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Modification of serine 392 is a critical event in the regulation of p53 nuclear export and stability

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Abstract Although it has been shown that phosphorylations of p53 serine its residues are critical events for the regulation of their function, the specific biological effects of each of these phosphorylations, especially at serine 392, remain to be elucidated. Serine 392 has been proposed to play a role in the tetramerization of p53 and in the enhancement of its DNA-binding affinity. However, this is not consistent with other reports showing that substitution of serine 392 does not disrupt p53 function. These discrepancies suggest that modification of serine 392 may contribute to p53 activity through other transactivating pathways. In this study, we demonstrate that this C-terminal serine residue (p53-392S) in fact plays a critical role in the regulation of p53 stability such that substitution with alanine (p53-392A) strongly enhances p53 stability without disrupting mouse double minute 2 binding. Additionally, the p53-392A mutant is localized mainly in the nucleus, whereas both wild-type p53 and a glutamic acid mutant, p53-392E, are evenly distributed throughout the cytoplasm and nucleus. However, each of these p53 species had similar effects on both cell cycle inhibition and apoptosis, in response to either UV or adriamycin treatment. Moreover, p53-392A protein was resistant to E6-mediated degradation. Our results suggest that although serine 392 is not essential for the transactivation and nuclear import of p53, it exerts important effects upon p53 stability via the inhibition of its nuclear export mechanism.

Keywords: p53; Phosphorylation; Nuclear export; Serine 392

1. Introduction

The p53 tumor suppressor protein is a crucial component of cellular mechanisms that are initiated by a variety of cellular stresses [1,2]. Under normal conditions, p53 is a short-lived protein that is highly regulated and maintained at low or undetectable levels. However, in response to genotoxic stress or oncogenic signaling, p53 levels rapidly increase, mainly through protein stabilization [3]. Recent findings suggest that p53 stabilization and accumulation can occur via the inhibition of nuclear export, implicating nuclear import–export as a potential mechanism for controlling p53 stability [4]. The levels and activity of p53 are controlled largely by mouse double minute 2 (MDM2), which is amplified or overexpressed in a variety of human tumors and can function as an oncogene in tissue culture systems [5,6]. MDM2 can bind directly to p53 and promote its ubiquitination and subsequently its degradation by the proteasome [6,7]. The ability of MDM2 to degrade p53 depends on both its ubiquitin E3 ligase activity and its nuclear localization signal (NLS) and nuclear export signal, which are required for MDM2 to shuttle between the nucleus and the cytoplasm [5,6].

The induction of p53 involves several mechanisms including post-translational modification such as phosphorylation and acetylation [8]. DNA-damage-induced phosphorylation of serines and threonines within the p53 amino terminus contributes to p53 stability by preventing MDM2 from binding and by rendering p53 more resistant to MDM2 [9–11]. Five serine residues in the carboxy-terminal region, and seven serines and one threonine within the N-terminal 46 residues, are known to be inducibly phosphorylated [12], but the exact order of phosphorylation and their individual contributions to p53 effector function still remain to be elucidated. For example, serine residues at positions 6, 9, 15, 20 and 37 are phosphorylated in response to DNA damage, probably by various protein kinases including ATM, Chk2, DNA-PK and ATR [13–15]. Phosphorylation of Ser 15 is observed only in response to DNA damage [16], but not following p53 activation in response to E1A expression [17], indicating that the specific roles of individual phosphorylation sites depend on the specific stimulus and also probably the cell type. In addition, phosphorylation of Ser 46 is functionally important, because mutation of this site within the p53 protein decreases the sensitivity towards UV-induced apoptosis [18,19]. There are at least three phosphorylation sites near the C-terminus of human p53 at positions 315, 378 and 392. Ser 315 has been shown to be a target of both $p34^{cdc2}$ kinase and cyclin-dependent kinase 2 (cdk2) [20]. Ser 392 is phosphorylated in vitro by purified casein kinase II and Ser 378 is a site for phosphorylation by protein kinase C (PKC). Furthermore, phosphorylation of these C-terminal serines has been shown to enhance the in vitro specific DNA-binding activity of p53 [21].

