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# Phosphatidylinositol 3-kinase inhibitors, Wortmannin or LY294002, inhibited accumulation of p21 protein after γ-irradiation by stabilization of the protein

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**Abstract**

Expression of the cyclin kinase inhibitor, p21, is regulated both transcriptionally and posttranscriptionally by the ubiquitin-proteasome degradation pathway. Recently, we reported that DNA damage is required for efficient p21 expression by demonstrating that enhanced p21 mRNA expression induced by DNA damage results in increased p21 protein, but enhanced p21 mRNA without DNA damage does not. In addition, we demonstrated that DNA damage suppressed the ubiquitination of p21. In this study, we analyze the link between p21 stabilization and DNA damage. Enhanced p21 protein expression in ML-1 cells resulting from 15 Gy γ-irradiation was diminished by Wortmannin or LY294002 pretreatment of cells. However, the levels of p21 mRNA were not affected by inhibitor pretreatment. Wortmannin or LY294002 pretreatment reduces p53 expression after γ-irradiation to a lesser degree than that of p21. In addition, we examined the involvement of DNA-PK, whose activity is inhibited by Wortmannin or LY294002, in p21 stabilization using the SCID fibroblast cell line and a DNA-PK targeting ML-1 cell line. Accumulation of p21 protein by γ-irradiation was similar to that of DNA-PK intact cells and was reduced by Wortmannin or LY294002 pretreatment. Involvement of another DNA damage detecting enzyme, the ATM gene product, whose activity is also inhibited by Wortmannin or LY294002, was evaluated. ATM deficient cells induced p21 after γ-irradiation, γ-irradiation-induced p21 protein was diminished by pretreatment of cells with Wortmannin or LY294002. We conclude that the p21 stabilization mechanism functions after γ-irradiation, was sensitive to Wortmannin or LY294002, and required neither DNA-PK nor ATM gene product for activity. 

*Keywords:* p21; DNA damage; Wortmannin; Ataxia telangiectasia mutated; DNA dependent protein kinase

## 1. Introduction

The progression of the cell cycle is precisely controlled by cyclin-dependent kinases (CDKs). The sequential activation of CDKs is accomplished by association of each CDK with a specific cyclin subunit [1,2]. In the mammalian cell cycle, $G_1$ progression is driven by cyclin D associated with either CDK4 or CDK6, while $G_1$/S transition requires both cyclin E/CDK2 and cyclin A/CDK2 [1]. As the cell cycle

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progresses, each cyclin is degraded and the phase specific CDK activity is diminished [3,4]. However, to ensure the prevention of an unscheduled entry into S phase, the activity of the cyclin/CDK complex is suppressed by association with CDK inhibitors [5]. For control at the $G_1$-S checkpoint, there are two groups of CDK inhibitors, the p21 family and the p16INK4 family. Ample evidence suggests that the expression of cyclin kinase inhibitors is regulated both at the transcriptional level and at the protein level [5,6].

When dividing cells are exposed to genetic stress, the cell cycle must be arrested immediately to ensure the integrity of the DNA and/or cell cycle control. Cyclin kinase inhibitors should be constitutively expressed in cells to prepare their prompt response against genetic stress during $G_1$. If the cell cycle progresses from $G_1$ to S on schedule without DNA damage, those inhibitors are to be degraded through the ubiquitin proteasome pathway as are cyclins [7]. Consequently, it is conceivable that DNA damage brings about accumulation of cyclin kinase inhibitors by inhibiting their degradation.

The direct association of DNA damage and cell cycle regulation has been described through the phosphorylation of p53 by a phosphatidylinositol (PI) 3-kinase family member, either DNA-dependent protein kinase (DNA-PK) or the ataxia telangiectasia mutated (ATM) gene product. DNA-PK is known to phosphorylate a number of proteins in vitro, p53 and other proteins with roles in replication or regulation of the cell cycle (c-Myc, c-Fos, c-Jun, topoisomerases I and II) [8]. ATM gene product is thought to be involved in sensing DNA damage due to the variety of abnormalities in A-T cells [9]. p53 is a cell cycle regulatory protein whose degradation is inhibited by DNA damage-induced phosphorylation [10]. Recently, evidence has been presented that suggests the phosphorylation of p53, by DNA-PK or ATM at serine 15 in the amino terminal transactivation domain, leads to stabilization of p53 by inhibiting its recognition by Mdm2 E3 ubiquitin ligase [11–13]. Other reports, however, have demonstrated that DNA-PK in not required for the p53-dependent response to DNA damage [14,15]. These conflicting reports of the cellular response(s) to DNA damage are ambiguous and demonstrate the need for further study.

The expression of cyclin kinase inhibitor p21 has been shown to be regulated by the ubiquitin-proteasome degradation pathway [16–19]. We recently reported that the treatment of ML-1 cells with deferoxamine, an iron chelator that functions as a inhibitor of deoxyribonucleotide reductase, causes an accumulation of p53 and the induction of p21 mRNA but not p21 protein [20,21]. When ML-1 cells were treated with etoposide, a DNA damaging agent, p53 accumulation with an increase of p21 mRNA followed by induction of p21 protein was observed [19]. In an attempt to analyze the ubiquitination of p21 after DNA damage, we analyzed ubiquitinated p21 in cells treated with various agents. Ubiquitinated p21 is detectable after deferoxamine and proteasome inhibitor treatment, but not in cells treated with etoposide [19,21]. Others have reported that UV and ionizing radiation have no effect on either the stability or the in vivo ubiquitination of p21 in U2OS cells, a human osteosarcoma cell line that expresses wild-type p53 [17]. Further, no direct evidence exists that DNA damage stabilizes CDK inhibitors. However, in our previous experiments comparing DNA damaging agents with a non-DNA damaging stimulant that induces p53 and p21 mRNA, we demonstrated that DNA damage increases the expression of p21 protein through posttranscriptional regulation [19]. The mechanism(s) by which DNA damage results in the stabilization of p21 is unknown. However, given that p21 is degraded via the ubiquitin-proteasome degradation pathway, one possibility is that DNA damaging agents somehow signal a repression of the ubiquitin-mediated degradation of p21. Involvement of DNA damage in regulation of the ubiquitin-proteasome pathway was demonstrated as the contribution of DNA damage-sensing protein kinases, DNA-PK or ATM, in the regulation of p53-ubiquitination by phosphorylation. According to these observations, in the present study, we examined the involvement of DNA-PK and ATM in p21 stabilization.

