Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation

Dmitry V.Bulavin[1], Shin'ichi Saito[2], M.Christine Hollander[1], Kazuyasu Sakaguchi[2,3], Carl W.Anderson[4], Ettore Appella[2] and Albert J.Fornace Jr[1,5]

[1]Division of Basic Science and [2]Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892 and [4]Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA

[3]Present address: Laboratory of Structure-Function Biochemistry, Department of Molecular Science, Kyushu University, Fukuoka 812-8581, Japan

[5]Corresponding author
e-mail: fornace@nih.gov

Components of the ras signaling pathway contribute to activation of cellular p53. In MCF-7 cells, p38 kinase activated p53 more effectively than other members of the ras pathway. p53 and p38 kinase exist in the same physical complex, and co-expression of p38 stabilized p53 protein. *In vitro*, p38 kinase phosphorylated p53 at Ser33 and Ser46, a newly identified site. Mutation of these sites decreased p53-mediated and UV-induced apoptosis, and the reduction correlated with total abrogation of UV-induced phosphorylation on Ser37 and a significant decrease in Ser15 phosphorylation in mutant p53 containing alanine at Ser33 and Ser46. Inhibition of p38 activation after UV irradiation decreased phosphorylation of Ser33, Ser37 and Ser15, and also markedly reduced UV-induced apoptosis in a p53-dependent manner. These results suggest that p38 kinase plays a prominent role in an integrated regulation of N-terminal phosphorylation that regulates p53-mediated apoptosis after UV radiation.
Keywords: apoptosis/DNA damage/p38/p53/ phosphorylation

Introduction

The tumor suppressor p53 plays a central role in preserving genomic integrity by arresting cell cycle progression or activating apoptosis after genotoxic stress (Levine, 1997; Prives and Hall, 1999). The regulation of p53 activity is complex and includes post-translational events such as phosphorylation and acetylation (Meek, 1998a; Sakaguchi et al., 1998). Phosphorylation at several different serine and threonine residues in p53 has been shown to occur after cells are exposed to DNA-damaging agents including UV radiation (Sakaguchi et al., 1998; Shieh et al., 1999), and recent reports suggest that phosphorylation of Ser392 after UV exposure (Kapoor and Lozano, 1998; Lu et al., 1998) may be important for p53 oligomerization (Sakaguchi et al., 1997), while phosphorylation of several N-terminal residues, including Ser15, Ser20 and Ser37,

after both ionizing (IR) and UV radiation (Shieh et al., 1997; Siliciano et al., 1997) is important for stabilizing p53 (Shieh et al., 1997; Unger et al., 1999). In addition, N-terminal serines may be important for p53 interactions with the transcriptional coactivators p300/CBP (CREB-binding protein) and PCAF (p300/CBP associated factor), and also for regulating p53 acetylation in the C-terminal domain (Lambert et al., 1998; Sakaguchi et al., 1998). Nevertheless, the picture of p53 phosphorylation at N-terminal sites in response to genotoxic stress is incomplete.

The ras pathway is a key signal transducing system that mediates several extracellular signals through a cascade of protein phosphorylation. In mammalian cells, this pathway (ras/MAPK/SAPK) has three distinct components: the extracellular signal-regulated kinase (ERK) pathway, the stress-activated c-Jun N-terminal kinase (JNK) and the p38/HOG kinase pathways. Thus, ras pathway members participate in regulating cell proliferation, differentiation, stress responses and apoptosis (Xia et al., 1995). Interestingly, genotoxic stress agents, such as UV radiation, activate p53 as well as the JNK and p38 kinase pathways (Kapoor and Lozano, 1998). Serrano et al. (1997) demonstrated a striking correlation between the overexpression of ras and activation of p53 in experiments in which ras triggered apparent cell senescence in normal (primary) human and rodent fibroblasts. A functional wild-type (wt) p53 was required for this ras-induced senescence. A further connection with p53 was shown for JNK and MEKK1, which regulate p53 stability in an Mdm2-dependent manner (Fuchs et al., 1998).

Several studies point to important differences in the regulation of stress responses in response to agents that produce primarily DNA strand breaks (e.g. IR) versus base-damaging agents such as UV radiation or DNA alkylation (Holbrook et al., 1996). For example, both IR and UV radiation induced increases in p53 protein, but p53 transcriptional activity, as measured by reporter constructs, was induced to a much greater extent by UV radiation and the alkylating agent methylmethane sulfonate (MMS) than by IR (Zhan et al., 1993). Interestingly, in many cell types UV radiation and MMS are far more effective than IR in inducing AP-1 activity and apoptosis, which are regulated by JNK and p38 kinase. Analysis of the role of these and other Ras/MAPK/SAPK kinases in p53-mediated responses has been problematic because the transfection procedures required to introduce expression vectors into cells can activate p53 (Renzing and Lane, 1995). Recently, Bai et al. (1997) used psoralen-inactivated adenovirus coupled with polylysine to introduce plasmid DNA into mammalian cells without chemical treatment of the cells. Here we used this approach, which did not by itself strongly activate p53, to transfect various Ras/MAPK/ SAPK expression vectors in several human cell lines. We found that p38 kinase activated p53-mediated transcription

more efficiently than other members of the ras pathway in MCF-7 cells. Activation resulted from phosphorylation by p38 kinase of p53 at Ser33 and Ser46, a site previously not known to be phosphorylated. After UV radiation, phosphorylation of these two sites was crucial for the subsequent phosphorylation of Ser15 and Ser37, two other N-terminal residues that are phosphorylated in response to UV radiation. Furthermore, UV-induced phosphorylation of Ser33 and Ser46 was associated with activation of p53-mediated apoptosis. We conclude that p38 kinase plays an important role in regulating p53 activity following exposure to UV radiation.

Results

p38 kinase, a member of the MAP kinase family, activates transcription from p53-responsive elements

To identify members of the Ras/MAPK/SAPK pathway that activate p53, we co-transfected cells with expression vectors for different family members and reporter constructs for either p53 or control (AP1-like) transcription factors. An adenovirus-mediated transfection method was used to avoid transfection-mediated activation of endogenous wt-p53, as occurs with some DNA transfection protocols such as calcium phosphate (Renzing and Lane, 1995) or lipid transfection reagents (data not shown). The Ras/MAPK/SAPK family members examined were c-Raf1, MKK1, ERK2 and the antisense orientated MKP1 (MAP kinase phosphatase) from the ERK branch of MAP kinases, MEKK1, SEK1 and JNK1, ASK1, and p38 kinase from the JNK and p38 branches of MAP kinases. We used two p53 reporter constructs: pP53RECAT2, which contains 5-repeats of the p53 consensus sequence from the *GADD45* gene, and pWWP-CAT with the entire 2.3 kbp *CIP1/WAF1* promoter (see Materials and methods). To assess activation of the ras-dependent system, we used two AP-1 reporter plasmids: TRE-CAT, which contains a TRE-responsive element, and *jun*-CAT, encompassing the entire promoter of *JUN*. We co-transfected either MCF7 cells, a human breast carcinoma cell line with a wt-p53 that activates the G_1 checkpoint and undergoes apoptosis after DNA damage, or H1299, a p53-null lung adenocarcinoma cell line, which served as a control for p53-dependent activation. Overexpression of members of the ERK pathway were less effective in inducing TRE-CAT and JUN-CAT than MEKK1 and ASK1, members of the JNK and p38 kinase-dependent pathways, respectively (Figure 1A and B). Similar experiments with p53 reporters showed that, in addition to p53 itself, only members of the JNK and p38 kinases branches of the MAPK families strongly activated p53-responsive elements (Figure 1C and D). MEKK1, an upstream activator of JNK and p38 kinase, and p38 kinase itself most effectively activated p53-mediated transcription; JNK, the downstream kinase for MEKK1, was much less effective than MEKK1. Increasing the amount of each kinase expression plasmid did not significantly change the amount of activation, indicating that the amount of expressed kinase was not limiting (data not shown).

