p38 Kinase Mediates UV-induced Phosphorylation of p53 Protein at Serine 389*

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The p53 tumor suppressor protein is a transcription factor that plays a key role in the process of apoptosis and the cell’s defense against tumor development. Activation of p53 occurs, at least in part, by phosphorylation of its protein. Very recently it has been reported that UV induced a functional activation of p53 via phosphorylation at serine 389. Here, we report that the UV-induced phosphorylation of p53 at serine 389 is mediated by p38 kinase. UVC-induced phosphorylation of p53 at serine 389 was markedly impaired by either pretreatment of cells with p38 kinase inhibitor, SB202190, or stable expression of a dominant negative mutant of p38 kinase. In contrast, there was no inhibition observed in cells treated with specific MEK1 inhibitor, PD98059, or with stable expression of a dominant negative mutant of ERK2 or JNK1. Most importantly, p38 kinase could be co-immunoprecipitated with p53 by using antibodies against p53. Incubation of active p38 kinase with p53 protein caused the phosphorylation of p53 protein at serine 389 in vitro, while no phosphorylation of p53 at serine 389 was observed when p53 was incubated with activated JNK2 or ERK2. Furthermore, pretreatment of cells with SB202190 blocked the p53 DNA binding activity and p53-dependent transcription. These results strongly suggest that the p38 kinase is at least one of the most important mediators of p53 phosphorylation at serine 389 induced by UVC radiation.

The p53 tumor suppressor protein is a transcription factor that enhances the transcriptional rate of several known genes, which play a critical role in transducing a signal from damaged DNA to specific cellular response (1–5). Previous studies have demonstrated that p53 protein contains four major functional domains (6, 7). At the N terminus is a transcriptional activation domain (amino acids 1–43) and within the central part of p53 is the sequence-specific DNA-binding domain (amino acids 100–300) (8). The C-terminal portion contains an oligomerization domain and a regulatory domain (amino acids 319–393) (9). These domains of p53 were defined in terms of separable activities that contribute to its overall functions. A transcriptional activation domain has been found for several regulatory proteins, such as GADD45 (10), mdm (11), WAF1/p21/CIP1 (12), and cyclin G (13). The DNA-binding domain accommodates most of the mutations found so far (6), and a regulatory domain has been implicated in binding to damaged DNA (14) and in apoptosis (15).

The activation of p53 has been implicated in cell cycle control, DNA repair, and apoptosis (1, 2, 16–18). Its function is controlled at the levels of transcription, translation, protein turnover, and cellular compartmentalization, as well as association with other proteins (19). In addition to these conditions, growing evidence indicates that the ability of p53 to inhibit diverse regulatory functions is also likely to depend on its phosphorylation, which is conformation-dependent (7, 20, 21). There are at least seven phosphorylation sites within the N terminus of the p53 protein and several phosphorylation sites in the C terminus (19). It was reported that some of these sites may influence DNA binding (20, 22), transactivation (20, 23), and growth arrest (24). p53 phosphorylation is mediated by a variety of protein kinases, including casein kinase (CK1) I, CK II, protein kinase A, CDK7, DNA-activated protein kinase, JNKs, ERKs, or protein kinase C (7, 19, 21, 24–27). It was found that phosphorylation of murine p53 protein at serine 389 (or homolog serine 392 of the human p53 protein) caused the enhancement of p53 DNA binding activity in vitro (28). Very recently, two different research groups reported that UV radiation induced the phosphorylation of p53 at serine 389 and that this phosphorylation is important for p53-mediated transcriptional activation in vivo (29, 30). Furthermore, Kapoor et al. (30) found that CKII could phosphorylate the p53 at serine 389 in vitro. Here, we demonstrated that UVC-induced p53 phosphorylation at serine 389 is mediated by p38 kinase by using a dominant negative mutant of p38 kinase and the p53 kinase inhibitor, SB202190, both in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials—Activated p38 kinase and active JNK2 were from Upstate Biotechnology; activated ERK2, Elk-1 fusion protein, c-Jun fusion protein, phospho-specific c-Jun (Ser63) antibody, phospho-specific Elk-1 antibody (Ser113), p38 kinase antibody, phospho-specific p38 kinase (Tyr182) antibody, phospho-specific p53 (Ser156) antibody, and p38 kinase assay kit were purchased from New England Biolabs; monoclonal mouse IgG against p53 antibody (Ab1) was from Oncogene Research Products; MEK1 specific inhibitor, PD98059, was from Biomol; p38 kinase inhibitor, SB202190, was from Calbiochem; dominant negative mutant of p38 kinase was a generous gift from Dr. Mercedes Rincon, Department of Medicine, University of Vermont, Burlington, VT (31, 32); Eagle’s minimal essential medium (MEM), Dulbecco’s modified Eagle’s medium, and RPMI 1640 were from Calbiochem; fetal bovine serum (FBS) was from Life Technologies, Inc.; and luciferase substrate was from Promega. Recombinant p53 was produced in insect cells infected with baculovirus vector carrying human p53 cDNA and partially purified through DNA affinity chromatography (33).