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Abbreviations: Adr, adriamycin; CHX, cycloheximide; PBS, phosphate-buffered saline; WB, Western blot; WT, wild-type; MDM2, mouse double minute 2

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In this study, we demonstrate that p53 stability is differentially regulated by the substitution of serine 392 but that this mutation does not affect either p53-induced cell cycle inhibition or apoptosis. The increase in p53 stability was also found to be independent of MDM2 activity and the modification of serine 392 also confers resistance to E6-mediated p53 degradation. We suggest, therefore, that the regulation of p53 stability by modification of its serine 392 residue is due to the dysregulation of the nuclear export mechanism.

2. Materials and methods

2.1. Cell culture and reagents

The human prostate cancer cell line, PC3, was obtained from ATCC and maintained in RPMI-1640, supplemented with 10% heat inactivated fetal bovine serum (Gibco BRL), at 37 °C in a humidified chamber with 5% CO_2. All of the reagents used in these experiments were purchased from Sigma–Aldrich and antibodies were obtained from Santa Cruz Biotech.

2.2. Construction of vectors and transfectiom

p53 wild-type (WT), 392A and 392E constructs were generated by RT-PCR based cloning using the pcDNA 3.1 topo His/V5 vector (Invitrogen). The primers used are shown in Table 1. The HPV-6 clone was kindly provided by S.G. Chi (Kyung Hee University, Seoul). The transfection reagent used was Gemeporter, which was purchased from Gene Therapy Systems. Transfection procedures were performed following the manufacturer's protocol. Briefly, cells were seeded in 6 well plates at a concentration of 1×10^5 cells/well. 4 μg of DNA per well was mixed with serum-free media containing Geneporter (10 μl/well), incubated for 30 min at room temperature, and added to cells that were previously washed twice with phosphate-buffered saline (PBS). After 3–5 h, media (1 ml/well) containing 20% FBS were added to each well. All experiments, including thymidine incorporation assays, Western blotting, immunoprecipitations and cell death assays were performed after incubation for 24 h. To monitor transfection efficiency, cells were co-transfected with GFP-pcDNA.

2.3. Cell proliferation and DNA synthesis assays

Cells were seeded at a concentration of 1×10^4 cells/well in 24 well plates and transfected with p53 constructs on the following day. The cells were then washed twice with PBS, followed by the addition of medium containing 0.1 μg/ml adriamycin (Adr) or 5 ng/ml actinomycin D, and incubated for 20 h. Cells were then pulse-labeled for 4 h with [^{3}H]thymidine, which was added to the cells at a final concentration of 1 μCi/ ml. Cells were then washed with PBS twice and incubated with 70% TCA at 4 °C for 0.5 h to precipitate nucleic acids. After two additional washes with PBS, precipitates were dissolved with DNA lysis buffer (2% Na_2C0_3, 2% SDS, and 0.1% NP-40) and mixed with 2 volumes of scintillation Cocktail solution to measure the levels of incorporated isotope using the liquid β-counter. To examine the effect of UV, cells were harvested after 20 h of 100 J/m^2 UV exposure and subjected to the same procedure as described above. Each experiment was performed in triplicate.

2.4. Indirect immunofluorescent analysis of p53

To determine the localization of p53, we transfected PC3 cells with either WT p53, p53-392A or p53-392E that had been V5-His-tagged for 24 h. In addition, to examine resistance to E6-mediated p53 degradation, cells were co-transfected with each p53 construct and an E6 construct. Following transfection, the cells were either incubated with Adr or exposed to UV, and then fixed with 2% paraformaldehyde for 2 h at RT, followed by incubation with both permeable and blocking buffers. The cells were then incubated with anti-V5 antibody, diluted in permeable buffer (1:2000) for 2 h at RT and subsequently treated with anti-mouse IgG-Rhodamine for 2 h. The antibody incubations were followed by staining with DAPI for 10 min and the cells were then washed twice with ice cold PBS and examined under fluorescence microscopy.

2.5. Apoptosis assays

Cells were seeded (4×10^5 cells/well) in 96 well plates and incubated with 10 μM BrdU labeling solution (Cellular DNA Fragmentation ELISA kit, Roche) at 37 °C for 24 h. After the first incubation, cells were either treated with Adr or UV irradiated at 37 °C for 6 h prior to harvesting. Cells were lysed in incubation solution and were then incubated in precoated plates with anti-BrdU POD-conjugate solution according to the manufacturer's guidelines. These experiments were repeated at least three times. The absorbance level of each sample was measured by an ELISA microplate reader.

