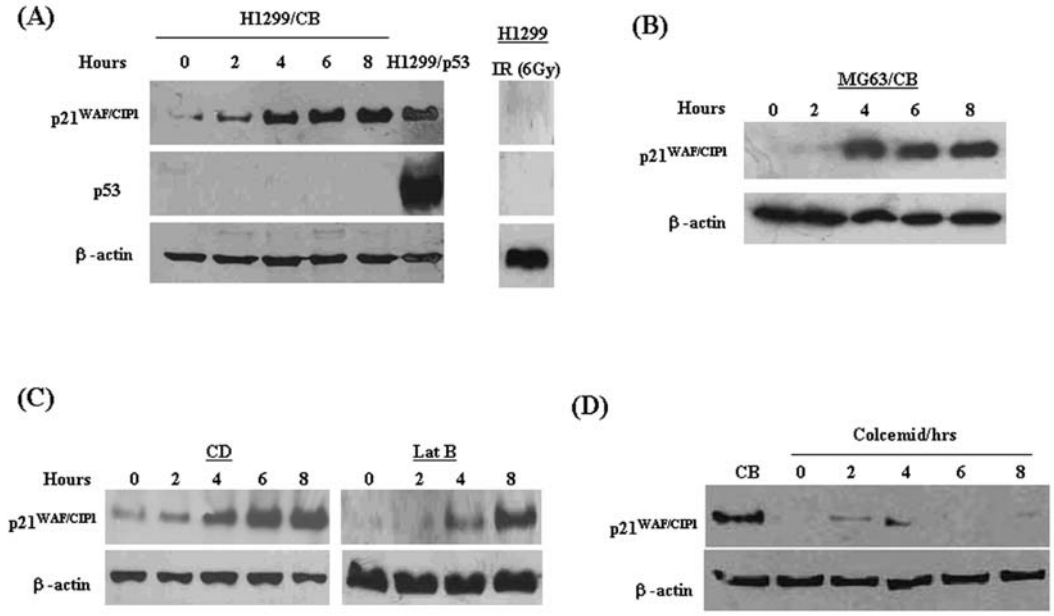


Figure 2. Up-regulation of p21$^{WAF/CIP1}$ in p53-null cells after destabilization of actin cytoskeleton. (A) H1299 cells were exposed to 10 μM cytochalasin B for the time-points as indicated and p21$^{WAF/CIP1}$ levels were then determined by Western blot analysis. P53-dependent up-regulation of p21$^{WAF/CIP1}$ in H1299 cells was demonstrated by cells transfected with wild-type p53 (H1299/p53, see Materials and methods). Without p53, p21$^{WAF/CIP1}$ could not be induced after H1299 cells were exposed to γ-rays (6 Gy); (B) Up-regulation of p21$^{WAF/CIP1}$ was also detected in p53-null MG63 cells exposed to 10 μM cytochalasin B; (C) Up-regulation of p21$^{WAF/CIP1}$ was detected after H1299 cells were exposed to other actin targeting agents cytochalasin D (CD, 2 μM) and latrunculin B (Lat B, 1 μM); (D) p21$^{WAF/CIP1}$ was not up-regulated by colcemid (Col, 1 μg/ml) treatment. The lysate sample from H1299 cells treated with 4 h of cytochalasin B was used as a positive control of p21$^{WAF/CIP1}$. Each blotting result was the representative of duplicate experiments.