## 2. Materials and methods

### 2.1. Cells and chemicals

ML-1, a human myelocytic leukemia cell line

(wild-type functional p53), and NIH3T3 mouse fibroblast were supplied by the Health Science Research Resource Bank (HSRRB). The SV40 transformed severe combined immunodeficiency (SCID) mouse fibroblast cell line, SC3VA4, is kindly supplied by Dr. M. Abe (National Institute of Radiological Science) [22]. Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines from patients with ataxia telangiectasia (A-T), AT1ABR and AT3ABR, were kindly provided by Dr. Martin Lavin (Queensland Inst. Med. Res., Brisbane, Australia). The AT1ABR cell produces a near full length ATM gene product with a deletion of three amino acids (2546–2548) upstream from the PI 3 kinase domain [23,24]. The AT3ABR cell has a mutation, A8266T, and the 350 kDa ATM protein is not detected [24]. ML-1, AT1ABR and AT3ABR cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum at 37°C in a humidified 5% $CO_2$ incubator. Cells were cultured at a concentration between $2\times10^5$ and $1\times10^6$/ml. Exponentially growing ML-1 cells were prepared by seeding at a concentration of $2\times10^5$/ml 24 h prior to treatment. NIH3T3 cells were cultured in Dulbecco's modified Eagle's essential medium (DMEM) containing 10% calf serum at 37°C in a humidified 5% $CO_2$ incubator, and SC3VA4 cells were cultured in DMEM containing 10% fetal calf serum under the same conditions. Cells were seeded at $1\times10^6$ cells in 10 cm diameter dishes and passaged every third day. Exponentially growing NIH3T3 cells or SC3VA4 cells were prepared by seeding at a concentration of $3\times10^5$ cells in 25 $cm^2$ culture flask 24 h prior to treatment.

Either Wortmannin (Sigma) or LY294002 (Sigma) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of $1\times10^{-2}$ M. γ-Irradiation (IR) was performed at room temperature with a Gammacell 3000 Elan (Nordion International, Canada) with a $^{137}Cs$ source emitting at a fixed dose rate of 3.1 Gy $min^{-1}$.

### 2.2. Protein extraction and Western blot analysis

Cells ($2\times10^7$) were harvested, washed with phosphate buffered saline (PBS) and then lysed in 1 ml of lysis buffer (1% Nonidet P-40, $1\times10^{-2}$ M sodium phosphate pH 7.2, $1.5\times10^{-1}$ M NaCl, $1\times10^{-3}$ M EDTA, $5\times10^{-2}$ M sodium fluoride, $1\times10^{-2}$ M sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 5 μg/ml phenylmethylsulfonyl fluoride and 60 μg/ml *N*-ethylmaleimide). After incubation on ice for 30 min, the lysate was clarified by ultracentrifugation at $105\,000\times g$ for 1 h at 4°C. The protein concentration of the cell lysate was determined with the Bradford method [25]. For Western blot analysis, 1% sodium dodecyl sulfate (SDS) and 2.5% β-mercaptoethanol were added to the cell lysate (30 μg) and heated for 5 min at 100°C. Denatured samples were applied on 10–20% or 4–15% SDS-polyacrylamide gel electrophoresis using a running buffer of $2.5\times10^{-2}$ M Tris-HCl pH 8.3, $1.92\times10^{-1}$ M glycine, 0.1% SDS. The gels were electroblotted (5 mA/$cm^2$, 2 h) onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, CA) in a transfer buffer of $2.5\times10^{-2}$ M Tris-HCl pH 8.3, $1.92\times10^{-1}$ M glycine and 20% methanol. The membranes were incubated with 10% nonfat dry milk in PBS, then incubated with either anti-p21 rabbit polyclonal antibody (1:200, H164; 1:200, C-19, Santa Cruz Biotechnology, CA), anti-p53 monoclonal antibody (1:500, DO-1, Santa Cruz) or anti DNA-PKcs rabbit polyclonal antibody (1:200, C-19, Santa Cruz) diluted in $1\times10^{-2}$ M Tris-HCl pH 8.3, $5\times10^{-1}$ M NaCl (TBS) for 1 h at room temperature. 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, Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) substrate was used to visualize the bands. The same blot was probed with an anti-β-tubulin monoclonal antibody (5H1, Pharmingen, CA) to control the amount of protein loaded onto the gel. The band density was quantified by Image Master (Pharmacia, Sweden). The fold increase in protein expression in relation to the expression in untreated control cells was indicated.

### 2.3. Northern blot analysis

RNA extraction and Northern blot hybridization were performed as described previously [20]. Cells were lysed in of 5 M guanidine isothiocyanate then

extracted with phenol-chloroform. RNA (20 μg) was treated with 50% formamide and separated on a 1.0% agarose gel in $6.6\times10^{-1}$ M formaldehyde, $2\times10^{-2}$ M 3-morpholinopropanesulfonic acid (MOPS) pH 7.0 and transferred to a positive charged nylon membrane, Hybond $N^+$ (Amersham, UK). p21 cDNA (provided by Dr. Noda, Meiji Cell Technology Center, Japan) and β-actin DNA were digoxygenin-labeled by the random primer method. Hybridization was performed in 50% formamide, 5×SSC (1×SSC: $1.5\times10^{-1}$ M NaCl, $1.5\times10^{-2}$ M sodium citrate), sodium phosphate pH 7.0, 7% SDS, at 50°C for 16 h. After washing with 2×SSC, 0.1% SDS at room temperature then with 0.1×SSC, 0.1% SDS at 68°C with gentle agitation, the membrane was incubated with 1% nonfat dry milk in maleic acid buffer ($1\times10^{-2}$ M maleic acid, $1.5\times10^{-2}$ M NaCl pH 7.5) for 1 h at 20°C. The membrane was washed with maleic acid buffer followed by incubation with alkaline phosphatase-conjugated anti-digoxygenin antibody in maleic acid buffer (1:5000, Boehringer Mannheim, Germany) for 1 h at 20°C. After washing with maleic acid buffer thoroughly, the hybridized bands were visualized using NBT/BCIP. The level of expression was normalized by comparative hybridization using digoxygenin-labeled β-actin probe.

### 2.4. Generation of DNA-PKcs targeting plasmid

The DNA fragment for DNA-PKcs was constructed from a human spleen cell cDNA library using polymerase chain reaction (PCR). PCR were performed at 94°C 90 s, 58°C 90 s, 72°C 90 s for 30 cycles. Primers for PCR were forward 5′-4321 AACAGCACAGAGCATTGAGG-3′ and reverse 5′-4803 TGCATGAGCTCCAATACAGC-3′ based on the human DNA-PK catalytic subunit (DNA-PKcs) cDNA sequence [26]. The produced fragment of 483 bp was inserted into pTargeT vector (Promega, WI), downstream from the cytomegalovirus immediate early promoter, and transfected into JM109 according to manufacturer's instruction. The transfectant was propagated in LB and the plasmid DNA was extracted as described previously [27]. A portion of plasmid DNA (3 μg) was digested with 6 units *Sal*I restriction endonuclease for DNA sequencing. The sequencing reaction was performed by the dideoxynucleotide termination method using M13 universal primer 5′-CGACGTTGTAAAACGACGGCCAGT-3′ and analyzed by ALF sequencer (Pharmacia, Sweden) as described previously [28]. The sequence was compared with human DNA-PKcs sequence [26] and the confirmed fragment was inserted into the pTargeT vector. pΔDNA-PKcs was isolated and identified as an antisense DNA-PKcs RNA expressing plasmid. The plasmid DNA (100 μg) was linearized by digesting with 100 units *Sal*I restriction endonuclease, then precipitated with 70% ethanol at −20°C for 8 h. Linearized plasmid DNA was collected by centrifugation $10\,000\times g$ at 4°C, dissolved in sterile water at a concentration of 10 μg/μl.