In addition to p53, several proteins are now known to bind and activate p53-responsive elements, including p73, NBP (non-p53RE binding protein) and p53CP. To confirm
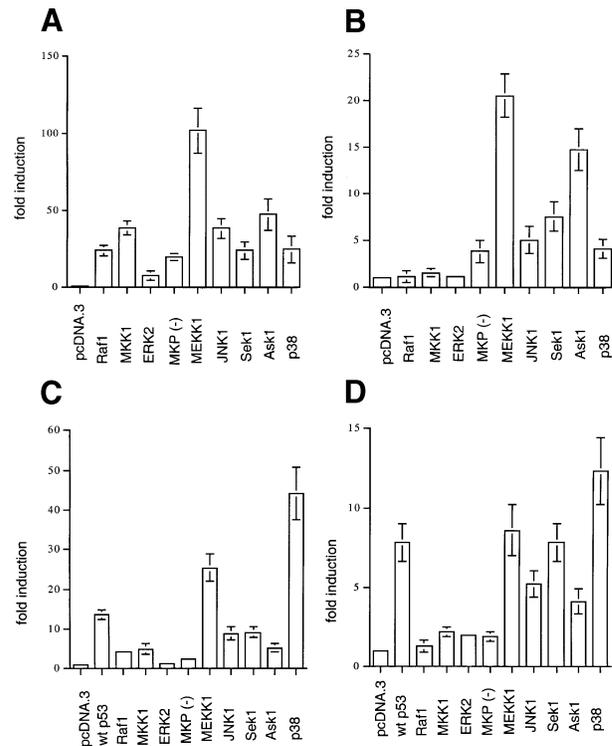
Fig. 1. Activation of p53-mediated transcription by Ras/MAPK/SAPK pathway members in MCF7 cells. MCF7 cells, which express wt-p53, were transfected with expression vectors for MAP kinase family members (0.6 μg) as indicated, and with reporters for AP-1 or p53 activity (0.2 μg). As controls, transcription activity was assessed with chloramphenicol acetyltransferase (CAT) reporters bearing (**A**) a TRE-binding site (TRE-CAT, TPA-responsive element) or (**B**) the entire *JUN* promoter (JUN-CAT). p53 activity was assessed with CAT reporters containing p53-responsive elements (**C**) linked to a basal promoter reporter (pP53RECAT2) or (**D**) from the entire *CIP1/WAF1* promoter (pWWP-CAT). Cultures were harvested 24 h after adenovirus-mediated transfection and processed for CAT activity as described in Materials and methods.

the p53-dependent mechanism of pWWP-CAT and pP53RECAT2 activation after co-transfection with MAPK family members, we analyzed activation of these reporters after transfection into H1299 cells. Transcription from TRE-CAT and JUN-CAT in H1299 cells was activated by the same kinases as in MCF-7 (Figure 2A and B). In contrast, activation of transcription from p53 reporters was significantly reduced (Figure 2C and D). wt-p53 increased expression from pP53RECAT2 in H1299 cells up to 42 times, but MEKK1 and p38 increased it by only 2.2 and 5.5 times, respectively. Thus, activation of p53-responsive elements by the MAPK family members seems to be largely p53 dependent, although we do not exclude small contributions from other p53 family members.

p38 kinase interacts with, and phosphorylates, Ser33 and Ser46 of human p53

Overexpression of MEKK1 and activation of JNK1 were shown to stabilize p53, thereby increasing expression from p53-dependent reporters (Fuchs *et al.*, 1998). To investigate the way in which p38 kinase activates p53, we examined the ability of activated p38 kinase to phosphorylate p53 *in vitro*. p38 kinase immunoprecipitates were prepared from H1299 cells transiently transfected with the pCMV-p38-Flag expression vector previously
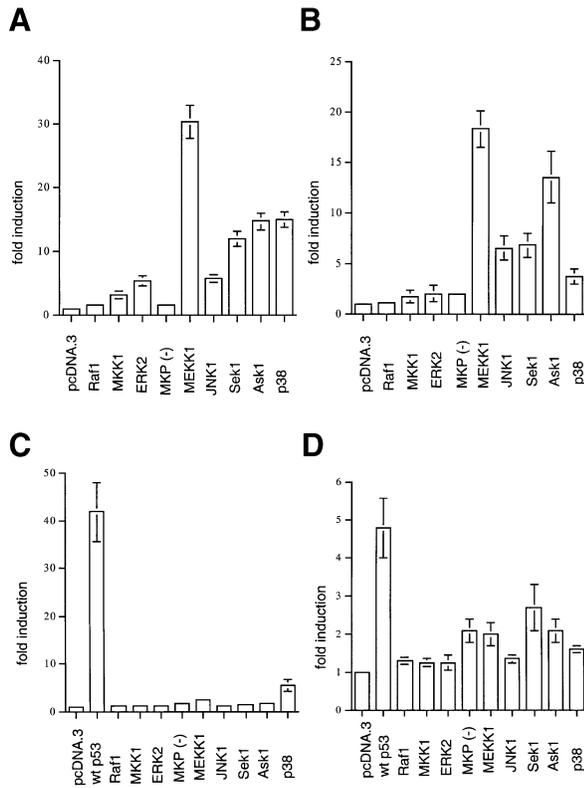
Fig. 2. Activation of p53-mediated transcription by Ras/MAPK/SAPK pathway members in H1299 cells. H1299 cells, which do not express p53, were transfected with expression vectors for Ras/MAPK/SAPK family members (0.6 µg) as indicated, and with reporters for AP-1 or p53 activity (0.2 µg). AP-1 and p53 activity was assessed as described in the legend to Figure 1 with control CAT reporters containing (**A**) a TRE-binding site (TRE-CAT) or (**B**) the entire *JUN* promoter (JUN-CAT), or with CAT reporters containing p53-responsive elements (**C**) (pP53RECAT2) or (**D**) the entire *CIP1/WAF1* promoter (pWWP-CAT).

activated by treatment with UV radiation or not (see Materials and methods). Initial experiments using GST–ATF2 as substrate showed that Flag-tagged p38 immuno-precipitates from UV-activated cells containing active p38 kinase (Figure 3A, right), but not JNK activity, as judged by an inability to phosphorylate GST–JUN (data not shown). To identify possible p53 phosphorylation sites, we incubated the UV-activated p38 kinase with four groups of synthetic human p53 peptides, three from the N-terminal domain and one from the C-terminal domain. None of the C-terminal peptides p53(319–393), p53(319–358) or p53(385–393) were phosphorylated by p38 kinase, but several N-terminal peptides, including p53(1–39) and p53(25–65), but not p53(1–25), were readily phosphorylated (Figure 3A; data not shown), suggesting that serines or threonines between Ser33 and Thr55 might be substrates for p38 kinase. To define the p38 kinase site(s) further, we used several N-terminal peptides as p38 kinase substrates in which phosphate was incorporated chemically at different sites. The peptide p53(1–39) with phosphorylated Ser37 was phosphorylated as well as the non-phosphorylated peptide, but the peptide with phosphate on Ser33 was a much poorer p38 kinase substrate (Figure 3A). Similar results were obtained with peptide p53(25–65); nevertheless, with phosphate on Ser33 and Ser37 it was still phosphorylated well by p38 kinase, suggesting that Ser33

and one other site distal to Ser37 are p38 kinase sites. To confirm this possibility we synthesized three peptides, substituting alanine for Ser33 in one, alanine for Ser46 in another and finally for both serines. Substitution at either Ser33 or Ser46 in the p53(25–65) peptide significantly reduced phosphorylation by p38 kinase, but substitution at both serines abolished phosphorylation (Figure 3A). A similar result was obtained with GST–p53; substitution at both Ser33 and Ser46 reduced phosphorylation by 90% (Figure 3B). The resulting membrane did not stain with an antibody that recognizes p53 phosphorylated at Ser392 (data not shown), indicating that Ser392 of the human GST–p53 was not phosphorylated by p38 kinase. Thus, Ser33 and Ser46 in human p53 are the major sites phosphorylated by p38 kinase *in vitro*.

Pulse–chase analyses were used to determine whether p38 kinase expression changed the half-life of p53. HCT116 p53$^{-/-}$ cells were co-transfected with wt-p53 and pcDNA.3 vector or with the p38 kinase expression construct, and 24 h later one set of cells was treated with UV (14 J/m^2). A pulse–chase experiment was performed as described in Materials and methods, and the results are presented in Figure 3C. Quantitation of the autoradiograms from replicate experiments indicated that the half-life of wt-p53 was ~70 min. Co-transfection with p38 at a p53:p38 molar plasmid ratio of 1:3 increased the half-life of wt-p53 to >4 h, a value comparable to that caused by UV treatment (Figure 3C). Thus, overexpression of p38 kinase increases the stability of p53.

