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A truncated isoform of the PP2A B56γ regulatory subunit reduces irradiation-induced Mdm2 phosphorylation and could contribute to metastatic melanoma cell radioresistance

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Summary. F10, a subline of the B16 mouse melanoma cell line, is itself the parent of the more metastatic BL6 line. BL6 cells differ from F10 cells by an alteration of the gene encoding the B56γ regulatory subunit of protein phosphatase 2A (PP2A), which results in mRNA encoding a truncated variant of the subunit (Δγ1). Expression of Δγ1 protein is detectable only when BL6 cells are transplanted into mice and then γ-irradiated. Recently, B56γ subunit-containing PP2A holoenzymes have shown to dephosphorylate Mdm2, a negative regulator of p53. Thus, we assessed whether the expression of Δγ1 affects irradiation-induced phosphorylation of Mdm2 and radioresistance of melanoma cells by perturbing the regulation of p53. Western blot analyses revealed that irradiated COS-7 and NIH3T3 cells stably expressing Δγ1 showed significantly less irradiation-induced Mdm2 phosphorylation. Mdm2 phosphorylation reduces the ability of Mdm2 to target p53 for degradation, which probably explained why p53 protein levels in NIH3T3 cells expressing Δγ1 were not significantly elevated by irradiation, unlike in wild-type cells. This was also true for F10 cells transfected with Δγ1 ($F10^{\Delta\gamma1}$) when the cells expressed Δγ1 after being irradiated *in vivo*. p53 mRNA levels in irradiated wild-type and Δγ1-expressing cells were both only slightly elevated, suggesting that Mdm2 regulates p53 levels by a post-transcriptional mechanism. p53-mediated induction of the pro-apoptotic gene encoding Bax was also significantly lower in $F10^{\Delta\gamma1}$ cells irradiated *in vivo*. Moreover, $F10^{\Delta\gamma1}$ and BL6 cells were less apoptotic than F10 cells when the cells were irradiated *in vivo*. The p53 in F10 cells appears to be as functional as that in NIH3T3 cells because irradiation-induced expression of p53-target genes was comparable in both cells. Collectively, Δγ1 appears to reduce irradiation-induced Mdm2 phosphorylation, which then blocks irradiation-stimulated p53 accumulation. Defects, such as Δγ1, in PP2A may thus contribute to melanoma cell radioresistance.

Key words: B16 mouse melanoma, Apoptosis, p53, Bax

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Introduction

The sensitivity of tumor cells to DNA damage often depends on the status of the p53 tumor suppressor protein (Fisher, 1994; Lowe et al., 1994). p53 is a nuclear phosphoprotein that functions as a sequence-specific DNA-binding transcription factor and whose expression is induced by DNA damage (Levine, 1997). In response to DNA damage, p53 induces cell cycle arrest or apoptosis (Kastan et al., 1991; Kuerbitz et al., 1992; Lowe et al., 1993; Clarke et al., 1993). To fulfill this role, p53 becomes active by elevating its protein levels and being phosphorylated and dephosphorylated on particular amino acid residues. Many human tumors have accumulated mutations in their genomes that allow the tumors to resist p53-dependent apoptosis. The most common mutations occur in the p53 gene itself, resulting in the expression of inactive p53 protein that can not induce apoptosis in response to DNA damage (Levine, 1997).

Melanoma cells typically express wild-type p53 (Volkenandt et al., 1991; Weiss et al., 1993; Albino et al., 1994; Lubbe et al., 1994; Montano et al., 1994) and would therefore be expected to be sensitive to the DNA-damaging agents used for cancer therapy. However, most melanoma cells are actually extremely resistant to radiation (Jenrette, 1996; Geara and Ang, 1996). Moreover, it appears that as melanoma cells progress to

increased levels of malignancy, the tumors become more radioresistant (Rofstad, 1992). Although these phenotypes have significant implications in treating melanoma with ionizing radiation, the molecular bases underlying melanoma radioresistance are still not fully understood.

The B16 mouse melanoma cell line has given rise to a variety of sublines with differing metastatic properties (Hart, 1979; Poste et al., 1980). Of these, the BL6 subline, which was selected from the F10 subline, is one of the most malignant (Poste et al., 1980). While both the BL6 and F10 cells metastasize to the lungs after being injected intravenously into mice, BL6 cells can metastasize to the lungs even after being injected subcutaneously (Poste et al., 1980). We analyzed the difference in gene expression between the two sublines, and found that in the BL6 cells, a retrotransposon had been inserted into an intronic region of the gene encoding the B56γ regulatory subunit of protein phosphatase type 2A (PP2A) (Nakaji et al., 1999; Kataoka et al., 2000; Ito et al., 2000a,b; Nakamoto et al., 2001; Watabe et al., 2001).

PP2A consists of a series of serine/threonine phosphatase holoenzymes that are composed of a common dimeric core of invariable catalytic (C) and structural (A) subunits associated with a variable regulatory (B) subunit (Usui et al., 1988). The regulatory subunit is extremely diverse because it is constituted by members from at least three unrelated families, namely, PR55 (or simply B), B56 (B'), and PR72 (B'') (Virshup, 2000). Each of these families in turn consists of several subfamilies, each of which contains several proteins resembling each other structurally. For example, the B56γ subfamily belongs to the B56 family and consists of three alternative splicing isoforms, B56γ1, γ2 and γ3. In BL6 cells, the rearrangement of the gene encoding the B56γ regulatory subunit resulted in the abundant expression of a chimeric mRNA in which the 5' part of the original B56γ subunit mRNA had been replaced with the retrotransposon sequence (Ito et al., 2000a). The chimeric mRNA thus encodes a mutant protein, termed Δγ1, that lacks the N-terminal 65 amino acid residues of B56γ1 (Ito et al., 2000a).

