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The p16^{INK4a} tumor suppressor controls p21^{WAF1} induction in response to ultraviolet light

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ABSTRACT

p16^{INK4a} and p21^{WAF1}, two major cyclin-dependent kinase inhibitors, are the products of two tumor suppressor genes that play important roles in various cellular metabolic pathways. p21^{WAF1} is up-regulated in response to different DNA damaging agents. While the activation of p21^{WAF1} is p53-dependent following γ-rays, the effect of ultraviolet (UV) light on p21^{WAF1} protein level is still unclear. In the present report, we show that the level of the p21^{WAF1} protein augments in response to low UVC fluences in different mammalian cells. This up-regulation is mediated through the stabilization of p21^{WAF1} mRNA in a p16^{INK4a}-dependent manner in both human and mouse cells. Furthermore, using p16-siRNA treated human skin fibroblast; we have shown that p16 controls the UV-dependent cytoplasmic accumulation of the mRNA binding HuR protein. In addition, HuR immunoprecipitations showed that UV-dependent binding of HuR to p21 mRNA is p16-related. This suggests that p16 induces p21 by enabling the relocalization of HuR from the nucleus to the cytoplasm. Accordingly, we have also shown that p16 is necessary for efficient UV-dependent p53 up-regulation, which also requires HuR. These results indicate that, in addition to its role in cell proliferation, p16^{INK4a} is also an important regulator of the cellular response to UV damage.

INTRODUCTION

Eukaryotic organisms are under continuous stress of environmental as well as intracellular origins. The cellular response to these stresses is complex and involves different metabolic pathways. These include the activation of a battery of genes that guarantee efficient repair and impose a tight negative regulation on the progression of the cell cycle, in order to prevent premature entry of the cell with damaged DNA into the next phase (1). These delays of cellular proliferation at specific stages, called cell cycle checkpoints, integrate DNA repair with cell cycle progression through a network of genes and pathways (2). Many of these genes are mutated in a broad range of human cancers, which reveals their pivotal role in the cellular defense against neoplastic transformation. The function of the major tumor suppressor genes is to inhibit the action of specific cyclin-dependant kinases (CDK), whose oscillations in activity play a key role in the regulation of the cell cycle clock (3). Based on the primary sequences, two distinct families of CDK inhibitors (CKIs) have been identified in mammalian cells [for a review, see (4)]. These families are commonly referred to as CIP/KIP and INK4, represented by p21^{WAF1} (hereafter denoted as p21) and p16^{INK4a} (hereafter denoted as p16), respectively. Although these two CKIs belong to two independent pathways, recent data have revealed the existence of important interactions between them, including their binding to the common targets, CDK4 and CDK6 (4,5). p21 protein is activated in response to different DNA damaging agents, including ultraviolet (UV) light and ionizing radiation. Following γ-rays, p21 protein is activated in p53-dependent manner (6). On the other hand, the effect of UV light on p21 protein level is still puzzling. Several reports have shown that p21 protein level is up-regulated in response to low UV fluences in both normal human cell strains (7–10) and mouse embryonic fibroblasts (11). However, other publications have reported reduction and ubiquitin-dependent degradation of p21 protein level following UV irradiation in different cell lines (12–18). It is noteworthy, however, that most of these studies showing UV-dependent reduction in p21 level were performed on cancer cell lines. To shed light on these conflicting results, Itoh and Linn used a human normal primary lung fibroblast strain (IMR-90), which has been also used by Bendjennat *et al*. (12), and showed that p21 protein level is indeed up-regulated in response to low UV doses (1.2–6 J m^{-2}) in different experimental conditions (19).

Other conflicting conclusions regarding UV-dependent p21 induction concern the role of p53 in this process. While different laboratories have reported that p21 protein as well

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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as cell cycle arrest are not p53-related in various cell types (7,11,20–22), two papers described p53-dependent activation of p21 (23,24). These conflicting conclusions indicate that p21 up-regulation in response to UV light is still unclear and thereby, further studies are required to clarify this issue.

p16 is a key tumor suppressor that blocks the progression of the cell cycle by binding to either CDK4 or CDK6 and inhibiting the action of cyclin D (25–28). The p16 coding gene has been found homozygously deleted, mutated or transcriptionally inhibited by methylation in a large number of different human tumor types (26–29). Furthermore, mice lacking p16 are tumor prone and develop different types of cancer, particularly after exposure to carcinogens (30,31). p16 has been also found to be linked to familial melanoma and is considered the most important melanoma susceptibility gene (27,32). Compelling epidemiological and basic science data support a critical causal role of sunlight exposure in the development of melanoma (33). However, the functional relationship between p16 and UV radiation in the pathogenesis of cutaneous malignant melanoma is largely unknown.

In the present report, we have shown that p21 protein level increases following low UV fluences in different human and mouse cell lines, and that this increase is under the control of p16. We have also shown that the UV-dependent nucleocytoplasmic shuttling of the HuR protein is under the control of p16, which led us to suggest that p16 controls p21 up-regulation by enabling the cytoplasmic accumulation of HuR.

MATERIALS AND METHODS

Cell lines and cell culture

U2OS, human osteosarcoma cell line, which does not express endogenous p16 due to hypermethylation, EH1 and EH2, derived from U2OS, which express p16 under the control of isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter (34). (The three cell lines are a generous gift from Dr G. Peters). The mouse embryo fibroblasts used were p16 (WT) and their p16-specific knockout counterpart (30) (MEF cell lines are a generous gift from Dr R. A. DePinho). Mouse skin fibroblast cells, $p53^{+/+}$ and $p53^{-/-}$ were derived, respectively, from B6129F2/J 101045 and B6129-$Trp53^{tm1Tyj}$ strains (35). MM470, is a melanoma cell line (gift from Dr N. Hayward and Dr P. Parsons); MEN1, meningioma-derived primary cell line (36); HSF1 and HFSN1 are normal human skin fibroblasts and MCF-12, 'normal' epithelial cells (ATCC). These cells were routinely cultured in DMEM supplemented with 10% CBS.

Irradiation

Cells were grown to confluence in DMEM with supplements. The medium was removed and the monolayers in dishes were covered with phosphate-buffered saline (PBS) and exposed to a germicidal UV lamp (254 nm) at a fixed distance. The UV dosimetry was performed using an UV meter (Spectronics Corporation, NY). For γ-ray treatment, a Co source was used at a dose rate of 0.60 Gy/min.