The p53 protein functions as a transcription factor in the nucleus, whereas under normal conditions, p38 kinase localizes primarily in the cytoplasm. Several kinases involved in mitogenic signaling are known to move from the cytoplasm to the nucleus; therefore, we examined whether p38 kinase does so after UV irradiation in A549 lung carcinoma cells. Figure 3D shows that before exposure p38 kinase was primarily cytoplasmic, although concentrated around the nucleus. In contrast, p53 was primarily nuclear, although some staining was seen in the cytoplasm. By 1 h after treatment with 14 J/m^2 UV radiation, practically all nuclei stained for p38 kinase, indicating that the kinase had moved to the nucleus. Between 2 and 4 h after treatment, p38 kinase was redistributed in the cytoplasm (data not shown). Thus, both p53 and p38 kinase have a similar distribution 1 h after UV irradiation when p38 kinase is most active.

To determine whether p38 kinase and p53 are associated, we performed co-immunoprecipitation experiments. We were unable to detect endogenous p53 by Western blotting in immunoprecipitates of p38 kinase from A549 or MCF7 cells (data not shown); however, p53 was readily observed in Western blots of anti-Flag precipitates from H1299 cells that were harvested 24 h after co-transfection with a wt-p53 expression vector and p38-Flag (Figure 3E). As expected, no p53 was present if the H1299 cells were transfected with p38-Flag alone. These results indicate that p53 and p38 kinase can be found in the same complex. The amount of p53 in the p38 precipitates was not increased after UV treatment. The apparent abundance of p38–p53 complexes in untreated cells may reflect the fact that p38 kinase is both nuclear and cytoplasmic when overexpressed in H1299 cells (data not shown) and COS-1 cells (Raingeaud *et al.*, 1995). This result is
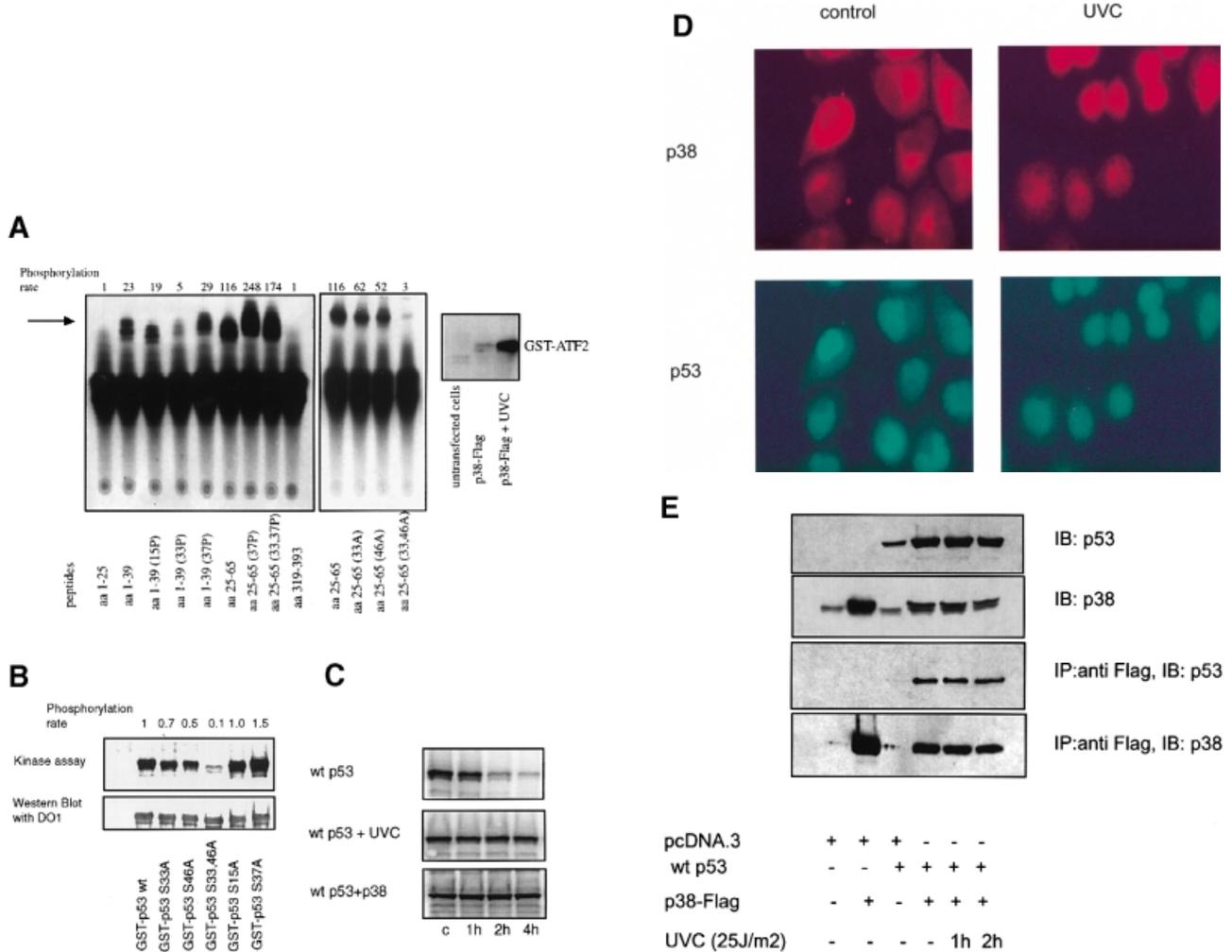
Fig. 3. p38 kinase phosphorylates, stabilizes and binds human p53. (**A**) Phosphorylation of N- and C-terminal p53 peptides by p38 kinase. Immunoprecipitates of exogenous p38-Flag kinase with or without UV activation were used to phosphorylate GST–ATF2 (right panel). UV-activated p38 kinase immunoprecipitates were used to phosphorylate peptides corresponding to N- or C-terminal regions of human p53, and the phosphorylated peptides were fractionated by TLC as described in Materials and methods. Some peptides were synthesized with phosphate (P) at the indicated p53 serine residue; others had alanine (A) in place of the indicated serine. The position of the phosphorylated peptide is indicated by an arrow to the left of the autoradiograms. (**B**) Phosphorylation of GST–p53 by p38 kinase. GST–wt-p53 and different mutants (see figure) were phosphorylated by p38 kinase *in vitro*. The amount of GST–p53 was verified by staining the same membrane with DO-1. (**C**) p38 kinase prolongs the half-life of p53. HCT116 p53$^{-/-}$ cells were co-transfected with expression vectors for wt-p53 and pcDNA.3 (vector) or p38 kinase; 18 h later, one set of cells was treated with UV (14 J/m^2). Six hours later, all sets of cells were labeled with [^{35}S]methionine and then chased with cold methionine for 0–4 h. p53 was immunoprecipitated from cell extracts and fractionated by SDS–PAGE. (**D**) Co-localization of p53 and p38 kinase. A549 cells were treated with UV radiation (25 J/m^2) and fixed in methanol 1 h later. p38 kinase and p53 were detected by indirect immuno-fluorescence with the specific antibodies C-20 and DO-1 (Santa Cruz), respectively. Control, no UV treatment. (**E**) Binding of p53 and p38 kinase. H1299 cells were transfected with pcDNA.3, pCMV-p38-Flag or pCMV-p38-Flag and pCMV-wt-p53 together, and 24 h after transfection the cells were UV irradiated with 25 J/m^2. p53 in complex with p38 kinase was immunoprecipitated with anti-Flag antibodies and was visualized with AB-7 (Calbiochem). p38 kinase was detected with C-20 (Santa Cruz) (the upper band is transfected and the lower band is endogenous p38); p53 was visualized in whole-cell extracts with AB-7.

consistent with the stimulation of p53 transcriptional activity after overexpression of p38 kinase without UV activation (Figure 1C and D).

