

Mechanisms of Cr(VI)-induced p53 activation: the role of phosphorylation, mdm2 and ERK

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The present study investigated the molecular mechanisms of p53 activation induced by Cr(VI), using human lung epithelial A549 cells. Cr(VI) increased both protein level and transactivation ability of p53 protein. The activation of p53 is at the protein level instead of the transcriptional level. The degradation of p53 was dramatically decreased upon stimulation by Cr(VI). In addition, Cr(VI) treatment decreased the interaction of p53 with mdm2 proto-oncoprotein, which blocks the transactivation ability of p53 and promotes the degradation of p53 protein. In response to Cr(VI) treatment, p53 protein became phosphorylated and acetylated at Ser15 and Lys382, respectively. The phosphorylation levels at either Ser20 or Ser392 did not show any significant alterations. Since previous studies report that it is Ser20 and not Ser15 phosphorylation that contributes to mdm2 dissociation from p53, the results obtained from the current investigation suggest a different mechanism: Ser15 instead of Ser20 may play a key role in the dissociation of mdm2 in response to Cr(VI). Erk, a member of mitogen-activated protein kinase, acts as the upstream kinase for the phosphorylation of the p53 Ser15 site.

Introduction

The p53 tumor suppressor protein plays an important role in protecting cells from fatal genetic injury (1,2). Mutational inactivation of p53 protein has been found to be involved in various cancers in humans and experimental animals (2). A variety of mutations in the *p53* gene have been reported in >50% of human cancers, suggesting a crucial role for p53 protein in maintaining normal cell growth (2,3).

Normally, p53 has a very short half-life and protein levels are low (4). A lengthening of the half-life and rapid accumulation of p53 protein were observed in response to a variety of stimuli, such as UV light, γ radiation, hypoxia and nucleotide deprivation (5–8). A key player in this modulation is the murine double minute-2 (mdm2) proto-oncoprotein. mdm2 binds to p53 within its N-terminal transactivation domain, blocks its transcriptional activity, shuttles p53 out of the nucleus and mediates p53 degradation by ubiquitin-protease (9–13). Since the expression of mdm2 is regulated directly by the p53

Abbreviations: ATM, ataxia telangiectasia mutated; cdk, cyclin-dependent kinase; CHX, cycloheximide; DNA-PK, DNA-dependent protein kinase; Erk, extracellular-signal regulated kinase; FBS, fetal bovine serum; JNK, c-jun N-terminal kinase; MAP, mitogen-activated protein; mdm2, murine double minute-2; PKC, protein kinase C; ROS, reactive oxygen species.

transcription factor, they form a negative autoregulatory feedback loop in quiescent cells. Therefore, the balance between the association and the disassociation of mdm2 is a major factor in p53 activation.

In addition to the interaction between mdm2 and p53, post-translational modifications of p53 protein, including multiple phosphorylations and acetylations, also play a key role in regulating p53 activity (14–16). The modifications affect several aspects of p53 protein, such as transactivation and transrepression abilities, sequence-specific DNA binding ability and mdm2–p53 interaction (17–21). In response to various stimuli, several different protein kinases are reported to phosphorylate p53 at distinct sites, such as N-terminal phosphorylation by DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM) protein, mitogen-activated protein (MAP) kinase, Raf-1, casein kinase I, C-terminal by protein kinase C (PKC), casein kinase II, cyclin-dependent kinase (cdk) and p38 kinase (22–26). Recently, several groups reported the acetylation of the p53 C-terminal domain by histone acetyltransferases p300, CBP (CREB binding protein) and PCAF (p300/CBP-associated factor) (27–29). N-terminal phosphorylation is responsible for both enhanced transactivation activity and the dissociation of mdm2. Modifications at the C-terminal end are associated with an increase in sequence-specific DNA binding ability. Phosphorylation and acetylation events have a synergistic effect on the activation of p53 protein.