Cellular lysate preparation

Cells were washed and scraped in lysis buffer [150 mM NaCl, 1% Triton X-100 and 50 mM Tris–HCl (pH 7.5)], supplemented with 40 μg/ml aprotinin, 20 μg/ml leupeptin and 5 μg/ml pepstatin. Lysates were homogenized using a polytron homogenizer and then centrifuged at 14 000 r.p.m. in an Eppendorf microcentrifuge tube for 20 min. The supernatant was removed, aliquoted and stored at −80°C.

Immunoblotting

SDS–PAGE was performed using 12% separating minigels. Equal amounts of protein from different samples were placed in boiling water for 10 min in the presence of SDS gel sample buffer (0.5 mM Tris (pH 6.8), 10% glycerol, 10% SDS, 5% 2-mercaptoethanol and 1% bromophenol) and electrophoresed for 2 h at 125 V. After transfer on to polyvinylidene difluoride membrane (PVDF), the membrane was first blocked with 5% powdered skimmed milk in PBST (138 mM NaCl, 2.7 mM KCl (pH 7.4) and 0.1% Tween) for 2 h and then incubated with the appropriate first antibody overnight. Visualization of the second antibody was performed using a chemiluminescence detection procedure according to the manufacturer's protocol (Amersham-Pharmacia Biotech).

Monoclonal antibodies directed against mouse p21 (F5), mouse p53 (R-19), human p21 (187), p14 (C-18), p16 (50.1), p53 (DO-I), HuR (3A2), β-actin (C-11), α-tubulin (TU-02) and PCNA (PC10) were purchased from Santa Cruz (USA).

Analysis of protein half-life

UV-irradiated and non-irradiated confluent cells were treated with 20 μg/ml cycloheximide (Sigma) for various periods of time (0–24 h) and then lysed. Cellular extracts from each time point were subjected to immunobloting using the appropriate antibodies. The intensity of the bands was determined by densitometry.

Analysis of p21 mRNA stability

UV-irradiated and non-irradiated confluent cells were treated with 5 μg/ml Actinomycin D (Sigma) for various periods of time (0–6 h) and then total RNA was purified and used to quantify p21 mRNA.

siRNA transfection

Specific p16 siRNA with the sequence (Fw: TACGATACAAGGCTGTTAGAGAG, Rev: TAGAAGGCACAGTCGAGG) targeting the p16 exon 1α and a control siRNA were integrated in a expression vector (GenScript). Stable transfection was carried out using human dermal fibroblast nucleofector kit (Amaxa Biosystems) following the protocol recommended by the manufacturer. Briefly, 95% confluent HFSN1 cells were passaged 2 days before nucleofection and 3 μg of p16 siRNA or the control plasmids was electroported into 0.7×10^6 cells using amaxa electroporation instrument. After electroporation, cells were plated in a 6-well plate containing 1 ml of pre-warmed DMEM media and incubated for 24 h. In the next day, stable transfectant were selected using DMEM media containing 1.5 mg/ml neomycin. The concentration of neomycin was reduced thereafter

to 100 μg/ml and single colonies were isolated and p16 expression level has been assessed by immunoblotting.

RNA purification and RT–PCR

Total RNA was purified using the TRI reagent (Sigma) according to the manufacturer's instructions. The concentration of RNA was determined using Gene Quant II, RNA/DNA calculator (Pharmacia Biotech). Single-stranded complementary DNA (cDNA) was obtained from reverse transcription of 1 μg of RNA using RT–PCR kit (BD Biosciences) and following the manufacturer protocol.

cDNA was then amplified with 1 U *Taq* polymerase, dNTPs (50 mM) and primers (25 pmol each). The mixture was first heated at 94°C for 5 min and then 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, then 72°C for 10 min. PCR products were seen on 2% agarose gel electrophoresis stained by ethidium bromide. The respective primers were: p21: 5′-CAGAGGAGGCGCCAAGACA-G-3′ (forward) and 5′-CCTGACGGCGGAAAACGC-3′ (reverse), and β-actin: 5′-CCCAGCACAATGAAGATCAA-GATCAT-3′ (forward) and 5′-ATCTGCTGGAAGGTGGA-CAGCGA-3′ (reverse). The intensity of the bands was determined with the Quantity One program (Bio-Rad) and was normalized against β-actin.

Immunoprecipitation and detection of p21 mRNA

The immunoprecipiation experiment has been performed as described previously (37). Briefly, cell lysates were prepared from confluent cells, and then centrifuged at 10 000 *g* at 4°C. Subsequently, 200 μg of protein extracts were incubated in buffer (50 mM Tris (pH 8), 100 mM NaCl, 10% glycerol, 1× protease inhibitors, 5 mM DTT and 2 U/μl RNasin) and 7.5 μg HuR mouse monoclonal antibody (mouse IgG1 was used as control) was added and mixed at 4°C for 4 h. Equal volume of protein A agarose was added per immunoprecipitation and mixed overnight at 4°C. After centrifugation, the pellet was resuspended in 1 ml TRI reagent used for RNA extraction. Total RNA was resuspended in 20 μl DEPC water, heated at 75°C for 5 min and then chilled on ice. The RT–PCR reactions were performed as described above.

Subcellular fractionation

Nuclear and cytoplasmic extracts were prepared as previously described (38).

Fluorescent RT–PCR

Total RNA from cultured cells was prepared by using Trizol. Single-stranded cDNA were obtained from reverse transcription of 3 μg of total RNA using random hexanucleotides as primer (50 μM), in the presence of dNTP (250 μM), DTT (10 μM) and M-MLV (10 U/μl), 1 h at 37°C. cDNA was then amplified by PCR with *Taq* polymerase (0.01 U/μl), dNTP (250 μM) and specific 6-FAM labeled primers (10 μM). The mixture was first heated at 94°C for 5 min, and then 20 cycles at 92°C for 30 s, 55°C for 30 s, 70°C for 30 s, then 72°C for 5 min. PCR products were analyzed using the ABI genetic analyzer. This approach was used to quantify the p21 mRNA in different conditions. The primers used are:p21: 5′-6FAM-CTTTCTAGGAGGGAGACAC-3′ (forward) and 5′-GTTCCGCTGCTAATCAAAG-3′ (reverse). Actin: 5′-6FAM-GATCCACATCTGCT-3′ (forward) and 5′-GACAGGATGCAGAAGGAGAT-3′ (reverse).