Substitution of Ser33 and Ser46 with alanine decreased p53-mediated apoptosis

To explore the role of Ser33 and Ser46 in regulating p53 functions, we constructed p53 mutants that encode alanine in their place, and analyzed those by transient transfection of H1299 cells. First, we checked the p53 level after transient transfection. Wild-type and all mutant (S33A, S46A and S33,46A) p53s were expressed at essentially the same level (Figure 4A). Next, we assessed the ability

of different mutants to transactivate the reporter plasmid pP53RECAT2, and found that alanine substitution for either Ser33 or Ser46 had no significant effect on the ability of p53 to transactivate this reporter. In contrast, p53-S33,46A was much less able to activate such transcription (Figure 4A). To determine whether the loss of transactiv-ation potential was due to a decrease in p53's half-life, we then analyzed the half-life of wt and p53-S33,46A by pulse–chase analysis (Figure 4B). Both had similar half-lives of ~73 min, consistent with the fact that their protein levels determined by Western blotting were similar. Nevertheless, co-transfection with p38 kinase as well as UV treatment (Figure 4B) still stabilized p53-S33,46A
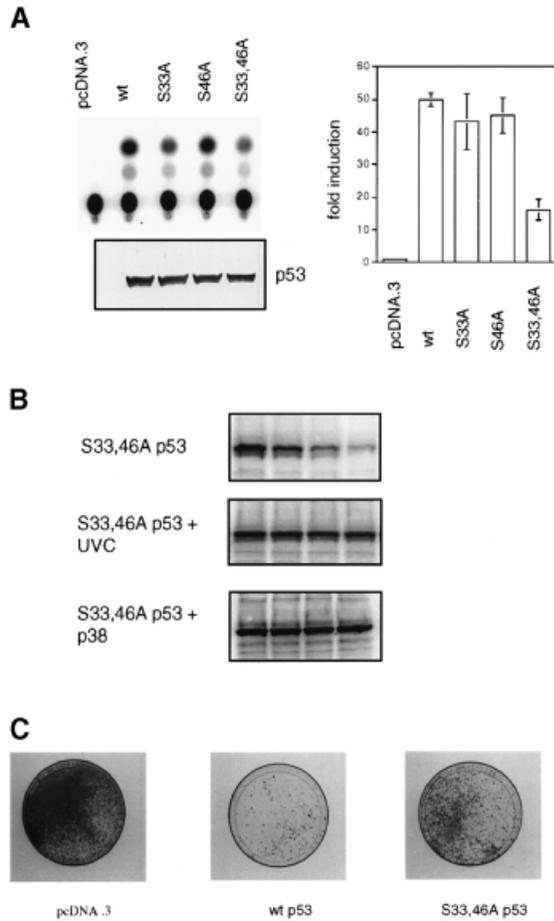
Fig. 4. Ser33 and Ser46 of human p53 are required for transactivation and growth suppression. (**A**) p53-S33,46A is defective in transactivation. H1299 cells were transiently transfected with pP53RECAT2 and wt or mutant p53 expression vectors, and CAT activity was determined 24 h later as described in Materials and methods. The left panel shows the image of a CAT assay after transfection with vector (pcDNA.3), p53-SN3 (wt), p53-S33A, p53-S46A, or p53-S33,46A. Western immunoblot analysis with the p53-specific DO-1 antibody (below CAT assay) showed that expression levels of wt and mutant p53 were the same. The graph to the right presents the relative fold induction of CAT from pP53RECAT2 in response to wt and mutant p53. Data are averages of four independent experiments. (**B**) Substitution of alanine for Ser33 and Ser46 does not block the stabilization of p53 by UV or p38 kinase. HCT116 p53$^{-/-}$ cells were transiently transfected with the p53-S33,46A expression vector as described above and then pulse-labeled with [^{35}S]methionine as described in Materials and methods. (**C**) p53-S33,46A is defective in growth suppression. H1299 cultures were transfected with pcDNA.3, wt-p53 or p53-S33,46A and then subjected to G418 selection. Colonies were stained with crystal violet 2 weeks later.

even though p38 kinase could not phosphorylate the mutant p53. These results indicate that p38 kinase regulates p53 by at least two mechanisms: one is connected with p53 phosphorylation and the other, which does not require p53 phosphorylation, regulates p53 stability.

To determine whether Ser33 and Ser46 were required for p53-mediated apoptosis, we compared the ability of wt-p53 and p53-S33,46A to suppress colony formation (Figure 4C). Five to seven times more colonies were obtained from cells transfected with p53-S33,46A and selected for G418 resistance than from similarly selected cells transfected with wt-p53, affirming that phosphoryla-

tion of Ser33 and Ser46 may be important for the induction of apoptosis. However, S33A and S46A single mutants were able to suppress colony formation to the same extent as wt-p53 (data not shown).

To analyze further the ability of p53-S33,46A to activate cell cycle arrest and apoptosis, we transiently co-transfected H1299 cells with a plasmid that expresses the surface marker CD20 and with wt-p53 or p53-S33,46A. Eighteen hours later, one set of cultures was treated with 14 J/m^2 UV radiation; the cultures were harvested 48 h after transfection, and stained with anti-CD20 antibodies and propidium iodide. FACS analysis was used to determine the DNA content of CD20-positive cells (Figure 5A). Without UV exposure and p53, ~4% of the H1299 cells were in apoptosis as determined from the fraction of cells with a sub-G$_1$ DNA content, and UV increased this fraction to 8–10%. In non-irradiated cells transfected with wt-p53, 16% of cells were apoptotic, and UV increased this fraction to 51%. In contrast, only 8.3% of unexposed, p53-S33,46A-transfected cells were apoptotic, while UV irradiation increased this fraction to 16–18%.

We then analyzed the level of phosphorylation at Ser15, Ser33, Ser37 and Ser392 in H1299 cells transfected with wt-p53 or with p53 mutants that had alanine substituted at Ser33, Ser46 or both residues, with and without UV irradiation. To detect phosphorylation, we used polyclonal antibodies specific for p53 phosphorylated at each of these sites to probe Western blots of p53 immunoprecipitates as described previously (Sakaguchi *et al.*, 1998). Cultures were harvested either before or 4 or 8 h after UV irradiation with 25 J/m^2. Under these conditions, Ser392 was phosphorylated to an equal extent in both wt-p53 and the p53-Ser33,46A mutant after UV treatment (data not shown). As shown previously (Sakaguchi *et al.*, 1998; Shieh *et al.*, 1999), phosphorylation of Ser15 and Ser37 in wt-p53 was strongly induced by exposure to UV light, while the increase at Ser33 was more modest as a consequence of a significant basal level of phosphorylation. Substituting alanine for Ser46 did not change this pattern of response (Figure 5B), but a significant deregulation in N-terminal phosphorylation was observed when alanine was substituted for Ser33 alone or when both Ser33 and Ser46 were changed. Substitution at Ser33 completely blocked UV-induced phosphorylation at Ser37 but did not decrease phosphorylation at Ser15 (Figure 5B). In the p53-S33,46A double mutant, not only was phosphorylation of Ser37 abrogated, but phosphorylation at Ser15 was also dramatically reduced. Thus, unexpectedly, either the presence of Ser33 and Ser46 themselves, or their ability to be phosphorylated, is important for a full N-terminal phosphorylation of p53 after cells are exposed to UV light.

Inhibition of p38 kinase reduced p53-mediated transactivation, N-terminal phosphorylation and apoptosis in response to UV radiation

To investigate whether phosphorylation by p38 kinase at Ser33 and Ser46 of human p53 is a prerequisite for UV-mediated phosphorylation at other N-terminal p53 sites, we used the specific inhibitors of p38 kinase, SB203580 and SB202190 (Hazzalin *et al.*, 1996). We first determined that both inhibitors were effective *in vivo* by treating A549 cells with 10, 20 or 40 µM inhibitor for 30 min before exposure to 25 J/m^2 UV, and continuing incubation in the

A

pCDNA3   wt p53   S33,46A p53

control

apoptosis - 4.2 %   apoptosis -16.1%   apoptosis - 8.3%
G1 - 57.0%   G1 - 72.1%   G1 - 78.9%

UVC (14J/m2)

apoptosis - 8.5%   apoptosis - 51.4 %   apoptosis -16.9%
G1 - 65.1%   G1 - 45.6%   G1 - 68.7%