Chromium [Cr(VI)] compounds are used widely in industry and are also found in the environment (30). Exposure to Cr(VI)-containing compounds is known to induce lung toxicity and increased incidence of cancers of the respiratory system (31–34). Although the mechanisms of Cr(VI)-induced carcinogenesis are not fully understood, the reactive metabolic products of Cr(VI), including reactive oxygen species (ROS), Cr(V) and Cr(IV), are considered to play major roles in the carcinogenic process (35–40). Previous work from our laboratory showed that Cr(VI) activates p53 protein via hydroxyl radical-mediated reactions (36). In the present study, we extend our investigation to elucidate the possible mechanisms of activation of p53 induced by Cr(VI).

Materials and methods

Materials

Sodium dichromate ($\text{Na}_2\text{Cr}_2\text{O}_7$) was purchased from Aldrich (Milwaukee, WI). Cycloheximide, actinomycin D and p38 inhibitor (SB 202190) were purchased from Calbiochem (La Jolla, CA). MEK1 inhibitor, PD 98059, was purchased from New England Biolabs (Beverly, MA).

Cell culture

A human lung epithelial cell line (A549 cells) was obtained from American Type Culture Collection (ATCC) (Rockville, MD). The cells were maintained in a nutrient mixture (Kaighn's Modification) medium (F-12K) (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 1000 U/ml penicillin/streptomycin. The cells were cultured in 75 cm² cell culture flasks and trypsinized with 0.25% trypsin/EDTA for plating and passaging.

Luciferase assay

Human lung epithelial A549 cells (3×10^5) suspended in 1 ml 10% FBS F-12K medium were seeded into each well of a 6-well plate. Following incubation at 37°C for 24 h, the cells were transiently transfected with 1 µg reporter plasmid and 1 µg β-gal plasmid in reduced-serum medium. The cells were incubated overnight, washed and fresh 10% FBS F-12K medium was added. The cells were exposed to various treatments after an additional 24 h of culture. The cells were then extracted with 400 µl reporter lysis buffer at 4°C for 2 h. The luciferase activity was measured with 80 µl cell lysate, using a Monolight luminometer, Model 3010 (Analytical Luminescence Laboratory, Sparks, MD). β-Gal activity was determined as described previously (41). The results are expressed as relative p53 activity compared with controls after normalizing by β-gal activity.

Western blot

The whole cell lysate was used for western blot analysis. Aliquots of 2×10^5 cells were cultured in each well of 6-well plates to 95% of confluency. After treatment, the cells were washed once with ice-cold PBS and extracted with 100 µl SDS-sample buffer. The extracts were then sonicated to shear DNA and reduce the viscosity. A 20 µl aliquot of lysate was heated to 95°C for 5 min and microcentrifuged for another 5 min. Proteins were electrophoretically separated on 10% Tris-glycine gels (Novex, San Diego, CA) and were transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was treated with a blocking buffer [Tris-buffered saline Tween (TBS-T) containing 5% non-fat milk] for 1 h and exposed to primary antibody at 4°C overnight. The membrane was rinsed and incubated with a 1:2000 dilution of secondary antibody for 1 h. The membrane was then washed with TBS-T and antibody binding sites were visualized by ECL western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). P53 mouse monoclonal antibody (DO-1) and mdm2 mouse monoclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA); phospho-p53, phospho-p38 kinase (Thr180/Tyr182) and phospho-Erk kinase (Thr202/Tyr204) antibodies were from New England Biolabs (Beverly, MA); acetylated p53 (Lys382) antibody was from Oncogene (Cambridge, MA).