RESULTS

Low UV fluences up-regulate p21 protein in different normal human cell lines

To investigate the effect of low UV doses on p21 protein level, human skin fibroblast cell line (HSF1), normal breast epithelial cell line (MCF-12) and benign primary meningioma cell line (MEN1) were irradiated with 5 J m^{-2} and reincubabed for different periods of time and then whole cell extracts were prepared and used for immunoblotting analysis. Specific antibodies against p21 and PCNA (used as internal control) were utilized. p21 level was up-regulated, following the cellular treatment with this low UV fluence, reaching 7-fold induction after 12 h in HSF1 cells, and almost the same level was maintained throughout the subsequent 12 h of incubation (Figure 1A). Similar result was obtained with the MCF-12 cell line, wherein p21 level reached 6-fold induction 8 h following the treatment, and MEN1 cells that showed a 3.3-fold induction in p21 level 12 h following the treatment (Figure 1A). These results show that p21 protein level increases in response to low UVC fluences in different human cell types. It is noteworthy that similar results were obtained with other human skin fibroblast, epithelial and brain tumor cell lines (data not shown).

Low UV fluences do not up-regulate p21 protein in p16-deficient cell lines

It has been previously reported that p21 protein level does not increase in response to UV light in the p16-defective U2OS cell line (12,13). Likewise, when the p16-deleted MM470 skin cancer cell line (39) was treated with 5 J m^{-2} of UV light, p21 protein level did not increase (Figure 1B). In contrast, the p21 level decreased during the first 16 h (Figure 1B), like in U2OS [Figure 2B and (12,13)]. These results suggested a potential role for p16 in the up-regulation of p21 in response to UV light.

p16 controls UV-dependent up-regulation of p21 in human cells

To further investigate the possible role of p16 in UV-dependent up-regulation of p21, we made use of the U2OS cell line, which does not express p16 due to promoter methylation, and its derivative cell lines EH1 and EH2, which contain p16 under the control of IPTG inducible promoter (34). It is noteworthy, however, that in the absence of IPTG, EH1 and EH2 cells express a low basal level of p16, comparable to the low amount detected in early-passage human diploid fibroblasts for EH1, but almost undetectable in EH2 (Figure 2A). p16 expression in these cells does not exert any measurable effect on cellular growth (12,13). Using immunoblot analysis, we confirmed the lack of p21 up-regulation following the treatment of U2OS cells with low UV fluence of 5 J m^{-2} (Figure 2B). When EH1 cells were similarly treated, p21 protein level increased reaching at time 16 h a level 3-fold higher than that in non-treated

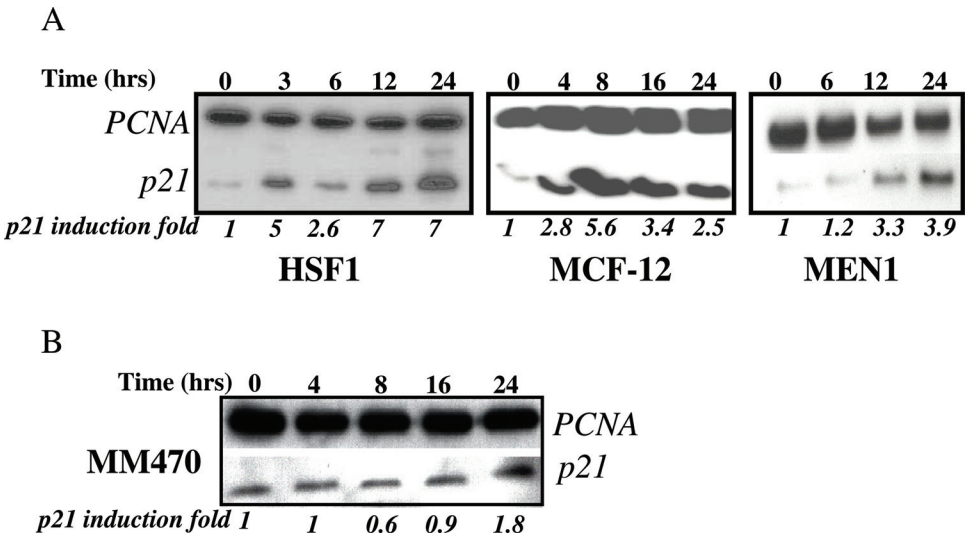

Figure 1. Effect of low UVC fluences on p21 protein levels in normal and p16-defective human cell lines. Cells were either mock-treated or irradiated with UV light (5 J m^{-2}) and reincubated for different periods of time, as indicated. Thirty mg of extracted proteins were used for western blot analysis. p21 protein fold induction following UV treatment was determined by normalizing the values against PCNA. (**A**) p16-proficient cells. (**B**) p16 defective cell line.

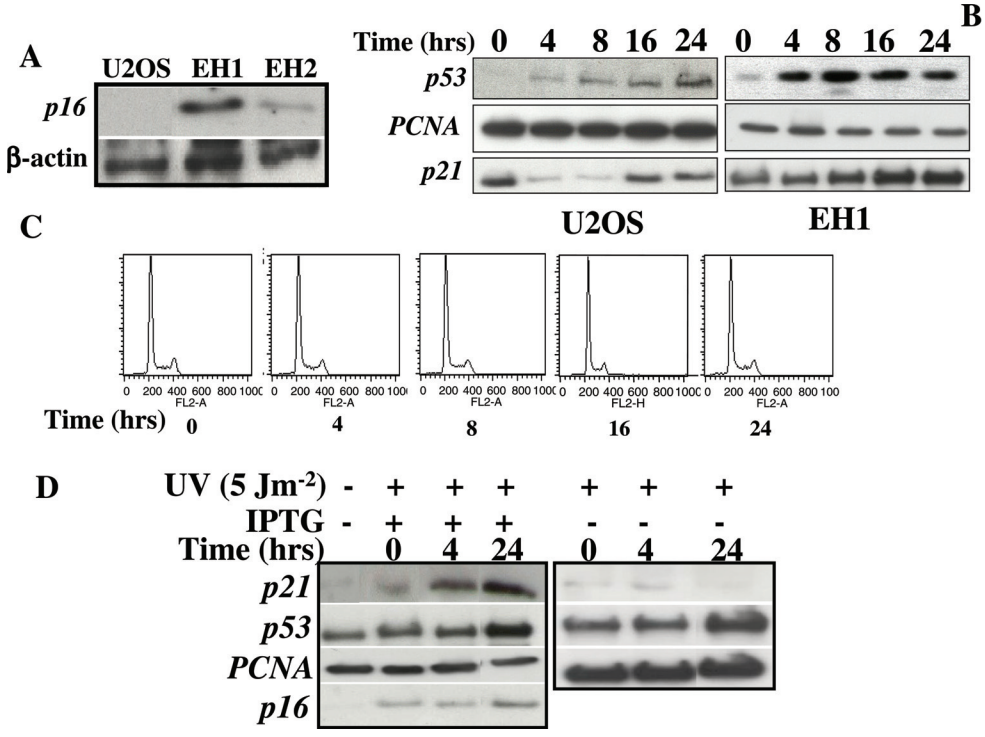

Figure 2. Effect of UV light on p21 and p53 protein levels in U2OS, EH1 and EH2 strains. Cells were mock-treated or challenged with 5 J m^{-2}, reincubated and harvested after the indicated time periods for protein purification or FACScan analysis. Thirty microgram of proteins was used for western blot analysis using the appropriate antibody, as indicated. PCNA and β-actin were used as internal controls. (**A**) p16 protein basal levels in U2OS, EH1 and EH2. (**B**) p21 and p53 protein levels following UV irradiation. (**C**) FACScan analysis of UV-treated EH1 cells. (**D**) IPTG-treated and non-treated EH2 cells.