2.6. Real-Time PCR

Real-time PCRs were performed using a TaqMan detection system, consisting of 12.5 μl of TaqMan Universal PCR Masler Mix, 1.25 μl of Assay-on-Demand Gene Expression probe (Applied Biosystems, USA), TaqMan probe, 100 ng of each cDNA template and water, in a final volume of 25 μl. All PCR samples and controls were prepared in identical tubes in triplicate using 0.2 ml Micro-Amp Optical reaction tubes and MicroAmp Optical tube caps (Applied Biosystems, USA). Each PCR mixture was held at 50 °C for 5 min and denatured at 95 °C for 10 min. 40 amplification cycles were carried out at 95 °C for 20 s followed by 60 °C for 1 min. All PCRs were performed using an ABI PRISM® 7900HT (Applied Biosystems) and PCR products were analyzed using the corresponding sequence detector software (Applied Biosystems).

2.7. TUNEL assay

To detect DNA breaks in apoptotic cells in situ, we adopted a terminal deoxynucleotidyl transferase (TdT)-mediuted DIG-nucleotide Nick end labeling (TUNEL) method using a Cell Death Detection Kit, TMR-Red (Roche, Germany). Negative controls were prepared by omitting TdT and DNase-treated cells were used as positive controls. TUNEL fluorescence of individual nuclei, in a final volume of 700 μl, was analyzed by an FACS Calibur (Becton–Dickinson, USA), while gating on physical parameters was enacted to exclude cell debris. A minimum of 10 000 events was counted per sample.

3. Results

3.1. Substitution of serine 392 alters p53 stability

To study the functional role of the p53 serine residue at position 392, we generated two different p53 mutant constructs

Table 1
Sequences of the primers used in this study

Name		Sequence (5′–3′)	Usage
p53-wt-l	S	CATGGAGGAGCCGCAGTCAG	C
p53-wt-2	AS	GTCTGAGTCAGGCCCTTCTG	C
p53-S392A	AS	GTCTGCGTCAGGCCCTTCTG	C
p53-S392E	AS	GTCCTCGTCAGGCCCTTCTG	C
p53-AS	AS	CAAGTCACAGACTTGSGCTGTCC	Q
GAPDH-1	S	AACCATGAGAAGTATGACAACAGC	Q
GAPDH-2	AS	CATGTGGGCCATGAGGTCCACCAC	Q
T7		TAATACGACTCACTATAGGG	C and Q

S and AS indicate the orientation of sense and antisense primers, respectively. Q and C represent their usage in semi-quantitative gene cloning and ChIP, respectively. All sequences are oriented in the 5′ to 3′ direction.

by substitution with either alanine (p53-392A) or glutamic acid (p53-392E). To examine the pattern of expression of these constructs, the p53-null cell line PC3 was transiently transfected with normalized levels of either WT p53, p53-392A, p53-392E or appropriate control vectors. We were able to specifically detect and quantify exogenous p53 mRNA levels using real-tune PCR, all of which were normalized to GAPDH mRNA expression, and found them to be comparable (Fig. 1A). The p53-392A protein levels, however, were higher than either WT p53 or p53-392E under the same experimental conditions (Fig. 1B). These results indicate that the C-terminal serine residue of p53 has an important role in the regulation of p53 stability. To explore the molecular mechanism by which serine 392 affects p53 stability, we tested whether the replacement of this residue directly controls p53-mediated cell cycle inhibition and apoptosis.