Immunoprecipitation

Aliquots of 2×10^5 cells were cultured in each well of 6-well plates to 95% of confluency. After treatment, the cells were washed once with ice-cold PBS and collected in 100 µl PBS. After addition of 100 µl 2× reducing buffer (0.125 M Tris, pH 6.8, 20% glycerol, 2% SDS and 10% 2-mercaptoethanol), the extracts were boiled at 95°C for 5 min and 500 µl TNT buffer (20 mM Tris, pH 7.4, 0.2 M NaCl and 1% Triton 100) was added. The cell lysate, 20 µl Recin/TNT (1:10) and 10 µg antibody were incubated at 4°C overnight on a rotor mixer. Recin was spun down and washed with TNT buffer followed by boiling with 20 µl reducing buffer for 5 min. The supernatant was collected for western blot analysis.

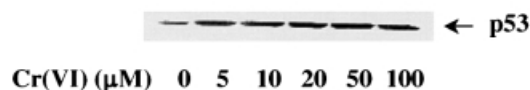
Results

Cr(VI)-induced p53 activation

Human lung epithelial A549 cells were used to study induction of p53 activation by Cr(VI). The cells were incubated with Cr(VI) at different concentrations for 3 h. P53 protein level was analyzed in the whole cell lysates. As shown in Figure 1a, p53 protein levels increased following exposure to Cr(VI) compared with control. Increasing the Cr(VI) dosage had little effect on the p53 protein level.

Since p53 is a transcription factor, its transactivation activity can be determined by a luciferase assay. Briefly, the cells were co-transfected with p53-luciferase reporter plasmid and β-gal plasmid, and incubated with different concentrations of Cr(VI) for 3 h. After washing the cells, fresh F-12K medium supplemented with 10% FBS was added. The cells were then incubated at 37°C overnight. P53 activity was analyzed by luciferase assay as described in Materials and methods. Figure 1b shows the results of the luciferase assay for p53 activity. P53 activity increased following exposure to Cr(VI). Increasing the Cr(VI) concentration up to 50 µM enhanced p53 activity. Increasing Cr(VI) concentration further resulted in a slightly lower p53 activation, possibly due to Cr(VI)-induced apoptosis.

a



b

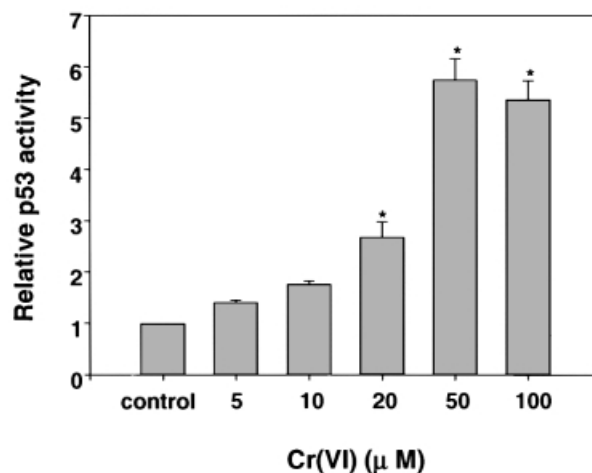


Fig. 1. p53 activation induced by Cr(VI). (a) Western blot of p53 protein level. Human lung epithelial A549 cells (2×10^5) were cultured in each well of 6-well plates to 95% of confluency. After 3 h of treatment with Cr(VI) at various concentrations, the cells were collected and whole cell lysates were obtained. The p53 protein levels were determined by western blot, using specific antibodies for p53 protein. (b) Luciferase assay for Cr(VI)-induced p53 activation. Human lung epithelial A549 cells (3×10^5) suspended in 1 ml 10% FBS F-12K medium were seeded into a 6-well plate. After being incubated at 37°C in a humidified atmosphere of 5% CO₂ for 24 h, the cells were transiently transfected with a p53-luciferase reporter plasmid and a β-gal plasmid in reduced-serum medium. The cells were incubated overnight, washed, added to fresh 10% FBS F-12K medium and incubated for 24 h. The cells were exposed to Cr(VI) at various concentrations for 3 h, washed, added to fresh F-12K medium and incubated overnight. The p53 activity was measured by luciferase activity assay as described in the Materials and methods. Results are presented as relative p53 induction compared with the untreated control cells (means and standard deviation of three separated experiments). Asterisks indicate a significant increase from control ($P < 0.05$), using Student *t*-test for data analysis.