It has long been known that PP2A plays an important role in cell growth regulation (Yamashita et al., 1990; Kawabe et al., 1997). However, it is not clear how PP2A participates in this and which of the PP2A holoenzymes are involved. Recently, Okamoto et al. (2002) provided the evidence that the B56γ subunit-containing PP2A holoenzymes might be involved in cell growth control by regulating the phosphorylation of Mdm2, a key negative regulator of p53 (Oliner et al., 1993; Haupt et al., 1997; Kubbutat et al., 1997; Thut et al., 1997). They also suggested that this type of holoenzymes dephosphorylates Mdm2 on the Thr216 residue (Okamoto et al., 2002). Dephosphorylated Mdm2 on this residue can bind to p53 and thereby blocks the ability of the p53 protein to activate transcription (Oliner et al., 1993; Thut et al., 1997). The binding of Mdm2 also promotes the degradation of the p53 protein (Haupt et al., 1997; Kubbutat et al., 1997). Mdm2 is thus considered to be important in keeping p53 activity very low under non-stressed conditions. A separate series of reports have shown that when DNA damage occurs, Mdm2 becomes phosphorylated on another residue, namely, Ser395, and that this phosphorylation also counteracts the ability of Mdm2 to target p53 for degradation (Khosravi et al., 1999; Maya et al., 2001; Zhang et al., 2001). Thus, there are two separate phosphorylation events that attenuate the ability of Mdm2 to bind to p53, namely, phosphorylation on Ser395 and Thr216. The B56γ-containing PP2A holoenzymes may play crucial roles in these events as they may block the kinases that phosphorylate Mdm2.

We have shown previously that Δγ1 inhibits the ability of the B56γ-containing PP2A holoenzyme to dephosphorylate its targets (Ito et al., 2000a) and thus it is possible that Δγ1 in BL6 cells may lead to the aberrant phosphorylation of Mdm2 in response to DNA damage. We examined this possibility in the present study and found that in COS-7 and NIH3T3 cells that express Δγ1 exogenously, Mdm2 does not become fully phosphorylated on its Thr216 and Ser395 residues in response to γ-irradiation. This difference was also associated with a reduction in p53 accumulation after γ-irradiation. Moreover, F10 cells expressing Δγ1 and BL6 cells were both less apoptotic than F10 cells when the cells were transplanted into mice and then γ-irradiated. These observations support the notion that Δγ1 can contribute to melanoma radioresistance by perturbing the regulation of p53 by Mdm2.

Materials and methods

Plasmid construction

The *Asc*I-*Not*I cDNA fragments of Δγ1 has been described previously (Ito et al., 2000a). The pCX4bsr vector is derived from the pCXbsr vector (Akagi et al., 2000) with a minor modification, and was a kind gift from Dr. T. Akagi (Osaka Bioscience Institute, Osaka, Japan). The pCX4bsr vector was digested with *Eco*RI, blunted with T4 DNA polymerase, digested with *Not*I, and ligated with the *Asc*I-*Not*I cDNA fragment of Δγ1 that had been blunted at its 5' end by T4 DNA polymerase (pCX4bsr-Δγ1).

Antibodies

The production and specificity of the anti-B56γ Ab has been described previously (Ito et al., 2000a, 2003b). Other primary Abs included those specific for Mdm2 (2A10; Oncogene, Boston, MA, and SMP14; Sigma, St. Louis, MO), p53 (PAb240; Oncogene), and α-tubulin (DM 1A; Sigma). Peroxidase-labeled secondary Abs included anti-mouse IgG_1 Ab (A85-1; Pharmingen, San Diego, CA), anti-rabbit IgG Ab (MBL Co. Ltd., Nagoya, Japan), and anti-mouse IgG Ab (MBL Co. Ltd.).

Western blot analysis

To detect p53, cells and tumor masses were lysed in RIPA buffer [50 mM Tris-HCl (pH7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride]. In other cases, cells were lysed in a buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride. Equal quantities of cell lysates were denatured in SDS gel loading buffer (Takara, Ohtus, Japan), separated on 10% SDS-acrylamide gels, transferred to Immobilon (Millipore, Bedford, MA), and reacted with the primary Ab indicated. After washing, the blots were incubated with peroxidase-labeled secondary Ab and then reacted with Renaissance reagents (NEN, Boston, MA) before exposure.

Cell culture and transfection

The B16 melanoma sublines F10 and BL6 were kindly provided by Dr. I.J. Fidler (University of Texas). NIH3T3 mouse fibroblastic cells and COS-7 monkey kidney cells were purchased from the American Type Culture Collection (Manassas, VA). All cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. COS-7 cells were transfected with either the empty pCX4bsr vector or the pCX4bsr-Δγ1 construct by using the Fugene 6 transfection regent (Roche Diagnostics Co. Ltd., Indianapolis, IN) according to the manufacturer's instructions. After transfection, cells were selected by resistance to blasticidin (3 μg/ml; Invitrogen, Carlsbad, CA) for 3 weeks to obtain single colonies. The transfectant subclones of F10 and NIH3T3 cells used in the experiments here have been described previously (Ito et al., 2000a).

γ-irradiation and tumor transplantation

Cells growing exponentially in culture dishes were γ-irradiated using a ^{137}Cs AECL Gamma cell 40 γ-irradiator (Nordion, Ottawa, Ont, Canada) at a dose rate of 1.1 Gy/minute. To irradiate F10 and its subclone cells growing *in vivo*, $1x10^5$ cells were injected subcutaneously into the right footpad of a 4-week-old male C57BL/6 mouse. Tumor size was monitored by measuring the anteroposterior diameter by calipers every day. On the day the footpad tumors reached a diameter of 4 mm, the tumor-bearing mice were anesthetized and placed in a lead box that shields the entire trunk and head. The right foot was extended outside through a hole in the side of the box. Radiation was delivered over the box with a collimated beam in a Radioflex 350 irradiator (Rigaku Co. Ltd., Osaka, Japan) at a dose rate of 1.5 Gy/minute. At the indicated time points, tumor cell masses were removed from the footpads by cutting out the skin tissue.