### 2.5. Electroporation

Exponentially growing ML-1 cells ($1\times10^7$) were harvested, washed with ice-cold PBS, then resuspended in 0.5 ml of RPMI on ice. Cell suspensions were transferred to electroporation cuvettes (Bio-Rad) chilled on ice. Linearized plasmid DNA (50 μg) was added to the cell suspension. 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 6 ml RPMI with 10% FCS containing 400 μg/ml G418 and cultured at 37°C in a humidified 5% $CO_2$ incubator. Culture medium was changed every 2 days, and a transfectant was obtained after a 2 week culture.

## 3. Results and discussion

### 3.1. IR-induced p21 protein expression in ML-1 cells is inhibited by either Wortmannin or LY294002

In order to begin analysis of the early cellular response to DNA damage, we examined the protein expression levels of p53 and p21 in ML-1 cells to determine the time of the initial increase of p21 after induction with 15 Gy IR. The expression of both p53 and p21 began to increase after 2 h. The expression of p53 reached a plateau at 3 h, while p21 expression reached maximum level at 4 h (Fig. 1A). At 4 h, a

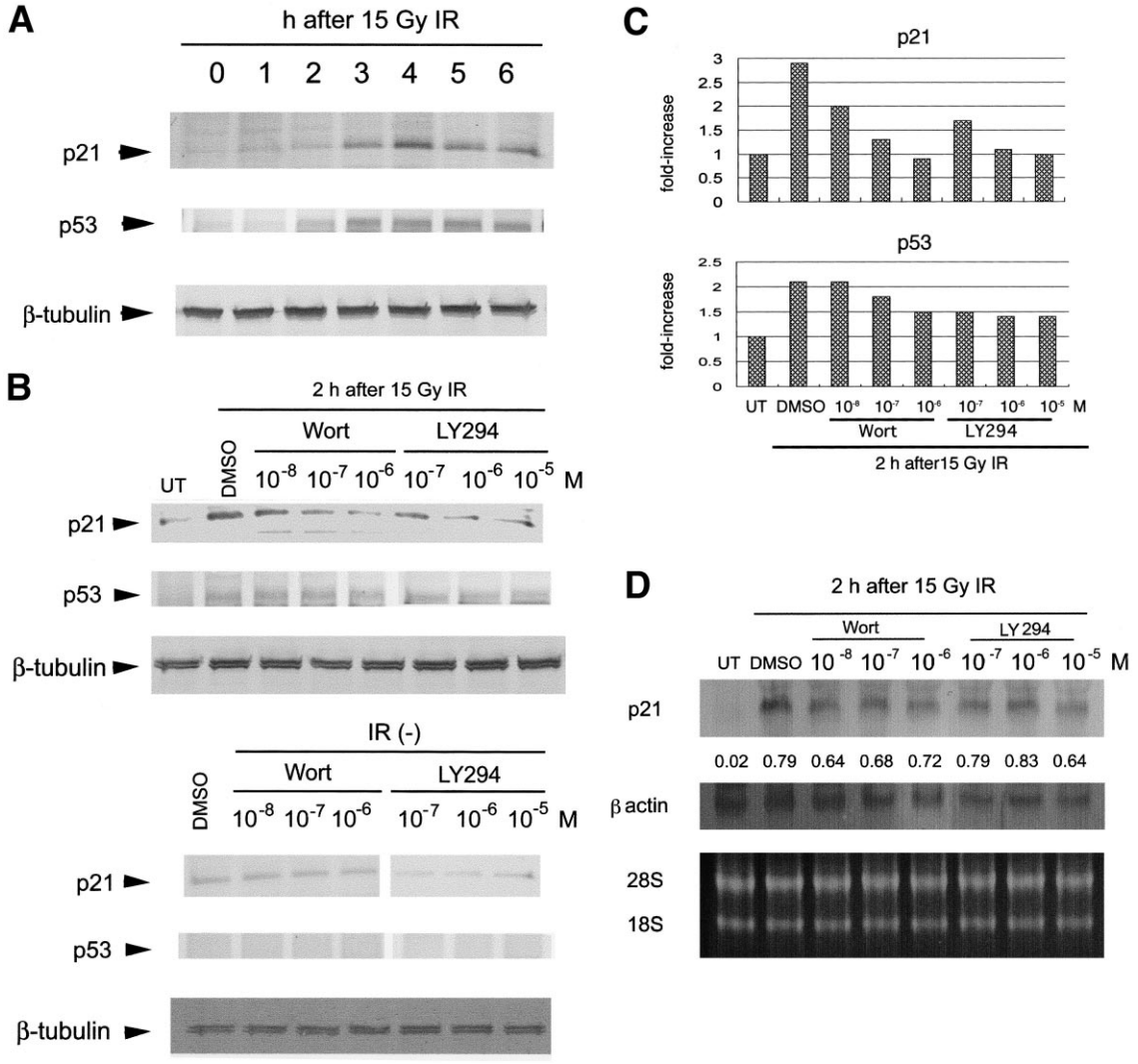


Fig. 1. Wortmannin or LY294002 pretreatment diminished γ-irradiation-induced p21 expression at the protein level in ML-1 cells. (A) Time course of p53 and p21 expression in ML-1 in response to γ-irradiation were examined. Exponentially growing ML-1 cells were irradiated with 15 Gy. Cell lysates were extracted after incubation for 1–6 h as indicated. Cell lysates (30 μg) were electrophoresed on 10–20% SDS-PAGE and blotted onto PVDF membrane. The membrane was probed with anti-p53 monoclonal antibody (DO-1) or with anti-p21 polyclonal antibody (H164) as described in Section 2. (B) Exponentially growing ML-1 cells were treated with $1\times10^{-8}$–$1\times10^{-6}$ M Wortmannin or $1\times10^{-7}$–$1\times10^{-5}$ M LY294002 for 30 min, irradiated with 15 Gy or unirradiated, then incubated for 2 h. Cell lysates (30 μg) were electrophoresed on 10–20% SDS-PAGE and blotted onto PVDF membrane. The membrane was probed with either anti-p21 polyclonal antibody (H-164) or anti-p53 monoclonal antibody (DO-1). In addition, the membrane was probed with anti-β-tubulin monoclonal antibody (5H1) as an internal control. UT is an untreated control lysate and DMSO is a lysate obtained from irradiated cells treated with vehicle solution only. (C) Histogram of p21 and p53 band intensities quantified by densitometric scanning. The fold increase in protein expression, a comparison of the expression with that in untreated control cells, is indicated. (D) Twenty micrograms of total RNA were subjected to electrophoresis on formaldehyde agarose gel, transferred to a Hybond $N^+$ membrane, and hybridized with digoxigenin-labeled probes for p21 mRNA. The level of expression was normalized by comparison with β-actin and indicated as a relative intensity. Ethidium bromide staining of total RNA is shown at the bottom of the figure. Experiments were repeated independently at least 3 times. Typical blots are shown in the figures. The relative expressions of both protein and mRNA were reproducible (within ±10%).

portion of the ML-1 cells began apoptosis (data not shown). Therefore, we chose the time point of 2 h after IR, which is prior to the start of cell death, for further analysis of p21 expression.