Transcription is not an important factor for p53 activation by Cr(VI)

In general, protein expression can be regulated at several different levels: the transcriptional level (mRNA), the translational level (protein synthesis) or the post-translational level (protein modification such as phosphorylation and acetylation). To investigate whether p53 protein activation is due to an increased mRNA level, actinomycin D (AMD), an inhibitor of transcription, was used to pre-treat cells before the 3 h incubation with Cr(VI). As is shown from western blots, inhibition of transcription did not decrease levels of p53 protein (Figure 2a, lanes 3 and 6). Addition of cycloheximide (CHX), an inhibitor of protein synthesis, dramatically reduced levels of p53 protein (Figure 2a, lanes 2 and 5). These results suggest that the Cr(VI)-induced increase in p53 protein level is not due to an increase in transcription.

To further confirm the above conclusion, a p53-promoter luciferase plasmid (a generous gift from Dr Finian Martin, University College Dublin, Ireland), which can be used to

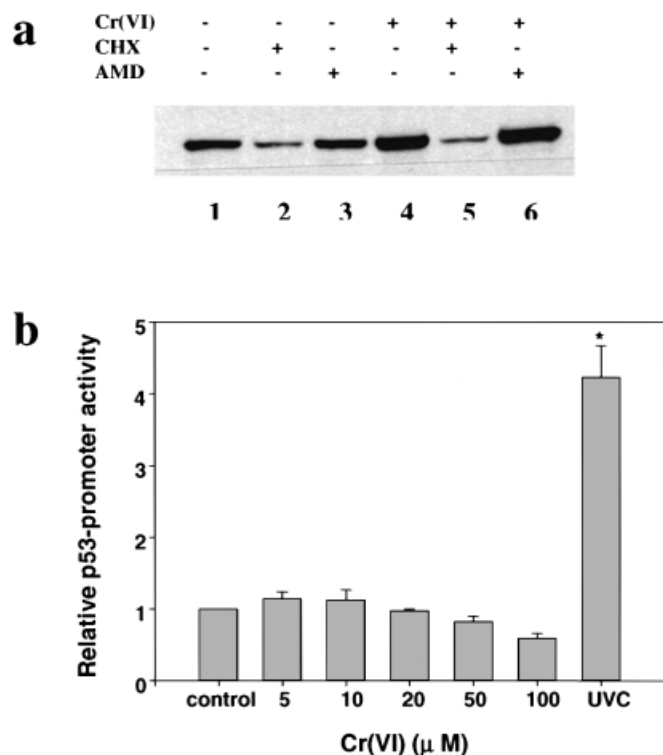


Fig. 2. Transcription is not a major factor in p53 activation. (a) A549 cells were pre-treated with 10 μg/ml cycloheximide (CHX) or 1 μg/ml actinomycin D (AMD) for 30 min, followed by incubation with or without 50 μM Cr(VI) for 3 h. The whole cell lysates were collected for western blot analysis, using a specific p53 antibody. (b) Luciferase assay for p53 promoter activity. A549 cells were transiently transfected with p53-promoter luciferase plasmid. Then the cells were exposed to various concentrations of Cr(VI) for 3 h, washed, added to fresh F-12K medium and incubated overnight. The p53 activity was measured by the luciferase activity assay. UVC light was used as a positive control. Results are presented as relative p53 promoter induction compared with the untreated control cells (means and standard deviation of three separate assays). Asterisks indicate a significant increase from control ($P < 0.05$), using Student *t*-test for data analysis.

measure p53 gene expression, was transiently transfected into A549 cells (42). The cells were then exposed to various concentrations of Cr(VI). As shown in Figure 2b, treatment with Cr(VI) did not result in an increase in p53 gene expression. The results obtained from both western blot and luciferase assays demonstrate that p53 activation is due to enhanced protein synthesis, rather than increased gene expression.