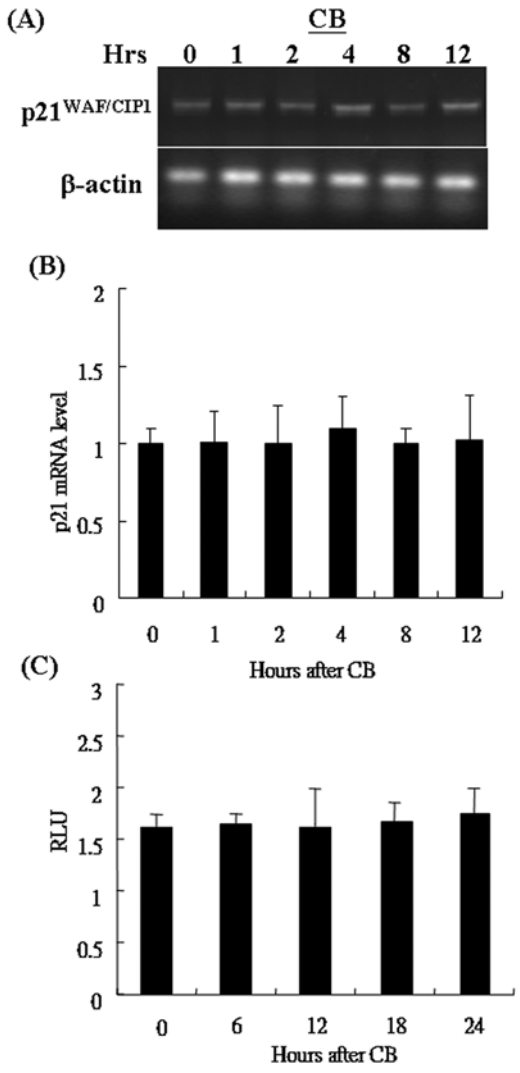


Figure 3. The expression of p21$^{WAF/CIP1}$ mRNA is not affected following destabilization of actin cytoskeleton. (A) The RT-PCR was used to determine the expression of p21$^{WAF/CIP1}$ mRNA in H1299 cells exposed to 10 μM cytochalasin B for the time-points as indicated. Three independent experiments were conducted; (B) The effect of cytochalasin B on p21$^{WAF/CIP1}$ mRNA expression was determined by the quantitative real-time PCR (qPCR). Values are means of three independent experiments ± SD; (C) The luciferase activity was not induced in H1299 cells transiently transfected with WWP-Luc reporter plasmid (see Materials and methods) after cytochalasin B. Each value is the average of three independent experiments normalized by the value obtained by the co-transfected pRL-SV40 plasmid.

H1299 cells. Firstly, semi-quantitative RT-PCR showed that p21$^{WAF/CIP1}$ transcripts in cytochalasin B-treated cells were not increased compared to the untreated control (Fig. 3A). Similar result was obtained using the quantitative real-time PCR (Fig. 3B). The promoter activity using the WWP-Luc reporter plasmid containing 2.4 kb p21$^{WAF/CIP1}$ gene promoter containing p53 binding elements was not either significantly stimulated by cytochalasin B up to 24 h (Fig. 3C). Thus, it suggests that up-regulation of p21$^{WAF/CIP1}$ is not dependent on the increase of mRNA level after destabilization of actin cytoskeleton.

Enhancement of the p21$^{WAF/CIP1}$ protein stability after destabilization of actin cytoskeleton. In order to resolve the transcript-independent up-regulation of p21$^{WAF/CIP1}$, we used cycloheximide (CHX) blocking new protein synthesis to examine the degradation rate of p21$^{WAF/CIP1}$ protein after destabilization of actin cytoskeleton. H1299 cells were exposed to 10 μM cytochalasin B for 2 or 4 h, and then washed and replaced with CHX (10 μg/ml) for additional 0.5-2 h. Compared to cells treated with CHX only, delay of p21$^{WAF/CIP1}$ protein degradation was detected in cytochalasin B-treated cells (Fig. 4A). Densitometric measurement showed that the half-life of p21$^{WAF/CIP1}$ protein was about 30 min, and that this was extended to more than 2 h in cytochalasin B-treated cells (Fig. 4B). To determine if the change of protein stability is associated with the ubiquitination, we used the immunoprecipitation assay to pull down endogenous p21$^{WAF/CIP1}$ using anti- p21$^{WAF/CIP1}$ antibody and then detected the states of bound ubiquitins by Western blot analysis. The result showed that the p21$^{WAF/CIP1}$ ubiquitination was decreased after 4 h of cytochalasin B treatment, suggested that the delay of p21$^{WAF/CIP1}$ degradation was associated with reduction of protein ubiquitination (Fig. 4C).

Up-regulation of p21$^{WAF/CIP1}$ is not accompanied by cytochalasin B mediated reduction of cell viability. To determine if p21$^{WAF/CIP1}$ is associated with the cell stress responding to destabilization of actin cytoskeleton, we exposed A549 and H1299 cells to cytochalasin B up to 24 h to detect the states of cell viability. Significant reduction of plating efficiencies that represented impair of cell proliferative ability was detected only after 24 h of treatment (Fig. 5A). Neither cell line exhibited apparent DNA degradation until 24 h of treatment (Fig. 5B). These results suggest that up-regulation of p21$^{WAF/CIP1}$ is not associated with the cyto-toxicity caused by cytochalasin B.