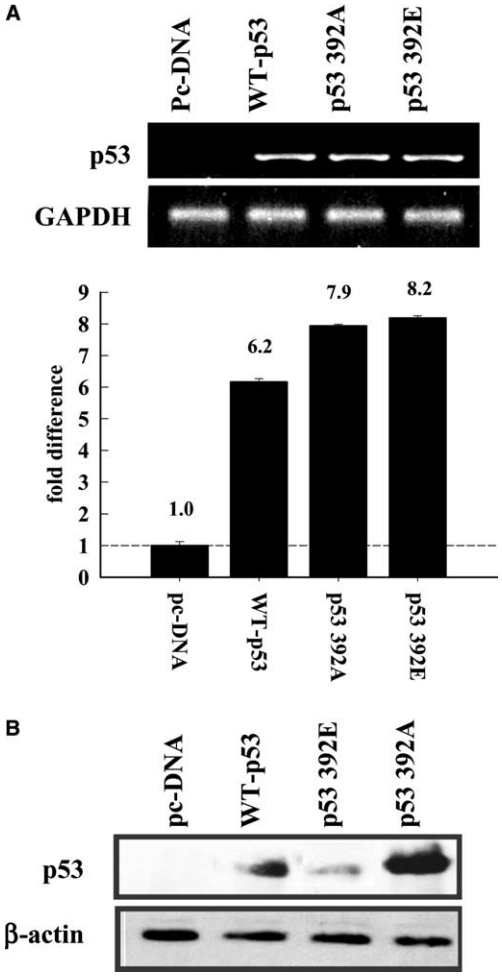

Fig. 1. Modification of serine 392 affects the stability of p53. (A) PC3 cells were transfected with either a control pcDNA vector or the indicated p53 constructs (4 μg). To ensure comparable transfection efficiencies, a sample of the cells was transfected with GFP and monitored for expression levels. PC3 cells transfected with the p53 constructs were analyzed by real time-PCR and p53 levels were normalized to GAPDH. Data shown are the average of two assays. Error bars, SEM. (B) A fraction of PCS cells from the experiment described in (A) was lysed and tested by Western blotting for p53 expression. β-actin was usied as a loading control.

3.2. Substitution of p53 serine 392 does not disrupt either UV- or Adr-induced cell cycle inhibition and apoptosis

To examine the impact of our p53 mutants on p53-mediated cell cycle inhibition, we determined the impact of each transfected construct upon cell cycle regulation in response to Adr (0.2 μg/ml) using a [^{3}H]thymidine incorporation assay following transient transfection. The p53 variants did not show significant differences in their effects upon cell cycle regulation, with inhibition ranging from 40% to 60% of the control (Fig. 2A). Next, we examined Adr-induced apoptosis using both DNA-fragmentation and TUNEL assays and again found no significant differences between the 3 p53 species (Fig. 2B). These results suggest that Ser-392 does not play a role in Adr-induced cell cycle inhibition and apoptosis. Parental PC3 cells were resistant to apoptosis, therefore Adr-induced apoptosis and cell cycle inhibition are mediated by p53 (Fig. 2A and B). We then examined whether these p53 variants show any discrepancies in their ability to mediate UV-induced apoptosis or cell cycle inhibition. Under the same Adr-induced apoptotic conditions, transfectants were exposed to 100 J/m^2 UV amd cell cycle inhibition and apoptosis levels were determined using the same method. UV irradiation was found to have an effect upon p53-induced cell cycle arrest and apoptosis but this did not vary between the different transfected p53 species (Fig. 2C and D). These results demonstrate that neither UV-induced cell cycle arrest nor apoptosis are regulated by modification of p53 serine 392, although UV exposure is known to cause phosphorylation of this residue [23]. The role of serine 392 is, therefore, likely to be the maintenance of p53 stability but this is not related to the induction of either apoptosis or cell Cycle inhibition. Additionally, these results are also consistent with a previous study showing that replacement of serine 392 does not disrupt p53 function [24].