Post-translational modifications are important for p53 activation by Cr(VI)

In addition to protein synthesis, protein degradation is also an important factor in regulating total protein levels. CHX was utilized to investigate the rate of degradation of p53 protein with or without Cr(VI) stimulation. A549 cells were exposed to Cr(VI) for 3 h followed by addition of CHX. At different time points after cessation of protein synthesis, whole cell lysates were collected to perform western blots. In normal cells, the p53 half-life was approximately 1 h. After stimulation with Cr(VI), the rate of degradation of p53 protein was reduced dramatically with no significant decrease in protein levels even at 4 h (Figure 3), indicating enhanced stability of the p53 protein after Cr(VI) stimulation.

It is generally believed that post-translational modifications, including phosphorylation and acetylation, play a role in

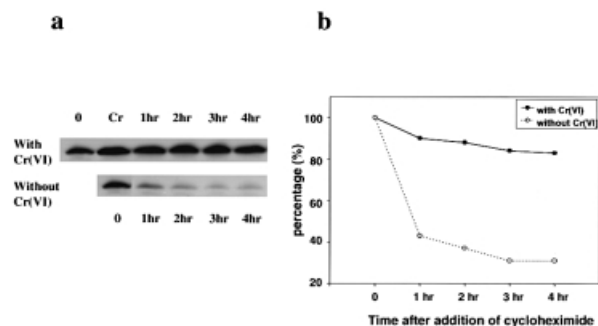


Fig. 3. p53 degradation is reduced with Cr(VI) stimulation. A549 cells were incubated with or without 50 μM Cr(VI) for 3 h, followed by the addition of 10 μg/ml CHX. The whole cell lysates were collected at different time points after treatment with CHX. Western blot analysis was performed using a specific p53 antibody. (a) The results from the western blot. (b) Percentage of p53 protein level at different times based on the quantitation of (a).

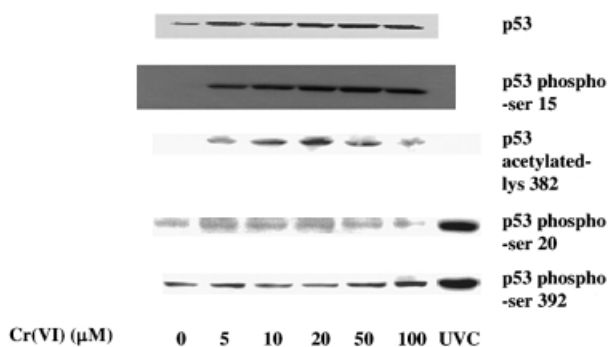


Fig. 4. Phosphorylation and acetylation of p53 induced by Cr(VI). A549 cells were incubated with various concentrations of Cr(VI) for 3 h. The whole cell lysates were collected for western blot analysis using specific antibodies for p53 protein, Ser15 and Ser392 phospho-p53 and Lys382 acetylated p53 protein, respectively. UVC light was used as a positive control.

stabilizing p53 protein. With the stimulation of Cr(VI), while the level of total p53 protein remained constant with increase in Cr(VI) concentrations, phosphorylation of p53 at Ser15 exhibited a clear concentration-dependent increase (Figure 4). No phosphorylation was detected at Ser15 in Control cells. With the addition of Cr(VI), Ser15 phosphorylation increased and peaked at 50 μM Cr(VI) exposure. Acetylation of Lys382 at the C-terminus of p53 protein exhibited a similar dose-dependent pattern, but with a peak at 20 μM Cr(VI). In contrast, phosphorylation of N-terminal residue Ser20 and C-terminal residue Ser392 showed little change in response to stimulation with Cr(VI) (Figure 4).