Previous studies suggest that p21 protein expression was enhanced only after DNA damage [19]. We proposed the existence of a protein stabilization mechanism that is induced by DNA damage. In order to access the involvement of DNA-PK or ATM in the stabilization mechanism, γ-irradiation-induced p21 expression was examined in cells pretreated with either Wortmannin or LY294002, PI 3-kinase inhibitors. ML-1 cells were treated with $1\times10^{-8}$–$1\times10^{-6}$ M Wortmannin or $1\times10^{-7}$–$1\times10^{-5}$ M LY294002 for 30 min followed by 15 Gy IR. Cells were incubated for 2 h more, then p21 protein expression was examined (Fig. 1B). IR resulted in a 2.9-fold increase in p21 expression (Fig. 1C). Pretreatment with either Wortmannin or LY294002 reduced the IR-induced p21 expression dose-dependently, with levels reduced to that of untreated controls at either $1\times10^{-6}$ M Wortmannin or $1\times10^{-6}$ M LY294002 (Fig. 1B,C). Incubation of the cells in DMSO, the vehicle solution for Wortmannin or LY294002, had no effect on increased p21 expression after IR (Fig. 1B,C). The treatment of the ML-1 cells with either Wortmannin or LY294002 in the absence of IR did not affect p21 expression (Fig. 1B). These results suggest that the inhibition of IR-induced p21 expression was the result of the activity of both Wortmannin and LY294002.

p53, an upstream p21 transcription factor, is stabilized by phosphorylation through the activity of ATM or DNA-PK when ML-1 cells are exposed to

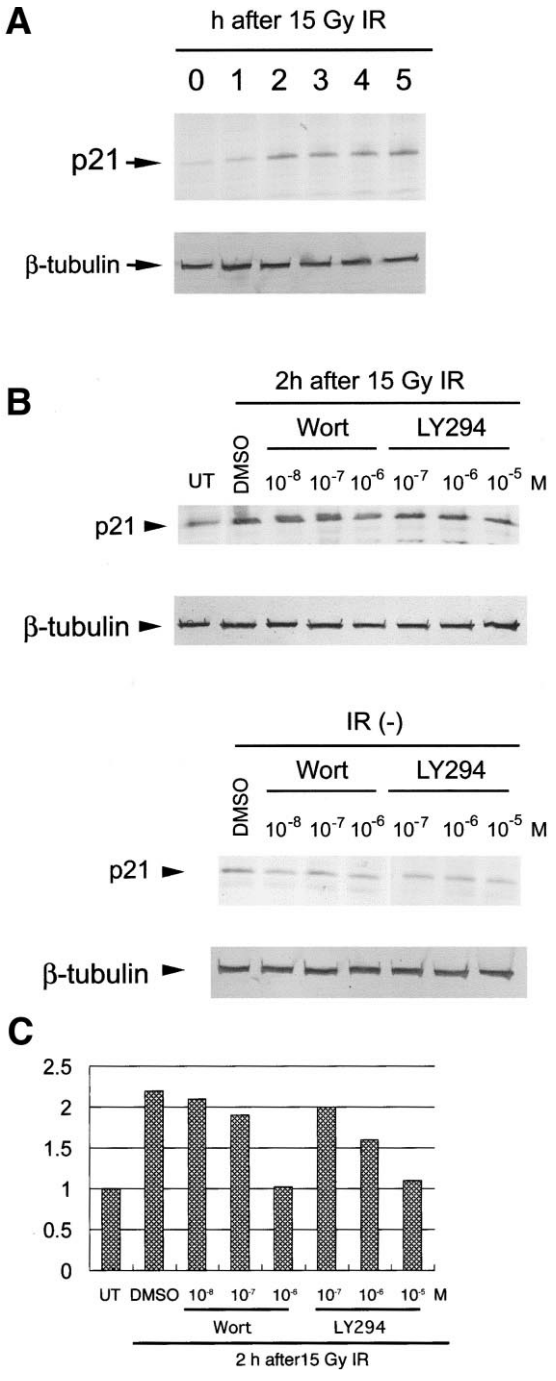


Fig. 2. Wortmannin or LY294002 pretreatment diminished the γ-irradiation-induced p21 expression at the protein level in NIH3T3 cells. (A) The p21 levels increased in response to γ-irradiation in NIH3T3. Exponentially growing NIH3T3 cells were irradiated at a dose of 15 Gy. Cell lysates were prepared at the indicated times after irradiation and examined by Western blotting with the anti-p21 polyclonal antibody (C-19). (B) Exponentially growing NIH3T3 cells were treated with $1\times10^{-8}$–$1\times10^{-6}$ M Wortmannin or $1\times10^{-7}$–$1\times10^{-5}$ M LY294002 for 30 min, irradiated with 15 Gy or unirradiated, then incubated for 2 h. Cell lysates (30 μg) were electrophoresed on 10–20% SDS-PAGE and blotted onto PVDF membrane. The membrane was probed with anti-p21 polyclonal antibody (C-19), or with anti-β-tubulin monoclonal antibody (5H1) as an internal control. (C) The intensities of p21 bands after IR were quantified by densitometric scanning. The fold increase in protein expression, a comparison of the expression with that in untreated control cells, is indicated. Experiments were repeated independently at least 3 times. Typical blots are shown in the figures. The relative expressions of both protein and mRNA were reproducible (within ±10%).