In situ detection of DNA fragmentation

Tumor cell masses were removed from the footpads by cutting out the skin tissue, which was then fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm thick slices. DNA fragmentation was detected with the fluorescein-based TUNEL assay using the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, sections were stained with the TUNEL regent and PI and observed through a 40x objective of a confocal laser scanning microscope (LSM510; Carl Zeiss, OberKochen, Germany). The high-power field contained about 100 tumor cells. After images were created on the computer monitor of the LSM510 system, sections were washed with distilled water and stained with hematoxylin and eosin (H & E). To quantify the apoptosis of the tumor cells, the areas on the sections with a relatively high density of tumor cells were observed. Tumor samples were obtained from two individual mice before and after irradiation, and 10 randomly selected high-power fields were examined in each sample. The proportions of TUNEL-positive cells to the total cell number were pooled and the mean and standard error (SE) were calculated. All experiments were repeated twice.

Northern blot analysis

Northern blotting and hybridization was performed using standard methods. Total RNA was prepared from cultured cells using 3 M lithium chloride and 6 M Urea, and from tumor tissues by the guanidine thiocyanate/cesium method. For probe preparation, a 500-base pair region of the $p21^{Waf1}$ cDNA was polymerase chain reaction (PCR)-amplified using the following set of primers: sense, 5'-ATTGGAGTCAGGC GCAGATCCACAG-3'; antisense, 5'-GGGCACTTCA GGGTTTTCTCTTGC-3'. The PCR-amplified cDNA fragment was labeled with [α-^{32}P] dCTP by the random labeling method. Preparation of the p53 (Tsujimura et al., 1997), Bax (Tsujimura et al., 1997), cyclin G1 (Kimura et al., 1997) and ß-actin (Tsujimura et al., 1997) probes have been described previously. Signal intensity was quantified with the BAS 2000 system (Fuji Photo Film Co., Tokyo, Japan) to compare Bax mRNA levels after irradition. Relative intensities of Bax signals were obtained by dividing intensities of Bax signals by those of ß-actin signals and were expressed as relative values to the Bax signal intensity on 0 day of irradition. Experiments were repeated three times to calculate the mean and SE of relative intensities of Bax signals at each time point.

Sequencing of p53 cDNA

The total RNA from F10 cells (2 μg) was reverse-transcribed in a reaction mixture containing 200 U of Superscript II (Invitrogen) and 0.2 μg of oligo (dT)

primer. The resulting first strand cDNA was used as a template in a PCR reaction to amplify three overlapping cDNA fragments of p53: Fragment I containing nucleotides (nts) 77 to 571 [numbering is according to the deposited sequence of the mouse p53 mRNA (GeneBank accession no. X01237)], Fragment II containing nts 420 to 980, and Fragment III containing nts 854 to 1423. 25-nt-long primers annealing to the 5'- and 3'-ends of the three fragments were synthesized and used in PCR consisting of 30 cycles of denaturation at 94 °C for 30 seconds, reannealing at 55 °C for 30 seconds, and polymerization at 72 °C for 1 minute. PCR-amplified DNA fragments were purified with columns containing Bio-Gel P30 (Bio-Rad Loboratories, Hercules, CA), mixed with the regents of the BigDye DNA sequencing kit (Applied Biosystems, Foster City, CA), and then subjected to PCR according to the manufacturer's instructions. Reaction products were analyzed with the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). In another experiment, PCR-amplified Fragment II was subcloned into the pBluescript II vector (Startagene, La Jolla, CA). Plasmids from independent colonies were purified with an automated plasmid purification machine (PI-100; Kurabo, Osaka, Japan) and subjected to the same sequencing reaction described above.

Results

Δγ1 blocks irradiation-induced Mdm2 phosphorylation

To determine whether Δγ1 influences the phosphorylation status of Mdm2 before and after γ-irradiation, we stably expressed the exogenous Δγ1 protein in COS-7 and NIH3T3 cells (COS-7$^{\Delta\gamma1}$ and NIH3T3$^{\Delta\gamma1}$). In these cells, the expression of Δγ1 did not alter the expression levels of the endogenous B56g isoforms (Fig. 1A and data not shown). Similar results were shown by cells transfected with the vector only (COS-7^{Vec} and NIH3T3^{Vec}). The logarithmically growing cells were γ-irradiated and cell lysates were prepared at several time points later and immunoblotted with two antibodies (Abs) against Mdm2, 2A10 and SMP14. The 2A10 (Khosravi et al., 1999) and SMP14 (Maya et al., 2001) Abs do not recognize the forms of Mdm2 in which Ser395 and Thr216 is phosphorylated, respectively. Before γ-irradiation, the untransfected COS-7, COS-7^{Vec} and COS-7$^{\Delta\gamma1}$ cells all reacted equivalently with the 2A10 Ab but the COS-7$^{\Delta\gamma1}$ cells reacted more prominently with the SMP14 Ab (Fig. 1B; 0 h). This suggests that the Thr216 residue of Mdm2 in the COS-7$^{\Delta\gamma1}$ cells is constitutively poorly phosphorylated.

Two hours after γ-irradiation, COS-7 and COS-7^{Vec} cell lysates showed a greatly reduced reactivity with 2A10, indicating rapid and heavy phosphorylation of Mdm2 on Ser395 (Fig. 1B). The 2A10 reactivity was restored to its original levels by 10 hours after irradiation. In contrast, the COS-7$^{\Delta\gamma1}$ cell lysates showed only a small attenuation of 2A10 reactivity, indicating minimal phosphorylation of Mdm2 on Ser395 (Fig. 1B). Furthermore, the 2A10 reactivity was restored to the 0 hour levels as early as 4 hours after irradiation.