3.3. p53 Ser-392 mutants have similar MDM2 binding affinities

To investigate whether MDM2 is required for the observed increases in p53 stability, we checked the interaction between each p53 Ser-392 mutant and MDM2. MDM2 is known to promote the downregulation of p53 through nuclear export and ubiquitin-dependent degradation [25,26]. Cell extracts from PC3 cells, expressing exogenous p53 mutants, were immunoprecipitated wilh MDM2 and subsequent Western blot analysis revealed that the binding affinity between each of the p53 mutants and MDM2 showed no significant differences (Fig. 3A). These results indicate that the enhanced stability of p53-392A occurs independently of MDM2 binding in vitro. We next examined the possibility that this increased p53 stability is due to a prolonged mRNA half life. Cells were transfected with p53 variants and treated with a high dose (2 mM) of the transcription inhibitor actinomycin D [27] over a specific tune course. As shown in Fig. 3B, each of the exogenous p53 transcript levels was reduced over time and did not show any significant differences in stability. Furthermore, the same experiments were also performed with an inhibitor of translation, cycloheximide (CHX) [28]. Similar results were obtained with the exception of p53-392A levels (Fig. 3C). Strikingly, we found that the steady-state levels of p53-392A were unchanged up to the 8 h timepoint, indicating that the modification of Ser-392 is a critical post-translational regulatory mechanism of p53. Our findings, therefore, indicate that the modification of Ser-392 regulates p53 stability through a post-translational mechanism that is independent of MDM2