cells (Figure 2B). Figure 2C shows that most of the EH1 cells were in the G_0/G_1 phase of the cell cycle throughout the 24 h that followed the irradiation, indicating that p21 up-regulation is not cell cycle-related. Since p53 is known to be inducible by UV light, we tested this inducibility in our conditions and cell lines. Figure 2B shows that in contrast to p21, p53 levels increased in response to UV light in both U2OS and EH1, indicating that the effect of p16 is specific for p21.

Next, we studied the kinetics and the extent of p21 and p53 induction in the EH2 cell strain, wherein p16 expression is very low. Confluent cells were split into two sub-populations, one was treated with 1 mM IPTG for 48 h and the other one was mock-treated, and then cells were irradiated with a UV

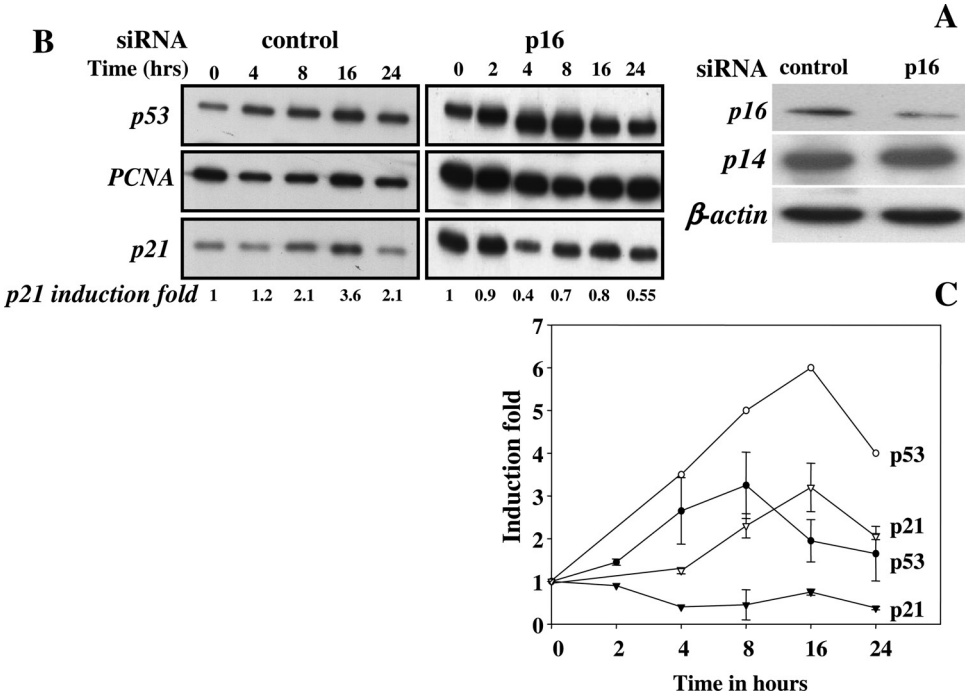

Figure 3. p16 knock-down by specific siRNA abrogates UV-dependent p21 up-regulation. HFSN1 cells expressing either specific p16 siRNA or a control sequence were treated as described in Figure 1, and then specific antibodies were used as indicated. (**A**) Basal p16 protein levels. (**B**) p21 and p53 protein levels. (**C**) Graph depicting p21 and p53 protein levels following UV-treatment of HFSN1 siRNA-control (open symbols) or HFSN1 p16 siRNA cells (dark symbols). Error bars indicate standard errors.

fluence of 5 J m^{-2}. In absence of IPTG, p21 level did not increase in response to UV-irradiation (Figure 2D). In contrast to p21, p53 level increased in EH2, like in EH1 (Figure 2B and D). Figure 2D shows also that the IPTG-treatment increased the level of p16 in EH2 cells. This increase, which did not have major effect on the distribution of cells in the different phases of the cell cycle (data not shown), led to UV-dependent p21 up-regulation. Indeed, 4 h after the treatment p21 level increased and reached a level 6-fold higher after 24 h (Figure 2D). This indicates that p16 plays a positive role in UV-dependent p21 protein up-regulation.

p16 specific siRNA abrogates p21 activation in response to UV light

To demonstrate the role of p16 in UV-dependent up-regulation of p21, the CDKN2A gene has been knocked-down by specific siRNA in the human skin fibroblast HFSN1. p16 siRNA was designed to target the p16 specific exon 1α and avoiding the $p14^{ARF}$ message. Cells were transfected with a vector expressing either p16 siRNA or a nonspecific control siRNA, and neomycin resistant clones were isolated and stable cell lines were established. Figure 3A shows that p16 protein level decreased >2.5 times in the p16 siRNA-treated cells as compared to their corresponding control, however, the $p14^{ARF}$ protein level was not affected. These cells were then challenged with 5 J m^{-2} and treated as described above. Importantly, the decrease in p16 level in HFSN1-p16 siRNA cells was accompanied by an absence of p21 up-regulation in response to UV treatment (Figure 3B). p21 level rather decreased with time as it was observed in MM470, U2OS and EH2 cell lines (Figures 1B and 2B, D). However, p21 protein level increased in UV-irradiated control cells reaching a level 2.6-fold higher in 16 h subsequent the treatment (Figure 3B and C). In contrast, the p53 protein levels were up-regulated in both p16-down-regulated and control cells (Figure 3B), indicating that the p16-knock down effect is specific for p21. These results show that UV-related p21 up-regulation is p16-dependent.