Up-regulation of p21$^{WAF/CIP1}$ is associated with G1 phase arrest induced by destabilization of actin cytoskeleton. P21$^{WAF/CIP1}$ is one of the cell cycle regulators controlling the G1-S phase progression, in which the retinoblastoma protein (Rb) phosphorylation is essential for such a process. Besides, actin cytoskeleton is important for normal G1 phase progression and cell proliferation. Here we found that in H1299 cells, up-regulation of p21$^{WAF/CIP1}$ was accompanied by dephosphorylation of Rb after cytochalasin B treatment (Fig. 6A). Also, the G1 phase arrest was determined using colcemid to block the recycle of M phase cells. It showed that cytochalasin B apparently delay the progression of G1 phase to S phase compared to colcemid alone (Fig. 6B). The quantification of G1 phase percentage in different time-points of treatment is summarized in Table I. These results suggested that destabilization of actin cytoskeleton mediated p53-independent up-regulation of p21$^{WAF/CIP1}$ may be associated with G1 phase arrest, at least in part.

Discussion

Although p21$^{WAF/CIP1}$ is rarely mutated in cancer formation, accumulated data have demonstrated that inefficient expression of p21$^{WAF/CIP1}$ contributes to the neoplastic progression (19,21,25,31,32). Transcription of p21$^{WAF/CIP1}$ gene via wild-type p53 activation following DNA damage is a well-known

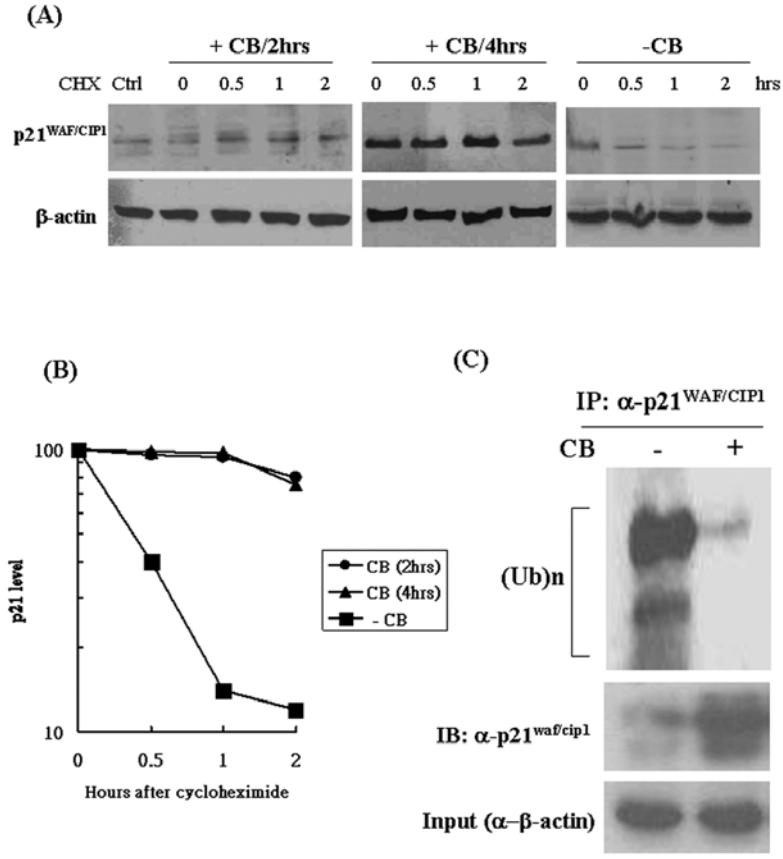
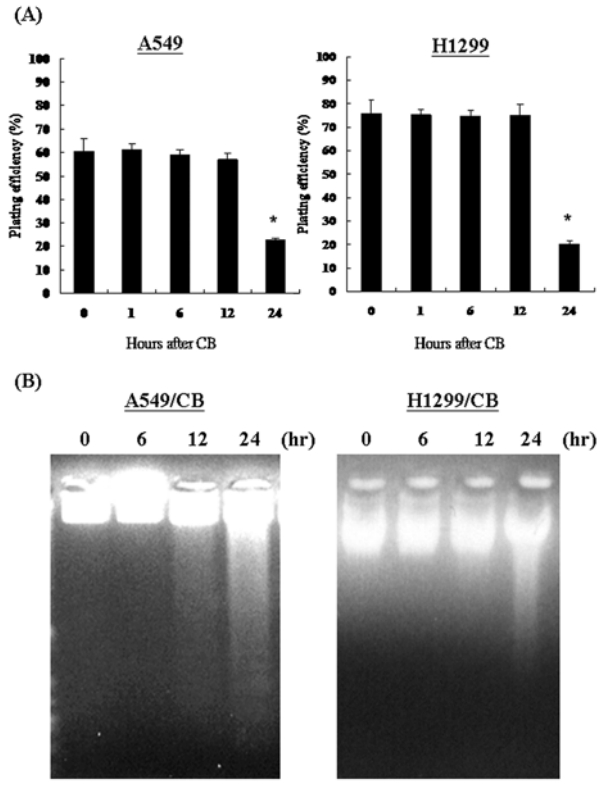