B

Ser 15

Ser 33

Ser 37

DO1

wt p53 | wt p53 +UVC 4h | wt p53 + UVC 8h | S33A p53 | S33A p53 + UVC 4h | S33A p53 + UVC 8h | S46A p53 | S46A p53 + UVC 4h | S46A p53 + UVC 8h | S33,46A p53 | S33,46A p53 + UVC 4h | S33,46A p53 + UVC 8h
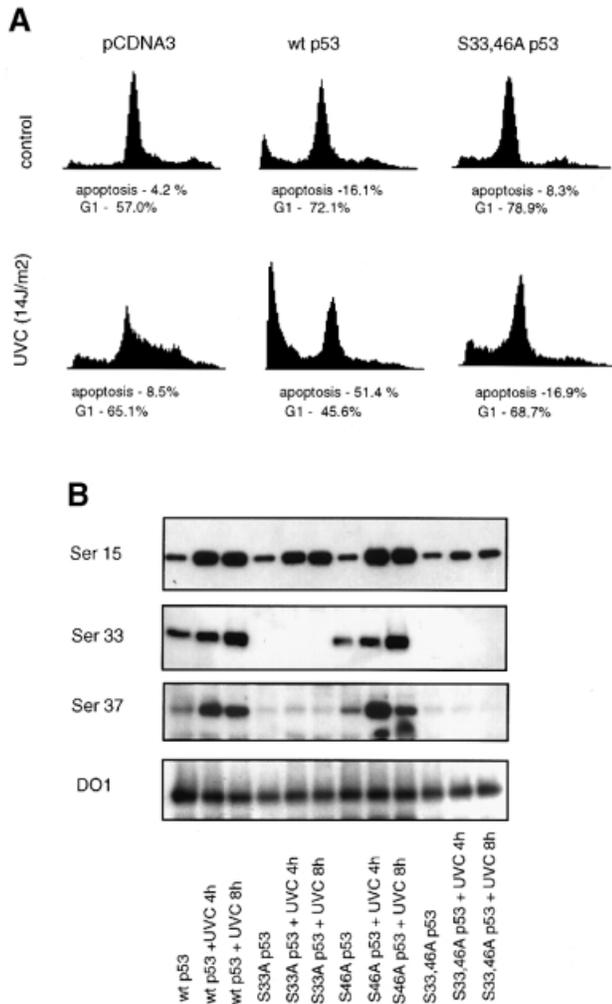
Fig. 5. p53 Ser33 and Ser46 modulate UV-induced apoptosis and phosphorylation of other N-terminal sites. (**A**) Requirement of Ser33 and Ser46 for UV-induced apoptosis. H1299 cells were transiently co-transfected with expression vectors for the surface marker protein CD20 and wt-p53 or a p53-S33,46A mutant and then treated 18 h later with 14 J/m^2 UV radiation or not (Control). Forty-eight hours after transfection, the cells were prepared for FACS analysis as described in Materials and methods, and the signals were gated for expression of CD20 (data not shown). The display of cell number versus DNA content for CD20-positive cells co-transfected with empty vector (pCDNA.3), wt-p53 or p53-S33,46A is shown. Apoptosis was determined from the number of cells with a sub-G$_1$ DNA content. (**B**) Mutation of Ser33 and Ser46 affects phosphorylation at Ser15 and Ser37 in response to UV. H1299 cells were transiently transfected with expression vectors for wt-p53 or phosphorylation site mutants p53-S33A, p53-S46A or p53-S33,46A and then UV irradiated with 25 J/m^2 18 h later. Immediately before treatment, and 4 and 8 h after treatment, cell extracts were prepared and analyzed for p53 phosphorylation by Western blot analysis (see Materials and methods). The Western blots of p53 immunoprecipitates probed with antibody specific for phosphorylation at Ser15, Ser33 or Ser37 are shown. DO-1 is a p53-specific monoclonal antibody that indicates the levels of p53 expression in the samples.

A

Fold activation   1   15.7   13.2   11.1   8.9   9.1   4.6   3.4

GST-ATF2

control | DMSO | SB203580, 10 µM | SB202190, 10 µM | SB203580, 20 µM | SB202190, 20 µM | SB203580, 40 µM | SB202190, 40 µM

UVC, 25J/m2

B

Ser 15

Ser 33

Ser 37

DO1

control | DMSO | SB203580, 20 µM | SB202190, 20 µM | SB203580, 40 µM | SB202190, 40 µM

UVC, 25J/m2

C

control

UVC

UVC + inhibitor

C   1h   2h   4h

Fig. 6. Inhibitors of p38 kinase block phosphorylation at Ser15, Ser33 and Ser37 in response to UV radiation but do not affect the half-life of wt-p53. (**A**) SB203580 and SB202190 were added to A549 cells at the concentrations indicated (10, 20 or 40 µM) 30 min before exposure to 25 J/m^2 UV radiation, and extracts were prepared 1 h later. p38 kinase was immunoprecipitated from cell extracts and assayed as described in Materials and methods using GST–ATF2 as the substrate. Shown is an autoradiogram of the assay gel. (**B**) SB203580 and SB202190, two p38 kinase-specific inhibitors, were added to A549 cells at the concentrations indicated (20 or 40 µM) 30 min before irradiation with 25 J/m^2, and extracts were prepared 12 h later. Endogenous wt-p53 was immunoprecipitated and analyzed for phosphorylation by Western blot analysis using antibodies specific for p53 phosphorylated at Ser15, Ser33 or Ser37 as described in the legend to Figure 5. The DO-1 antibody controls for the amount of p53 present. (**C**) A549 cells were treated with 40 µM SB202190 and UV as described above, and pulse-labeled with [^{35}S]methionine 12 h later, then chased with cold methionine as described in Materials and methods. Endogenous wt-p53 was immunoprecipitated with DO-1 antibody, and the half-life was analyzed after SDS–PAGE.

presence of the inhibitor. Extracts were then prepared and p38 kinase activity in immunoprecipitates was assayed with GST–ATF2 as substrate, as described earlier. At concentrations of 20 and 40 µM, both inhibitors significantly reduced p38 kinase activity (Figure 6A). Next, we analyzed p53 phosphorylation on Ser15, Ser33 and Ser37 in p53 immunoprecipitates from similarly prepared A549 extracts by Western blotting. Again, at 20 and 40 µM,

both inhibitors significantly reduced phosphorylation at all three N-terminal sites (Figure 6B). The same concentration of inhibitors did not affect p53 phosphorylation on Ser15, Ser33 and Ser37 after γ-irradiation (data not shown). These data strongly suggest that Ser33 and Ser46 of human p53 must be phosphorylated by p38 kinase for phosphorylation of Ser15 and Ser37 to occur in response to UV light *in vivo*. We also examined their ability to inhibit purified DNA-dependent protein kinase (DNA-PK) *in vitro*; at 40 µM, neither inhibitor reduced phosphoryla-

tion of Ser15 in the peptide p53(1–25) by DNA-PK. DNA-PK activity was also not affected by incubation with p38 kinase immune precipitates (data not shown). p38 kinase inhibitor (SB202190, 40 µM) also did not significantly alter the half-life of p53 in A549 cells after UVC treatment (Figure 6C), showing that several mechanisms regulate p53 stability after UVC.

We also asked whether the p38 kinase inhibitors would block activation of p53-mediated transcription and apoptosis after UV induction. In H1299 cells transiently co-transfected with the wt-p53 expression vector and the PG13-CAT reporter (containing p53 binding sites), as well as in RKO cells stably transfected with this reporter, either inhibitor at concentrations up to 40 µM decreased chloramphenicol acetyltransferase (CAT) activity by as much as 60% in cells harvested 24 h after treatment with 14 J/m^2 UV radiation (data not shown). In RKO cells, this decrease was accompanied by a decrease in *CIP1/WAF1* mRNA accumulation (data not shown). Thus, decreased phosphorylation of p53 at N-terminal residues was accompanied by a decrease in p53 transcription factor activity. To analyze the role of p38 kinase in regulating p53-mediated apoptosis, we examined cell death as determined from nuclear morphology after DAPI staining in several cell lines after inhibitor treatment. To increase cell survival, the amount of kinase inhibitor was decreased to 10 µM and the dose of UV to 14 J/m^2. Under these conditions, both inhibitors significantly reduced p38 kinase activation after UV (Figure 7A). Nevertheless, a strong reduction in apoptosis (up to 40%) occurred in A549 and RKO cells, both of which express wt p53, at 18 h after UV treatment in the presence of either p38 kinase inhibitor (Figure 7B). In contrast, no inhibition of apoptosis compared with control dimethylsulfoxide (DMSO)-treated cells was observed in RKO cells transformed with the human papillomavirus (HPV) E6 expression vector. To confirm that this effect did not reflect a delay in the activation of apoptosis, we analyzed long-term cell survival by colony formation. This assay revealed an even more dramatic protection from UV-induced cell death by the p38 kinase inhibitors (Figure 7C). Colony formation in A549 and RKO cells was 1.6–2.5 times that of control UV-treated cells grown in the presence of DMSO. However, the p38 kinase inhibitors had no effect on cell survival in H1299 cells, which lack p53, nor in RKO-E6 cells that rapidly degrade p53 (Figure 7C). In contrast, two inhibitors of the ERK pathway, apigenin and PD98059, had no effect on cell survival after UV radiation (data not shown). These data argue that p38 kinase plays a significant role in the p53-mediated transcriptional response to UV light as well as in p53-mediated induction of apoptosis in response to UV.