Another major factor that influences the stability of p53 protein is the binding of p53 with mdm2. In the cytoplasm, the binding of mdm2 to the N-terminus of p53 promotes the degradation of p53. Since we observed that the rate of p53 degradation was reduced upon stimulation with Cr(VI), the possible involvement of mdm2 in this process was investigated. A549 cells were incubated with Cr(VI) for 3 h and then collected for immunoprecipitation by p53 antibody. The binding of mdm2 was determined by western blot analysis. As shown in Figure 5, the binding of mdm2 to p53 dramatically decreased at a concentration of 50 μM Cr(VI) and disappeared at 100 μM Cr(VI).

These results suggest that phosphorylation of Ser15,

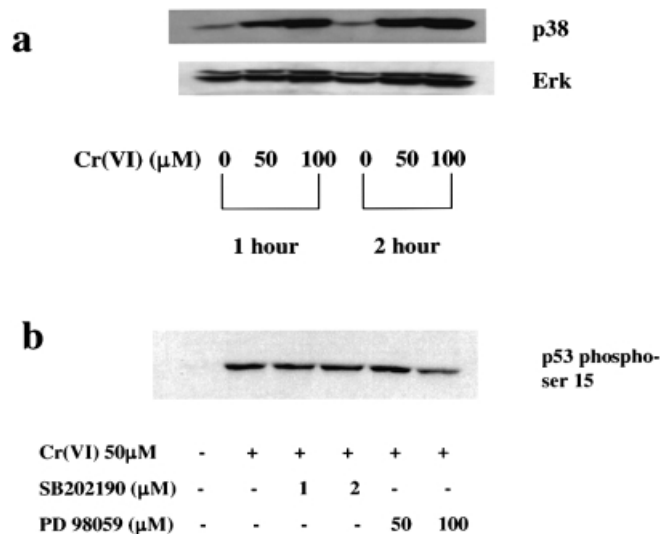
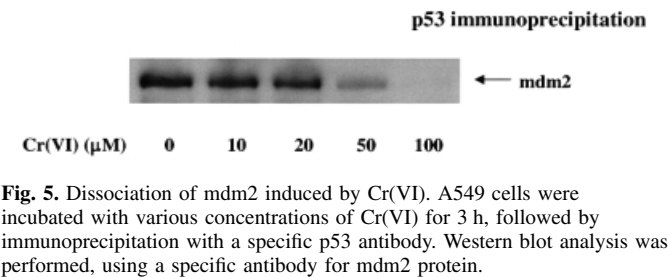


Fig. 6. (a) Activation of p38 and Erk with Cr(VI) stimulation. A549 cells were incubated with various concentrations of Cr(VI) for various times as indicated. The whole cell lysates were collected for western blot using specific antibodies for phospho-p38 and phospho-Erk, respectively. **(b)** Inhibition of Erk reduces Ser15 phosphorylation. A549 cells were pre-treated with p38 inhibitor SB202190 or MEK1 inhibitor PD98059 for 30 min, followed by incubation with or without 50 μM Cr(VI) for 3 h. The whole cell lysates were collected for western blot, using specific antibody for phospho-Ser15 p53 antibody.

acetylation of Lys382 and disassociation of mdm2 are jointly responsible for p53 activation induced by Cr(VI).

Erk is important in the phosphorylation of Ser15

It has been reported that MAP kinase family members are involved in p53 phosphorylation induced by UV light. P38 kinase was found to phosphorylate Ser392 of p53 protein, and c-jun N-terminal kinase (JNK) was reported to associate with p53 directly and stabilize the protein (26,43). To further understand the mechanisms of p53 activation by Cr(VI), we examined the possible roles of MAP kinase on p53 phosphorylation. Both p38 kinase and extracellular-signal regulated kinase (Erk) can be activated by Cr(VI) in a dose and time dependent manner (Figure 6a). Two chemical inhibitors, PD98059 (PD), a MEK1 inhibitor that is able to abolish Erk activity, and SB202190 (SB), a p38 kinase inhibitor, were pre-incubated with cells followed by Cr(VI) exposure. As shown in Figure 6b, inhibition of p38 kinase activity did not have any effect on Ser15 phosphorylation. In contrast, inhibition of Erk significantly decreased the phosphorylation of Ser15. In addition to the role of Erk in phosphorylation of the p53 Ser15 site, we examined the effects of Erk on the p53 transactivation ability by luciferase assay. As shown in Figure 7, addition of PD significantly reduced p53 activation induced by Cr(VI). The results suggest that Erk plays an important