p16 positively controls UV-dependent activation of p21 in MEF cells

To confirm this important relationship between the two main CKIs, p16 and p21, and show that its not cell type or species specific, use was made of the $p16^{-/-}$ and their isogenic normal counterpart $p16^{+/+}$ MEFs. These cells (passage 6) were challenged with a UV fluence of 10 J m^{-2}, and then p21 levels were assessed. Figure 4A shows that at passage 6 the level of p21 did not increase in both $p16^{-/-}$ and $p16^{+/+}$ cells, which is in contrast to what has been described above for different p16-proficient cell lines (Figures 1–3). Knowing that p16 levels augment with serial passages (ageing) (40) similar experiment was performed, but on MEF cells at passage 9. Figure 4D shows that p16 expression is indeed higher in these cells than in those at passage 6, as previously reported (30). Importantly, at passage 9, the level of p21 reached 2.6-fold increase 8 h following UV-treatment and attained 4-fold increase at 24 h (Figure 4B). On the other hand, the level of the p21 decreased following UV-irradiation in $p16^{-/-}$ as observed for the different p16-defective cell lines (Figures 1–3). This suggested that at higher passages the level of the p16 protein is elevated and

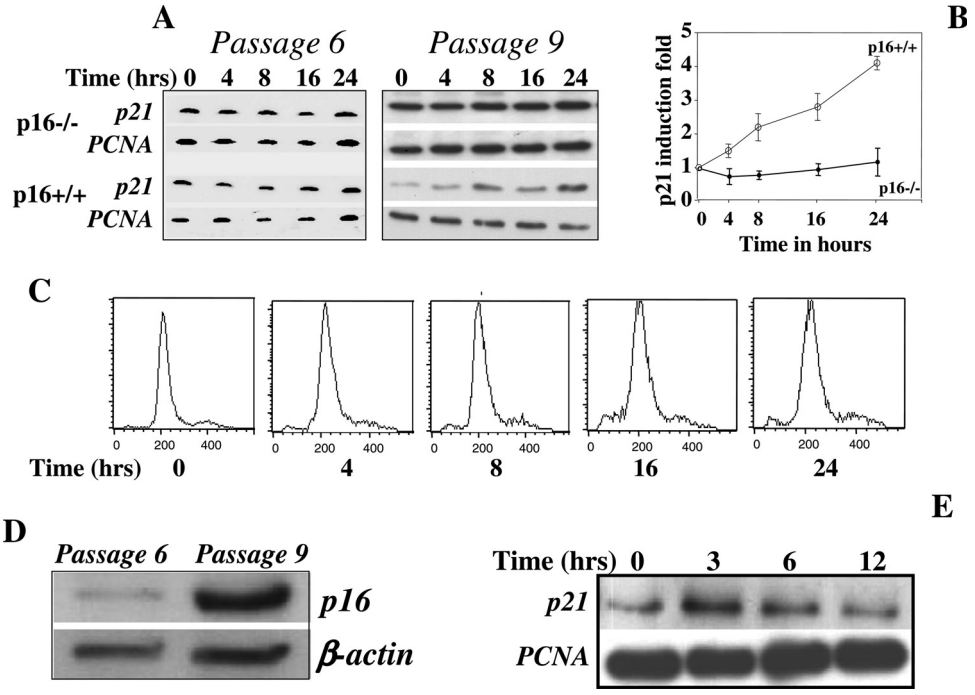

Figure 4. Effect of UV light and γ-rays on p21 protein levels in MEF cells. MEF cells at passage 6 and 9 were either mock-treated or irradiated with UV light (10 J m^{-2}) or γ-rays (5 Gy), reincubated and harvested after the indicated time periods for protein purification or FACScan analysis. Thirty microgram of proteins was used for western blot analysis using the appropriate antibody, as indicated. (**A**) Western blot. (**B**) Graph depicting p21 induction fold. Error bars indicate standard errors. (**C**) FACScan analysis of UV-treated $p16^{+/+}$ cells. (**D**) Western blot, level of p16 in different passages. (**E**) Western blot, p21 protein level following γ-rays in $p16^{-/-}$ cells.

hence, enables UV-dependent activation of p21. To show that this phenomenon is not cell cycle-related, the cell cycle status of $p16^{+/+}$ cells was analyzed by flow cytometry at different periods of time following UV-treatment. Figure 4C shows that most of these cells present a 2*n* DNA content pattern, indicating that p21 activation following UV-irradiation is not cell cycle-related. These findings corroborate the data obtained in human cells and show the role of p16 in UV-dependent activation of p21.

To show that the role of p16 in p21-induction is specific for UV light, $p16^{-/-}$ cells were treated with γ-rays (5 Gy) and p21 protein level was assessed at different periods of time. As expected, p21 level increased 3-fold at 3 h post-irradiation (Figure 4E). This shows that p21-activation, known to be mediated through p53, is not p16-dependent in response to γ-rays.

De novo protein synthesis is required for the p16-dependent induction of p21 following UV radiation

To address the mechanism whereby p16 up-regulates p21 protein levels in response to UV light, we examined the effect of UVC on p21 half-life. For this, UV-irradiated and mock-treated HFSN1 cells were incubated in the presence of cycloheximide (20 μg/ml) to inhibit protein synthesis, and then the relative amounts of p21 and p53 proteins remaining at various time points after cycloheximide treatment were assessed following immunoblotting analysis using densitometry. Figure 5 shows that while p21 protein basal half-life is ~2 h, p53 basal half life is ~1 h. However, following UV-irradiation p21 protein levels rapidly decreased like in

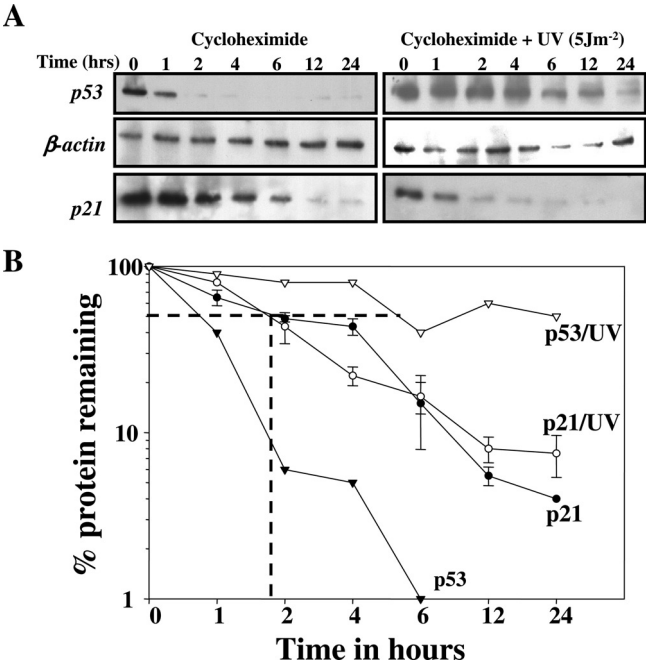

Figure 5. UV light does not stabilize p21 protein in HFSN1 cells. Cells were either UV-irradiated (5 J m^{-2}) or mock-treated, and then reincubated in the presence of cycloheximide (20 μg/ml). Next, cells were harvested at the indicated times for protein purification and western blotting. One hundred microgram of proteins was loaded and appropriate antibodies were used, as indicated. (**A**) Western blots. (**B**) Graph depicting p21 and p53 protein amounts. Signals were quantitated by densitometry and were normalized against PCNA, the amount present at time 0 is considered as 100%. Error bars indicate standard errors.