Discussion

Ras-mediated pathways play a major role in p53 signaling and stress responses, but less is known about their role in activating p53-mediated transcription. Here we demonstrated that while expression of several members of the p38 or JNK kinase signaling cascade activates p53-mediated transcription, the most pronounced activation was caused by p38 kinase and MEKK1. Importantly, stress-activated p38 kinase was shown to phosphorylate
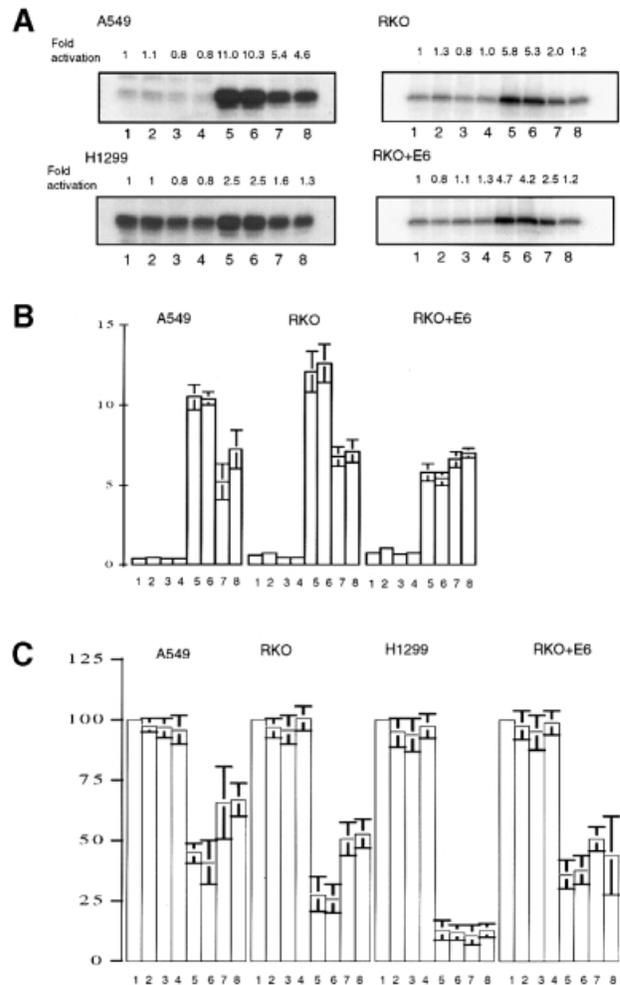
Fig. 7. Inhibition of p38 kinase prevents p53-mediated apoptotic cell death and increases clonogenic viability after exposure to UV radiation. (**A**) p38 kinase activity. A549, H1299, RKO or RKO+E6 cells were or were not exposed to 14 J/m^2 UV in the presence or absence of the specific p38 kinase inhibitors SB203580 and SB202190, and p38 kinase activity was determined using GST–ATF2 as described in Materials and methods. Lanes 1–4, no UV exposure; lanes 5–8, 14 J/m^2 UV. Additions were: lanes 1 and 5, no addition; lanes 2 and 6, DMSO; lanes 3 and 7, 10 µM SB203580; lanes 4 and 8, 10 µM SB202190. (**B**) Fraction of cells in apoptosis. A549, RKO and RKO+E6 cells were incubated with DMSO or the p38 kinase inhibitors for 30 min as shown in (A), the medium was removed and the cultures were treated with 14 J/m^2 UV radiation; the medium with inhibitors was then replaced. Four hours later, the medium was replaced with fresh medium without inhibitors, and the cultures were fixed with methanol 18 h after UV treatment. Nuclei were stained with DAPI, and cells with micronuclei were manually scored as apoptotic. Lane designations are as in (A). (**C**) Colony formation. A549, RKO, RKO+E6 and H1299 cells were incubated with the p38 kinase inhibitors or DMSO for 30 min before UV treatment (14 J/m^2). Five hundred cells (2000 cells for H1299 cell line) were then seeded on 60 mm dishes; after 10–14 days of incubation, colonies were stained with crystal violet and counted. Lane designations are as in (A). The means of triplicate samples from two independent experiments are shown; error bars indicate standard deviations.

p53 on Ser33 and Ser46, and phosphorylation at these sites was required for normal UV-induced phosphorylation of Ser37 and Ser15. Rapid nuclear localization of p38 kinase, as well as p53 stabilization, was also observed after UV irradiation. These findings are biologically relevant since UV-induced p53 transcriptional activity and

p53-dependent apoptosis were reduced by inhibiting p38 activity or mutating Ser33 and Ser46 of human p53.

Activation of p53 by p38 kinase or MEKK1 has important implications in stress signaling and aberrant oncogene expression. MEKK1 plays multiple roles in p53 activation that include stabilizing p53, activating apoptosis (Fuchs *et al.*, 1998) and activating *p19 ARF* gene expression (our unpublished results), and these effects are probably mediated by both the JNK and p38 downstream kinases. For example, we have found that MEKK1, but not p38, activates a reporter construct extending 5′ to –301 in the *p19 ARF* promoter (our unpublished results). Thus, activation of p53 by the expression of oncogenic Ras is complex, and the p38 pathway is only one of multiple effectors contributing to cellular senescence and cell death after aberrant oncogene expression (reviewed in Meek, 1998b; Prives and Hall, 1999). For stress signaling, our results directly support a role for p38 kinase in activating p53 after UV irradiation. Our results clearly demonstrate that induction of both p53 transcriptional activity and apoptosis by UV depends on p38 kinase activity and the p38 phosphorylation sites Ser33 and Ser46.

p38 kinase-mediated apoptosis

p38 kinase has been associated with apoptosis in several experimental systems, and our results point to its appreciable role in p53-mediated apoptosis after UV irradiation. Both the JNK and p38 kinase pathways are associated with increased apoptosis, while the ERK pathway was shown to suppress apoptosis (Xia *et al.*, 1995). Increased expression of upstream activators of p38 and JNK kinases such as ASK1 (Ichijo *et al.*, 1997) or MEKK1 (Fuchs *et al.*, 1998) also triggers apoptosis. In our studies, expressing mutant p53 with alanine at residues 33 and 46 caused less apoptosis and UV-induced apoptosis than wt-p53, and inhibiting p38 kinase reduced UV-induced apoptosis in a p53-dependent manner. At the C-terminus, Ser389 of murine p53 is required for normal p53-mediated transcription in growth-arrested cells (Hao *et al.*, 1996). Ser389 of murine p53 and Ser392 of human p53 are phosphorylated after UV irradiation but not after IR (Blaydes and Hupp, 1998; Kapoor and Lozano, 1998; Lu *et al.*, 1998), and p38 kinase was recently reported to phosphorylate this residue *in vitro* (Huang *et al.*, 1999; Keller *et al.*, 1999). In contrast, we observed no phosphorylation of a C-terminal p53 peptide by activated p38 kinase (Figure 3A) and little if any phosphorylation of GST–human p53 with Ser33 and Ser46 changed to alanine (Figure 3B). This difference may result from species or cell type differences that will require further investigation; however, our data clearly support a role for p38 kinase in the N-terminal phosphorylation of human p53 at Ser33 and Ser46 after UV radiation.

The role for p38 kinase in phosphorylation of the N-terminus of p53

A picture of p53 regulation is emerging that reveals a complex interplay of several protein kinases that contribute to p53 protein stability and transcriptional activity through phosphorylation of N-terminal residues (reviewed in Meek, 1998b; Prives and Hall, 1999). ATM, ATR, DNA-PK, CAK and JNK, as well as acetylation by PCAF and p300/ CBP (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998), have