Figure 4. Delay of p21$^{WAF/CIP1}$ protein degradation following destabilization of actin cytoskeleton. (A) P21$^{WAF/CIP1}$ protein stability was determined using cycloheximide (CHX, 10 μg/ml) that can block new protein synthesis. H1299 cells were exposed to cytochalasin B (CB, 10 μM) for 2 and 4 h, and then replaced with CHX for 0.5, 1 and h. Cells treated with CHX only (-CB) was used as a control. Western blot analysis was performed for detection of p21$^{WAF/CIP1}$ protein; (B) Quantification of protein degradation rate by densitometric analysis of the protein bands on the blot. The value of ß-actin was used to normalize that of p21$^{WAF/CIP1}$. The values were the average of duplicate experiments; (C) P21$^{WAF/CIP1}$ protein ubiquitination was determined using immunoprecipitation/immunoblotting analysis. Anti-ubiquitin antibody was used to detect the conjugated ubiquitins on the endogenous p21$^{WAF/CIP1}$ protein pulled down by anti- p21$^{WAF/CIP1}$ antibody (see Materials and methods).



mechanism that is important for chemotherapy and radiotherapy. However, the evidence that p21$^{WAF/CIP1}$ can be up-regulated in p53-independent manner provides important alternative approaches for controlling cancers that 50% of them lack functional p53 (3-6).

Actin cytoskeleton accounts for most abundant composition in cells. Destabilization of actin structures leads to impairing normal cell functions that may be accompanied by molecular responses. In this study, we find that the actin inhibitor induced destabilization of actin cytoskeleton can up-regulate p21$^{WAF/CIP1}$ in cultured cells. Rapid accumulation of p21$^{WAF/CIP1}$ proteins were detected in both A549 (p53$^{+/+}$) and H1299 (p53$^{-/-}$) cell lines exposed to cytochalasin, suggested that a p53-independent mechanism was involved. Additional evidence includes up-regulation of p21$^{WAF/CIP1}$ is not accompanied by the accumulation of total and ser-15 phosphorylated p53 in A549

Figure 5. The effects of cytochalasin B on cell viability. (A) A549 cells and H1299 cells were exposed to cytochalasin B (10 μM) for 0, 6, 12 and 24 h. The plating efficiency was then measured in each cell line after drug treatment. (B) The extents of DNA degradation in cells after cytochalasin B treatment. Values are means of three independent experiments ± SD. *p<0.05 for comparison between cells with or without drug treatment (24 h).