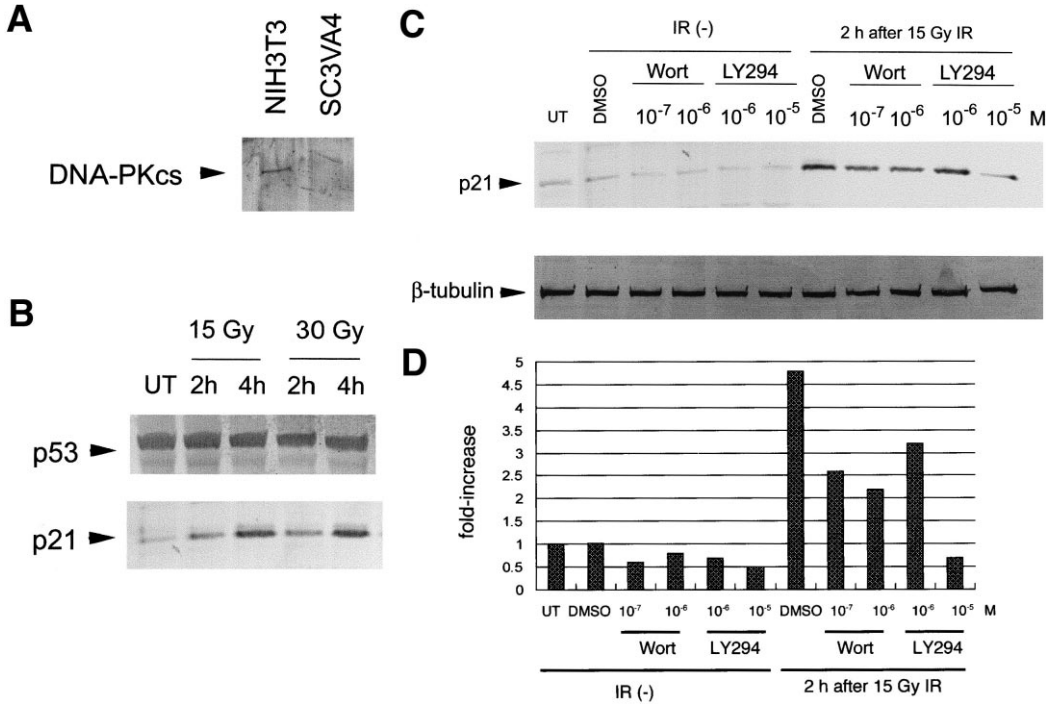


Fig. 3. γ-Irradiation-induced p21 was diminished by Wortmannin or LY294002 pretreatment in SC3VA4. (A) Expression of DNA-PKcs in SC3VA4 cells was examined by Western blotting. Cell lysates (30 μg) were electrophoresed on 4–15% SDS-PAGE and blotted onto PVDF membrane, then probed with anti-DNA-PKcs polyclonal antibody (C-19). DNA-PKcs 460 kDa band was observed in cell lysates of NIH3T3 but not in SC3VA4 lysates. (B) SC3VA4 cells were irradiated at a dose of 15 Gy or 30 Gy. Cell lysates were prepared at the indicated times after irradiation and electrophoresed on 10–20% SDS-PAGE and blotted onto PVDF membrane, then probed with anti-p53 monoclonal antibody (DO-1) or with anti-p21 polyclonal antibody (C-19). (C) Exponentially growing SC3VA4 cells were treated with $1\times10^{-8}$–$1\times10^{-6}$ M Wortmannin or $1\times10^{-7}$–$1\times10^{-5}$ M LY294002 for 30 min, irradiated with 15 Gy or unirradiated, then incubated for 2 h. Cell lysates (30 μg) were electrophoresed on 10–20% SDS-PAGE and blotted onto a PVDF membrane. The blot was probed with anti-p21 polyclonal antibody (C-19), or with anti-β-tubulin monoclonal antibody (5H1) as an internal control. (D) The intensities of p21 bands after IR were quantified by densitometric scanning. The fold increase in protein expression, a comparison of the expression with that in untreated control cells, is indicated. Experiments were repeated independently at least 3 times. Typical blots are shown in the figures. The relative expressions of both protein and mRNA were reproducible (within ± 10%).

a DNA damaging agent. Wortmannin or LY294002 inhibits the phosphorylation of p53 resulting in its destabilization. The induction of p53 in ML-1 cells by exposure to 15 Gy IR was decreased by Wortmannin or LY294002 pretreatment (Fig. 1B). p53 induction was reduced 30% by either $1\times10^{-6}$ M of Wortmannin or $1\times10^{-6}$ M LY294002 (Fig. 1C). The reduction of induced p53 was less than that of p21. Treatment of ML-1 cells with either Wortmannin or LY294002 in the absence of IR did not affect p53 expression (Fig. 1B). To confirm that the inhibition of p21 expression was not due to the inhibition of transcription through the diminution of p53, we analyzed the levels of p21 mRNA expression at the time point when Wortmannin or LY294002 pretreatment inhibits the IR-induced p21 protein expression. Results indicate that p21 mRNA was induced by IR in cells pretreated with either $1\times10^{-8}$–$1\times10^{-6}$ M Wortmannin or $1\times10^{-7}$–$1\times10^{-5}$ M LY294002. The levels of p21 mRNA induction were identical to that induced in cells pretreated with DMSO alone (Fig. 1D). Taken together, these results suggest that either Wortmannin or LY294002 specifically decreased the p21 protein expression while p21 mRNA expression remained unaffected in these experimental conditions.

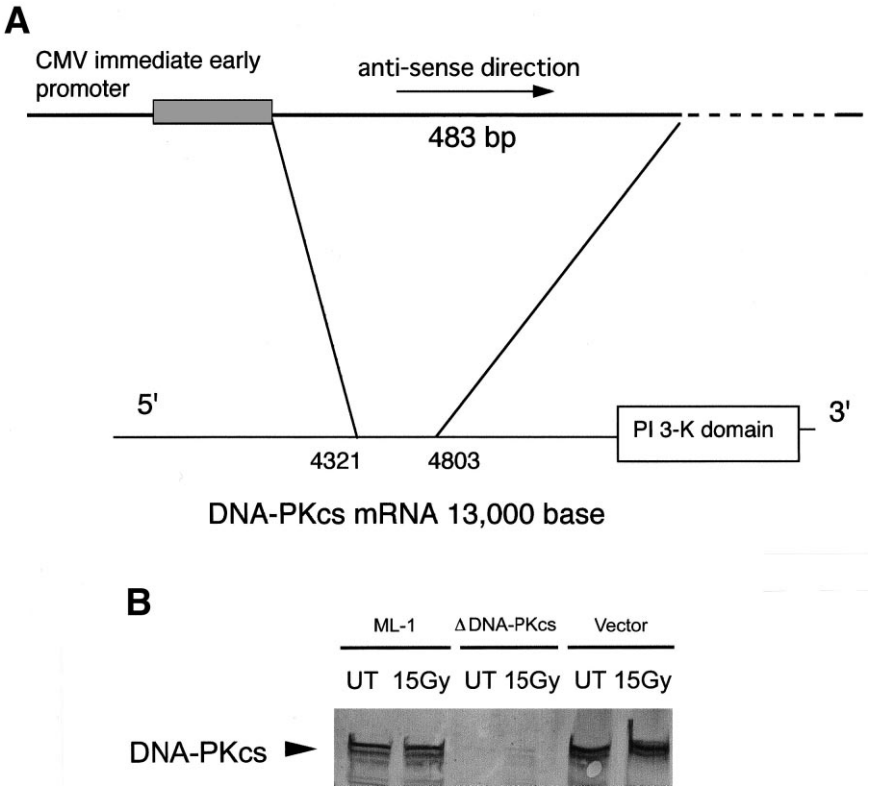


Fig. 4. Construction of DNA-PKcs targeting ML-1 cells. (A) A DNA fragment, encoding nucleotides 4321–4803 of the DNA-PKcs cDNA, was generated from a human spleen cell cDNA library by PCR and inserted into the pTargeT vector (Promega) at a position downstream from the cytomegalovirus immediate early promoter and in an antisense direction. The construct was designated pΔDNA-PKcs. (B) Linearized pΔDNA-PKcs was transfected into exponentially growing ML-1 and designated ML-1 ΔDNA-PKcs. The existence of DNA-PKcs in the transfectant was determined by Western blot analysis. ML-1, ML-1 ΔDNA-PKcs, and ML-1 transfected with vector alone (ML-1 vector) were irradiated with 15 Gy then incubated for 2 h. Cell lysates were prepared and 30 μg of each was electrophoresed on 4–15% SDS-PAGE and blotted onto PVDF membrane, then probed with anti-DNA-PKcs polyclonal antibody (C-19). DNA-PKcs 350 kDa band was observed in ML-1 and ML-1 vector but not in ML-1 ΔDNA-PKcs in either untreated cells or after irradiation.