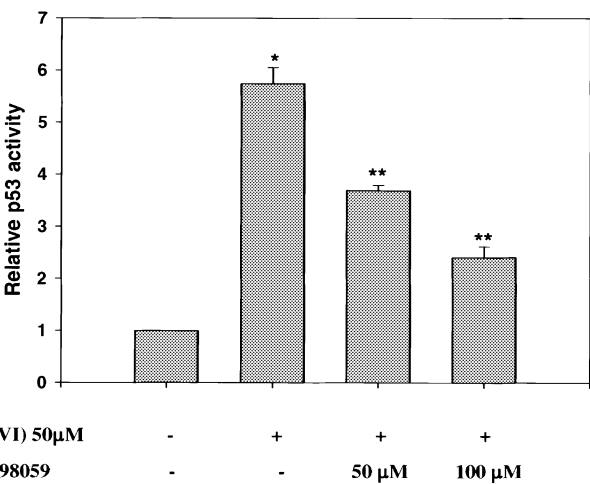


Fig. 7. Inhibition of Erk decreases Cr(VI)-induced p53 activation. A549 cells were transiently transfected with p53 luciferase plasmid. Then the cells were pre-treated with PD, followed by exposure to 50 μM Cr(VI) for 3 h. The cells were washed, added to fresh F-12K medium and incubated overnight. The p53 activity was measured by luciferase activity assay. Results are presented as relative p53 promoter induction compared with the untreated control cells (means and standard deviation of three repeated assays). Asterisks indicate a significant increase from control, and double asterisks indicate a significant decrease from the induction level ($P < 0.05$), using Student *t*-test for data analysis.

role in the phosphorylation of the Ser15 site of p53, which in turn plays a key role in the activation of p53 protein by Cr(VI).

The results shown above support the importance of post-translational modifications, including phosphorylation, acetylation and mdm2 dissociation, in Cr(VI)-induced activation of p53. During the activation process, Erk plays a crucial role by mediating the phosphorylation of the Ser15 site of the p53 protein.

Discussion

The present study shows that post-translational modifications, including phosphorylation at the N-terminus, acetylation at the C-terminus and segregation of mdm2, play a central role in Cr(VI)-induced p53 activation.

Previous studies from our group have demonstrated that after exposure to Cr(VI), p53 protein is activated at the protein level and via enhancement of both sequence-specific DNA binding ability and transactivation ability (26). In the present study, we further investigated the molecular mechanisms of p53 activation. Several groups have reported that increased mRNA expression or transcriptional activation was important for p53 activation (44–46). In our experiments, the addition of an inhibitor of transcription, actinomycin D, did not show any inhibitory effects on p53 activation. Consistent with this observation, the luciferase assay from cells transfected with p53 promoter–luciferase reporter plasmid demonstrates that p53 activation by Cr(VI) was not due to transcriptional activation.

P53 exists as a tetramer and rapidly degrades in normal cells. The degradation is mediated by proto-oncoprotein mdm2. The prerequisite for mdm2 to promote p53 degradation is its direct binding to the N-terminus of p53 protein. In addition to mediating p53 degradation, the binding of mdm2 also blocks the transactivation ability of p53. Therefore, breaking the p53–mdm2 interaction is a major mechanism to stabilize p53. Mdm2 protein immunoprecipitated with p53 decreased to

undetectable levels with Cr(VI) stimulation, demonstrating the dissociation of mdm2 from p53 induced by Cr(VI). The dissociation of mdm2 may be one explanation for the observed dramatic reduction in rate of p53 degradation after Cr(VI) stimulation.