With regard to the SMP14 Ab, the reactivity of the COS-7 and COS-7^{Vec} cells was reduced by half after 2 hours of γ-irradiation, and restored to the original level within 10 hours (Fig. 1B). In contrast, the reduction in the reactivity of irradiated COS-7$^{\Delta\gamma1}$ cells with SMP14 was quite small, indicating minimal phosphorylation of Mdm2 on Thr216 (Fig. 1B). These observations were reproduced when NIH3T3 transfectant cells were used (data not shown). Thus, Δγ1 appears to block the irradiation-induced phosphorylation of Mdm2 on its Thr216 and Ser395 residues.

Δγ1 expression is associated with reduced irradiation-induced accumulation of p53

Phosphorylation of Mdm2 on Ser395 abrogates the binding of Mdm2 to p53, which is then no longer targeted for destruction and can raise its levels to respond appropriately to γ-irradiation (Khosravi et al., 1999; Maya et al., 2001). As Δγ1 appears to block Ser395 phosphorylation, it may therefore also reduce the normal elevation of p53 levels in response to γ-

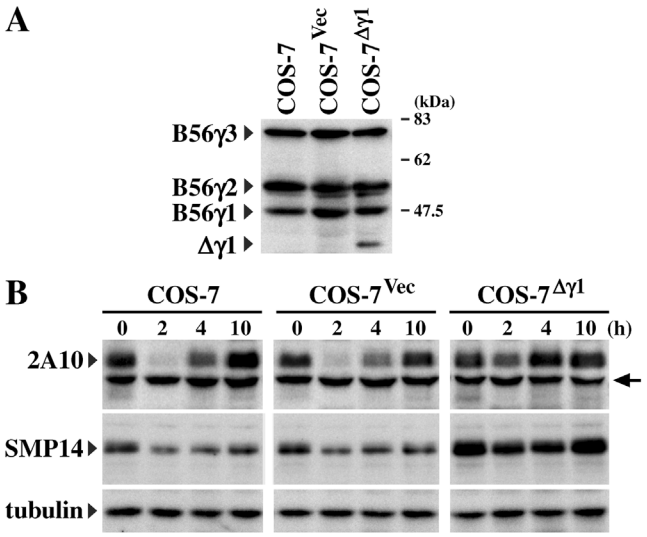

Fig. 1. Western blot analyses of COS-7 transfectant cells. **A.** Establishment of a COS-7 subclone stably expressing Δγ1. Cell lysates from COS-7 and its transfectants COS-7^{Vec} and COS-7$^{\Delta\gamma1}$ were separated on a 10% SDS-acrylamide gel and blotted with the anti-B56γ Ab. **B** Irradiation-induced phosphorylation of Ser395 and Thr216 on Mdm2 is reduced in COS-7$^{\Delta\gamma1}$ cells. COS-7 and its transfectants were grown in culture and γ-irradiated. Lysates were prepared from these cells at the time points after irradiation indicated (hour) and blotted with the 2A10 or SMP14 Ab. After stripping, the blot was probed with the anti-α-tubulin Ab to reveal the total amount of protein loaded per lane. Arrowheads indicate the specific signals and an arrow indicates nonspecific bands.

irradiation. We examined this issue with γ-irradiated logarithmically growing NIH3T3 and NIH3T3$^{\Delta\gamma1}$ cells. Denatured lysates from these cells were prepared at several time points after irradiation and immunoblotted with the PAb240 Ab, which is reactive with wild-type p53 under denaturing conditions (Gannon et al., 1990; Stephen and Lane, 1992). The PAb240 Ab reactivity of the denatured NIH3T3 cell lysates increased gradually after γ-irradiation, peaking at 24 hours and then declining slightly by 36 hours (Fig. 2A). Irradiated NIH3T3^{Vec} cells followed a similar pattern of PAb240 Ab reactivity (data not shown). In contrast, the PAb240 Ab reactivity of the denatured lysate of NIH3T3$^{\Delta\gamma1}$ cells showed a small increase 3 hours after irradiation and remained largely unchanged thereafter (Fig. 2A). Thus, Δγ1 expression reduces the irradiation-induced accumulation of p53.

We also examined changes in the mRNA levels of the p53 gene after irradiation. In both the NIH3T3 and NIH3T3$^{\Delta\gamma1}$ cells, p53 gene expression increased slightly 4 and 8 hours after irradiation but returned to the original levels by 24 hours (Fig. 2B). This indicates that the reduced irradiation-induced accumulation of p53 protein in NIH3T3$^{\Delta\gamma1}$ cells is due to a post-transcriptional event.