### *3.2. p21 expression in NIH3T3 cells and in SCID cells (SC3VA4) is induced by 15 Gy IR and is inhibited by Wortmannin or LY294002 in a dose-dependent manner*

The Wortmannin/LY294002-sensitive p21 stabilization that is induced by DNA damage may be mediated by DNA-PK, a PI 3-K family protein. To examine the role of DNA-PK in the stabilization of mouse p21 protein expression, we compared the levels of IR-induced p21 in SCID fibroblast cell line, whose DNA-PK activity is deleted, with that in NIH3T3, a mouse fibroblast with intact DNA-PK in the presence or absence of either Wortmannin or LY294002.

First, we tested the time course of p21 protein expression in NIH3T3 cells after 15 Gy IR. A marked increase of p21 was observed 2 h after 15 Gy irradiation (Fig. 2A). We chose these conditions for further analysis. As observed in the ML-1 cells, pretreatment of NIH3T3 cells with either Wortmannin or LY294002 decreased the IR-induced p21 protein level in a dose-dependent manner. The 2.2-fold p21 induction by IR was reduced to control level by pretreatment with either $1\times10^{-6}$ M Wortmannin or $1\times10^{-5}$ M LY294002 (Fig. 2B,C). Treatment of cells with an equal amount DMSO for dissolving inhibitors had no effect on p21 inhibition (Fig. 2B). The p21 protein level in cells pretreated with either Wortmannin or LY294002 without IR was not changed (Fig. 2B).

To determine whether DNA-PK, a possible target

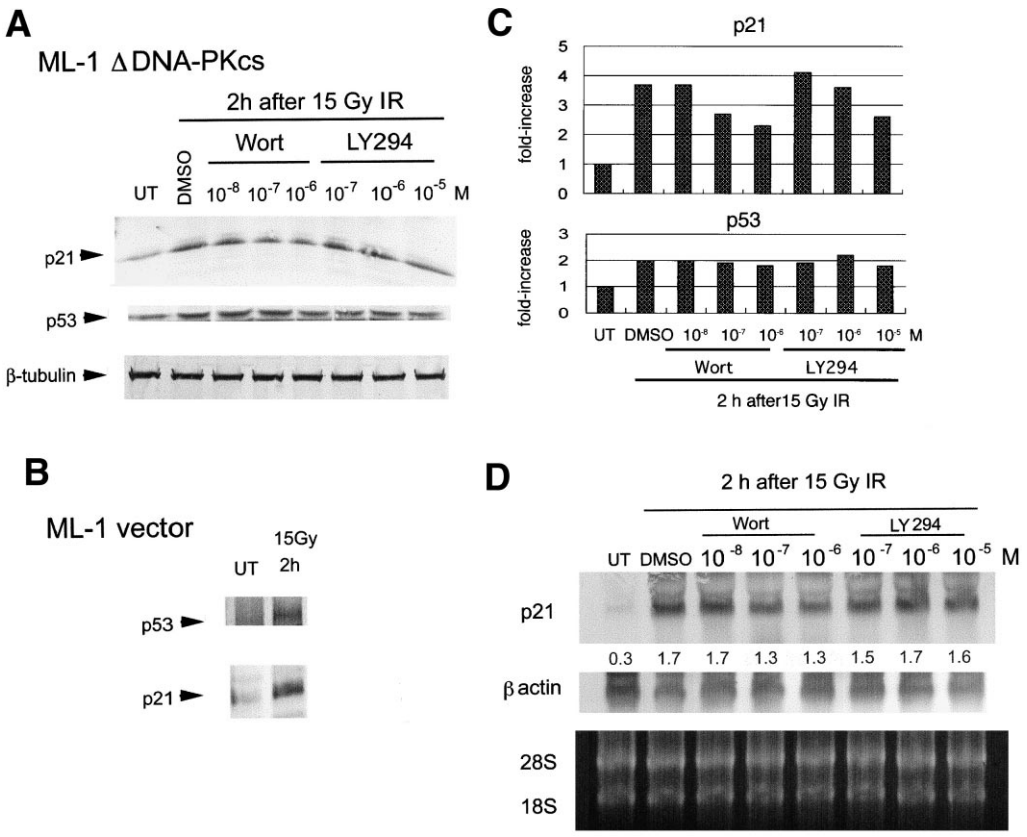


Fig. 5. γ-Irradiation-induced p21 was diminished by Wortmannin or LY294002 pretreatment in ML-1 ΔDNA-PKcs. (A) Exponentially growing ML-1 ΔDNA-PKcs cells were treated with $1\times10^{-8}$–$1\times10^{-6}$ M Wortmannin or $1\times10^{-7}$–$1\times10^{-5}$ M LY294002 for 30 min, irradiated with 15 Gy followed by a 2 h incubation. Cell lysates (30 μg) were electrophoresed on 10–20% SDS-PAGE and blotted onto PVDF membrane, then the blot was probed with either anti-p21 polyclonal antibody (C-19) or anti-p53 monoclonal antibody (DO-1). The blots were also probed with anti-β-tubulin monoclonal antibody (5H1) as an internal control. (B) Exponentially growing ML-1 vector cells were irradiated with 15 Gy followed by a 2 h incubation. Cell lysates (30 μg) were electrophoresed on 10–20% SDS-PAGE and blotted onto PVDF membrane, then the blot was probed with anti-p21 polyclonal antibody (C-19) or anti-p53 monoclonal antibody (DO-1). (C) The intensities of p21 and p53 bands in ML-1 ΔDNA-PKcs were quantified by densitometric scanning. The fold increase in protein expression, a comparison of the expression with that in untreated control cells, is indicated. (D) Total RNA was extracted from ML-1 ΔDNA-PKcs cells. Twenty micrograms of total RNA were subjected to electrophoresis on formaldehyde agarose gel, transferred to a Hybond $N^+$ membrane, and hybridized with digoxigenin-labeled probes. The level of expression was normalized by comparison with β-actin and indicated as a relative intensity. Ethidium bromide staining of total RNA is shown at the bottom of the figure. Experiments were repeated independently at least 3 times. Typical blots are shown in the figures. The relative expressions of both protein and mRNA were reproducible (within ±10%).

of Wortmannin or LY294002, is involved in p21 stabilization, we examined the IR-induced p21 expression using the SCID cell line, SC3VA4, whose DNA-PKcs is deleted (Fig. 3A). After treatment of SC3VA4 with either 15 Gy or 30 Gy IR, the expression level of p53, after either 2 or 4 h, was similar to untreated controls (Fig. 3B). In contrast, the level of p21 expression increased with both increasing IR dosage and time after treatment (Fig. 3B).