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1 The abbreviations used are: CK, casein kinase; JNK, c-Jun N-terminal kinase; MEM, Eagle’s minimal essential medium; FBS, fetal bovine serum; ERK, extracellular signal-regulated protein kinase; CMV, cytomegalovirus; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MEK1, mitogen-activated protein kinase; DTT, dithiothreitol.

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Role of p38 Kinase in UV-induced p53 Protein Phosphorylation

Cell Culture—JB6 mouse epidermal cell line Cl 41 and its stable transfectants, Cl 41 CMV-neu, Cl 41 DN-p38 G7, Cl 41 DN-JNK1 mass., Cl 41 MAPK-DN B3 mass., and Cl 41 p53 were cultured in monolayers at 37 °C and 5% CO2 using Eagle’s MEM containing 5% FBS, 2 mM L-glutamine, 25 μg/ml gentamicin (18, 34, 35).

Induction of p53 phosphorylation at serine 389 induced by UV radiation and the complex of p53 with p38 kinase was measured by Western blot for immunoprecipitation using specific antibodies against p53. Briefly, JB6 Cl 41 cells or its transfectants were cultured in 100-mm dishes with 5% FBS MEM until they reached 80% confluence. Then, the cells were starved by culturing them in 0.1% FBS MEM for 18 h. The cells were exposed to UV irradiation for induction of p53 phosphorylation at serine 389. The cells were lysed on ice for 1 h in the lysis buffer and spun at 14,000 rpm for 5 min. The lysates were immunoprecipitated using p53 antibodies (Ab1) and protein G plus protein A-agarose. The bands were washed, and the phosphorylated protein of p53 at serine 389 and the p38 kinase, as well as phosphorylated p38 kinase, were selectively measured by Western immunoblotting using a specific antibody and chemiluminescent detection system.

Generation of Stable Cotransfectants—JB6 Cl 41 cells were cultured in a six-well plate until they reached 85–90% confluence. We used 1 μg of CMV-neo vector with or without 12 μg of plasmid DNA of dominant negative mutant of p38 kinase and 15 μl of Lipofectamine reagent to transiently transfect as well in the absence of serum. After 10–12 h, the medium was replaced by 5% FBS MEM. Approximately 30–36 h after the beginning of the transfection, the cells were digested with 0.03% trypsin, and cell suspensions were plated into 75-ml culture flasks and cultured for 24–28 days with G418 selection (300 μg/ml). Stable transfectants were identified by using p38 kinase activity assay kit. Stable transfected Cl 41 CMV-neu and Cl 41 DN-p38 G7 were established and cultured in G418-free MEM for at least two passages before each experiment.

Assay for p38 Kinase Activity—p38 kinase assay was carried out as described by the protocol of New England Biolabs. In brief, JB6 Cl 41 CMV-neu or Cl 41 DN-p38 G7 cells were starved for 48 h in 0.1% FBS MEM until they reached 90% confluence. The cells were starved by culturing them in 0.1% FBS MEM for 12 h. Then, the cells were incubated with 6 × 103 viable cells suspended in 100 μl of kinase buffer per sample (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 mg/ml leupeptin). The lysates were sonicated and centrifuged, and the supernatant was incubated with a specific p38 kinase antibody to the N-terminal rocking for 4–10 h at 4 °C and then the protein A-Sepharose beads were added in the incubation for another 4 h. The beads were washed twice with 500 ml of lysis buffer with phenylmethylsulfonyl fluoride and twice with 500 μl of kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, 10 mM MgCl2). The kinase reactions were carried out in the presence of 100 μM ATP and 5 μM of ATP-2 at 30 °C for 30 min. ATP-2 phosphorylation is selectively measured by Western immunoblotting using a chemiluminescent detection system and specific antibodies against phosphorylation of ATP-2 at Thr71.