Δγ1 reduces p53 accumulation in irradiated F10 cells

We then assessed whether expressing Δγ1 in F10 cells would also reduce the irradiation-induced elevation of p53 protein levels. We transfected F10 cells with Δγ1 cDNA and obtained several stable clones, three of which were examined. All three showed essentially similar results and the results obtained with one clone (denoted F10$^{\Delta\gamma1}$) are shown in this paper. A vector-control F10 clone (F10^{Vec}) was also examined. When F10$^{\Delta\gamma1}$ cells are grown *in vitro*, the expression levels of the Δγ1 protein are quite low, as we reported previously (Ito et al., 2003a). However, when these cells are grown *in vivo* and then γ-irradiated, Δγ1 protein levels are significantly increased around 2 days after irradiation (Fig. 3A) (Ito et al., 2003a). This pattern of expression was also observed in BL6 cells (Fig. 3A). We thus compared the changes in p53 protein levels in the F10, F10^{Vec} and F10$^{\Delta\gamma1}$ cells after these cells had been transplanted into mice by subcutaneous injection into the mouse footpads. After 14 days, when the tumors had grown to about 4 mm in diameter, the footpads were irradiated by a single dose of 12 Gy, a dose that is sublethal for subcutaneous tumors of F10 cells (Nathanson et al., 1989). Denatured lysates from the primary tumor tissues were prepared at several time points after irradiation, and immunoblotted with the PAb240 Ab (Fig. 3B). In the F10 tumors, PAb240 reactivity increased gradually after γ-irradiation and peaked on day 3 (Fig. 3B). By day 7, this reactivity had declined markedly. The F10^{Vec} tumors showed an essentially similar pattern of PAb240 reactivity (data not shown). In contrast, the F10$^{\Delta\gamma1}$ tumors did not show an increase in p53 protein levels in the first 4 days after irradiation, although a transient increase was observed on day 5 (Fig. 3B). When p53 mRNA levels in the F10

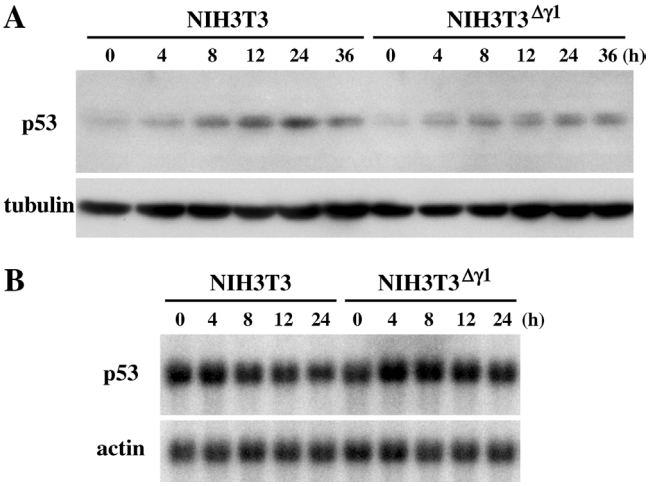

Fig. 2. p53 protein and mRNA levels in irradiated NIH3T3 transfectant cells. **A.** Irradiation-induced elevation of p53 protein levels is reduced in NIH3T3$^{\Delta\gamma1}$ cells. NIH3T3 and NIH3T3$^{\Delta\gamma1}$ cells were grown in culture and γ-irradiated. Lysates were prepared from these cells at the time points after irradiation indicated (hour) and blotted with the p53 (PAb240) Ab. After stripping, the blot was probed with the anti-α-tubulin Ab to reveal the total amount of protein loaded per lane. **B.** NIH3T3 and NIH3T3$^{\Delta\gamma1}$ cells do not differ in p53 mRNA expression after irradiation. Total RNA was prepared from irradiated cells at several time points and 10 μg was electrophoresed on 1% agarose gels. Blots were hybridized with p53 or ß-actin probes.

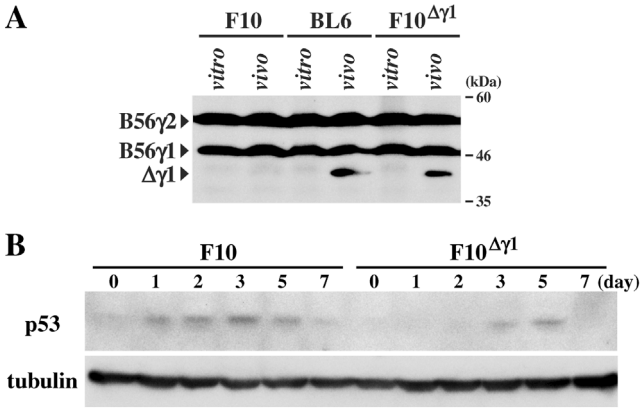

Fig. 3. Western blot analyses of F10, F10$^{\Delta g1}$, and BL6 cells. **A.** F10$^{\Delta\gamma1}$ cells express Δγ1 protein only when they are grown *in vivo*. Lysates prepared from cultured F10, BL6 and F10$^{\Delta\gamma1}$ cells (*vitro*) or from the *in vivo*-grown cells 2 days after they received γ-irradiation (*vivo*) were blotted with the anti-B56γ Ab. **B.** Irradiation-induced elevation of p53 protein levels is reduced in F10$^{\Delta\gamma1}$ tumor cells. F10 and F10$^{\Delta\gamma1}$ cells were transplanted into mice, γ-irradiated and lysates were prepared from the primary tumor masses at several time points after irradiation (day) and blotted with the p53 (PAb240) Ab. After stripping, the blot was probed with the anti-α-tubulin Ab to reveal the total amount of protein loaded per lane.