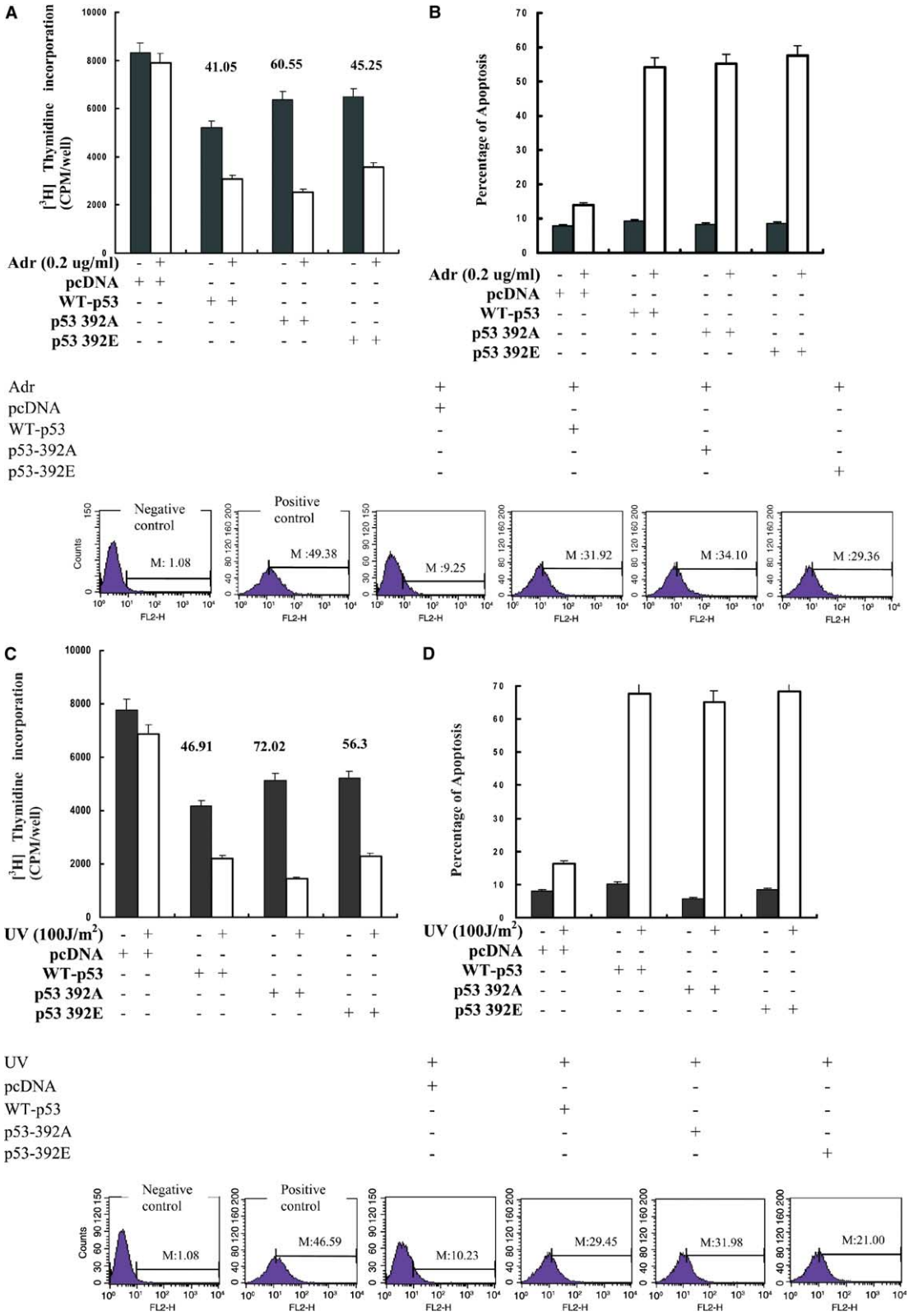

Fig. 2. Cells expressing exogenous WT, 392A and 392E p53 proteins show the same susceptibility to both UV- and Adr-induced apoptosis and cell cycle inhibition. (A) PC3 cells were transfected with pcDNA control and WT, 392A and 392E p53 constructs cells were either left untreated (filled column) or treated with Adr (Adr) (0.2 μg/ml, open column). Cell cycle regulation was quantified 24 h later by [^{3}H]thymidine incorporation; results shown are the means ± S.D. The numbers above the bar indicate percentage suppression. (B) Apoptosis, measured under the same cell cycle regulatory conditions, was quantified using both the DNA fragmentation and TUNEL assay methods (M, TUNEL positive). (C) PC3 cells were either left untreated or irradiated with UV (100 J/m^2) to induce apoptosis and analyzed 20 h later. The numbers on the bar indicate the percentage of apoptotic cells, when compared with the control. (D) UV-induced apoptosis was quantified by both DNA fragmentation and TUNEL assays.

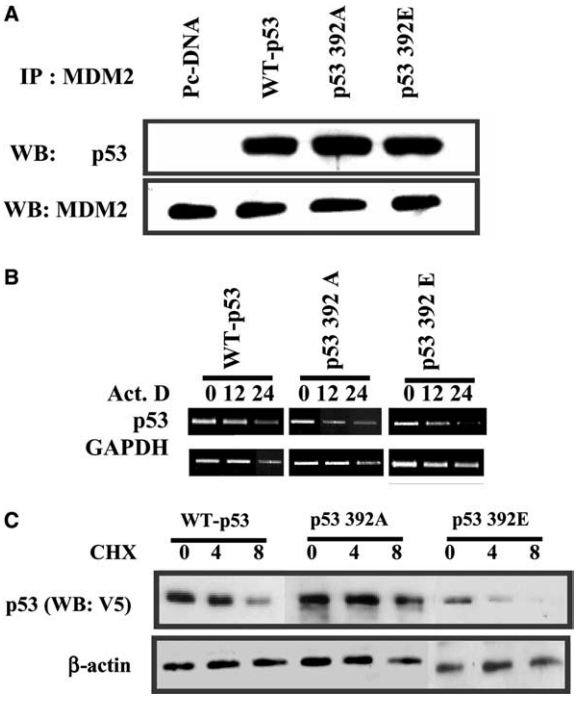

Fig. 3. Modification of serine 392 does not disrupt the interaction between MDM2 and p53. (A) PC3 cells were transfected with the WT, 392A and 392E p53 constructs and cells were harvested after 48 h. Cell lysates were immunoprecipitated with anti-MDM2 antibodies and co-precipitated exogenous p53 protein was then detected by Western blotting. The WBs were also tested using anti-MDM2 antibodies as a loading control. (B) PC3 cells were transfected with the same p53 constructs, the expression of which was measured by RT-PCR after treatment with actinomycin D at 0, 1.2 and 24 h. The GAPDH normalizing conlrol transcript levels also decreased over the same time course. (C) PC3 cells were transfiicted with p53 constructs and the cells were harvested after 0, 4 and 8 h treatment with CHX. Cell lysates were analyzed by Western blotting with V5 antibodies to measure the stability of the exogenous p53 expression. β-Actin was used as a loading control.