One of the factors inducing disassociation of mdm2 from p53 is phosphorylation of p53 protein at its N-terminus. N-terminal phosphorylation has been reported to activate the transactivation ability of p53 (22). It is generally believed that the phosphorylation sites close to or inside the mdm2 binding site (residues 18–23), such as Ser15 and Ser20, are capable of interfering with the association between mdm2 and p53. However, recent reports from several groups suggest that phosphorylation of the Ser15 site only affects p53's transactivation ability while Ser20 phosphorylation is believed to mediate the dissociation of mdm2 from p53 and the stabilization of p53 (47–49). In the present study, we find reduced mdm2 binding was associated with Ser15 instead of Ser20 phosphorylation. Therefore, it appears that mdm2 dissociation induced by Cr(VI) is not due to Ser20 phosphorylation, and that phosphorylation of other sites, such as Ser15, may play a role in the dissociation process. In addition to phosphorylation at the N-terminus of p53 protein, evidence is accumulating that mdm2 can be phosphorylated by DNA-PK and ATM following stimulation with DNA damaging reagents. These phosphorylation events are considered to be another important mechanism for modulating interactions between mdm2 and p53 (50,51).

Unlike N-terminal phosphorylation, modification at the C-terminus is involved in the regulation of the sequence-specific DNA binding ability of p53. p53 exists as a tetramer in the cytoplasm of quiescent cells, the C-terminus acts as a negative regulatory domain by folding and binding to the central specific DNA binding domain of another monomer unit. The phosphorylation and/or acetylation of the C-terminus results in the release of the latent specific DNA binding domain from the inhibitory domain. Several stimuli, including ultraviolet (UV) radiation, tumor necrosis factor (TNF) α and anisomycin, are reported to phosphorylate Ser392 (Ser389 in mouse) and so activate the specific DNA binding activity of p53 (26,52). In the present study, Cr(VI) did not affect Ser392 phosphorylation in A549 cells. Instead, significant acetylation at Lys382 was observed. This acetylation at the C-terminus is likely to be responsible for the increase in specific DNA binding activity of p53 induced by Cr(VI).

Several protein kinases are capable of phosphorylating distinct sites on the p53 protein. The kinases DNA-PK, ATM, p38 and Erk are thought to be responsible for Ser15 phosphorylation of p53. However, different groups present contradictory results. For example, p38 kinase was considered to phosphorylate Ser392 but not Ser15 in response to UV radiation (53). In contrast, another group reported that upon stimulation with UV light, p38 kinase was responsible for phosphorylation of the Ser15 site (54). The different effects of upstream kinases in phosphorylating the same sites might be due to different cellular environments. In our investigation, the results obtained demonstrate that it is Erk, and not p38, that is involved in the phosphorylation of Ser15.

Although Cr(VI) is a well established carcinogen, its mechanism of action is not fully understood. It is well established that p53 plays an important role in carcinogenesis. The two major functions of p53 protein are cell cycle arrest and apoptosis, which are responsible for repairing or removing

the damaged cells. Since these two processes have a crucial role in protecting cells from permanent genetic damage, which is mediated by p53, alterations in these pathways can contribute to cancer development. Therefore, understanding the mechanisms of p53 activation is essential for the understanding of the overall carcinogenic process induced not only by Cr(VI), but also by other chemical and particle carcinogens.

In conclusion, post-translational modifications, including Ser15 phosphorylation, Lys382 acetylation and mdm2 dissociation, play a crucial role in the process of Cr(VI)-induced p53 activation. Erk, and not p38 kinase, is responsible for the phosphorylation of Ser15 induced by Cr(VI).

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