Protein Phosphorylation Assay in Vitro—Phosphorylation of p53, c-Jun, or Elk-1 by activated ERK2, JNK2, or p38 kinase were carried out at 30 °C for 60 min in the presence of kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, 10 mM MgCl2) with 200 μM ATP and p53, c-Jun, or Elk-1 as substrate. The phosphorylation proteins were detected by Western immunoblotting using phospho-specific antibodies.

Gel Mobility Shift Assay—The p53 DNA-protein binding assay was carried out according to methods described previously (37). The synthetic oligonucleotide of p53-binding consensus sequence (5′-GACATGTCGCCCAATCTGTTGC-3′ or GADD45 5′-GACATGTCCTAAGC-ATGCTG-3′) was annealed and labeled with 32P using T4 polynucleotide kinase and γ-32P ATP. Nuclear extracts were prepared from confluent Cl 41 cells treated with various concentrations of SB202190 for 1 h, then exposed to UVC (60 J/m2) and cultured for 18 h. Cells were washed with phosphate-buffered saline and lysed in cold lysis buffer (25 mM HEPES, pH 7.8, 10% glycerol, 1 mM PMSF, 10 μM leupeptin, 20 μM aprotinin, and 100 μM DTT) for 5 min. After washing once with the lysis buffer without Nonidet P-40, the pellet was resuspended in cold extraction buffer (25 mM HEPES, pH 7.8, 500 mM KCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 20 μM aprotinin, and 100 μM DTT) and incubated on ice for 20 min with frequent mixing followed by spinning at 14,000 rpm for 5 min. The supernatant was then saved as the nuclear extract. Protein concentrations were determined using the Bio-Rad Protein Assay Kit. A DNA-binding reaction mixture of 24 μl contained 20 μM Tris-HCl, pH 7.5, 4% Ficoll-400, 2 mM EDTA, 0.5 mM DTT, 1 μg of poly(dI/dC), 32P-labeled oligonucleotide (20,000 cpm) was incubated with 6 μg of protein-containing nuclear extract. To determine the binding specificity, 100 × excess of cold oligonucleotide was included in some reactions. The mixture was incubated at room temperature for 45 min and then loaded onto a 3.5% polyacrylamide gel. The gel was run in 0.5 × Tris borate-EDTA buffer at 160 V, dried, and scanned.

RESULTS

Induction of p53 Phosphorylation at Serine 389 by UVC Radiation—P53 activity is regulated by multisite phosphorylation (7, 20, 21, 29, 30). It has been reported that phosphorylation of the murine p53 protein at serine 389 in UV response plays an important role in p53 sequence-specific DNA binding in vitro and in p53-dependent transcriptional activation in vitro (28–30). To investigate the signal transduction pathways leading to phosphorylation of p53 at serine 389 in UV response, we first measured the UV-induced phosphorylation of p53 at serine 389 in a mouse JB6 epidermal cell line, Cl 41. Previous studies indicated that Cl 41 cells contain high levels of wild-type p53 protein that could activate the p53-dependent transcriptional activation in UV response (18, 36–38). Cl 41 cells were exposed to UVC radiation; the p53 protein in the cell lysate was immunoprecipitated with specific antibodies against p53 (Ab1). The levels of p53 phosphorylation were analyzed by Western blotting using phospho-specific antibody against p53 at serine 389. The results show that UVC radiation caused phosphorylation of p53 at serine 389 (Fig. 1). This phosphorylation was observed in vivo in UV response.
Inhibition of UVC-induced p53 phosphorylation at serine 389 by pretreatment of cells with p38 kinase inhibitor, SB202190, but not with MEK1 inhibitor, PD98059. Cl 41 cells were cultured in monolayers in 100-mm diameter dishes until 90% confluent. The cells were starved by changing the medium with 0.1% FBS MEM for 36–48 h. Then, the cells were pretreated with either SB202190 (A) or PD98059 (B) for 1 h at the concentration indicated. The cells were exposed to UVC (60 J/m²) for 30-min incubation. Thirty minutes later, the cells were harvested, and phosphorylation levels of p53 at serine-389 were measured as described under “Experimental Procedures.”