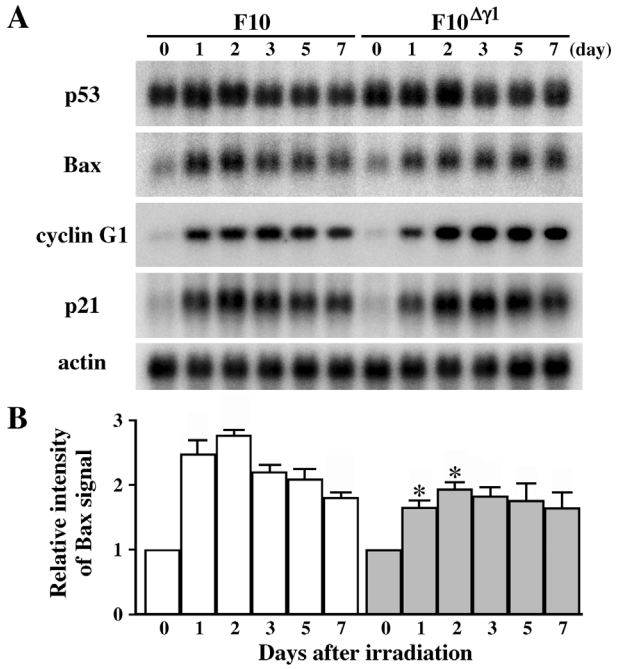

Fig. 4. Northern blot analyses of irradiated F10 and F10$^{\Delta\gamma1}$ cells. **A.** Changes in mRNA expression of p53 and its transcriptional target genes after γ-irradiation. F10 and F10$^{\Delta\gamma1}$ cells were transplanted into mice, γ-irradiated and 10 μg of total RNA was prepared from the tumor masses at several time points (day) after irradiation and electrophoresed on 1% agarose gels. Blots were hybridized with probes to p53, Bax, cyclin G1, p21^{Waf1}, or ß-actin. **B.** Irradiated F10$^{\Delta\gamma1}$ cells show reduced induction of Bax mRNA expression. The intensities of the hybridization signals with the Bax probe in **A** were quantified densitometrically and normalized against the intensity of the hybridization signal on 0 day. Experiments were repeated three times and the mean values of F10 (white) and F10$^{\Delta\gamma1}$ (gray) tumors were plotted with bars indicating SE. *: P <0.05 by t-test when compared with the values of F10 tumors.

and F10$^{\Delta\gamma1}$ tumors were measured, a similar slight increase was observed in both tumors after γ-irradiation (Fig. 4A). Thus, the irradiation-induced accumulation of p53 is also reduced in F10$^{\Delta\gamma1}$ tumors and appears to be due to a post-transcriptional event.

Apoptosis is decreased in F10$^{\Delta\gamma1}$ cells

It is possible that the reduced irradiation-induced accumulation of p53 in F10$^{\Delta\gamma1}$ tumors results in the reduced apoptosis of these tumors after γ-irradiation. We tested this by transplanting F10, BL6 and F10$^{\Delta\gamma1}$ cells into mice and γ-irradiating the primary tumor. Tumor tissues were excised from the footpads before and 4 days after the irradiation and then stained with terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) and propidium iodide (PI). As PI stains the nuclei of all cells red and TUNEL stains apoptotic nuclei green, apoptotic cells can be identified as the cells containing yellow nuclei (Fig. 5). Before irradiation, apoptotic cells were rarely detectable in any of the tumor cells (data not shown). However, 4 days after γ-irradiation, 16.4% of F10 cells were apoptotic while only a few percent of BL6 and F10$^{\Delta\gamma1}$ cells were apoptotic (Fig. 5).

The Bax gene in irradiated F10$^{\Delta\gamma1}$ cells is poorly transactivated

We examined the expression of several transcriptional target genes of p53 in irradiated F10 and F10$^{\Delta\gamma1}$ tumors. In the irradiated F10 cells, the mRNA levels of the genes encoding Bax (Levine, 1997), cyclin G1 (Okamoto et al., 1994) and p21^{Waf1} (Levine, 1997) were greatly increased on the day after irradiation and peaked 1 or 2 days later (Fig. 4A). In the irradiated

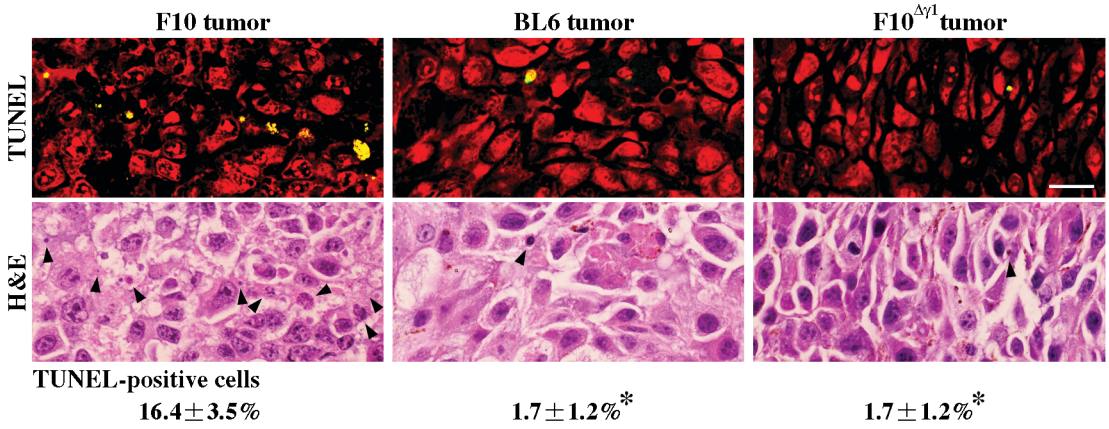

Fig. 5. *In situ* detection of DNA fragmentation in footpad tumors derived from F10, BL6 and F10$^{\Delta\gamma1}$ cells. Sections of tumor tissues taken 4 days after irradiation were stained with the fluorescein-based TUNEL reagent and PI and observed with the LSM510 microscope (**upper panels**). TUNEL-positive cells contain yellow nuclei. After washing, the same sections were stained with H&E (**lower panels**). Arrowheads indicate TUNEL-positive cells in the upper panels. The mean proportions of TUNEL-positive cells appear under the histological images. *: P <0.05 by t-test when compared with the value of F10 tumors. Bar: 50 mm (original magnification, x 400).