all been implicated in regulating p53 in response to stress. Stress activation is complex, and important differences exist in the sites and kinetics of phosphorylation after UV irradiation compared with IR (Prives and Hall, 1999). JNK or CAK have been shown to phosphorylate murine Ser34 or human Ser33 *in vitro* (Milne *et al.*, 1995; Hu *et al.*, 1997; Ko *et al.*, 1997); however, our results indicate that p38 kinase is a major contributor to phosphorylation of this site after UV irradiation (Figure 6B). Furthermore, we show that both Ser33 and Ser46 are important for establishing the normal pattern of N-terminal phosphorylation after UV irradiation, and that p38 kinase contributes to both p53 stabilization and transcriptional activation. We demonstrate for the first time the existence of the Ser46 phosphorylation site in human p53. Interestingly, mutation of both Ser33 and Ser46 was required to reduce p53 transcriptional activity appreciably (Figure 4A). This finding highlights an interplay between different N-terminal stress-induced phosphorylation sites, although Ashcroft *et al.* (1999) reported that mutation of Ser6, Ser9, Ser15, Ser20, Ser33 and Ser37 and Thr18 to alanine did not substantially attenuate p53 transcriptional activity. The cooperativity of Ser33 and Ser46 is also highlighted by our finding that Ser15 phosphorylation was attenuated in the Ser33/46 double mutant (Figure 5B). In the case of Ser37, which can be phosphorylated by ATR and DNA-PK *in vitro* (Lees-Miller *et al.*, 1992; Tibbetts *et al.*, 1999), its phosphorylation was virtually abrogated by mutation of Ser33 even though phosphorylation of Ser37 by DNA-PK *in vitro* was unaffected (data not shown). Ser15, which can be phosphorylated *in vitro* by DNA-PK (Lees-Miller *et al.*, 1992), ATM (Banin *et al.*, 1998; Canman *et al.*, 1998) and ATR (Tibbetts *et al.*, 1999), and *in vivo* in response to both IR and UV radiation (Shieh *et al.*, 1997), is an important site for p53 regulation. While our results suggest that p38 does not directly phosphorylate either Ser15 or Ser37, they point to an integrated regulation of N-terminal phosphorylation centered on the phosphorylation at Ser33 and Ser46 by p38 kinase. Our data are consistent with the recent proposal of Shieh *et al.* (1999) that a coordinated phosphorylation of p53 N-terminal sites may result from an association with a complex containing several kinases and support the concept of a phosphorylation cascade following UV irradiation in which p38 is critically involved.

Materials and methods

Cell cultures

A549 cells (ATCC CCL-185), a human lung carcinoma cell line that expresses wt-p53, were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2 in air. HCT116 p53$^{-/-}$ cells were kindly provided by B.Vogelstein and were similarly grown in McCoy's 5A medium. MCF7 (ATCC HTB-22), which expresses wt-p53, H1299 (ATCC CRL-5803), which is deleted in both *p53* alleles, RKO, which expresses wt-p53 and RKO cells containing stably transformed HPV E6 oncogene were grown in RPMI medium supplemented with 10% FCS. UVC radiation with germicidal bulbs (254 nm) was carried out as described (Zhan *et al.*, 1993).

Plasmids

The following cDNA expression vectors were used: RAF-BxB, a dominant-positive mutant for c-RAF, was kindly provided by U.Rapp; pEXV3-MKK1 expressing full-length MKK1, and pEXV3-ERK2 expressing a full-length ERK2, were obtained from C.Marshall; MKP1(–),

an antisense for MKP1, was obtained from N.Holbrook; and pEBG-SEK1, a full-length SEK1, was kindly provided by J.Kyriakis. pCMV-MEKK1 was provided by A.Giaccia (Stanford University), HA-JNK1 by M.Karin, pcDNA.3- ASK1 by H.Ichijo, and pCMV-Flag-p38 by R.J.Davis. The reporter plasmid pWWP-CAT was provided by W.el-Deiry; pP53RECAT2 was from M.Todd (Xenometrix, Boulder, CO), pJUN-CAT and p3XTREtkCAT were kindly provided by P.Angel; pC53-SN3 expressing wild-type human p53 from a cytomegalovirus (CMV) promoter and a neomycin-resistance gene from the herpes simplex virus (HSV) thymidine kinase promoter was from B.Vogelstein; p53-S33A expressing human p53 with codon 33 (serine) changed to encode alanine was derived from pC53-SN3 as described below. GST–ATF2 was kindly provided by R.Davis, and the GST–ATF2 fusion protein substrate was purified for use as a p38 kinase substrate from BL21 *Escherichia coli* cells.

Transient transfections and CAT assays

Transient transfections for reporter studies were conducted by an adenovirus-mediated delivery of genes as described (Bai *et al.*, 1997). Briefly, a transfection mixture was prepared by incubating for 30 min 0.2 μg of reporter plasmid, 0.6 μg of expression vector, 0.3 μg of the CMV-β-gal plasmid, 1.5 μg of poly-lysine and an appropriate number of virus particles (the virus particle to cell ratio was 10:1) in HBS buffer (20 mM HEPES, 0.14 M NaCl pH 7.3). Cultures were harvested 24 h after transfection and assayed for CAT and β-gal activity. The fraction of acetylated chloramphenicol was determined using a PhosphorImager. After correction for yields as determined from the β-gal activity, the ratio of CAT activity relative to that from cultures co-transfected with reporter and empty pcDNA.3 vector was calculated. For all biochemical studies requiring higher transfection efficiency, plasmids were introduced using the Effectene Transfection Reagent (Qiagen) as recommended by the manufacturer.

In vitro kinase assays

Endogenous p38 kinase was immunoprecipitated from A549 cells by incubating with the specific antibody C20 (Santa Cruz) for 1 h, followed by incubation with protein A/G–Sepharose. Flag-tagged p38 kinase complex was immunoprecipitated with anti-Flag beads (Kodak) from H1299 cells transfected with pCMV-Flag-p38. Where indicated, p38 was activated by exposing cultures to 14 J/m^2 UV radiation 1 h before harvesting, and cells were treated with the p38-specific inhibitors SB203580 and SB202190 or the MAPK-specific inhibitors apigenin and PD98059 (Calbiochem, Inc.). Immunoprecipitates were washed twice with lysis buffer and then with kinase buffer (20 mM HEPES pH 7.9, 20 mM MgCl$_2$, 25 mM β-glycerophosphate). Kinase reactions were performed in 50 μl of kinase buffer containing p38 immunoprecipitates from 0.5 mg of cell supernatant protein, 10% glycerol, 1 mM dithiothreitol (DTT), 50 μM unlabeled ATP, 5 μCi of [^{32}P]ATP and 2 μg of GST–ATF2 as substrate. Reactions were incubated for 20 min at 30°C and then stopped by adding 50 μl of 2× sample buffer and boiling for 5 min. Proteins were analyzed by SDS–PAGE in 4–20% gradient gels, and the dried gels were exposed to X-ray film (Kodak). For peptide phosphorylation, activated Flag-tagged p38 immunoprecipitates were incubated at 30°C for 60 min in buffer containing 20 mM Tris–HCl pH 7.5, 200 μM unlabeled ATP, 20 mM MgCl$_2$, 5 mM DTT, 2 μCi [γ-^{32}P]ATP and the indicated peptide at 200 μM. Phosphorylated peptides were separated by thin-layer chromatography (TLC) on cellulose plates (Merck) in *n*-butanol:pyridine:acetic acid:water (3:2:1:4).

Pulse–chase experiments

HCT 116 p53$^{-/-}$ cells were transfected as described above with different plasmids and 24 h later were incubated with 300 μCi of [^{35}S]methionine per milliliter of EasyTag Express Protein Labeling Mix (NEN) for 30 min. The culture medium was then replaced with fresh McCoy's 5A medium containing 2 mM cold methionine. At the times indicated, cultures were harvested, and protein extracts were obtained and used for subsequent immunoprecipitation with anti-p53 antibodies and analyzed by SDS–PAGE.

Immunoblot, immunoprecipitation and immunofluorescence analyses

Cultures were washed twice in ice-cold phosphate-buffered saline (PBS), lysed in ice-cold RIPA buffer (50 mM Tris–HCl pH 8.0, 0.14 mM NaCl, 1% NP-40, 20 mM β-glycerophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), and centrifuged at 14 000 *g* for 15 min. For immunoprecipitation, 0.5–2 mg of total supernatant protein were incubated with antibodies as indicated below.

The antibody complexes were isolated using protein A/G–beads, washed three times with RIPA buffer and boiled in SDS sample buffer. SDS–PAGE and electroblotting were as described (Zhan *et al.*, 1993). For immunofluorescence, cells were seeded on coverslips 24 h before UV treatment. One hour after UV treatment, the coverslips were washed twice in PBS and fixed in methanol:acetic acid (95:5) for 10 min at room temperature. Slides were incubated with the primary antibodies anti-p38 (C-20; Santa Cruz) and anti-p53 (DO-1; Calbiochem), washed, incubated with Cy3 and Fluor-X-labeled secondary antibodies (Jackson Immunochemicals), and examined on an Olympus AX 70 microscope.