We investigated whether the inhibitory effect of Wortmannin or LY294002 on p21 expression in the NIH3T3 cells would also occur in the SCID cell. The IR-induced p21 expression in SC3VA4, 4.8-fold of control, was reduced by pretreatment of cells with either Wortmannin or LY294002 (Fig. 3C). The reduction appeared dependent upon inhibitor dosage with $1\times10^{-6}$ M Wortmannin reducing expression to 2.2-fold of control, and $1\times10^{-5}$ M LY294002 pretreatment reducing expression to control levels (Fig. 3C,D). The p21 protein level in cells pretreated with Wortmannin or LY294002 followed by a 2 h incubation without IR was unchanged (Fig. 3C).

These results suggest that p21 expression induced by DNA damage is sensitive to Wortmannin or LY294002 at the protein level, and the target of inhibition by those inhibitors is not DNA-PK. Therefore, DNA-PK is not required for stabilization of the p21 protein.

### 3.3. The induction of p21 expression by IR is not affected by DNA-PKcs targeting in ML-1 cells

In order to confirm that the target of inhibition by Wortmannin or LY294002 is not DNA-PK, we constructed a mammalian expression plasmid encoding antisense DNA-PKcs RNA driven by CMV immediate early promoter, pΔDNA-PKcs (Fig. 4A). After transfection of ML-1 cells with the plasmid, we examined the cells for specific antisense targeting of DNA-PKcs. The efficiency of targeting was determined by Western blot analysis using anti-DNA-PKcs antibody. DNA-PKcs expression, a 350 kDa protein, was not detectable in ML-1 cells transfected with pΔDNA-PKcs, designated ML-1 ΔDNA-PKcs (Fig. 4B).

We examined p21 expression in ML-1 ΔDNA-PKcs cells 2 h after 15 Gy IR. On Western blots, the induction of p21 in ML-1 ΔDNA-PKcs cells appeared similar to that of both ML-1 cells transfected with vector alone (ML-1 vector) and parental ML-1 cells (Figs. 1 and 5A,B). The expression of p53 was also similar in parental ML-1, ML-1 ΔDNA-PKcs and ML-1 vector cells (Figs. 1 and 5A,B). Next, we examined the effect of the Wortmannin or LY294002 pretreatment of ML-1 ΔDNA-PKcs on p21 and p53 expression. By Western blot analysis, the IR-induced p21 expression, 3.7-fold of control level, was diminished with increasing dosage of either Wortmannin or LY294002 pretreatment of ML-1 ΔDNA-PKcs (Fig. 5A,C). To determine whether the decrease of p21 occurred at the protein level, p21 mRNA expression was examined (Fig. 5D). Wortmannin pretreatment ($1\times10^{-7}$ M and $1\times10^{-6}$ M) reduced both p21 mRNA induction by IR to 77% of the p21 expression by IR-treated cells without inhibitors (Fig. 5D), and p21 protein expression from 3.7-fold to 2.7-fold (28% reduction) at $1\times10^{-7}$ M and to 2.3-fold (40% reduction) at $1\times10^{-6}$ M of control cells (Fig. 5C). In contrast, LY294002 pretreatment did not affect induction of p21 mRNA (Fig. 5D), but reduced p21 protein expression from 3.7-fold to 2.6-fold (40% reduction) at $1\times10^{-5}$ M (Fig. 5A,C). In the parent ML-1 cells, the p21 protein induction by IR was fully inhibited by $1\times10^{-6}$ M Wortmannin or $1\times10^{-6}$ M LY294002 (Fig. 1). Taken together, these results suggest that DNA-PK is not involved in p21 stabilization.

### 3.4. Involvement of the ATM gene product in p21 stabilization

Failure of IR to activate the $G_1$/S checkpoint in A-T cells is due to a defective p53 response [29,30]. In addition, it has been demonstrated that this downstream pathway, functioning through p21 CDK2, cyclin E and the retinoblastoma protein (RB), is defective in A-T cells [31,32]. We questioned whether the ATM gene product, another DNA damage-detecting