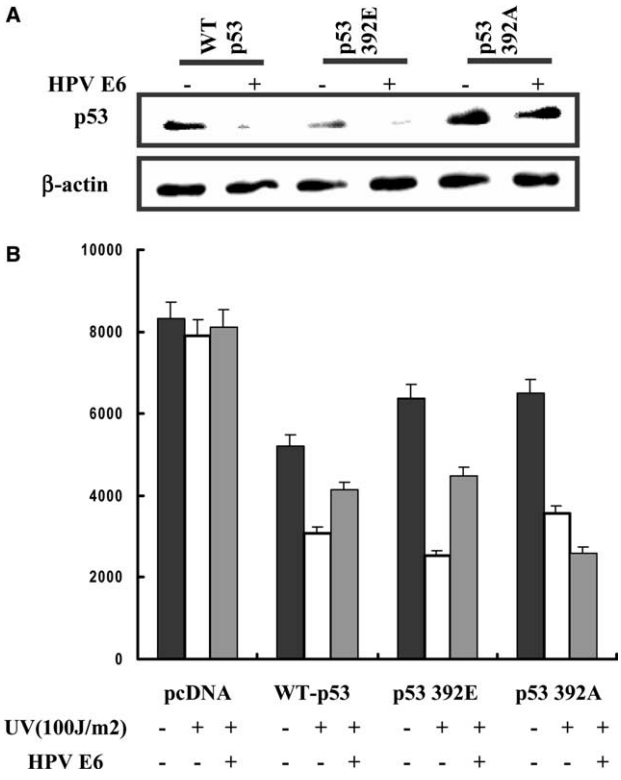

Fig. 4. p53-392A mutants are resistant to E6-mediated degradation. HPV protein E6 was co-expressed with WT, 3 92A and 392E p53 constructs in PC3 cells. (A) Cell lysates were analyzed by Western blotting for exogenous p53 stability. (B) Cells were either left untreated (filled column) or treated with a UV dose (100 J/m^2). Cell cycle regulation was quantified 24 h later by [^{3}H]thymidine incorporation; results are means ± S.D. under the same conditions as shown in Fig. 2.

binding. Consequently, we speculated that the modification of Ser-392 blocks the nuclear export of p53, as this is essential for proteasome-mediated degradation via MDM2 [5].

3.4. p53-392A protein is resistant to E6-mediated degradation

To examine the possible role of serine 392 in p53 nuclear export, we transiently co-transfected PC3 cells with HPV-E6 and each of the p53 Ser-392 mutants, as the mechanism of E6-induced p53 degradation is similar to MDM2 [29–31]. We predicted, therefore, that if p53 nuclear export is disrupted by modification of Ser-392, this should also affect E6-induced p53 destruction. Interestingly, p53-392A protein was found to be resistant to E6-mediated destruction, whereas both WT p53 and p53-392E proteins were degraded (Fig. 4A). To further explore the functional relationship between p53-392A and E6, we tested the effects of E6 expression on the cell cycle. As shown in Fig. 4B, p53-392A transfected cells are susceptible to cell cycle inhibition, whereas both WT p53 and p53-392E transfected PC3 cells showed no comparable cell cycle block under the same conditions. These results strongly suggested that modification of serine 392 inhibits E6-mediated p53 degradation by regulating its subcellular localization.

3.5. p53-392A is localized in the nucleus

Based on our observations that p53 is likely to be stabilized by blocking nuclear export, we investigated this directly by determining whether the modification of Ser-392 directly promotes cytoplaismic retention of p53. As indicated in Fig. 5, ectopic expression of p53-392A yields a predominantly nuclear stain, with less than 5% of cells showing clear cytoplasmic localization. In contrast, however, WT p53 was distributed diffusely throughout the cell and p53-392E was sequestered in the cytoplasm. These results raise the possibility that modification of Ser-392 regulates both the nuclear localization and nuclear export of p53. According to previous reports, p53 contains a NLS in its C-terminus, whereas nuclear export is accomplished through the MDM2 pathway [31,32]. Although our previous results indicate that modification of Ser-392 does not affect p53-mediated functions, we cannot exclude the possibility that this modification is required for localization of p53 in the nucleus.