Blocking p53 phosphorylation at serine 389 by expressing a dominant negative mutant of p38 kinase, but not by dominant negative mutants of ERK2 or JNK1—To directly investigate the role of p38 kinase in UVC-induced phosphorylation of p53 at serine 389, we established a stable transfectant with a dominant negative mutant of p38 kinase (31, 32). The dominant negative mutant of p38 kinase was generated by replacing Thr180 and Tyr182 by Ala and Phe, respectively (31, 32). Cl 41 cells were transfected with dominant negative mutant of p38 kinase and selected with G418 as described under “Experimental Procedures.” G418-resistant transfectants were analyzed for the expression of dominant negative mutant by assay for UVC-induced p38 kinase activity. The results showed that expression of dominant negative mutant of p38 kinase specifically blocked UVC-induced p38 kinase activity (Fig. 3), while it did not inhibit the activation of ERKs and JNKs (data not shown). The expression of dominant negative mutant of p38 kinase markedly blocked p53 phosphorylation at serine 389 in UVC response (Fig. 4A). In contrast, overexpression of dominant negative ERK2 or JNK1 did not show significant inhibition of p53 phosphorylation at serine 389 (Fig. 4, B and C). The time course and dose response studies further indicated that p38 kinase appears to be the mediator of p53 phosphorylation at serine 389 in UVC response (Fig. 5).

p38 Kinase Was Present in the Immunoprecipitation Proteins from Cl 41 Cells Lysate with p53-specific Monoclonal Antibodies—Because the above data revealed the important role of p38 kinase in the signaling pathway leading to phosphorylation of
p53 at serine 389, we explored whether a direct interaction might exist between p38 kinase and p53. We exposed the C1 41 cells to UVC radiation for the different times and incubated the cell extract with specific monoclonal antibodies against p53 and protein G plus protein A-agarose. The beads were washed extensively to eliminate nonspecific binding. The bead-coupled proteins were eluted with SDS sample buffer and measured by Western blot using specific antibodies against phosphorylation of p53 at serine 389 and phosphorylation of p38 kinase at threonine 182 or nonphosphorylated p38 kinase. We found that the p38 kinase binding to p53 was detected in the immunoprecipitation complex (Fig. 6). Furthermore, the kinetics of p53 phosphorylation at serine 389 were well correlated with that of p38 kinase phosphorylation at threonine 182 after UVC exposure (Fig. 6). Very interestingly, the total p38 kinase in all the immunoprecipitation samples was almost the same (Fig. 6). These data suggested that nonphosphorylated p38 kinase could bind to p53, and the phosphorylated p38 kinase may be responsible for p53 phosphorylation.

**P53 Is Phosphorylated at Serine 389 in Vitro by Active p38 Kinase, but Not by Active ERK2 or JNK2**—The above data strongly suggest that p38 kinase is the possible direct mediator of UVC-induced p53 phosphorylation at serine 389. If this is the case, the active p38 kinase should phosphorylate p53 at serine 389 in vitro. To test this, we incubated partially purified p53 protein with one of activated MAP kinase family in the presence of 200 μM ATP. The phosphorylation level of p53 was detected by Western blot using specific antibodies against phosphorylation of p53 at serine 389 (Fig. 7A), but not by active ERK2 or JNK2 (Fig. 7, B and C), while active ERK2 and JNK2 show their ability to phosphorylate Elk-1 or c-Jun, respectively (Fig. 7, B and C). Because the p53 used in this study consists of the purified baculovirus-expressed proteins that are a mixture of phosphorylated and unphosphorylated p53 at serine 389, a relatively high basal level of phosphorylation of p53 at serine 389 in the control group is probably due to a mixture of phosphorylated and unphosphorylated p53 at serine 389 in this baculovirus-expressed protein. These data, taken together with other results from this study, strongly suggest that p38 kinase...
is the direct mediator of UV-induced phosphorylation of p53 at serine 389.

**Blockade of p53-dependent Transcription Activity and p53 DNA Binding Activity by Pretreatment of Cells with SB202190**—To investigate the functional requirement of p38 kinase mediating the phosphorylation of p53 at serine 389 in...
UV response, we determined the inhibitory effect of SB202190, a p38 kinase inhibitor, on p53-dependent transcription activity and DNA binding activity. The results showed that UVC-induced p53-dependent transcription activity and DNA binding activity could be blocked by pretreatment of cells with SB202190 (Fig. 8). This band was shown to be compatible with the unlabeled p53 binding oligonucleotides and was not detectable in p53−/− cells derived from p53 knockout mouse (Fig. 8, A and B, and data not shown). This result suggests that phosphorylation of p53 at serine 389 is functionally required for p53-dependent transcription.