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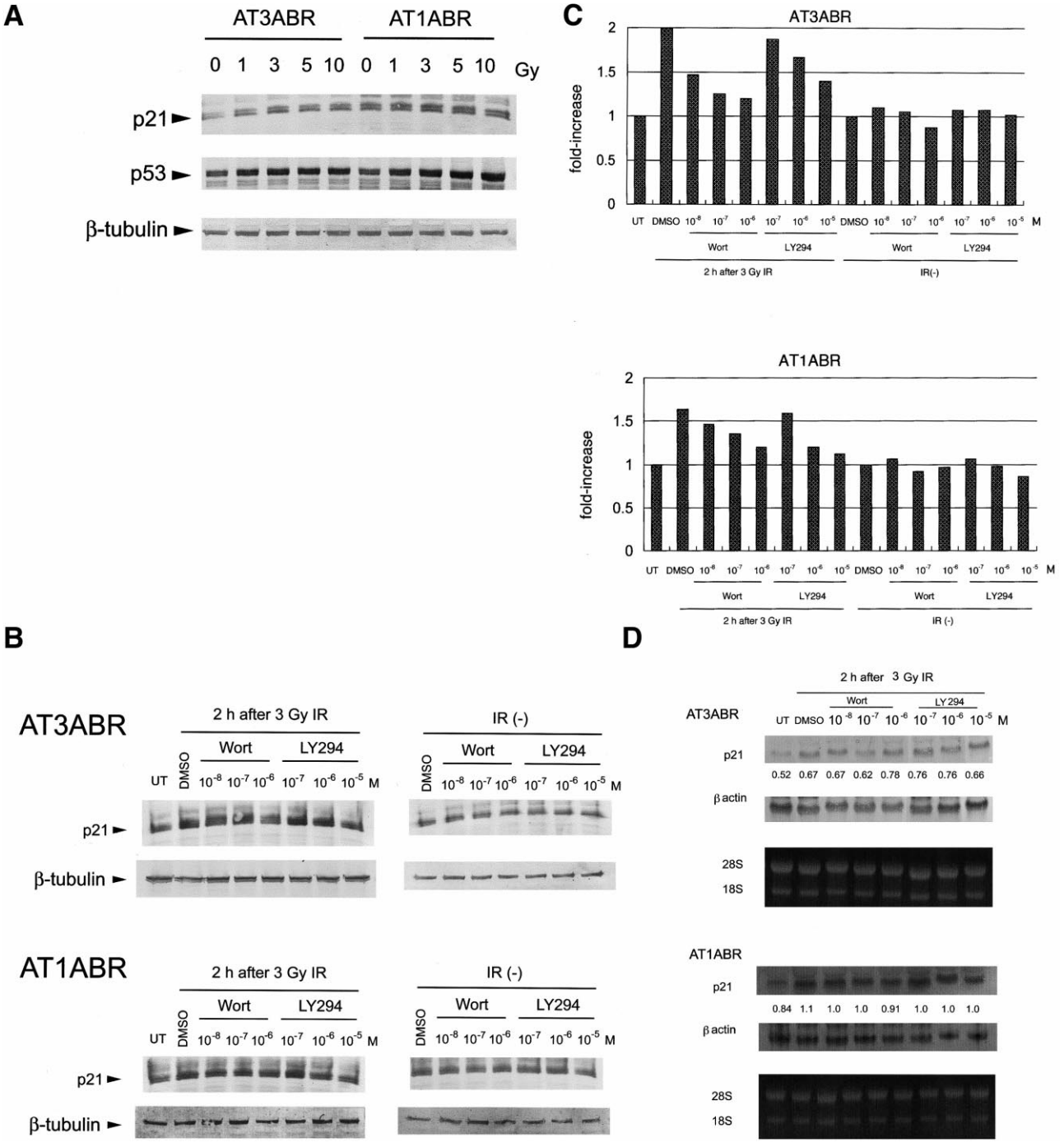
Fig. 6. γ-Irradiation-induced p21 was diminished by Wortmannin or LY294002 pretreatment in AT3ABR or AT1ABR. (A) p53 and p21 levels increased in response to γ-irradiation in A-T cell lines. Exponentially growing AT3ABR and AT1ABR cells were irradiated at the indicated dose followed by a 2 h incubation. Cell lysates (30 μg) were electrophoresed on 10–20% SDS-PAGE and blotted onto PVDF membrane, then the blot was probed with either anti-p21 polyclonal antibody (C-19) or anti-p53 monoclonal antibody (DO-1). The blots were also probed with anti-β-tubulin monoclonal antibody (5H1) as an internal control. (B) Exponentially growing AT3ABR or AT1ABR cells were treated with $1\times10^{-8}$–$1\times10^{-6}$ M Wortmannin or $1\times10^{-7}$–$1\times10^{-5}$ M LY294002 for 30 min, irradiated with 3 Gy followed by a 2 h incubation. Cell lysates (30 μg) were electrophoresed on 10–20% SDS-PAGE and blotted onto PVDF membrane, then the blot was probed with anti-p21 polyclonal antibody (H-164), or with anti-β-tubulin monoclonal antibody (5H1) as an internal control: top: AT3ABR; bottom: AT1ABR. (C) The intensities of p21 bands were quantified by densitometric scanning. The fold increase in protein expression, a comparison of the expression with that in untreated control cells, is indicated. (D) Total RNA was extracted from AT3ABR or AT1ABR cells. Twenty micrograms of total RNA were subjected to electrophoresis on formaldehyde agarose gel, transferred to a Hybond $N^+$ membrane, and hybridized with digoxigenin-labeled probes. The level of expression was normalized by comparison with β-actin and indicated as a relative intensity. Ethidium bromide staining of total RNA is shown at the bottom of the figure. Experiments were repeated independently at least 3 times. Typical blots are shown in the figures. The relative expressions of both protein and mRNA were reproducible (within ±10%).

enzyme, is involved in p21 stabilization. For this purpose, we used two EBV-transformed lymphoblastoid cell lines, AT1ABR and AT3ABR, derived from A-T patients. AT1ABR and AT3ABR have been shown by others to be devoid of ATM activity [24]. Western blot analysis of p53 and p21 expression in both cell lines, 2 h after 1–10 Gy IR, revealed an increase in the level of p53 expression by 1 Gy IR. Increasing p53 expression plateaued by 3 Gy IR (Fig. 6A). In addition, the level of p21 expression was



increased by 1 Gy IR and also plateaued 3 Gy IR (Fig. 6A). In these A-T cells, it is conceivable that p21 expression was induced, not through transactivation by p53 stabilized by the ATM gene product activity.

We examined whether the p21 expression is inhibited by pretreatment of cells with Wortmannin or LY294002. In AT3ABR cells, Wortmannin alone inhibited p21 protein expression, while LY294002 alone was less effective (Fig. 6B,C). When AT3ABR cells were subjected to 3 Gy IR after either Wortmannin or LY294002 pretreatment, the IR-induced p21, 1.6-fold of control, was diminished in a dose-dependent manner. Wortmannin at $10^{-6}$ M reduced p21 expression from 1.6-fold to 1.2-fold, while $10^{-5}$ M LY294002 reduced p21 expression from 1.6-fold to 1.1-fold (Fig. 6C). In AT1ABR cells, neither Wortmannin nor LY294002 pretreatment alone had any effect on p21 expression (Fig. 6B). However, the induction of p21 by 3 Gy IR, 2.0-fold of control, was clearly decreased by either Wortmannin or LY294002 pretreatment in a dose-dependent manner, with $10^{-6}$ M Wortmannin reducing p21 expression from 2.0-fold to 1.2-fold and $10^{-5}$ M LY294002 reducing p21 expression from 2.0-fold to 1.4-fold (Fig. 6C). In both cell lines, Wortmannin was more effective than LY294002 (Fig. 6B,C).

To confirm that the reduction of p21 expression in both AT1ABR and AT3ABR by either Wortmannin or LY294002 is due to the inhibition of protein stabilization, p21 mRNA expression was examined under these conditions. Induction of p21 mRNA by 3 Gy IR was observed in both AT1ABR and AT3ABR cells (Fig. 6D). The p21 mRNA induced by 3 Gy IR was not diminished by pretreatment with either Wortmannin or LY294002 in both cells.

In the present study, we used EBV-transformed A-T lymphoblastoid cells. Thus, p53 in these cells might form a complex with either EBNA5 or BZLF1 and could not function [33,34]. Hence, although IR induced p53 levels in both AT1ABR and AT3ABR cells, it is unclear that the increased p53 functions properly. Regardless of the status of p53, either Wortmannin or LY294002 pretreatment diminished the level of IR-induced p21 protein. Further, p21 mRNA was increased by IR and its level was not suppressed by either Wortmannin or LY294002 pretreatment. These results confirm that the expression of p21 was inhibited at the protein level by these inhibitors.

### 3.5. Conclusion

In this report, we demonstrate that the accumulation of p21 protein after irradiation is diminished at the protein level by pretreatment of cells with either Wortmannin or LY294002. Our data also indicate that neither DNA-PK nor the ATM gene product is the responsive enzyme for stabilization of p21. Taken together, these results suggest that the increase of p21 protein expression by DNA damage is accomplished by unidentified enzymes that are sensitive to either Wortmannin or LY294002. It is noteworthy that neither Wortmannin nor LY294002 is specific for the PI 3-kinase family alone. Their inhibitory effects are reported for other enzymes including the UV-stimulated activation of JNK kinase 1 [35], myosin light chain kinase [36], and bombesin-stimulated phospholipase $A_2$ activity [37]. We cannot tell whether the DNA damage targets p21 to inhibit either the association of E3 ligase or the apparatus for ubiquitination. Further experiments are planned to identify the target site on p21 by an in vitro ubiquitination competition assay using a peptide containing the possible phosphorylation site of p21.

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