To further evaluate the role of Ser-392 in p53 nuclear localization, we transiently transfected PC3 cells with our p53 constructs. As expected, indirect immunofluorescent analysis showed that p53 accumulates in the nucleus, regardless of serine 392 mutations, at 1 h, but that each p53 species had a different subcellular localization after 12 h. In contrast to WT p53 and p53-392E, which showed a dispersed localization, a large portion of exogenously expressed p53-392A was detected in the nucleus (data are not shown). This finding was consis-

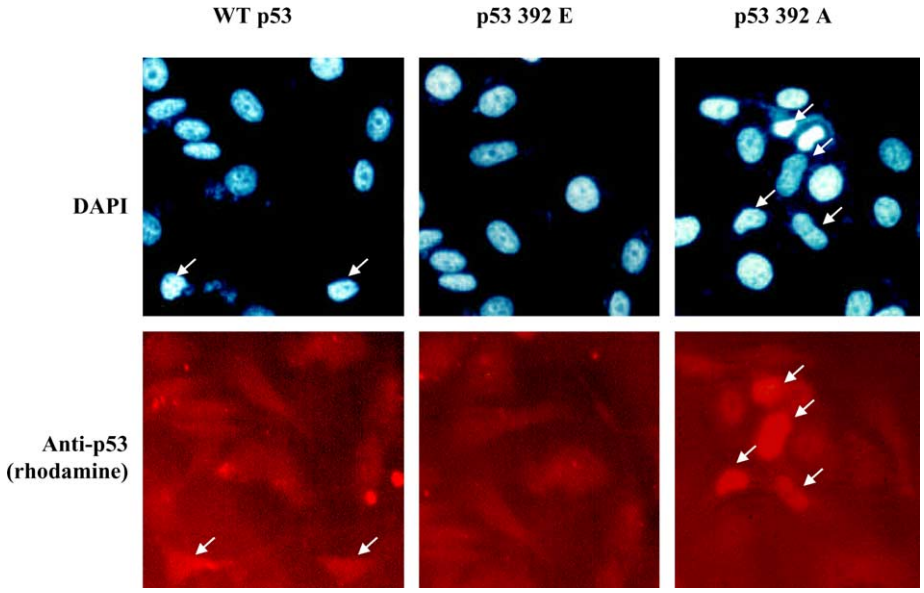

Fig. 5. p53 serine 392 mutants show different cellular distributions. PC3 cells were transfected with WT, 392A and 392E p53 constructs and the localization of p53 was detected by indirect immunofluorescence using anti-V5 antibody and rhodamine conjugated mouse IgG. Nuclear DNA was counterstained with DAPI (blue). Areas of p53 localization are indicated by arrowheads.

tent with our previous result that p53-392A is resistant to degradation. Finally, we examined the subcellular localization of p53 after co-transfection with E6 and determined that p53-392A was resistant to E6-mediated nuclear export.

4. Discussion

We propose that the serine residue at position 392 in the p53 protein functions as a regulator of p53 nuclear export and that it is critically involved in the regulation of p53 subcellular localization. The nuclear localization of p53 is essential for its role as a transcription factor in tumor suppression. Moreover, phosphorylation of serine 392 has been proposed as the regulating motif of p53 tetramerization, leading to enhancement of target gene expression [23,33]. However, this hypothesis is not consistent with other results showing that IR-induced p53 activation initiates both apoptosis and cell cycle arrest in the absence of any Ser-392 modification, accompanied by the transcriptional induction of p53 target genes such as p21/wafl and Bax. In addition, modification of Ser-392 does not affect either Adr or UV-induced cell cycle arrest and apoptosis [22] (Fig. 2), which is induced via the upregulation of p53 target genes. Moreover, we tested the transcriptional variation of each of our WT, 392A and 392E p53 constructs and no significant differences were detected (data not shown). In contrast, we showed that the modification of Ser-392 is required to maintain the localization of p53 in the nucleus. Hence, the regulation of p53 nuclear export by modification of Ser-392 has an important role in the control of p53 function, in addition to its role in p53 tetramerization.

The effects of the modification of p53 serine 392 are not necessarily related to its charge, such as is the case with the N-terminal nuclear export mechanisms [34], as both serine to alanine substitutions and the phosphorylation of Ser-392 were found to increase p53 stability [23,33]. Moreover, modification of Ser-392 regulates both p53 nuclear export and protein stability. Using several lines of investigation, therefore, our studies indicate that the modification of Ser-392 increases p53 protein stability through its retention in the nucleus. Although we have not yet fully elucidated the nuclear export mechanism of p53, our results suggest the eistence of a new p53 regulatory pathway.

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