**DISCUSSION**

DNA damage-induced activation of the p53 tumor suppressor is suggested to be central in cellular damage response pathways. One of the major roles of p53 in normal cells is to trigger cell cycle arrest or apoptosis in response to DNA damage by acting as a sequence-specific transcription factor that activated genes involved in control of cell cycle and apoptosis (20). Mutations in the p53 gene have been found in over half of all human cancers (20). Loss of p53 suppressor function, by mutation, is a universal step in the development of human cancer (42). For these reasons, it was very interesting to investigate the signal transduction pathways leading to p53 phosphorylation and activation. UV radiation has been shown previously to induce p53 phosphorylation at serine 389. The critical kinase that mediates this UV-responsive phosphorylation is, however, not well documented. In this study, we determined the role of p38 kinase in UVC-induced p53 phosphorylation at serine-389. Exposure of C1 41 cells to UVC radiation leads to activation of p38 kinase and the phosphorylation of p53 at serine 389 in C1 41 cells. Pretreatment of cells with SB202190, a p38 kinase inhibitor, or expressing of dominant negative mutant of p38 kinase, impaired the phosphorylation of p53 at serine 389 and p53-dependent transactivation and DNA binding activity in UVC response. Most importantly, we found that p38 kinase was present in the immunoprecipitation proteins from a cell extract using specific antibodies against p53, and active p38 kinase was shown to phosphorylate the p53 protein at serine 389 in vitro. All these data demonstrated that p38 kinase plays a critical role in UVC-induced phosphorylation of p53 protein at serine 389, suggesting that there is a functional requirement of p38 kinase for UV-induced p53 activation.

Exposure of cells to UV irradiation elicits a complex set of acute cellular responses called “UV responses.” Generally, UV responses serve to protect the cells. The initial signal triggering the UV response is in large part independent of DNA damage, but rather appears to be mediated by a membrane-associated component of the Ras pathway with activation of MAPKs (39, 40, 43, 44). However, others argue that even UV-induced activation of MAPKs may have a DNA damage signal component (45). Very recently, Bender et al. (46) reported that UV-induced activation of NFκB is through DNA damage-dependent and -independent pathways. In the case of p53, activating signals clearly involve both DNA damage signals and other nongenotoxic stresses (47). It is accepted that p53 function is regulated at the levels of its transcription, translation, protein turnover, cellular compartmentalization, stabilization, and association with other proteins, as well as phosphorylation (19). p53 has multiple sites for phosphorylation in both N- and C-terminal domains, and p53 phosphorylation has been found to be mediated by several cellular kinases, including CKI, CKII, PKA, CDK7, ERKs, and JNKs (7, 19, 21, 24–27). Among these protein kinases, those that are expected to produce stress-activated phosphorylation of p53 are JNKs and DNA-activated protein kinase, which reportedly phosphorylates residues of p53 within the N-terminal domain of p53 (19, 21). DNA-activated protein kinase mediates phosphorylation of p53 at serine 15 and serine 37 (48). This phosphorylation was identified as one of the major sites on p53 in cellular stress response and contributed to p53 accumulation (48). JNKs phosphorylate the murine p53 at serine 34 (19, 26, 27). JNKs signaling contributes to the ability of p53 to mediate apoptosis through stabilization and activation of p53 (7, 21). It was also reported that the DNA binding function of p53 is activated by phosphorylation of the C-terminal serine by purified CKII in vitro (28). The studies from two different groups indicated that UV radiation results in functional activation of p53 through phosphorylation of p53 protein at serine 389 (29, 30) and that p53 could be phosphorylated at serine 389 by CKII in vitro (30). In this study, we found that inhibition of p38 kinase by either chemical p38 kinase inhibitor SB202190, or the biological inhibitor, dominant negative mutant of p38 kinase, results in blocking phosphorylation of p53 at serine 389 in UV radiation. Pretreatment of cells with SB202190 also impaired the UV-induced p53-dependent transcription and DNA binding activity. Most importantly, the p38 kinase not only phosphorylates the p53 at serine 389 but also is present in the immunoprecipitation proteins from cell lysate using specific antibodies against p53. Furthermore, the phosphorylation at serine 389 is well correlated with the phosphorylation of p38 kinase, which is considered essential for activation of p38 kinase, in the time course studied. Taken together, our results provide evidence, both in vitro and in vivo, that p38 kinase is a mediator of p33 phosphorylation at serine 389 in UVC response, and p38 kinase is functionally required for UV-induced p53-dependent transcription.

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