FACS analysis and colony formation assay

Apoptosis was assessed by FACS to determine the fraction of cells with a sub-G$_1$ DNA content. H1299 cells were co-transfected with expression vectors for wt-p53 or p53 mutants and a CD20 surface marker. Forty-eight hours after UV radiation, the cells were harvested with 0.1% EDTA, stained with fluorescein isothiocyanate-conjugated anti-CD20 antibodies (Sigma), fixed in ethanol, and stained with propidium iodide. CD20-positive cells were gated using the Cell Quest program, and the fraction of these cells with sub-G$_1$ DNA content was determined. Suppression of colony formation was determined 2–3 weeks after transfection of H1299 cells in 100 mm dishes using Effectene Transfection Reagent (Qiagen) with 2 μg of pcDNA.3 vector, pC53-SN3 expressing wt-p53, or p53-S33,46A, which expresses human p53 with codons 33 and 46 (serine) changed to encode alanine. Colonies were selected with 500 μg/ml G418, fixed, and stained with 0.1% crystal violet.

Site-directed mutagenesis

The QuikChangeTM Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer's instructions with the oligonucleotides 5′-ggatgatttgatgctggccccggacgatattg-3′ and 5′-caatatcgtccggggcca-gcatcaaatcatcc-3′ to introduce a single nucleotide change in codon 46 of human p53 in the expression vectors pC53-SN3 for wt-p53 and pCMV-p53S33A, which has codon 33 changed to encode alanine. The presence of the changes was confirmed by DNA sequence analysis.

Acknowledgements

References

Ashcroft,M., Kubbutat,M.H. and Vousden,K.H. (1999) Regulation of p53 function and stability by phosphorylation. *Mol. Cell. Biol.*, **19**, 1751–1758.

Bai,W., Rowan,B.G., Allgood,V.E., O'Malley,B.W. and Weigel,N.L. (1997) Differential phosphorylation of chicken progesterone receptor in hormone-dependent and ligand-independent activation. *J. Biol. Chem.*, **272**, 10457–10463.

Banin,S. *et al.* (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science*, **281**, 1674–1677.

Blaydes,J.P. and Hupp,T.R. (1998) DNA damage triggers DRB-resistant phosphorylation of human p53 at the CK2 site. *Oncogene*, **17**, 1045–1052.

Canman,C.E., Lim,D.S., Cimprich,K.A., Taya,Y., Tamai,K., Sakaguchi,K., Appella,E., Kastan,M.B. and Siliciano,J.D. (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science*, **281**, 1677–1679.

Fuchs,S.Y., Adler,V., Pincus,M.R. and Ronai,Z. (1998) MEKK1/JNK signaling stabilizes and activates p53. *Proc. Natl Acad. Sci. USA*, **95**, 10541–10546.

Gu,W. and Roeder,R.G. (1997) Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell*, **90**, 595–606.

Hao,M., Lowy,A.M., Kapoor,M., Deffie,A., Liu,G. and Lozano,G. (1996) Mutation of phosphoserine 389 affects p53 function *in vivo*. *J. Biol. Chem.*, **271**, 29380–29385.

Hazzalin,C.A., Cano,E., Cuenda,A., Barratt,M.J., Cohen,P. and Mahadevan,L.C. (1996) p38/RK is essential for stress-induced nuclear responses: JNK/SAPKs and c-Jun/ATF-2 phosphorylation are insufficient. *Curr. Biol.*, **6**, 1028–1031.

Holbrook,N.J., Liu,Y. and Fornace,A.J.,Jr (1996) Signaling events controlling the molecular response to genotoxic stress. *EXS*, **77**, 273–288.

Hu,M.C., Qiu,W.R. and Wang,Y.P. (1997) JNK1, JNK2 and JNK3 are p53 N-terminal serine 34 kinases. *Oncogene*, **15**, 2277–2287.

Huang,C., Ma,W.Y., Maxiner,A., Sun,Y. and Dong,Z. (1999) p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389. *J. Biol. Chem.*, **274**, 12229–12235.

Ichijo,H. *et al*. (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science*, **275**, 90–94.

Kapoor,M. and Lozano,G. (1998) Functional activation of p53 via phosphorylation following DNA damage by UV but not γ radiation. *Proc. Natl Acad. Sci. USA*, **95**, 2834–2837.

Keller,D., Zeng,X., Li,X., Kapoor,M., Iordanov,M.S., Taya,Y., Lozano,G., Magun,B. and Lu,H. (1999) The p38MAPK inhibitor SB203580 alleviates ultraviolet-induced phosphorylation at serine 389 but not serine 15 and activation of p53. *Biochem. Biophys. Res. Commun.*, **261**, 464–471.

Ko,L.J., Shieh,S.Y., Chen,X., Jayaraman,L., Tamai,K., Taya,Y., Prives,C. and Pan,Z.Q. (1997) p53 is phosphorylated by CDK7-cyclin H in a p36MAT1-dependent manner. *Mol. Cell. Biol.*, **17**, 7220–7229.

Lambert,P.F., Kashanchi,F., Radonovich,M.F., Shiekhattar,R. and Brady,J.N. (1998) Phosphorylation of p53 serine 15 increases interaction with CBP. *J. Biol. Chem.*, **273**, 33048–33053.

Lees-Miller,S.P., Sakaguchi,K., Ullrich,S.J., Appella,E. and Anderson,C.W. (1992) Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. *Mol. Cell. Biol.*, **12**, 5041–5049.

Levine,A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331.

Lu,H., Taya,Y., Ikeda,M. and Levine,A.J. (1998) Ultraviolet radiation, but not γ radiation or etoposide-induced DNA damage, results in the phosphorylation of the murine p53 protein at serine-389. *Proc. Natl Acad. Sci. USA*, **95**, 6399–6402.

Meek,D.W. (1998a) Multisite phosphorylation and the integration of stress signals at p53. *Cell. Signal.*, **10**, 159–166.

Meek,D.W. (1998b) New developments in the multi-site phosphorylation and integration of stress signalling at p53. *Int. J. Radiat. Biol.*, **74**, 729–737.

Milne,D.M., Campbell,L.E., Campbell,D.G. and Meek,D.W. (1995) p53 is phosphorylated *in vitro* and *in vivo* by an ultraviolet radiation-induced protein kinase characteristic of the c-Jun kinase, JNK1. *J. Biol. Chem.*, **270**, 5511–5518.

Prives,C. and Hall,P.A. (1999) The p53 pathway. *J. Pathol.*, **187**, 112–126.

Raingeaud,J., Gupta,S., Rogers,J.S., Dickens,M., Han,J., Ulevitch,R.J. and Davis,R.J. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.*, **270**, 7420–7426.

Renzing,J. and Lane,D.P. (1995) p53-dependent growth arrest following calcium phosphate-mediated transfection of murine fibroblasts. *Oncogene*, **10**, 1865–1868.

Sakaguchi,K., Sakamoto,H., Lewis,M.S., Anderson,C.W., Erickson,J.W., Appella,E. and Xie,D. (1997) Phosphorylation of serine 392 stabilizes the tetramer formation of tumor suppressor protein p53. *Biochemistry*, **36**, 10117–10124.

Sakaguchi,K., Herrera,J.E., Saito,S., Miki,T., Bustin,M., Vassilev,A., Anderson,C.W. and Appella,E. (1998) DNA damage activates p53 through a phosphorylation–acetylation cascade. *Genes Dev.*, **12**, 2831–2841.

Serrano,M., Lin,A.W., McCurrach,M.E., Beach,D. and Lowe,S.W. (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*, **88**, 593–602.

Shieh,S.Y., Ikeda,M., Taya,Y. and Prives,C. (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell*, **91**, 325–334.

Shieh,S.Y., Taya,Y. and Prives,C. (1999) DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, ser20, requires tetramerization. *EMBO J.*, **18**, 1815–1823.

Siliciano,J.D., Canman,C.E., Taya,Y., Sakaguchi,K., Appella,E. and Kastan,M.B. (1997) DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev.*, **11**, 3471–3481.

Tibbetts,R.S., Brumbaugh,K.M., Williams,J.M., Sarkaria,J.N., Cliby,W.A., Shieh,S.Y., Taya,Y., Prives,C. and Abraham,R.T. (1999) A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes Dev.*, **13**, 152–157.

Unger,T., Juven-Gershon,T., Moallem,E., Berger,M., Vogt Sionov,R., Lozano,G., Oren,M. and Haupt,Y. (1999) Critical role for ser20 of human p53 in the negative regulation of p53 by mdm2. *EMBO J.*, **18**, 1805–1814.

Xia,Z., Dickens,M., Raingeaud,J., Davis,R.J. and Greenberg,M.E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*, **270**, 1326–1331.

Zhan,Q., Carrier,F. and Fornace,A.J.,Jr (1993) Induction of cellular p53 activity by DNA-damaging agents and growth arrest. *Mol. Cell. Biol.*, **13**, 4242–4250.