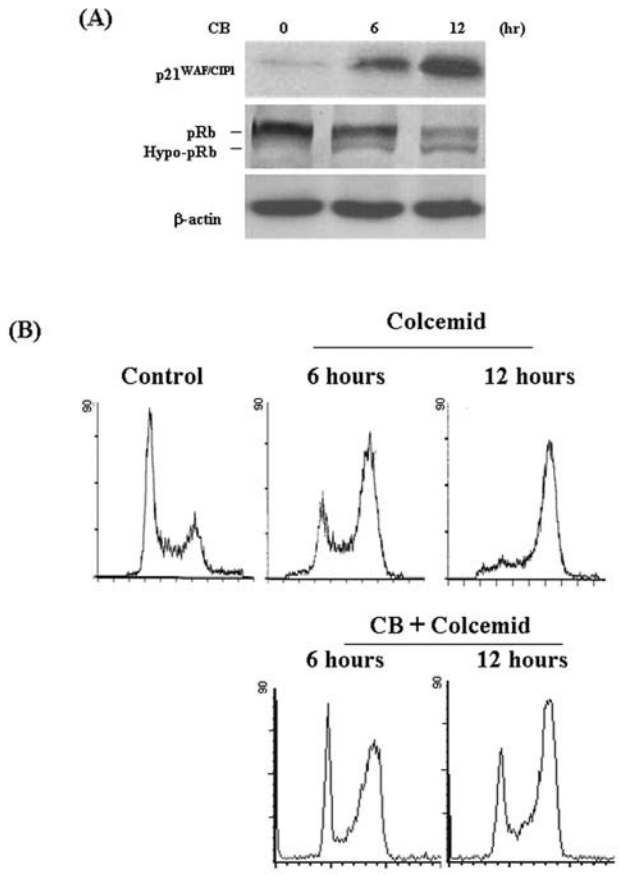


Figure 6. The association between p21$^{WAF/CIP1}$ and G1 phase arrest induced by actin cytoskeletal destabilization. (A) Western blot analysis showed that up-regulation of p21$^{WAF/CIP1}$ was accompanied by decrease of hyper-phosphorylated Rb (pRb) proteins after H1299 cells were exposed to cytochalasin B up to 24 h; (B) Flow cytometric analysis showed that G1 phase progression from 0 to 12 h was blocked in cells treated with cytochalasin B plus colcemid. Cells treated with colcemid alone were used for demonstrating the G1 phase progression. CB, cytochalasin B, 10 μM. Col, clocemid, 10 μg/ml.

Table I. The G1 percentage of H1299 cells measured by flow cytometry.

	Colcemid (10 μg/ml)			Cytochalasin B[a] + colcemid	
	Control	6 h	12 h	6 h	12 h
H1299[b]	56.3%	19.8%	3.5%	38.2%	25.4%

[a]Concentration, 10 μM. [b]The values were an average of two independent experiments.

cells. Also, no p53 activity was detectable in A549 cells using the 2.4kb p21$^{WAF/CIP1}$ promoter containing p53 binding elements for reporter gene assay (data not shown). Though the p21$^{WAF/CIP1}$ up-regulation of A549 cells was slightly earlier than that of H1299 cells under the same dose of cytochalasin B (1 vs. 2 h), the maximum levels of p21$^{WAF/CIP1}$ up-regulation remained similar in both cell lines after 4 h of treatment. It is speculated that the little kinetic difference is due to cell-type specific effect.

One of the most important questions is whether cytochalasin B can trigger other effects to up-regulate p21$^{WAF/CIP1}$ rather than actin destabilization. Cytochalasin B is also a specific inhibitor of GLUT transporters that can limit the uptake of glucose in cells (33,34). Using the drugs cytochalasin D and latrunculin B that do not inhibit basal glucose transport, they were still able to up-regulate p21$^{WAF/CIP1}$ in p53-null H1299 cells. A parallel experiment using colcemid showed no effect on up-regulation of p21$^{WAF/CIP1}$. These results further confirm that actin-targeting agents can specifically up-regulate p21$^{WAF/CIP1}$ without p53 activation.

The expression of p21$^{WAF/CIP1}$ level in the absence of p53 is either dependent on gene transcription or post-translational machinery. P53-independent transcription of p21$^{WAF/CIP1}$ can be mediated by mitogenic stimulation, transformation growth factor-ß, oxidative stress or differentiating signals (2-4,6). Our data reveal that no essential increase of p21$^{WAF/CIP1}$ mRNA after cells are treated with cytochalasin B, suggesting that the signaling pathways for p21$^{WAF/CIP1}$ gene transactivation is not involved. Indeed, under the same experimental condition, we did not find increase of phosphorylation on p44/p42 mitogenic activated protein kinase (MAPK) in cells treated with cytochalasin B (data not shown). On the other hand, the p21$^{WAF/CIP1}$ protein stability was enhanced after destabilization of actin cytoskeleton. Cytochalasin B induced relatively lower amount and approximately maximum amount of p21$^{WAF/CIP1}$ in H1299 cells after 2 and 4 h of treatment, respectively (Fig. 2). The degradation rate of p21$^{WAF/CIP1}$ protein was examined at these two time-points following cycloheximide treatment, and similar results were obtained. That is, compared to the untreated controls, apparent delay of endogenous p21$^{WAF/CIP1}$ protein degradation in cytochalasin B treated cells was detected. Such an observation is consistent with previous report that inhibition of Rho GTPase activity leads to disruption of actin cytoskeleton and p21$^{WAF/CIP1}$ stabilization (26). On the other hand, we cannot exclude an indirect effect using cycloheximide to explore p21$^{WAF/CIP1}$ degradation rate since it may block proteins that affect its turnover rate. A pulse-chase analysis will be helpful to confirm this observation.