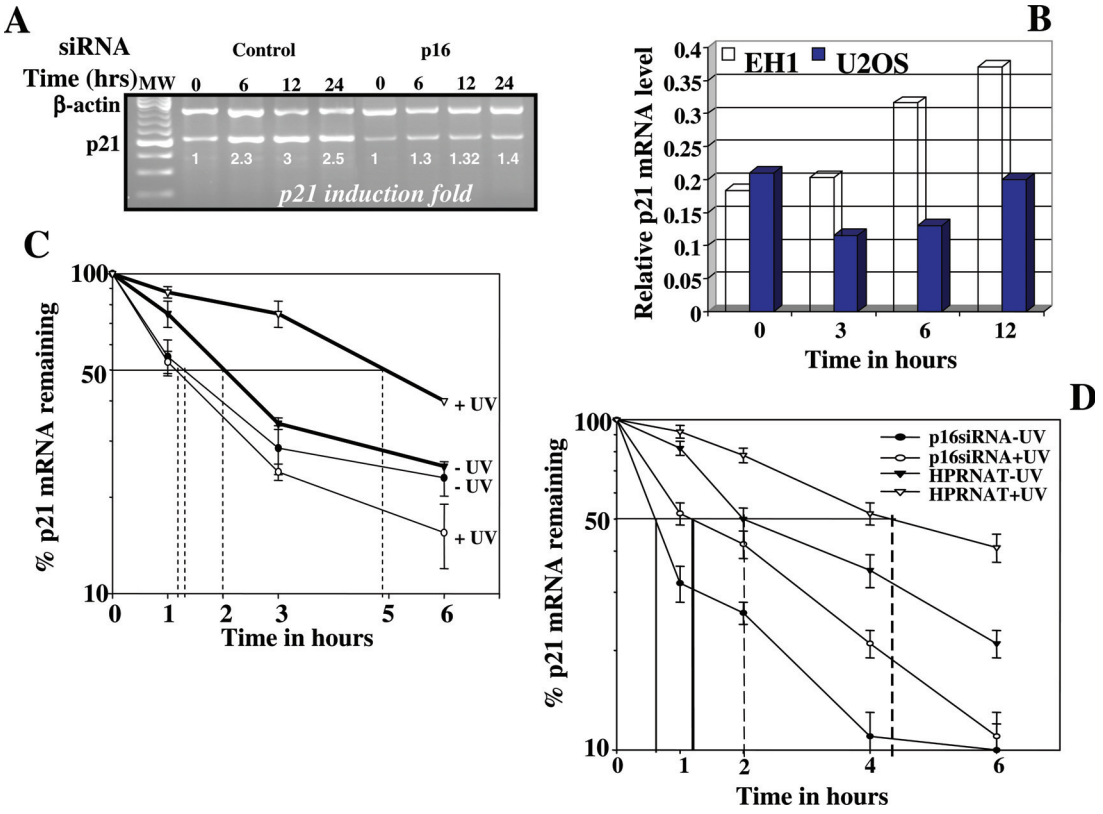

Figure 6. UV light stabilizes p21 mRNA in a p16-dependent manner. Cells were either mock-treated or UV-irradiated (5 J m^{-2}) and then reincubated for different periods of time. Total mRNA was extracted and the amount of p21 mRNA was assessed using RT–PCR technique and normalized against actin. (**A**) Levels of p21 mRNA in UV-treated p16siRNA and control siRNA expressing HFSN1 cells. (**B**) Levels of p21 mRNA in UV-treated U2OS and EH1 cells. (**C**) Levels of p21 mRNA in UV-irradiated and non-irradiated U2OS (circles) and EH1 (triangles), treated with ActD. (**D**) p21 mRNA levels in UV-irradiated and non-irradiated HFSN1 cells expressing either p16 siRNA or control siRNA treated with ActD. Error bars indicate standard errors.

non-irradiated cells, whilst p53 levels were stabilized and the p53 half-life reached 5 h (Figure 5). This indicates that UV-treatment did not augment p21 half-life in HFSN1 cells. Similar results were obtained with the p16-proficient EH1 and $p16^{+/+}$ MEF cell lines (data not shown). This shows that *de novo* protein synthesis is required for the induction of p21 in response to UV light, indicating that by contrast to p53, the p16-dependent increase in p21 protein level is not the result of post-translational modifications of the p21 protein.

p16 is required for UVC-dependent stabilization of p21 mRNA

To investigate the mechanism whereby UVC augments p21 protein level and the role of p16 in this process, we studied the level of p21 mRNA in HFSN1 expressing either the control siRNA or the p16 siRNA. Cells were challenged with a UV fluence of 5 J m^{-2}, and then were reincubated for different periods of time. Subsequently, total RNA was purified and the amounts of p21 and actin mRNAs were assessed in both cell strains using RT–PCR technique. In control cells 3-fold increase in p21 mRNA was obtained 12 h subsequent UV treatment (Figure 6A). In contrast, p21 mRNA did not change significantly during the 24 h of incubation that followed UV-treatment (Figure 6A). Similar results were obtained when fluorescent RT–PCR was used to assess the role of p16 in the activation of p21 mRNA in the U2OS and EH1 cells (Figure 6B). Comparable results were previously reported for U2OS cells treated with 15 J m^{-2} (13). Together, these data indicate that p16 is required for the up-regulation of p21 mRNA in response to UV light.