F10$^{\Delta\gamma1}$ tumors, while p21^{Waf1} and cyclin G1 gene expression was similar to that in F10 tumors (Fig. 4A), Bax gene expression seemed somewhat poor (Fig. 4A). We quantified the degree of Bax gene induction by densitometric analysis of the band intensity and found that in F10 tumors, Bax gene expression increased 2.8-fold 2 days after irradiation, after which expression declined to 1.7-fold by 7 days (Fig. 4B). In contrast, F10$^{\Delta\gamma1}$ tumors showed at most a 1.8-fold increase in Bax gene expression in the 7 days after irradiation.

F10 cells appear to possess a functional form of p53

To determine whether the p53 gene in F10 cells is properly functional, we first assessed whether the p21^{Waf1}, Bax and cyclin G1 target genes of p53 are equally transcriptionally upregulated in F10 and F10$^{\Delta\gamma1}$ cells when they are grown *in vitro* and then γ-irradiated. In these growth conditions, Δγ1 expression is undetectable in F10$^{\Delta\gamma1}$ cells (Fig. 3A) and thus should have no effect on p53 transactivation activity. In both cell types, the expression of all three genes occurred as early as 4 hours after irradiation and continued up to 24 hours (Fig. 6A). A similar induction profile was observed when NIH3T3 cells were grown *in vitro* and then γ-irradiated (Fig. 6A). Thus, the p53 in F10 cells seemed functionally normal.

We then determined the p53 genotype of F10 cells by sequencing the coding region of its p53 cDNA. The first nucleotide of codon 128 showed a major (A) and a minor (G) peak (Fig. 6B). The former agrees with the sequence of wild-type p53 and the latter results in the substitution of Asn128 to Asp (N128D). Thus, F10 cells bear one normal and one mutated p53 gene but it appears that the mutated gene is less frequently expressed. To assess whether the wild-type message is indeed predominantly expressed, an approximately 500-nt-long fragment of the p53 cDNA containing the N128D mutation was PCR-amplified and subcloned into a plasmid vector. Sequencing of the twenty independent clones revealed that only 3 clones (15%) carried the N128D mutation. F10 cells thus appear to predominantly express wild-type p53.

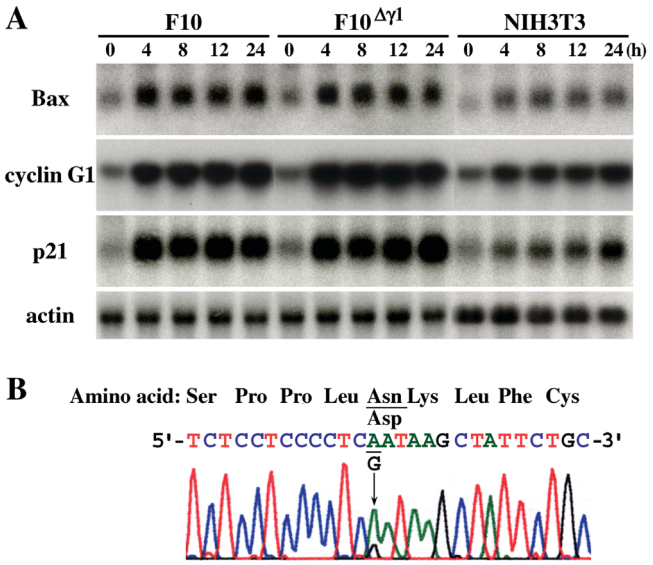

Fig. 6. p53 appears to be functional in F10 cells. **(A)** Irradiated *in vitro* grown F10, F10$^{\Delta\gamma1}$ and NIH3T3 cells show equivalent changes in mRNA expression of the p53 gene and its transcriptional target genes. F10, F10$^{\Delta\gamma1}$ and NIH3T3 cells were grown in culture, γ-irradiated, and 10 μg of total RNA was prepared from these cells at several time points (hour) after irradiation and electrophoresed on a 1% agarose gel. Blots were hybridized with probes to Bax, cyclin G1, p21^{Waf1} and ß-actin. **(B)** Sequencing of the p53 cDNA derived from F10 cells. Total RNA from F10 cells were reverse-transcribed and the resulting first strand cDNA was used as a template of PCR to amplify the coding region of p53 cDNA. PCR-amplified cDNA fragments were sequenced with the ABI Prism 3100 Genetic Analyzer. The first nucleotide of codon 128 was represented by one major (A) and one minor (G) peak. The former peak agrees with the sequence of wild-type p53 while the latter results in the substitution of Asn128 to Asp.

Discussion

In the present study, we assessed whether expression of Δγ1 affects the irradiation-induced phosphorylation of Mdm2 and whether this could contribute to the increased radioresistance of melanoma cells. This working hypothesis was inspired by the recent reports showing that the B56γ-containing PP2A holoenzyme dephosphorylates Mdm2 on the Thr216 residue (Okamoto et al., 2002). As the dephosphorylated Mdm2 protein can then bind to p53 and thereby target p53 for degradation and prevent it from inducing apoptosis (Oliner et al., 1993; Haupt et al., 1997; Kubbutat et al., 1997; Thut et al., 1997; Khosravi et al., 1999; Maya et al., 2001; Zhang et al., 2001), we speculated that Δγ1 may block irradiation-induced Mdm2 phosphorylation, thus downregulating p53 apoptotic activity, and enhancing the cell's resistance to irradiation.

We firstly examined Mdm2 phosphorylation in COS-7 and NIH3T3 cells that stably express Δγ1. This is because Δγ1 protein expression in BL6 and F10$^{\Delta\gamma1}$ cells is detectable only when they are grown *in vivo* (Ito et al., 2003a). We found that irradiation-induced phosphorylation on the Ser395 and Thr216 residues of Mdm2 was observed in vector-transfected and wild type COS-7 and NIH3T3 cells but not in those expressing Δγ1. Furthermore, a lower level of constitutive Thr216 phosphorylation was observed in the non-irradiated Δγ1-transfected cells. Thus, the B56γ-containing PP2A holoenzymes may be involved in regulating Mdm2 phosphorylation both in normal situations and after irradiation and Δγ1 may stimulate this activity, leading to greater overall dephosphorylation of Mdm2. We have previously found that Δγ1 prevents B56γ-containing PP2A holoenzymes from dephosphorylating the paxillin cytoskeletal molecules (Ito et al., 2000a), which is a reverse effect as compared to the observation here that Δγ1 activates B56γ-containing PP2A holoenzymes. It

seems that the influence of Δγ1 on B56γ-containing PP2A holoenzymes differs with the substrate being examined.