P21$^{WAF/CIP1}$ is a short-lived protein that can be degraded by either the ubiquitination-dependent or -independent pathways (14,16). P21$^{WAF/CIP1}$ protein harbors six lysine residues that can be ubiquitinated for 26S proteasome mediated degradation (15,35,36). The E3 ubiquitin ligase SCF^{Skp2} complex that consists of Rbx1, Cul1, Skp1, and Skp2 subunits is essential for p21$^{Cip1/WAF1}$ ubiquitin-proteasome degradation (14,15). Our data showed that polyubiquitinated p21$^{WAF/CIP1}$ was reduced in response to cytochalasin B mediated destabilization of actin cytoskeleton. Interestingly, we also found that the total polyubiquitinated protein was reduced to half of the original amount at the same treatment (data not shown). Whether destabilization of actin cytoskeleton will influence the activity of SCF^{Skp2} complex or other ubiquitination machinery remains largely unknown and requires further investigation.

The ubiquitination-independent pathway is demonstrated by finding that mutant p21$^{WAF/CIP1}$ lacking lysine residues

(p21K6R) for ubiquitination is degraded normally (16). Furthermore, Touitou *et al* demonstrated that the C-terminal of $p21^{WAF/CIP1}$ protein directly binds to the C8 α-subunit of 20S proteasome for degradation without ubiquitination (17). Our current data can not exclude the existence of the ubiquitination-independent pathway. Especially, actin organization is associated with cell attachment to extracellular matrix (ECM) for entry of cell cycle and growth (37). ECM has been reported to induce degradation of mutant $p21^{WAF/CIP1}$ (p21K6R), suggesting that ubiquitin-proteasome degradation of $p21^{WAF/CIP1}$ is not necessary during cell anchorage and spreading (38). It will be possible to identify whether destabilization of actin cytoskeleton can up-regulate $p21^{WAF/CIP1}$ via suppression of ubiquitination-independent machinery using p21K6R chimera.

The other question is the association between the up-regulated $p21^{WAF/CIP1}$ and cell viability after cytochalasin B treatment. A previous report shows that disruption of actin microfilaments by cytochalasin D induces apoptosis in a p53-dependent manner (39). In contrast to their results, our data revealed that both A549 ($p53^{+/+}$) cells and H1299 ($p53^{-/-}$) cells exposed to cytochalasin B exhibit significant reduction of cell viability after 24 h of treatment. These differences are possibly due to cell-type specific responses. Given that the timing of $p21^{WAF/CIP1}$ up-regulation is distinct from that of the loss of cell viability by cytochalasin B, it is speculated that $p21^{WAF/CIP1}$ is not associated with cytochalasin B induced cytotoxicity. Nevertheless, further evidence is required. For instance, a gene knockdown approach using RNA interference may at least in part clarify the role of $p21^{WAF/CIP1}$ responding to pharmaceutically-mediated destabilization of actin cytoskeleton.

Destabilization of the actin cytoskeleton has been reported to inhibit early G1 phase progression (40,41). It has been reported that inhibition of actin organization in mammalian fibroblast leads to G1 phase arrest depending on retinoblastoma (Rb) pocket proteins but not p53 (42). Besides, the $p21^{WAF/CIP1}$ level was not elevated, whereas $p27^{KIP1}$ protein was up-regulated in their study. Although we found that the hypophosphorylated Rb was increased accompanied by up-regulated $p21^{WAF/CIP1}$ in H1299 cells exposed to cytochalasin B, we did not detect apparent elevation of $p27^{KIP1}$ under the same condition (data not shown). These controversial results suggest that the molecular regulations following destabilization of actin cytoskeleton for G1 phase arrest are cell-type specific. However, it remains to address whether $p21^{WAF/CIP1}$ or $p27^{KIP1}$ can directly influence cyclin-dependent kinase (CDK) activity and result in hypophosphorylation of Rb for G1 phase arrest after destabilization of actin cytoskeleton.

In conclusion, current results have demonstrated that destabilization of actin cytoskeleton is able to up-regulate $p21^{WAF/CIP1}$ via a p53-independent pathway. Up-regulation of $p21^{WAF/CIP1}$ is mainly dependent on post-translational mechanism rather than transcriptional pathway. Also, the function of the up-regulated $p21^{WAF/CIP1}$ is potentially associated with G1 phase arrest prior to significant cell death. The underlying mechanisms regarding how destabilization of actin cytoskeleton is able to enhance $p21^{WAF/CIP1}$ protein stability and influence CDK activity, if any, will require further investigation.

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