Next, we sought to study the stability of p21 mRNA following UV-irradiation in both U2OS and EH1 cells using fluorescent RT–PCR. Both cell lines were treated with the transcription inhibitor ActD and then either mock-treated or irradiated with a UV fluence of 5 J m^{-2}. Figure 6C shows that p21 mRNA level decreased sharply in ActD treated U2OS cells, both non-treated and UV-irradiated, with a p21 mRNA half-life of ~70 min. This indicates that UV-irradiation did not stabilize p21 mRNA in U2OS cell strain. However, a great mRNA stabilization was observed in UV-irradiated EH1 cells (p21 mRNA half-life ~5 h), compared to the non-irradiated cells (p21 mRNA half-life ~2 h) (Figure 6C). Likewise, when p16 was down-regulated using specific siRNA in the skin fibroblast HFSN1 cells, p21 half-life in non-treated and irradiated cells was ~40 and 70 min, respectively (Figure 6D). This 30 min increase in p21 mRNA turnover following UV treatment could be due to the presence of a small amount of p16 (Figures 6D and 3A). On the other hand, UV-irradiation increased p21 half-life in the control cells from 2 to 4 h 30 min. These results indicate that p16 is required for the UV-dependent stabilization of p21 mRNA. Figure 6C and D show also that, even in absence of DNA damage,

p21 mRNA half-life is longer in the presence than in the absence of p16, indicating a role of this protein in the stabilization of p21 mRNA.

p16 is required for UV-dependent HuR cytoplasmic localization and binding to p21 mRNA

The HuR protein has been identified by its ability to bind p21 mRNA. HuR is principally a nuclear protein that becomes cytoplasmic following UVC radiation and is required for the stabilization of p21 mRNA (38). Therefore, it is possible that p16 and HuR proteins participate in UV-dependent p21 stabilization through the same pathway. p16 may also be directly or indirectly involved in the HuR function or channeling from the nucleus to the cytoplasm in response to UV-irradiation. To explore these possibilities, we examined the sub-cellular distribution of the HuR protein in the nuclear and cytoplasmic extracts of mock-treated and UV-irradiated (5 J m^{-2}) p16-knocked down HFSN1 cells and their corresponding control. As previously reported, Figure 7 shows that HuR is predominantly nuclear, while tubulin is cytoplasmic. Following UV-irradiation, HuR protein level starts increasing in the cytoplasmic fraction 6 h following the treatment and reached a 5.8-fold increase 12 h later. This significant increase in the HuR level correlated with the peak of p21 induction (Figure 6A). However, no significant change has been observed in the nuclear fraction (Figure 7). In contrast, in p16-down-regulated cells, the HuR level decreased 10-fold in the cytoplasmic fraction, 12 h subsequent UV-treatment (Figure 7).

Intreresting, similar results were obtained when confluent U2OS and EH1 cells were used. Indeed, following UV treatment (5 J m^{-2}) the cytoplasmic HuR level increased up to 6.2 times in the p16-proficient EH1 cells, but decreased in the p16-deficient U2OS cells (Figure 7). This indicates that the UV-dependent increase in the level of HuR in the cytoplasm is under the control of p16.

To further elucidate the role of p16 in the HuR-dependent stabilization of p21 mRNA following UV light, we studied the binding of HuR to p21 mRNA following UV-treatment in p16-defective versus p16-proficient cells. To this end, U2OS and EH1 were either not treated or UV challenged with a UV fluence of 5 J m^{-2}, and then reincubated for 3 and 6 h. Protein lysates were prepared, and then anti-HuR monoclonal antibody was used to immunoprecipitate HuR. RNA that coprecipitated with HuR was isolated and used for RT–PCR reactions using specific p21 primers. The house keeping β-actin was also amplified and used as internal control. Figure 8 shows that p21 mRNA was associated with HuR in non-treated cells. Interestingly, the amount of p21 mRNA that coimmunoprecipated with HuR increased >4 times 6 h after the UV-treatment of EH1 cells. However, a slight decrease in the coimmunoprecipitated p21 mRNA occurred in the U2OS cells (Figure 8). This shows that HuR binding to p21 mRNA following UV light is controlled by p16 protein.

p16 is required for efficient p53-induction in response to UV light

It has been recently shown that HuR binds p53 mRNA and enhances its translation following UV irradiation (41). Thereby, after showing that the UV-related nuclear/cytoplasmic relocalization of HuR is under the control of p16, we asked whether p16 enables efficient p53 induction following UV light. In fact, we noticed that, although p53 is induced following UV light, this induction is not optimal in all the p16-defective cell lines as compared to normal ones (Figures 2 and 3). Next, we studied UV-dependent p53 up-regulation in MEFs ($p16^{-/-}$ and $p16^{+/+}$) treated as described above. Figure 9 shows that p53 level increased in both genotypes, however, not to the same extent. Indeed, while UV treatment enhanced p53 level 8-fold in $p16^{+/+}$ cells, the induction was only 2.7-fold in p16-defective cells (Figure 9). This shows that p16 knockout attenuated the up-regulation of p53 protein following UV light.

DISCUSSION

The p21 gene product integrates several extracellular stress signals into cellular responses that lead to proliferation or cell cycle arrest (42). These signals are mediated through different, but overlapping, pathways that are governed by many proteins. The p21 tumor suppressor protein is activated in response to different DNA damaging agents and is the effector of p53 in the ionizing radiation-mediated signaling route (6). However, the role of p21 and p53 in the UV-dependent signaling pathway remains elusive. In the present report, we present evidence that p21 protein is up-regulated in response to low UV fluences in different mammalian cell lines that express p16 protein. These include mouse embryonic fibroblasts and mouse skin fibroblasts ($p53^{-/-}$ and $p53^{+/+}$) and various types of human cells, EH1, two different skin fibroblasts: HSF1 and HFSN1, MCF-12 (epithelial cells) and MEN1 (meningioma-derived cell line). In addition, the IPTG activation of p16 up-regulated p21 in response to UV light in EH2 cells. On the other hand, p21 protein levels did not augment following UV light in the cell lines wherein p16 is defective or down regulated, MEFs, EH2, U2OS,

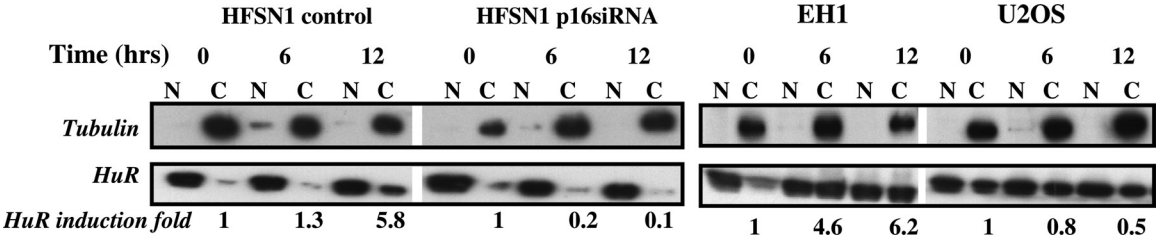

Figure 7. Effect of p16 on the UV-dependent nucleocytoplasmic shuttling of HuR. Confluent cells were mock- or UV-treated (5 J m^{-2}) and reincubated for the indicated periods of time. Cytoplasmic (C) and nuclear (N) cell lysates were prepared and subjected to western blot analysis using the indicated antibodies.