We found that p53 levels, which are normally elevated by irradiation, were only marginally raised in γ-irradiated NIH3T3$^{\Delta\gamma1}$ cells. This may be attributed to the poor phosphorylation of Mdm2 on Thr216 and Ser395 in the presence of Δγ1. We showed here that when cells are γ-irradiated, Mdm2 becomes normally phosphorylated on, at least, these two amino acid residues (Fig. 1). Such phosphorylation results in the elevation of p53 levels, because Mdm2 phosphorylated on Thr216 can no longer bind to p53 (Zhang et al., 2001), and Mdm2 phosphorylated on Ser395 loses its ability to target p53 for degradation (Khosravi et al., 1999; Maya et al., 2001). When Δγ1 was present, however, the phosphorylation of Mdm2 on Thr216 and Ser395 was abrogated and thus the Mdm2 in irradiated Δγ1-bearing cells seemed still highly potent to promote the degradation of p53 protein levels (Figs. 1, 2). When we measured the p53 mRNA levels in γ-irradiated NIH3T3$^{\Delta\gamma1}$ cells, we found that there was only a small increase that was also seen in irradiated untransfected NIH3T3 cells. This suggests that the significant accumulation of p53 protein levels in irradiated NIH3T3 cells and the poorer accumulation in irradiated NIH3T3$^{\Delta\gamma1}$ cells are due to a post-transcriptional event. This is also consistent with the notion that the poorer p53 protein accumulation in irradiated NIH3T3$^{\Delta\gamma1}$ cells may be attributable to the inadequate phosphorylation of Mdm2 because Mdm2 mainly regulates p53 at a protein level (Oliner et al., 1993; Thut et al., 1997; Haupt et al., 1997; Kubbutat et al., 1997).

When we transplanted F10$^{\Delta\gamma1}$ cells into mice and then γ-irradiated, we again found that irradiation of these cells resulted in poorer accumulation of p53 protein compared to irradiated F10 cells. Furthermore, examination of the p53 mRNA levels in these cells revealed that the induction of the p53 gene after γ-irradiation was only slight in both cells, supporting the notion that the inadequate phosphorylation of Mdm2 mediated by Δγ1 may reduce p53 protein accumulation through a post-transcriptional event. We found that the reduced accumulation of p53 protein in irradiated F10$^{\Delta\gamma1}$ cells was also associated with the poor induction of the Bax gene. p53 protein accumulation and Bax gene expression both occur in the three days following γ-irradiation, which suggests that the poor Bax induction in irradiated F10$^{\Delta\gamma1}$ cells is a direct consequence of the reduced p53 protein accumulation in these cells. Thus, taken together, the results presented here revealed that Δγ1 could be the cause of the following sequence of events in response to γ-irradiation: (1) poor phosphorylation of Mdm2 on Thr216 and Ser395, (2) reduction in the amount of p53 accumulation, and (3) poor induction of the Bax gene.

The end result of this sequence of events could be that irradiation-induced apoptosis is reduced in F10$^{\Delta\gamma1}$ cells. The fact that Bax is a pro-apoptotic protein (Oltvai et al., 1993) supports this possibility. Confirming this notion, we indeed found that both F10$^{\Delta\gamma1}$ and BL6 cells undergo less apoptosis than F10 cells when the cells were transplanted into mice and then γ-irradiated. This effect was observed on the fourth day after irradiation. As the reduced accumulation of p53 and the poor induction of Bax are already evident 3 days after irradiation, it is quite possible that these two events may have led to the reduced rates of irradiated F10$^{\Delta\gamma1}$ cell apoptosis.

F10 cells have a heterozygous mutation in the p53 gene (p53$^{+/N128D}$). This type of p53 heterozygous mutation has been reported in a patient with non-small-cell lung carcinoma (Liloglou et al., 1997), although its physiologic role remains unknown. Regardless of its role, F10 cells appeared to express the wild-type p53 as a predominant form, because the three p53-target genes Bax, cyclin G1 and p21^{Waf1} were expressed at similar levels in irradiated F10 cells as in irradiated NIH3T3 cells (Fig 6). These observations of F10$^{\Delta\gamma1}$ cells are reminiscent of the paradox that melanoma cells in the clinic are often highly resistant to radiation despite the fact that they typically express wild-type p53. This paradox suggests that proper p53 function is blocked somehow in these cells. Supporting this is the study by Satyamoorthy et al. (2000), who reported that in several human melanoma cell lines, the activation of wild-type p53 is defective due to its aberrant phosphorylation after DNA damage. This indicates that melanoma cells may have defects in the DNA damage-induced processes that activate p53. Our observations suggest that mutations that lead to the expression of Δγ1 or other defective PP2A proteins that can block irradiation-induced p53 function could be a cause of melanoma radioresistance. To date the mutations resulting in Δγ1 or similarly truncated forms of the B56γ subunit have not been found in human melanoma cells. But, it has been recently reported that expression of the PP2A B56γ gene is markedly suppressed in melanoma cells when compared with benign nevus cells derived from the same patients (Deichmann et al., 2001). Thus, reduced expression of the B56γ subunit might contribute to radioresistance of human melanoma cells by perturbing the regulation of p53 by Mdm2.

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