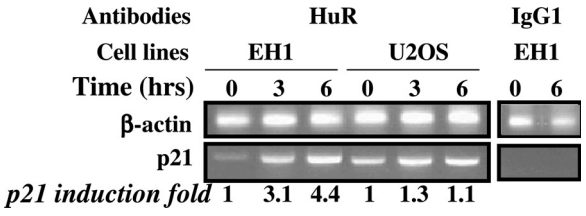

Figure 8. Role of p16 in the UV-dependent binding of HuR to p21 mRNA. Confluent cells were mock- or UV-treated (5 J m^{-2}) and reincubated for the indicated periods of time. Cell lysates were immunoprecipitaed with HuR mouse monoclonal antibody and mouse IgG1. Coprecipitated RNA was purified and used for RT–PCR reactions.

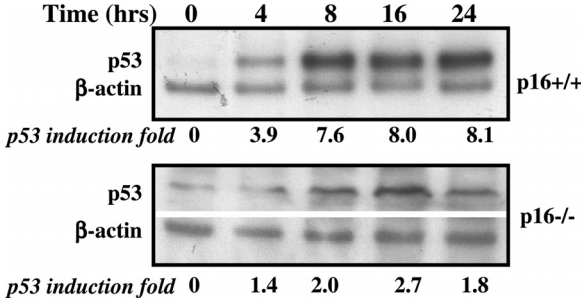

Figure 9. Effect of p16 on the UV-dependent p53 up-regulation in MEF cells. Cells were treated as described in Figure 4 and western blots using the indicated antibodies were performed.

HFSN1-expressing p16siRNA and MM470 cells. Collectively, these results clearly show that p21 protein levels increase in response to low UV fluences, but in a p16-dependent manner. These data offer further support for what has been previously reported concerning the up-regulation of p21 protein in response to UV light (7–11,19), and suggest that the lack of p21 up-regulation that has been previously found in different human cell lines could be, at least is part, due to a defect in p16. In fact, in these studies several cancer cell lines, including U2OS, were used. For the human normal primary fibroblast IMR-90 strain, it has been recently shown that p21 protein increases in response to low UV fluences using different growing and irradiation conditions (19). Furthermore, it has been recently shown that UV induced the level of p21 protein at low fluences, but reduced its level at higher fluences (43). We have also found that UV fluences >5 J m^{-2} (human cells) and 10 J m^{-2} (MEFs) decreases p21 level (data not shown). Therefore, the decrease in p21 levels following low UV fluences reported by Bendjennat *et al.* (12) may have several reasons, such as UV light calibration and/or the conditions of UV irradiation (presence/absence of medium) (19) or the antibody that has been utilized (43).

Furthermore, our data show that p16 protein level is critical for p21 induction in both human and mouse cells. Indeed, in the same cell line, cells that express low level of p16 such as MEF $p16^{+/+}$ at passage 6, p21 level did not increase after UV-treatment. However, when the same cell line reached passage 9 during which the expression of p16 is higher, p21 protein level increased in response to UV light (Figure 4). Likewise, no p21 up-regulation was observed in HFSN1-p16 siRNA and EH2 cells wherein p21 levels are only ~2 times lower than in their corresponding 'normal' counterparts, which showed p21 induction in response to UV damage. This indicates that p16 level is a critical factor that needs to be tightly controlled. Indeed, it has been recently shown that a modest increase in p16 (1.5-fold) had significant impact on cancer resistance (44).

Is p21 up-regulation following UV damage p53-dependent or not? This is another puzzling question that needs clarification. We have found that UV damage up-regulates p21 in mouse skin fibroblasts independently of p53 (data not shown). Similar results were previously obtained in different cell lines (7,11,20,21,45). However, two reports described a p53-dependent activation of p21 (23,24). In these papers, the p21 basal mRNA levels (24) or the p21 basal protein levels (23) were much lower in p53-deficient cells than in p53-proficient ones. However, in both genotypes p21 levels increased following UV-treatment. Therefore, it seems more likely that p21 induction in response to UV light is p53-independent. Therefore, based on the nature of DNA damage, p21 is activated either through p53 (ionizing radiation), or in a p16-dependent manner (UV light). This may suggest that p21 acts as an effector of p16 in the cellular response to UV damage, while it acts under the control of p53 following γ-rays. In fact, in response to UV light, both p21 and p16 act as antiapoptosis factors (13,46). Furthermore, p16- and p21-defective cell lines do not display UV-dependent cell cycle arrest at G_1 phase (12,13,47–49) and are sensitive to UV-irradiation (50,51). Moreover, it has been previously found that p21 is required for efficient p16-mediated cell cycle arrest in HCT116 cells (5). Together, these results indicate a functional relationship between p16 and p21 during the cellular response to UV damage.

Evidence presented herein shows also that p16-dependent activation of p21 following UV light occurs by post-transcriptional stabilization of p21 mRNA (Figure 6), but not from post-translational modifications (Figure 5). These results are in keeping with the previously reported data showing that the elevation of p21 expression by UVC is mediated through mRNA stabilization in both MEFs and human colorectal carcinoma cells (24). This stabilization requires the nuclear HuR protein that accumulates in the cytoplasm following UVC radiation (38). We have shown here that the increase in the HuR cytoplasmic abundance and its binding to p21 mRNA in response to UV light are under the control of p16 (Figures 7 and 8). Accordingly, we are led to conclude that p16 controls UV-related p21 induction by regulating the nucleo-cytoplasmic translocation of the mRNA binding HuR protein. If this is the case, p16 should also be important for UV-dependent up-regulation of p53 protein, since HuR enhances p53 translation following UV (41). Indeed, we have found that p16 is required for efficient p53 induction following UV light (Figure 9). In addition, in response to UV damage, HuR stabilizes the mRNA of the gene coding for the small GTP binding RhoB protein (37). This indicates that HuR play important roles in the cellular response to UV light.

In conclusion, the data presented in the present report vividly show that low UVC fluences up-regulate p21 protein in different mammalian cells, and that this induction is under the control of the other important CDK inhibitor and tumor suppressor p16 protein. Moreover, it is shown here that p16 controls also the UV-dependent cytoplasmic accumulation

of the mRNA binding HuR protein, which is required for p21 induction, suggesting an indirect effect of p16 on the UV-mediated p21 mRNA stability through the HuR protein. Since HuR has a broad post-transcriptional function, we assume that p16 has major roles in the cellular response to the carcinogenic UV light, the most important etiological cause of skin cancer.

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